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The Role of Complement in Antibody-Dependent Acquired Immunity to *Plasmodium falciparum* Malaria

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THE ROLE OF COMPLEMENT IN ANTIBODY-DEPENDENT ACQUIRED IMMUNITY TO *PLASMODIUM FALCIPARUM* MALARIA

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ABSTRACT

Many pathogens evade complement recognition by binding to complement regulatory proteins, such as factor H (FH), that protect host cells from complement attack. Asexual *Plasmodium falciparum* stages evade complement destruction by capturing FH on their surface. An initial study identified Pf92 as the only protein that interacts with FH; however, the Pf92 knockout transgenic parasites did not wholly abolish FH recruitment to the merozoite. Thus, I hypothesized that potentially, more merozoite proteins are involved in complement evasion.

I used a combination of modified ELISA binding assay and surface plasmon resonance and tested a library of 110 recombinant merozoite proteins for interaction with FH. Protein microarray was used to measure antibody responses to the merozoite antigens that interacted with FH in a malaria longitudinal cohort of children. Additionally, I tested whether circulating levels of FH and the Y402H variant in the FH gene influence malaria severity.

In addition to Pf92, I found seven additional merozoite proteins that interact with the FH, i.e. PF3D7_1105800, PF3D7_0206200, RH5, P12, PF3D7_1252300, PF3D7_0629500 (SEG) and P12p. Majority of the proteins bound to complement control protein modules (CCP) 5-7 of FH. Antibody responses against a combination of some of these merozoite FH receptor antigens were found to be associated with protective immunity. Children with severe malaria (SM) had markedly lower circulating FH levels compared to uncomplicated malaria (UM) controls (median: SM=269 ug/ml, UM=378 ug/ml, P=<0.0001). Besides, children homozygous for the Y402H variant had a slightly increased risk of severe malaria (OR 1.08 95% CI: 0.46-2.58 P=0.854) while those who were heterozygous had reduced risk (OR 0.62 95% CI: 0.30-1.28 P=0.195) when compared with normal individuals.

These findings suggest that the *Plasmodium falciparum* parasite uses many proteins to interact with FH to avoid complement destruction and that FH protein, could be a vital host determinant for malaria severity. This study offers insights into the mechanisms of malaria parasite complement immune evasion and provides a rationale for future studies to evaluate stage-specific parasite proteins for interaction with complement regulatory proteins.

ACKNOWLEDGMENTS

DEDICATION

This thesis is dedicated to;

My mum Rosemary, dad Joseph, my siblings, Faith and Steve for your love and support

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ACT	artemisinin-based combination therapy
aHUS	atypical haemolytic-uraemic syndrome
AMD	age-related macular degeneration
AQ	amodiaquine
CCPs	complement control protein repeats
CFH	complement factor H
CFHR	complement factor H-related
CSP	circumsporozoite protein
CT	cell traversal
DAF	decay accelerating factor (CD55)
DCs	dendritic cells
DDT	dichloro-diphenyl-trichloroethane
DNA	deoxyribonucleic acid
ECs	endothelial cells
ELISA	enzyme-linked immunosorbent assay
FH	factor H
FHR	factor H-related
IFA	indirect immunofluorescence
IPTc	Intermittent Preventive Treatment in children
iRBCs	infected red blood cells
KC	kupffer cells
KCH	Kilifi County Hospital
KEMRI	Kenya Medical Research Institute
KHDSS	Kilifi Health Demographic Surveillance System
LRP-1	low-density lipoprotein receptor-related protein
MCP	membrane cofactor protein (CD46)
NK	natural killer cells
PCR	polymerase chain reaction
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PLP1	perforin-like protein 1
QBC	quantitative buffy coat
RBCs	red blood cells
RCA	regulators of complement activation
RIFINs	repetitive interspersed families of polypeptides
SCRs	short consensus repeats
SERU	Scientific and Ethics Review Unit
SM	severe malaria without anaemia
SMA	severe malaria anaemia
SMC	seasonal malaria chemoprevention

SNPs	single nucleotide polymorphisms
SP	sulfadoxine pyrimethamine
SPR	Surface plasmon resonance
SPEC-1	Spororozoite microneme protein essential for cell traversal
STEVOR	Subtelomeric variant open reading frame
TBVs	Transmission blocking vaccines
TCC	Terminal complement complex
UM	uncomplicated malaria
VSA	Variant surface antigens
WHO	World Health Organization

THESIS OUTLINE

I start in **chapter one** by giving a broad introduction to malaria, its epidemiology and control strategies. I then briefly discuss malaria vaccine development and innate immunity to malaria. Thereafter, I introduce the complement system, mechanisms that pathogen use to evade complement activation and the role of complement in immunity to malaria. I close the discussion on complement by focusing how it contributes to the development of severe malaria.

In **chapter two** I comprehensively describe the malaria longitudinal cohort of children and the hospital case control study whose samples I used. I then provide detailed explanations for the experimental procedures that I conducted.

In **chapter three** I test the hypothesis that many merozoite proteins interact with complement regulatory protein FH in order to facilitate immune evasion. I began by expressing a library of recombinant merozoite antigens. I then used a combination of ELISA binding assay and surface plasmon resonance to narrow down the merozoite antigens that bind to FH. I observed that, contrary to what is known that only one merozoite antigen binds to FH, many more proteins are involved.

In **chapter four** I explore whether antibody responses to merozoite proteins that bind FH (merozoite FH receptor proteins) are associated with protection. In the longitudinal cohort I observe that children, who make antibody responses to a combination of these proteins, have some protective immunity.

In **chapter five** I determine, whether variation in FH levels within individuals influence malaria susceptibility and severity. In the longitudinal cohort there was a five-fold range in FH levels. This variation did not statistically influence the prospective risk of

developing malaria. Using the case-control study, there was a marked difference in FH levels in children with severe malaria compared to the uncomplicated malaria controls. Children, who had the CC AMD risk allele of the Y402H variant of FH had a slightly, but not significantly, increased risk of having severe malaria than those who are normal, TT. In addition, children, who were CC homozygotes had reduced FH levels compared to the TT homozygote children.

Chapter six summarizes key finding and offers recommendation for future studies

1   Introduction

1.1   Summary and aims

This introductory chapter provides background information to the thesis project. It is split into two parts. In the first section, I discuss the public health significance of malaria, control strategies, innate immunity to malaria, and parasite immune evasion mechanisms. In the second section, I review the complement system and its role in disease pathogenesis with a focus on infectious diseases.

1.2   Malaria is a global health problem

The global burden of malaria is massive. In 2017 alone, an estimated 219 million cases occurred globally. Approximately 92% of these cases occurred in Africa, followed by Southeast Asia with 5% and Eastern Mediterranean with 2%. There were 435,000 deaths reported, 61% occurred in children under the age of five years. Sub-Saharan Africa accounted for 93% of all malaria deaths (WHO 2018b). Of note, between 2010 and 2017, malaria incidence declined by 18% from 72 to 59 cases per 1000 population at risk. However, the number of cases per 1000 population has remained at 59 for the last three years indicating that much more remains to be done, if the goal of moving towards malaria elimination is to be achieved. Furthermore, with many countries still having ongoing malaria transmission **Figure 1.1** (WHO 2018b), new tools are urgently needed to contribute to malaria prevention and control.
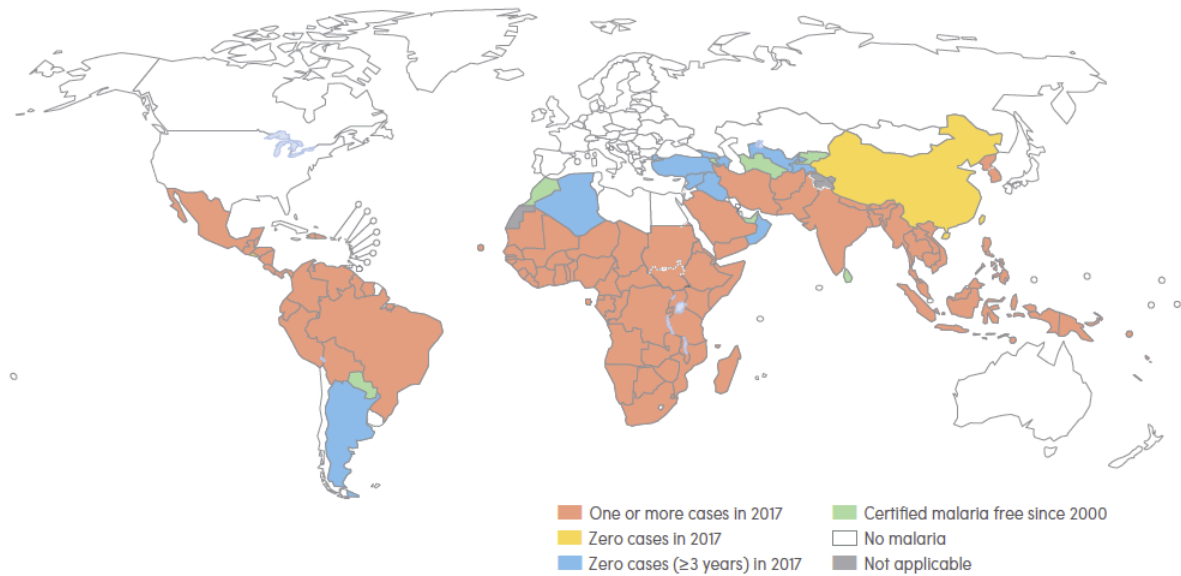
Figure 1.1: A global map of countries with ongoing malaria transmission.

Countries with indigenous malaria cases in 2000 and their status as of 2017. Countries with zero indigenous cases over at least the past three consecutive years are considered to be malaria free. Figure from (WHO 2018b).

1.3   Five species of *Plasmodium* infect humans

Malaria in humans is caused by five species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae, P. ovale* and *P. knowlesi*. The current distribution of *Plasmodium* species shows the preponderance of *P. falciparum* in tropical parts of Africa, while *P. vivax* is common in tropical areas outside Africa. Most Africans lack the Duffy blood group antigen that is expressed on the surface of erythrocytes (Howes et al. 2011), and is considered as an essential receptor for *P. vivax* invasion (Miller et al. 1976). However, this long held-notion is being challenged by more recent evidence that suggests that *P. vivax* can infect, and cause disease in some Duffy-negative individuals (Menard et al. 2010; Mendes et al. 2011). This suggests that the parasite can invade erythrocytes using other receptors. However, it remains to be determined, whether these are true *P. vivax* or variants. Both *P. falciparum* and *P. vivax* are prevalent in Southeast Asia and the Western Pacific. Infections with *P. vivax* were rarely thought to lead to death

in contrast to those with *P. falciparum* (Baird 2007). On the contrary, it has now been demonstrated that infections with *P. vivax* could be associated with severe and fatal illness (Kochar et al. 2005; Genton et al. 2008; Tjitra et al. 2008). Although *P. malariae* can occur in all malaria areas, its prevalence as a mono-infection or co-infection with other plasmodia is generally low. In tropical Africa, *P. falciparum* and *P. malariae* co-infections are sometimes encountered. Unlike *P. malariae*, *P. ovale* is more widespread primarily in tropical Africa whereas human *P. knowlesi* infections have been reported in Southeast Asia (Cox-Singh et al. 2008; Autino et al. 2012).

1.4 *Plasmodium spp.* have a complex life cycle

The life cycle of *Plasmodium* parasites is complex. It requires an anopheline mosquito vector and a vertebrate host **Figure 1.2** (White et al. 2014). In the human host, infection begins with the bite of an infected *Anopheles* mosquito that inoculates the individual with sporozoites (Greenwood et al. 2008). It is estimated that one mosquito can inject up to 10-100 sporozoites per bite (Vanderberg and Frevert 2004; Gueirard et al. 2010). The sporozoites migrate to the capillaries, then travel via the bloodstream to drain into the liver, where they invade hepatocytes and initiate the pre-erythrocytic cycle (Jones and Good 2006). The pre-erythrocytic phase of infection is clinically silent, and lasts approximately 5-14 days depending on the *Plasmodium spp.* involved (Antinori et al. 2012). Upon invasion of hepatocytes, sporozoites transform inside a parasite-induced cellular compartment (also called the parasitophorous vacuole), where they undergo replication, also called schizogony, at the end of which thousands of daughter cells are formed. These cells differentiate into invasive blood stage parasites called merozoites that are released in 'batches' into the bloodstream within membranous remnants of their host cells (merosomes). The transformation from sporozoites to merozoites is driven by the expression of a discrete set of genes (Sturm et al. 2006; Bijker et al. 2015).

In the bloodstream, the merozoites quickly invade red blood cells (RBCs) to initiate the pathogenic erythrocytic stage of the parasite life cycle, also called asexual cycle. To achieve this, the merozoite makes initial contact with the red blood cell and reorients to bring its apical tip close to the cell surface. Parasite proteins in the apical prominence bind to red blood cell proteins, triggering commitment to entry (Gilson and Crabb 2009). Subsequently, a tight junction forms between parasite and red blood cell membranes, propelling the parasite into the cell (Dvorak *et al.*, 1975). Within the RBCs, the merozoites mature to form rings, then trophozoites and eventually schizonts that burst to release a new generation of merozoites thereby starting the process of invading new RBCs. Asexual *P. falciparum* blood stage parasites produce 8-20 new merozoites every 48 hrs (72 hrs for *P. malariae*), causing parasite numbers to rapidly rise to levels as high as 10^{13} per host (Greenwood et al. 2008).

A variety of parasite products are released upon lysis of infected red blood cells (iRBCs) that correlate with the onset of malaria symptoms including headaches, fever, and lethargy (Crompton et al. 2014). The asexual life cycle is accompanied by gametocytogenesis, whereby a fraction of merozoites differentiate into male and female gametocytes that are taken up during a mosquito's blood meal. In the mosquito midgut, male and female gametes fuse to form ookinetes that cross the midgut epithelium, where they develop into oocysts, which ultimately differentiate into sporozoites that invade the mosquito's salivary glands (Crompton et al. 2014). Depending on the external temperatures, the mosquito becomes infectious to its next susceptible host approximately two weeks after ingesting gametes. Of note is that *P. vivax* can develop within the mosquito at lower temperatures than *P. falciparum*, explaining the preponderance of *P. vivax* infections outside tropical and subtropical regions (Greenwood et al. 2008).
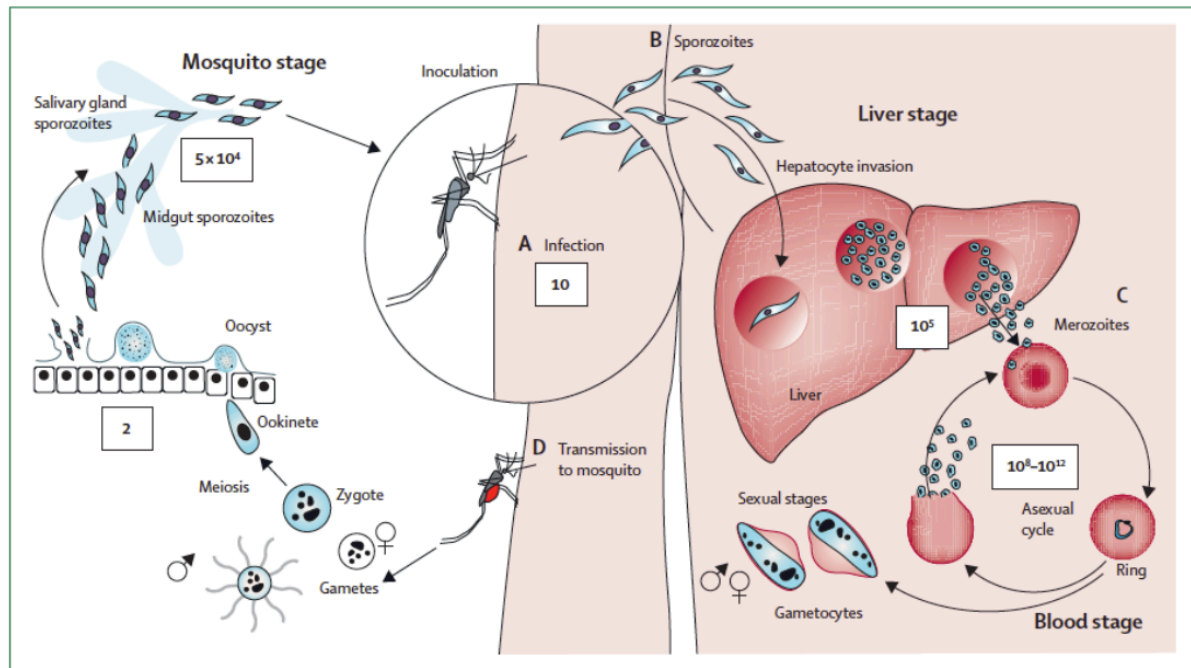
Figure 1.2 *Plasmodium falciparum* life cycle in the human host and the anopheline mosquito

The cycle starts when a mosquito injects of sporozoites into the dermis of the human host during a blood meal (A), the sporozoites then travel to the liver (B), where they invade the hepatocytes and then multiply. After about one week, the liver schizonts burst releasing merozoites into the bloodstream that invade red blood cells to begin the asexual cycle (C), also called the blood stage. Some parasites can develop into sexual forms (gametocytes). Gametocytes are taken up by a blood-feeding anopheline mosquito (D) where they reproduce sexually, forming an ookinete and then an oocyst within the mosquito mid-gut. The oocyst bursts to release sporozoites, which migrate to the salivary glands awaiting inoculation during the next blood meal. The entire cycle can take approximately one month. Estimated numbers of parasites are shown in the boxes. Figure from (White et al. 2014).

1.5   Clinical presentation of malaria

When an individual gets infected with *Plasmodium falciparum* malaria parasites, a variety of clinical manifestations may follow. Depending on the host innate or acquired immunity, these range from asymptomatic parasitemia to uncomplicated malaria, which can lead to severe malaria and ultimately death. Uncomplicated malaria usually presents with fever and other nonspecific symptoms such as vomiting and diarrhoea. In adults, severe malaria can sometimes lead to complications including acute

pulmonary oedema, jaundice, and renal failure. Such complications are not common in young children with severe malaria, who mostly present with prostration, respiratory distress, severe anaemia, or cerebral malaria (White et al. 2014). Coma which is as a result of cerebral malaria and acidosis, occurs in all age groups **Figure 1.3** (Greenwood et al. 2008). Age is closely linked to the manifestations of severe malaria (Dondorp et al. 2008). Severe anaemia tends to occurs in children in areas of high transmission and is usually a result of repeated infections, whereas cerebral malaria is common in older children in low transmission areas (Calis et al. 2008; White, 2014a; Snow et al. 1994)
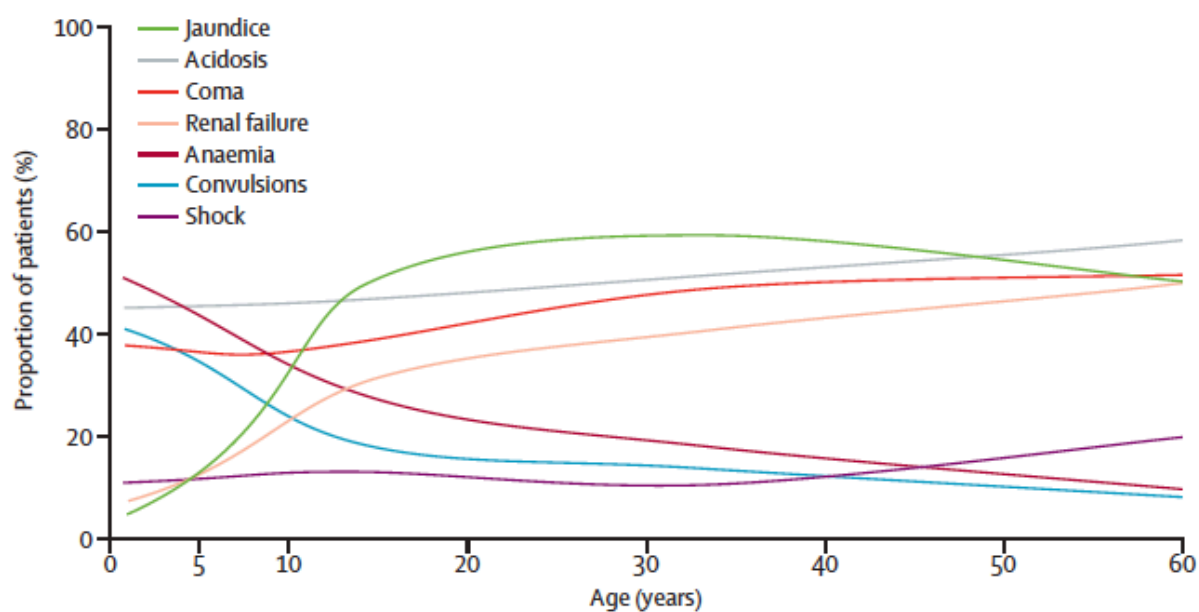
Figure 1.3 Manifestations of severe *Plasmodium falciparum* malaria by age.

Severe malaria anaemia and coma affect mostly young children, while jaundice and renal failure occur in adults. Figure from (White et al. 2014).

The most commonly encountered clinical syndromes that define severe malaria in children fall into three main categories: severe anaemia, cerebral malaria/ impaired consciousness and metabolic acidosis/respiratory distress. These syndromes may

occur separately or in any combination **Figure 1.4** (Marsh *et al.*, 1995). Although syndromes of malaria are known, and their age distribution have been described, it remains unclear why some individuals develop an asymptomatic infection while others proceed to severe malaria. However, it has been shown that genetic factors (Kwiatkowski 2005), and both the age of the patient and the intensity of transmission influence susceptibility to severe anaemia and cerebral malaria (Snow et al. 1997; Reyburn et al. 2005)-
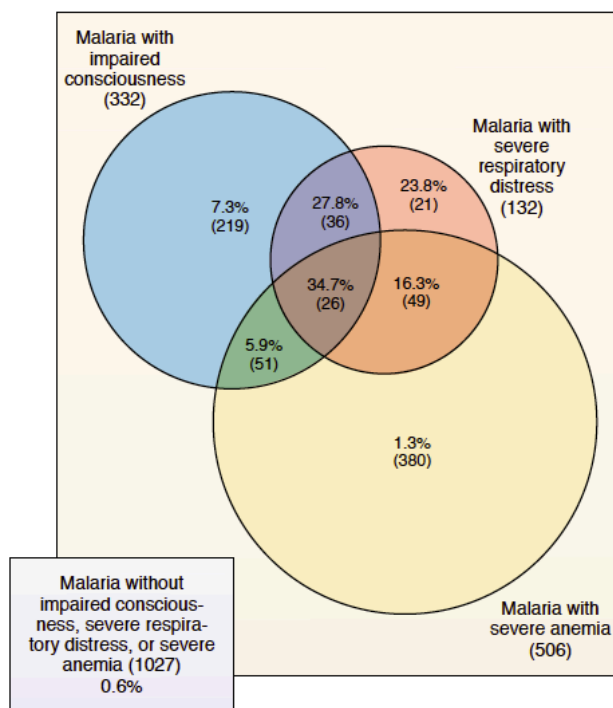
Figure 1.4 Prevalence overlap and mortality for major subgroups of 1844 Kenyan children with severe malaria.

Total numbers are given in parenthesis, and mortality is given in percentages, mean age of the children was 2.2 years. Figure from (Marsh *et al.*, 1995).

1.6 Epidemiology of malaria

Infections with *Plasmodium falciparum* are limited primarily to the tropics (WHO 2018b). In sub-Saharan Africa, malaria transmission can occur either throughout the

year (perennial) or can be highly seasonal, but primarily confined to the 3-4 month-long rainy season **Figure 1.5** below (Autino et al. 2012).

Definition of malaria transmission. Malaria transmission can be classified as either stable or unstable, which is also closely related to endemicity levels. The stable-unstable classification was first proposed by Sir Ronald Ross and adapted by George Macdonald for the measurement of malaria endemicity. Stability was defined quantitatively by the average number of feeds that a mosquito takes on a man during its lifetime (Macdonald 1957). The limitation of Macdonald's stability index measurement was that it required detailed entomological data that is rarely available (Snow et al. 2012). Constant, frequent, year-round infection was termed as stable transmission, whereas in areas where transmission is low, erratic or focal is termed as unstable transmission. Another metric used to define malaria transmission relies on using parasite prevalence in the 2-10 year-olds ($PfPP_{2-10}$) (Hay et al. 2008). Based on this metric, four different endemicity areas can be distinguished: holoendemic, when transmission occurs all year round; hyperendemic, where intense transmission occurs but with periods of remission during the dry season; mesoendemic indicating regular seasonal transmission; and hypoendemic with very intermittent transmission.

Presentation of malaria in areas of stable/unstable transmission: In stable malaria transmission areas, anaemia and childhood mortality are common, parasitemia is high in children aged 6-15 years, and adults often carry asymptomatic infections (Griffin et al. 2015). In areas where transmission is low/unstable, protective immunity preventing malaria symptoms is not acquired, but the symptomatic disease affects all ages. In such regions, changes in environmental, economic, or social conditions can result in an epidemic, with substantial mortality in all age groups (White et al. 2014). While these are general considerations, there are special groups of people for whom the normal patterns are not observed e.g., pregnant women.

Pregnant women remain at increased risk whether residing in stable or unstable transmission areas. In stable transmission areas, where adult women are considered to have developed acquired immunity due to continuous exposure, *P. falciparum* infection during pregnancy does not cause symptomatic malaria, but it may lead to placental malaria infection and low birth weight (LBW) in newborns (Steketee et al. 2001; Parise et al. 1998). In these areas, primigravidae and, to a lesser extent, secundigravidae are at highest risk for malaria infection and LBW (Jelliffe 1968; Brabin 1983; McGregor et al. 1983; Steketee et al. 1988). In unstable transmission areas, women do not acquire substantial antimalarial immunity hence infection with *P. falciparum* can lead to clinical illness in the woman, and this has been linked to anemia in the women and low birth weight (McCormick 1985).
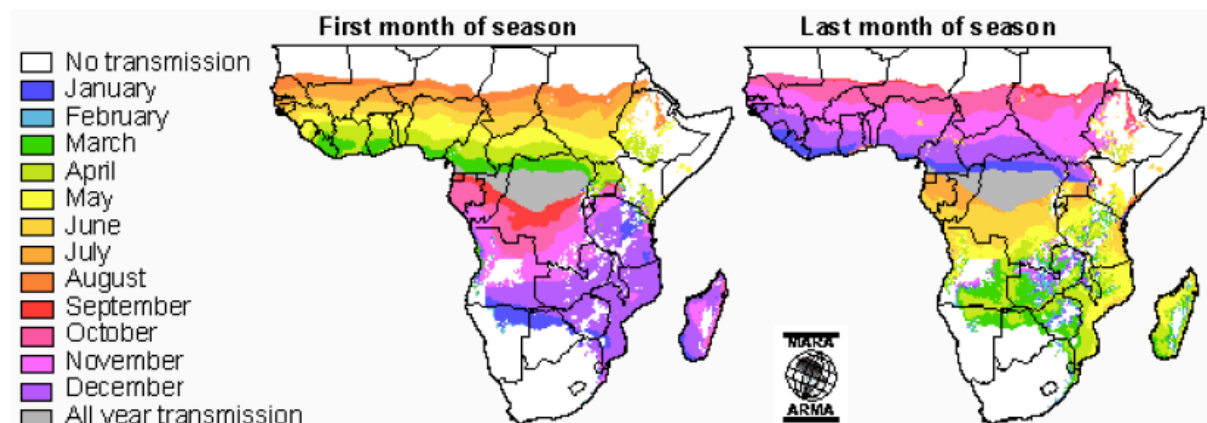
Figure 1.5 Illustration showing the start/end of transmission season on the Africa continent over a year.

While few countries have all year transmission, most countries have two rainy seasons that lead to fluctuations in malaria incidence according to seasonality. Figure from (Autino et al. 2012).

1.7 Malaria control efforts

Historical perspectives The first malaria control efforts date back to 1942-1951.

During this period household spraying of the residual insecticide dichloro-diphenyl-trichloroethane (DDT) led to the elimination of malaria in the south eastern states of America (Zucker 1996). After that, the WHO launched an ambitious Global Malaria Eradication Programme in 1955 that aimed to use chloroquine for malaria treatment and prevention and DDT for vector control. This programme achieved some success as it led to a reduction of malaria in low transmission areas but suffered lack of sustained political good will. This was later compounded by emergence of chloroquine-resistant *Plasmodium* parasites and DDT-resistant *Anopheles* mosquitoes (Greenwood et al. 2008; WHO 2013). Furthermore, the campaign did not attempt to eradicate malaria in many parts of Africa, where transmission is intense. In 1998, WHO launched yet another large-scale attempt to fight malaria, called the Roll Back Malaria Initiative, with a focus on the African continent. The goal of the programme was to ensure that at least 60% of the continent's at-risk population were protected or treated with appropriate methods (Snow and Marsh 2010).

Current perspectives At present, substantial resources have been mobilized for malaria control with many programs working towards the ambitious goal of malaria elimination. For instance, in 2017 an estimated US$ 3.1 billion was invested in malaria control and elimination efforts globally by governments of malaria-endemic countries and international partners (WHO 2018b), although mostly contributed by the latter. The modern control tools to which these resources are being channeled to include: i) vector control, ii) chemoprevention and iii) rapid diagnosis, testing, and treatment.

1.7.1 Vector Control

Vector control involves stopping mosquitoes from biting human beings. The most commonly used methods for prevention of mosquito bites are insecticide-treated bed

nets (ITNs) and indoor residual spraying (IRS) (World Health Organization 2016). Pyrethroids are by far the most commonly used insecticides for IRS and are the only ones currently recommended by the WHO for treatment of bed nets (World Health Organization 2016). Three other classes of WHO-recommended adulticides (organophosphates, carbamates, and organochlorines) are also used in IRS to differing extents throughout Africa (Knox et al. 2014). The evidence suggests that in areas with a stable transmission, ITNs reduce the incidence of uncomplicated malarial episodes by 50% compared to no nets at all, and by 39% when compared to untreated nets. In areas of unstable transmission, the equivalent reduction in uncomplicated malaria was 62% compared to no nets and 43% compared to untreated nets (Lengeler 2004).

Indoor residual spraying has been shown to reduce the incidence of malaria in unstable malaria settings in randomized clinical trials. However, similar data from settings with stable malaria transmission are scanty. A limited amount of data suggests that ITNs provide better protection than IRS in unstable areas. However, more trials are needed to compare the effects of ITNs with IRS, as well as to quantify their combined effects (Pluess et al. 2010). For both ITNs and IRS, insecticide resistance in countries with ongoing malaria transmission is concerning (WHO 2018a).

1.7.2   Chemoprevention

The WHO defines seasonal malaria chemoprevention (SMC), (previously referred to as Intermittent Preventive Treatment in children (IPTc)), as the periodic administration of full treatment courses of an effective antimalarial medicine during the malaria season to prevent malarial illness. The objective is to maintain a therapeutic antimalarial drug concentration in the blood throughout the time of greatest malarial

risk. The recommended medicines are sulfadoxine pyrimethamine (SP) and amodiaquine (AQ), which should be administered if the parasite is sensitive to them (WHO 2012). This recommendation is based on a previous clinical trial, which suggested that SMC was highly effective, reducing the incidence of malaria by 82% and also contributing to a reduction in childhood deaths (Wilson and IPTc Taskforce 2011). Slightly reduced estimates of the degree of protection provided by SMC were reported in a Cochrane review of the efficacy of SMC (Meremikwu et al. 2012). Although SMC is beneficial and is highly recommended by the WHO, it has not been fully implemented due to its associated high costs and logistical challenges. It requires twelve contacts per year between child and health care provider so that each dose of anti-malarial is given under direct observation, which is rarely practicable and, if this is not done, there is a danger that children may not get a full course (Greenwood et al. 2017).

A potential future threat to the implementation of SMC is the observation of an increase in the proportion of malaria-causing parasites carrying markers of resistance to SP at the end of the malaria transmission season in some early trials (Wilson and IPTc Taskforce 2011; Cissé et al. 2016). Except for dihydroartemisinin-piperaquine there is currently no agreed drug combination that can be used in place of SP and AQ in the areas where SMC is being delivered (Zongo et al. 2015). The option of using artemisinin-based combination (ACT) therapy widely for chemoprevention in Africa would be controversial given the threat of the emergence of parasite resistance to ACTs (Takala-Harrison et al. 2015; Talundzic et al. 2015; Wang et al. 2015; Greenwood et al. 2017).

1.7.3   Rapid malaria diagnosis

Prompt detection and treatment of malaria patients has been argued to be the most effective intervention to ensure that a mild case of malaria does not develop into severe disease. High levels of access for effective malaria case management may also help to reduce the pool of individuals who contribute to onward transmission (WHO 2010). Malaria diagnosis involves identifying malaria parasites, antigens or their products in patient blood. Although this may seem relatively simple, the diagnostic efficacy of any tool may be affected by multiple factors such as: the different malaria species; the different stages of the parasite, the endemicity of different species, parasitemia levels, immunity, and the use of chemoprophylaxis. All of these factors can influence parasite identification and interpretation of malaria diagnostics (Tangpukdee et al. 2009). Malaria diagnosis combines both clinical and laboratory components.

A clinical diagnosis of malaria is based on the patient's symptoms and physical findings at examination. However, the considerable overlap of malaria symptoms with other febrile diseases makes accurate clinical diagnosis challenging. For example, initial symptoms of malaria (fever, chills, sweats, headaches, muscle pains, nausea and vomiting) are often non-specific and are also shared by many infectious diseases. Likewise, the physical findings are often not specific (elevated temperature, perspiration, tiredness). This overlap of malaria symptoms with those commonly present in other tropical diseases impedes diagnostic specificity, which can promote the indiscriminate use of anti-malarial. It can also compromise the quality of care for patients with non-malarial fevers in endemic areas (Reyburn et al. 2004; Mwangi et al. 2005a; McMorrow et al. 2008). However, it's worthwhile to note that in severe malaria (caused by *Plasmodium falciparum*), clinical findings (confusion, coma, neurologic focal signs, severe anaemia, respiratory difficulties) are more striking and may increase the index of suspicion for malaria.

Malaria diagnosis in the laboratory is achieved using different techniques. These include: microscopy (Ngasala et al. 2008), quantitative buffy coat (QBC) method (Bhandari et al. 2008), rapid diagnostics tests such as optiMAL (Tagbor et al. 2008; Zerpa et al. 2008), paraScreen (Endeshaw et al. 2008), and molecular techniques such as polymerase chain reaction (PCR) (Holland and Kiechle 2005; Vo et al. 2007). Some advantages and disadvantages of these methods relate to sensitivity, specificity, accuracy, precision, the time consumed, cost-effectiveness, the need for highly skilled microscopists (Tangpukdee et al. 2009). There are also indirect serological techniques. Serological kits detect antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA) approaches. In endemic areas, these serological techniques are used mostly for epidemiological surveillance and vaccine research. Moreover, in non-endemic areas, such as in Europe, antibody detection is used mostly for diagnosis and for checking the safety of blood transfusion. However, the detection of antibodies is not suitable for the diagnosis of acute malaria (https://www.cdc.gov/malaria/diagnosis_treatment/diagnosis.html). The gold standard for malaria diagnosis is microscopy using either thin or thick blood smears, and RDTs where laboratory facitilies are unavailable (WHO 2014).

1.7.4 Treatment for malaria

The WHO recommends treatment of uncomplicated malaria using artemisinin-based combination treatments (ACTs) which are a combination of artemisinin derivatives and other structurally unrelated and more slowly eliminated antimalarials. The most commonly used ACTs are artemether-lumefantrine, artesunate-amodiaquine, dihydroartemisinin-piperaquine, artesunate-mefloquine, and artesunate plus sulfadoxine-pyrimethamine (WHO 2018b). Although highly effective, their continued

usage is threatened by reports of the emergence of resistance to artemisinin. Artemisinin resistance is characterized by slow parasite clearance *in vivo* without corresponding reductions in conventional *in vitro* susceptibility (Dondorp et al. 2009). The resistance is linked to mutations in the "propeller" region of the *P. falciparum* Kelch protein (Ariey et al. 2014). Emergence of clinical resistance to artemisinin was first reported in Cambodia (Denis et al. 2006; Noedl et al. 2008; Dondorp et al. 2009) but has now spread across Southeast Asia and South China (Ashley et al. 2014; Huang et al. 2015; Ménard et al. 2016). So far there is no reported evidence that artemisin resistance has spread to Africa (WWARN K13 Genotype-Phenotype Study Group 2019). Nevertheless, continuous spread of artemisinin resistance as currently reported would have dramatic consequences since replacement therapies are currently limited (Ménard et al. 2016).

1.8   Malaria vaccines in clinical trials

The development of an efficacious vaccine against *Plasmodium falciparum* malaria remains a key research priority. Malaria vaccine development has primarily focused on pre-erythrocytic, blood-stage or transmission-blocking vaccine targets (Ouattara and Laurens 2015). An ideal pre-erythrocytic vaccine would have to aim at either preventing sporozoites from reaching the liver or prevent maturation of liver stage parasites and hence prevent clinical disease as well as transmission (Riley and Stewart 2013). Blood stage vaccines would aim to reduce the severity of the disease. The scientific rationale supporting the development of asexual blood-stage vaccines is rooted in the observation that naturally acquired immunity can be passively transferred to susceptible individuals (Cohen et al. 1961). Finally, the transmission-blocking vaccines aim to target sexual stages, thereby preventing parasite development within the mosquito and subsequent transmission to the human host (Birkett 2016).

1.8.1 Pre-erythrocytic vaccines

Pre-erythrocytic vaccine targets include the circumsporozoite protein(CSP), a protein expressed on the surface of sporozoites (Kappe et al. 2004). The most advanced CSP-based vaccine is RTS, S. It is comprised of hepatitis B surface antigen (HBsAg) particles fused to the *P. falciparum* CSP central repeat and thrombospondin domains formulated in the adjuvant ASO1 (Ouattara and Laurens 2014). Among the numerous vaccines thus far tested, RTS, S was the first one to undergo evaluation in a phase 3 clinical trial involving 15,460 children at 11 sites in 7 African countries (Agnandji et al. 2011; Leach et al. 2011). During the first 18 months of follow-up, three doses of RTS, S/AS01 induced protective efficacy against clinical malaria of 46% in 5–17 month old children, and 27% in 6–12 week old infants (RTS,S Clinical Trials Partnership 2014). This protective efficacy declined during the 38–48 month follow up period and at the end of the trial was 28.3% and 18.3% for the children and infants, respectively. Other pre-erythrocytic vaccine candidates include those that achieve protective immunity through irradiated/chemo-attenuated *P. falciparum* sporozoites, e.g. P*f*SPZ vaccines (Seder et al. 2013; Mordmüller et al. 2017) and the next-generation RTS, S-like vaccine, R21 (Collins et al. 2017).

1.8.2 Asexual stage vaccines

Asexual blood-stage antigen-based vaccines are aimed to stop parasite invasion of the red blood cells and hopefully translate to offering protection against clinical disease. At present several blood-stage antigens have or are being tried and these include apical membrane antigen 1 (AMA1), erythrocyte-binding antigen175 (EBA-175), glutamate-rich protein (GLURP), merozoite surface protein (MSP) 1, MSP2 and MSP3 and serine repeat antigen 5 (SERA5) (Ellis et al. 2010; Ntege et al. 2017). So far most of these vaccines have shown limited efficacy and this has been thought to

be due to antigenic variation, extensive polymorphism, conformation-dependence, and in some instances safety concerns (Schwartz et al. 2012; Ntege et al. 2017). To overcome some of these challenges various approaches have been tried including: i) incorporation of multiple alleles of same antigens into one vaccines such as the example of PfAMA1-DiCo (Sirima et al. 2017) and ii) use of relatively conserved regions of antigens targets such as BK-SE36 vaccine (Palacpac et al. 2013). However, none of these vaccines has reached advanced stages.

1.8.3   Transmission blocking vaccines

Transmission blocking vaccines (TBVs) are designed using antigens expressed by the transmissible sexual-stages of the parasite, as well as those expressed by the mosquito, and aim to prevent sporozoite development and onward transmission (Carter et al. 2000). Notably, while TBV would not directly offer protection to an individual, it could have a substantial impact: in an endemic population of asymptomatic or submicroscopic carriers, a TBV will serve to arrest onward transmission and thus provide protection to the community through herd immunity (Draper et al. 2018). Transmission-blocking immunity is thought to be mediated by antibodies against surface protein antigens of sexual stages and acting within the midgut of a blood-fed mosquito (Carter 2001). A number of different potential antigenic targets of transmission immunity have been investigated. Other antigens that have been tested as potential TBVs include P*fs*47 that is involved in parasite immune evasion in the mosquito vector (Molina-Cruz et al. 2013), P*f*HAP2 that is expressed on the male gametocyte and microgamete (Angrisano et al. 2017) and P*fs*25 which is expressed during macro-gametogenesis within the mosquito midgut and persists throughout zygote, ookinete and early oocyst development (Wu et al. 2008).

1.8.4   Multi-stage, multi-component vaccines

Historically, vaccine development has focused on a small number of antigens. Thus far, of the more than 5000 parasite proteins, approximately 20 or so have reached preclinical development (Riley and Stewart 2013). It is now believed that it may be necessary to combine parasite antigens from the same stage (Osier *et al.*, 2014) or those selected from different stages of malaria parasites to achieve synergistic effects. For example, partially effective pre-erythrocytic and blood-stage components might allow the development of a multicomponent vaccine that can reduce malaria transmission as well as provide protection against malaria (malERA Consultative Group on Vaccines 2011). Nevertheless, a previous attempt to use multistage antigens did not give encouraging results. For example, the NYVAC-PF7 attenuated vaccine containing candidates from various stages: pre-erythrocytic (CSP, SSP, and LSA1), blood stage (MSP1, AMA1, and SERA), and mosquito-stage (Pfs25) antigens only showed modest results (Ockenhouse et al. 1998).

Another example that has been tested is PEV3A containing peptides from both the pre-erythrocytic (circumsporozoite protein) and the blood- stage antigen (AMA-1). Although the vaccine did not induce sterile protection, lower rates of parasite growth were observed in volunteers compared to unvaccinated controls (Thompson et al. 2008). There have also been attempts to design a cocktail of multistage and multicomponent vaccines (Boes et al. 2015) although this is yet to move to advanced stages of vaccine testing.

1.9   Malaria vaccine development: challenges and opportunities

Effective vaccines constitute one of the greatest success stories within the public health sector. The most notable examples include the eradication of smallpox and the virtual elimination of polio (Hussein *et al.*, 2015). For malaria, many factors have

hindered the development of a highly efficacious vaccine for widespread use. First, there is a lack of reliable correlates of protection. It has been known that antibodies play a crucial role in acquired immunity to malaria (Cohen et al. 1961); however, there has been inconsistency in the literature as to the precise antigens that induce protective immunity (Tuju et al. 2017). Second, it is the complex biology of the life cycle of the parasite, which exists in different forms (and each form with a different pattern of antigen expression) in different tissues of the body and the mosquito. It has been shown that the parasite has well over 5, 000 genes (Gardner et al. 2002) and it remains unclear which of the respective proteins should be selected for vaccine development (Tuju et al. 2017). Lastly, among other factors, antigenic variation and polymorphism, have rendered subunit vaccine development challenging, especially given that many of the sequence alterations in malaria proteins occur in regions that appear to be crucial to immunity (Good 2001). Nevertheless, these challenges also provide opportunities to find new ways to interrupt the parasite life cycle and potentially contribute to the design of a next-generation malaria vaccine.

1.10 Immunity to malaria

Epidemiological observations suggest that in areas where there is continuous exposure to malaria, children become susceptible to clinical episodes of malaria including severe malaria. However, the incidence of severe malaria declines with age becoming rare in children over five year of age.This indicates that immunity to this life-threatening illness can be acquired relatively early in life (Baird JK and Baird 1995). In contrast, adults carry parasites but remain asymptomatic and rarely experience severe disease (Marsh and Kinyanjui 2006). Sterile immunity to infection with parasites is rarely achieved. In the subsequent few sections below, I discuss specific aspects of immunity to malaria in more depth including innate immunity, genetically based

resistance, naturally acquired immunity (NAI) and antibodies as mediators of NAI. The discussion focuses on blood stage immunity.

1.10.1 Innate immunity to malaria

Unlike other infections with intracellular pathogens, such as viruses, bacteria and some protozoan parasites, in which the role of the innate immune responses has been well investigated (Scharton-Kersten et al. 1995; Biron 1997; Scharton-Kersten and Sher 1997; Unanue 1997) relatively few studies have examined the role of innate immunity to malaria. In these studies, the focus has been on macrophages, dendritic cells, $\gamma\delta$ T cells and natural killer cells (NK).

Macrophages. Studies in humans and mouse models have shown that macrophages function as antigen-presenting cells and can also phagocytose infected erythrocytes (Serghides et al. 2003). Following chronic or acute infections in semi-immune individuals, proteins on the surface of iRBCs induce the development of malaria-specific antibodies (Bull et al. 1998) leading to Fc-receptor-mediated uptake of blood-stage parasites by phagocytic cells. However, during acute infection in non-immune hosts, antigen-specific antibody responses are not yet developed, and effector cells must rely on alternative mechanisms to recognize and clear iRBCs. Monocytes or macrophages often use pattern recognition receptors (PRRs), including scavenger receptors such as CD36, to recognize and phagocytose invading microbes before a pathogen-specific immune response has been generated (Peiser et al. 2002). *In vitro,* studies have pointed out the importance of CD36-iRBCs interaction in malaria. Macrophages from CD36 knockout mice phagocytosed significantly less iRBCs (2.9%) compared to macrophages from CD36 wild type (WT) mice (19.3%) (Patel et al. 2004). Moreover, macrophages can also mediate antibody-dependent cellular inhibition or the

production of anti-parasite molecules, such as nitric oxide, after their activation by CD4+ T-cell-derived INF-γ (Good and Doolan 1999; Good 2001).

Dendritic cells. DCs form a distinct lineage of mononuclear phagocytic cells specialized in antigen presentation. They have a unique ability to capture, process, and present antigens to T cells (Nussenzweig and Steinman 1980), directly activate B cells (Avery et al. 2003) and are also involved in the activation NK cells (Ferlazzo et al. 2002). Additionally, DCs are involved in tolerance development in the thymus by negative selection of autoreactive lymphocytes (Brocker et al. 1997) and in the periphery, where they present self-antigens in the absence of inflammation (Steinman and Nussenzweig 2002). This entire range of DC functions have been associated with their capacity to recognize pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively) through pattern recognition receptors (PRRs) (Iwasaki and Medzhitov 2015).

Evidence that malaria parasites interact with DCs to promote inflammatory responses is limited and controversial. Urban and colleagues showed that intact malaria-infected erythrocytes; specifically, PfEMP1(discussed in the immune evasion section) adhere to dendritic cells via CD36 or CD51, inhibit the maturation of dendritic cells and subsequently reduce their capacity to stimulate T cells (Urban et al. 1999). Conversely, Elliot and colleagues showed that DC modulation does not require CD36 binding, PfEMP1, or contact between DCs and infected RBCs but depends on the iRBC dose. The iRBCs expressing a PfEMP1 variant that binds chondroitin sulfate A (CSA) but not CD36 were phagocytosed. In addition there was inhibition of lipopolysaccharide (LPS)-induced phenotypic maturation and cytokine secretion, and abrogation of the ability of DCs to stimulate allogeneic T-cell proliferation (Elliott et al. 2007). The possible reasons for such contradictions could include the use of different parasite species and stages, and also differences in the DC activation status (Amorim et al. 2016).

γδ T cells. Human T cells express two different types of antigen T-cell receptors (TCRs) in a molecular complex with CD3 (Brenner et al. 1986). Most mature T cells express the $\alpha\beta$ TCR, which recognizes antigens in association with major histocompatibility complex antigens (Davis and Bjorkman 1988) and is responsible for both regulatory functions and cell-mediated immune responses. A small subpopulation of T cells expresses the $\gamma\delta$ TCR, which can recognize an antigen in an MHC-restricted or non-MHC-restricted manner (Meuer et al. 1983). In healthy human adults, the majority of γδ T cells in peripheral blood are Vγ9V δ2$^+$ T cells, representing between 1-10% of circulating lymphocytes. These cells can be found as the minority in the gut, liver and other peripheral tissues, whereas V δ1$^+$ γ δ cells are present at higher frequencies at these sites (Pang et al. 2012). Until recently, it was thought that γδ T cells were simply innate immune T cells with limited or somewhat redundant functions. The current view is that these cells complement many different arms of the innate immune system (Hayday and Pennington 2007) and, it is becoming clear that they are heterogeneous populations of cells with important unique roles in many infections, autoimmune diseases, allergies and in immunoregulation (Deroost and Langhorne 2018). To date, several studies have investigated the role of these cells during malaria infections. These studies, done both *in vivo* and *in vitro,* suggest that activation of γδ T cells during malaria infection contributes to parasite clearance while others argue that they could be responsible for immune-mediated pathology **Table 1.1** (Deroost and Langhorne 2018).

Table 1.1 γδ T cells in human and mouse malaria

	Human	Mouse
Known γδ T subsets expanded during *Plasmodium* infection in		
Skin (sporozoites entry)	Not known	Not known
Liver (liver stage infection)	Not known	Not known
Peripheral blood and lymphoid organs (blood-stage infection)	Vγ9Vδ2 (Ho et al. 1994; Roussilhon et al. 1994; Schwartz et al. 1996)	Vγ1 (Mamedov et al. 2018), Vγ2, Vγ4 (Taniguchi et al. 2007)
γδ T cell expanded by		
Irradiated / Live sporozoites	Yes (Seder et al. 2013) (Zaidi et al. 2017)	Yes (Zaidi et al. 2017)
Liver-stage parasites	Not known	Not known
Blood-stage parasites	Yes- *in vitro* by *P. falciparum* (Goodier et al. 1995)	Yes- *in vivo* (Inoue et al. 2017)
Potential effector functions against		
Sporozoites	Not known	Not known
Liver-stages	Not known	Not known
Blood-stages	Vδ1, Vγ9Vδ2: degranulation and granulysin decrease *P. falciparum* replication *in vitro* (Costa et al. 2011)	IFNγ- production during blood stage infection (Seixas and Langhorne 1999)
Protective against infection by		
Sporozoites	Vγ9V δ2 cell expansion associated with protection in irradiated sporozoite vaccination (Seder et al. 2013)	Yes – by recruitment of CD8α+ dendritic cells which cross-present to effector CD8+ T cells (Zaidi et al. 2017)
Blood stage	Not known	Vγ1 γδ T cells and M-CSF protect against chronic *P. chabaudi* (Mamedov et al. 2018)

Table modified from (Deroost and Langhorne 2018)

Natural Killer cells. NK cells represent an early source of IFN-γ during primary murine malaria infections and NK depletion using an anti-NK1.1 monoclonal antibody leads to higher parasitemia and mortality (Kitaguchi et al. 1996). *In vitro,* human NK cells form stable conjugates with iRBCs but not with uninfected RBCs and the induction of IFN-γ synthesis is dependent on direct contact between the NK cell and the iRBC. NK cells

respond to iRBCs only in the presence of a source of IL-12/IL-18, and the subset of NK cells that preferentially respond to iRBCs expresses high levels of the lectin-like receptor CD94/NKG2A (Artavanis-Tsakonas et al. 2003). Recently, it has been shown that, in addition to IFN-γ, NK cells can also produce perforin and granzyme against parasite iRBCs (Korbel et al. 2005).

1.10.2 Innate immunity to malaria: genetic mutations that confer resistance

Malaria parasites have co-evolved together with the human host for many years (Jongwutiwes et al. 2005; Kwiatkowski 2005). This has resulted in the selection or fixation of inherited traits within the human population that confer survival advantages against malaria, as evidenced by their high frequencies in malaria-endemic regions (Kwiatkowski 2005). Host-genetic factors alone have been shown to account for approximately 25% of the variation in protection seen with malaria in Africa (Mackinnon et al. 2005). To date, some of the best-described malaria-protective polymorphisms relate to genes that affect the structure or function of the RBCs (Williams et al.2006). These hereditary red cell disorders are broadly classified into three groups: the haemoglobinopathies (structural and functional aberrations of haemoglobin), red cell membrane proteins and red cell enzymes **Table 1.2** (Williams et al. 2006). In addition to determining these genetic disorders and their frequencies, great strides have also been made in understanding their mechanisms of action in order to find new approaches for the prevention and treatment of malaria **Table 1.2**.

Table 1.2 Genetic traits that affect immunity to malaria

Component	Mechanism	References
Membrane proteins		
Gerbich blood group negativity	Protection against EBA-140 mediated invasion by *P. falciparum* parasites	(Thompson et al. 2001) (Maier et al. 2003)
Southeast Asian ovalocytosis	Resistance to invasion by a subset of virulent *P. falciparum* parasites (Cortes et al. 2004) Increased adhesion of *P. falciparum* infected ovalocytes to CD36 (Rowe et al. 2009)	
Complement receptor 1 (CR1)	Reduced ability of *P. falciparum* infected CR-deficient red blood cells to form rosettes	(Cockburn et al. 2004a)
Red blood cells enzymes		
Glucose-6-phosphate dehydrogenase deficiency	Accelerated removal of ring-stage infected G6PD-deficient red blood cells through improved opsono-phagocytosis	(Ayi et al. 2004)
Pyruvate kinase deficiency	Major determinant of resistance to *P. chabaudi* in the mouse system. No confirmatory evidence yet available in humans	(Min-Oo et al. 2003)
Haemoglobinopathies		
Haemoglobin C (HbC)	Greatest degree of protection seen in homozygotes (Modiano et al. 2001; Mockenhaupt et al. 2004) Reduced surface expression of PfEMP1 in HbCC homozygotes(Fairhurst et al. 2003, 2005)	
Haemoglobin S (HbS)	Accelerated removal of ring-stage infected HbAS red blood cells through improved opsono-phagocytosis. (Ayi et al. 2004) Improved acquisition of malaria-specific immunity in HbAS children (Marsh et al.; Cabrera et al. 2005; Williams et al. 2005)	
Haemoglobin E (HbE)	Reduced invasion of HbAE red blood cells by *P. falciparum* parasites *in vitro*(Chotivanich et al. 2002)	

Table from (Williams 2006)

1.10.3 Naturally acquired immunity to malaria

More than 110 years ago, Robert Koch demonstrated that individuals living in malaria-endemic areas develop naturally acquire immunity (NAI) to malaria. Koch based his observations on microscopically detectable parasitemia in children, adults and trans migrants in highly endemic areas of Papua New Guinea and Indonesia where people were exposed to many infectious bites each year. By observing higher rates of parasitemia in exposed adults and malaria symptoms more prevalent in children, he concluded that immunity develops slowly after many years of exposure, but that sterile immunity was never really achieved (Ewers, 1972). Years later, the essential features of NAI was been described. It is now generally accepted that natural immunity to malaria was (i) effective in adults after uninterrupted lifelong continuous exposure, (ii) lost upon cessation of exposure, (iii) species-specific, (iv) somewhat stage-specific, and (v) acquired at the rate which was dependent upon the degree of exposure (Schwartz et al. 2001; Doolan et al. 2009). The incomplete understanding of how NAI to malaria is achieved and maintained remains a significant hurdle in progress towards the development of an efficacious malaria vaccine.

1.10.3.1 Antibodies as important mediators of naturally acquired immunity

Antibodies are known to be important components of naturally acquired immunity to malaria. The strongest evidence came from Cohen *et a*l. in which passive transfer of purified gamma immunoglobulins from immune African adults to children admitted with clinical malaria was shown to significantly reduce parasitemia and led to the resolution of fever (Cohen et al. 1961). Serum antibodies, which are made by plasma cells, mediate protection by acting predominantly against parasites of the asexual blood-stages that cause the clinical symptoms of malaria (Fowkes et al. 2016). However, a knowledge gap remains regarding which specific antigens induce protective immunity,

and which antibody effector functions are the most important in protection from disease.

Prospective population-based immuno-epidemiological studies have been useful in assessing the temporal association between the presence of antibodies and subsequent association with disease (Osier et al. 2007) offering insights into targets of protective immunity. In a systemic review of longitudinal studies, the presence of IgG antibodies against vaccine candidates such as $MSP1_{19}$, MSP3 and apical membrane antigen 1 (AMA1), was associated with protection from symptomatic malaria (Fowkes et al. 2010). Other studies have also suggested that members of the *P. falciparum* reticulocyte-binding homologues (PfRHs) and erythrocyte binding families (EBAs) would be potential targets (Richards et al. 2013; Weiss et al. 2015; Beeson et al. 2016). Noteworthy, most of these studies have only evaluated these antigens within a limited geographical area. Future studies, done across multiple countries/sites in sub-Saharan Africa with differing malaria transmission, may accelerate the rational selection and subsequent validation of antigen targets.

Furthermore, the effector mechanisms that are important for immunity are largely unknown. However, studies suggest that some of these effector functions include antibody-dependent cellular killing mediated by cytophilic antibodies, binding of antibodies to parasite-induced molecules on the surface of RBCs leading to clearance of iRBCs, antibody blockage of RBC invasion by merozoites as well as antibody-induced complement-mediated lysis **Figure 1.6**. Of note, the relative importance of each mechanism remains unclear as the results of different studies have been inconsistent (Langhorne et al. 2008; Duncan et al. 2014; Blackman et al. 1990; Boyle et al. 2015). For instance, there is inconsistency across studies of naturally acquired and vaccine-induced immunity on whether growth inhibitory antibodies are associated with protection or not (Duncan et al. 2014). Differences in methodologies do not seem

to comprehensively explain this variability (Dent et al. 2008) suggesting that other mechanisms may be involved.
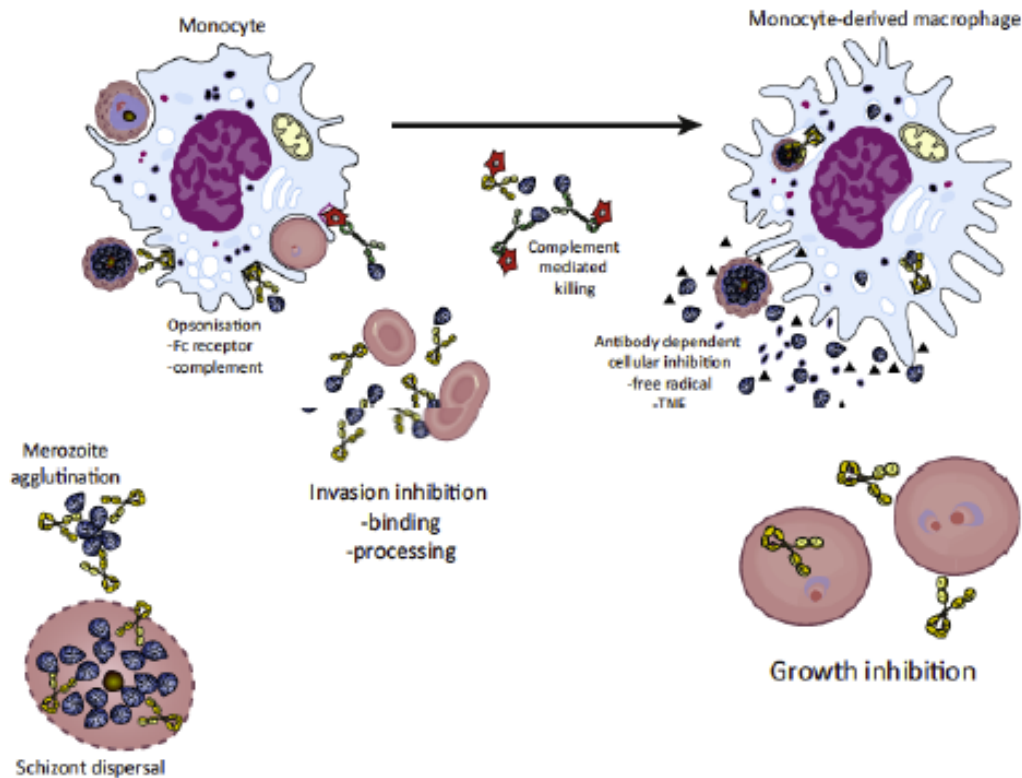
Figure 1.6 Antibody-mediated effector functions in immunity to *P. falciparum* malaria.

Antibodies against merozoites can prevent invasion of erythrocytes, inhibit schizont rupture (growth inhibitory antibodies), fix complement leading to merozoite lysis, opsonize merozoites for uptake by phagocytic cells (opsonic phagocytosis), and stimulate monocytes and macrophages to release molecules that kill parasites (antibody-dependent cellular inhibition). Abbreviations: TNF, tumor necrosis factor. Figure modified from (Teo et al. 2016).

1.11 *Plasmodium falciparum* immune evasion mechanisms

Malaria parasites have evolved to acquire strategies that evoke poor immune responses and allow an infection to be established even in previously exposed individuals (Hisaeda et al. 2005). While some of these mechanisms are essential for

parasite pathogenesis, they have hindered efforts to generate an effective malaria vaccine. Below is a brief discussion of stage-specific evasion mechanisms.

1.11.1 Immune evasion strategies during the pre-erythrocytic stages

The first barrier that sporozoites encounter upon transmission to the host is the skin. Thus, the sporozoites have evolved to overcome this barrier by various mechanical strategies such as motility and cell traversal (Tavares et al. 2013). Sporozoites are equipped with specialized mechanical proteins that help them achieve successful passage before reaching the liver. The sporozoite microneme protein essential for cell traversal (SPECT-1) and perforin-like protein 1 (PLP1) are critical for *Plasmodium falciparum* sporozoite cell traversal and migration to the liver (Sinnis and Zavala 2012). The thrombospondin-related anonymous protein (TRAP) is another protein important for motility of sporozoites and it functions to allow the parasite to interact with host surface molecules which is critical for gliding motility to exit the dermis. It can also bind to sulfated glycoconjugate motifs that aid in the recognition and entry of hepatocytes (Müller et al. 1993).

To invade hepatocytes, sporozoites cross a barrier lined with endothelial cells (ECs) and Kupffer cells (KCs) (Frevert et al. 2005; Tavares et al. 2013). To overcome this barrier, the circumsporozoite protein (CSP) interacts with low-density lipoprotein receptor-related protein 1 (LRP-1) and proteoglycans of KCs, which increases the level of cAMP/EPAC and prevents the formation of reactive oxygen species (ROS). The ROS are a natural by-product produced during environmental stress and can cause cellular damage or parasite killing (Ikarashi et al. 2013). In some cases, the binding of sporozoites can induce KC apoptosis and reduce expression of major histocompatibility complex (MHC)-1 which results in the induction of T cell tolerance (Ikarashi et al. 2013; Belachew 2018).

1.11.2 Immune evasion strategies during the intraerythrocytic stage

In most instances, immune evasion by intraerythrocytic parasites is the result of (1) antigenic variation, (2) sequestration and cytoadherence or (3) rosetting.

1.11.2.1 Antigenic variation

Antigenic variation is the expression of variable and distinct proteins at different life-cycle stages of the parasites which changes the proteins exposed to and recognized by the immune system. This enables the parasite to evade immune clearance and establish chronic infections. Antigenic diversity has been shown to occur as a result of at least two mechanisms: 1) the presence of multicopy gene families encoding variant surface antigens (VSA), and 2) the presence of polymorphic alleles in the parasite population. While the former is prominent in studies of the infected red cell surface antigens, the latter is better studied for merozoites (Dinko and Pradel 2016).

Variant surface antigens (VSA) are encoded by many diverse multigene families whose variants are exposed on the surface iRBCs (Su et al. 1995). Three major VSA multigene families have been identified in *P. falciparum*: *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by the *var* genes (Smith et al. 1995; Su et al. 1995), repetitive interspersed families of polypeptides (RIFINs) encoded by the *rif* genes and subtelomeric variant open reading frame proteins (STEVOR) encoded by the *stevor* genes (Cheng et al. 1998). Among these families, the *var* genes are the most extensively studied and have been shown to be important in the pathogenesis of malaria. There are about 60 distinct copies of *var* in the *P. falciparum* 3D7 reference clone (Gardner et al. 2002). The expression of *var* is monoallelic with only a single *var* being transcribed and expressed by a single parasite while the remaining *var* genes are maintained in a transcriptionally silent state (Chen et al. 1998; Voss et al. 2006; Scherf et al. 2008). Also, the parasites can switch the expression of *var* genes leading

to different PfEMP1 molecules within a clonal population. While each variant of PfEMP1 appears to be a major target of human acquired humoral immunity (Bull et al. 1998; Chan et al. 2012; Bull and Abdi 2015), the clonal switching of *var* genes enables the parasite to evade the host immune response that has been already acquired against a previously surface expressed P*f*EMP1 variant **Figure 1.7** (Bull and Marsh 2002). The other multi-gene families, *rif* and *stevor* genes, are also involved in antigenic variation and immune evasion (Dinko and Pradel 2016) through switching of their gene expression.

Antigenic diversity/ polymorphism involves the expression of antigenically different alleles of a gene in different parasite populations and is thought to arise due to immune selection pressure. In the presence of specific immunity, parasites expressing mutated alleles that are not recognized by the immune system would selectively expand. In most instances, antigens eliciting immune responses often show extensive polymorphism (Hisaeda et al. 2005). For example, merozoite surface protein-1 (MSP-1) has many alleles (Qari et al. 1998), and some antibodies to one MSP-1 allele do not recognize the others (Ekala et al. 2002). Also, apical membrane antigen 1 (AMA-1) is known to elicit a robust immune response (Osier et al. 2008), although it has numerous distinct haplotypes (Polley et al. 2003). Thus, the polymorphic nature of many malaria antigens poses challenges for vaccine development.

1.11.2.2 Sequestration and cytoadherence

The spleen is essential for the identification and clearance of old and abnormal RBCs and therefore represents a severe challenge for circulating iRBC. To avoid the spleen, *P. falciparum* has developed an immune evasion strategy whereby the parasite iRBCs adhere to human endothelium cells thereby sequestering in the microvasculature of various organs, a process called cytoadherence (Yam and Preiser 2017).

Sequestration can occur in the heart, lung, brain, liver, kidney, subcutaneous tissues and placenta. The various endothelial cells in these organs and syncytiotrophoblasts in the placenta express different and variable amounts of the host receptor. To successfully adhere to these cells, the parasites can bind to several receptors **Figure 1.7**. The adhesion phenotype is not homogenous, and different parasites can bind to variable numbers and combinations of host receptors (Newbold et al. 1997). This variability is believed to affect the tissue distribution and pathogenesis of parasites. For example, PfEMP1, which is expressed on the surface of infected erythrocytes, mediates parasite binding to various receptors through multiple adhesion domains (Newbold et al. 1997; Chen et al. 2000b).

It is important to note that the binding to host receptors does not necessarily lead to pathogenesis. Indeed compared to infection and mild disease, severe complications and deaths are rare (Snow and Marsh 1998). It remains unclear what causes the transition from uncomplicated to severe forms of malaria. One possibility is that the expression of certain binding properties leads to distinct patterns of sequestration and to pathogenic consequences (Miller et al. 2002). An example is the sequestration of iRBCs in the placenta, which causes premature delivery, as well as low birth weight. Parasitized RBCs isolated from placentas have a unique adhesion property that is different from parasites collected from non-pregnant women. These parasites can bind to CSA but not to CD36 (Fried and Duffy 1996; Beeson et al. 1999).

1.11.2.3 Rosetting

Rosetting is a phenomenon where an iRBC binds to many uninfected RBCs. Rosetting is thought to protect iRBCs from the host immune response while at the same time protecting the newly released merozoites from host invasion-inhibitory antibodies. It also provides a favorable environment for the emerging merozoites enabling them to

rapidly invade the bound uninfected RBCs (Wahlgren et al. 1989; Deans and Rowe 2006). Most VSAs have been shown to be mediators of rosetting including P*f*EMP1 **(Figure 7)** (Rowe et al. 1997), STEVOR (Niang et al. 2014) and RIFINs (Goel et al. 2015). Although the role of rosetting remains unclear, there is evidence to suggest that it would be important in the pathogenesis of severe malaria. *P. falciparum* field isolates have been shown to vary in the extent to which they form rosettes with higher levels of rosette formation being strongly associated with severe malaria in numerous studies in African children (Carlson and Wahlgren 1992; Ringwald et al. 1993; Rowe et al. 1995). A polymorphism associated with deficiency of the red blood cell rosetting receptor CR1 that reduces the ability to form rosettes (Rowe et al. 1997) has also been shown to confer protection from severe malaria (Cockburn *et al.*, 2004).
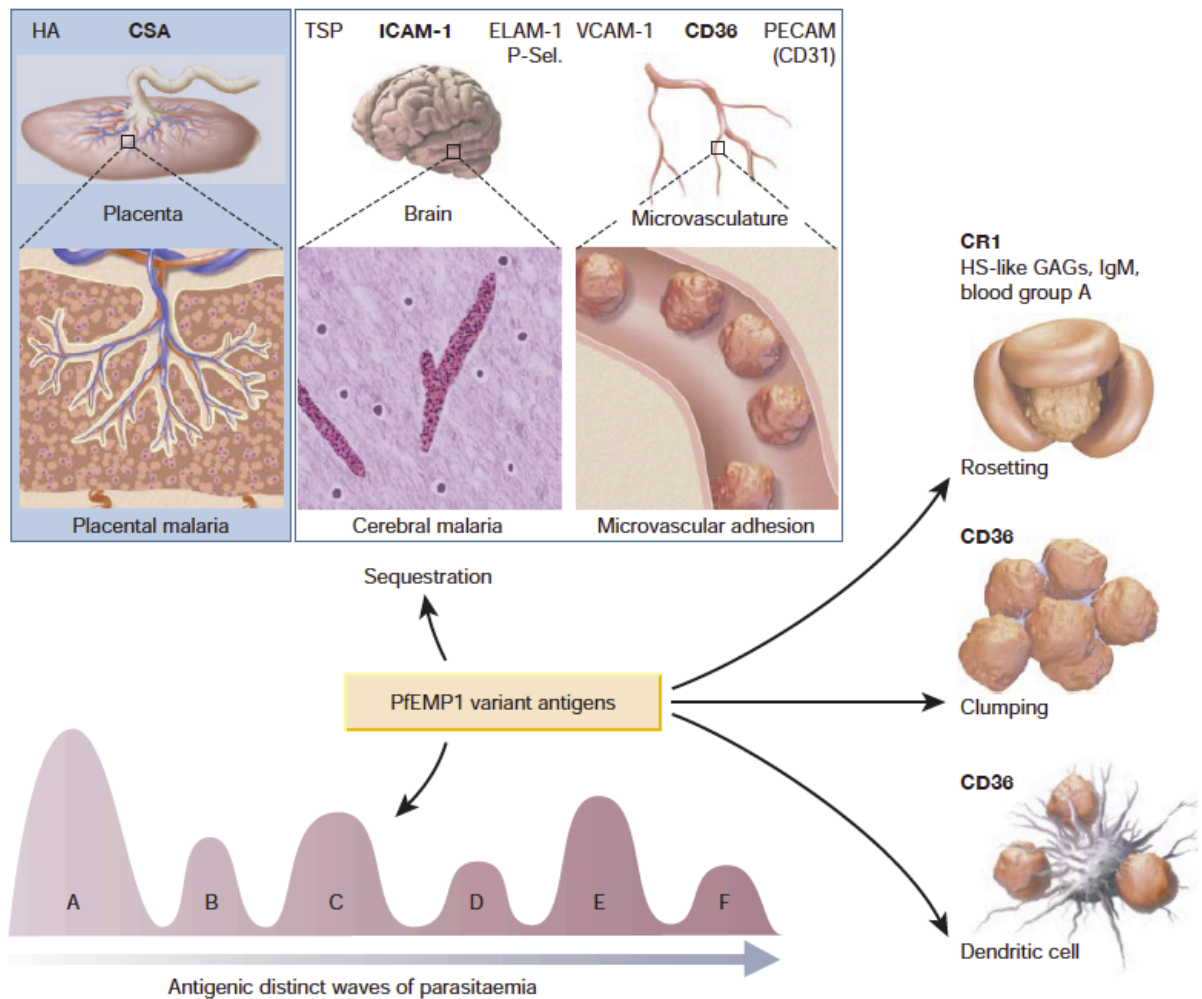
Figure 1.7 PfEMP1 expressed on the surface of P. falciparum-infected red blood cells is involved in clonal antigenic variation and can bind to many host receptors through its multiple adhesion domains.

Sequestration of the parasites in the brain and placenta are thought to contribute to the development of cerebral malaria and placental malaria respectively. Additionally, binding of uninfected erythrocytes (rosetting), and clumping of infected erythrocytes through platelets are also associated with the pathogenesis of malaria. Parasite-infected RBC binding to dendritic cells is thought to contribute to downregulation of the immune response. HA, hyaluronic acid; TSP, thrombospondin; ELAM-1, endothelial/leukocyte adhesion molecule 1; P-Sel., P-selectin; VCAM-1, vascular cell adhesion molecule 1; PECAM (CD31), platelet endothelial cell adhesion molecule 1; CR1, complement receptor 1; HS-like GAGs, heparin sulphate-like glycosaminoglycans; IgM, immunoglobulin M. Figure from (Miller et al. 2002)

1.11.3 Immune evasion by merozoites

The mechanisms used by the merozoites to evade immunity include extensive diversity as well as complexity in the number of proteins that are involved in the invasion process, also referred to as redundancy of the invasion pathways (Dinko and Pradel 2016). Antigenic diversity/polymorphism has been discussed briefly above (on antigenic variation **section 1.11.2.1**). Two major merozoite invasion ligands have been described: 1) merozoite surface proteins (MSPs) (Boyle et al. 2010a), and 2) alternative pathway ligands, so-called due to their functional redundancy. The latter include two major protein families of reticulocyte binding homologues (PfRh2a, PfRH2b and PfRh4) and erythrocytes-binding like homologues (EBA-175, EBA-140, EBA-181 and EBL1) (Galinski et al. 1992; Adams et al. 2001; Tham et al. 2012). These proteins bind to receptors on the red blood cell surface. While some ligands have known receptors, for others, their respective receptors are yet to be determined **Figure 1.8**. The redundancy displayed by the EBL/PfRh proteins allows parasites to modify their usage of ligands, conferring a broad host range and adaptability to polymorphisms on the red blood cells (Harvey et al. 2012). Another mechanism that has recently been described for merozoite immune evasion is the hijacking of complement regulatory protein factor H (FH). FH functions to protect host cells from complement activation (Zipfel et al. 2002). By recruiting FH the parasite is able to disguise itself as host and hence gets protected from complement attack (Kennedy et al. 2016).
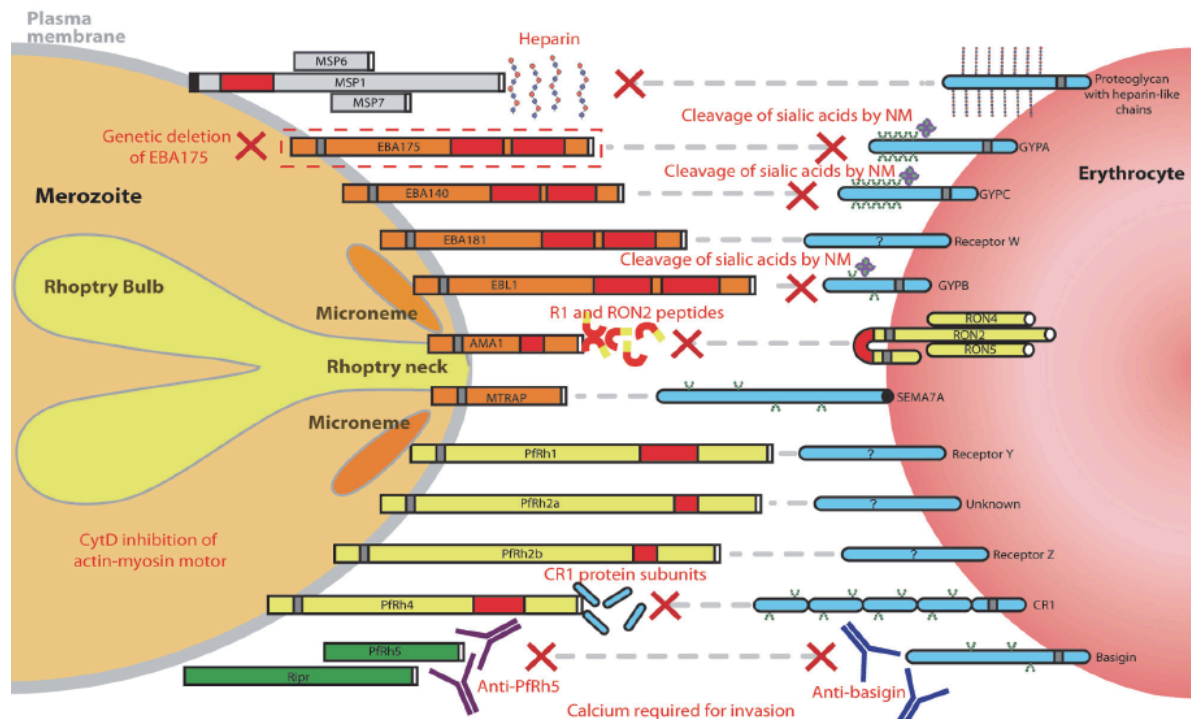
Figure 1.8 Merozoite ligands, their receptors, and methods that have so far been used to ablate their interactions.

1) heparin sulphate blocks MSP1$_{42}$ binding to unknown erythrocyte glycoproteins, 2) treatment with neuraminidase (NM) removes sialic acids on glycophorin A (GYPA) hence preventing binding of EBAs, 3) genetic deletion of EBA175, NM treatment and complement receptor 1 (CR1) fragments inhibits PfRh4 binding to CR1, 4) anti-PfRh5 and anti-basigin IgGs blocks the binding of PfRh5 to basigin, and 5) peptides of R1 and RON2 inhibits AMA1–RON2 interactions, figure from (Weiss et al. 2015).

1.12 The complement system

Complement was originally discovered in the 1880s by scientists Nuttal and Buchner. They observed that blood depleted of white blood cells was still capable of killing infecting bacteria (Buchner et al.1894; Nuttall et al. 1988). At that time, complement was termed "Alexin" from the Greek meaning to "ward off." Thus, along with phagocytic cells, complement was one of the first components identified in the innate immune system. Within a few years of its discovery, the term "Alexin" was changed to complement when Border (a Nobel Prize winner) showed that it could "complement"

the bactericidal activity of serum antibodies (Bordet et al. 1899; Bordet et al. 1900) and the term complement was thereafter universally adopted.

The complexity of complement as we now know it with multiple proteins (over 50 proteins) involved in the three major pathways was unraveled slowly with the advancement of technology. For example, improvement in molecular technology provided insight into the role of complement outside the traditional definition of complement as merely "the first line of defense against invading pathogens." There is now plenty of data to suggest that complement is involved in normal homeostatic processes including the mobilization of hematopoietic cells, tissue regeneration, and clearance of immune complexes. Apart from these, complement has been shown to play an essential role in B cell activation and antibody production and regulation of T cell responses (Müller-Eberhard 1988; Lachmann 2006; Carroll 2008; Dunkelberger and Song 2010; Mastellos et al. 2013; Phieler et al. 2013; Morgan and Harris 2015; Sim et al. 2016). Moreover, it is now known that there is crosstalk between the complement system and some cell effector systems (Kolev et al. 2014)

1.12.1 Complement pathways

Three major complement pathways exist: the classical pathway, the lectin pathway and the alternative pathway **Figure 1.9**. All three share the common step of activating the central component C3, but they differ according to the nature of recognition. They ultimately lead to the formation of the membrane attack complex which is responsible for the lysis of invading pathogens **Figure 1.9**.

1.12.1.1 Classical pathway

The classical complement pathway is triggered by the recognition of antibody-antigen complexes on foreign cell surfaces. Initiation of the pathway occurs when C1q, in

complex with C1r and C1s serine proteases (the C1 complex), binds to the Fc region of complement-fixing antibodies (mostly IgG1 and IgM) attached to pathogenic surfaces (Dunkelberger and Song 2010). During the last two decades it became clear that, in addition to IgG and IgM, C1q also recognizes a variety of structurally different self- and non-self-target molecules, such as C-reactive protein (CRP), pentraxin 3 (PTX3), bacterial porins (e.g. OmpK36 from Klebsiella pneumoniae), a large number of extracellular membrane-associated receptors, among others (Kishore et al. 2004; Ghai et al. 2007; Kojouharova et al. 2010). Autocatalytic activation of C1r and C1s, in turn, cleaves C4 and C2 into larger (C4b, C2a) and smaller (C4a, C2b) fragments. The larger fragments associate to form C4bC2a on pathogenic surfaces and this complex is termed the C3 convertase (Dunkelberger and Song 2010). C3 convertase cleaves C3 into anaphylatoxin C3a and the opsonin C3b. In addition, C3b complexes with the C3 convertases to form the C5 convertases (C4bC2aC3b). The C5 convertases then cleave C5 to form C5a and C5b. The membrane attack complex (C5b-9, MAC) also termed as the terminal complement complex (TCC) is then initiated by C6 and C7 binding to C5b and then C8 and multiple molecules of C9 binding to the C5bC6C7 complex. The MAC complex forms a pore by inserting itself into cell membranes, resulting in cell lysis (Dunkelberger and Song 2010; Sarma et al. 2011).

1.12.1.2 Lectin pathway

The lectin pathway uses germline-encoded pattern recognition receptors (PPRs), such as mannose-binding lectin (MBL) and ficolins, for pathogen recognition. MBL is structurally similar to the classical pathway recognition protein C1q. However, MBL can form carbohydrate-recognition domains and binds N-acetyl glucosamine and mannose, which are common among invading pathogens. Ficolins like MBL recognizes N-acetyl glucosamine and mannose structures. Both MBL and ficolins are associated with serine proteases, which activate C2 and C4 leading to the formation

of central lectin and classical pathway C3 convertase C4bC2a (Carroll 2004). The subsequent steps are the same as described for the classical pathway (Degn et al. 2007).

1.12.1.3 Alternative pathway

The alternative pathway (AP) is able, in a robust manner, to discriminate normal human cells from microbes and damaged or transformed human cells in an antibody-independent manner. Another critical feature of the AP is that it can amplify its activation regardless of which pathway was initiated (Meri 2016). The AP is initiated by low-level, spontaneous hydrolysis of C3 to the C3b analog, $C3(H_2O)$ which binds to Factor B and in turn allows cleavage of Factor B into Bb and Ba in the presence of Factor D and hence form the alternative C3 convertase, $C3(H_2O)$ Bb. The formation of C3 convertase is the basis of an amplification loop by which C3 is broken down to C3b and C3a, similar to the C3 convertase (C4bC2a) of the classical and the lectin pathways. C3b triggered in such a manner can bind to surfaces nearby and associate with Factor B, which can, in turn, be activated by Factor D to form C3bBb, the predominant alternative pathway C3 convertase. As a result of the continuous activation and the ability to form a feedback amplification pathway, there are many regulatory proteins which function to confine complement activation to appropriate contexts to prevent unintended complement activation in healthy cells (Dunkelberger and Song 2010) discussed in more details on the complement regulation **section 1.12.2**. The AP C5 convertase is formed by the association of a second C3b molecule to the AP C3 convertase. The subsequent steps leading ultimately to the formation of MAC are identical to the other pathways.

1.12.2 Complement regulation

The complement system is tightly controlled by fluid phase regulators (e.g. factor H, FHL-1, C4b binding protein, and C1 inhibitor) and also on cell membranes (e.g., by CR1/CD35, MCP/CD46, DAF/CD55, and protectin/CD59) to prevent inappropriate complement activation and cell destruction **Figure 1.9** (Merle et al. 2015; Hovingh et al. 2016)**.** Complement receptor type 1 (CR1/CD35), membrane cofactor protein (MCP/CD46), decay-accelerating factor (DAF/CD55) and CRIg inhibit complement activation at the C3 convertase level. Protectin/CD59 prevents complement activation by binding to C8 and C9, thereby preventing C9 from polymerizing and forming the MAC (Farkas et al. 2002; Zipfel and Skerka 2009; Ricklin et al. 2010; Thielen et al. 2018). Of the soluble regulators, C1-inhibitor (C1-INH) and C4b binding protein (C4BP) inhibit the initial steps of the classical pathway while clusterin and vitronectin inhibit the terminal pathway. While factor H and Factor H-like 1 (FHL-1), an alternatively spliced product of the factor H gene, regulate the amplification loop of the alternative pathway (Meri et al. 2008).
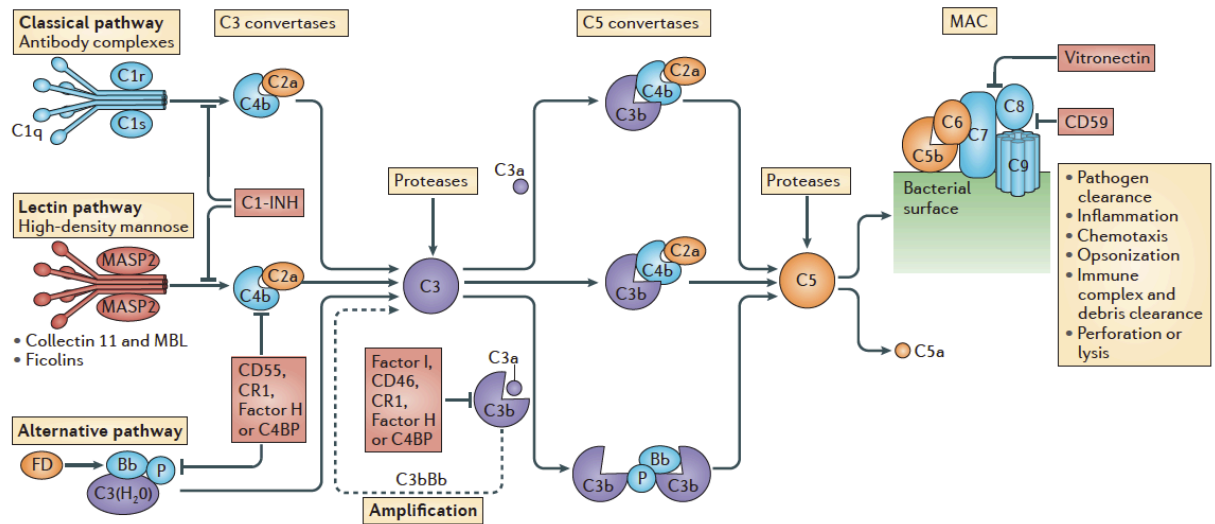
Figure 1.9 The complement system activation and regulation.

The complement system forms the first line of defense against invading pathogens. It can be activated through three major pathways: the classical pathway, the lectin pathway, and the alternative pathway. Upon activation there is the initial deposition of C3b on a surface which can also initiate a feedback amplification loop. Through the formation of C3 convertases, these pathways culminate in the formation of the opsonin C3b and the anaphylatoxin C3a. Subsequent C5 convertase formation leads to C5b and anaphylatoxin C5a generation, with C5b initiating the formation of the membrane attack complex (MAC) or also called terminal complement complex (TCC) which is inserted into target membranes. Host tissues and cells are protected from complement deposition through fluid-phase and cell-bound regulators. C1 inhibitor (C1-INH) inhibits the functions of C1r, C1s, and mannan-binding lectin-associated serine protease 2 (MASP2). C3b (and C4b) are inactivated by complement Factor I and one of several cofactor proteins (surface-bound CD46 and complement receptor type 1 (CR1) or fluid phase Factor H and C4b-binding protein (C4BP). Convertases are regulated through disassembly by regulators that have decay-accelerating activity, surface-bound CD55 and CR1 or fluid-phase Factor H and C4BP, and the formation of the MAC is controlled by the activities of CD59 and vitronectin. Figure from (Kolev et al. 2014).

1.12.3 Complement factor H/CFHR gene family

Factor H is encoded by a single gene (*HF1*) located on human chromosome 1q32 within the regulators of complement activation (RCA) gene cluster. *HF1* is closely related to the *FHR1, FHR2, FHR3, FHR4* and *FHR5* genes encoding five complement

factor H-related human plasma (FHR) proteins **Figure 1.10** (Rodríguez De Córdoba et al. 2004). The *FHR1-5* genes originated from the *HF1* gene by tandem duplication events (Pérez-Caballero et al. 2001). More than 550 single nucleotide polymorphisms (SNPs) in the human *HF1* gene are known, that may sometimes lead to an amino acid substitution in the FH protein (De Córdoba and De Jorge 2007). Some of these mutations have been associated with increased risk for diseases such as renal disorders type II membranoprofilic glomerulonephiritis and atypical haemolytic-uraemic syndrome (aHUS) (Pickering and Cook 2008), and also age-related macular degeneration (Ansari et al. 2013). The RCA family also encodes complement receptor 1 (CR1 or CD35), decay accelerating factor (DAF or CD55), and membrane cofactor protein (MCP or CD46), which are expressed on the surface of host cells (Skerka et al. 2013).
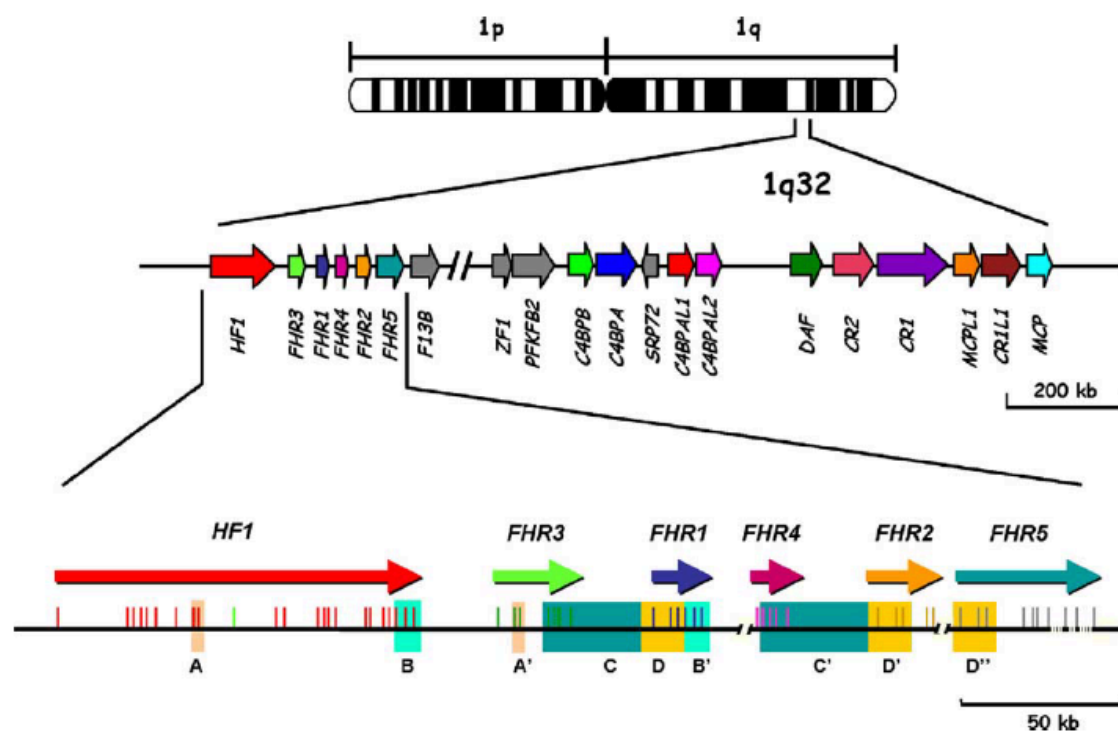
Figure 1.10 Factor H is a member of the regulators of complement activation (RCA) gene cluster.

The human RCA gene cluster is located on human chromosome 1q32 and includes more than 60 genes, of which 15 are complement-related. All of the complement-related genes are arranged in

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tandem within two groups. The two groupings are a telomeric DNA segment which contains the C4BPB, C4BPA, C4BPAL1, C4BPAL2, DAF, CR2, CR1, MCPL1, CR1L1 and MCP genes and a centromeric DNA segment that contains HF1, FHR3, FHR1, FHR4, FHR2 and FHR5, as well as the gene coding for the B subunit of the coagulation Factor XIII, F13B. These two gene groups are separated by DNA-containing genes that are unrelated to complement and that have diverse functions. The bottom part of the figure illustrates the existence within the RCA centromeric region of many large genomic duplications (A–D) including different exons of the HF1/FHR1–5 genes. Figure from (Rodríguez De Córdoba et al. 2004).

1.12.4 The factor H/FHR protein family

The FH/FHR proteins comprise repetitive units of approximately 60 amino acids named short consensus repeats (SCRs) or also called complement control proteins (CCPs), arranged continuously **Figure 1.11**. The C- terminal region (CCPs 18-20) and CCPs 6/7 of FH, harbouring the major surface recognition sites of human complement FH, are retained with differing degrees of conservation in all FHRs, explaining their ability to interact with most of the FH ligands, although with varying avidity, and immunological cross-reactivity (Zipfel et al. 1999; Józsi and Zipfel 2008; Skerka and Zipfel 2008). However, none of the FHRs contains regions similar to the FH CCPs 1-4, which has cast doubt on the conservation of the complement regulatory activities of FH in the FHRs.

The most striking characteristics of FHR-1, FHR-2, and FHR-5 is that the two N-terminal CCPs, which are almost identical in these FHRs, contains a dimerization domain (Goicoechea de Jorge et al. 2013). The dimerization domain means that they always circulate in the plasma as dimers or tetramers (Goicoechea de Jorge et al. 2013; Tortajada et al. 2013). Besides, FHR-1, FHR-2, and FHR-5 have higher affinity to surface-bound C3b, resulting in more efficient competition with factor H. For several of them the ability to competitively inhibit factor H binding to specific host ligands has

been demonstrated. Examples include surface-bound C3b and the pentraxins CRP and PTX3 (Goicoechea de Jorge et al. 2013; Chen et al. 2014; Csincsi et al. 2015, 2017). In addition, FHR-3 was shown to compete with factor H for a bacterial ligand, the fHbp protein of *Neisseria meningitides* (Caesar et al. 2014), and most recently, FHR1 has been shown to compete with FH for binding to the malaria parasite, *Plasmodium falciparum* merozoites and intra-erythrocytic schizonts (Reiss et al. 2018).

Generally, the circulating plasma levels of complement FH related proteins (FHR) are 100-fold lower than FH (Abarrategui-Garrido et al. 2009; Pouw et al. 2016). The competition *in vivo* between FH and FHR is thought to be as a result of similarities between these proteins; the conserved domains of FHR proteins are homologous to CCPs 6-9 and 19-20 of FH **Figure 1.11**. As a result of this similarity, they can bind to the same ligands, albeit to varying degrees (Józsi and Zipfel 2008). For example, FH and FHR3 bind to the same region in *N. meningitidis* fHbp and hence competition for binding between these two and their circulating levels can influence disease susceptibility (Caesar et al. 2014).

An alternative splice version of the FH gene encodes the Factor H-like 1 protein (FHL-1), which consists of FH CCPs1-7 plus four additional residues at its C-terminus. The main difference between FHL-1 and FH is that while FHL-1 regulates complement at the fluid phase using its CCP1-4 domain, FH is both a fluid phase and membrane regulator due to its additional CCP19-20 which functions in host recognition (Schmidt et al. 2016).
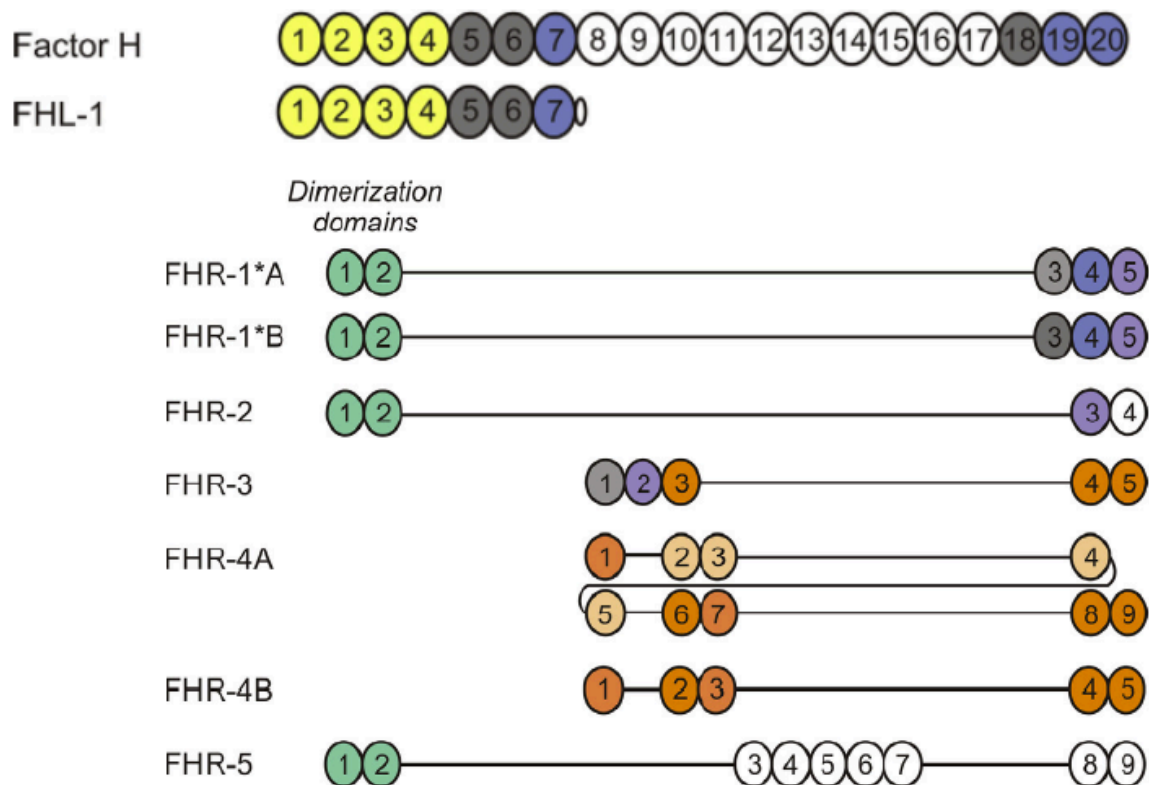
Figure 1.11 Schematic presentation of the human factor H family proteins.

Factor H consists of 20 complement control protein (CCP) domains, also called short consensus repeats (SCR). Factor H-like 1 (FHL-1) is identical with the CCPs 1–7 of factor H but has four unique amino acids at its C-terminus. The factor H-related (FHR) proteins are composed of 4–9 CCP domains, with varying degrees of amino acid sequence identity to specific factor H domains (same colors indicate a complete or very high degree of sequence identity). The CCPs 1-2 of FHR-1, FHR-2, and FHR-5 (shown in green) are closely related to each other and are responsible for dimerization of these FHRs. Due to the conservation of domains homologous to factor H CCPs 6-9 and 18-20 among the FHRs, FHRs can bind some of the ligands of factor H; thus, factor H and the FHRs can be argued to have partly overlapping ligand-binding profiles. GAGs, glycosaminoglycans; MDA, malondialdehyde; CRP, C-reactive protein; PTX3, pentraxin 3; CR, complement receptor. Figure modified from (Józsi et al. 2018).

1.12.5 FH as a complement regulator

The FH protein is a 155 kDa glycoprotein composed of 1213 amino acid residues (Ripoche et al. 1988) and is the primary central fluid phase regulator of the alternative pathway. It consists of 20 complement control protein (CCP) modules also called short

consensus repeats (SCR) **Figure 1.12**. Domains 1-4 of FH function to dissociate the C3 convertase (C3bBb), also called decay-accelerating activity. FH also acts as a co-factor for factor I-mediated cleavage and inactivation of formed C3b. Besides, factor H also can recognize specific markers on host cells and control complement on the cell surfaces using domains 6-8 and 19-20 **Figure 1.12**.

Continuous activation of the alternative pathway means that there is always deposition of C3b on host cells. Factor H detects and binds to C3b deposits in combination with specific polyanionic markers, primarily sialic acid, and sulfated polysaccharides such as heparin sulphate on host cells. The ability to detect C3b binding allows alternative pathway activation to proceed on any other surface to which factor H cannot bind efficiently due to lack of host or host-like markers (Pangburn 2002; Ferreira et al. 2010). The FH protein is mainly synthesized by the liver (Fagerberg et al. 2014). It is found in human plasma at highly variable concentrations which is thought to influence disease susceptibility (Esparza-Gordillo et al. 2004; Scholl et al. 2008). These variable concentrations appear to be a result of both environmental and genetic conditions (Esparza-Gordillo et al. 2004).

Previous studies have measured circulating FH levels both in healthy adults (Pouw et al. 2016) and children (Davis et al. 1979; Sonntag et al. 1998) and even during disease state (Ansari et al. 2013). What has been observed in many studies is that there is variation in FH levels across different populations. In a large study by Ansari et al. investigating the genetic influence on plasma FH and its role in age-related macular degeneration (AMD), they reported a three-fold variation in circulating FH levels mean, min-max (400.2, 218-654.1) ug/ml in their cross-sectional survey of Croatians (n=1004). In the same study, their Scottish AMD cases (n=382) had a four-fold variation in plasma FH levels, mean, min-max (412.6, 127.8-540.2) ug/ml (Ansari et al. 2013). In a Spanish population (n=358), plasma levels of FH showed a five-fold range

(116-562 ug/ml) (Esparza-Gordillo et al. 2004). Furthermore, a recent study by van Beek et al. measured FH levels in healthy Dutch children with a mean age 10.3 years observed a nearly three-fold variation in the levels, median 281ug/ml 95% CI (153.5-432.5 ug/ml).
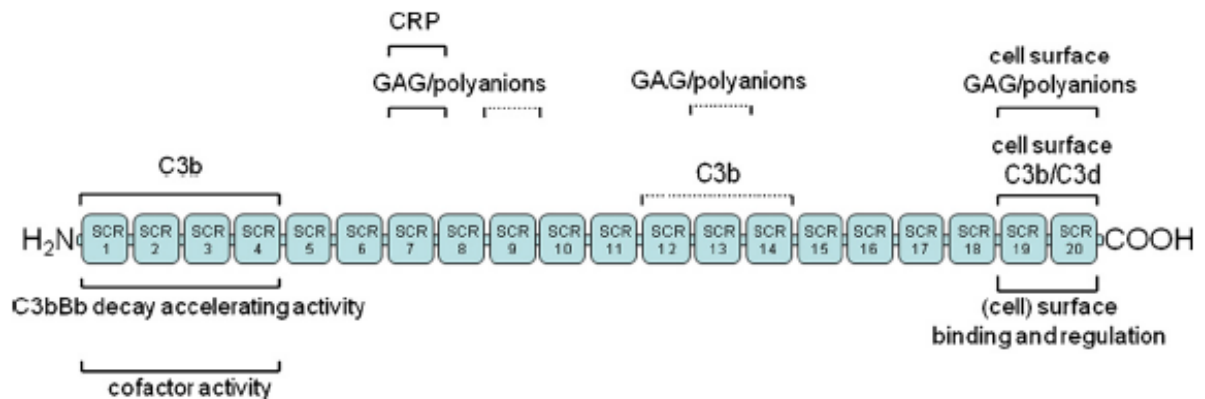
Figure 1.12 Functional domains of human complement factor H protein.

The complement FH protein consists of 20 short consensus repeats (SCRs) or complement control protein (CCP) modules that show a certain degree of similarity. However different clusters of SCRs have different functional properties as indicated in the figure. Abbreviations: CRP, C-reactive protein; GAG, glycosaminoglycans (Boon et al. 2009).

1.12.6 Complement evasion by pathogenic microbes

Pathogens exploit multiple strategies to interfere with complement immune recognition and effector functions in order to survive in the human host (Zipfel et al. 2007; Blom et al. 2009). They express diverse and multiple surface proteins and secrete additional molecules to avoid complement attack **Figure 1.13** (Zipfel et al. 2007). Commonly shared strategies include interference at the activation level, interference at C3 and C5 convertase level, and inactivation of the membrane attack complex (C5b-9). Additionally, multiple pathogens can interact with complement regulators such as FH to inhibit complement activation. These strategies are briefly discussed below:

1.12.6.1 Modulation at the complement activation level

Pathogenic microbes can influence complement activation (Zipfel et al. 2007). A notable example is an interaction between *Staphylococcal protein A* (*SpA*) and the Fc part of IgG. The *SpA* is a type I membrane protein that is bound to the cell wall of *Staphylococcus aureus* via its C-terminal cell-wall binding region X. In the N-terminal half of the protein are its IgG-binding domains E, D, A, B, and C. Through binding of IgG, protein A blocks Fc-receptor mediated phagocytosis and also interferes with binding of C1q (Goward et al. 1993). Interference at the early stage of complement activation is an evasion strategy as it leads to a blockage of downstream effects.

1.12.6.2 Interference at the C3 and C5 convertase level

Pathogenic microbes can influence the functioning of C3 and C5 convertases in multiple ways. For C3 convertase, they can block the enzyme directly, adjust enzyme activity, or modulate the strength of the amplification loop (Rooijakkers et al. 2005; Laarman et al. 2011). Examples include the immune evasion protein Sbi of *S. aureus* which forms a tripartite complex with C3b and Factor H and allows for tripartite complexed C3b processing and inactivation by the complement protease Factor I (Haupt et al. 2008). Another example is the *Staphylococcal complement inhibitor (SCIN).* SCIN is an excreted 9.8-kilodalton protein that blocks human complement by specific interaction with C4b2a and C3bBb. Staphylococcal complement inhibitor binds and stabilizes C3 convertases, interfering with additional C3b deposition leading to a considerable decrease in phagocytosis and killing of *Staphylococcus aureus* by human neutrophils (Rooijakkers et al. 2005).

Pathogens can also use proteases that cleave and degrade host C5. The staphylococcal SSL7 (staphylococcal superantigen-like protein 7) binds C5 and thereby blocks cleavage by the C5 convertase (Langley et al. 2005; Laursen et al.

2010).   Glyceraldehyde-3-phosphate   dehydrogenase   (GAPDH)   from *S. pyogenes* binds to C5a, and the inhibitory action is assisted by ScpA, the streptococcal C5a peptidase, which degrades C5a (Terao et al. 2006).

1.12.6.3 Interference at the terminal pathway

More pathogens control and exploit the terminal pathway of complement. The Gram-positive bacterium *S. pyogenes* expresses Streptococcal inhibitor of complement (SIC). SIC has been identified as a terminal complement pathway inhibitor since it binds the soluble C5b-7 complex and thereby prevents its insertion into the cell membrane (Fernie-King et al. 2001). *S. mansoni* uses schistosoma C inhibitory protein type 1 (SCIP-1) as a TCC inhibiting protein (Parizade et al. 1994). *Borrelia burgdorferi* confers resistance to the terminal pathway by an 80 kDa surface protein that shares both antigenic and functional similarities with human CD59, a natural membrane-bound inhibitor of MAC. Both CD59 and Borrelial CD59-like inhibit cell lysis by preventing the polymerisation of C9 and the formation of MAC. Despite its similarities, the CD59-like molecule exhibits many structural and functional differences from human CD59. The CD59-like molecule is much larger than CD59, indicating that it is not an acquired regulator.

Furthermore, CD59-like interacts with native C8 and C9 while human CD59 binds the same molecules only in the context of the assembling MAC. Inhibition of MAC formation by membrane-bound CD59-like represents an important survival mechanism in *B. burgdorferi* (Pausa et al. 2003). Some Gram-negative, *Haemophilus influenzae, Moraxella catarrhalis and P. aeruginosa* and Gram-positive bacteria *S. aureus, S. pneumoniae,* and *S. pyogenes,* as well as yeast *C. albicans* bind vitronectin, the soluble human TCC inhibitor (Singh et al. 2010). Vitronectin binds to

and blocks the membrane binding site of C5b-7 and the polymerization of C9 and thereby blocks TCC action.

1.12.6.4 Interactions with host regulators for complement immune evasion

Many bacterial evade complement attack by sequestration of human complement regulators to their surface. Well-described examples are bacterial surface proteins that bind human C4BP and FH/FHL-1. The regulators are captured in such a way that they are still able to interact with C3 convertases and function as cofactors in factor I cleavage of C3b/C4b. By binding C4BP, bacteria disturb activation of the classical/lectin pathway (Rooijakkers and van Strijp 2007).

Among the most virulent factors of *Streptococcus pyogenes* (group A streptococcus; GAS) is the M proteins. Studies have shown that the M proteins have a remarkable ability to bind with high affinity to the N-terminal region of C4BP (Johnsson et al. 1996). These M proteins interact with CCP10 and CCP2 of the C4BP α chain (Blom et al. 2000). Most importantly, the ability to bind C4BP was recently correlated with phagocytosis resistance of these bacteria (Carlsson et al. 2003). Furthermore, the binding of outer membrane protein A (OmpA) of *E. coli* K1 to C4BP has been shown. C4BP bounding to *E. coli* K1 acts as a cofactor to factor I in the breakdown of both C3b and C4b, hence limiting the ensuing complement cascade. Therefore, a peptide corresponding to the complement control protein domain 3 of the C4BP sequence, was shown to compete with C4BP binding to OmpA and cause increased deposition of C3b. Thus, the binding of C4BP appears to be responsible for the survival of *E. coli* K1 in human serum (Wooster et al. 2006).

Many pathogens capture FH and/or FHL-1 to their surface resulting in alternative pathway C3 convertase decay and inactivation of C3b by factor I. To evade the

alternative pathway, pathogens commonly recruit FH to their surfaces by binding to complement control protein (CCP) 6-7 or CCP 19-20 leaving the critical active site of FH CCP1-4 functional (Meri et al. 2013). Bacterial proteins that bind FH via CCP 6-7 can also recruit FHL-1 due to the shared conserved CCP domains between FH and FHL-1 (Meri et al. 2013).

Haemophilus influenzae clinical isolate, Hib 541, has been shown to bind to both FH and FHL-1 with higher affinity. The isolate showed increased survival in normal human serum and bound to FH using CCP 6–7, and CCP 18–20. Two *H. influenzae* membrane proteins of approximately 32 and 40 kDa were detected with radiolabeled FH in far Western blot as the receptors for FH (Hallström et al. 2008). Further characterization of these proteins is needed. Fba is an FHL-1 and FH binding protein expressed on the surface of the human pathogenic bacterium, *Streptococcus pyogenes.* The *Fba* binding site was localized to CCP 7, a domain common to FHL-1 and FH. Binding to FHL-1 was shown to promote the entry of streptococci into human epithelial cells in a dose-dependent manner (Pandiripally et al. 2003). *Borrelia burgdoferi* produces several proteins that bind to FH including complement regulator-acquiring surface protein 1 (CRASP-1*)* and OspE (Hellwage et al. 2001a; Brooks et al. 2005)*.* The binding site for OspE was localized at CCP 15-20 (Hellwage et al. 2001a). Importantly, some pathogens bind FH using other CCP modules. Factor H binding inhibitor of complement (Hic) of *Streptococcus pneumoniae* recruits FH via CCP 8-11 and CCP 12-14 (Kohler et al. 2015).

Perhaps the most extensively studied protein with respect to FH binding is the human-specific lipoprotein factor H binding protein (fHbp) of *Neisseria meningitidis*. Binding of FH to fHbp is a high-affinity interaction involving both N- and C- terminal parts of fHbp and CCP 6-7 of FH (Schneider et al. 2009). Besides recruitment of FH, fHbp is also capable of binding to FHR3 (Caesar et al. 2014), which is a known competitive

antagonist of FH (Józsi et al. 2015). It is thought that recruitment of FHR-3 over FH would render a strain more susceptible to alternative pathway complement-mediated killing (Hovingh et al. 2016). Of note is that *P. falciparum* has also been shown to recruit complement regulatory proteins (Simon et al. 2013; Rosa et al. 2015; Kennedy et al. 2015) in order to escape complement activation.

It is important to note that although binding to FH has long been considered to be beneficial for the pathogen, it is possible that the host can use it to its advantage. For example, FH binding to *Candida albicans* mediates phagocytosis and killing via interaction with CR3 on neutrophils (Losse et al. 2010). Therefore, future studies will benefit by interrogating host and pathogen benefits of recruitment of FH. Elucidating the molecular mechanisms underlying complement evasion may help to identify novel vaccine antigens and also improve our understanding of the molecular and functional basis of good vaccine antigens (Serruto et al. 2010).
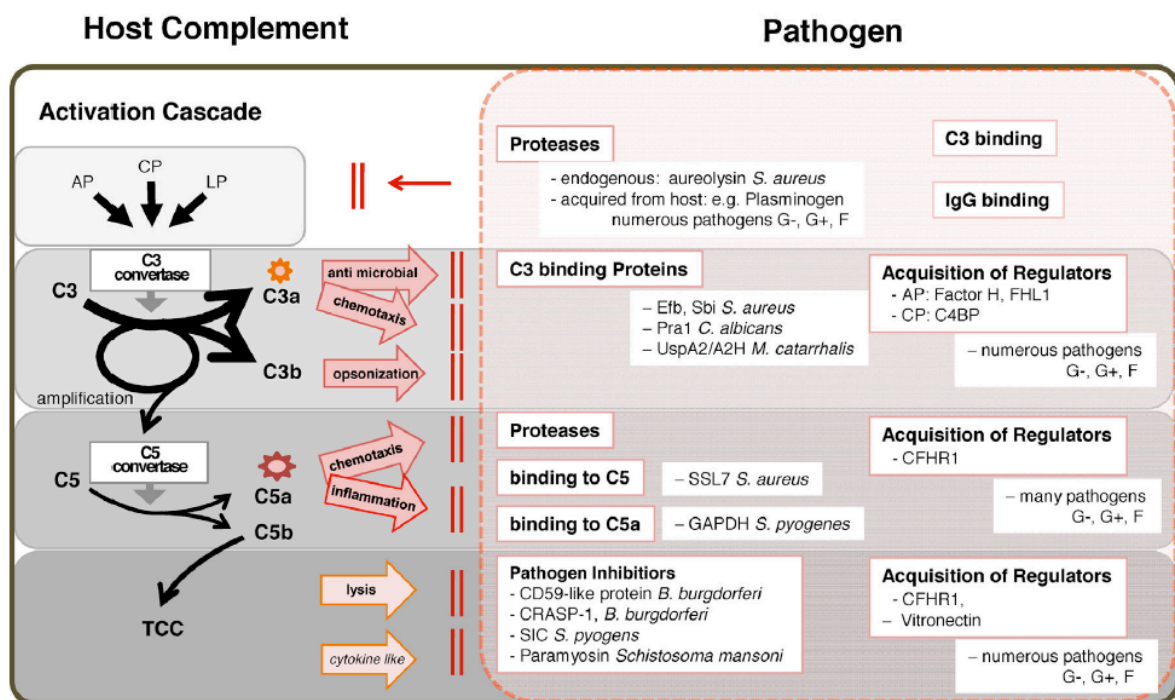
Figure 1.13 Complement evasion proteins of pathogenic microbes interfere with cascade progression and amplification.

Complement activation can be controlled at the activation level, e.g., by proteases, C3 acquiring proteins, as well as host bound pre-proteases like plasminogen. Complement evasion proteins bind to C3b, interfere with C3a function. Besides, many pathogenic microbes bind host plasma proteins and complement regulators like Factor H, FHL1 and C4BP, which influence C3 convertase activity and favor dissociation of the assembled cascade. Microbial proteins interfere with the C5 circuit by degrading C5a, binding and blocking C5b. Similarly, microbial pathogens acquire CFHR1 a human C5 convertase inhibitor. Microbial proteins interfere with the terminal pathway by binding to components like C7 and by acquiring the human TCC inhibitors clusterin and CFHR1. G−, Gram-negative bacteria; G+, Gram-positive bacteria; F, fungi. Figure from (Zipfel et al. 2013).

1.12.7 Complement evasion molecules as vaccine candidates

The current "complement vaccine" research, in particular for infectious diseases, has mostly focused on recognition of pathogenic proteins that bind to complement regulatory proteins. It is argued that if binding of the complement regulators is prevented, complement activation will proceed on or near the microbial surface leading to opsonization, phagocytosis or lysis of the microbes (Meri et al. 2008). This concept has been explored, and a successful component vaccine developed and licensed for use. Factor H binding protein (fHbp), a lipoprotein present on the surface of *Neisseria meningitidis* is a component of Bexsero (Novartis) and Trumenba (Pfizer), two recently approved vaccines against meningococcus. Bexsero contains fHbp var1.1 in combination with two additional recombinant proteins (NHBA and NadA) and membrane vesicles from the New Zealand outbreak strain NZ98/254 (Giuliani et al. 2006). Trumenba contains two recombinant lipidated fHbp subvariants (var1.55 and var3.45) (Jiang et al. 2010). Antibodies directed against fHbp can act in many ways. Those with serum bactericidal activity can mediate direct bacteriolysis and can also promote phagocytosis and subsequent intracellular killing. Additionally, fHbp-specific antibodies can block binding of factor H, and hence increase bacterial susceptibility to killing via the alternative pathway (Madico et al. 2006; Schneider et al. 2006; Welsch

et al. 2008). In fact, the killing of the bacteria by anti-fHbp antibodies in the presence of human complement (serum bactericidal assay) is used to predict the efficacy of the vaccine in humans (Biagini et al. 2016).

1.12.8 Complement dysfunction and disease susceptibility

Numerous studies have associated genetic variation in complement regulatory proteins with some chronic and infectious diseases (de Cordoba et al. 2012; Rodriguez et al. 2014; K. Liszewski and Atkinson 2015; de Córdoba 2016). Genetic mutations in complement factor H (FH) have extensively been implicated in renal disorders type II membranoprofilic glomerulonephiritis, atypical haemolytic-uraemic syndrome (aHUS) (Pickering and Cook 2008; Ricklin et al. 2016) and age-related macular degeneration (AMD) (Ansari et al. 2013). Notably, aHUS is strongly associated with polymorphisms and mutations of activators (C3 and FB) and regulators (CD46, FH, and FI) of the alternative pathway (Mele et al. 2014). More than 300 aHUS-associated genetic alterations are listed in the FH aHUS mutations to date, with mutational hotspots primarily in the C-terminus of complement factor H (Rodriguez et al. 2014). **Table 1.3** below indicates common factor H genetic variants and their respective disease phenotypes.

Table 1.3 Complement factor H genetic variants and disease phenotypes

Disease association	Genetic variant	CCP	Effect	Reference
Hemolytic uraemic syndrome	C-257T A2089G G2881T	Promoter CCP11 CCP16	- Silent Glutamic acid-to-Aspartic acid change	(Caprioli et al. 2003)
	W1183L L1189R V1197A	CCP20	Tryptophan-to-leucine change Leucine-to-arginine change Valine-to-alanine change	(Pérez-Caballero et al. 2001)

Dense deposit disease	T431A	CCP7	Cysteine-to-serine change	(Dragon-Durey et al. 2004)
	K224	CCP4	Deletion of a single Lysine residue	(Licht et al. 2006)
	G184A	CCP1	Valine-to-isoleucine change	(Abrera-Abeleda et al. 2011)
	T1277C	CCP7	Tyrosine-to-histidine change	(Abrera-Abeleda et al. 2011)
age-related macular degeneration	T1277C	CCP7	Tyrosine-to-histidine change	(Sofat et al. 2012)

Although the mechanism of how these mutations affect disease outcome are yet to be fully defined, some are known. It has been shown that fH62I alters the formation of alternative pathway convertase or its regulation (Heurich et al. 2011). Others such as fH402H haven been implicated in the reduction of the binding of FH to its natural ligand such as malondialdehyde (Weismann et al. 2011), a phenomenon implicated in the development of AMD. The CCP 7 domain where Y402H mutation occurs mediates recruitment of *Streptococcus pyogenes* (Parente et al. 2017a). *In vitro* experiments have shown that the Y402H polymorphism causes reduced binding of FH to this group A streptococcus leading to its increased killing via phagocytosis in the blood (Haapasalo et al. 2008). Future mechanistic studies can evaluate how the Y402H polymorphism affects malaria parasite binding to FH and how this affects its survival in the human host. On the other hand, delLys224 impedes FH recognition of C3b, resulting in loss of its function (Wu et al. 2009). The work presented in this thesis was the first to attempt to link whether the FH variant the Y402H is associated with malaria disease. Only one study has examined whether FH levels vary within different malaria disease state (van Beek et al. 2018). Still, the study findings were not linked to genetic changes in the FH gene.

For infectious diseases, it is now well appreciated that some single nucleotide polymorphisms (SNPs) have been associated with susceptibility or protection from

infection and disease. For example, a genome-wide association study (GWAS) of Caucasians has found variations within the complement FH gene family that are linked to host susceptibility to meningococcal disease. This study identified SNPs within complement FH and in complement Factor H-related protein 3 as being responsible for the susceptibility (Davila et al. 2010). A similar study will need to be done in an African population to corroborate these findings. It has also been shown that genetic variation could influence the circulating levels of FH. Individuals possessing some polymorphisms within FH have been shown to have increased serum FH protein levels, reduced bactericidal activity against meningococci and an increased risk of meningococcal disease (Haralambous et al. 2006). Put together these findings implicate complement control in setting disease risk.

With regards to malaria, previous studies have focused on the Mannose-Binding Lectin (MBL) gene and the complement receptor 1 gene. Three polymorphisms of the MBL gene (*MBL2*) exon 1 that result in non-synonymous substitutions have been linked to decreased serum levels of MBL ('B' G54G, 'C' G57E, and ''D R52C) (Lipscombe et al. 1995). Of these alleles, the *MBL2* C allele is found almost exclusively in the African population suggesting that it might provide a survival advantage in that population (Lipscombe et al. 1992; Madsen et al. 1998; Biryukov et al. 2014). Importantly, of the several studies that have studied the association of MBL polymorphisms with parasitemia and disease, while some have shown an association (Luty et al. 1998; Holmberg et al. 2008, 2012; Boldt et al. 2009) others have not (Garred et al. 2003; Mombo et al. 2003; Thevénon et al. 2009). The lack of consistent results among these studies is probably due to differences in experimental design, population demographics such as age and gender, differences in transmission intensity, and statistical rigour (Biryukov and Stoute 2014). Nevertheless, these results suggest that the role of MBL in protection against *P. falciparum* malaria would be significant (Biryukov and Stoute 2014). A carefully, well-designed case-control study, done across

multiple sites in malaria-endemic regions with varying malaria transmission intensities, will be essential to elucidate if there is an association of the MBL specific alleles with protection from or susceptibility to *P. falciparum* infections and disease.

Several studies have related complement receptor 1 to malaria pathogenesis. CR1 has three major polymorphisms: those affecting the polypeptide size variants; quantitative variants that affect the number of CR1 molecules on RBCs; and missense mutations of CR1. CR1 mutations form the basis of the Knops blood group system of antigens that includes the antithetical antigen pairs of Swain-Langley 1 and 2 (Sl1 and Sl2) and McCoy a and b (McC[a] and McC[b]) (Krych-Goldberg and Atkinson 2001; Schmidt et al. 2015). Quantitative blood group polymorphisms impact on CR1's ability to form rosettes (Chen et al. 2000). Rosetting is mediated either by PfEMP1 binding to ABO blood groups antigens (Rowe et al. 2007) or to CR1 (Rowe et al. 1997). Although it has been argued that rosetting might mediate severe malaria and hence CR1 deficient individuals should be protected from severe forms, studies investigating this phenotype have given inconsistent findings. While some show that low CR1 numbers are associated with protection (Cockburn et al. 2004b; Thomas et al. 2005), others have not (Nagayasu et al. 2001; Teeranaipong et al. 2008). Intriguingly the knobs antigens McC[b] and S12 are unusually high in the African population (Figure 2) in (Opi et al. 2018), which might suggest a possible survival advantage against malaria (Rowe et al. 1997; Opi et al. 2018). Put together, the role of the CR1 molecule in the pathogenesis of severe malaria remains unresolved and further studies are indeed. It remains unclear whether there are any genetic polymorphisms in FH that would influence the complement evasion efficiency of the malaria parasite. Also, whether they have a geographical distribution coinciding with varying malaria transmission intensity especially in Africa.

1.12.9 The Y402H variant of FH

The most widely studied mutation in FH is the Y402H variant, which is a histidine replacement of tyrosine at position 402 (using the pro-protein sequence numbering). Structurally, the Y402H polymorphism occurs in the seventh of FH's twenty complement control protein (CCP) domains (Schramm et al. 2014) and does not alter the overall conformation of the protein (Herbert et al. 2007). However, the Y402H alters the binding of FH to many ligands, most notably C-reactive protein, Streptococcus M protein and sulfated polyanions, such as glycosaminoglycan (GAG) chains of proteoglycans (Clark, Bishop, & Day, 2010). It is important to note that this SNP has been linked strongly to AMD. Heterozygous individuals for the Y402H polymorphisms have a 2.3- fold increased risk of developing the disease, and homozygous 5.2 fold (Sofat et al. 2012). Around 30% of people of European descent carry at least one copy of the 402H risk allele (Sofat et al. 2012). A proposed explanation for the high prevalence of the Y402H polymorphism is that the 402H allele provides a survival advantage against streptococcal infections in early life (Haapasalo et al. 2008). The FH binding protein of streptococcus has a lower affinity for 402H than 402Y, which would enhance activation of the alternative pathway on these bacteria.

1.12.10   Complement and naturally acquired immunity to malaria

Several lines of evidence support the idea that acquired and vaccine-induced human antibodies recruit complement and interfere with RBC invasion by the malaria parasite (Boyle et al. 2015; Feng et al. 2018). Boyle *et al.* have described an antibody effector function known as antibody-mediated complement dependent (Ab-C') inhibition, in which antibodies use the complement to limit parasite growth *in vitro*. In some individuals, the antibodies were shown to be non-inhibitory in the absence of complement. Antibodies that fix C1q were shown to be associated with protection from

clinical episodes of malaria (Boyle et al. 2015). The importance of complement is further supported by the association of protection from malaria with levels of cytophilic IgG1 and IgG3 that activate complement via C1q (Roussilhon et al. 2007; Stanisic et al. 2009; Richards et al. 2010). Furthermore, antibodies that fix complement have been shown to inhibit *P. falciparum* sporozoite invasion of hepatocytes (Behet et al. 2018; Kurtovic et al. 2018; Zenklusen et al. 2018) implying that antibody complement interactions have many roles in immunity against different stages of malaria.

Perhaps a significant step forward in our understanding of the antibody-complement effector function is that the specific targets for complement-fixing antibodies are now known (Reiling et al. 2019). The work of Reiling *et al.* has shown that EBA140 RIII-V, RASPI, GAMA, PfRH2, MSP-DBL1, PfRH5, EBA 175-RIII-V, and MSP2-3D7 merozoite proteins are targets of complement-fixing antibodies. Importantly, antigen-specific complement-fixing antibodies were strongly associated with protection from malaria in a longitudinal study of children. Also, using statistical modeling, they observed that combining three antigen targets of complement-fixing antibodies could increase the potential protective effect to over 95%. These findings support antibody-complement interactions against merozoite antigens as important antimalaria immune mechanisms (Reiling et al. 2019).

In contrast, Biryukov *et al.* have shown that anti-merozoite antibodies and complement activation can paradoxically aid the malaria parasite to invade red blood cells. Using a monoclonal antibody directed against the merozoites and human polyclonal IgG from merozoite vaccine recipients in a standard invasion inhibition assay, they showed that in the presence of complement and antibodies, there was enhanced invasion (Biryukov et al. 2016). However, unlike the previous studies, this study suffers one major limitation of not testing the enhancement of invasion using sera from immune adults that have traditionally been used to measure antibody effector functions.

1.12.11   Complement and severe malaria anaemia

The pathogenesis of severe malaria anaemia (SMA) remains poorly understood although it is thought to arise as a result of direct destruction of RBCs during schizont rupture, increased destruction of uninfected RBCs and impaired erythropoiesis due to bone marrow suppression (Jakeman et al. 1999; Awah et al. 2011). A significant body of evidence suggests that complement activation may be responsible for the loss of uninfected red blood cells hence contributing to SMA. Children with severe malaria anaemia tend to have higher levels of immune complexes (Mibei et al. 2005) and show a considerably lower complement hemolytic activity (CH50) along with the decreased activity of all the major complement pathways (Nyakoe et al. 2009). Additionally, it has been observed that red blood cells in children with SMA have increased surface IgG and deficiencies in complement regulatory proteins CR1 and CD55 (Waitumbi et al. 2015). Erythrocyte deficiencies of CR1 and CD55 in children with SMA result in reduced immune complex (IC) binding capacity and are associated with increased deposition of C3b on erythrocytes during malaria infection (Odhiambo et al. 2008; Owuor et al. 2008). It, therefore, can be postulated that in the absence of CR1 and CD55, there would be increased deposition of complement products C3b marking these RBCs for phagocytosis or complement-mediated lysis. Parasite antigens can also cause erythropoietic dysfunction. *In vitro* work by Layez *et al.* indicated that rhoptry protein RSP2 had a higher affinity for erythroid precursors in the bone marrow pointing to the possibility that parasite antigens may limit the production of erythrocytes (Layez et al. 2005).

1.12.12   Complement and cerebral malaria

There is evidence from animal models and human studies that suggest that complement could be implicated in the development of cerebral malaria (CM), although

this is mostly correlational (Silver et al. 2010). Infection of mice with *Plasmodium berghei* ANKA is the most commonly used animal model for CM. One study observed up-regulation of complement factors C1q and C5 in the brain of mice with CM (Lackner et al. 2008). Susceptibility of *P. berghei*-induced CM correlated with the expression of C5, as C5 deficiency has been shown to cause resistance to CM. Transfer of the *C5*-defective allele from A/J (CM resistant) into C57BL/6 (CM-susceptible) mice confers increased resistance to CM (Patel et al. 2008).

Furthermore, the treatment of wild-type mice with anti-C5aR or anti-C5a Ab protected against the development of experimental CM, and anti-C5aR Ab significantly inhibited monocyte-derived cytokine production (Conroy et al. 2009). Conversely, in another study C5aR$^{-/-}$ mice were found to be fully susceptible to disease (Ramos et al. 2011). Nevertheless, these data suggest that C5 is essential in the pathogenesis of CM. On the other hand, C3$^{-/-}$ mice have also been shown to be highly susceptible to experimental cerebral malaria (Ramos et al. 2012). In humans, a case-control study observed that the median levels of C5a at presentation were significantly higher in children with CM versus those in children with UM (43.7 versus 22.4 ng/ml; *P* < 0.001) (Kim et al. 2014). Thus, overactive complement in the form of C5 fragment production may contribute to severe malarial disease and efforts to regulate this could lead to better patient outcomes (Schmidt et al. 2015).

1.13 Scope

Many pathogens can establish infections despite exposure to the complement system. They have evolved mechanisms to evade complement-mediated clearance through, among others, the ability to hijack human complement regulatory protein factor H (FH) (Lambris et al. 2008). Numerous studies have shown that *Plasmodium falciparum* recruits FH for immune evasion (Simon *et al.*, 2013; Kennedy *et al.*, 2015; Khattab *et*

al., 2015a; Rosa *et al.*, 2016). Specifically, within the blood stages, merozoite protein Pf92 has so far been identified as the merozoite receptor for FH (Kennedy et al. 2015). I hypothesized that potentially more merozoite proteins are involved in complement evasion. I used a combination of modified ELISA binding assay and surface plasmon resonance and tested a library of recombinant merozoite proteins to discover new proteins that interact with FH other than Pf92 (Kennedy et al. 2015). Next, I tested whether antibody responses to the newly identified merozoite FH receptor proteins were associated with protection using a malaria cohort study. I also tested whether FH levels influence susceptibility to malaria infection and whether a common FH variant, Y402H (Sofat et al. 2012), influences FH levels and is associated with clinical malaria using a longitudinal and case-control study in children respectively.

I hypothesized that many merozoite proteins are involved in the interaction with FH because of previous observations in *Plasmodium falciparum* and also in bacteria. In studies published by Kennedy et al. Pf92 was characterized as an interacting partner with FH using a parasite knockout. They showed only a modest reduction in growth in the presence or absence of complement. This implies two possibilities, either Pf92 is not the only protein involved, or the parasite evades complement by interacting with other complement regulatory proteins (Kennedy et al. 2015). Indeed in follow-up studies, Kennedy et al. and colleagues identified MSP3.1 as another protein that interacts with C1-INH for parasite complement immune evasion (Kennedy et al. 2017). A similar mechanism of redundancy had been observed in bacteria. Gram-negative bacterium *Borrelia burgdorferi* has been shown to have two or three FH binding proteins: CspA, CspZ; and OspE (Hellwage et al., 2001; Brooks et al., 2005, Kraiczy and Stevenson, 2013) for FH interaction. Gram-positive bacterium *Streptococcus pyogenes* has also been shown to have three FH binding proteins, M1 protein, Fba and Scl1.6 (Pandiripally et al. 2002, 2003; Reuter et al. 2010).

Lastly, it is known that the parasite has many proteins, its genome encodes over 5000 proteins. Given what is known of parasite biology, it is highly unlikely that an important parasite survival strategy is entrusted to only one protein. For example, for parasite invasion, we know multiple parasite proteins have been shown to engage different ligands on erythrocytes and that the parasites can switch between different invasion pathways (Weiss et al. 2015). With variant surface proteins, again the parasite uses a complex strategy of switching sequential and variable parasite proteins to avoid immune clearance (Dinko and Pradel 2016) presumably. These examples show a pattern of the parasite using an array of proteins rather than single proteins to promote its survival.

1.14 Composition of the thesis

Chapter 1 provides a broad introduction to malaria, immunity and the complement system.

In **chapter 2** I outline the cohorts analyzed in this project and the range of laboratory methods applied. Subsequently, the data chapters are:

Chapter 3: Identification of blood stage *Plasmodium falciparum* merozoite antigens that interact with human complement factor H for complement evasion

Chapter 4: Analysis of antibody responses to merozoite FH receptor proteins and their association with protection against clinical malaria

Chapter 5: Association of human complement FH levels and Y402H variant with susceptibility and severity to *Plasmodium falciparum* malaria

Each chapter contains summary and aims, a brief introduction, methods, results, and discussion.

Chapter six contains the overall discussion of the findings, further areas of research and conclusions.

2   Materials and Methods

2.1   Summary and aims

This chapter describes the laboratory methods used in the subsequent chapters of this thesis (Chapter 3,4 and 5), the data source population, and the analysis methods. The experimental work was conducted at the Immunology Laboratory, KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya and at the Department of Bacteriology and Immunology, University of Helsinki, Finland. The recombinant merozoite proteins used in this study have been previously described (Zenonos et al. 2014; Kamuyu et al. 2018; Crosnier et al. 2013) and were part of a bigger study led by Prof Faith Osier with the aim to define the merozoite targets of protective immunity against *Plasmodium falciparum* malaria. The specimens analyzed are continuously collected and are archived as part of on-going studies to understand acquired immunity to malaria in children in Kilifi County, Kenya.

The current study involved the systematic screening of a recombinant merozoite proteins library (Crosnier et al. 2013; Zenonos et al. 2014; Kamuyu et al. 2018) to determine interaction partners of complement regulatory protein factor H (FH). The aim is to improve our understanding of complement immune evasion by the malaria parasites. Human serum and blood EDTA samples were used to: analyze antibody responses to merozoite FH receptors and their association with protection against malaria; inter-individual variation in FH levels and its association with malaria susceptibility and severity; and the influence of FH Y402H polymorphism on the risk of developing the disease.

Samples were processed according to established standard operating procedures and stored at −80°C within an hour of collection. During subsequent handling by individual

investigators, samples are at all times kept on ice and immediately returned to −80°C freezers. Sample handling is controlled as the sample request is managed centrally. Since the samples are managed and owned by the KEMRI-Wellcome Trust Research Programme, they are a shared resource and several investigators have requested for them over the years. These samples have previously been requested 10 times (according to the records with the Biobank Manager). As long as samples are appropriately handled, complement protein measurement should not be affected (Yang et al. 2015).

2.2   Study location

The epidemiological studies, which were the source of the specimen analyzed in this thesis project, were part of a series of studies to understand naturally acquired immunity to malaria. The studies were conducted at Kilifi County, located along coastal Kenya. Specifically, this project used archived serum and blood EDTA samples collected from residents of the Kilifi Health and Demographic Surveillance System (KHDSS) area (Scott et al. 2012; Nyiro et al. 2018) attending Kilifi County Hospital (KCH), and children presenting with uncomplicated malaria at the Junju dispensary (located in the south of KHDSS) **Figure 2.1**. Archived serum samples from the Junju malaria longitudinal study of children (described in the next section 2.4) were also used. KHDSS spans an area of 891 km^2, 50 km north and south, and 30 km west with reference to KCH. KCH is the largest and only referral hospital within the KHDSS offering both outpatient and in-patient services while Junju dispensary offers outpatient services only. **Figure 2.1** indicates the location of KHDSS in relation to KCH and other major health facilities.
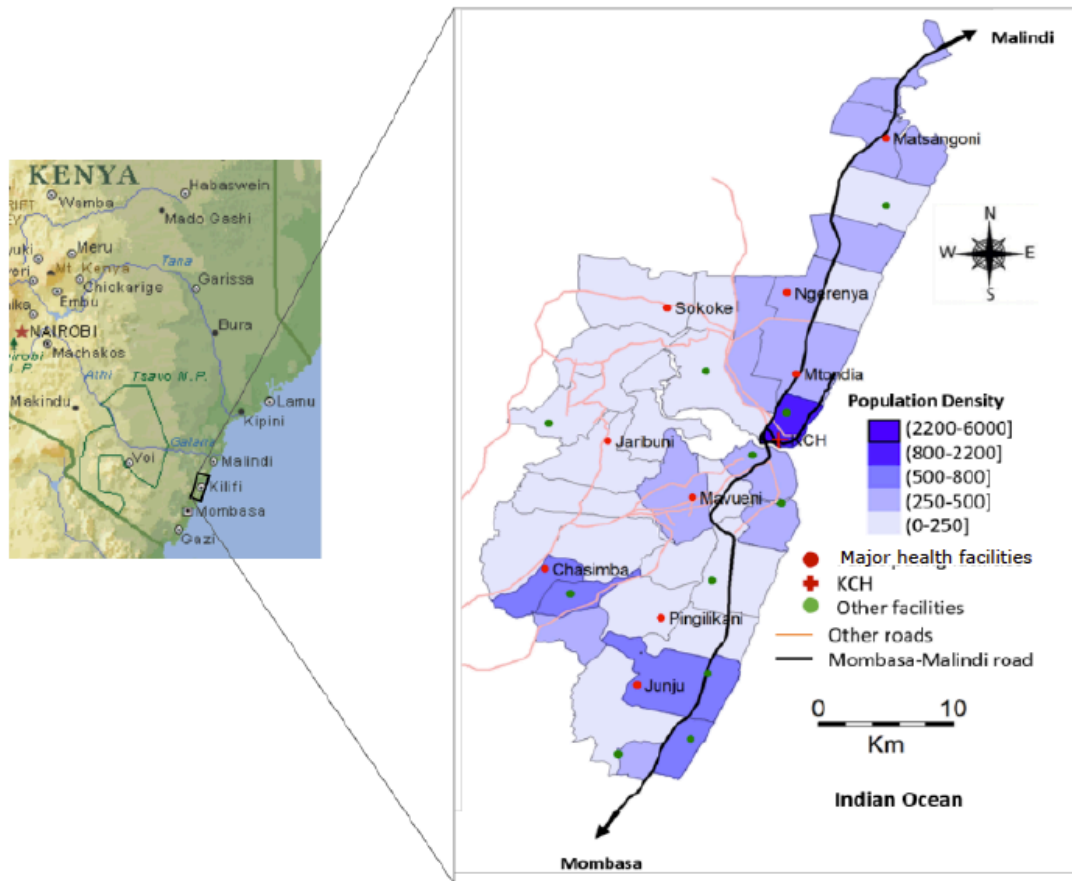
Figure 2.1. A map of the Kilifi Health and Demographic Surveillance System (KHDSS) area, coastal Kenya

The map is expanded from a map of Kenya showing population density (person per km^2) and the health facilities. Figure modified from (Nyiro *et al.*, 2018)

2.3 Study population

The population of Kilifi County according to the last Kenyan government census of 2009 is approximately 1 million, of which around 260,000 are within the KHDSS (Scott et al. 2012; Nyiro et al. 2018). The majority resides in rural areas. The population is predominantly of the "Mijikenda" ethnic group and consists of mainly small-scale subsistence farmers of maize, cassava, cashew nuts, and coconuts, as well as goats and dairy cattle. The majority of malaria infections in this region are due to *Plasmodium falciparum* (Mwangi *et al.*, 2005).

2.4   Junju longitudinal study of children

The study comprised an initial cross-sectional survey and subsequent longitudinal monitoring for six months **Figure 2.2.** The cross-sectional survey was conducted at the start of the malaria transmission period to enable the prospective monitoring of clinical cases of malaria **Figure 2.2**. At the cross-sectional survey, a blood sample was collected to test for malaria infection using a rapid diagnostic test (RDT) in the field. Samples that were positive by RDT were re-tested in the laboratory using microscopy and PCR. Active surveillance was done through weekly home visits by trained fieldworkers where children are tested for fevers associated with *Plasmodium falciparum* malaria. The fieldworkers are also available to assess any fevers occurring in-between the weekly visits. Children found with fevers and positive using the RDT were referred to the nearest health dispensary where a clinical officer reviewed them and administered anti-malaria drugs. Since the cohort is under active surveillance, children who develop malaria, including having multiple clinical episodes during the follow-up period are treated and recorded.

Severe cases with complications are referred to the Kilifi County Hospital for admission. On admission, an electronic case record was filled capturing age, residence, vital signs, clinical history and examination and Blantyre coma score. In addition, the following tests were done: malaria blood slide, full blood count, blood glucose and blood culture tests (Njuguna et al. 2019). In this region of Junju sub-location, the peak malaria transmission occurs during the rainy months (long rains from May to July and short rains from November to December). The *Plasmodium falciparum* parasite prevalence in 2-10-year-olds ($PfPR_{2-10}$) is around 30% (Ndungu et al. 2015; Wamae et al. 2018). A clinical episode of malaria was defined as a body temperature of >37.5 $^{\circ}$C with a parasite density of ≥ 2500 parasites/ µL (Smith, Schellenberg and Hayes, 1994; Mwangi *et al.*, 2005).
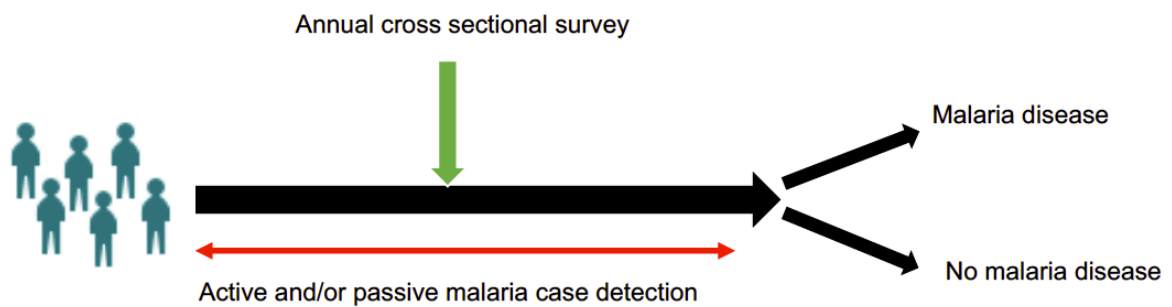
Figure 2.2. Illustration of the Junju malaria longitudinal study of children

Children aged 0-15 years are recruited into the study. Infants born into the participating households are also continuously recruited. Malaria case detection is done both actively through visits by trained fieldworkers and passively at selected participating health facilities

2.5   Kilifi County Hospital case-control study

A case-control study of well-defined severe malaria cases was designed. The KEMRI hospital surveillance study draws its participants from children presenting at KCH and who need admission. Children are included in the study only if their parents' consent. The timing for the collection of blood samples was prior to admission. Children meeting the clinical definition for malaria (see **section 2.5.1 and 2.5.2**) were used for the work presented in this thesis. Upon admission, basic demographic data such as age and residence were electronically recorded in addition to vital signs, clinical history and examination and Blantyre coma score. The following laboratory tests were also routinely done: malaria blood slide, full blood count, blood glucose and blood culture test (Njuguna et al. 2019). Any child that developed another clinical episode of malaria and re-admitted was captured in the Kilifi Health Demographic Surveillance System as long as they remained residents at the time of hospital presentation. Hence their multiple malaria clinical episodes were treated and recorded. In this PhD work, I sampled cases only once irrespective of the number of times the child was admitted.

Three categories of children were selected according to the presenting symptoms: severe malaria anaemia (SMA); severe malaria without anaemia (SM); and uncomplicated malaria (UM).

Cases were residents of Kilifi Health Demographic Surveillance Systems (KHDSS) presenting at Kilifi County Hospital (KCH) with severe malaria. KCH is the largest referral hospital within KHDSS **Figure 2.1**. Controls were children presenting with mild malaria at Junju dispensary outpatient clinic located within the KHDSS. The KHDSS has several outpatient's health facilities **Figure 2.1**, but the KEMRI Wellcome Trust Research Programme has established an active malaria cohort within the Junju dispensary catchment area. There was no direct matching of the controls to the cases but they were both recruited from the KCH catchment area and under the age of 5 years old (Hayes et al. 1992).

2.5.1   Severe malaria case definition

Severe malaria anaemia (SMA) was defined as Hb <5 grams per deciliter (g/dl) with parasitemia of ≥ 10,000 parasites/µL of blood (Marsh *et al.*, 1995). Severe malaria without anaemia (SM) was defined as children with impaired consciousness defined as Blantyre coma score (BCS) of < 3, chest in-drawing or deep breathing (English et al. 1996). To avoid overlap with SMA, children with severe malaria without anaemia (SM) were defined by a haemoglobin of >5 g/dl. The SM group enabled an equivalent comparison of severity that did not include SMA.

2.5.2   Uncomplicated malaria case definition

Criteria for selecting uncomplicated malaria included children with a parasite density of ≥ 2500 parasites/ µL of blood in children 1–15 years old and temperature of >37.5

°C (Smith, Schellenberg and Hayes, 1994; Mwangi *et al.*, 2005). Selection of this third group was necessary in order to determine whether the differences in antibody responses could be attributed to lower parasite densities.

2.6   Detection of *Plasmodium falciparum* infections

P. falciparum detection in blood samples collected at hospitalization or during the cross-sectional survey was achieved principally with light microscopy. Briefly, finger prick blood was collected, and thick and thin blood films prepared and stained using Giemsa. Slides were examined under a light microscope. Parasite density was determined as the number of parasites per 8,000 white blood cells per microliter of blood. Real-Time PCR was used to measure submicroscopic infections (Lundblom et al. 2013; Murungi et al. 2016).

2.7   Ethics statement

The samples in this study were collected after receiving informed written consent from each participant over the age of 18 years or through a guardian or parent for participants under 18 years. All children assented to participate if aged >13 and <18 years. The KEMRI Scientific and Ethical Review Unit (SERU) approved the protocols covering work for this thesis project (SERU/017/3149).

2.8   Key resources for the laboratory experiments

Details of the key reagents used for various laboratory experiments are listed in **Table 2.1** below.

Table 2.1. Key resources

Reagent	Source	Identifier (Cat No.)
Antibodies		
anti-factor H goat polyclonal antibody, IgG	Calbiochem	341276
Donkey anti-goat IgG Antibody, HRP	Calbiochem	AP180P
Monoclonal anti-FH 3D11	Seppo Meri Lab	-
Monoclonal anti-FH 196X	Seppo Meri Lab	-
Monoclonal anti-FH 131X	Seppo Meri Lab	-
Monoclonal anti-FH 86X	Seppo Meri Lab	-
Peroxidase Affinipure rabbit anti-mouse IgG (H+L)	Jackson ImmunoResearch Europe Ltd	315-035-003
Anti-His(C-term)-HRP Antibody	Invitrogen	R931-25
Complement C3b Antibody (10C7), IgG1	ThermoFisher Scientific	MA1-70054
Goat anti-mouse HRP conjugate	Bio-red	170-5047
Donkey anti-human IgG-Alexafluor 647 conjugated	Jackson ImmunoResearch Europe Ltd	709-606-098
Complement proteins/serum		
Complement C3b, human	Calbiochem	204860-250UG
Factor H, Human	Calbiochem	341274
FH Depleted serum	CompTech	A337
Complement commercial ELISA assays		
MicroVue SC5b-9 Plus EIA	Quidel	A020
MicroVue Complement Factor H EIA	Quidel	A039
Factor H, 402H/Y variant detection, Human, ELISA kit	HycultBiotech	HK353
Protein expression reagents		
QIAGEN Plasmid midi kit	Qiagen	12143
Opti-MEM® I Reduced-Serum Medium	ThermoFisher Scientific	31985062
Expifectamine 293 Transfection	ThermoFisher Scientific	A14525
Expi293™ Expression Medium	ThermoFisher Scientific	A1435102
Ni-NTA agarose (100 ml)	ThermoFisher Scientific	R90110
InstantBlue	Expedeon	ISB1L
Novex® ECL Chemiluminescent Substrate Reagent Kit	Invitrogen	WP20005
Western blot reagents		
Tris-glycine SDS sample buffer	Novex by life technologies	LC2676
Proto-gel quick cast	National diagnostics	EC-895
PVDF Transfer Membrane	Thermo Scientific™	88518
Surface Plasmon resonance reagents		
N-hydroxysuccinimide (NHS)	GE Healthcare	BR-1000-50
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	GE Healthcare	BR-1000-50
Ethanolamine	GE Healthcare	BR-1000-50
HEPES buffer	Gibco, Life technologies	15-630-080
Experimental strain		
Plasmodium falciparum 3D7 strain	-	
Software and algorithms		
Graphpad Prism	San Diego, CA, USA	Version 7

| BiaEvaluation | GE Healthcare | |
| R | R Core Team (2013) | |

2.9 Parasite culture

3D7 *P. falciparum* strain was routinely cultured in complete medium (incomplete medium **Table 2.2** supplemented with 10% Albumax) added to O+ erythrocytes. The parasites were maintained under an atmosphere of 1% O_2, 3% CO_2 and 96% N_2 and at <10% parasitemia and 2% hematocrit. Parasite synchronization to early stages (rings) was achieved using 5% (w/v) D-sorbitol (Sigma) (Lambros and Vanderberg 1979) for 20 minutes at $37^{\circ}C$ in a water bath with gentle hand agitation (two times during incubation) or using magnetic isolation with MACs LD columns (Miltenyi Biotech).

Table 2.2 Composition of incomplete medium

Reagent	Volume
RPMI 1640	500ml
HEPES 1M (Gibco)	18.75ml
L-glutamine 200nM (Gibco)	5.0ml
Gentamicin 10mg/ml (Gibco)	1.25ml
20% glucose (VWR)	5.0ml
Sodium hypoxanthine (Sigma)	3.0ml

To prepare complete medium, 10% Albumax (Gibco) was added to 90% incomplete medium.

2.10 Modified growth inhibition assay

Growth inhibition assay (GIA) (Persson et al. 2006; Rono et al. 2012; Murungi et al. 2016) was modified to test the effect of presence or absence of FH on parasite growth. Briefly, 40 µL of highly synchronous *P. falciparum* 3D7 mid-trophozoite parasites at 0.5% parasitemia and 1% hematocrit (resuspended in incomplete media) was added

to individual wells of sterile 96-well U-bottom plates (Falcon). Thereafter, 5 µL of serum depleted of FH (Complement Technologies, Inc.) and 5 µL of 1X sterile PBS were added in duplicate, alongside experimental controls, and the plates were placed in a sealed humidified gas chamber and incubated at 37°C in 5% O_2, 5% CO_2, and 90% N_2. At the end of the second growth cycle (after 96hrs), 10 µg/ml of ethidium bromide (molecular grade) diluted in 1X sterile PBS was added to each well, and the plates were incubated for 30 mins at room temperature in the dark. The plates were centrifuged at 1,000 rpm for 1 min, and the pellet was resuspended in 200 µL of 1X PBS. Parasitemia was determined by analyzing 50,000 events on a FACS Canto (BD) flow cytometer.

2.11 Isolation of *Plasmodium falciparum* merozoites

Merozoites were prepared as described by Boyle et al. (Boyle et al. 2010b) **Figure 2.3**. Parasite cultures were centrifuged at 2000rpm for 5 minutes (TOTANTA 460R) and resuspended into 30 ml complete medium. MACs columns (Miltenyi Biotech) were pre-wetted, and the resuspended parasite cultures were passed through the columns. Unbound parasites and uninfected blood cells were washed out of the column before removing the column from the magnet and trophozoites collected and centrifuged at 1800rpm to concentrate the trophozoites. The pellet was re-suspended in the culture medium, without the addition of red blood cells and incubated with 10µM of *trans*-Epoxysuccinyl-L-leucylamino (4-guanidino) butane (E- 64) protease inhibitor until distinct merozoites were visible under the light microscope. Segmented schizonts were centrifuged at 2000g for 5 minutes and re-suspended in 20ml of the incomplete medium before passing them through a pre-wetted 1.2 µm filter to release merozoites. Trapped merozoites were removed by rinsing the filter and the merozoites centrifuged at 2000g for 5 minutes. The merozoites were resuspended in 1X Pierce protease inhibitor (Thermo Fisher Scientific) and stored at -20°C.
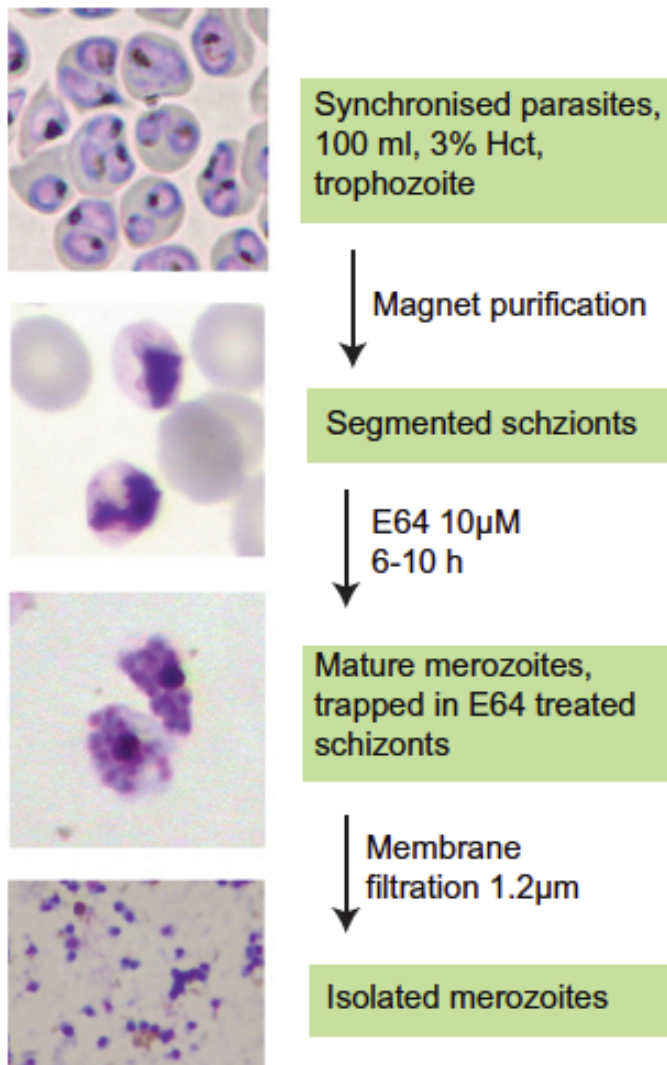
Figure 2.3. Isolation of *Plasmodium falciparum* merozoites by membrane filtration.

Magnetic purification was used to remove uninfected red blood cells from highly synchronized trophozoites. When the parasite develops to segmented schizonts, they are treated with trans-Epoxysuccinyl-L-leucylamido (4-guanido) butane (E64) for 6-10h until mature merozoites have formed. Merozoites are then released by membrane filtration through a 1.2 μm filter. Figure from (Boyle et al. 2013).

2.12 Maintenance of Expi293 mammalian cells

To start the cell culture, frozen vials containing human embryonic kidney cells (Gibco Expi293 cells) were obtained from liquid nitrogen. The cells were thawed rapidly in a 37°C water bath and added to 30ml of pre-warmed Expi293 expression media

(ThermoFisher Scientific). Cells were grown in standard corning flasks (120-500 ml; Sigma) and maintained in a humidified incubator with 8% CO_2 supply at 37^oC, free of any antibiotics. Cells were regularly checked to ensure that the viability (determined using countess automated cell counter (Invitrogen)) was maintained at 90%. Cells expressing recombinant proteins (see section **2.14** on transfection) were left to grow and secrete proteins for six days post-transfection. For the preservation of Expi293 cells, the cells were washed and re-suspended in FCS with 10% DMSO and transferred into cryo-vials (ThermoFisher Scientific). Cells were frozen at -70^oC before transfer to liquid nitrogen.

2.13 Protein expression

Expression plasmids containing recombinant *Plasmodium falciparum* antigens have been previously described and are available at Addgene (Crosnier et al. 2013; Zenonos et al. 2014). Briefly, regions of the genes corresponding to the ectodomain fragments of proteins from the *P. falciparum* 3D7 strain were predicted using trans-membrane, GPI- anchor or signal peptide prediction software (Sonnhammer et al. 1998; Eisenhaber et al. 1999; Dyrløv Bendtsen et al. 2004). Sequences encoding the extracellular domains were codon-optimized for expression in human cells and synthesized at Geneart AG, Germany. Furthermore, due to previous data showing that *Plasmodium* proteins are not N-glycosylated *in vivo* (Dieckmann-Schuppert et al. 1992; Kimura et al. 2000), these sites were mutated to prevent inappropriate glycosylation. Protein coding sequences were flanked with unique NotI and AscI restriction sites and subcloned into a derivative of the pTT3 expression plasmid between a 5' mouse variable κ light chain signal peptide (Crosnier et al. 2010) and a 3' tag consisting of the rat Cd4 domains 3 and 4 (Cd4 tag) (Brown and Barclay 1994) followed by a hexa-histidine tag (Zenonos et al. 2014; Crosnier et al. 2013). Plasmids were transformed into One Shot[TM] TOP10 Chemically Competent *E. coli* culture (Invitrogen) and

incubated overnight in LB broth media (BD) supplemented with ampicillin sodium salts (Sigma). Subsequently, amplified plasmids were purified using the Qiagen Plasmid Mini kit. The purified plasmid DNA was assessed for quantity and quality by measuring the absorbance at 260 nm (A260) and 280 nm (A280) using a Nanodrop spectrophotometer (ThermoFisher Scientific).

2.14 Transient transfection of protein expression plasmids

The recombinant protein expression plasmids were transiently transfected using human embryonic kidney Expi293 cells. Briefly, cells were split into 30-240 ml of fresh Expi293™ expression medium at a density of 2.0×10^6 cells/ml. Density and viability were determined by staining the cells with 0.4% trypan blue stain (Life technologies) and placing them in the cell counting chamber slide (Invitrogen) followed by counting using the countess automated cell counter (Invitrogen). Only cells with viability of higher than 85% were used for transfection. For each 30-mL transfection or an adjusted volume depending on the protein quantity required, the preparation was done as follows. Plasmid DNA was diluted in 1.5ml of Opti-MEM® I Reduced Serum Medium and mixed gently. A volume of 80ul of ExpiFectamine™ 293 Reagent were added to 1.5ml Opti-MEM® I medium and mixed gently. Diluted DNA and ExpiFectamine™ 293 Reagent were incubated separately for 5 minutes at room temperature. Diluted DNA and diluted ExpiFectamine™ 293 reagents were then mixed and incubated for 20 minutes at room temperature so that the DNA-ExpiFectamine™ 293 Reagent complexes could form, and finally, the complexes were added to the flasks containing viable cells. After 16-18 h incubation in a humidified incubator with 8% CO_2 and an orbital shaker platform, a mixture of 150 µL of ExpiFectamine™ 293 Transfection Enhancer 1 and 1.5 mL of ExpiFectamine™ 293 Transfection Enhancer 2 were added to the flask. The protein was harvested six days post-transfection.

2.15 Purification of His-tagged recombinant proteins using Ni-NTA Purification System

His-tagged proteins were purified using Ni^{2+}NTA resins (novex, life technologies) according to the manufacturer's instructions with slight modifications. Briefly, the Ni-NTA resin was prepared by centrifuging the required amount of resin at 800rpm at 4°C for 5 minutes. The supernatant was discarded, and the resin underwent 3 washing steps with distilled water. For the final washing a 1X native binding buffer with imidazole was used. In a 50ml culture supernatant, 50 µL of Nickel(II) chloride were added together with the resin. The mixture was incubated at 4°C overnight while rotating. To purify the proteins, the mixture (resin with Nickel (II) chloride and harvested culture supernatant) was centrifuged at 800rpm for 10 minutes at 4°C and supernatant removed, and the resin transferred to the filter columns (Qiagen). The resin was washed 3 times with native wash buffer. The proteins were eluted using native elution buffer and quantification of the expressed proteins was performed using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

5X Native purification buffer
250mM sodium phosphate monobasic 7g
2.5M NaCl 29.2g
Water
pH 8.0
1X Native purification buffer
5X native purification buffer
Water
pH 8.0

Native binding buffer
With Imidazole
1X Native Purification Buffer
3 M Imidazole, pH 6.0
pH 8.0

Native wash buffer
1X Native Purification Buffer
20mM Imidazole, pH 6.0
pH 8.0

Native elution buffer
1X Native Purification Buffer
3 M Imidazole, pH 6.0

pH 8.0

2.16 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out based on the method described by Laemmli (Laemmli, 1970). Samples were mixed with reducing sample-buffer (β-mercaptoethanol) and Tris-glycine SDS sample buffer (Novex by Life Technologies) in a 1:1 ratio and boiled for 5 min at 95 °C in a heating block. Samples were also run under non-reducing conditions depending on the aim of the experiment. Freshly prepared 10% resolving gels and 2% stacking gels using proto-gel quick cast (National Diagnostics) were used for all experiments. A prestained broad range protein ladder (Page Ruler plus, Fermentas) was used as a size standard. Gels were electrophoresed in running buffer at a maximum voltage of 140V for 1h. Staining was carried out for 5 min using instant blue (Expedeon).

2.17 Western blotting

Western blotting was applied: to confirm the presence/absence and quality of the expressed recombinant merozoite proteins; to test merozoite interaction with FH and to determine complement activation by merozoites leading to C3b formation. Depending on the experimental goal, samples were mixed with reducing sample buffer (β-mercaptoethanol) in a ratio of 1:1 and heated for 10 minutes at 60°C. The samples were fractionated by 12% SDS-PAGE under reducing conditions (140V for 1hr). Wet (tank) transfers were conducted to transfer the separated proteins from the gel onto an activated nitrocellulose membrane (activation was done using 100% methanol for 30 sec followed by soaking in transfer buffer).

Pre-soaked sponge pads, blotting paper, resolved SDS gel, PVDF membrane, blotting paper, and additional pre-soaked sponge pads were assembled in that order on the

cathode core of the XCell II Blot module (Invitrogen) and sealed and assembled onto the X-cell Surelock mini-cell system. The blot module was filled with transfer buffer and an outer chamber filled with distilled water or ice. Transfer was done at 100V for 45 minutes. Following transfer, the PVDF membrane was incubated with 4% Blotto, non-fat dry milk (Santa Cruz Biotechnology) prepared in TBS-T for 1 h. The membrane was then incubated with the primary and secondary antibody with in-between washes using TBS-T. Detection was done using Novex® ECL Chemiluminescent Substrate (ThermoFisher Scientific).

2.18 Mass spectrometry

Mass spectrometry technique was used to confirm the identity of the recombinantly expressed proteins. Briefly, proteins were treated with dithiothreitol (DTT) and iodoacetamide followed by trypsin digestion overnight at $37^{\circ}C$. The peptides were then purified using C18 Zip-Tips (Millipore, Germany) and injected into the liquid chromatography-mass spectrometry Q Exactive. Peptide identification was done using MASCOT search engine against the combined database of PlasmoDB.

2.19 ELISA binding assay using polyclonal anti-FH antibody ("Kilifi ELISA binding assay")

To narrow down merozoite proteins that interact with FH, a library of recombinant merozoite proteins (over 100 proteins) was systematically screened using an ELISA binding assay developed at Kilifi (also referred to as "Kilifi ELISA binding assay"). The assay was performed as follows. Individual 96-well plates, Dynex 4HBX Immunolon plates (Dynex Technologies Inc.), were coated with a starting concentration of 128 µg/ml or less of the recombinant proteins, followed by a twofold serial dilution in 100 µL of carbonate coating buffer. The plates were incubated overnight at $4^{\circ}C$ before washing four times with PBS/ 0.05%Tween (PBS-T; also called wash buffer). The

plates were then blocked using 1% skimmed milk in wash buffer and incubated at room temperature for 5 hours.

After another round of four times washing with PBS-T, the plates were incubated with 100 µL of FH (Calbiochem) diluted at 0.42 ng/mL in blocking buffer and incubated overnight at 4°C. Plates were washed four times again and incubated for three hours at room temperature with 100 µL anti-Factor H Goat pAb (Calbiochem, USA) at 1/2500 in blocking buffer. Plates were again washed followed by addition of 100 µL of Horseradish Peroxidase (HRP) conjugated donkey anti-goat IgG antibody, (Chemicon, USA) at 1/5000 in blocking buffer and incubated at room temperature for three hours. A final wash was done followed by detection using o-phenylenediamine dihydrochloride (OPD) (Sigma). The reaction was stopped with the addition of 25 µL of 2M H_2SO_4 per well and absorbance read at 492nm.

Coating buffer (Carbonate buffer pH 9.4-9.6)
1.59g Na2CO3
2.93g NaHCO3
1L distilled water

2.20 ELISA binding assay using monoclonal anti-FH antibody ("Helsinki ELISA binding assay")

The ELISA binding assay developed in Kilifi ("Kilifi ELISA binding assay") had some limitations discussed later in chapter three. I, therefore, improved this assay in Helsinki (also referred to as "Helsinki ELISA binding assay") by using different buffers and detection antibodies. "Helsinki ELISA binding assay" was conducted as follows. Recombinant merozoite proteins or Cd4 tag (16 ug/ml) in coating buffer (carbonate buffer pH 9.4) were coated onto MaxiSorp microtiter plates (Nunc) at 4°C overnight. After washing three times using 1X PBS-T blocking was done using 1% BSA in 1X

PBS-T for 1 hour followed by addition of FH (Calbiochem) in 0.1% BSA in 0.25X VBS++ was added and another round of incubation for 1 hr and after that washing was done with PBS-0.01% Tween. Afterwards monoclonal anti-FH antibody (isotype IgG2b$_k$; clone 131X, directed against CCP8-15) diluted at (1:500 0.1% BSA in 0.5X VBS++) was added and after an hour of incubation HRP rabbit anti-mouse antibody (at 1:2000 in 0.1% BSA in 0.5X VBS++) was added. The reaction was developed with OPD (Sigma) and stopped by the addition of 25 µL of 2M H_2SO_4 per well and absorbance read at 492nm.

5X VBS buffer
83.0 g NaCl
10.19 g Sodium 5,5-diethylbarbiturate
Water
Adjust pH to 7.3

2.21 Surface plasmon resonance

Surface plasmon resonance (SPR) is a label-free, real-time method that allows the determination of the affinity and kinetics of a wide range of molecular interactions (such as protein-protein interactions, DNA-DNA, DNA-protein, lipid-protein) (Cooper 2002). It remains the gold standard for measuring protein-protein interactions (Yang et al. 2016) and is extensively applied in multiple stages of drug discovery and development (Myszka and Rich 2000). The main advantage of SPR is that it allows highly sensitive measurement of low-affinity interactions among macromolecules that could otherwise be missed if using other techniques (Hoffrogge et al. 2003; Hornemann et al. 2003). SPR can also be used as a confirmatory test downstream of an avidity-based extracellular interaction screen (AVEXIS) (Crosnier et al. 2011; Dundas et al. 2018) and yeast two-hybrid screen (Hoffrogge et al. 2003; Hornemann et al. 2003). For this study, SPR was used to confirm the interaction observed with the "Helsinki ELISA binding assay." The SPR experiments were all conducted using a Biacore T100 and the amine coupling kit (GE Healthcare).

2.21.1 Buffer exchange for the recombinant merozoite proteins

Before running the merozoite recombinant proteins on the SPR platform, buffer exchange was performed to allow for compatibility with the SPR buffers. Zebra spin desalting columns (Thermo Scientific) were used for buffer exchange. Briefly, the procedure entailed inverting the spin column to suspend the slurry, removing the cap of the column, placing the column in a 1ml microcentrifuge collection tube. The columns were centrifuged at 1500g for one minute. The flow-through discarded and 300ul of 0.5X VBS++ added for a three times wash. 40-100ul of the recombinant antigen was added and collected in a new centrifuge tube after spinning at 1500 g for 2 minutes. The eluted protein was quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific).

2.21.2 Ligand immobilization

Human complement factor H (FH) (Calbiochem) and FH fragments produced in-house at Prof Seppo Meri's laboratory at the University of Helsinki were immobilized onto flow cells in the CM5 sensor chip using a standard amine coupling protocol **Figure 2.4**. First, the carboxyl groups on the sensor surface were activated by injecting a fresh mixture of (1-ethyl-3-3-dimethylaminopropyl) carbodiimide (0.4M) mixed 1:1 with N-hydroxysuccinimide (0.1M). Second, FH or FH fragments prepared at 30 mg/mL in 10 mM sodium acetate (pH 4.5) were injected over the activated surface, to allow for covalent attachment through their primary amines. Lastly, the excess reactive esters were blocked with 1 M ethanolamine, a process called regeneration. The goal of regeneration is to elute any non-covalently bound analyte without disrupting the activity of the ligand. Regeneration allows the sensor chip flow cell to be reused many times (Mullett et al. 2000; Ahmed 2008; Drescher et al. 2018). Each step was performed with a 7-min injection at a 5 ml/min flow rate. Flow cell one was left blank to provide a

reference surface. In a typical SPR experiment what is immobilized is called a ligand and the analytes (i.e. the samples) are injected into.
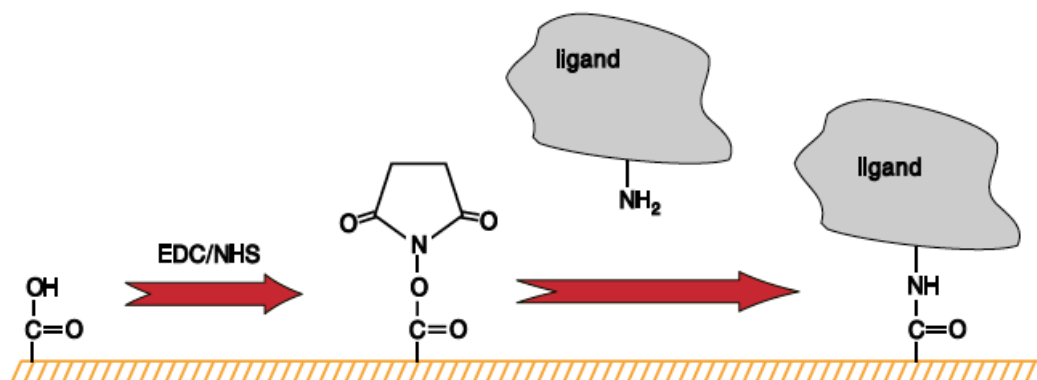
Figure 2.4 Schematic of a typical amine coupling of ligands to the sensor chip.

Activation of the dextran matrix on the sensor chip is done using a mixture of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) and N- hydroxysuccinimide (NHS) to expose the reactive esters. When the ligand is passed over the surface and the esters react spontaneously with amino so that the ligand covalently attach to the matrix. After injection of ligand, ethanolamine is passed over the sensor surface to deactivate remaining active esters.

2.21.3 Analysis of protein-protein interactions by using CM5 sensor chip

Analysis of protein-protein (FH-merozoite proteins) interactions was conducted as follows. The CM5 sensor chip was docked on the Biacore T100 instrument and after immobilization of the ligand (FH) as stated above, 0.5 uM of the analyte (recombinant merozoite proteins) prepared in running buffer (10mM HEPES pH7.4, 150 mM NaCl, 0.005% Tween20) were injected into the experimental and reference flow cells at a flow rate of 20 ul/ml. For each experiment, a blank flow cell was used as the reference surface. The NaCl solution (1 M) was used to regenerate the surface after each injection cycle. All buffer solutions were freshly prepared, degassed, and passed through a 0.22 mm pore size filter. Results were analyzed using the BiaEvaluation

software (GE Healthcare). An example of the expected real-time SPR response data, which is referred to as a sensorgram is illustrated in **Figure 2.5**.
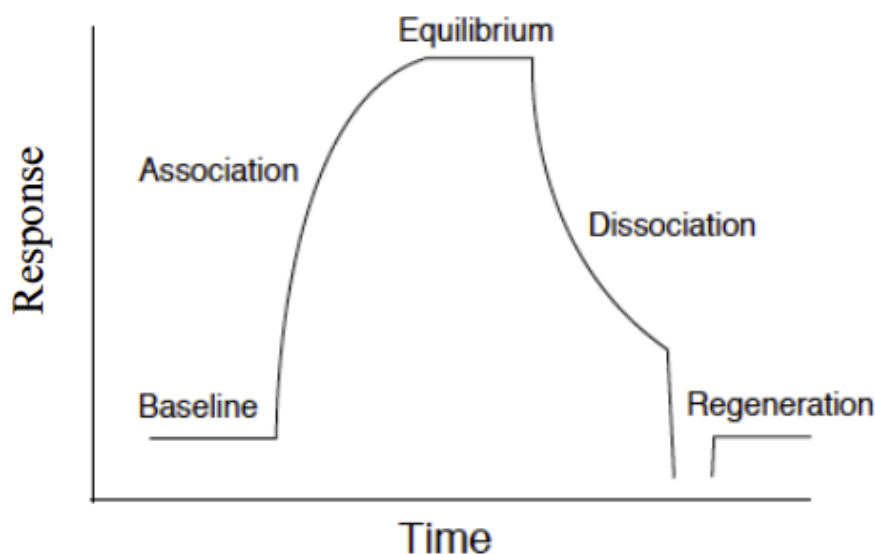
Figure 2.5. Schematic of a typical surface plasmon resonance sensorgram.

Before injecting an analyte, the baseline response should be stable. An increase in response during the association phase represents protein-protein interaction. Equilibrium is achieved when an equal number of analyte molecules associate with and dissociate from the surface at the same time. After washing the surface, the decay rate of the complex can then be obtained during the dissociation phase. Following regeneration, the binding response should return to the initial baseline position. Figure adapted from (Myszka and Rich 2000).

2.22 Protein microarray

Antibody responses to merozoite FH receptor proteins were analyzed using KILchip v1.0 protein microarray (Kamuyu et al. 2018). Briefly, each well in the slide wa washed 3 times with 250 µL of wash buffer 1 with a 5-minute incubation following each wash. The wells were additionally washed 3 times with 250 µL wash buffer 2. Non-specific binding was blocked using blocking buffer for 2 hours at room temperature in the dark while rotating. Wells were then washed 3X with 250 µL of wash buffer 1 with a 5-minute incubation followed by a single wash in wash buffer 2. Serum was then added to the wells after a 1:400 dilution in the diluent buffer and incubated overnight at 4°C while

rotating. Wells were subsequently washed 3X with 250 µL of wash buffer 1 with a 5-minute incubation followed by a single wash in wash buffer 2. Afterwards, 150 µL of secondary antibody (Donkey anti-human IgG-Alexafluor 647 conjugated) at a 1:400 dilution in diluent buffer was added. The samples were incubated for 3 hours at room temperature while rotating in the dark. After the incubation three washes were performed using wash buffer 1 with a 5-minute incubation and subsequently two washes with wash buffer 2. Slides were carefully disassembled from hybridization cassettes and rinsed using wash buffer 3 in coplin jars. Slides were dried by centrifugation using a blotting paper-lined Eppendorf combiSlide adapter for 5 minutes at 300g. Finally, slides were scanned using a genepix 4000B scanner coupled to GenePix pro7 Microarray acquisition and analysis software. Data processing and further analysis were performed in R **(**R Core Team, 2013). **Figure 2.6** illustrates the steps involved in the microarray experiment.
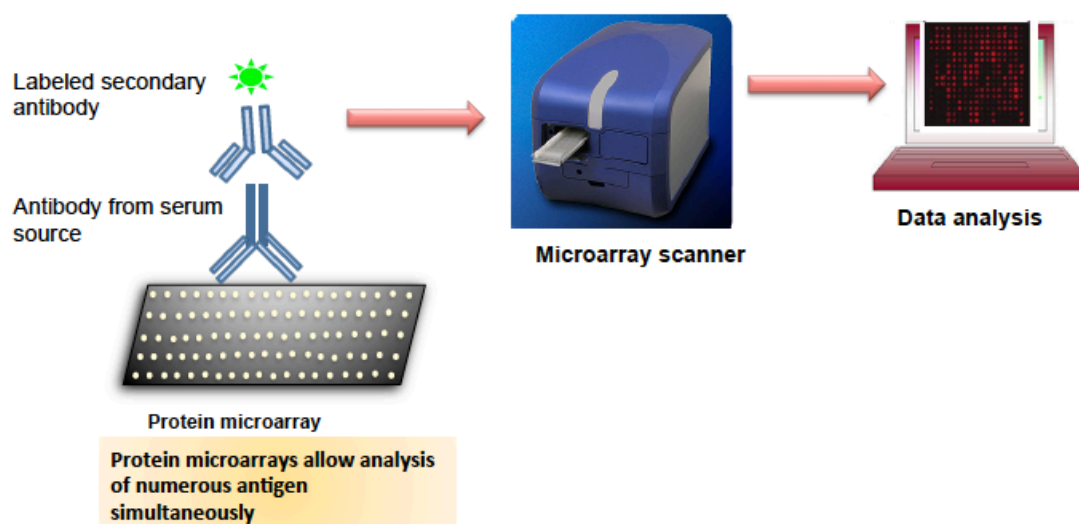
Figure 2.6. Overview of protein microarray procedure

After initial washing and blocking, serum is added to the wells and incubated overnight at 4°C while rotating followed by additional washing steps. Thereafter a secondary antibody (donkey anti-human IgG-Alexafluor 647-conjugated) is added. After another round of washing the slides are scanned using genepix 4000B scanner coupled to GenePix pro7 Microarray acquisition analysis software and data analyzed using R (R core Team (2013)).

10X HEPES buffer saline (HBS) buffer
1.4M NaCl, 50mM KCl, 20mM CaCl2, 10mM MgCl2, 100mM HEPES

Wash buffer 1
0.1 % Tween 20/1X HBS

Wash buffer 2
1 X HBS

Wash buffer 3
MilliQ water

Block and diluent buffer
2% IgG free Bovine Serum albumin (BSA)/0.1 % Tween 20/ 1X HBS

2.23 Quantification of the soluble terminal complement complex (sC5b-9)

To determine the level of complement activation in children with severe malaria and uncomplicated malaria, MicroVue sC5b-9 Plus Enzyme Immunoassay kit (Quidel) was used. The kit measures the concentration of soluble terminal complement complex (sC5b-9) hence showing the status of the terminal complement pathway in the analyzed specimen. The plates come coated with a monoclonal antibody to the C9 ring of the sC5b-9 to capture the complex. The trapped sC5b-9 is subsequently detected with HRP-conjugated antibodies that bind to antigens of the sC5b-9 complex. All the experiments were conducted according to the manufacturer's instructions.

2.24 ELISA for TCC activation by merozoites

Potential merozoite-dependent activation of complement leading to terminal complement complex formation (TCC) was tested (Rosa *et al.*, 2015). Merozoites were incubated with cell culture medium containing 20 vol% NHS or HIS for 1 hr at 37°C (Rosa et al. 2015b). The merozoites were spun down, and supernatant collected for TCC quantification using MicroVue sC5b-9 Plus Enzyme Immunoassay kit (Quidel, USA) following the manufacturer's instruction. Absorbance at 450 nm was measured.

2.25 Quantification of FH using ELISA

FH was measured using an available commercial kit, MicroVue Factor H EIA (Quidel, USA). The kit is a three-step procedure utilizing: a micro assay plate coated with a monoclonal antibody that binds specifically to human Factor H **Figure 2.7**; an HRP-conjugated murine anti-human Factor H; and a chromogenic substrate. The kit comes complete with standards of known concentrations and controls. The specimen diluent was applied to select few wells as blanks, to control for background. Serum samples were suspended in specimen diluent kit buffer and the procedure then followed the manufacturer's instructions. OD was measured for absorbance at 450 nm. Due to the cross-reactivity of FH with other FH related proteins (Józsi et al. 2018) the kit uses antibodies that are very specific for FH, shown in **Figure 2.7**.

Figure 2.7. FH and FH related proteins

Circled in red are the specific regions that are targeted by antibodies of the MicroVue Factor H

EIA kit (Quidel, USA). Figure adapted from Józsi et al. 2018.

2.26 DNA extraction

Archived EDTA blood samples (stored at -80°C) were used to extract DNA using the QIAmp DNA blood kit (Qiagen). Due to the limited available starting materials for some samples, adjustments were made to extract from 10 µL of the EDTA blood samples.

The sample volume was topped up to 50ul using 1X filtered sterile PBS. 10ul of proteinase K and 50ul of buffer AL were added, vortexed and incubated at 56°C for 10 minutes. After the addition of 50ul of 100% ethanol, the contents were transferred to QIAamp Mini spin column, washed twice and eluted in 40uL of buffer AE. DNA material was quantified using Nanodrop.

2.27 Polymerase chain reaction

The Polymerase Chain Reaction (PCR) was performed in order to genotype the Y402H variant in the severe malaria cohort. The T to C transition at nucleotide position 1277 in exon 9 of complement factor H results in a tyrosine to histidine substitution at codon 402 of the protein (Rodríguez De Córdoba et al. 2004). PCR was performed with the use of 5'- TCATTGTTATGGTCCTTAGGAAA -3' as the forward primer and 5'-TTAGAAAGACATGAACATGCTAGG-3' as the reverse primer (Lau et al. 2006). The composition of a typical 10 µL PCR reaction using the Expand™ High Fidelity PCR System (ThermoFisher Scientific) is shown in **Table 2.3** while the standard cycling parameters are shown in **Table 2.4**.

Table 2.3. Composition of a typical PCR reaction

Ingredients	10ul reaction
Mix1	
dH$_2$O	2.7 µL
dNTPs	0.2 µL
MgCl$_2$	1.0 µL
Forward primer	0.3 µL (10 pmol)
Reverse primer	0.3 µL (10 pmol)
Mix 2	
dH$_2$O	3.86 µL
Expand High Fidelity Buffer (10×) with 15 mM MgCl2	1.0 µL
Expand High Fidelity Enzyme	0.14 µL
DNA	0.5 µL

Table 2.4. Standard PCR cycling parameters

Step	Temperature	Duration	No. of cycles
Initial melting	94°C	2 min	1
Melting	94°C	15 sec	35
Annealing	56°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	7 min	1

2.28 Agarose gel electrophoresis

Generated PCR products were analyzed by agarose gel electrophoresis. Two percent of agarose gels were prepared in 1X 0.5% TBE buffer (Invitrogen). After cooling Red Safe (iNtRON Biotechnology) was added into the melted agarose for visualization of the DNA under ultraviolet (UV) light. The set gels were run in TBE buffer after samples were loaded with loading dye (6x from NEB). Gels were run at 90-100V for approximately an hour. The DNA was visualized using the Molecular Imager ChemiDox XRS imaging system (BioRad).

2.29 Sequencing for Y402H positive variants

Products of PCR reaction were cleaned up using ExoSAP-IT (Affymetrix) in order to remove unused primers and nucleotides. Five microliters of the post-PCR reaction product were added with 1ul of ExoSAP-IT. This was followed by an incubation at 37°C for 20 minutes to degrade remaining primers and nucleotides and an additional incubation at 80°C for 20 minutes to inactivate ExoSAP-IT reagent. The cleaned PCR products were diluted by addition of 45ul of PCR water (SIGMA-ALDRICH). The cycle sequencing reaction was then set up. **Table 2.5** below shows the reaction volumes per sample and **Table 2.6** shows the cycling conditions.

Table 2.5. Composition for the cycle sequencing reaction

Ingredients	Reaction vol. (µL)
Big dye enzyme mix	0.5 µL
5X BigDye sequencing buffer	1.75 µL
dH$_2$O	4.75 µL
DNA PCR product	1.0 µL
Forward primer	1.0 µL (5 pmol)
Reverse primer	1.0 µL (5 pmol)

Table 2.6. Cycle sequencing PCR thermal cycling condition

Step	Temperature	Duration	No. of cycles
Initial melting	95°C	30 sec	1
Melting	95°C	10 sec	25
Annealing	50°C	0.05 sec	
Extension	60°C	4 min	
Final extension	15°C	7 min	1

To remove excess dye terminators, cycle sequencing PCR products, ethanol/EDTA precipitation was executed. Briefly, 1ul of 125mM EDTA (Sigma) and 1ul 3M sodium acetate were added to the cycle sequencing PCR product. After incubating for 15 minutes, the samples were centrifuged/spun at maximum speed for 20 minutes at 20°C. The plates were gently inverted on a paper towel in order to drain off the supernatant and spinning was done briefly at 400rpm for one minute to ensure complete drain off. Next, 100ul of 70% ethanol was added to each sample well and centrifugation done at a maximum speed of 20°C for 5 minutes. Supernatant was drained off and the plates were left to air dry at room temperatures for 30 minutes. Hi-Di at 10ul was then added and samples were denaturated at 95°C for 3 minutes. Capillary electrophoresis was done in the ABI 3130xL (Applied Biosystems).

2.30 Human Complement Factor H, 402H variant detection using ELISA

In addition to sequencing, ELISA was implemented to detect the 402H or Y402 variant using Factor H, 402H/Y variant detection, Human, ELISA kit (Hycult Biotech). This was possible due to the availability of paired EDTA blood and heparin serum samples for most of the severe malaria hospital case-control subjects. The ELISA is based on a sandwich principle in which the plates come coated with a monoclonal antibody to the 402H or Y402 variants, and the samples are incubated together with the peroxidase-conjugated antibody. The conjugated antibody binds to the captured factor H variants and tetramethylbenzidine (TMB) substrate is used for detection. Absorbance was read at 450 nM.

3   Multiple blood stage *Plasmodium falciparum* merozoite antigens interact with human complement factor H and may facilitate complement evasion

3.1   Summary and aims

In this chapter, I sought to identify merozoite proteins other than the previously described Pf92 (Kennedy et al. 2016) that interact with human complement regulatory protein factor H and may, therefore, allow *Plasmodium falciparum* to evade complement-mediated destruction. To achieve this, I used two techniques: i) a modified ELISA binding assay and ii) surface plasmon resonance (SPR), to screen over 100 recombinant merozoite proteins. The merozoite proteins have previously been described (Crosnier et al. 2013; Zenonos et al. 2014; Kamuyu et al. 2018) and were part of a larger study to define antibody targets of protective immunity led by Prof Faith Osier. Thereafter, I localized the binding sites of these proteins in FH and subsequently used a modified growth inhibition assay to assess the significance of the interaction between FH and the malaria parasite. For the modified ELISA binding assay and SPR, I used Pf92 (Kennedy et al. 2016) as the positive control and Cd4 tag (present in each antigen) as the negative control.

3.2   Introduction

Malaria remains among the leading causes of childhood morbidity and mortality globally (WHO 2018b). Scaling up of malaria control interventions such as insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), larvicides, chemoprophylaxis and artemisinin-based combination therapies has led to reduction in malaria-associated hospitalization and deaths (Bhatt et al. 2015; WHO 2015; Murray et al. 2012; Ceesay et al. 2008; O'Meara et al. 2008; Aregawi et al. 2011). However, these

gains may be thwarted by the rapid spread of antimalarial drug resistance (Ashley et al. 2014). The most advanced malaria vaccine, RTS,S is only partially protective against disease, and the obtained immunity wanes over time (White et al. 2015). Therefore, designing the next second-generation vaccine will benefit from understanding how to interrupt the complex parasite life cycle and the discovery of new vaccine targets.

Complement forms part of the innate immune system and consists of a tightly regulated network of proteins that are important in host defense and inflammation. Complement activation results in opsonization of target microbes and their subsequent removal by phagocytosis, as well as cell lysis (Sarma and Ward 2011). There are three established mechanisms of complement activation: classical, lectin, and alternative pathways. All the three pathways converge on the C3 molecule, where activation leads to covalent deposition of the opsonins C3b and iC3b, generation of inflammatory anaphylatoxins C3a and C5a and formation of the membrane attack complex (Lambris et al. 2008; Dunkelberger and Song 2010). Under normal circumstances, activation of complement is controlled by the coordinated action of soluble (e.g. C1-INH, C4bp, factor H, factor I, S-protein and clusterin) and membrane-associated (DAF, CR1, MCP, CRIg, and CD59) complement regulatory proteins (Lambris et al. 2008; Serruto et al. 2010). Complement regulation ensures that activation is focused on surfaces of invading microorganisms and, on the other hand, limited against host cells and tissues (Walport et al. 2010).

Pathogens exploit multiple strategies to interfere with complement immune recognition and effector functions (Zipfel et al. 2007; Blom et al. 2009). A common strategy employed by many pathogens for immune evasion is the hijacking of human complement regulatory proteins such as FH, essential for alternative pathway regulation and C1- esterase inhibitor (C1-INH) that regulate both the classical and the

lectin pathways. Once they recruit the complement regulatory proteins to their surfaces, microbial surfaces disguise themselves as "self" and are protected from complement activation and amplification. Identifying pathogen proteins that act to inhibit complement activation can lead to the discovery of new vaccine antigens and enhance our understanding of pathogen biology.

Recent studies suggest that *Plasmodium falciparum* avoids complement activation by recruiting complement regulatory proteins C1-INH (Kennedy et al. 2017) and FH (Simon et al. 2013; Rosa et al. 2015; Kennedy et al. 2015; Simon et al. 2018). It has been suggested that merozoite proteins actively recruit C1-INH to their surfaces, allowing the parasite to limit the alternative and the classical pathway proteases and hence avoid downstream complement activation (Kennedy et al. 2017). PfMSP3.1 was identified as the C1-INH interacting partner. Interestingly, although PfMSP3.1 parasite knockouts showed reduced C1-INH recruitment and increased C3b deposition, which would imply that they were more susceptible to complement-mediated killing, the knockout paradoxically had enhanced red blood cell invasion in the presence of active complement (Kennedy et al. 2017). More studies are needed to interrogate the host benefit of pathogen recruitment of regulatory proteins. However, parasite hijacking of FH for immune evasion has recently gained prominence.

During the mosquito stage of development, *Plasmodium falciparum* gametes recruit factor H (from the human blood ingested during a blood meal) to their surface to evade human complement attack within the mosquito midgut. Gamete surface protein GAP50 binds factor H (FH) and uses bound FH to inactivate the complement protein C3b. Furthermore, a monoclonal antibody directed against GAP50, when taken with the infected blood meal, led to impaired gametogenesis and blocked parasite transmission to the mosquito, pointing to the possibility of using GAP50 as a potential transmission-blocking vaccine candidate (Simon et al. 2013). Additionally, the *Anopheles* mosquito

midgut cells protect themselves from active complement in the blood meal by capturing factor H. Factor H inhibited complement on the midgut cells by promoting inactivation of C3b to iC3b and preventing the activity of the alternative pathway amplification C3 convertase enzyme. Interference of the FH regulatory activity by monoclonal antibodies, carried to the midgut via blood, resulted in increased mosquito mortality and reduced fecundity (Khattab *et al.*, 2015). The study by *Khattab et al.* also identified a hitherto unknown mosquito midgut FH receptor (Khattab *et al.*, 2015) that warrants further investigation.

Within the human host, there is evidence that intraerythrocytic schizonts and free merozoites bind FH and FH-like protein (FHL-1) to their surfaces. This binding inactivates C3b to ensure survival during the erythrocytic replication phase (Rosa et al. 2015). Indeed, of all the parasite asexual stages, free merozoites and intracellular mature schizonts have been shown to significantly bind more FH molecules than trophozoites and rings (Simon et al. 2018). So far only merozoite protein Pf92 has been characterized as the protein responsible for interaction with FH. Deletion of the Pf92 gene led to somewhat increased complement-mediated destruction of merozoites (Kennedy et al. 2016). I hypothesized that more merozoite proteins are involved in complement evasion.

3.3 Specific aims

a) Expression of a library of recombinant merozoite proteins

b) Develop an ELISA-binding assay to test for binding between FH and merozoite proteins

c) Use Surface Plasmon Resonance (SPR) to confirm the binding observed in (b) above

d) Localize the FH CCP modules involved in binding

e) Demonstrate the consequences of the binding

3.4   Materials and methods

To determine which merozoite proteins interact with human complement FH for parasite survival during asexual blood stage, recombinant proteins were expressed, and a modified ELISA binding assay used to down-select antigens that bound to FH. The initial modified ELISA binding assay (also referred to as the Kilifi binding assay) was used to screen over 100 proteins. However, the Kilifi ELISA binding assay had some limitations and was subsequently modified. This latter assay is referred to as the Helsinki ELISA binding assay. Surface Plasmon Resonance (SPR) was used to confirm the interactions observed with the Helsinki ELISA binding assay. Both the modified ELISA binding assays and SPR are described in detail in the materials and methods chapter **section 2.19, 2.20 and 2.21.**

Previous studies that identified merozoite receptors, Pf92 and MSP3 as antigens that interact with complement regulatory proteins FH and C1-INH respectively, used pull-down assays, co-immunoprecipitation, followed by identification using mass spectrometry (Kennedy et al. 2015, 2017). The same methods were used to identify PfGAP50 as a gamete protein receptor for FH (Simon et al. 2013) during parasite sexual stage development in the mosquito. I used modified ELISA binding assay and SPR as these techniques would allow me to test each antigen for interaction with FH individually.

Similar approaches have been used to detect interacting partners between proteins. Galaway *et al.* used Avidity-based Extracellular Interaction Screen (AVEXIS) to screen a library of recombinant merozoite proteins and showed that P113 binds to the N-terminal domain of RH5, RH5Nt (Galaway et al. 2017). Furthermore, SPR was used

to quantify the biophysical properties of P113 and RH5Nt (Galaway et al. 2017). AVEXIS uses the same principle as the ELISA binding assay, but the only difference is that in the former the recombinant proteins need to be produced in two forms; as a biotinylated bait and a pentamerized enzyme-tagged prey (Kerr and Wright 2012). Other examples include the use of an ELISA binding assay to demonstrate that RH4 bound better to CCP1-3 compared to CCP10-11, 15-17, 21-22 and 15-25 of CR1 (Tham et al. 2011) and the mapping of the binding site of vitronectin to peptide fragments of SERA5 (Tou et al. 2018).

Multiple alignment of merozoite antigens that bound well to FH was done using MUSCLE software https://www.ebi.ac.uk/Tools/msa/muscle/ to determine whether there are shared motifs within the amino acid sequences of these antigens.

3.4.1   Statistical analysis

Spearman correlations were calculated using the GraphPad Prism (San Diego, CA, USA) version 7. Surface Plasmon Resonance (SPR) data were analyzed using BIAevaluation software (GE Healthcare).

3.5   Results

3.5.1   Merozoites activate complement causing deposition of C3b and TCC formation

During the asexual stage parasite development, malaria antigens either on infected red blood cells, antigens released during schizont rupture or membrane-bound organelles containing malaria pigment hemozoin (digestive vacuoles) can all activate complement (Greenwood and Brueton 1974; Glew et al. 1975; Adam et al. 1981;

Dasari et al. 2012). I determined whether merozoites specifically activate complement causing C3b deposition **Figure 3.1 A,** which can lead to the formation of the terminal complement complex (TCC). Merozoites were prepared as described in (Boyle et al. 2010b). They were then incubated with normal human serum (NHS), or heat-inactivated complement/serum (at 56°C for 30 minutes) or using EDTA of 500 mM or 125 mM concentration. Merozoite incubation in PBS was used as a negative control. After incubation for one hour at 37°C, the pellet was tested for C3b deposition using Western Blotting under reducing conditions while the supernatant was tested for TCC formation using a commercial ELISA kit (Quidel).

Complement C3b and its breakdown product iC3b containing fragments α'_1 and α'_2 were observed to accumulate on the merozoites in the presence of active complement (NHS). C3b accumulation was not observed in the negative control **Figure 3.1 B**. A previous study had observed that indeed C3b gets deposited on the merozoites, but its binding was reduced in HIS and completely absent when complement was inactivated by 10 mM EDTA (Kennedy et al. 2015). To interrogate the results further, I used schizonts to test whether they could also activate complement and lead to C3b formation, and whether there could be differences between C3b deposition in NHS or HIS. I observed no differences in C3b deposition on schizonts whether using NHS or HIS, similar to what I found out with merozoites **Appendices Figure 3.1**. Differences in experimental procedures could account for the contradictory results with what was seen with Kennedy et al. work.

Merozoites also activated complement and led to TCC formation **Figure 3.1 C**. Heat inactivation of serum to reduce complement activity has traditionally been done in various laboratories (Soltis et al. 1979), however, there is no clear evidence to show how much of complement activity is reduced by heating *per se*. I speculate that the current observation of C3b deposition and TCC formation might imply that the

complement inactivation by heating or treatment with EDTA did not completely reduce complement activity. More likely, however, since there was no difference between the active and inactivated serum, the result indicates that the merozoites can defend themselves against complement attack.
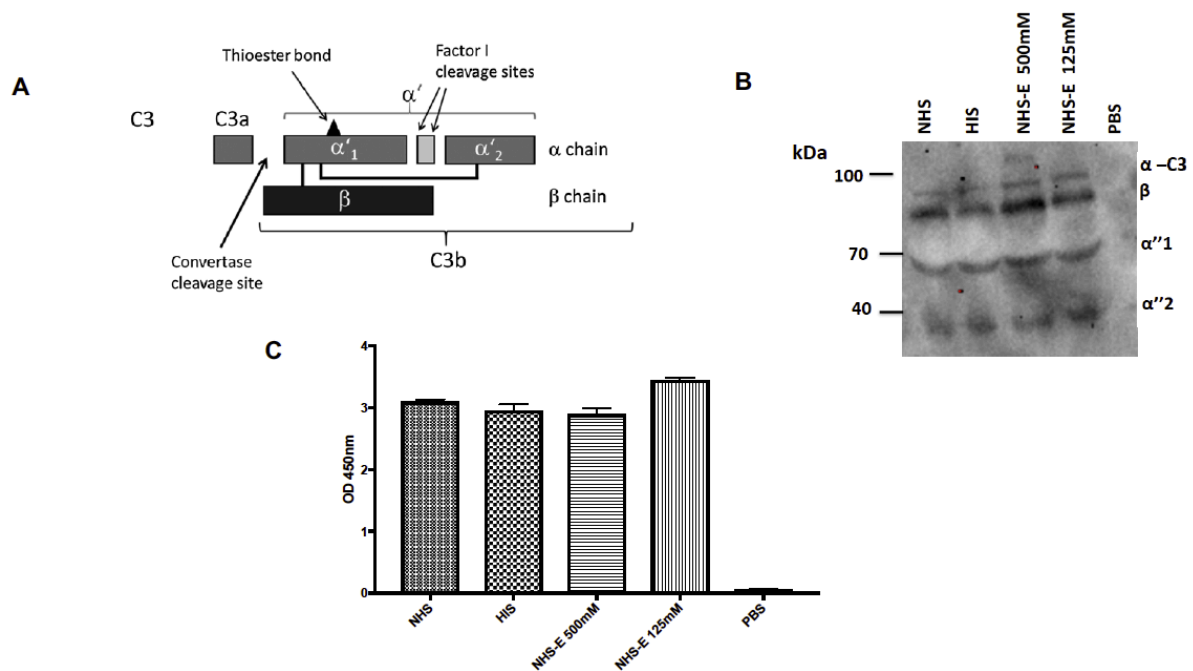
Figure 3.1 C3b deposition and TCC formation by merozoites

(A) Schematic depiction of C3 molecule structure. The polypeptide sizes are: C3 (180 kDa); C3a (9 kDa); C3b α (109 kDa), β (75 kDa); α' (101 kDa), α'$_1$ (67 kDa) and α'$_2$ (40 kDa). **(B)** Merozoites were incubated with normal human serum (NHS), heat inactivated serum (HIS) or NHS inactivated using EDTA (NHS-E 500 mM or NHS-E 125 mM) at 37°C for 1 hr. The proteins from the pellet were washed, and separated on SDS-PAGE under reducing conditions, transferred to an activated nitrocellulose membrane and probed for C3b using anti-C3b antibody and HRP detection antibody. Merozoite incubation with PBS was used as a negative control. **(C)** Merozoites were incubated with same conditions as **(B)**. The supernatant was used to detect TCC formation using a commercial MicroVue sC5b-9 Plus Enzyme Immunoassay kit.

3.5.2 *Merozoites bind FH from human serum as the complement source*

The previous observation of merozoites resisting activation of complement **Figure 3.1** could imply involvement of FH in order to facilitate the rapid breakdown of C3b, a process important for complement immune evasion. To test this assumption, merozoites were incubated with 20 vol% normal human serum (NHS), 20 vol% heat-inactivated serum (HIS), complement inactivated by EDTA (NHS-E) or with PBS and incubated at 37°C for one hour. Thereafter the merozoites were washed three times and the pellet proteins resolved by SDS-PAGE under reducing conditions followed by western blotting. Recombinant FH diluted at 1:400 in sterile PBS was loaded as a control. As shown in **Figure 3.2** merozoites bound FH from NHS and heat-inactivated serum and the band was resolved at the molecular weight of 155 kDa similar to recombinant FH. Some studies have suggested that, due to the close similarities between FH and FHL-1 (Józsi et al. 2018), pathogens that recruit FH also bind FHL-1 to their surfaces (Hellwage et al. 2001; Pandiripally et al. 2002). Apparently, the sensitivity of the assay used was not high enough to detect the small amount of serum FHL-1 or the FH binding proteins bound to domains other than those present in FHL-1.
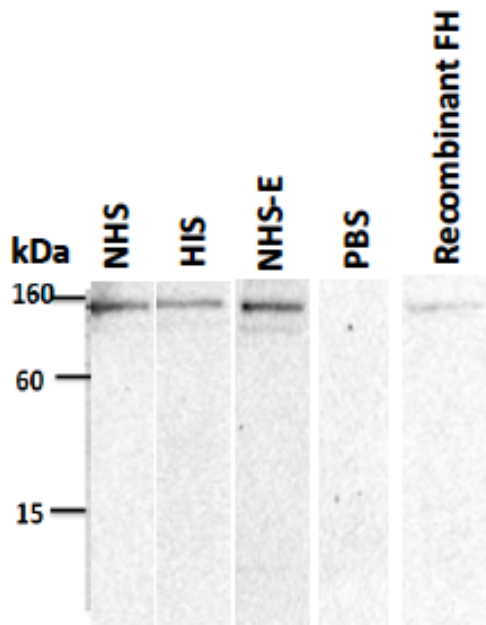
Figure 3.2 Merozoites recruit FH to their surfaces from human serum.

Merozoites were incubated with NHS, HIS, NHS-E (500 mM EDTA) or PBS buffer only for one hour at the 37 degrees in the incubator. After three washes, the pellet proteins were resolved by SDS-PAGE and analyzed by western blotting using polyclonal anti-FH antibody and HRP antibody. Recombinant FH diluted in 1:400 in sterile PBS was also loaded as a control for the western blotting experiment.

3.5.3   Expression of Plasmodium falciparum merozoite proteins

The selection of genes of interest, cloning, and production of plasmids has been previously published (Kamuyu et al. 2018). The criteria for selection of the proteins was based on whether they were known to be on the surface of the merozoites, were involved in erythrocyte invasion and had previously been associated with protection from malaria. Included on the list were also newly discovered antigens identified using a combination of immuno-proteomics and bioinformatics and that have not previously been tested in the context of naturally acquired immunity to malaria (Kamuyu et al. 2018). A complete list of the proteins used in this study is included in Supplementary Table 1 of (Kamuyu et al. 2018). Plasmids were already available from a previous study (Kamuyu et al. 2018) and I used these to express a selection of proteins. The

proteins were expressed using the Expi293 mammalian expression system and purified them using Ni-NTA purification system (Novex, life technologies). **Figure 3.3** below shows a western blot for a subset of randomly selected antigens. For some of them, 12/29 proteins demonstrated a single, distinct band of expected size, while the rest 17/29 had a band of expected size as well as smaller non-specific or degradation bands, which are not uncommon (Zenonos 2013).
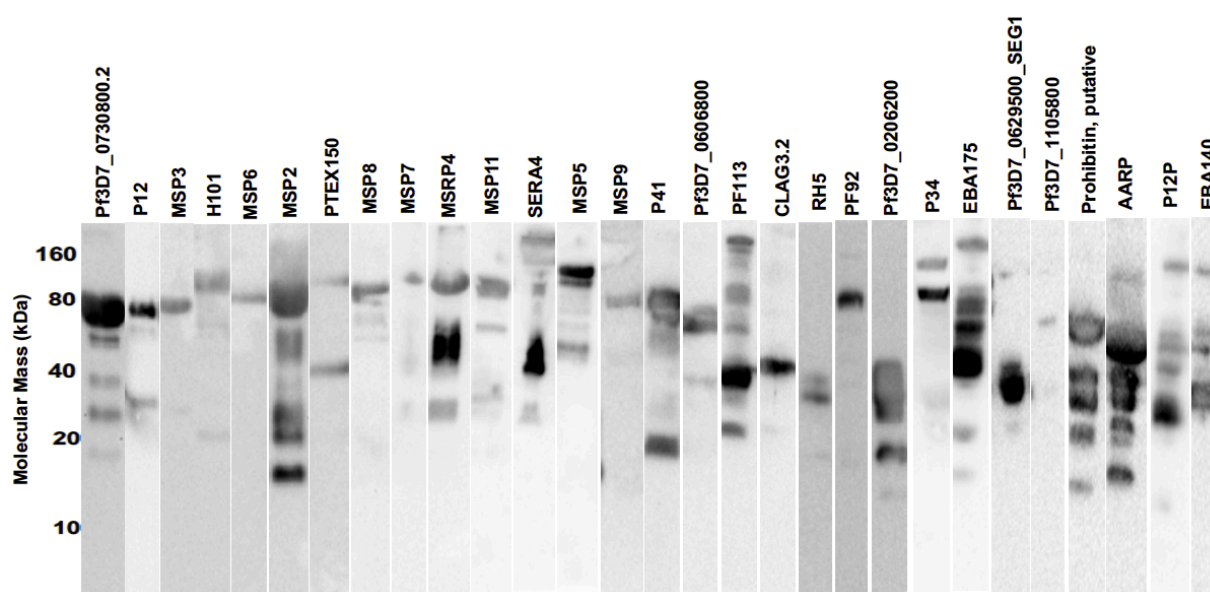
Figure 3.3 A selection of *Plasmodium falciparum* recombinant merozoite proteins that were expressed.

After expression of *Plasmodium falciparum* merozoite proteins, they were resolved by SDS-PAGE under reducing conditions, wet transferred to an activated nitrocellulose membrane and probed with anti-His antibody. Each protein contains a Cd4 tag of approximately 25kDa.

3.5.4   Development of the Kilifi ELISA binding assay

3.5.4.1   Checkerboard titration to optimize antigen coating concentration

I used an existing ELISA protocol (Osier et al. 2008) to develop the Kilifi ELISA binding assay **Figure 3.4 A**. I began by testing whether there would be recognition of FH by

Pf92 as previously shown (Kennedy et al. 2015), and subsequently used this as a positive control. The Cd4tag (present in all antigens), a non-parasite antigen, was used as a negative control. A checkerboard titration using varying concentrations of antigen was performed to determine the optimal concentration. A starting concentration of 128 ug/ml of Pf92 or Cd4 tag in two-fold dilutions was used. As shown in **Figure 3.4** Pf92 bound to FH in a dose dependent manner and antigen saturation was achieved from 16 ug/ml. There was low reactivity detected with the negative control.
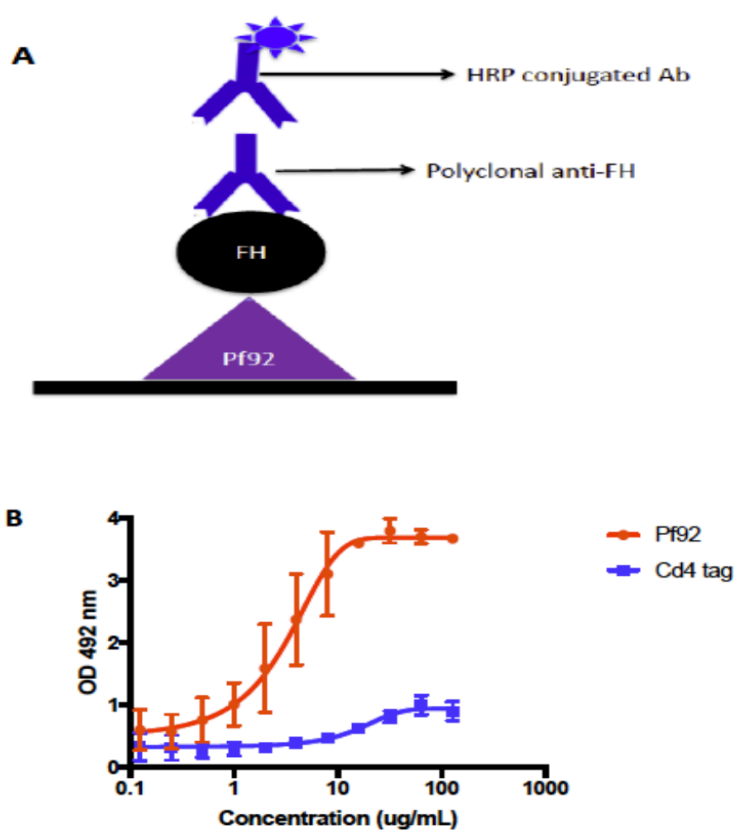
Figure 3.4 Binding of FH to immobilized Pf92 occurs in a dose-dependent manner and is saturable

(A) Schematic presentation of Kilifi ELISA binding assay **(B)** Microtiter wells were coated with Pf92 or control Cd4-tag. FH was added, and interaction detected using polyclonal anti-FH and HRP-conjugated antibody.

3.5.4.2 Checkerboard titration to optimize FH dilution

A checkerboard titration was performed to optimize the dilution of FH. The Pf92 and Cd4 proteins were immobilized on the microtiter plate. A varying concentration of FH, ranging from 0.525-0.131 ug/ml was added. Concentrations of the primary and secondary antibodies were kept constant at 38.3 and 0.16 ug/ml, respectively. As shown in **Figure 3.5**, no differential binding was observed in the range of dilutions tested for either Pf92 or Cd4 tag.
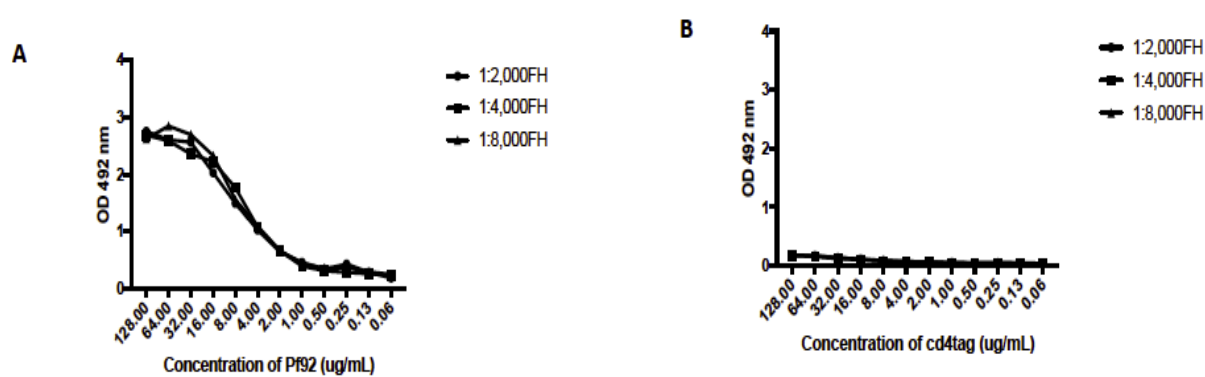
Figure 3.5 Checkerboard titration to optimize FH dilution.

Pf92 **(A)** and Cd4 tag **(B)** was immobilized on microtitrate plate and varying concentration of FH tested. The interaction was detected using polyclonal anti-FH and HRP-conjugated antibody

3.5.4.3 Checkerboard titration to optimize primary and secondary antibody dilutions

A checkerboard titration using varying primary (polyclonal anti-FH) and secondary (HRP) antibody were performed to determine the optimal concentrations. The Pf92 and Cd4 tag proteins were immobilized on microtiter plates. FH was added followed by varying primary antibody (starting with 1000 ug/ml) and secondary antibody (starting with 0.16 ug/ml). There was reduction in reactivity with reduction of both the primary and secondary antibody **Figure 3.6.**
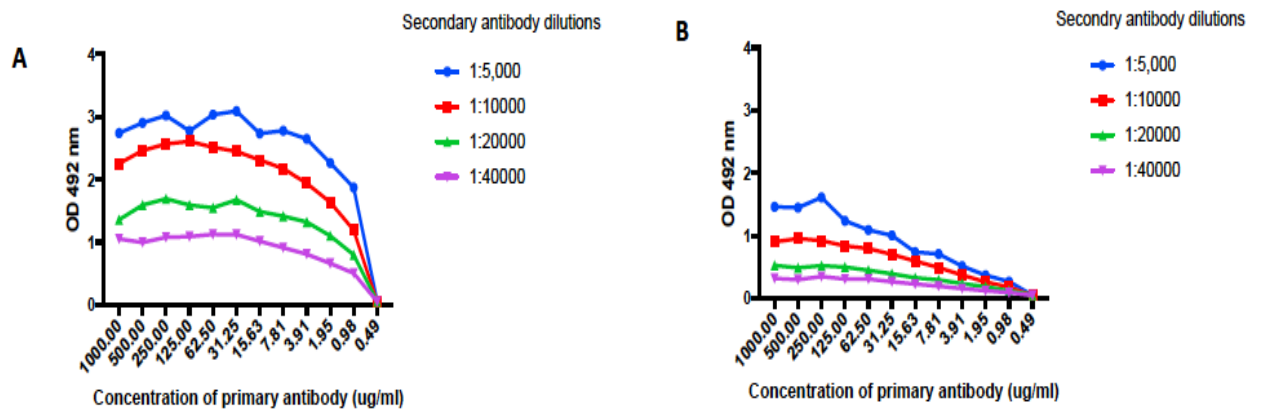
Figure 3.6 Checkerboard titration to optimize antibody dilution for Kilifi ELISA binding assay Pf92 (A) and Cd4 tag (B) was immobilized on an ELISA plate. FH was added and varied concentration of primary and secondary antibody added. Detection was done using OPD substrate and reading done at 492 nm.

In summary, based on the results obtained from **Figures, 3.4, 3.5** and **3.6** I used the following dilutions for the subsequent Kilifi ELISA binding assay: FH in 1:2500, primary antibody 1:2000 and secondary antibody of 1:5000.

3.5.5 Characterizing additional Plasmodium falciparum merozoite antigens that bind FH using Kilifi ELISA binding assay

Although the original publication indicated that Pf92 was the only merozoite protein that bound to FH (Kennedy et al. 2016), I hypothesized that the parasite would likely have additional proteins that interact with FH as a survival strategy. Having optimized the conditions to detect merozoite FH receptors **Figure 3.4, 3.5 & 3.6**, I therefore screened additional antigens to determine which ones bind to FH. An arbitrary cut off was used to classify the antigens as high FH binders; those that bound to FH as close to or equal to Pf92 (OD 3.64-2.36) **Figure 3.7 A**; intermediate FH binders bound to FH lower than Pf92 and above the negative control of Cd4 tag and have OD reading of

2.36-1.20 **Figure 3.7 B** and low FH binders as antigens that bound to FH as equal to the Cd4 tag or lower **Figure 3.7 C** with OD range of 0.98-0.29. In summary, the Kilifi ELISA binding assay identified antigens: PF34, PF3D7_1105800, PF3D7_0629500 (SEG1), EBA140, AARP, Prohibitin, MSP8 and P12p as probable merozoite FH receptors, also termed as high FH binders.
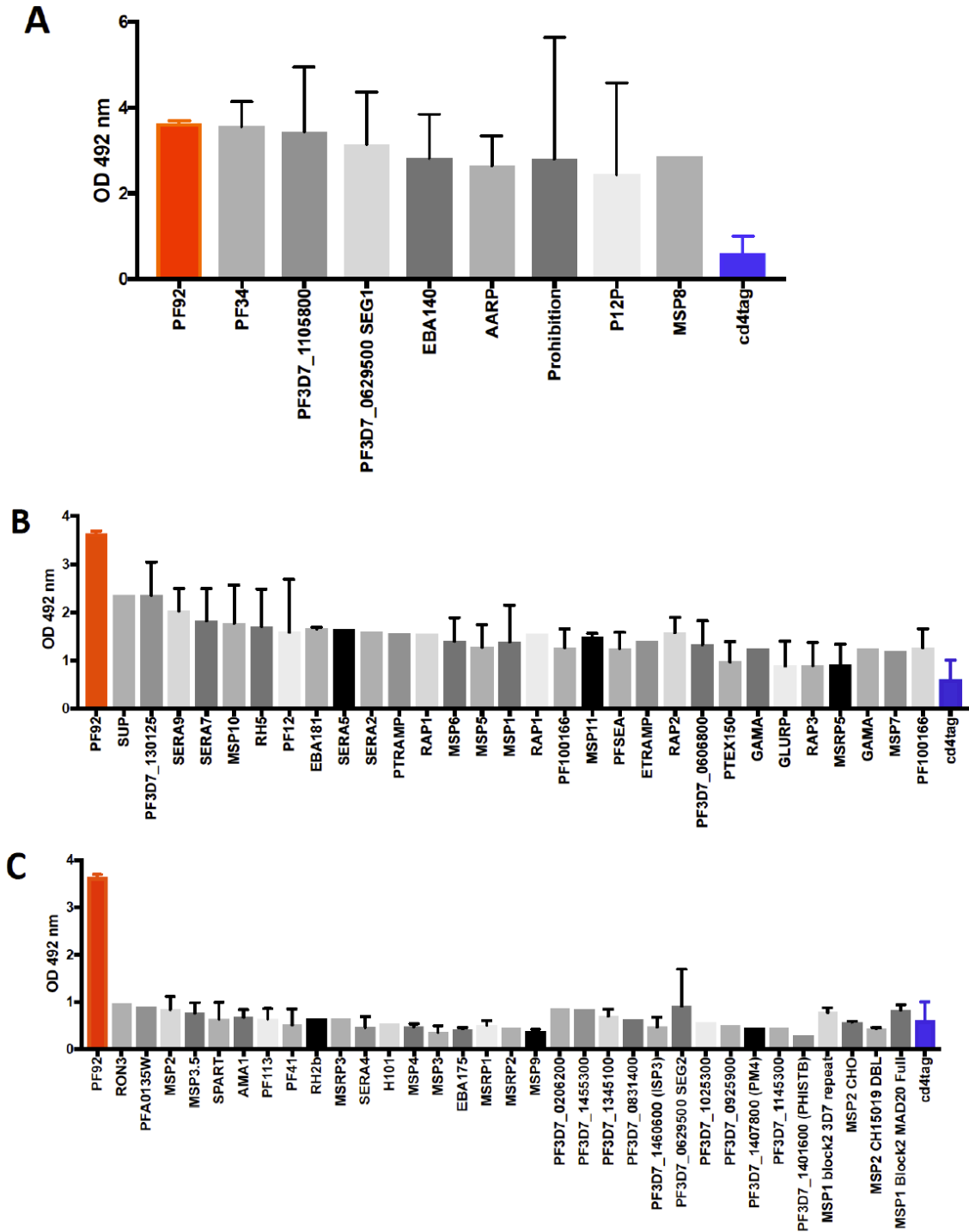
Figure 3.7 Differential binding of merozoite proteins to complement regulatory protein FH

Microtiter wells were coated with merozoite antigens at 128 ug/ml. FH at 1:2500 was added, and binding detected using anti-FH (1:2000) and HRP-conjugated antibody at 1:5000. **(A)** shows antigens that recognized FH as well as Pf92 or close to Pf92 also called high FH binders, **(B)** antigens that recognized FH at OD slightly lower than Pf92 but above the Cd4 tag also referred to

as intermediate FH binders or **(C)** antigens whose OD value was equal or lower than Cd4 tag, which are referred to as not binding/low FH. The data is representative of two independent experiments.

3.5.6   *Limitations of the Kilifi ELISA binding assay*

One of the major limitations of the Kilifi ELISA binding assay was that FH could not be completely titrated out as shown in **Figure 3.5** even when using varying concentrations of Pf92 **Appendices Figure 3.2**. To investigate possible explanations, I developed another checkerboard titration and tested a range of conditions including: a) no coating b) coating no blocking c) coating, blocking (using 1% milk in 1$\times$PBS-0.005% T), FH addition but no primary antibody d) coating, blocking, FH addition, primary antibody (anti-factor H goat polyclonal antibody, IgG), no secondary antibody and e) coating, blocking, FH addition, primary (anti-factor H goat polyclonal antibody, IgG) and secondary antibody. These conditions were first tested for both Pf92 and Cd4 tag coated at a constant concentration of 8ug/ml. **Figure 3.8 A** illustrates the plate map for the checkerboard. I observed that blocking did not affect reactivity, but binding occurred in the presence or absence of FH **Figure 3.8 B and 3.8 C** implying direct recognition of the Pf92 by the primary antibody. In the absence of primary antibody, there was very low, almost background OD reading.

A

FH	No FH	FH	No FH
No coating	**No coating**	**Coating, blocking, primary, secondary**	**Coating, blocking, primary, secondary**
No coating	No coating	Coating, blocking, primary, secondary	Coating, blocking, primary, secondary
Coating , no blocking	Coating , no blocking	BLANK	BLANK
Coating , no blocking	Coating , no blocking	BLANK	BLANK
Coating, blocking, no primary antibody	Coating, blocking, no primary antibody	BLANK	BLANK
Coating, blocking, no primary antibody	Coating, blocking, no primary antibody	BLANK	BLANK
Coating, blocking, no secondary antibody	Coating, blocking, no secondary antibody	BLANK	BLANK
Coating, blocking, no secondary antibody	Coating, blocking, no secondary antibody	BLANK	BLANK

Figure 3.8 Limitation of the Kilifi ELISA binding assay.

(A) Checkerboard titration testing various conditions for the Kilifi ELISA binding assay. **(B)** and **(C)** no blocking or blocking did not affect OD reading and signal observed both in the presence or absence of FH. Circled in red in A is where an OD reading was detected.

Next, to check whether the same effect occurred with the other antigens, I tested a subset of merozoite antigens that I had in Helsinki under the same conditions but in the presence or absence of FH. As shown in **Figure 3.9** below, there was no difference in reactivity in the presence or absence of FH in the antigens tested still using the same anti-factor H goat polyclonal antibody, IgG.
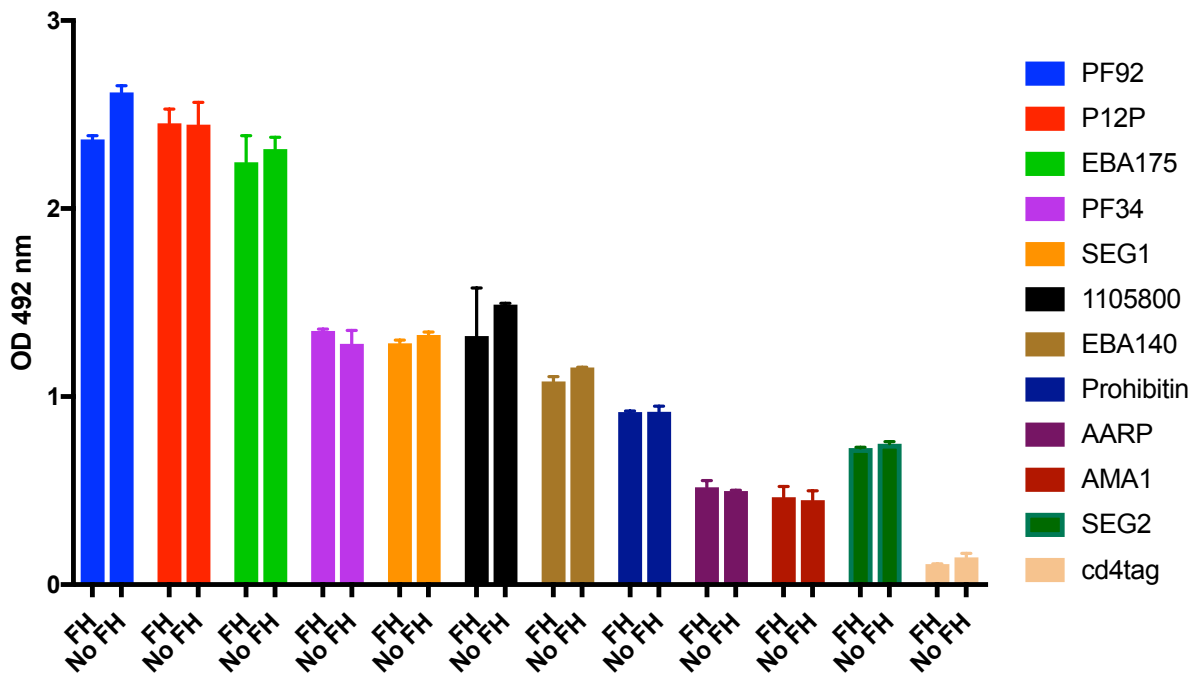
Figure 3.9 Polyclonal anti-FH antibody in the Kilifi ELISA binding assay directly recognized merozoite antigens

Antigens at a concentration of 8 ug/ml were coated on the ELISA plates. FH at 1:2500 was added and after incubation, polyclonal anti-FH at 1:2000 was added followed by HRP antibody at 1:5000. Each antigen was run in duplicate.

3.5.7 Development of the Helsinki ELISA binding assay

Although the Kilifi ELISA binding assay showed that more merozoite antigens bind to FH, the limitations presented above meant that the initial binding experiments were inconclusive. I therefore tried to improve this assay by developing a second ELISA binding assay, at Prof Seppo Meri's Complement laboratory at University of Helsinki, hence the name Helsinki ELISA binding assay. I began by changing from a polyclonal antibody against FH to a monoclonal one. I started off by testing a list of monoclonal antibodies 196X, 86X, 3D11 and 131X (Fontaine et al. 1989; Jokiranta et al. 1996; Rosa et al. 2015b) that were available *in-house* for recognition of FH. I included in the test the anti-FH polyclonal antibody. **Figure 3.10 A** below shows the binding sites for

the monoclonal antibodies in FH and their reactivity when tested against immobilized FH **Figure 3.10 B**. The results show that polyclonal anti-FH antibody had the highest recognition for FH. Monoclonal anti-FH clone 196X and clone 3D11 showed almost similar recognition. Monoclonal anti-FH 131X showed the lowest FH recognition. Since clone 131X is very specific to FH - its binding sites do not include any regions in FH that is closely related to FH like 1 or FH related proteins (CFHR1-5) **Figure 3.10 A** – and it doesn't bind to regions that have been shown to bind to pathogens such as CCP5-7 or CCP 19-20 (Ferreira, Pangburn and Cortés, 2010), I chose to use it in the subsequent experiments.
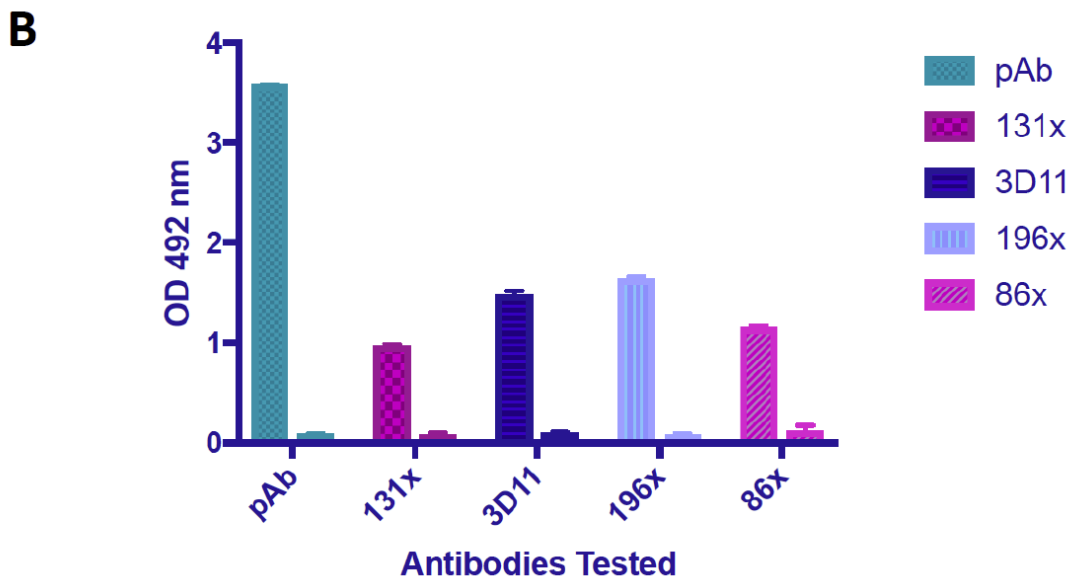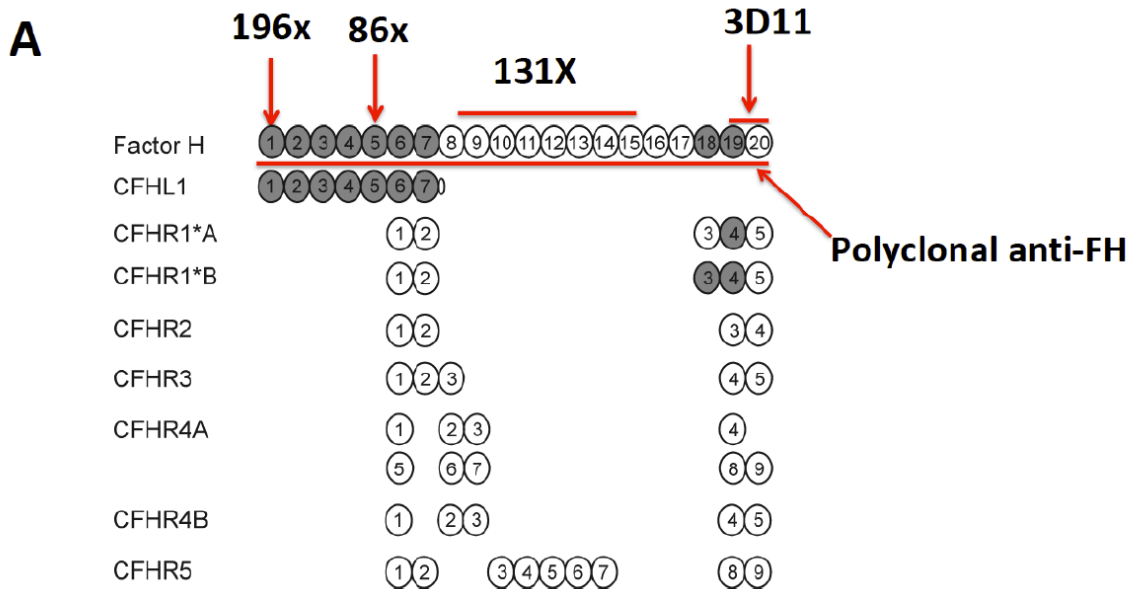
Figure 3.10 Recognition of FH by the various monoclonal antibodies and the polyclonal antibody

(A) schematic overview of the various monoclonal antibodies plus the polyclonal antibody on FH (Fontaine et al. 1989; Jokiranta et al. 1996; Rosa et al. 2015b) (B) FH at 1 ug/ml was coated unto an ELISA plate, various monoclonal/polyclonal antibody was subsequently added and HRP conjugated antibody used for detection. Second bar for each sample represents the blank well, where no FH was added.

Next, I tested various experimental conditions to determine the optimal binding between FH and the merozoite proteins. I started by testing, whether the set of monoclonal antibodies could recognize Pf92 directly i.e. in the presence or absence of FH. As shown **in Figure 3.11**, only the polyclonal antibody had reactivity in the presence or absence of FH confirming that that antibody directly recognized the antigen.
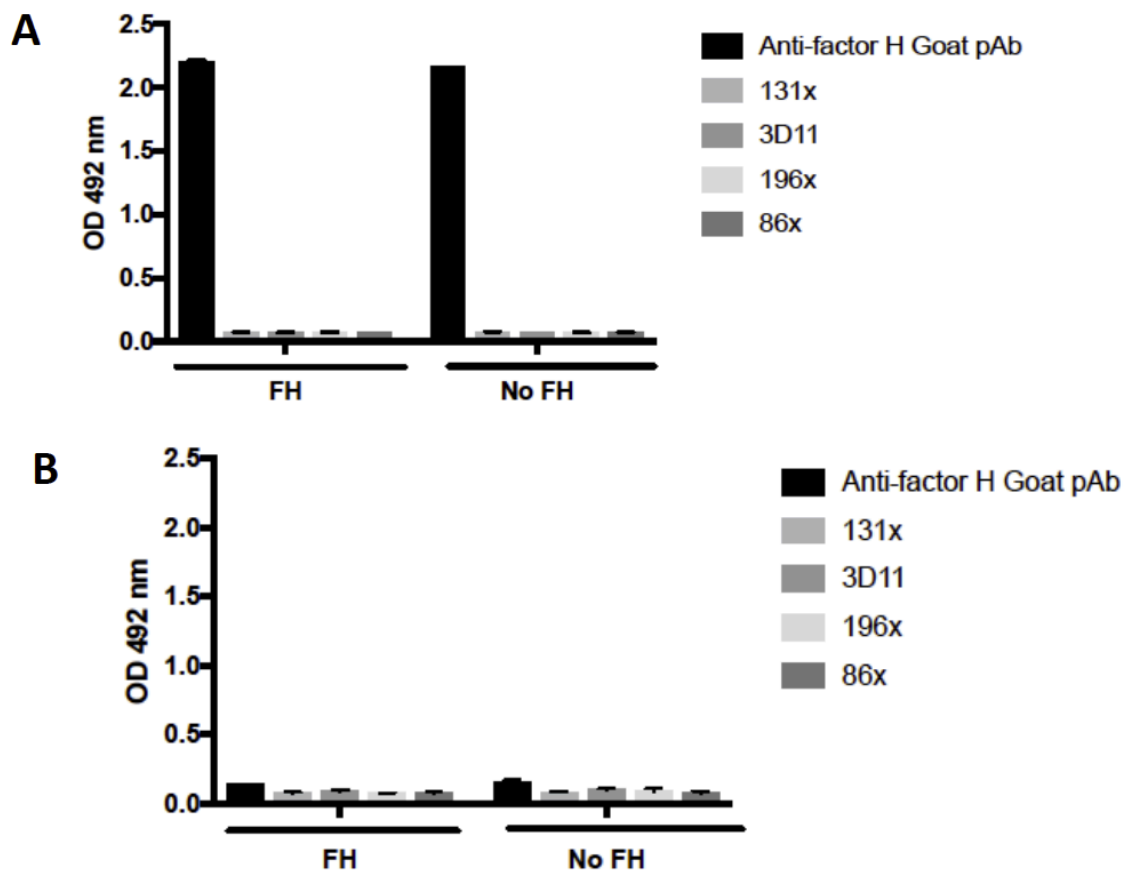
Figure 3.11 Only the polyclonal anti-FH antibody binds to Pf92 in the presence or absence of FH.

A concentration of 8 ug/ml for Pf92 **(A)** and Cd4 tag **(B)** were coated on the ELISA plate and incubated overnight. FH in 1:2500 dilution in block buffer (1% milk in 1X PBS-T). Primary antibodies were added followed by the secondary antibodies, all diluted in blocking buffer. Washes were done using 1 X PBS-0.005%T

Next, I tested a series of conditions to determine the optimal reactivity using monoclonal anti-FH antibody clone 131X. The optimization included, increasing the concentration of FH, changing from PBS-T to using VBS++ buffer, varying the concentration of VBS++ buffer and reducing the concentration of PBS-T used for washing. Increasing the concentration of FH did not improve the reactivity as the OD was very low at 0.15 and there was no distinction between the positive and negative control **Figure 3.12 A**. I reasoned that this could be a low affinity interaction that could potentially be improved by a change of buffer. Diluting FH in 0.5X VBS++ buffer showed a slight change in OD reading with the negative control now having an OD reading of above 0.2, although this was still very low. There was no overlap in OD reading at least for FH concentration above 3.75 ug/ml **Figure 3.12 B**. I observed modest increase in OD reading when FH and primary and secondary antibodies were diluted in 0.5X VBS++ buffer **Figure 3.12 C**. The effect was retained even at increased concentration of FH **Figure 3.12 D**.
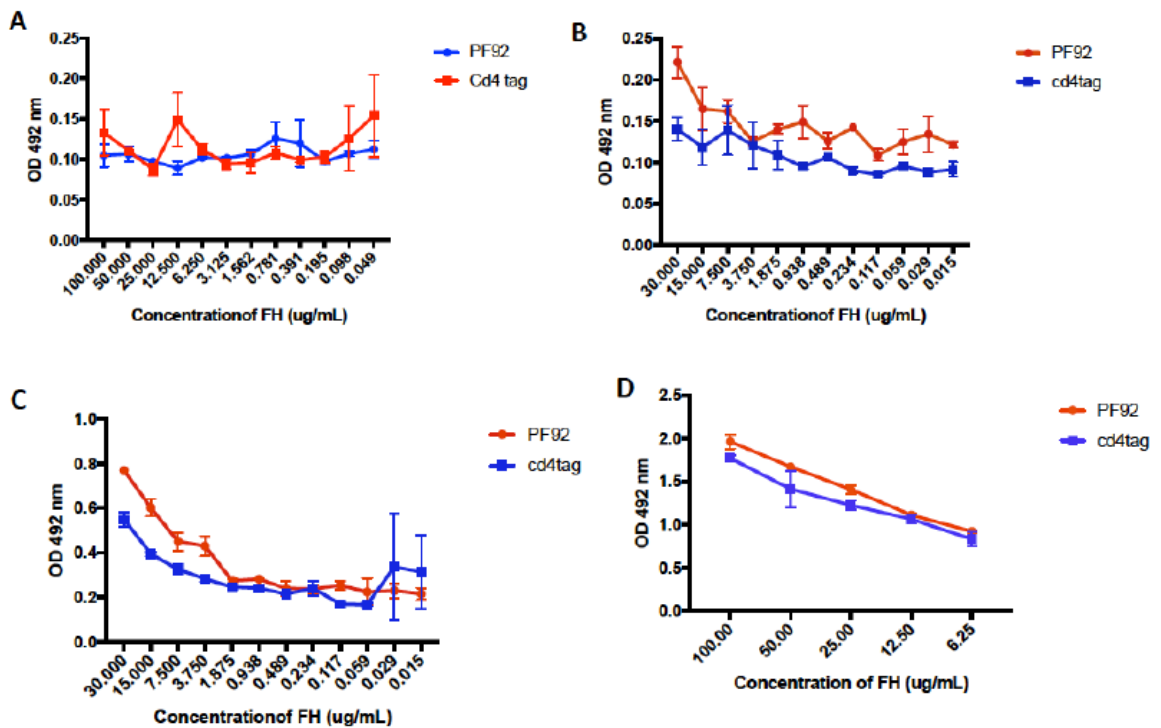
Figure 3.12 Optimization of Helsinki ELISA binding assay.

(A) Increasing the concentration of FH did not improve binding between FH and Pf92. **(B)** Dilution of FH in 0.5X VBS++ instead of 1 X PBS-0.001% Tween showed slight improvement in OD reading. **(C)** Using 0.5X VBS++ buffer to dilute both FH and antibodies improved the OD reading with a clear distinction of positive and negative control at high FH levels. **(D)** Further increasing the concentration of FH and lowering concentration of VBS++ from 0.5X to 0.25X for FH increased OD reading, but there was no difference between positive and negative control. Each antigen was run in duplicate.

In summary, the main difference between the Kilifi ELISA binding assay and the Helsinki ELISA binding assay is that different antibodies against FH were used. The binding detected using the monoclonal antibody in the Helsinki ELISA binding assay was credible and dependent on FH unlike that by the polyclonal antibody in the Kilifi assay. Changing buffers made it possible for the detection of low affinity binding that may have been easily overlooked. **Table 3.1** summarizes the differences between the Helsinki and Kilifi ELISA binding assays.

Table 3.1 Differences between Helsinki ELISA binding assay and Kilifi ELISA binding assay

Experimental conditions	Helsinki ELISA binding assay	Kilifi ELISA binding assay
Washing buffer	1X PBS – Tween 20 (0.01-0.05%)	1X PBS in 0.05% Tween 20
Blocking buffer	1% BSA in 1X PBS	1% skimmed milk in 1X PBS-T
Concentration of FH used	60 ug/ml	0.42 ug/ml
Dilution buffer for FH	0.1% BSA in 0.25X VBS ++	1% skimmed milk in 1X PBS-T
Primary antibody used	Monoclonal anti-FH antibody (clone 131X)	anti-factor H goat polyclonal antibody
Dilution buffer for primary and secondary antibody	0.1% BSA in 0.5X VBS ++	1% skimmed milk in 1X PBS-
Time period for the ELISA	One-day ELISA	Three-day ELISA

3.5.8   *Characterizing additional Plasmodium falciparum merozoite antigens that bind FH using the Helsinki ELISA binding assay*

Having overcome the challenges of the initial Kilifi ELISA binding assay and developed an improved version of the assay, I used the Helsinki ELISA binding assay to screen 18 merozoite proteins, to determine those that interacted with FH. The proteins screened were the same as the top hits on the Kilifi ELISA binding assay; either high FH binders or the best of the intermediate FH binders. For the Helsinki ELISA binding assay, antigens were defined as high FH binders based on an arbitrary cut off of having an OD above the negative control (Cd4 tag). Based on this cut off, merozoite proteins PF3D7_ 1105800, PF3D7_ 0206200, PF3D7_0629500 (SEG1), PF3D7_0629500 (SEG2), RH5, Pf34, EBA140 and P12p showed good binding to FH **Figure 2.13.**
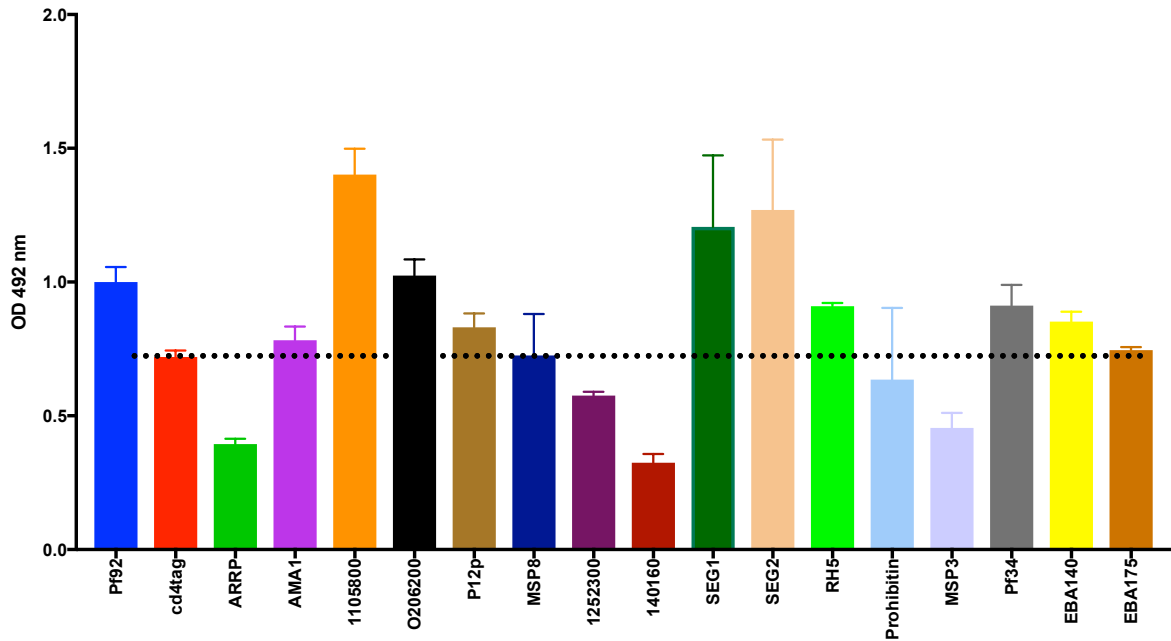
Figure 3.13 Differential binding of merozoite proteins to FH using Helsinki ELISA binding assay.

A concentration of 16 ug/ml of each antigen was coated on an ELISA plate for an overnight incubation. The following day the plates were washed using PBS-0.05%T and blocked using 1% BSA in 1X PBS for 1hr. FH at 60 ug/ml in 0.1% BSA in 0.25X VBS++ was added and incubated for 2hr at room temperature, thereafter the plates were washed using 1X PBS-0.05%. Primary antibody, monoclonal 131X in 1:500 dilution was added and incubated for 1 hr, followed by rabbit anti-mouse IgG HRP. The antibodies were diluted in 0.1% BSA in 0.5X VBS++. After addition of primary and secondary antibody washing was done using 1X PBS-0.05% T. Detection was done using OPD and the reaction was stopped using 0.5M H_2SO_2

3.5.9 *Using surface plasmon resonance (SPR) to confirm interaction between FH and selected merozoite proteins*

One general limitation of ELISA binding assay is that it is possible to overlook low affinity interactions (Hoffrogge et al. 2003; Hornemann et al. 2003). As shown in **Figure 3.12** the difference in OD value between the positive control (Pf92) and the negative control (Cd4 tag), is not major. Surface plasmon resonance (SPR) allows for real time

detection of low affinity interactions and remains the gold standard for measuring protein-protein interactions (Yang et al. 2016). **Table 3.2** below highlights the advantages of using SPR over ELISA binding assays (Cooper 2002; Yang et al. 2016).

Table 3.2 Advantages of SPR over ELISA binding assay

Surface Plasmon Resonance	ELISA binding assay (protein-protein binding assay)
Label free detection of interaction	Requires antibodies
Allows for real time monitoring of interaction	-
Lower sample consumption- requires smaller volumes, lower concentration	Low affinity binding may require higher sample volume at higher concentrations
Reusable sensor chips	ELISA plates once used cannot be reused
Can test interactions from crude samples	Requires sample purification to reduce background signal
Replicate injection of same sample to test reproducibility	Duplicate/ triplicate sample analysis needed to test reproducibility

3.5.9.1   Immobilization of FH ligand on the sensor chip

A typical SPR experiment starts with immobilization of ligand on a sensor chip. Each sensor chip has four flow cells. Depending on the nature of the experiment, immobilization can be done on two or all the flow cells, as long as one flow cell is left as a blank to act as a reference flow cell. After immobilization, a confirmatory test is done to test whether the immobilization was successfully before the actual test samples are run. With regard to the current study, I immobilized FH on one flow cell using 10 mM sodium acetate, pH 5.0, via amine coupling chemistry. Buffer was injected into another flow cell to act as a blank and as also a reference flow cell to account for background signal. Next, I tested whether the immobilization worked using C3b of varying concentration. C3b is a host-generated protein regulated by FH (Boon

et al. 2009). As shown in **Figure 3.14 B**, nothing was bound to the blank flow cell, while

FH was immobilized on the flow cell **Figure 3.14 C**. Dose-dependent binding of C3b

to the immobilized FH confirmed that the immobilization had indeed worked.
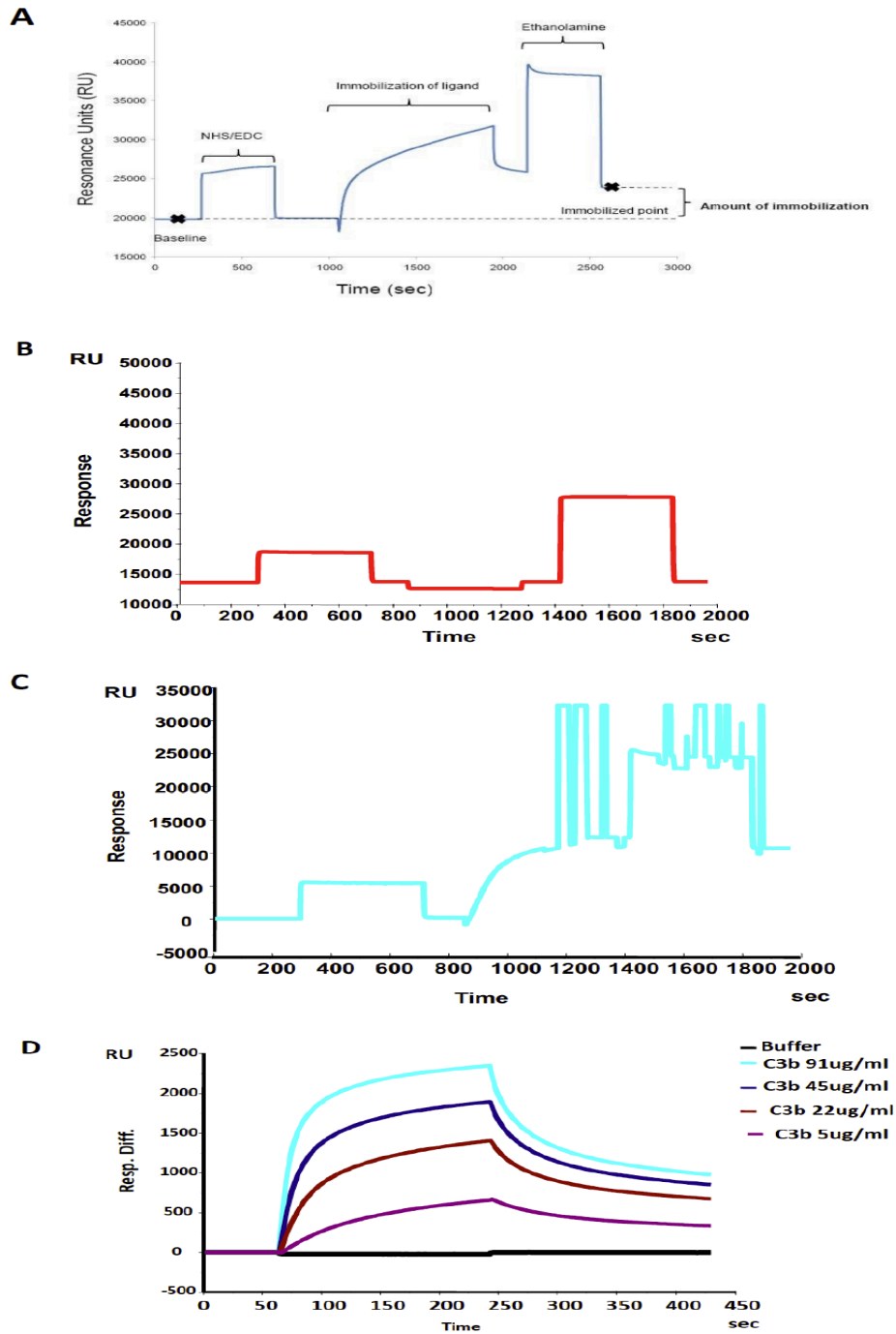
Figure 3.14 Amine coupling of FH ligand to the flow cell on the sensor chip.

(A) schematic sensorgram of a typical amine coupling of ligands to the flow cell of a sensor chip. The procedure involves activation of the matrix on the sensor chip with a mixture of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) and N- hydroxysuccinimide for activation. Ligand is then passed over the surface, which is then covalently attached to the matrix. After injection of ligand, ethanolamine is passed over the sensor surface to deactivate remaining active binding sites. A distinction between the amount bound and the amount immobilized is also shown on the schematic

sensorgram. **(B)** No ligand was attached to the blank **(C)** FH bound to the flow cell **(D)** Dose-dependent binding of C3b to FH.

3.5.9.2 *Optimization of the merozoite protein binding conditions on the flow cell*

To determine the right experimental conditions that could allow for interactions between merozoite proteins and FH, different running buffers were tested. They included: 0.5X VBS++, 10 mM HEPES-0.01% TWEEN and 1/3 of 10 mM HEPES with no TWEEN. As shown in **Figure 3.15** below, 0.5X VBS++ and 10 mM HEPES-0.01% TWEEN gave a negative and low signal sensorgram reading respectively **Figure 3.15 A** and **Figure 3.15 B.** On the other hand, using 1/3 of 10 mM HEPES with no TWEEN gave a positive sensorgram reading implying that it allowed the interactions to occur **Figure 3.15 C**. All the subsequent test experiments were conducted using 1/3 of 10 mM HEPES with no TWEEN in the running buffer **Figure 3.15 C**.
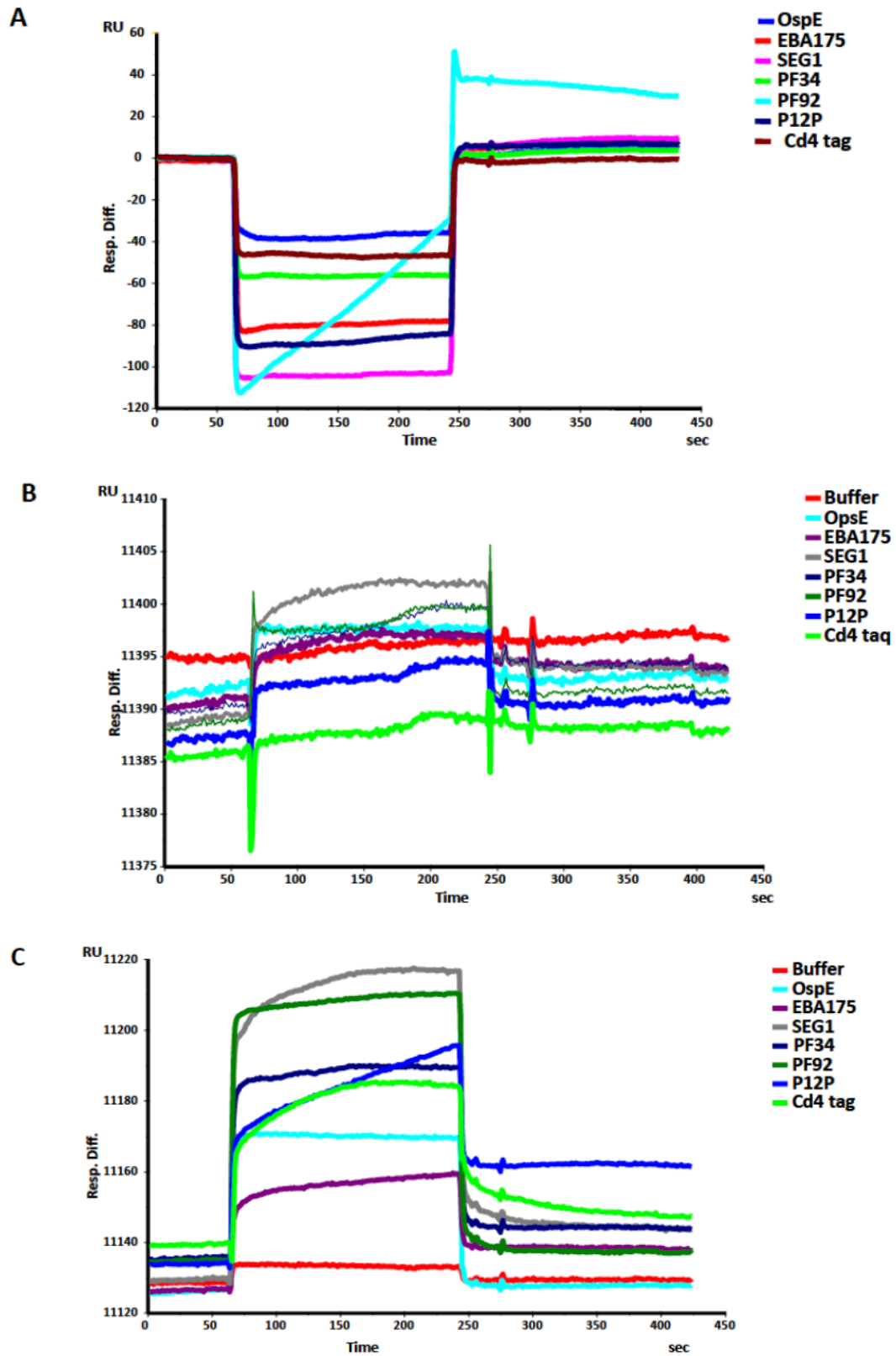
Figure 3.15 Optimization of surface plasmon resonance experimental binding buffers

(A) 0.5 VBS++ buffer gives a negative sensorgram. **(B)** Using HEPES-Tween slightly improved the binding **(C)** 1/3 of 10mM HEPES with no Tween in the buffer enhanced binding.

3.5.10 Surface plasmon resonance confirms binding of a subset of merozoite

proteins to FH and reveals new FH binders

After optimization of the running buffer conditions **Figure 3.15**, the 18 merozoite proteins were tested for an interaction with FH. All antigens were normalized to a concentration of 0.56 uM. I also included another *in-house* recombinantly expressed protein Outer surface protein E (OspE) as an internal positive control. OspE has been shown to bind to FH and hence protect *Borrelia burgdorferi* from complement mediated opsonophagocytosis and killing (Hellwage et al. 2001; Alitalo et al. 2002). SPR was used to confirme the binding that was observed with the Helsinki ELISA binding assay.

The SPR results showed interaction between FH and merozoite proteins (**Figure 3.16 A** and **Figure 3.16 B)**. The figures are split for illustration purposes. In fact, PF3D7_1105800 bound to FH almost as well as OspE. The other antigens PF3D7_0206200, RH5, Pf12, PF3D7_1252300, PF3D7_0629500 (SEG1) and P12p bound to FH even better than Pf92 **Figure 3.16 C**. The sensorgram for the second independent experiment is in **Appendices Figure 3.3**. I observed a weak positive correlation between SPR and Helsinki ELISA binding assay, although not significant (Spearman correlation r= 0.333 P= 0.177) using SPR response 1 and Spearman correlation r= 0.185 P= 0.463 using SPR response 2 **Appendices Figure 3.4**. It is important to note that only a small subset of the 18 antigens were available for testing for both the Helsinki ELISA binding assay and SPR. Head to head comparison between ELISA binding assay and SPR using a bigger sample set will be more informative.
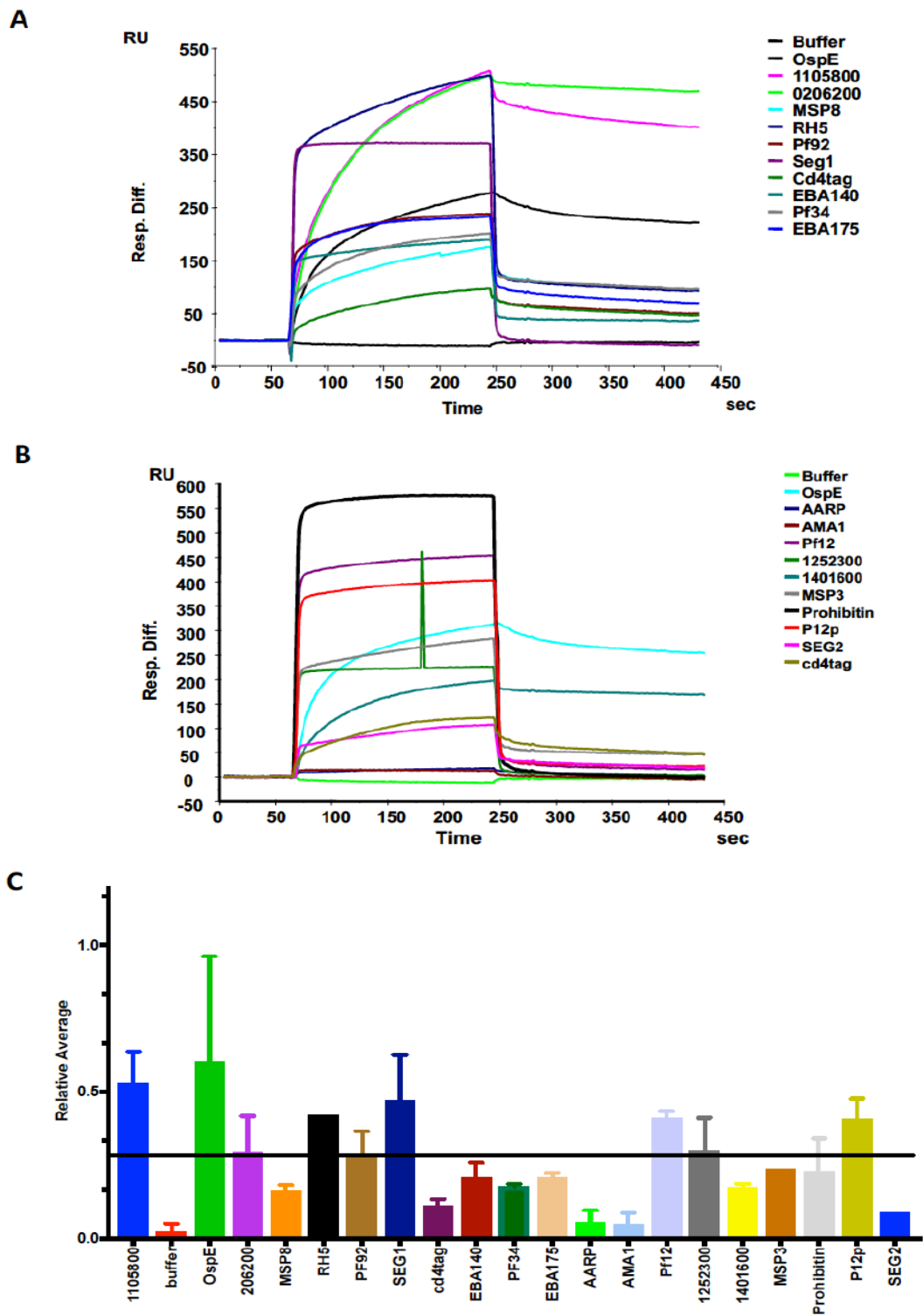
Figure 3.16 SPR confirms that many merozoite proteins interact with FH.

(A) and **(B)** are sensograms showing interactions between merozoite proteins and immobilized FH. The graphs are split for illustration purposes. **(C)** A summary graph of the RU responses for the merozoite proteins.

3.5.10.1 Reproducibility of the SPR experiments

The reproducibility of measuring binding affinity of merozoite protein to FH for the SPR experiment was assessed by injecting varying concentration of different antigens and running same antigens on different days using different sensor chips. Due to limited antigen availability, only two antigens (PF3D7_0206200 and PF3D7_1105800) using varying concentration were tested on the same flow cell. PF3D7_0206200 and PF3D7_1105800 interacted with immobilized FH in a dose-dependent manner as shown in **Appendices Figure 3.5**. Correlation analysis done for two independent experiments of merozoite FH interaction showed that there was strong correlation for the experiments done on different days using different sensor chips (Spearman correlation r= 0.593 P= 0.009; **Figure 3.17**, indicating that the data are highly reproducible.
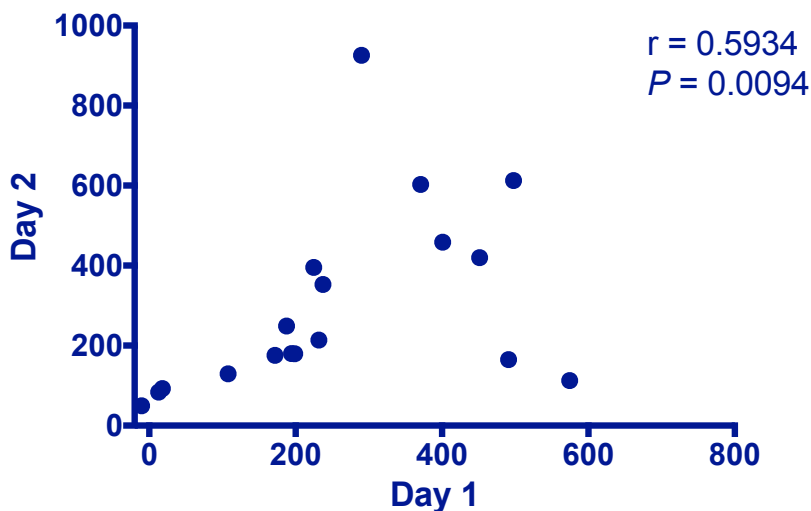
Figure 3.17 Correlation for the SPR experiments done using the same antigens but on different days, using different sensor chips.

FH was immobilized on the flow cell and merozoite antigens n=18 tested for interaction with FH on different days using different sensor chips.

In summary, the interaction of merozoite proteins with complement FH was performed using a series of assays. Since the ELISA binding assay offers a chance for high-throughput analysis, I used it to screen over a 100 recombinantly expressed merozoite proteins. However, the ELISA binding assay that was initially developed – Kilifi ELISA binding assay - had some limitations. The polyclonal anti-FH used bound to the antigens directly **Figure 3.8 and 3.9. I** improved the assay and developed Helsinki ELISA binding assay in which a monoclonal antibody replaced the polyclonal anti-FH. The SPR platform was used as a confirmatory test. **Figure 3.18** below shows a summary of binding characterized of high FH binding antigens across the different assays used.

No. of proteins screened: 110

Kilifi ELISA binding assay	Helsinki ELISA binding assay	Confirmed by SPR
Pf92	Pf92	1105800
Pf34	1105800	0206200
1105800	0206200	SEG
SEG1	SEG	Pf12
EBA140	RH5	P12p
AARP	Pf34	RH5
Prohibitin	EBA140	Pf92
P12p	EBA175	1252300
MSP8	P12p	
RH5		
Pf12		
1252300		
RH5		
EBA175		

Merozoite proteins that bound well to FH (high FH binders) using the different assays

Figure 3.18 Summary of merozoite proteins that were identified using different assay.
The assays used included Kilifi ELISA binding assay, Helsinki ELISA binding assay and the surface plasmon resonance test.

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Majority of the antigens that were observed to be high binders with the Helsinki ELISA binding assay were identified as high binders on the SPR platform **Figure 3.18**. However, some other antigens such as Pf34, EBA 140 and EBA 175 that were high binders with Helsinki ELISA binding assay failed binding on the SPR. Indeed, I observed a weak positive correlation between these two assays **Appendices Figure 3.4** which, however, was not significant. Although the number of antigens tested were few and could in part account for the results, it points to the fact that ELISA binding assay is not a robust way to assess protein-protein interactions. With advancement in technology, there are now superior SPR machines that allow for high throughput analysis (Nguyen et al. 2015). Future studies should therefore consider using high-throughput SPR platforms that allow for real time protein-protein interaction detection.

3.5.11 Localization of the FH CCP modules involved in binding to merozoite proteins

I used a series of truncated FH fragments (CCP1-5, CCP1-6, CCP1-7, CCP5-7 and CCP1-20) to localize CCP binding modules of high FH binders. The FH CCP modules were already expressed and available *in-house* at Prof Seppo Meri's Complement Laboratory at the University of Helsinki. Each CCP module is approximately 7kDa. As shown in **Figure 3.19** on the SDS-PAGE, all the modules were of the expected molecular weight. CCP 1-6, CCP 1-7 were available in small volumes and at lower concentrations, and therefore were not used for the subsequent SPR experiments. Recombinant FH (CCP1-20) was loaded as a control.
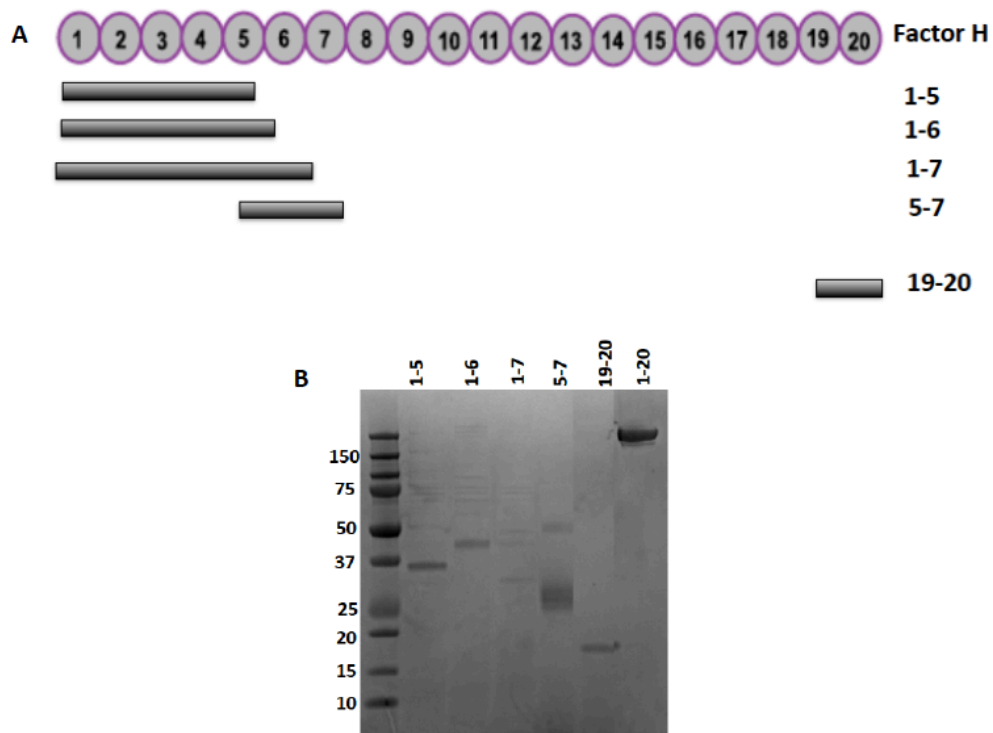
Figure 3.19 Complement control protein (CCP) modules for the complement regulatory protein FH.

Illustrated in **(A)** is the schematic of the 20 CCP domains (grey circles) that represent FH. Black bars beneath the schematic span the different recombinant FH fragments, and the numbers on the right side refer to the CCP domains that are within them. **(B)** SDS_PAGE of FH recombinant fragments.

Complement FH fragments CCP 1-5, CCP 5-7 and CCP 19-20 were first immobilized unto the flow cells. A blank flow cell was prepared to be used as a reference cell. Sensorgram for the immobilized FH fragment is shown in **Appendices Figure 3.6.** As OspE and C3b binding sites in FH are known, I used those to test whether immobilization to the FH fragment had worked. OspE binds to FH CCP19-20 (Hellwage et al. 2001; Alitalo et al. 2002), while C3b has two binding sites, CCP 1-4 and CCP 19-20 (Boon et al. 2009). As shown in **Figure 3.20**, OspE bound to CCP 19-20 and not CCP 1-5 or CCP 5-7. Similarly, C3b bound to CCP 1-5 and CCP 19-20 and not CCP 5-7. Although there was no known protein to test for CCP 5-7 immobilization, the fact that OspE did not bind to that region but bound to CCP19-20, and they were in the

same sensor chip and injected into all the flow cells was used as confirmation that immobilization was specific.
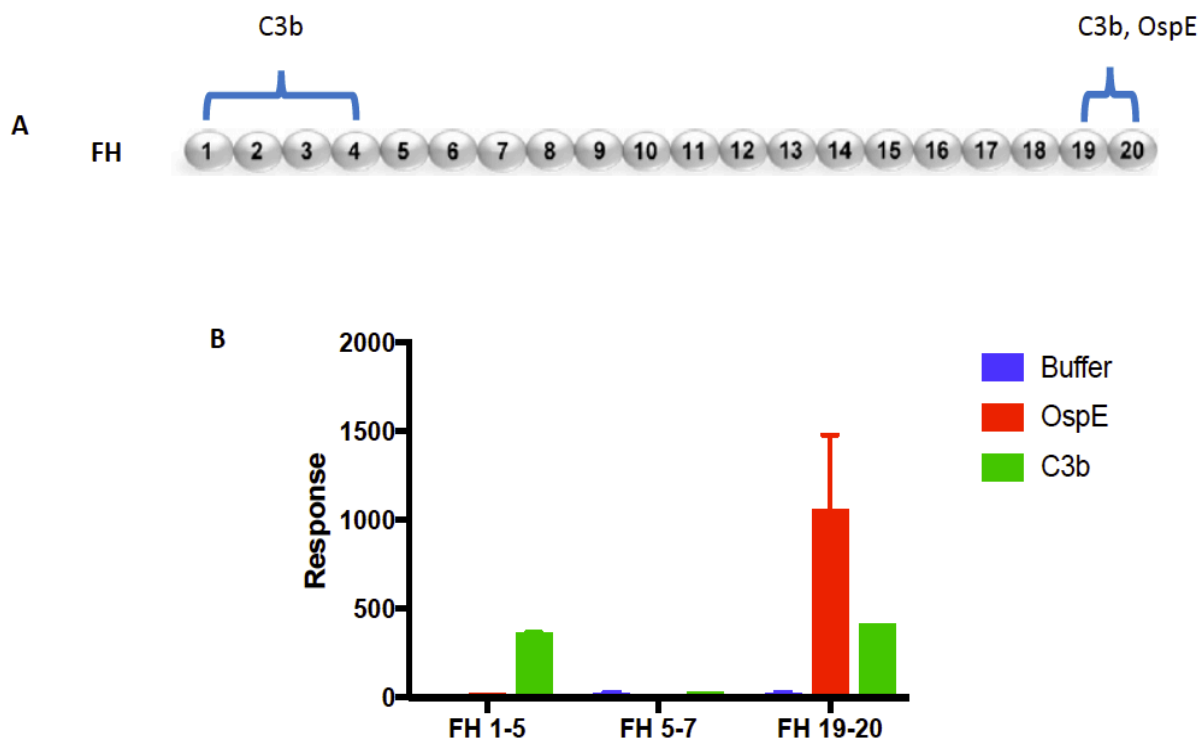
Figure 3.20 Validation of FH fragment immobilization to the flow cells on the sensor chip.

(A) A schematic of OspE and C3b binding sites in FH. **(B)** OspE and C3b bound to the expected CCP modules in FH.

Next, the merozoite proteins were tested against the immobilized FH fragments to localize their binding sites in FH. Initially, CCP 1-5, CCP 5-7 and CCP 19-20 were immobilized on the same sensor chip, but CCP 1-5 failed and was therefore repeated using a different sensor chip. Due to this fact, the data is presented as the original response units, and the experiments were not pooled together. Although the correlation for the response for the same antigen done on different days on the same flow cell is high (Pearson correlation r= 0.847 P= <0.001) **Appendices Figure 3.7,** there is an equally good correlation for same antigens tested on different days using different sensor chips **Figure 3.17**. As shown in **Figure 3.21** below, antigens

PF3D7_0206200, PF3D7_0629500 (SEG1) and Pf12 bound more strongly to FH CCP 5-7 compared to CCP 1-5 or CCP 19-20. Although the antigen P12p bound slightly better to FH CCP 5-7 compared to CCP 1-5 or CCP 19-20, it had generally lower responses compared to the rest of the antigens. There was no preferential binding for Pf92 to any of the three tested CCP module fragments. The wide confidence intervals could be due to protein expression batch to batch effects. Due to volume limitations antigens RH5 and PF3D7_1252300 were only tested for binding to FH CCP 5-7 and CCP 19-20. Hence, the results are inconclusive
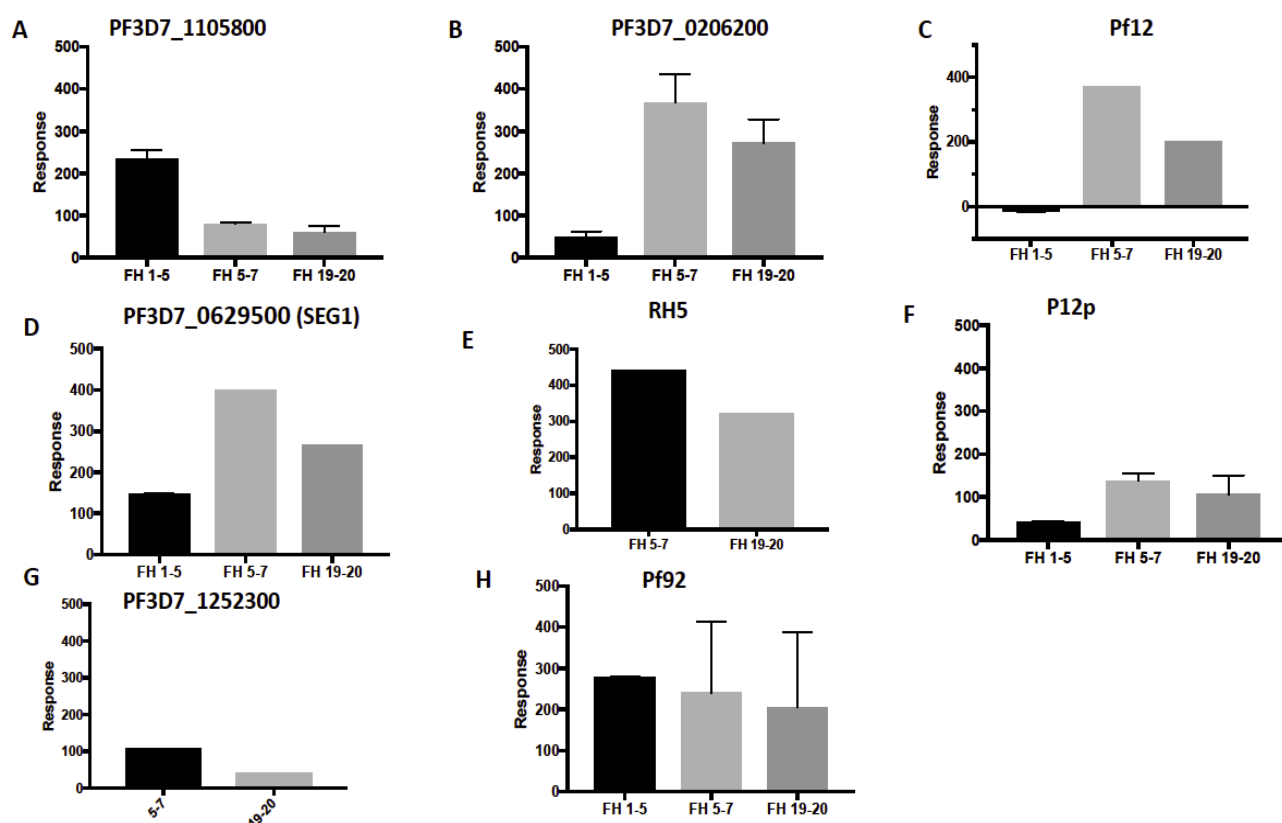
Figure 3.21 Merozoite protein interactions with the various FH CCP fragment modules

From the top **(A)** PF3D7_1105800, **(B)** PF3D7_0206200 **(C)** Pf12 (D) PF3D7_0629500 **(E)** RH5 **(F)** P12p **(G)** PF3D7_1252300 and **(H)** Pf92. FH fragments CCP1-5, CCP5-7 and CCP19-20 were immobilized on the flow cells and tested for interaction with the selected merozoite antigens.

3.5.12 Reduced parasite growth in serum depleted of FH

I have demonstrated that multiple *Plasmodium falciparum* parasite proteins bind to complement regulatory proteins FH **Figure 3.16**. It has been argued that parasite binding to FH is important for immune evasion (Rosa et al. 2015b). Therefore, I hypothesized that there would be reduced parasite growth in the absence of FH. I tested this assumption using a modified growth inhibition assay. Parasites at a starting parasitemia of 0.5% were allowed to grow for 2 cycles (96 hrs) in the presence of complement (using normal human serum), heat-inactivated serum, serum depleted of FH (CompTech) and serum depleted of FH but reconstituted by the addition of varying concentrations of FH. Thereafter, the parasites were stained with ethidium bromide and parasitemia determined using flow cytometry. There was significant reduction in parasite growth in serum depleted of FH compared to either NHS or Albumax. There was no difference in parasite growth in NHS or HIS (at 56°C for 30 minutes) conditions, although a slightly better growth was observed in NHS compared to Albumax (mean parasitemia was 12.5% for NHS and 10.4% for Albumax). Reconstitution of serum depleted of FH by the addition of FH only resulted in a modest gain in parasite growth using 50 ug/ml to 250 ug/ml of FH but no further gain in growth was achieved when 500 ug/ml of FH was added **Figure 3.22**.
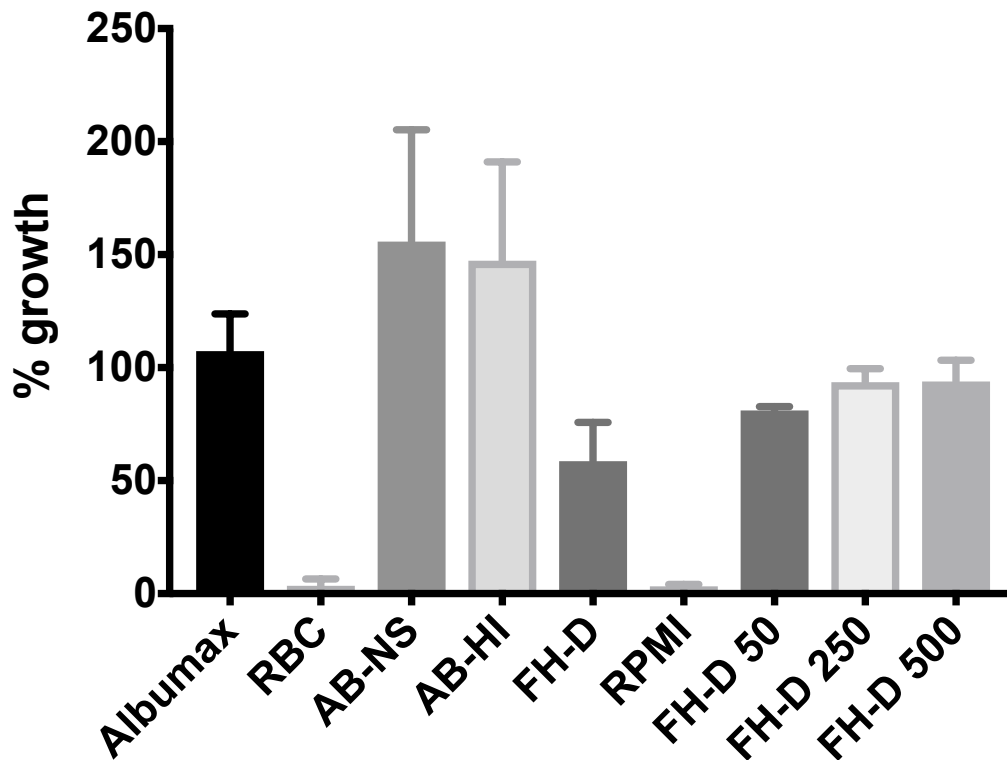
Figure 3.22 Parasite growth in the presence or absence of FH.

Parasites were diluted in incomplete media to a starting parasitemia of 0.5%. A reaction mix of 50 ul was used with 40 ul being diluted parasites and 10ul of the experimental conditions of RBCs, Albumax, AB-NS (AB normal human serum). AB-HI (AB serum heat inactivated) and serum depleted of FH (FH-D). FH-D was reconstituted by the addition of 50 ug/ml, 250 ug/ml and 500 ug/ml of FH denoted as FH-D 50, FH-D 250 and FH-D 500, respectively. Parasites were allowed to grow for two cycles and parasitemia measured using flow cytometry. The data are representative of four independent experiments. Complement FH was obtained commercially, from a pool of healthy adults (Sigma). The same serum batch was used for these experiments and was also heat-inactivated to get the HI serum. FH depleted serum is normal donor serum but devoid of FH and was obtained commercially (Complement Technology). Unfortunately, FH concentration in this commercially obtained serum was not measured; omitting a crucial step.

3.5.13 Amino acid alignment of merozoite FH receptor antigens

Next, I tested whether there were any similarities in the merozoite FH receptor proteins that could explain why they were all binding to FH. A multiple sequence alignment of

the merozoite FH receptor protein amino acid sequences indicated that three short regions were aligned across all the sequences without gaps, although there was no shared short amino acid stretch among the regions **Figure 3.23.** Further analysis using Nuclear Magnetic Resonance (NMR) and X-ray crystallography will be required to characterise in detail the nature of binding and specifically the binding sites between FH and these antigens (Bhattacharjee et al. 2013).
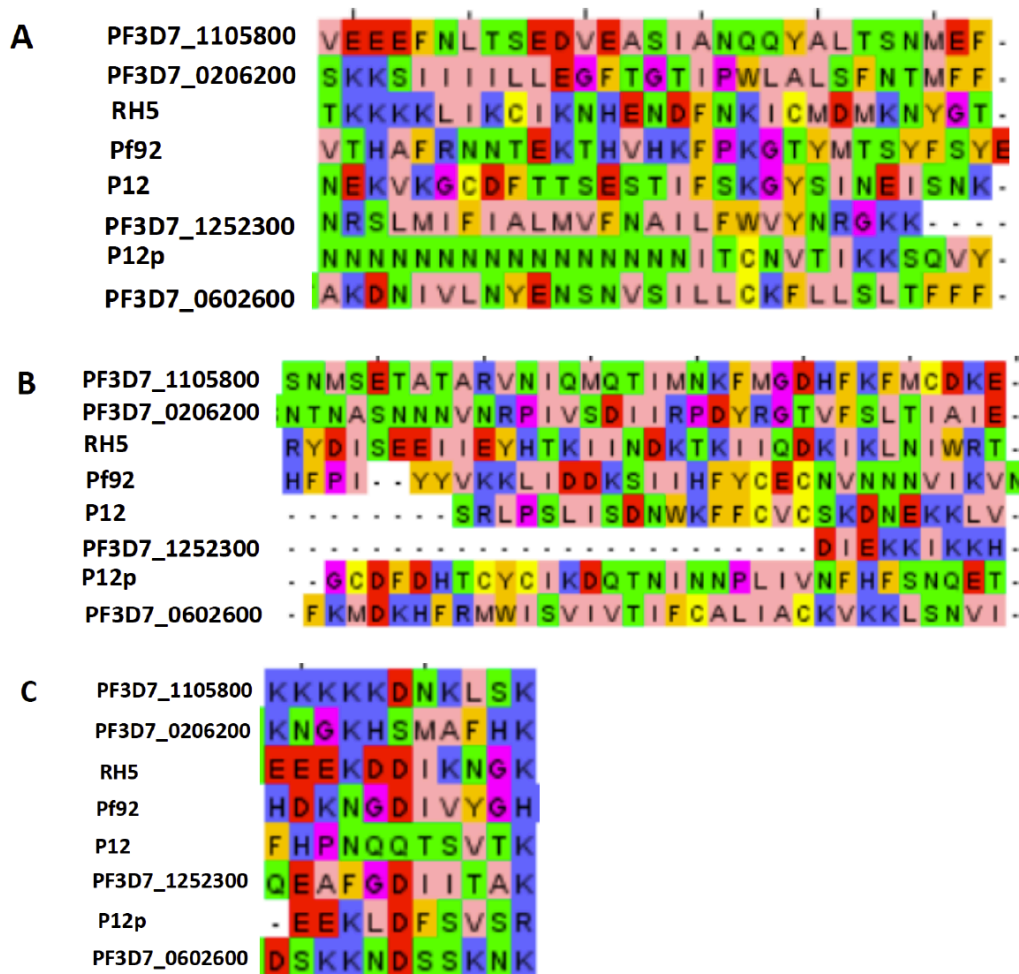
Figure 3.23 Illustration of the amino acid regions alignment

Regions A, B and C of the merozoite FH receptor proteins are the truncated sections that aligned together. They are truncated from the entire amino acid alignment.

3.6 Discussion

Complement immune evasion strategies have been extensively described for bacterial pathogens (Hellwage *et al.*, 2001; Zipfel, Würzner and Skerka, 2007; Serruto *et al.*, 2010; Luo *et al.*, 2013). For the malaria parasite *Plasmodium falciparum* there is far less evidence, most of this having been described quite recently. We now know that the merozoite antigen Pf92 interacts with FH to avoid complement-mediated lysis (Kennedy et al. 2015). The results obtained from my study suggest that in addition to Pf92, there are other merozoite proteins including PF3D7_1105800, PF3D7_0206200, RH5, Pf12, PF3D7_1252300, PF3D7_0629500 (SEG1) and P12p that interact with FH for potential immune evasion. These results support my hypothesis that multiple merozoite antigens may be involved.

The observation that *Plasmodium falciparum* uses several proteins to acquire complement regulatory protein FH suggests a mechanism of redundancy. A similar phenomenon has been observed in other pathogens. For example, the Gram-negative bacterium *Borrelia burgdorferi* has been shown to have two or three FH binding proteins: CspA, CspZ; and OspE (Hellwage *et al.*, 2001; Brooks *et al.*, 2005, Kraiczy and Stevenson, 2013) for FH interaction. Another example is the Gram-positive bacterium *Streptococcus pyogenes,* which has been shown to have three FH binding proteins, M1 protein, Fba and Scl1.6 (Pandiripally et al. 2002, 2003; Reuter et al. 2010). On the other hand, there are instances, where pathogens use only one molecule to inhibit several complement regulatory proteins. An example is Pra1 (pH-regulated antigen 1) in yeast *Candida albicans* that binds FH, FHL1, C4bP and fibrinogen (Zipfel et al. 2011) and hence limits complement activation at several stages. Taken together, this highlights the complexity of complement and immune evasion mechanisms used by several pathogens.

The majority of the additional antigens that interact with FH (merozoite FH receptors) were observed to use CCP 5-7 modules. Interestingly, this is the same region that gametes use (Simon et al. 2013), while schizont-infected red blood cells have been shown to use CCP5 and CCP 20 (Rosa et al. 2015b). Studies on other pathogens indicate preferential usage of CCP 5-7. *Haemophilus influenzae, Streptococcus pyogenes* and *Neisseria meningitidis* have been shown to bind to FH using sites located along CCP 5-7 (Pandiripally et al. 2003; Hallström et al. 2008; Schneider et al. 2009). It has been argued that microbial proteins which bind FH via CCP 6-7 can also recruit FHL-1 due to the shared conserved CCP domains between FH and FHL-1 (Meri et al. 2013). However, this study did not directly test the merozoite FH receptors for binding to FHL-1. FH CCP19-20 has also been shown to be an essential binding site for other pathogens (Meri et al. 2013). However, none of the merozoite FH receptors showed preferential binding to CCP 19-20, although those that bound to CCP 5-7 also had some binding to CCP 19-20. Both CCP 5-7 and CCP 19-20 have glycosaminoglycan binding sites (Schmidt et al. 2008) and it can be speculated that there could be some similarities in these modules allowing pathogens to recognize them with varying degrees.

Out of the eight identified merozoite FH receptors, three are known to be surface glycosylphosphatidylinositol (GPI)-anchored proteins: P12, P12p and Pf92. RH5 is a member of the reticulocyte binding homologue family and plays an important role in the invasion of the parasites to the red blood cells (Baum et al. 2009). PF3D7_0206200 is known to be a panthothenate transporter proposed to be essential for erythrocyte invasion (Hu et al. 2010), while PF3D7_0629500 is an amino acid transporter. The other proteins PF3D7_1105800 and PF3D7_1252300 are of unknown function (Kamuyu, PhD thesis 2017). Of note is that other than Pf92 (Kennedy et al. 2015), none of these proteins have been studied in the context of involvement with complement either for immune evasion or mediating invasion.

The standard growth inhibition assays are normally conducted in complement-free media and form part of a set of assays used to asses antibody effector functions (Dent et al. 2008; McCallum et al. 2008; Wilson et al. 2011; Murungi et al. 2016). I modified the GIA and tested for parasite growth in the presence of complement (NHS) or in serum depleted of FH. The GIA was modified in that I did not use serum from malaria immune individuals as traditionally used for this assay. Using this modified GIA, I observed reduced parasite growth in serum depleted of FH compared NHS. The difference between parasite growth in albumax and NHS was not significant. Albumax does not contain FH **Appendices Figure 3.8**. This could imply that the current parasite culture protocols that use albumax allow the parasite to grow in the absence of FH. On the other hand, work done by Rosa et al. in which they cultured parasites in NHS, HIS, NIS supplemented with monoclonal antibody (mAb) to FH and also albumax showed reduced parasite growth in NHS with mAb compared to NHS without FH mAb or albumax (Rosa et al. 2015b). Their results somewhat agree with what this study found out in terms of reduction in parasite growth in the absence of FH. However, in Rosa et al, work, they did not show a titration curve to indicate whether by addition of mAb to NHS they were able to deplete out FH. They also determined parasitaemia using microscopy, which is not as robust as flow cytometry. A clearer way to assess the role of FH will be to use antibodies to merozoite FH receptors individually or in a "cocktail" and test whether blocking this interaction would have an effect on parasite growth.

The current study has a number of limitations. As mentioned previously the Kilifi ELISA binding assay that was used to down select the antigens before further analysis with SPR proved to be non-specific; the polyclonal antibody directly recognized the recombinant merozoite proteins. In addition to the limitations of Kilifi ELISA binding assay discussed, the Helsinki ELISA binding assay was not optimal. It could not clearly distinguish between negative and positive controls. As these two assays were used to

down select the parasite merozoite antigens that were confirmed using the surface plasmon resonance (SPR), it is possible that potential antigens that interact with FH were missed out. In addition, since there is a weak correlation between ELISA binding assay and SPR **Appendices 3.4**, it is possible that the former selected some antigens that are not true FH binders. However, since the majority of the proteins from ELISA binding assay were taken through SPR, which is the gold standard for protein-protein interaction, the data produced is robust and shows that multiple proteins interact with FH. Due to limitations in the quantity of recombinant proteins, the SPR experiments for binding to FH CCP fragments were not robustly assessed.

In conclusion, this study provides evidence that *Plasmodium falciparum* uses multiple merozoite proteins to bind to complement FH for possible immune evasion. Indeed, an analysis of stage-specific FH receptor proteins revealed that merozoites and schizonts bind significantly more to FH molecules than other parasites stages (Simon et al. 2018), lending support to the current study observation. With the recent observation that the malaria parasite could also bind C1-INH (Kennedy et al. 2017), future study that comprehensively screens known and even stage-specific parasite proteins against complement inhibitors will give a clearer picture of the complexity of the parasite complement immune evasion mechanism. Nevertheless, the current study provides the first major evidence of multiple interaction partners for malaria parasites and could be a major addition to the field.

4   Antibody responses to merozoite FH receptor proteins and association with protection against malaria

4.1   Summary and aims

Pathogenic proteins or antigens responsible for limiting complement activation may be attractive as vaccine candidates because (i) immune responses against them could prevent the establishment of infection and (ii) they provide an opportunity to neutralize virulence factors (Meri et al. 2008). The same approach has been applied in a successful vaccine against meningococcus (Giuliani et al. 2006).

Neisseria meningitidis is among the leading causes of bacterial meningitidis and sepsis (Jafri et al. 2013; Borrow et al. 2017). Pathogenic strains are classified based on the antigenic structure of their polysaccharide capsule. So far thirteen types have been described, six of these serotypes (A, B, C, W135, X, and Y) are responsible for the invasive disease (Pollard 2004). One of serogroup B's main virulence protein is Factor H binding protein (FHbp). FHbp is responsible for the recruitment of FH and enables the bacteria to resist complement-mediated killing (Madico et al. 2006; Schneider et al. 2006). Interestingly, FHbp is now a component of Bexsero (Norvatis) and Trumenba (Pfizer), two recently approved vaccines against meningococcus (Giuliani et al. 2006) and fHbp-specific antibodies block binding of factor H, and hence increase bacterial susceptibility to killing via the alternative pathway (Madico et al. 2006; Schneider et al. 2006; Welsch et al. 2008). For malaria, we hypothesize that similarly, parasite antigens that help to recruit FH to avoid complement destruction may be blocked by antibodies, and thus could be potential vaccine candidates.

In chapter 3, I narrowed down to a subset of merozoites proteins that are responsible for interaction with FH (also referred to merozoite FH receptors) and potentially may

enable the parasite to establish infection. The antigens identified included PF3D7_1105800, PF3D7_0206200, RH5, Pf12, PF3D7_1252300, PF3D7_0629500 (SEG), P12p and Pf92 (Kennedy et al. 2015). P12p was among the antigens available but did not get printed onto the KILchip v1.0 Microarray, Supplementary Table 1 of (Kamuyu et al. 2018) and hence was not included in the subsequent antibody measurement and analysis. In this chapter, I test the hypothesis that the ability to make antibody responses to these antigens is associated with protection from clinical malaria.

4.2   Introduction

Antibodies targeting merozoites have been known to play a role in naturally acquired immunity to malaria (Sabchareon et al. 1991b; Doolan et al. 2009b; Fowkes et al. 2010). The strongest evidence comes from seminal passive transfer studies that showed that transfer of IgG from immune adults cleared parasitemia and led to a resolution of clinical symptoms in children admitted to hospital with *P. falciparum* malaria (Cohen et al. 1961). These studies provide a rationale for designing malaria vaccines that target the merozoites proteins as they are thought to be able to limit parasite growth and hence prevent clinical disease (Richards and Beeson 2009). Although knowledge has been sought on the targets and mechanisms of protective antibodies, and advances made (Beeson et al. 2008; Richards and Beeson 2009) our understanding is still limited.

The general approach used for identifying targets of protective antibodies is to assess how antibodies are acquired and their association with protection from malaria. Immuno-epidemiological studies involving longitudinal observation offer a powerful tool to examine relationships between measured antibodies and malaria outcomes (Fowkes et al. 2010; Osier et al. 2007). Evidence of merozoite antigens and

association with protection has so far been inconsistent. While some studies show evidence supporting the role of specific antibodies with protection, others have not (Cavanagh et al. 2004; Polley et al. 2006; Stanisic et al. 2009; Fowkes et al. 2010; Dodoo et al. 2008). Additionally, antibodies that target blood-stage malaria function in many ways. These protective antibodies can recognize merozoite antigens on the surface of merozoites or on infected red blood cells, can act to block parasite adhesion and invasion or can act in conjunction with immune cells such as neutrophils (Teo et al. 2016). Somewhat surprisingly, virtually no attention has been paid to the role of antibodies in blocking essential parasite functions or virulence factors.

Recent studies have demonstrated that complement could be an important mediator of immunity to malaria. It has been shown that acquired and vaccine-induced human antibodies recruit complement and interfere with malaria parasite RBC invasion (Boyle et al. 2015; Feng et al. 2018). Boyle *et al* showed that antibodies that fix C1q were associated with protection from clinical episodes of malaria in most individuals while in others, antibodies were shown to be non-inhibitory in the absence of complement (Boyle et al. 2015) suggesting that other mechanisms could be involved. There is also evidence that antibodies that fix complement can inhibit *P. falciparum* sporozoite invasion of hepatocytes (Behet et al. 2018; Kurtovic et al. 2018; Zenklusen et al. 2018). Furthermore, many specific targets for complement-fixing antibodies are now known (Reiling et al. 2019). They include merozoite proteins EBA140 RIII-V, RASPI, GAMA, PfRH2, MSP-DBL1, PfRH5, EBA 175-RIII-V and MSP2-3D7 (Reiling et al. 2019).

To the contrary, very little is known of the role of antibodies in blocking parasite virulence factors and how this relates to the acquisition of immunity to malaria. The prior findings that the parasite may be using merozoites antigens such as Pf92 and MSP3 to interact with complement regulatory proteins in order to inhibit complement activation (Kennedy et al. 2015, 2017) and the identification of additional merozoite

proteins by the current study provides a rationale to investigate the potential role of antibodies against parasite proteins important for its survival.

4.3   Specific aims

- Determine whether antibody responses to merozoite FH receptor proteins are associated with protection against clinical malaria
- Test whether antibodies to merozoite FH receptor proteins differ quantitatively or qualitatively in children developing severe malaria versus uncomplicated malaria controls

4.4   Methods

The Junju longitudinal cohort of malaria in children and the Kilifi County Hospital case control study have been described, as have the antibody responses measurements using the microarray platform (see materials and methods section).

4.4.1   Microarray data cleaning and processing

The KILchip microarray is a high throughput platform. It has 21 mini-arrays in a slide, 4 slides are assembled in a hybridization cassette, and 4 cassettes can be run in a hybridization workstation. A total of up to 522 samples per run per day can be analyzed. Each run has an allocation for positive controls: pooled human immune serum (PHIS) and purified malaria immunoglobulin (MIG), and negative controls: malaria naïve sera and blanks (Kamuyu et al. 2018). Samples used for this thesis work were done in one day utilizing the two hybridization workstations. PHIS, which was included in each slide, was used to adjust for variation between slides. Besides, sample allocation per slide was done randomly; as the laboratory investigators were blinded to age and location.

Data was acquired from scanned image files using the GenePix Pro 7 software (Molecular Devices). R version 3.4.4 was used for data processing, cleaning, and normalization as well as for statistical analyses (Mwai K et al. manuscript in preparation). The median fluorescent intensities (MFI) of local spot background surrounding each spot was subtracted from the MFI of each antigen spot. To account for technical slide-to-slide and batch-to-batch variation, a two-step normalisation process was performed according to previously described methods (Huber et al. 2002; Delfani et al. 2016). To account for within batch slide-to-slide effects, a ComBat model (SVA package in R) was fitted to the MFI data, and after obtaining the batch corrected data for the slide effect, a variance stabilization normalization transformation was done (Huber et al. 2002; Sundaresh et al. 2006; Johnson et al. 2007; Sboner et al. 2009).

4.4.2   Statistical analysis

All the analysis was done in STATA (StataCorp, version 13), R version 3.4.4. and GraphPad PRISM (version 7). Unpaired t-test was used to compare the mean parasite densities between the two age group categories (0-4 yrs, and > 5 yrs.). Mann-Whitney test was used to compare median normalized MFI between the age groups and between parasite positive and parasite negative children. For determination of association between antibody levels and development of clinical episode of *Plasmodium falciparum* malaria, antibody levels were classified into three tertiles based on the responses to the antigen measured as MFI values (high, medium or low). Medium and low responses were combined into one (low). The association between having a high and new "low response" were tested against protection to at least one clinical episode of malaria in the follow-up period using a modified Poisson regression model (Zou 2004). Previous analysis had validated the use of this regression analysis for similar studies (Osier et al. 2008). A clinical episode was defined as having

parasites densities of over 2500 (p/μl) and temperatures of over 37°C (Mwangi et al. 2005).

Another variable accounting for the individual's overall response to all the antigens was generated and called reactivity score. This variable used a Principal Component (PC) analysis to map the position of test samples with reference to the positive/negative/blank control spots and accounted for an individual's samples' inherent reactivity (i.e. characterizing the degree to which samples tended to be reactive to the panel of antigens tested). The individuals 4 SD's away from the mean of PC1 values of negative/blank control spots were categorized as high responders while the remainder were categorized as low responders. High responder's PC positions were close to the Malaria Immune Serum (PHIS / positive control). This variable thus takes into account age (antibody reactivities usually increase with age) as well as transmission intensity (more exposure to infectious mosquitoes, more antibodies are produced) (Mwai K et al. manuscript in preparation).

I investigated the protective efficacy for all possible combination of antibody responses to the merozoite FH receptor antigens. In the first instance, I identified a combination of 2, 3, and 4 antigens out of the total 8 antigens, which resulted in 28, 56 and 70 antigens combinations, respectively. In the second instance, I identified a combination of 3 and 4 antigens, but this time from the 5 antigens that showed protection in the single antigen analysis. The latter 3 and 4 antigens resulted in 10 and 5 combinations, respectively. The combinations (or also referred to as breadth score) ranged from 0 to the number of antigen combination used, in this case, either 2, 3 or 4 antigens. The breadth score was created for each child and was then fitted as a continuous variable in the modified Poisson model while adjusting for the reactivity score variable with the clinical episode of malaria in the 6 months follow up period fitted in the model as the outcome variable. The protective efficacy was calculated as (1 - IRR) *100, whereby

100% represented complete protection. The analysis on protective efficacy or combination of antigens was restricted to children who were parasite positive at the time of sampling (n=79) and was done as previously described (Osier *et al.*, 2014).

Kruskal Wallis test was used to compare differences in median antibody levels in children with severe malaria anaemia (SMA), severe malaria without anaemia (SM) and uncomplicated malaria (UM). A logistic regression model was used to assess the association between antibody levels and severe malaria by comparing odds of severe malaria in tertile 1 (high) and tertile 0 (low) and measuring the effects using odds ratios. In the logistic regression model, age and parasite density were fitted as continuous variables.

4.5   Results

4.5.1   *Junju cohort description*

I used the Junju 2008 cross-sectional survey in which children were sampled before the start of malaria transmission in May and actively followed up for malaria case detection through a weekly home visit by trained fieldworkers. The *Plasmodium falciparum* parasite prevalence in 2-10-year-olds (PfPR $_{2\text{-}10}$) in this area was around 30% (Ndungu et al. 2015; Wamae et al. 2018). Of worthwhile to note is that the area has witnessed a general decline in malaria transmission over the last few years (Okiro et al. 2010; Mogeni et al. 2016). A total of 286 individuals with complete antibody responses measurement were available for further analysis. Out of which, 27.62% (79/286) were parasite positive at the time of sampling. Of note is that the parasite prevalence increased with age (4.1% at 0-2yrs, 28.1% at 3-5yrs and 36.6% at 6-10yrs) **Table 4.1**. The older age group had lower mean parasite density compared to the younger age group, although not significantly different (> 5 yrs at $\log_{10}$ parasite

densities 7.55 while 0-4 yrs at 8.56, *P*= 0.058, **Appendices Figure 4.1**). **Table 4.1**

below shows the baseline characteristics for the study participants.

Table 4.1 Table showing the demographic characteristics for the Junju 2008 cohort of children

Characteristics	Parasite Positive		Parasite Negative		Total
	N	%	N	%	N
Age groups (yrs)					
0-2 yrs	2	4.1	47	95.9	49
3-5 yrs	32	28.1	82	71.9	114
6-10 yrs	45	36.6	78	63.4	123
Sex					
Females	44	30.6	100	69.4	173
Males	35	24.6	107	75.5	113
Parasite prevalence % (95% CI) binomial exact			27.6% (22.5 - 33.2)		
Median parasite densities ((p/µl) min-max, IQR)			1920 (40 - 480000, IQR 6680)		

4.5.2 Antibody responses to the merozoite FH receptor antigens by parasite status and age

I measured antibody response in children in the Junju longitudinal cohort of children whose ages ranged between 0 to 10 years **Table 4.1**. As shown in **Figure 4.1** below, antibody responses to P12, PF3D7_062900_SEG2, PF3D7_1105800, Pf92 were significantly higher in children who had parasites than those who did not. Furthermore, antibody responses to PF3D7_0206200, PF3D7_0629500_SEG1, and RH5 were higher though not significant in the same category of children suggesting that antibody levels were boosted by parasite infection. On the contrary, antibody responses to PF3D7_1252300 were significantly lower in children who had parasites than those who did not.
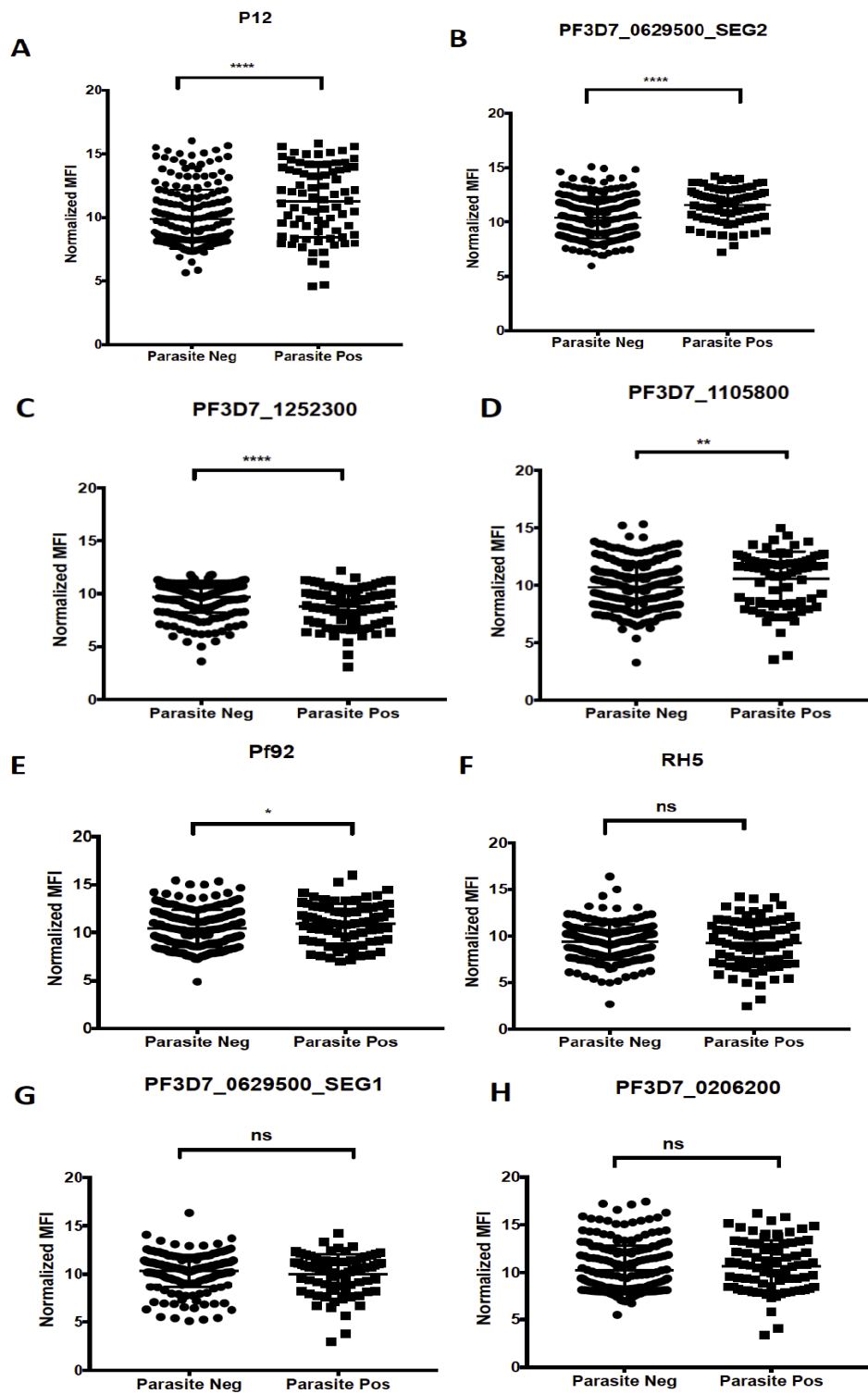
Figure 4.1 Antibody responses to merozoite FH receptor antigens by parasite status

A comparison of antibody levels in individuals who did not have parasites (Parasite Neg) to those who had parasites (Parasite Pos) at sampling to A) P12 B) PF3D7_0629500_SEG1 C) PF3D7_1252300 D) PF3D7_1105800 E) Pf92 F) RH5 G) PF3D7_0629500_SEG1 and H)

PF3D7_0206200. Data is from the Junju longitudinal children at the pre-transmission cross-sectional survey

Next, I tested the magnitude of antibody responses to these same antigens by age. I categorized the children into two main groups, 0-4 yrs and above 5 years in order to have sufficient numbers to compare. As shown in **Table 4.2** below antibody responses to P12, PF3D7_0206200, PF3D7_0629500_SEG2, PF3D7_1105800, and RH5 in parasite negative children increased with age and were significantly so for PF3D7_0629500_SEG2 in line with acquisition of malaria immunity with age and cumulative exposure. Antibody responses to PF3D7_1252300, PF3D7_0629500_SEG1 and Pf92 tended to decrease with an increase in age in the same category of children. In those children who had parasites at the time of sampling, responses to PF3D7_0206200, PF3D7_0629500_SEG1, PF3D7_1105800_PF3D7_1252300 and RH5 were lower in older children. However, antibody responses to P12, Pf92 and PF3D7_0629500_SEG2 increased with age in the same category of parasite positive children.

Table 4.2 Magnitude of antibody responses to merozoite FH receptor antigens

Antigen				Parasite positive by microscopy		
	0-4 yrs	**> 5yrs**	**P**	**0-4 yrs**	**> 5yrs**	**P**
P12	8.49 (8.10-11.00)	9.68 (8.25-11.75)	0.05	10.58(9.58-12.17)	11.95 (8.63-13.95)	0.54
PF3D7_0206200	9.02 (8.18-12.00)	9.40 (8.17-12.19)	0.95	11.71 (9.11-12.96)	10.33 (8.20-12.99)	0.14
PF3D7_0629500_SEG1	10.75 (9.64-11.46)	10.39 (9.35-11.00)	**0.01**	11.06 (10.61-11.63)	10.21 (8.26-11.27)	0.06
PF3D7_0629500_SEG2	9.86 (8.46-11.23)	11.12 (9.19-12.36)	**<0.01**	11.16 (9.98-12.22)	12.00 (10.67-12.95)	0.11
PF3D7_1105800	9.47 (8.09-11.21)	9.94 (8.30-11.35)	0.52	11.70 (11.31-12.56)	11.17 (8.38-12.03)	0.13
PF3D7_1252300	10.40 (9.44-10.80)	10.00 (8.58-10.75)	0.05	10.04 (9.36-10.39)	8.67 (7.23-10.07)	0.06
Pf92	10.75 (9.64-11.46)	10.53 (8.86-12.00)	0.08	10.84 (8.49-12.06)	11.13 (9.53-12.74)	0.24

| RH5 | 9.44 (8.16-10.72) | 9.53 (7.98-10.48) | 0.78 | 10.60 (8.92-11.22) | 8.95 (7.00-11.11) | 0.05 |

Median MFI values and inter quartile range are shown. Mann-Whitney test was used to compare median MFI between the two age groups in the parasite negative and parasite positive children. Data is from the Junju longitudinal children at the pre-transmission cross-sectional survey.

4.5.3 Association between merozoite FH receptor proteins and protection against clinical malaria

I investigated the association between antibody responses to merozoite FH receptor antigens and association with protection with clinical malaria in the longitudinal cohort of Junju children. Univariate analysis for parasite negative children showed that those who had high level antibody responses to PF3D7_1252300, Pf92 and PF3D7_0629500_SEG1 were protected against risk of developing clinical malaria, although this was not statistically significant. On the other hand, children who had high antibody responses to PF3D7_0629500_SEG2, P12, PF3D7_0206200 and PF3D7_1105800 had an increased prospective risk of *P. falciparum* malaria compared with children with low-level responses, although not statistically significant except for PF3D7_1105800. A similar increase risk was observed in children with high responses to PF3D7_1252300, PF3D7_0206200, and PF3D7_1105800 although this was in children who had parasites (parasite positive). Combining all the children, whether parasite positive or negative, increased prospective risk of developing clinical malaria was observed for children who made high antibody responses to PF3D7_1105800 and PF3D7_0206200 **Table 4.3.**

When adjustments were made using the reactivity score variable to control for potential confounders, the strength of association between antibodies and the prospective risk of developing at least one clinical episode of malaria during the 6 months follow up period was mostly reduced when parasite positive and parasite negative children were

combined. Controlling for the confounders had no effect on the IRR for PF3D7_0629500_SEG1 in parasite positive or RH5 and PF3D7_1252300 when parasite positive and parasite negative children were combined together **Table 4.3**. Almost similar IRR estimates were obtained when age instead of reactivity score variable was adjusted for in the modified Poisson regression model **Appendices Table 4.1. In addition to reactivity score, baseline FH levels was also added to the model.**

Table 4.3 Antibody responses to merozoite FH receptor antigens and as sociation with prospective risk of clinical malaria

Antigen	No adjustment		Reactivity score and factor H adjusted	
	IRR (95% CI)	*P*	IRR (95% CI)	*P*
Parasite negative children				
PF3D7_1252300	0.85 (0.61-1.17)	0.32	0.93 (0.66-1.31)	0.691
Pf92	0.95 (0.68-1.34)	0.79	0.99 (0.69-1.43)	0.972
PF3D7_0629500_SEG1	0.91 (0.67-1.24)	0.57	0.97 (0.71-1.34)	0.858
RH5	1.01 (0.71-1.44)	0.95	1.00 (0.69-1.45)	0.993
PF3D7_0629500_SEG2	1.11 (0.81-1.54)	0.52	0.96 (0.66-1.41)	0.842
P12	1.12 (0.81-1.54)	0.50	0.97 (0.67-1.42)	0.882
PF3D7_0206200	1.27 (0.95-1.71)	0.11	1.27 (0.83-1.96)	0.275
PF3D7_1105800	1.47 (1.09-1.97)	**0.01**	1.51 (1.03-2.21)	**0.036**
Parasite positive children				
PF3D7_0629500_SEG2	0.64 (0.29-1.43)	0.28	0.58 (0.26-1.32)	0.199
P12	0.67 (0.30-1.50)	0.34	0.60 (0.26-1.40)	0.234
PF3D7_0629500_SEG1	0.90 (0.40-2.05)	0.81	0.86 (0.37-1.98)	0.720
Pf92	0.96 (0.43-2.14)	0.93	1.06 (0.47-2.42)	0.881
RH5	1.00 (0.43-2.33)	0.99	1.99 (0.41-2.39)	0.982
PF3D7_1252300	1.30 (0.54-3.12)	0.55	1.28 (0.50-3.30)	0.611
PF3D7_0206200	1.25 (0.57-2.76)	0.57	1.45 (0.65-3.24)	0.362
PF3D7_1105800	1.36 (0.60-3.11)	0.46	1.82 (0.47-7.12)	0.386
All children irrespective of parasite status				
PF3D7_0629500_SEG2	0.87 (0.64-1.20)	0.40	0.78 (0.54-1.12)	0.179
P12	0.90 (0.66-1.23)	0.49	0.80 (0.56-1.15)	0.226
PF92	0.88 (0.64-1.22)	0.45	0.98 (0.72-1.37)	0.886
PF3D7_0629500_SEG1	0.92 (0.68-1.24)	0.59	0.97 (0.71-1.40)	0.918
RH5	0.96 (0.69-1.34)	0.82	0.98 (0.69-1.35)	0.80
PF3D7_1252300	0.97 (0.71-1.32)	0.85	1.03 (0.74-1.42)	0.880
PF3D7_1105800	1.22 (0.91-1.63)	0.19	1.35 (0.92-2.00)	0.122
PF3D7_0206200	1.23 (0.92-1.64)	0.16	1.41 (0.97-2.06)	0.072

Association between antibody levels and risk of developing clinical malaria in the ensuing 6 months period using modified Poisson regression model. Data is from the Junju longitudinal children at the pre-transmission cross-sectional survey

4.5.4 Combination of merozoite FH receptor antigen antibody responses and association with protection from malaria

Previously it has been shown that the breadth of antibody responses was associated with protection from clinical episodes of malaria (Osier et al. 2008). The 8 antigens were grouped into 2, 3 and 4 combinations and each parasite positive children assigned a breadth score. The breadth score was then fitted into a modified Poisson model as a categorical variable while adjusting for confounders using the reactivity score and clinical malaria episode in the ensuing 6 months period used as the outcome variable in the model. Thereafter, the protective efficacy was derived. I found a trend towards a slight increase in protective efficacy with increasing breadth of antibody responses. In addition, the median protective efficacy of the top best 5 protective antibodies (ranked based on individual antigen IRR) also slightly rose with increasing combination size **Figure 4.2.** However, on average (median) the protective efficacy was low, <50%, for any of the combination tested. The top 5 most protective antigens analyzed include, PF3D7_0629500_SEG2, P12, PF3D7_0629500_SEG1, Pf92, and RH5 **Table 4.3**. Together, these findings suggest that in this cohort of young children, the most protective of the four antigens offers the highest protective efficacy.
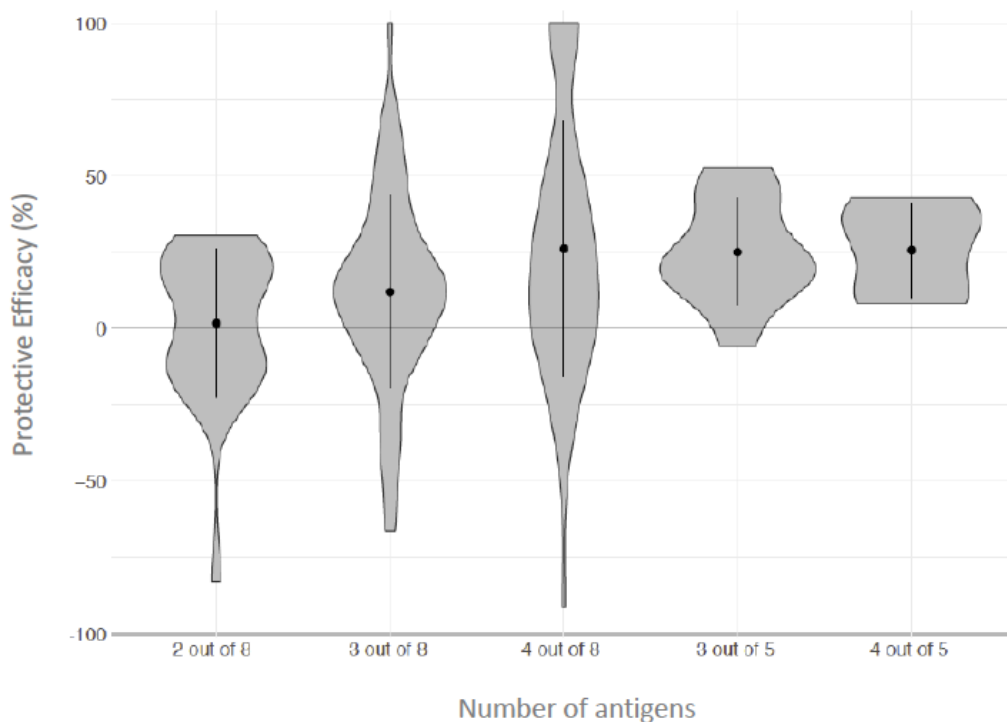
Figure 4.2 Protective efficacy of combination of antibody responses to the merozoite FH receptor antigens.

The IRR for clinical malaria episode obtained from the modified Poisson model were converted to protective efficacy (PE= (1-aIRR) *100). The combinations tested are shown on the x axis while the y axis shows the protective efficacy. Data is from the Junju longitudinal children at the pre-transmission cross-sectional survey

4.5.5   Comparison of antibody responses to merozoite FH receptor proteins in severe versus mild malaria

Baseline characteristic of severe malaria cases and uncomplicated malaria controls

The case-control study included children presenting with severe malaria; severe malaria anaemia (SMA) and severe malaria without anaemia (SM), and uncomplicated malaria (UM). A total of 131 children had SMA and presented to the hospital with Hb <5 grams per deciliter (g/dl), parasitemia of ≥ 10,000 parasites/µL of blood (Marsh et al. 1995a) while 87 had SM with symptoms that included; impaired consciousness defined as Blantyre coma score (BCS) of < 3, chest in-drawing or deep breathing

(English et al. 1996). On the other hand, 96 children had uncomplicated malaria defined as children presenting at an outpatient clinic with parasite density of ≥ 2500 parasites/ µL of blood and temperature of >37.5 °C (Smith, Schellenberg and Hayes, 1994; Mwangi *et al.*, 2005) but didn't have malaria severe enough to warrant admission. Hemoglobin level measurement was not done at the outpatient clinics; hence the data for the children in the UM group are not available. Only children under the age of 5 years were selected. **Table 4.4** shows the baseline characteristics for these categories of children. Clinical parameters were not available for the UM group as they are recruited from an outpatient dispensary clinic which conducts only basic patient observations.

Table 4.4 Baseline characteristics for the severe malaria cases and uncomplicated malaria controls

Characteristics	SM	SMA	UM	Total
	N (%)	N (%)	N (%)	
Age groups (yrs)				
0-1 yr	36 (41.38)	66 (50.38)	44 (45.83)	146
2-5 yrs	51 (58.62)	65 (49.62)	52 (54.17)	168
Sex				
Females	46 (52.87)	67 (51.15)	47 (48.96)	160
Males	41 (47.13)	64 (48.85)	49 (51.04)	154
Median parasite density (IQR	214480 (16848 – 470400)	235980 (74460 -361660)	18000 (8280 - 170000)	
Median Hb levels (IQR)	7.70 (6.50 - 9.70)	4.20 (3.70 - 4.60)	-	
Thal gene				
Het	24 (27.59)	28 (18.54)	-	
Homo	30 (34.48)	62 (41.06)	-	
No results	8 (9.19)	7 (4.64)	-	
Missing	25 (28.74)	54 (35.76)	-	
Sickle cell trait				
AA	61 (100)	79 (98.75)	-	
AS	0 (0)	1 (1.25)	-	
Co-infection				
Bacteraemia	20 (22.99)	20 (15.27)	-	
Missing bacteria results	67 (77.01)	111 (84.73)	-	
Median wbc levels (IQR)	13.5 (10.40-19.00)	12.70 (8.70-20.10)	-	
Median rbc levels (IQR)	3.5 (2.80-4.40)	1.80 (1.60-2.00)	-	

Median hct (IQR)	24.40 (20.70-29.90)	12.90 (11.50-14.10)	-
Median platelet (IQR)	103 (58.00-251.00)	96.00 (64.00-127.00)	-

Next, I tested whether there were differences in antibody responses to merozoite FH receptor antigens in this cohort of SM, SMA and UM. I observed a differential pattern in median antibody levels in the clinically different forms of malaria **Table 4.5**. For antigens P12, PF3D7_0629500_SEG1, Pf92, and RH5 the antibody responses did not differ among the groups. Statistically significant differences were observed for responses to PF3D7_0206200, PF3D7_0629500_SEG2, PF3D7_1105800, and PF3D7_1252300. When the children were stratified by age groups, the median antibody levels were significantly different for more antigens in children under the age of 2 yrs than the 2-5 yrs age category **Table 4.5** below.

Table 4.5 Antibody levels in children presenting with SMA, SM or UM

Median antibody levels for all children			
Antigen	**SM**	**SMA**	**UM**	***P***
P12	10.42	10.23	9.73	0.05
PF3D7_0206200	9.61	10.34	10.30	**<0.01**
PF3D7_0629500_SEG1	10.42	10.55	10.14	0.2113
PF3D7_0629500_SEG2	10.96	10.45	10.80	**<0.01**
PF3D7_1105800	11.13	10.46	10.05	**<0.01**
PF3D7_1252300	10.65	10.92	10.82	**<0.01**
Pf92	10.64	10.14	10.83	0.1219
RH5	10.14	10.09	9.79	0.1447
Median antibody levels for children under the age of 2 yrs			
Antigen	**SM**	**SMA**	**UM**	***P***
P12	9.90	9.73	9.26	0.17
PF3D7_0206200	9.38	10.11	10.04	**<0.01**
PF3D7_0629500_SEG1	10.20	10.31	9.80	0.17
PF3D7_0629500_SEG2	10.60	10.13	10.99	**0.02**
PF3D7_1105800	11.06	10.11	9.79	**0.03**
PF3D7_1252300	10.72	11.00	10.75	**<0.01**
Pf92	10.12	9.74	10.95	**0.03**
RH5	9.97	9.81	9.09	**<0.01**
Median antibody levels for children aged 2-5 yrs			

Antigen	SM	SMA	UM	*P*
P12	11.12	10.73	10.16	0.18
PF3D7_0206200	9.72	10.55	10.67	**0.02**
PF3D7_0629500_SEG1	10.56	10.72	10.55	0.49
PF3D7_0629500_SEG2	11.10	10.47	10.75	0.05
PF3D7_1105800	11.13	10.82	10.28	0.09
PF3D7_1252300	10.40	10.83	10.83	**<0.01**
Pf92	10.90	10.61	10.64	0.72
RH5	10.18	10.61	10.18	0.67

Median MFI value as measured using the protein microarray platform are displayed. The 3D7 reference strain was used for all antigens. Differences in the antibody responses among the groups were tested using the Kruskal Wallis test. The differences were again tested when children were stratified by ages (<2 yrs and 2-5 yrs). N=310 children. Data is from the Kilifi Hospital case control study.

I further interrogated the association between antibody levels and odds of having severe malaria using a logistic regression model. Children with either SMA or SM were pooled together into one group of severe malaria. Age, sex and parasite density were adjusted for, first by testing the effect of age alone then combining age, sex and parasite density together. High antibody responses to P12, PF3D7_0629500_SEG1, PF3D7_1105800, and RH5 were associated with increased odds of having severe malaria but only responses to PF3D7_1105800 were statistically significant when age was accounted for but lost when age, sex and parasite density were included in the model **Table 4.6**. Antibody responses to Pf92 were the only ones significantly associated with reduced odds of developing severe malaria and remained so after accounting for age, sex and parasite density **Table 4.6**.

Table 4.6 Association between antibody levels and odds of having severe malaria

Antibody	Univariate analysis		Adjusted for age		Adjusted for age, sex and parasite density	
	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
P12	1.47 (0.87-2.48)	0.15	1.56 (0.91-2.67)	0.11	1.60 (0.91-2.82)	0.10

PF3D7_0206200	0.74 (0.45-1.23)	0.25	0.76 (0.46-1.26)	0.28	0.67 (0.39-1.16)	0.15
PF3D7_0629500_SEG1	1.31 (0.78-2.21)	0.31	1.36 (0.80-2.32)	0.25	1.17 (0.66-2.06)	0.58
PF3D7_0629500_SEG2	0.76 (0.46-1.25)	0.28	0.76 (0.46-1.26)	0.28	0.78 (0.46-1.33)	0.36
PF3D7_1105800	1.83 (1.07-3.13)	**0.03**	1.87 (1.09-3.22)	**0.02**	1.74 (0.99-3.08)	0.06
PF3D7_1252300	0.98 (0.58-1.64)	0.93	0.95 (0.56-1.60)	0.84	1.02 (0.59-1.76)	0.96
Pf92	0.56 (0.34-0.92)	**0.02**	0.57 (0.34-0.94)	**0.03**	0.56 (0.33-0.95)	**0.03**
RH5	1.41 (0.83-2.38)	0.20	1.45 (0.85-2.47)	0.17	1.40 (0.80-2.46)	0.24

A logistic regression model used to calculate the odds of developing severe malaria in children under the age of 5. Age and parasite density were fitted as continuous variable. Antibody levels were fitted as a binary variable. Data is from the Kilifi Hospital case control study.

4.6   Discussion

The main aim of this study was to test whether antigens that bind to FH (merozoite FH receptor proteins) identified in chapter 3 were associated with protection against clinical malaria. The list of antigens tested included PF3D7_1105800, PF3D7_0206200, RH5, Pf12, PF3D7_1252300, PF3D7_0629500 (SEG1), PF3D7_0629500 (SEG2) and Pf92. In this Junju cohort of young children, most of these antigens were associated with protection, although none of the associations was statistically significant. However, there was an increase in protective efficacy with the increase in breadth responses. These data are in line with the previous observation that the breadth of antibody response is a good predictor of immunity against a clinical episode of malaria (Osier et al. 2008). However, it will be important that future studies are conducted in order to shed light on the generalizability of these findings and to identify important antigens across different populations that can be prioritized for vaccine development.

In a previous study where a similar cohort (Junju 2008 samples) were used, antibody levels were required to reach a defined protective threshold in order to be associated with protection. In fact, only a small proportion of children in Junju had antibody levels above the protective threshold (Murungi et al. 2013). Since the antigens tested were different from those used in the current study, a cut off for protective threshold was not applied. However, most of the antibody levels increased with age and were boosted in the presence of parasite infection as has been observed previously (Osier et al. 2008; Rono et al. 2013; Stanisic et al. 2015).

Majority of antigens described in my study are novel in the context of naturally acquired immunity to malaria (Kamuyu 2017, PhD Thesis) and have therefore not been previously evaluated in a cohort of children. However, in an adult cohort in Nyamisati, Tanzania sampled between 1993-1995, antibody responses to PF3D7_0629500 (SEG2), PF3D7_0206200 and PF3D7_1252300 were found to be associated with reduced odds of experiencing a clinical episode of malaria similar to what was observed for this current study **Table 4.6**. However, for the Nyamisati cohort the association was not statistically significant when adjustments were made to account for multiple comparisons (Kamuyu 2017, PhD Thesis). Other studies indicate that antibody responses to RH5 are associated with protection against malaria (Chiu et al. 2014; Tran et al. 2014). Very few studies have investigated antibody levels to Pf92 and its association with protection (Osier *et al.*, 2014).

Although immune-epidemiological studies are important in defining the targets of protective antibodies, inconsistencies are widely observed in many studies. While some show a protective role to a specific antigen, others do not (Fowkes et al. 2010). This can be partly be explained due to misclassification bias as to who is protected during the follow-up period. For instance, individuals can be classified as protected, but due to heterogeneity in exposure in endemic areas, these individuals could have

been not exposed to infectious bites (Bejon et al. 2009). I speculate that this could perhaps explain why the antigens that were associated with reduced risk of developing malaria differed between parasite positive and parasite negative children except for Pf92 in this study. To reduce this misclassification, it has been suggested that the analysis should be restricted to those children with parasites (asymptomatic) during sampling as they would have had equal exposure (Osier *et al.*, 2014). Based on this argument only antibody responses to PF3D7_0629500 (SEG2), P12, PF3D7_0629500 (SEG1) and Pf92 was associated with reduced risk of developing clinical malaria in the follow-up period. However, it is worthwhile to note that only a small subset of children, 28% in this cohort had parasites at the point of sampling.

In the group of children with severe malaria (SMA, SM) and UM, I tested whether there were differential antibody levels based on their clinical presentation. There were statistically significant differences in median antibody levels to PF3D7_0629500 (SEG2), PF3D7_ 0206200, PF3D7_1105800 and PF3D7_1252300. This is a line with a previous study that observed differential antibody levels in children presenting with similar clinical phenotypes (Dobaño et al. 2008) although they tested different antigens than those used in this current study. To account for the fact that the children in this study had different parasite densities **Table 4.4** and also age, I used a logistic regression model and observed that there was reduced odds of having severe malaria for responses to Pf92 even after adjusting for age, sex and parasite densities.

Studies assessing antibody responses using hospital-based case-control designs have widely been conducted (de Souza et al. 2002; Perraut et al. 2005; OKECH et al. 2006; TANGTEERAWATANA et al. 2007; Dobaño et al. 2008; Iriemenam et al. 2009; Ahmed Ismail et al. 2013). However, thus far these studies have failed to provide consistent results, and this can be attributed to partly due to differences in malaria case definition and methodological differences. Future studies with sufficient statistical

power and using carefully selected cases and control and head to head comparison using similar antigens are warranted to shed a light of the importance of antibody levels and susceptibility to severe malaria disease.

The current study is not without limitations. Although antibody levels were measured in the longitudinal cohort, potential mechanisms of protective immunity were not assessed due to time limitations. *In vitro* assays that are aimed at identifying antibody effector function continue to be developed and validated across different research groups (Boyle et al. 2017). What is more interesting is that some assays such as the complement-fixing antibody assay can now be specifically tested against an antigen (Reiling et al. 2019) unlike most of the other assays where whole merozoites/whole parasites are used. It will be interesting to test whether the antibodies to the antigens discovered by this thesis project fix complement or whether they work using different mechanisms.

In conclusion, this study provides evidence that antibodies against merozoite FH receptor antigens are targets of naturally acquired immunity to malaria. Although there was no evidence to suggest that antibody responses to these merozoite FH receptor antigens were individually associated with protection, some combination of these responses were found to be associated with protective immunity. Comprehensively characterizing *Plasmodium falciparum* stage specific antigens that interact with FH for parasite immune evasion will be important in determining which of these antigens could be good candidates for vaccine development.

5 Association of human complement FH levels and Y402H variant with susceptibility and severity to *Plasmodium falciparum* malaria

5.1 Summary and aims

Many pathogenic microbes have been shown to protect themselves against alternative pathway attack by acquiring host FH onto their surfaces (Ferreira et al. 2010; Dinko and Pradel, 2016). In the previous chapter, chapter 3, I showed that some *Plasmodium falciparum* merozoite proteins interact with FH, and this could be important in allowing the parasite to establish infection. Next, in chapter 4, I showed that these merozoite FH receptor proteins are important antibody targets of naturally acquired immunity to malaria. In this current chapter, I hypothesize that since *in vitro* experiments have shown that *P. falciparum* recruits FH unto its surface to protect the parasite from complement onslaught (Simon *et al.,* 2013; Kennedy *et al.,* 2016; Rosa *et al*., 2016), plasma levels of FH may influence susceptibility to malaria infection.

Long-standing observations point to the fact that mutations, variations and autoantibodies to FH predispose individuals to both infections (Davila et al. 2010), and autoimmune diseases (Pickering and Cook 2008; Ansari et al. 2013). Around 30% of people of European descent carry at least one copy of the 402H risk allele (Sofat et al. 2012). A proposed explanation for the high prevalence of the Y402H polymorphism is that the 402H allele provides a survival advantage against streptococcal infections in early life (Haapasalo et al. 2008). The FH binding protein of streptococcus has also been shown to have a lower affinity for 402H than 402Y, which would lead to enhanced alternative pathway activation on these bacteria. Here, I hypothesize that there would be polymorphisms in the *FH gene*, particularly the 402H allele that reduces the binding

between factor H and the malaria parasite, hence enhancing complement-mediated killing and ultimately conferring protection against malaria.

5.2   Introduction

Increased complement activation has been consistently observed in human malaria infections (Silver et al. 2010), with all the three major complement pathways activated. Malaria antigens expressed on the surface of infected erythrocytes, free antigens released from schizont rupture or by immune complexes formed by antibody interactions with antigens (Greenwood and Brueton 1974; Glew et al. 1975; Adam et al. 1981) can be recognized by IgG antibodies and this, in turn, can trigger the classical complement pathway (Roestenberg et al. 2007). Large amounts of hematin activate the alternative pathway and lead to increased deposition of C3b and its breakdown products on erythrocytes (Pawluczkowycz et al. 2007). Activities of the MBL pathway have also been found to be greatly reduced in children with severe malaria anaemia unlike their age-matched controls (Nyakoe et al. 2009).

Complement activity has been suggested to partly explain severe malaria anaemia (SMA) pathogenesis (Silver et al. 2010; Biryukov and Stoute 2014). SMA is thought to arise as a result of direct destruction of RBCs during schizont rupture, increased destruction of uninfected RBCs and impaired erythropoiesis due to bone marrow suppression (Jakeman et al. 1999; Awah et al. 2011). Children with severe malaria anemia have been shown to have higher levels of immune complexes (Mibei et al. 2005), lower complement hemolytic activity (CH50) in addition to decreased activity of all the major complement pathways suggesting increase complement consumption (Nyakoe et al. 2009). Likewise, other complement breakdown products such as C3a, C4a, and C5a have also been seen to be elevated in children with severe malaria (Silver et al. 2010). Similarly, children with SMA have been shown to have deficiencies

in complement regulatory proteins CR1 and CD55 (Waitumbi et al. 2015). Erythrocyte deficiencies of CR1 and CD55 in children with SMA result in reduced immune complex (IC) binding capacity and are associated with increased deposition of C3b on erythrocytes during malaria infection (Odhiambo et al. 2008; Owuor et al. 2008).

Complement activation has also been implicated in placental malaria (Alim et al. 2015; McDonald et al. 2015; Khattab et al. 2013). Levels of maternal plasma C5a and placental messenger RNA (mRNA) encoding the C5a receptor, C5aR, are increased in placental malaria infection (Conroy et al. 2009). Equally, the levels of C5a have been shown to be significantly higher in women with preeclampsia than in normal pregnant women. In contrast, the median maternal plasma concentration of C4a has been shown to be lower in women with preeclampsia than that of those with normal pregnancy with no changes in complement C3a observed (Soto et al. 2010). In summary, there is evidence to suggest that, 1) complement activation occurs during malaria infection, and 2) complement activation may be linked to the pathogenesis of malaria. However, little attention has been paid specifically to the role of plasma FH levels and polymorphisms in FH and whether they influence malaria outcomes.

Common polymorphisms in the *CFH gene* can be classified into three groups: (1) polymorphisms affecting the ability of FH to bind to either host or pathogens surfaces; (2) polymorphisms affecting how FH regulates alternative pathway activation; and (3) polymorphisms affecting FH expression and hence circulating levels (De Córdoba and De Jorge 2007; Harris et al. 2012; Van Der Maten et al. 2016). One of the FH variant that has been shown to be associated with circulating FH levels and affect disease susceptibility is the Y402H variant (Haralambous et al. 2006; Sharma et al. 2013). Therefore, I tested whether the Y402H variant influences FH circulating levels in a cohort of children and whether it relates to the risk of developing clinical malaria.

5.3   Specific aims

i.   Determine whether variation in FH levels within the population is associated with the development of clinical episodes of malaria

ii.   Test for correlation between FH levels and antibodies to merozoite FH receptor proteins

iii.   Determine whether complement factor H levels differ in children with severe malaria compared to uncomplicated malaria controls

iv.   Investigate whether the Y402H allele is a risk factor for clinical malaria

v.   Explore whether there is a relationship between FH genotype and plasma FH levels

5.4   Methods

5.4.1   Study design

The current study used archived heparinized plasma samples that are part of larger malaria immunological studies that are conducted at the KEMRI Wellcome Trust Research Programme started in 2005 (for the Junju longitudinal cohort of children) and 2000 (for the hospital case-control cohort) and are on-going to date (see methods **section 2.4 and 2.5** for more details). Baseline characteristics for the longitudinal cohort and the case-control are described in chapter 4 **Table 4.1** and **Table 4.4** respectively. For the Junju cohort, only 276 children had complete antibody and FH level measurements. Hence, only they were analyzed in the current chapter.

5.4.2   Laboratory procedures

Inappropriate sample handling after collection can influence the evaluation of complement biomarkers (Yang et al. 2015). EDTA-plasma is recommended for the determinations of complement proteins levels. EDTA blocks both classical and

alternative pathways by chelating Ca2+ and Mg2+ ions (Ca2+ is needed for the assembly of C1 complex and Mg2+ for the formation of the alternative pathway C3 convertase). This prevents artificial complement consumption during sample transport and handling, which may lead to false results (Roumenina et al. 2011). Although EDTA for reasons stated above is more efficient than say citrate and heparin in inhibiting *in vitro* activation, minimum activation has also been observed in all preparations (citrate and heparin) when samples are kept at 4°C for up to ten days. It has also been noted that a rapid increase in activation products can occur even in EDTA plasma when temperatures are elevated (Mollnes et al. 1988). Up to four freeze /thaw cycles on ice or room temperatures (RT) are known not to substantially increase the levels of C3a, factor Bb, C5a, and C5b-9 but can significantly affect C4d. Long term storage of plasma at -80°C has no significant effect on levels of complement factors (Yang et al. 2015). It is recommended that if using serum strict handling should be followed as stated by Lachmann (Lachmann 2010). The current study used heparinized archived plasma samples collected and stored at -80°C.

5.4.3 *Sample preparation for complement proteins measurement*

Venous blood was collected from all of the subjects into heparin tubes (BD Diagnostics, Oxford, England) and was transported from the hospital to the laboratory at 4°C in cooler boxes. Blood samples were centrifuged at 440g for 5 minutes at 4°C, and the plasma was aliquoted and stored at −80°C within an hour of collection and then thawed to room temperatures on ice when required by individual study investigators. The plasma FH levels and TCC levels were quantified by commercial MicroVue Factor H EIA (Quidel, USA) and MicroVue sC5b-9 Plus Enzyme Immunoassay kit (Quidel, USA) respectively. The FH ELISA kit determines the amount of free FH molecules in human serum (Simon et al. 2018).

5.4.4 Genotyping for the Y402H variant of FH

Genotyping of the Y402H variant was done using i) Sanger sequencing with published primers (Lau et al. 2006) and ii) using Factor H, 402H/Y variant detection, Human, ELISA kit (HycultBiotech) for a subset of samples. After sequencing, chromatograms were individually inspected using Ugene chromatogram viewer https://ugene.net/wiki/display/UUOUM15/Chromatogram+Viewer. To narrow down to the risk allele position, I used the nucleotide sequence; AAAAT**C**ATGGA (Dr. Ayman Khattab, personal communication) to search each sequence whereby the C in red denotes the presence of the variant nucleotide.

5.4.5 Statistical analysis

The correlation between FH levels and antibody MFI levels to merozoite FH receptor antigens were assessed using Spearman's rho. Kruskal-Wallis test was used to compare the median FH levels across the three age groups (0-2 yrs, 3-5yrs and >6 yrs). FH levels for all the children were grouped into ranked three tertiles from highest to the lowest as high, medium and low. Association between tertile and time to a first clinical episode of malaria (fever and > 2500 p/µl) was assessed using Cox proportional hazard model. The observation time was six months from the sampling time. Age as a categorical (0-2yrs, 3-5 yrs and 6+ yrs) variable was fitted into the model to account for its confounding effect on malaria. The Kruskal-Wallis test was also used to compared median FH levels in children with severe malaria anaemia (SMA), severe malaria without anaemia (SM) and uncomplicated malaria. A logistic regression model was used to test for the association between FH levels and risk of having severe malaria.

5.5 Results

5.5.1 Variation in FH levels as measured in a longitudinal cohort of children

Human complement FH is a 155kDa glycoprotein, composed of 1213 amino acid residues (Ripoche et al. 1988). It is the central fluid phase regulator of the alternative pathway (Pangburn 2002; Ferreira et al. 2010). It is mainly synthesized by the liver (Fagerberg et al. 2014) and is found in human plasma at highly variable concentrations (Esparza-Gordillo et al. 2004; Scholl et al. 2008). Similarly, in this cohort of young children (n=276) **Figure 5.1 A**, mean age 5.5 yrs, FH levels were observed to have a 5-fold range (117 - 699 ug/ml). In those children who had parasites at the time of sampling (n=79) the median FH levels was 245 ug/ml 95% CI (238-270 ug/ml) compared to 263 ug/ml 95% CI (251-275ug/ml) for the children who did not have parasites (n=197) **Appendices Figure 5.1**. Median FH levels did not vary across the age categories **Figure 5.1 B**. I observed a very weak negative correlation between FH levels and parasite density (r= -0.072, *P* = 0.523 **Appendices Figure 5.2**).
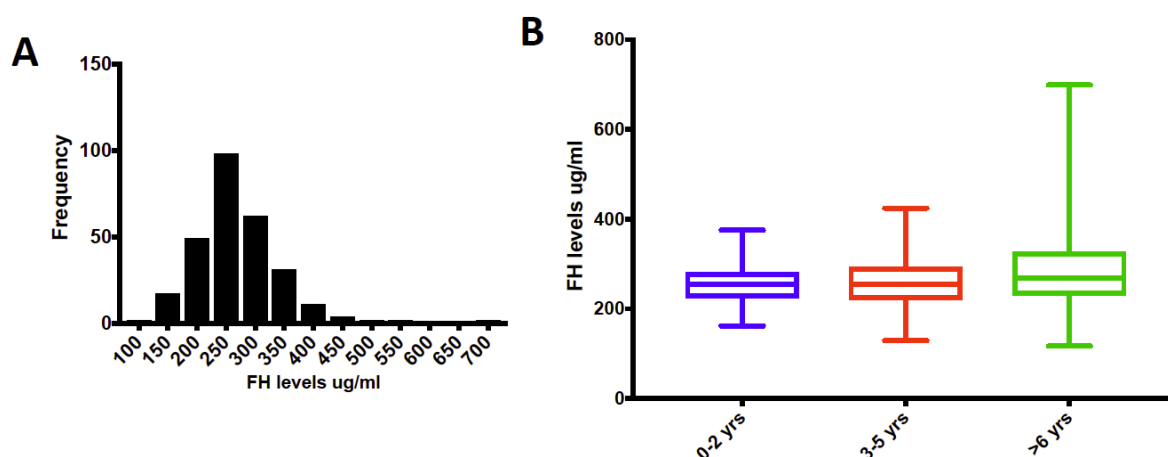
Figure 5.1 Distribution of FH levels (A) and comparison with age (B) in the malaria longitudinal cohort.

Children were divided into three age groups, 0-2yrs n=48, 3-5 yrs n=106 and >6 yrs n=122. Box plots show the medians with 25% and 75% percentiles and the total range of FH levels across the age groups.

5.5.2   FH levels and association with protection from malaria

Next, I tested whether the levels of FH measured during the cross-sectional survey would prospectively influence an individual's risk for the development of a clinical episode of malaria during the ensuing six months follow-up period. Individuals were grouped into three tertiles of high, medium, and low. I observed that FH levels (high, medium or low) were not associated with age **Figure 5.2 A**. Children who had high FH levels tended to have more clinical episodes **Figure 5.2 B**. The same trend was observed when children were stratified to whether they were parasite negative or parasite positive at the time of sampling **Appendices Figure 5.3**. Additionally, cox regression output shows that having high FH levels increases the risk of clinical malaria, but this is not statistically **Figure 5.2 C**. Similarly, it has been observed that high plasma levels of FH increase susceptibility to *Neisseria meningitidis* (Haralambous et al. 2006).

A

	0-2 yrs	3-5 yrs	> 6 yrs	Total
Low	16	39	37	92
Medium	21	33	38	92
High	11	34	47	92
Total	48	106	122	276

B

Fisher's exact=0.273

P= 0.2522
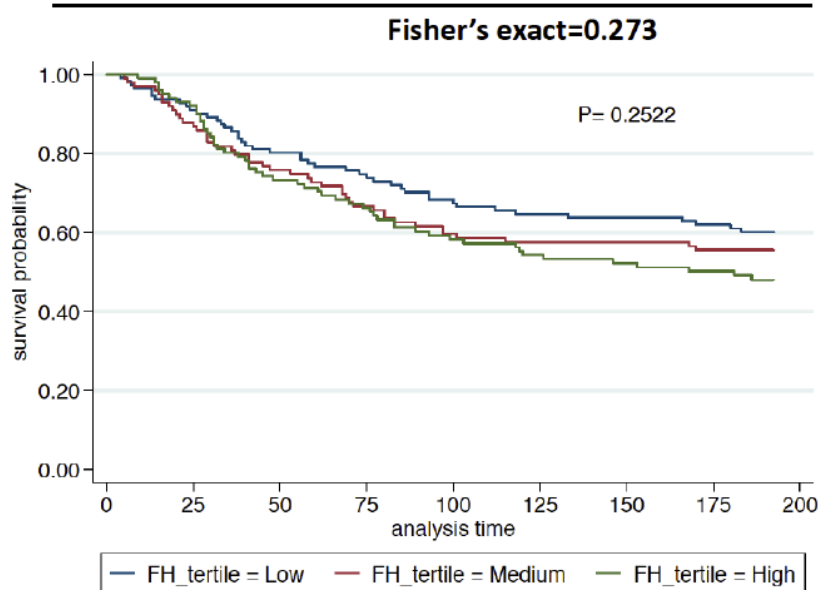
C

	uHR (95% CI)	p	Adjusted for age (95% CI)	p	Adjusted for age plus Age and FH level interaction	p
Low	1	-	1	-	1	-
Medium	1.19 (0.78-1.81)	0.41	1.22 (0.80-1.85)	0.91	1.47 (0.59-3.66)	0.41
High	1.40 (0.94-2.09)	0.10	1.55 (1.03-2.32)	2.11	2.25 (0.85-6.02)	0.10
	PH assumption (p value=0.3760)		PH assumption (p value=0.4100)		PH assumption (p value=0.6420)	

Figure 5.2 Risk of developing a clinical episode of malaria based on FH levels.

A) Frequency distribution of FH levels based on either low, medium or high based on rank, categorized by age B) Kaplan-Meier survival curves showing the risk of malaria stratified by whether the children had low (blue), medium (red) or high (green) FH levels, Statistical significance was determined by the log rank test for the differences between all three groups

C) Hazard ratio calculated using Cox proportional hazard model based while accounting for multiple clinical episodes of malaria during the six months follow up period. Unadjusted hazard ratio (uHR), adjusted HR (age adjusted) and interaction between age and FH were calculated. PH is the proportional hazard assumption test

5.5.3   Correlation between FH levels and antibodies to merozoite FH receptor antigens

Pathogenic proteins or antigens that bind complement regulatory protein FH are mostly surface exposed and have been shown to be targets of protective opsonophagocytic antibodies (Meri et al. 2008; Serruto et al. 2010). Specifically for *Neisseria meningitidis*, it has been shown that there is competition between FH and antibodies for binding to FH binding protein (fHbp) (Caesar et al. 2014; Biagini et al. 2016). It has been reported that there is an inverse correlation between FH in circulation and serum bactericidal antibody responses (Beernink et al. 2011). Here, I tested for correlation between circulating FH levels and protective antibody responses. I found no correlation between FH and antibody responses to merozoite FH receptor antigens **Figure 5.3**. The results obtained here suggest that the two could be acting independently of each other and highlight the complexity of malaria parasite-complement interactions.
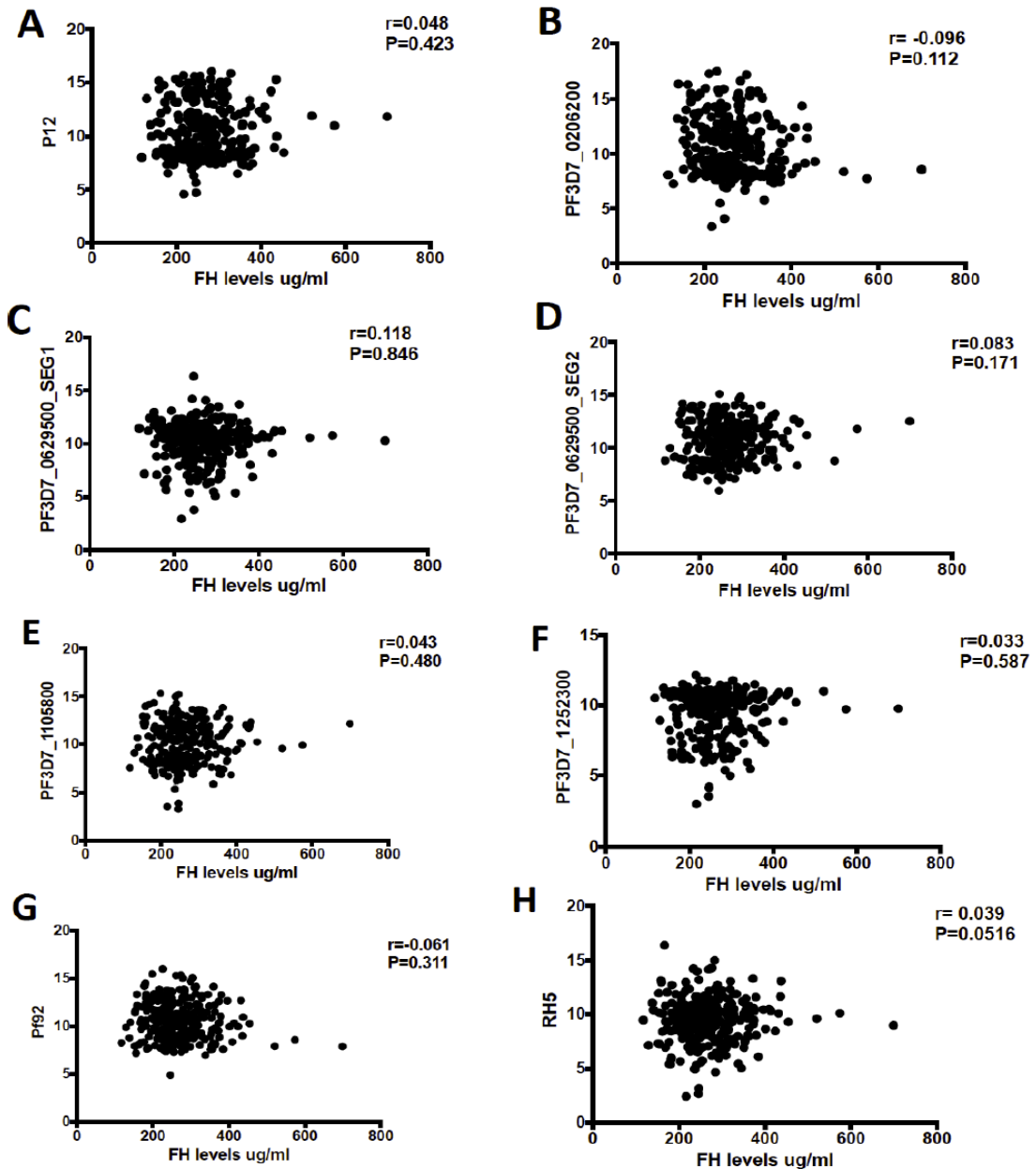
Figure 5.3 Correlation between FH levels and antibody responses

Antibody MFI measurements from all children in the Junju longitudinal cohort of children n=276 were correlated with FH levels. From the top A) P12 B) PF3D7_0206200 C) PF3D7_0629500_SEG1 D) PF3D7_0629500_SEG2 E) PF3D7_1105800 G) PF3D7_1252300 G) Pf92 and H) RH5 on the Y axis and FH levels on the X axis. Correlations were calculated using the Spearman's rho. Statistical significance is indicated as *p*-value for each comparison.

5.5.4 Complement activation occurs during malaria infection

There is evidence to suggest that complement activation occurs during malaria infection (Silver et al. 2010). I sought to determine whether there was differing complement activation in children with SMA, SM, and UM. Due to limited reagent availability, randomly selected samples of 29, 27, and 28 from UM, SMA and UM respectively were assessed. The median parasite density was significantly different among them (Kruskal Wallis test, P=0.025). The mean age was 2.3 yrs, 1.5 yrs and 1.8 yrs for UM, SMA and SM respectively. Children with SMA had markedly raised plasma levels of soluble terminal complement complex (TCC), compared with uncomplicated malaria (P=0.049). Children with SM also had raised complement activation compared with those with UM, although this was not significant (P=0.078). However, there was no significant difference between SMA and SM **Figure 5.4.** Overall there was increased complement activation in severe malaria compared to uncomplicated malaria.
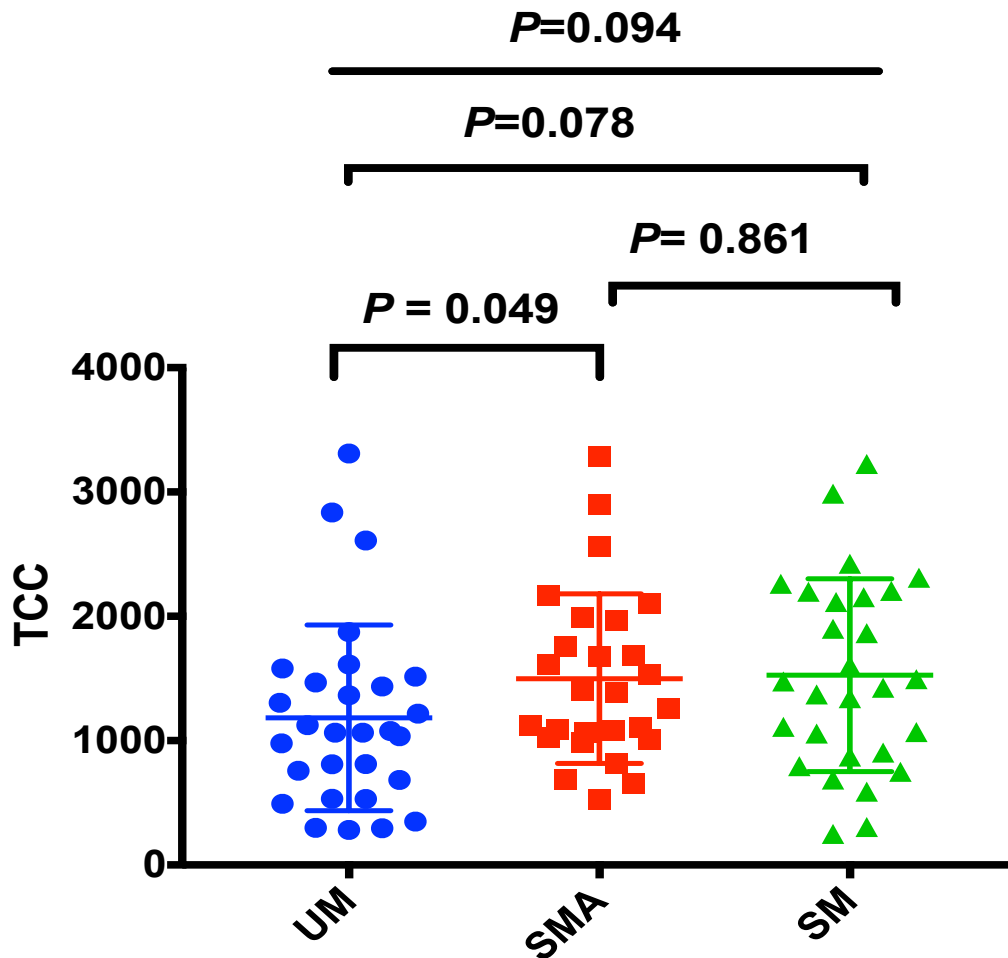
Figure 5.4 Complement activation in UM, SMA and SM.

Randomly selected samples of 29, 27, and 28 from children with UM, SMA and UM respectively were assessed for TCC activation levels. The Mann-Whitney test was used for comparison between groups. For comparison among groups Kruskal-Wallis test was used

5.5.5   Circulating levels of FH in severe and uncomplicated malaria

After observing that complement activation occurs in children with malaria **Figure 5.4**, next I tested whether there were differential FH levels in children with severe malaria without anaemia (SM, n=85), severe malaria anaemia (SMA, n=129) and uncomplicated malaria (UM, n=96). The median FH levels in SM was 316.29 ug/ml (range 252.65-366.87 ug/ml), for SMA it was 247.70 ug/ml (range (93.52-315.30 ug/ml) while UM children had median of 381.58 ug/ml (range 316.58- 450.46 ug/ml). The

difference in levels of FH among the groups was statistically significant (P= <0.0001)

Figure 5.5.
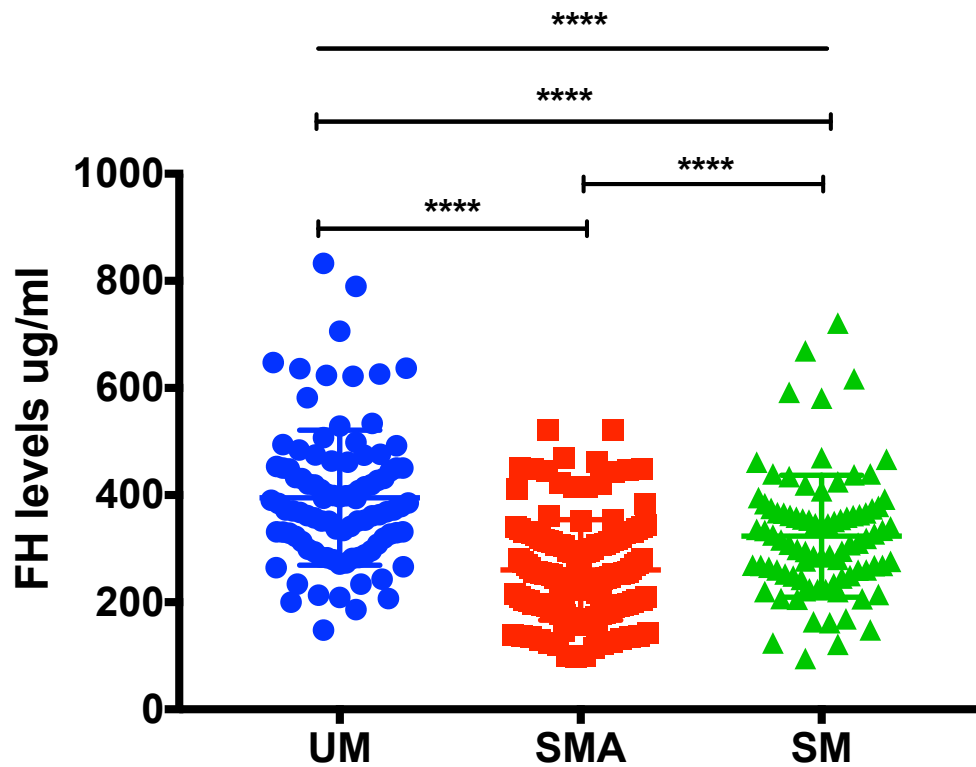
Figure 5.5 Factor H serum levels in children with severe malaria, SMA and SM compared to controls, UM

FH levels were measured in children presenting at the hospital with uncomplicated malaria (UM, n=96), severe malaria anaemia (SMA, n=129) and severe malaria without anaemia (SM, n=85). Mann-Whitney test was performed for the comparison between groups. For comparison among groups Kruskal-Wallis test was used.

Additionally, the median parasite density for children with SM was 214480 parasites/ µL range (16848- 470400 parasites/ µL), for those with SMA it was 241780 range (74925-364885 parasites/ µL) while children with UM had median parasite density of 18360 parasites/ µL (range 8280-170000 parasites/ µL). The median parasite density was significantly different among the groups (*P*= <0.0001). All the children were under the age of 5 years. Next, I used ordered logistic regression model to test for the

association between FH levels and the odds of having severe malaria. FH levels was used as the outcome variable and malaria severity, parasitaemia and age as covariates **Table 5.1**. The results indicate that children with severe malaria have a low probability of having high FH levels **Table 5.1**.

Table 5.1 Relationship between FH levels and severe malaria

Covariates	Total	Low (n %)	Medium (n %)	High (n %)	Crude odds ratio	*P* value	Adjusted odds ratio	*P* value
Malaria severity								
UM	96	9 (9.38)	28 (29.17)	59 (61.46)	1.00		1.00	
SMA/SM	214	95 (44.39)	75 (35.05)	44 (20.56)	0.15 (0.09-0.25)	<0.001	0.13 (0.08-0.24)	**<0.001**
Age								
<2 yrs	144	47 (32.64)	48 (33.33)	49 (34.03)	1.00			
2-5 yrs	166	57 (34.34)	55 (33.13)	54 (32.53)	0.93 (0.62-1.40)	0.731	0.95 (0.62-1.46)	0.813
Parasitemia mean (SD)								
Parasitemia	310	239278.4 (213284.9)	249278.5 (266606.8)	156733.2 (207942.8)	0.99 (0.98-1.00)	0.015	1 (0.99-1.00)	0.967

Total number of children included in the analysis is N=310 with children having severe malaria being 214 and those with uncomplicated malaria being 96

To further investigate the role of FH in with malaria, I assessed the relationship between FH and markers of malaria severity including parasite density and hemoglobin levels. There was a trend towards lower parasite density with increase in FH levels **Figure 5.6 A**. On the contrary there was a trend towards higher Hb as FH levels increased **Figure 5.6 B**. While there was a weak negative statistically significant correlation between parasite density and FH levels (r= -0.208, P=<0.001), there was a positive correlation between hemoglobin levels and FH (r=0.295) P=<0.001), which was also statistically significant.
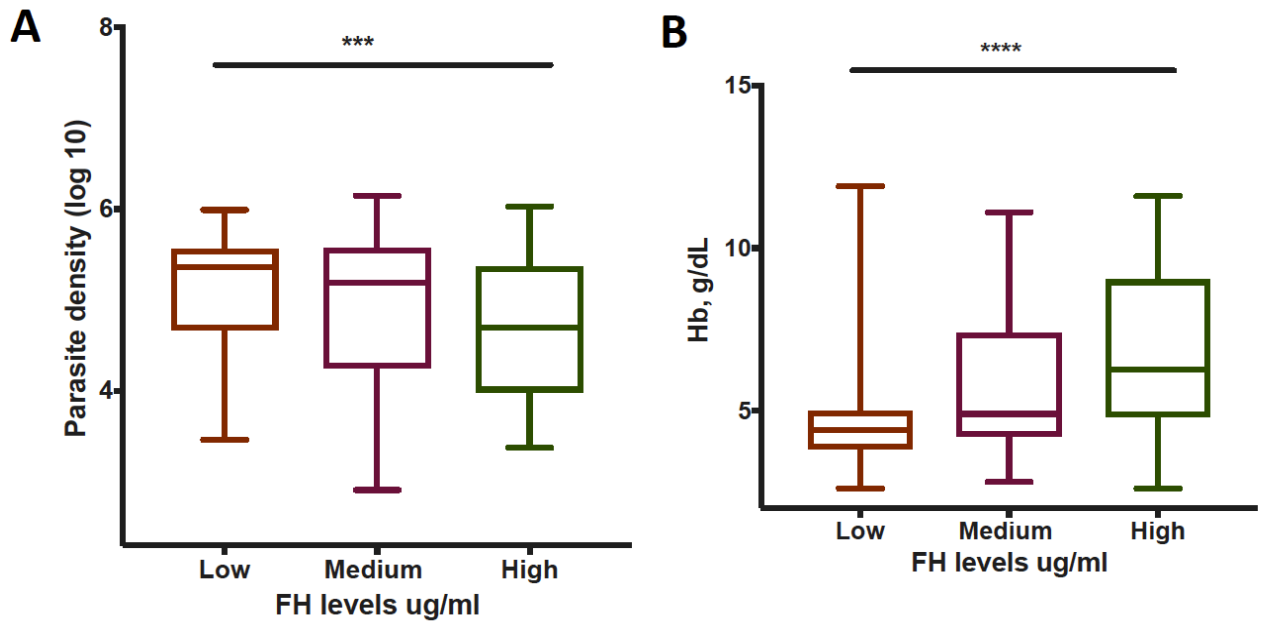
Figure 5.6 Relationship between FH levels and Parasite density (A) and Hemoglobin levels (B).

A total of N=310 children with either SMA, SM and UM were used for the comparison, with FH levels split into low, medium and high based on rank. Although all the children had complete parasite density data, only 161 had Hb data available. Kruskal-Wallis test was used for comparison among the groups.

I also investigated whether complement activation that leads to TCC formation as observed in **Figure 5.4** relates to complement regulation, in terms of FH levels. I found evidence of negative correlation between TCC and FH levels (r= -0.196 P=0.035). In addition, children with high FH levels tended to have less complement activation **Figure 5.7**.
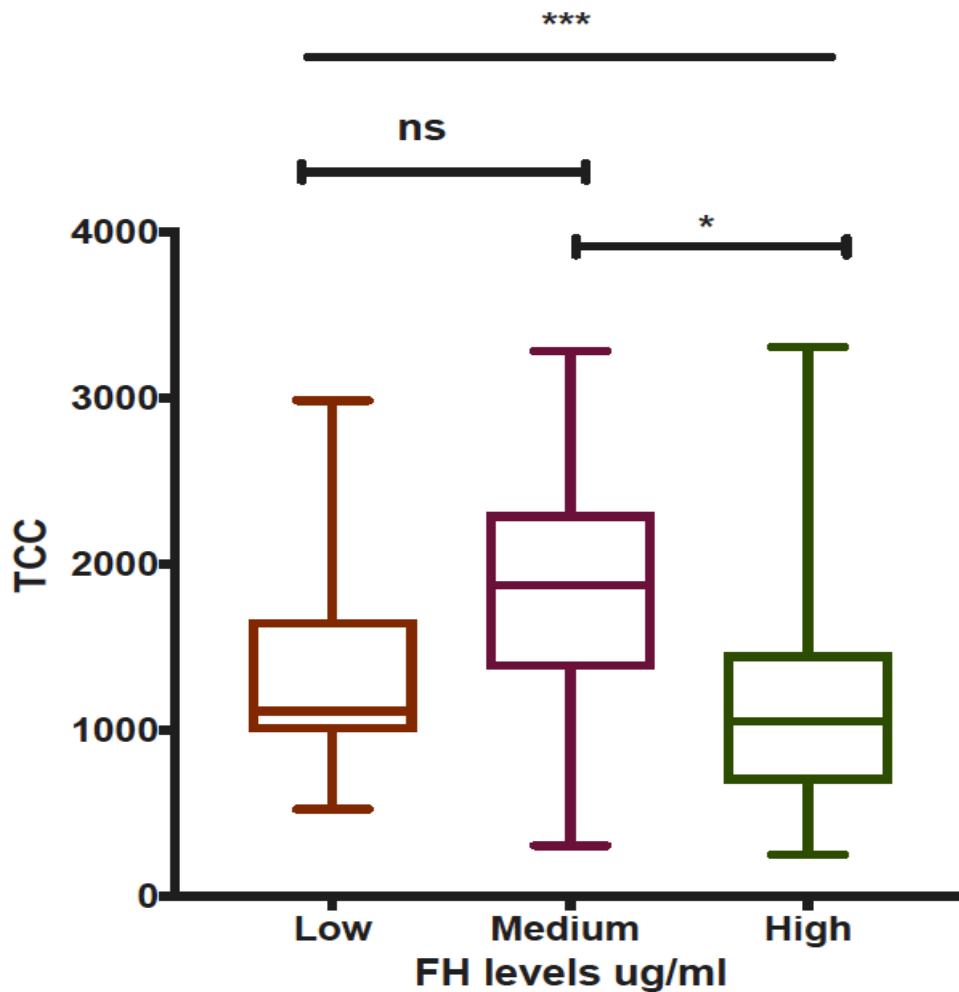
Figure 5.7 Relationship between complement activation and regulation

Only children with complete TCC and FH measurement were included in the analysis n=84, with FH split into low, medium and high based on rank. Kruskal-Wallis test was used for comparison among the three groups while Mann-Whitney test was performed for the comparison between groups

5.5.6   The Y402H variant of FH and risk of malaria

5.5.6.1   Genotyping for the Y402H variant in the malaria case control study using Sanger sequencing

The Y402H variant results in a histidine residue replacing a tyrosine residue at position 402 (using the pro-protein sequence numbering) of FH. Structurally, the Y402H polymorphism occurs in the seventh of FH's twenty complement control protein (CCP)

domains (Schramm et al. 2014). After Sanger sequencing was performed for all samples, chromatogram for each sample sequenced was individually inspected to check for the presence of the risk alleles (C) and the genotypes; TT, TC, and CC. Normal individuals have TT genotype at position 402, CT is heterozygous while the CC is homozygous for the AMD risk genotype. A total of 250 samples were sequenced, out of which 11 failed sequencing and 16 of them were undetermined, i.e. the chromatogram could not resolve the identity of the genotypes. Due to time limitations and reagent depletion, the failed, and undetermined samples were not repeated. Only 173 (101 with severe malaria and 71 with uncomplicated malaria) children who had complete genotype and FH level data were analyzed **Figure 5.8.**

5.5.6.2 *Confirmation of the Sanger sequencing genotyping using Factor H 402H/Y variant ELISA kit*

To confirm the genotyping done using Sanger sequencing a subset of samples were tested using Factor H, 402H/Y variant, Human ELISA kit. This ELISA kit uses a set of monoclonal antibodies to identify the Y402H allelic variant in plasma (Hakobyan et al. 2008). A total of 141 samples were run using the ELISA kit, three of which failed detection even after being repeated and varying dilution tested. Excluding the plasma samples that did not have paired EDTA blood and those that failed to sequence, only 120 samples had complete sequencing and ELISA genotyping results **Figure 5.8**. Due to time limitations, samples that did not sequence successfully were not repeated. There was a 94% agreement between the sequencing and ELISA genotyping data, I.e. 113/120 samples had the same genotypes identified through sequencing as the ELISA kit genotyping suggesting that either method can be used. However, due to the high cost of ELISA genotyping kit, studies with high throughput can consider relying on the relatively cheaper Sanger sequencing method.
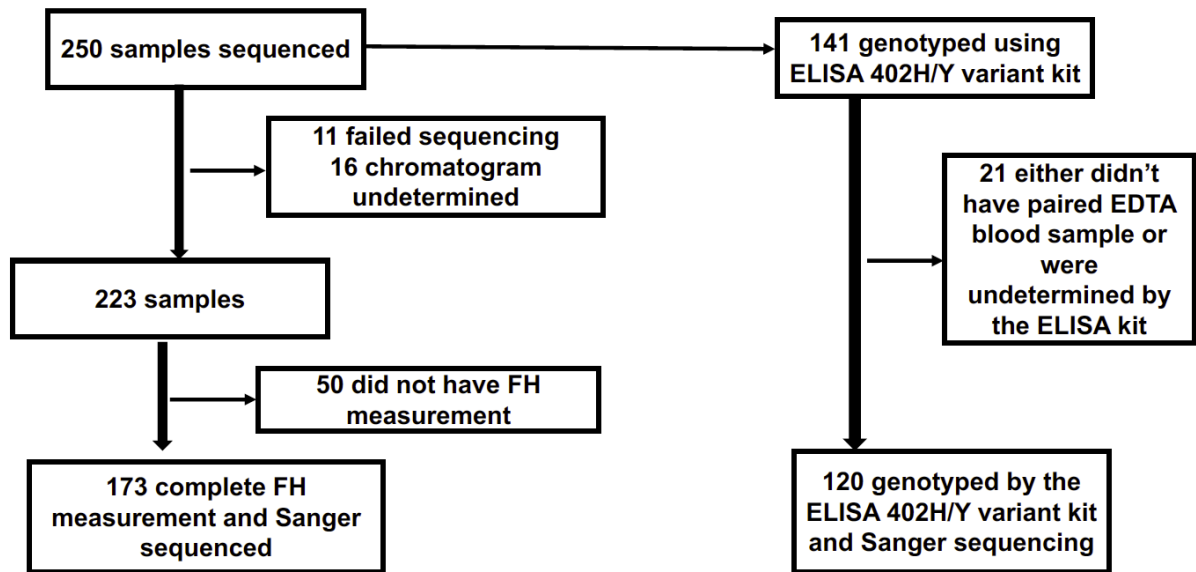
Figure 5.8 Flow chat of samples analyzed by Sanger sequencing and the ELISA 402H/Y variant for genotyping of the Y402H variant of FH

5.5.6.3 Association between the Y402H allele and genotype with severe malaria

There is evidence to suggest that the Y404H variants predispose individuals to age-related macular degeneration (AMD). A meta-analysis of 26 separate studies showed that individuals who are 402H heterozygous have a 2.3-fold increased risk of developing AMD, and the homozygotes have a 5.2-fold increased risk (Sofat et al. 2012) but these studies have been limited to populations of the Caucasian origin. Evidence for the association of the Y402H variant and infectious diseases is limited and thus far has only been investigated with regards to group A streptococcal infections (Haapasalo et al. 2008) and meningococcal disease (Haralambous et al. 2006) still in Caucasian population. Using a carefully designed case-control study of severe malaria and uncomplicated malaria, I observed that children with the CC genotype had an 8% increased risk of having severe malaria compared with the normal TT individuals, while those who were heterozygous had reduced odds of having malaria **Table 5.2**. Additionally, children with the risk allele, C, had a 19% increased

risk of having severe malaria if compared to the T allele. However, none of the odds ratios was statistically significant **Table 5.2** below.

Table 5.2 Genotype and allele frequency of Y402H by logistic regression analysis

	Severe malaria	Uncomplicated malaria	Unadjusted			Adjusted for age and parasite density		
	N (%)	N (%)	OR	95% CI	*P*	OR	95% CI	*P*
Y402H genotypes								
TT	32 (31.37)	18 (25.35	1	-	-	1	-	-
CT	43 (43.16)	39 (54.93	0.62	0.30-1.28	0.195	0.65	0.29-1.42	0.277
CC	27 (26.47)	14 (19.72)	1.08	0.46-2.58	0.854	1.04	0.41-2.61	0.941
Y402H alleles								
T	43 (42.16)	33 (46.48)	1	-	-	1	-	-
C	59 (57.84)	38 (53.52)	1.19	0.65-2.19	0.573	1.05	0.54-2.05	0.885

5.5.6.4   Relationship between genotypes and plasma FH levels

It has been shown that the *CFH* SNP [rs1061170 encoding Y402H), affects plasma circulating FH levels, which in return relates to susceptibility to AMD (Ansari et al. 2013). Here I tested whether there was variation in FH levels based on the Y402H genotypes. I found that overall, there were statistically significant differences in FH levels with regards to the genotype (*P* =0.019) **Figure 5.9**. Children with the AMD risk genotype CC had a statistically significant lower median FH levels compared to the normal individuals **Figure 5.9.** Based on the allele distribution, children who had the T allele had median FH levels of 330.37 ug/ml (range 121.48-832.80 ug/ml) while those

with the C allele had median FH levels of 308.39 ug/ml with a range of (123.38-706.22 ug/ml).
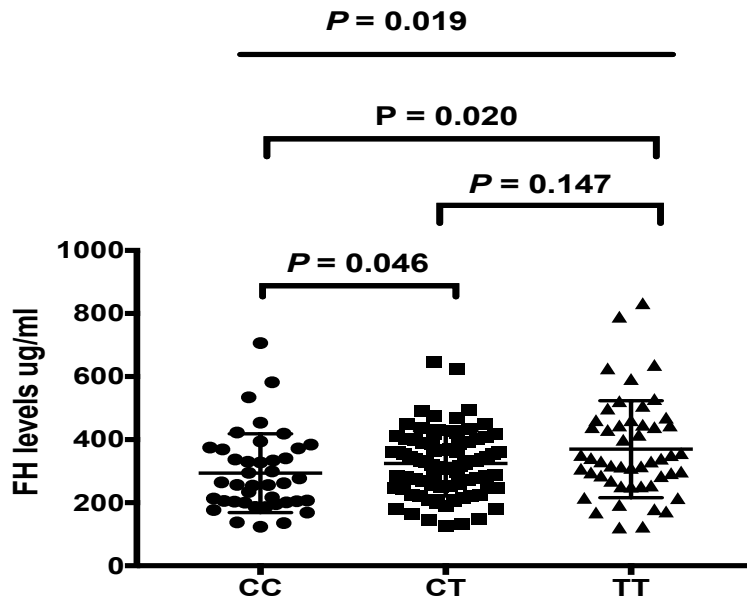
Figure 5.9 Levels of FH based on the genotypes of children with severe malaria and uncomplicated malaria with CC (n= 41), CT (n=82) and TT (n=50).

A total of 173 children from the cohort of SMA, SM and UM who had complete genotype and FH level measurements were included in the analysis. Mann-Whitney test was performed for the comparison between groups while Kruskal-Wallis test was used for comparison among the three groups.

As results from Figure 5.9 provides relationship between FH levels and genotypes, the analysis has one main limitation, it does not take into account malaria severity. Next I used the ordered logistic regression model so as to be able to control for the possible confounding effects of malaria severity **Table 5.3**. Being either homozygous (CC) or heterozygous (TC) increased the likelihood of having high FH levels even after accounting for parasitaemia but this was not statistically significant.

Table 5.3 Relationship between FH levels and the Y402H genotype

Covariates	Total	Lower (n %)	Medium (n %)	High (n %)	Crude Odds ratio (95% CI)	p value	Adjusted for severity	p value
Genotypes								
TT	50	12 (24.000	17 (34.00)	21 (42.00)	1		1	
TC	82	22 (26.83)	27 (32.93)	33 (40.24)	2.01 (1.00-4.06)	0.051	1.61 (0.77-3.35)	0.203
CC	41	18 (43.90)	12 (29.27)	11 (26.83)	2.22 (1.03-4.81)	0.043	2.10 (0.94-4.70)	0.071
Malaria severity								
Um	71	8 (11.27)	20 (28.17)	43 (60.56)	1		N/A	N/A
sma/sm	102	44 (43.14)	36 (35.29)	22 (21.57)	0.18 (0.09-0.33)	<0.001	N/A	N/A

Literature on the genetic model relating Y402H genotypes and FH levels is sparse. Of the few studies that have explored this area, the relationship had only been between the genotypes and disease outcomes. Zarewparsi et at., concluded that the additive model best accounted for the association with age macular degeneration (Zareparsi et al. 2005) while Kristel et al., concluded on the recessive genetic model for cardiovascular disease (Koeijvoets et al. 2009). The ordered logistic regression model in **Table 5.3** above used the additive model while in Table 5.4 below I have explored the recessive model. The results indicate compared to normal either having CT or CC increased the likelihood of having high FH levels, and is significant but the statistical significance is lost once malaria severity is adjusted for in the model.

Table 5.4 Association between FH levels and genotypes using recessive model

Covariates	Total (N)	Low (n %)	Medium (n %)	High (n %)	Crude odds ratio	*P* value	Adjusted for severity	*P* value
TT	41	18 (43.90)	12 (29.27)	11 (26.83)	1		1	
TC/CC	132	34 (25.76)	44 (33.33)	54 (40.91)	2.09 (1.08-4.04)	**0.028**	1.78 (0.90 - 3.54)	0.099

5.6 Discussion

The complement system plays an essential role in the host response to infectious processes (Walport, 2001). However, complement activation is tightly regulated, and the loss of this control can contribute to the pathogenesis of multiple diseases (Ricklin and Lambris 2013). Indeed complement activation has been implicated in the pathogenesis of malaria including, cerebral malaria (Patel et al. 2008), severe malaria anaemia (Nyakoe et al. 2009), and placental malaria (Silver et al. 2010). Although previous studies have looked at activation of complement pathways and the up-regulation or down-regulation of various complement pathways proteins, so far only one study has assessed the role of FH in the pathogenesis of malaria (van Beek et al. 2018). I have tested the effects of Y402H variant of FH, for the first time to the best of my knowledge, in carefully selected malaria cases and controls in addition to plasma FH levels. Homozygous, CC, children had increased odds of having malaria while those who were heterozygous, CT. had reduced odds of having severe malaria if compared to individuals with TT. Besides, the homozygous individuals had reduced FH levels compared to the normal. Since the children with severe malaria had markedly reduced FH levels compared to the uncomplicated malaria controls, it can be postulated that the reduced FH in children with severe malaria would be partly due to the Y402H polymorphism. Alternatively, the low FH levels could be malaria disease-driven.

Complement activation has been observed both during malaria challenge (Roestenberg et al. 2007) and during clinical infections (Silver et al. 2010). In line with these observations, there was an increased TCC level in children with severe forms of malaria (SM and SMA) compared to controls (UM). The increased complement activation could at least in part be due to the lower level of complement regulation because in the same case-control study, children with severe malaria anaemia (SMA) and severe malaria without anaemia (SM) had significantly lower FH levels compared to children with uncomplicated malaria (UM). Besides, children who had either medium or high FH levels had reduced odds of having severe malaria compared to those who had low FH levels **Table 5.1**. This essentially means that the higher complement activation in SMA and SM could, in some way, be related to lower FH levels.

The pathogenesis of malaria is complicated with parasite sequestering in tissues such as the brain for cerebral malaria and the loss of uninfected red blood cells during severe malaria anaemia (Perkins et al. 2011; Cowman et al. 2016). Specifically, during severe malaria anaemia, there is the formation of immune complexes (ICs) that get attached via the CR1 molecules to the red blood cells which can then be removed from the RBCs surfaces by macrophages in the liver and spleen. Continuous removal of the deposited ICs leads to a reduction in complement regulatory proteins on the RBCs and therefore the indiscriminate deposition of IC leads to their removal (Biryukov and Stoute 2014). In support of this argument is the observation that RBCs in patients with SMA have reduced CR1 and CD55 compared to their malaria controls (Waitumbi et al. 2004). Complement FH is important in the protection of host cells from unnecessary bystander complement attack (Parente et al. 2017b) and has been observed to be localized on the red blood cells (Rosa et al. 2015b). Therefore, it is possible that during SMA as there is an increased loss of both infected and uninfected red blood cells, the FH localized on these cells could also be lost leading to a reduction in FH levels. Factor

H could also be sequestered out of plasma by binding to malaria parasites or to cells infected or otherwise affected by malaria parasites.

Numerous reports have demonstrated the association of factor H deficiencies with chronic renal and infectious diseases, and that variation in plasma concentrations might influence disease susceptibility (Esparza-Gordillo et al. 2004). Within the longitudinal cohort, I observed a five-fold range in FH levels (117 - 699 ug/ml). This range remained the same, even after excluding children who had parasites at the time of sampling. The previously reported FH levels in individuals were about 350-700ug/ml (Oppermann et al. 1990); 350-750 ug/ml (Caprioli et al. 2003); 235-810 ug/ml (Neumann et al. 2003); 350-590 ug/ml (Venables et al. 2006; Moore et al. 2010); and 135-349 ug/ml (Hakobyan et al. 2008). Most of these studies were done on patients presenting with atypical haemolytic uraemic syndrome with controls using both children and adults. None of these measurements were done in an African population, and there are reports of genetic differences between African and Caucasian populations (Gurdasani et al. 2015). Therefore, the results on the wide range of FH concentrations obtained herein are comparable to other published data.

FH levels in the longitudinal cohort of children were divided into three equal tertiles, high, medium or low based on rank. The tertiles were used to determine whether starting FH levels influence susceptibility to clinical malaria. Overall there was no difference on whether a child had high, medium, or low FH levels and their prospective risk of developing malaria. However, children who had high FH levels tended to get more clinical episodes of malaria. There was an 80% increase in the number of episodes of malaria in children who had high FH levels compared to the low **Figure 5.2**. One possible explanation for these results in that high FH levels could imply the parasites has a higher chance to acquire FH hence protecting itself and surviving to establish an infection.

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Plasmodium falciparum merozoite proteins that bind FH can be considered as attractive vaccine candidates because blocking the interaction between FH and these antigens can prevent the parasite from establishing an infection. Alternatively, these antigens would be targets for protective antibodies. Since the protective antibodies and FH are targeting the same antigens on the parasites, I reasoned that there would be some correlation between the two as has been observed elsewhere (Beernink et al. 2011; Caesar et al. 2014; Biagini et al. 2016). The finding of no correlation between FH and antibody to merozoite FH receptor antigens **Figure 5.3** could imply that the two act independent of each other. Alternatively, the close relatedness between FH and FH related proteins could mean that they compete for binding to the same antigens (Caesar et al. 2014) and may not be related to how the antibodies interaction with these antigens.

In a previous study where FH levels were measured in children with different forms of malaria, children with severe forms of malaria had increased levels of FH compare to children with uncomplicated malaria (van Beek et al. 2018). This is contrary to what I observed in the current study. However, in the Van Beek et al. study, they measured FH levels in convalescent samples which were obtained 28 days after presentation with severe malaria. This may not reflect what could be happening during an active malaria infection. This is supported by the previous observation that complement regulatory proteins, specifically CR1 and CD55, are reduced during presentation with severe malaria but at convalescence following treatment, the expression of these molecules increased to levels comparable to those of matched control subjects (Waitumbi et al. 2004). However, it is not known whether the same happens to FH levels as they were only measured at enrollment in the current study. Nevertheless, the observation of lower FH in severe malaria in the current study could also simply mean that FH is also being consumed or being driven by malaria disease status. This

argument can be tested in future studies by measuring FH levels in children presenting at the hospital with severe malaria and same children tested at convalescence. It is also very much possible that the children with lower FH levels are genetically predisposed to having lower levels and perhaps also to severe malaria.

The Y402H variant of FH has a strong association with AMD (Sofat et al. 2012). The region of FH (402) containing this amino acid has also been shown to bind to heparin, CRP and the streptococcal M6 protein (Giannakis et al. 2003; Schmidt et al. 2008). In vitro work involving group A streptococcus showed reduced growth of GAS in human blood taken from 402H homozygous individuals. This observation leads to the thinking that the 402H variant individuals could have reduced binding to GAS, more complement activation and attack and hence be protected (Haapasalo et al. 2008). I, therefore, hypothesized that individuals homozygous for the AMD risk genotype, CC, could have less FH parasite interaction and therefore be protected from malaria. Contrary to this hypothesis, I observed that individuals with CC genotype had a modestly increased risk of having malaria compared to those individuals with the wildtype, TT, genotype. In addition, being either homozygous (CC) or heterozygous (TC) increased the likelihood of having high FH although this was not statistically significant. One possibility accounting for these results is that GAS might not necessarily behave the same way as malaria parasites. The current study did not also test for the interaction between the 402H variant and its binding to malaria parasites. Therefore, I was not able to systematically interrogate whether there is reduced binding between the two. Future studies with a bigger sample sizes will be helpful to robustly interrogate the relationship between Y402H genotypes and malaria severity.

In conclusion, I provide evidence that natural variation in FH levels is associated with malaria susceptibility and severity. I also show for the first time, to the best of my knowledge, that this variation could partly be explained by the genetic variation

occurring within FH. Future studies conducted in differing malaria transmission settings will be necessary for assessing the role of Y402H polymorphism and its interaction with malaria. Nevertheless, interrupting the malaria parasite FH interaction offers an alternative approach to limiting parasite growth and may contribute to the pool of tools required to fight malaria.

6   Concluding remarks and recommendations

6.1   Summary of findings

The complement system forms the first line of defense against invading pathogens (Walport, 2001). However, numerous microbes have evolved mechanisms to avoid complement-mediated destruction. They can achieve this through recruitment of complement regulatory proteins such as factor H (Zipfel et al. 2007; Blom et al. 2009). Although the mechanisms for complement immune evasion have been well described for pathogens such as bacteria (Serruto et al. 2010), evidence of the same for *Plasmodium falciparum* has only been shown recently (Simon et al. 2013; Rosa et al. 2015; Kennedy et al. 2015). The experiments and analysis presented in this thesis were aimed at: characterizing merozoite proteins important for interaction with FH and in part explain how the parasites escape complement attack; whether antibody responses to these merozoite FH receptor antigens are associated with protection; and finally whether host variation in FH levels and Y402H variant of FH influence malaria susceptibility and severity. Below is a summary of my findings.

First, I provide evidence, for the first time, to the best of my knowledge, that many merozoite proteins are involved in the interaction with complement regulator protein FH. This is contrary to what was reported previously that only one merozoite protein, Pf92, interacts with FH (Kennedy et al. 2015). The proteins identified in this thesis project and referred to as merozoite FH receptor proteins include, PF3D7_1105800, PF3D7_0206200, RH5, Pf12, PF3D7_1252300, PF3D7_0629500 (SEG), and P12p. In the Kennedy et al study, whole parasite materials obtained after saponin treatment in a co-immunoprecipitation assay to identified Pf92 as the only merozoite antigen binding to FH. However, in a growth inhibition assay, a parasite knockout of Pf92 only

showed a modest reduction in growth compared to the wildtype parasite. This could imply two possibilities, that the parasite either uses other merozoite proteins or that it could evade complement activation using other strategies that involving other complement pathways (Kennedy et al. 2016). It is not clear why the whole parasite approach identified only one protein. My approach of using individually expressed merozoite antigens in a simple ELISA binding assay allowed each antigen to be tested for binding to FH. Besides, the binding in ELISA binding assay was confirmed using surface plasmon resonance, which is the gold standard for protein-protein interaction.

Second, I used a longitudinal malaria cohort of young children and showed that the merozoite FH receptor proteins are targets of naturally acquired immunity to malaria. Although there was no evidence to suggest that antibody responses to these merozoite FH receptor antigens were individually associated with protection, some combinations of these responses were found to be associated with protective immunity. In the case-control study, antibody responses to some of these merozoite FH receptor proteins were associated with reduced odds of having severe malaria. Although antibodies to these merozoite FH receptor proteins are potential targets of protective antibodies, the mechanism by which they mediate their protection was not defined. Antigen-specific functional responses would provide valuable information required for the selection of vaccine candidates.

Lastly, I tested whether complement activation and regulation could in part, explain malaria susceptibility and severity. Children with severe malaria had increased complement activation compared to uncomplicated malaria controls. In the same set of severe malaria children, complement FH levels were statistically lower compared to uncomplicated malaria controls, supporting the idea of excessive complement consumption during severe malaria infection.

Malaria parasites have co-evolved together with the human host for many years (Jongwutiwes et al. 2005; Kwiatkowski 2005) exerting substantial evolutionary pressure on the human host. Thus far, the focus has been on changes on the red blood cell surface as the main determinant of malaria susceptibility and severity. For the complement system, the focus has been on the CR1 (Rowe et al. 1997) and the MBL protein (Lipscombe et al. 1995). I genotyped, for the first time to the best of my knowledge, the Y402H variant of FH, and showed that homozygous, CC, children, had increased odds of having malaria while those who were heterozygous, CT, had reduced odds of having severe malaria when compared to individuals, expressing the wild type (TT) alleles. Understanding the mechanism of how these variants confer protection may inform new approaches for the prevention and treatment of malaria.

6.2 Recommendation for future studies

The work described in this thesis can be improved or extended in future studies in three main ways; i) overcoming the challenges of ELISA binding assay, ii) systematic screening of *Plasmodium falciparum* parasite stage-specific antigens to determine which ones interact with FH and iii) clearly demonstrating the essence of this interaction in a functional assay in order to elucidate its importance to parasite survival.

6.2.1 Overcoming the challenges of ELISA binding assay

I used an ELISA binding assay to screen over 100 merozoite proteins to narrow down to antigens that interact with FH. The interaction was then confirmed using the surface plasmon resonance (SPR) platform. One major limitation of the ELISA binding assay is that it could not detect low-affinity binding, especially for interactions that are characterized by low/weak interaction strengths. Additionally, it is clear from the data presented in this thesis project that the choice of antibody in the ELISA binding assay

matters. I used a polyclonal anti-FH antibody from previous publications (Kennedy et al. 2015; Rosa et al. 2015a) that had been used in western blotting. Transferring this to the ELISA binding assay showed that this antibody could recognize the antigen in the presence or absence of FH. This background noise was corrected by using a monoclonal anti-FH antibody (Fontaine et al. 1989; Jokiranta et al. 1996; Rosa et al. 2015b). While using the ELISA binding assay as the first screening tool could mean that I might have missed other antigens involved in the interaction with FH, I was able to confirm a good proportion of these antigens for binding using SPR which is a gold standard for protein-protein interaction. These data are robust and showed that indeed many additional merozoite proteins bind to complement regulatory protein FH.

Additional methods that could be used in future include the yeast two-hybrid system and Avidity-based Extracellular Interaction Screen (AVEXIS) (Mehla et al. 2015; Galaway et al. 2017) which are not only highly sensitive but also allow for high throughput testing. However, these two methods demand that the proteins are tagged or labeled in one way or another. The yeast two-hybrid system requires that the protein of interest is fused to the DNA binding domain (BD) of a transcription factor (the 'bait'), while the target protein is fused to a transcriptional activation (AD) domain (the 'prey'). Interaction of the prey and bait occurs when the two reconstitutes a functional transcription factor and is detected via the expression of reporter genes controlled by a promoter bearing the DNA binding site (Mehla et al. 2015). On the other hand, AVEXIS assay needs recombinant proteins to be produced in two forms; as a biotinylated bait and a pentamerized enzyme-tagged prey (Kerr and Wright 2012).

One of the main advantages of the SPR platform is that it provides real-time label-free analysis of protein-protein interactions. Although the older platform of SPR allowed up to analysis of up to 24 samples, which can run for 8 hrs, advancement in technology has led to higher versions of SPR machines such as the Sierra SPR 32

https://www.bruker.com/products/surface-plasmon-resonance/sierra-spr-32/technical-details.html that can now analyze up to thousands of samples per day. In summary, future studies can benefit from using high throughput SPR platform, which can be used for both screening and a confirmatory test at the same time if many proteins are involved. It also offers the advantage of analyzing the kinetics of the interactions in terms of quantifying the strength of binding.

6.2.2 Screening of Plasmodium falciparum parasite stage specific antigens for interaction with FH

The work presented in this thesis looked at merozoite proteins that interact with FH and hence just to this stage of the parasite. This does not rule out that there are other antigens, possibly in the other developmental stages of the parasites that interact with FH. Indeed, it has been shown that during the mosquito stage of development, *Plasmodium falciparum* gametes recruit factor H (from the human blood ingested during a blood meal) to their surface to evade human complement attack within the mosquito midgut. Gamete surface protein GAP50 binds factor H (FH) and uses bound FH to inactivate the complement protein C3b (Simon et al. 2013). Within the human host, there is also evidence to suggest that other than the merozoites, trophozoites, and rings (Simon et al. 2018) also bind to FH. Together, there is evidence to suggest recruitment of FH by *Plasmodium falciparum* at different stages is crucial for parasite survival and therefore comprehensively identifying antigens involved will not only advance our understanding of parasite biology but will enable us to know how to interrupt the complex life cycle.

6.2.3 *Demonstrating the consequences of the parasite-complement interaction*

What is the implication of parasite recruitment of FH? To answer this question, I used a modified growth inhibition assay and showed that in serum depleted of FH there is reduced parasite growth compared to serum containing FH. However, this approach has some limitations. For instance, if the serum is depleted of FH, that means that there will be uncontrolled alternative pathway activation and formation of C3b, and therefore this serum is not stable (Dr Ayman Khattab, personal communication). Furthermore, it is difficult to measure how much of the complement activity is reduced per se if FH is removed and how this affects the other pathways. For these reasons, using serum depleted of FH might not be the appropriate way of assessing the effect of FH parasite interaction. A more rigorous way could be to use the growth inhibition assay where FH interaction with the parasite is blocked by using either antibodies to these merozoite FH receptor proteins or using parasite knockout of these genes. A comprehensive library of essential and non-essential genes for parasite growth has been recently described (Zhang et al. 2018) allowing future studies to obtain parasite knockout of most genes and assessing directly the effect parasite FH interaction on parasite growth in the presence or absence of these genes.

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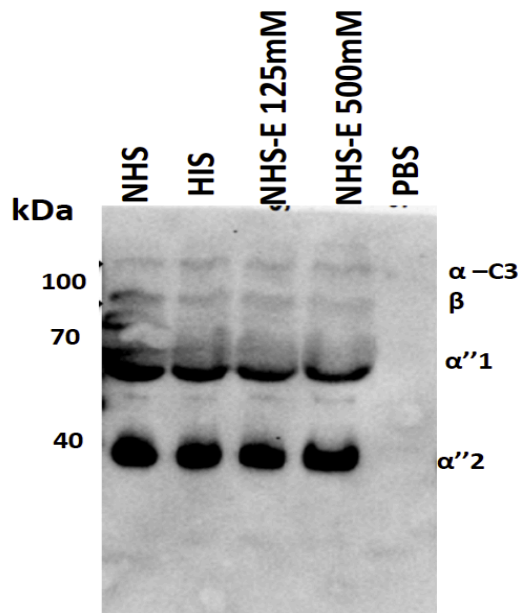
Appendices 3.1 C3b deposition by schizonts

Merozoites were incubated with normal human serum (NHS), heat inactivated serum (HIS) or NHS inactivated using EDTA (NHS-E 500mM or NHS-E 125mM) at 37oC for 1 hr. The proteins from the pellet were separated on SDS-PAGE under reducing conditions, transferred to an activated nitrocellulose membrane and probed for C3b using anti-C3b antibody and HRP detection antibody. Merozoite incubation with PBS was used as a negative control.
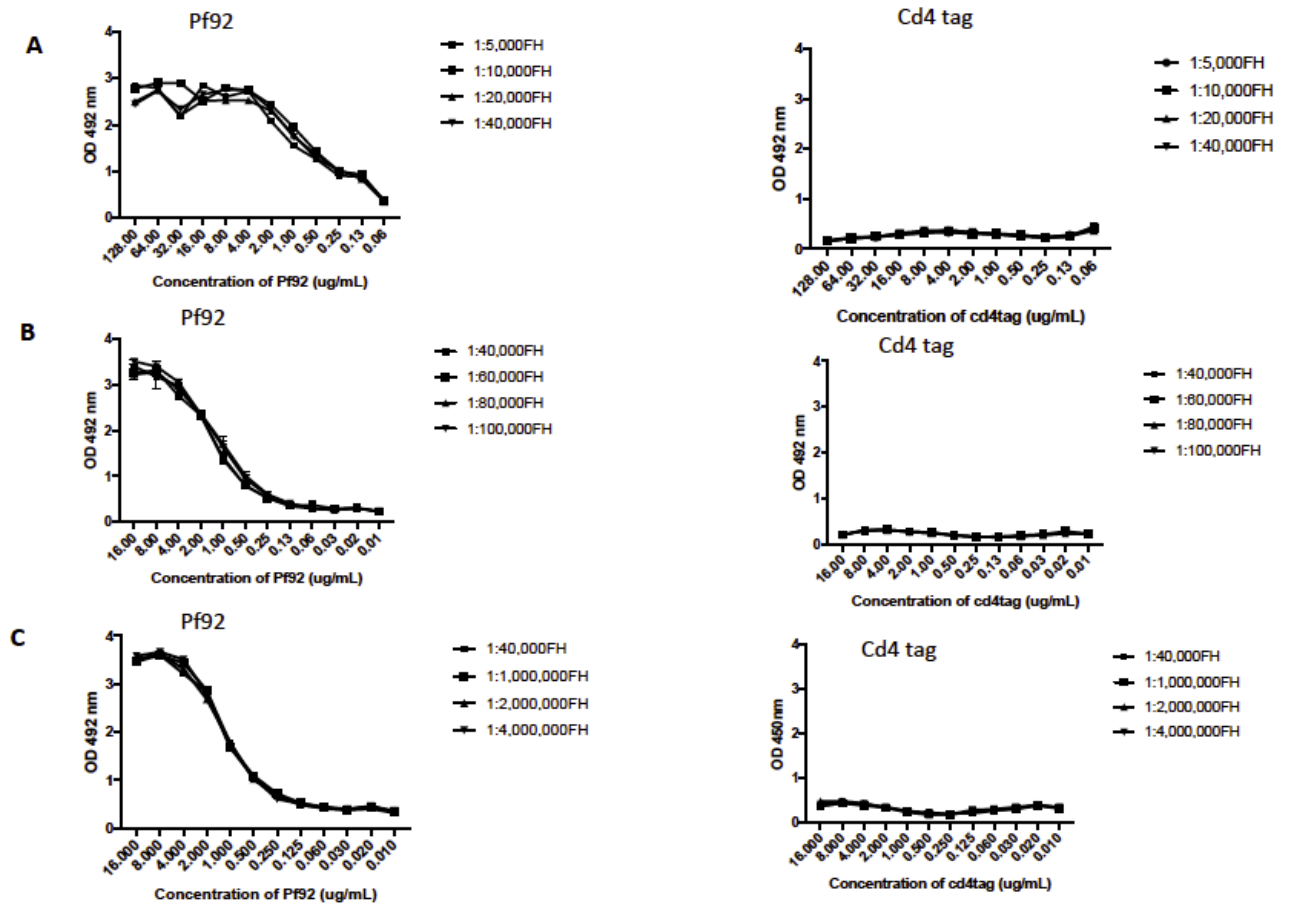
Appendices 3.2 Checkerboard titration for FH dilutions

A starting concentration of 16ug/ml for each of Pf92 (A) and Cd4 tag (B) was immobilized on microtitrate plate and varying concentration of FH tested for each duplication dilution of the coating antigen. Interaction was detected using polyclonal anti-FH and HRP conjugated antibody.
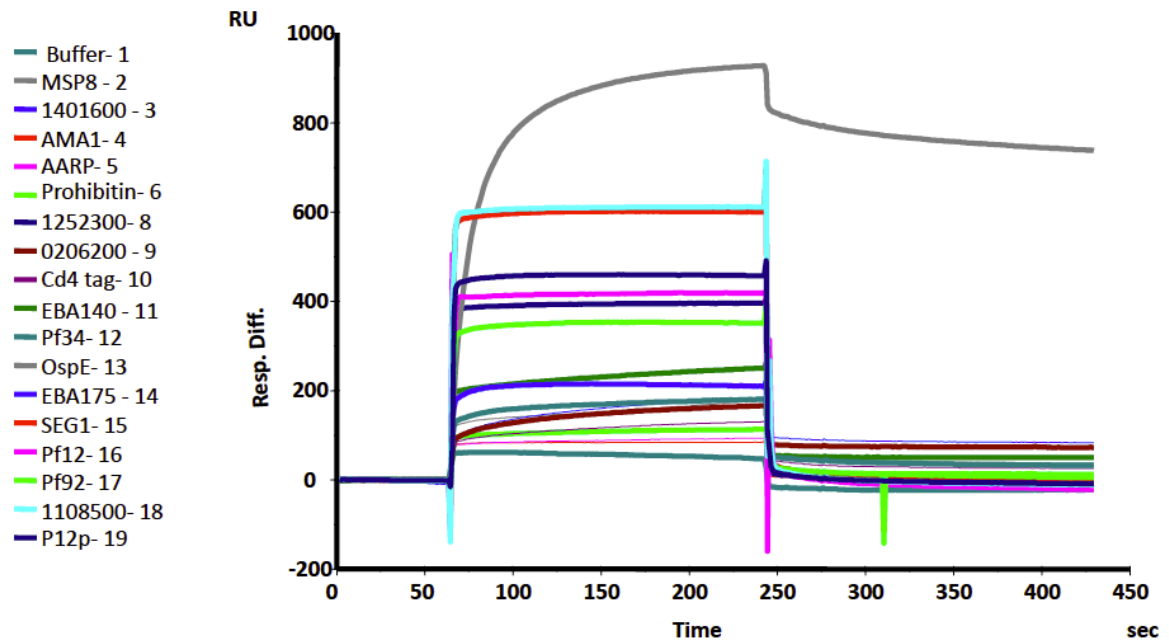
Appendices 3.3 Sensorgram for the second independent experiment to test for FH interactions with merozoite proteins using SPR

Merozoite proteins were injected over immobilized FH on the flow cell.
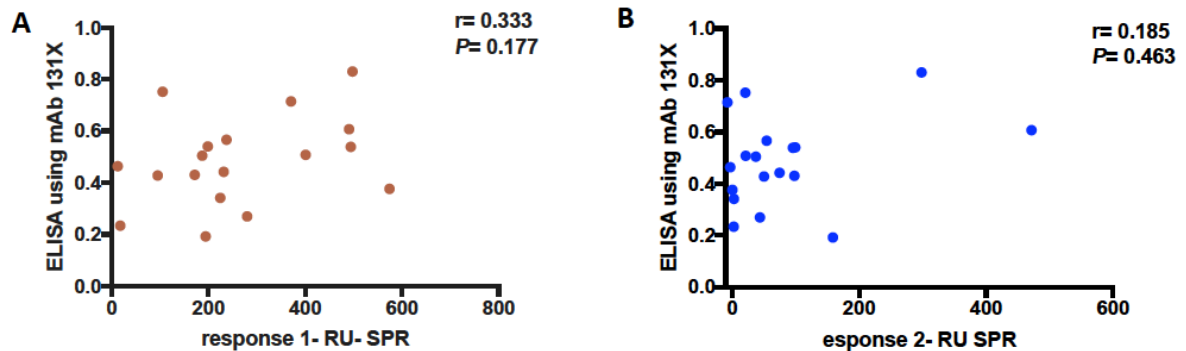
Appendices 3.4 Correlation between Helsinki ELISA binding assay and SPR (A) using SPR response 1 and (B) using SPR response 2

The same antigens were tested using Helsinki ELISA binding assay and SPR and correlation tested using Spearman's rho.
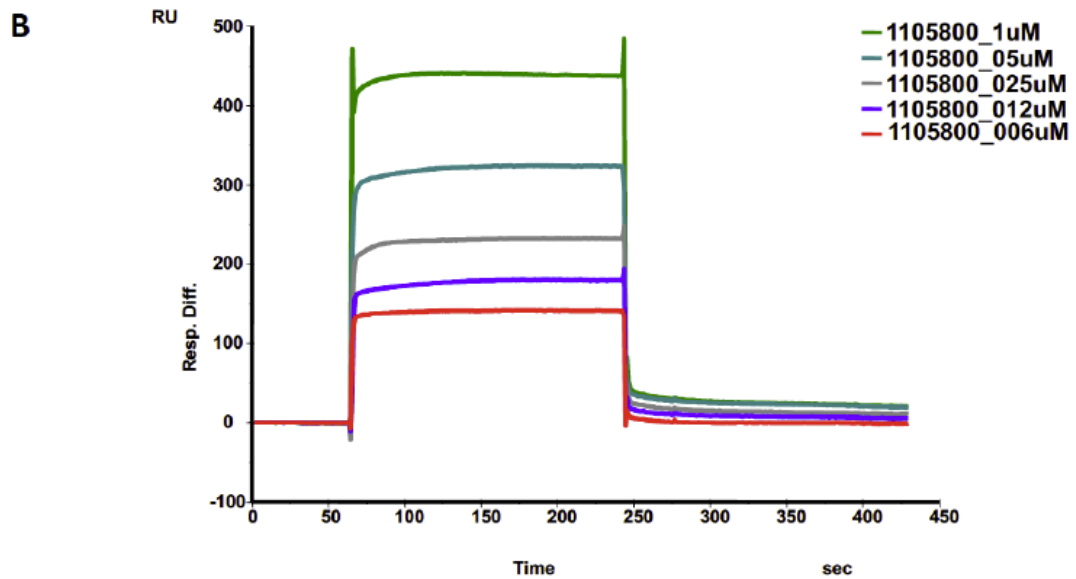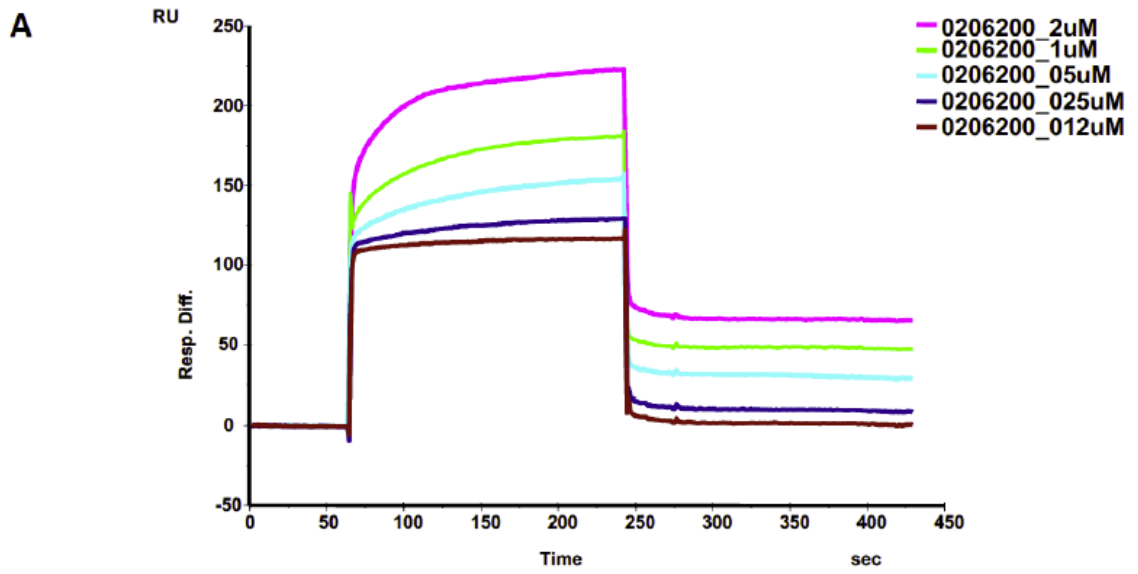
Appendices 3.5 Dose dependent interaction between FH and (A) PF_3D7_0206200 and (B) PF_3D7_1105800

Merozoite antigens **(A)** PF_3D7_0206200 and **(B)** PF3_D7_1105800 at a starting concentration of 2uM and 1uM respectively in tow fold dilution on FH immobilized on the flow cell.
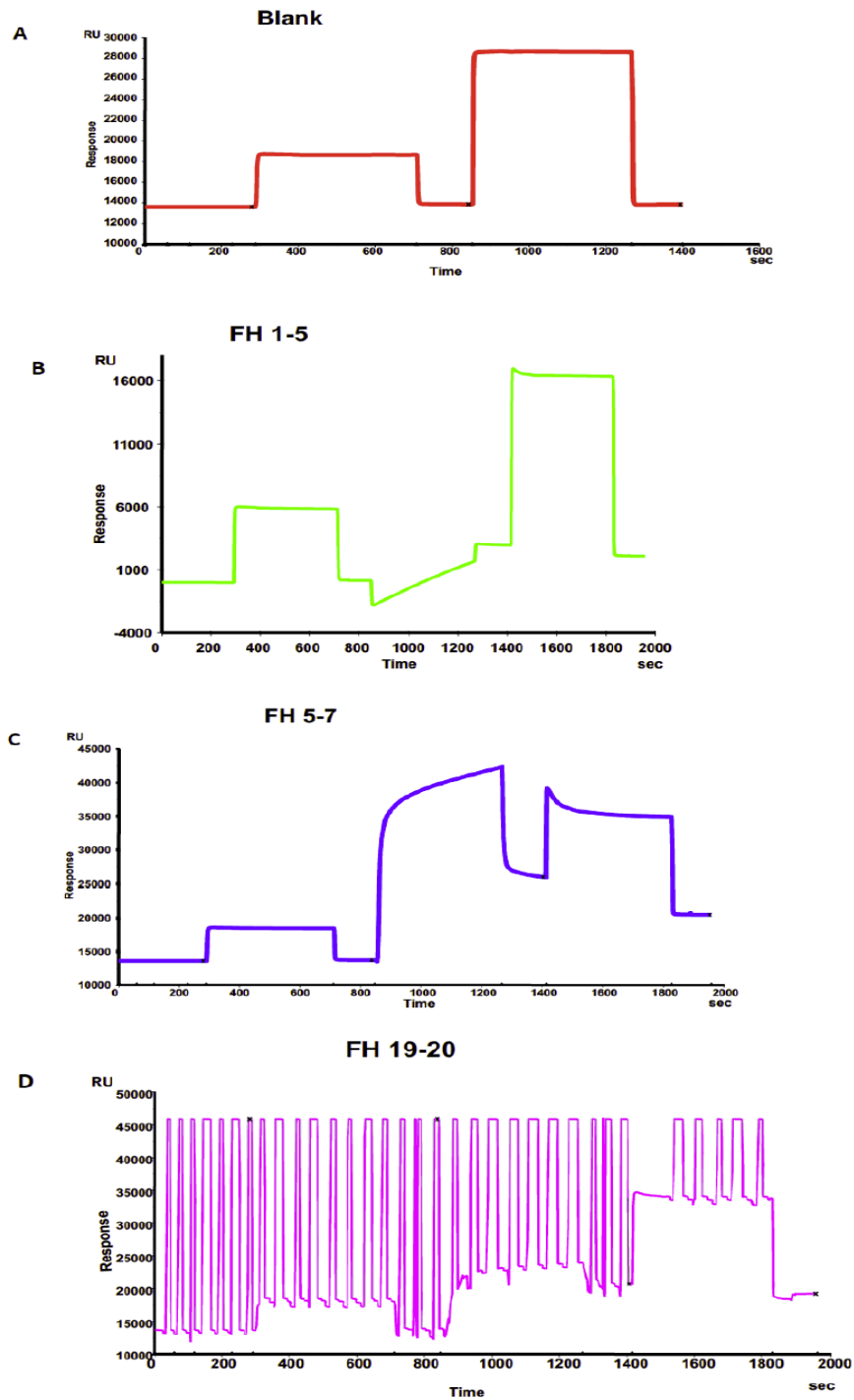
Appendices 3.6 Immobilization of FH fragments on the activated flow cell using amine coupling chemistry.

Different FH fragments were immobilized on the flow cell **(A)** Blank, **(B)** FH CCP 1-5, **(C)** FH CCP 5-7 and **(D)** FH CCP 19-20. Striking lines indicate presence of air bubbles during the injection of the ligand which has no effect on the immobilization
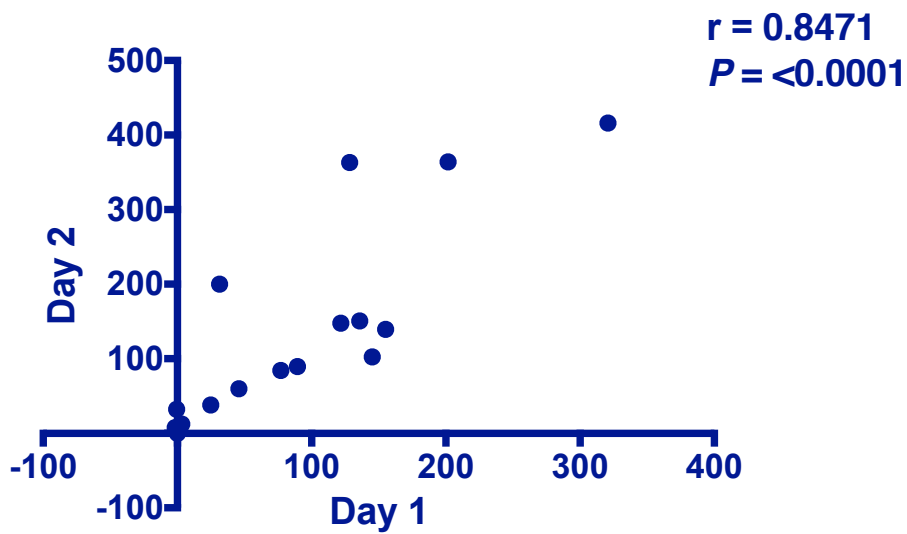
Appendices Error! Reference source not found. **Scatter plot of the response unit**

The same merozoite proteins were run on the same flow cell but different days on immobilized FH

CCP 5-7 fragment
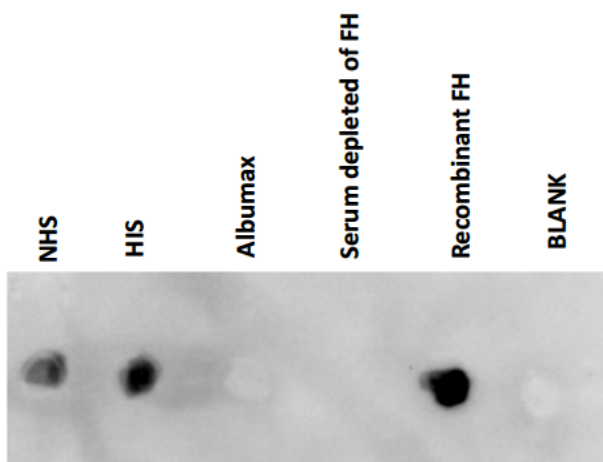
Appendices 3.6 Absence of FH in albumax and serum depleted of FH.

Two microliters of NHS, HIS, Albumax, serum depleted of FH, recombinant FH were blotted unto a

nitrocellulose membrane and allowed to air dry for at least 30 minutes. Blocking was done using

4% Blotto and polyclonal anti-FH added. After washing, an HRP antibody was added and

detection done using ECL (Invitrogen)
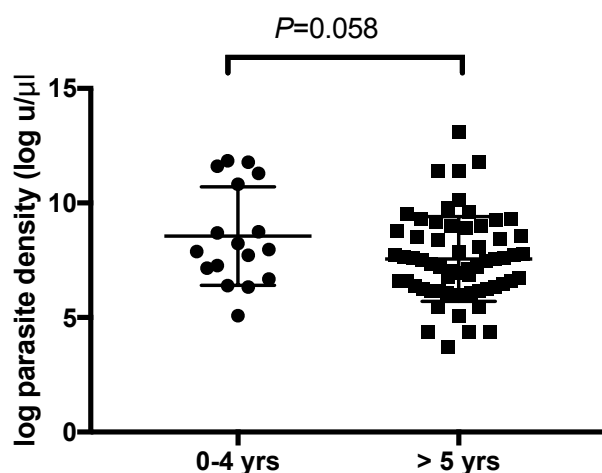
Appendices 4.1 Parasite density by age

Analysis is on parasite positive (by microscopy) in individuals in the Junju 2008 cohort by age

Appendices Table 4.1 Antibody responses and association with risk of clinical malaria

Antigen	No adjustment		Age adjusted	
	IRR (95% CI)	*P*	IRR (95% CI)	*P*
Parasite negative children				
PF3D7_1252300	0.85 (0.61-1.17)	0.32	0.86 (0.62-1.19)	0.35
Pf92	0.95 (0.68-1.34)	0.79	1.04 (0.74-1.46)	0.83
PF3D7_0629500_SEG1	0.91 (0.67-1.24)	0.57	0.86 (0.64-1.17)	0.34
RH5	1.01 (0.71-1.44)	0.95	1.04 (0.73-1.47)	0.85
PF3D7_0629500_SEG2	1.11 (0.81-1.54)	0.52	1.17 (0.86-1.60)	0.32
P12	1.12 (0.81-1.54)	0.50	1.10 (0.80-1.51)	0.57
PF3D7_0206200	1.27 (0.95-1.71)	0.11	1.26 (0.95-1.69)	0.11
PF3D7_1105800	1.47 (1.09-1.97)	**0.01**	1.42 (1.07-1.90)	**0.02**
Parasite positive children				
PF3D7_1252300	1.30 (0.54-3.12)	0.55	1.22 (0.50-2.95)	0.66
Pf92	0.96 (0.43-2.14)	0.93	1.17 (0.54-2.54)	0.69

PF3D7_0629500_SEG1	0.90 (0.40-2.05)	0.81	0.88 (0.38-2.05)	0.77
RH5	1.00 (0.43-2.33)	0.99	0.92 (0.41-2.04)	0.83
PF3D7_0629500_SEG2	0.64 (0.29-1.43)	0.28	0.76 (0.35-1.66)	0.49
P12	0.67 (0.30-1.50)	0.34	0.85 (0.40-1.78)	0.66
PF3D7_0206200	1.25 (0.57-2.76)	0.57	1.08 (0.51-2.30)	0.84
PF3D7_1105800	1.36 (0.60-3.11)	0.46	1.30 (0.59-2.86)	0.52
All children irrespective of parasite status				
PF3D7_1252300	0.97 (0.71-1.32)	0.85	0.95 (0.70-1.29)	0.75
Pf92	0.88 (0.64-1.22)	0.45	1.02 (0.74-1.40)	0.92
PF3D7_0629500_SEG1	0.92 (0.68-1.24)	0.59	0.87 (0.65-1.16)	0.33
RH5	0.96 (0.69-1.34)	0.82	0.96 (0.70-1.33)	0.83
PF3D7_0629500_SEG2	0.87 (0.64-1.20)	0.40	0.97 (0.71-1.33)	0.85
P12	0.90 (0.66-1.23)	0.49	0.96 (0.70-1.31)	0.80
PF3D7_0206200	1.32 (0.92-1.64)	0.16	1.21 (0.92-1.60)	0.18
PF3D7_1105800	1.22 (0.91-1.63)	0.19	1.22 (0.92-1.62)	0.17

Age categories used 0-2 yrs, 3-5yrs and >6 yrs

P= 0.0457
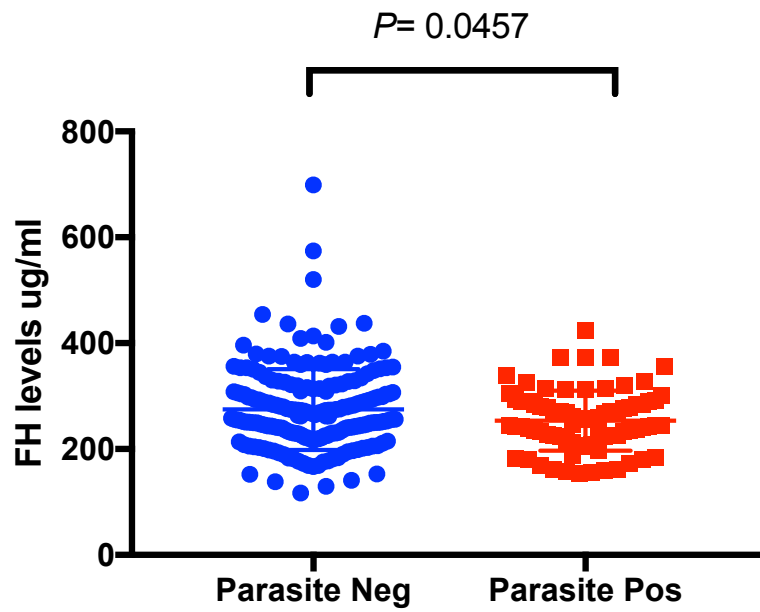
Appendices 5.1 Differences in FH levels in a group of children

The differences are between those children who did not have parasite (Parasite Neg) and those who had parasites (Parasite Pos) at the time of sampling from the Junju longitudinal cohort of children.
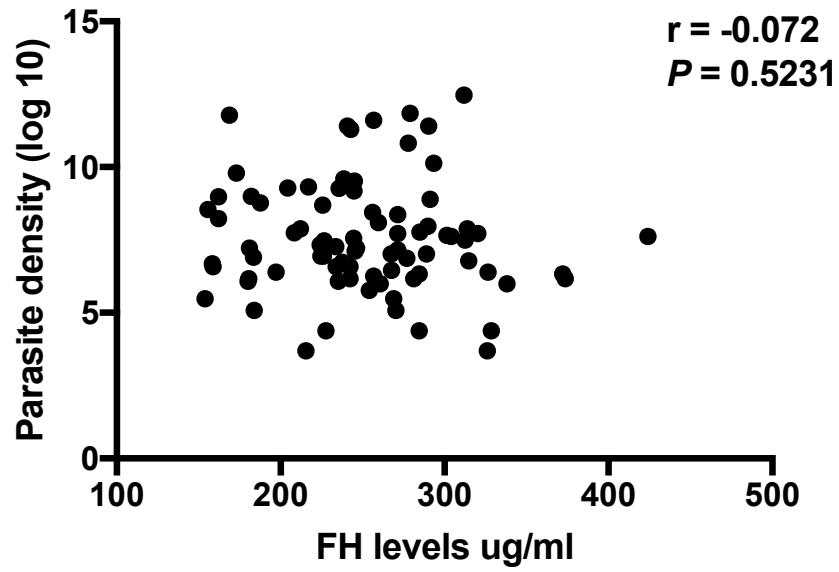
Appendices 5.2 Correlation between FH levels and parasite density

Parasite density and FH levels in parasite positive children at the time of the cross-sectional survey for the Junju longitudinal cohort of children
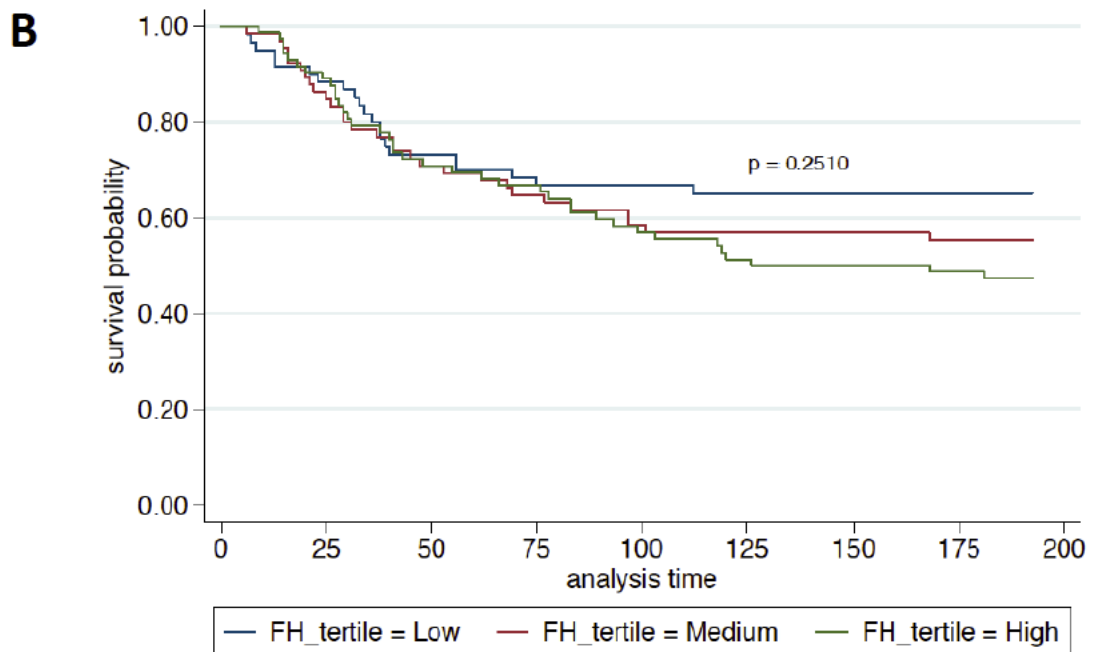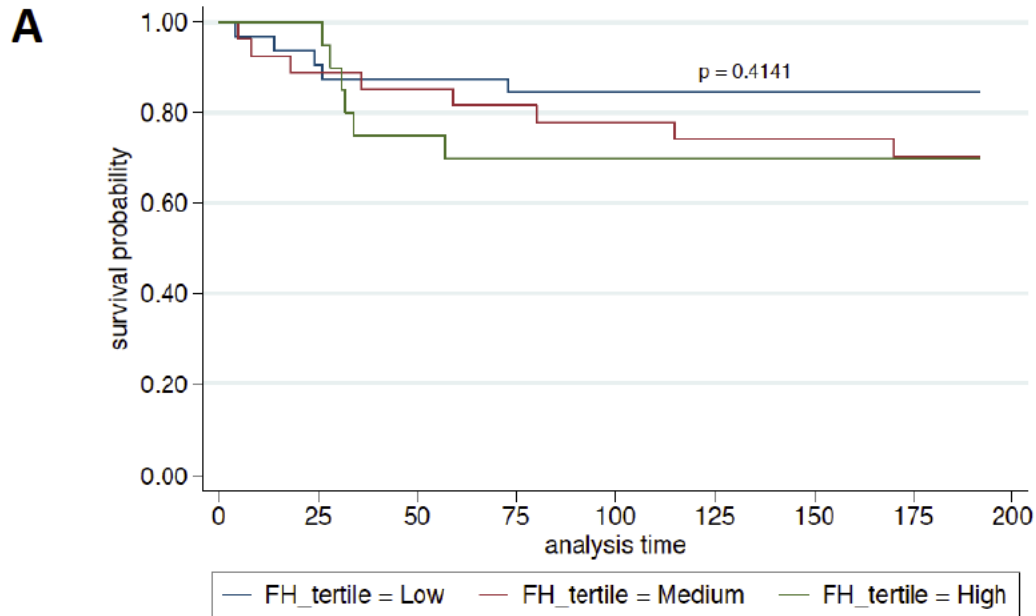
Appendices 5.3 Kaplan-Meier survival showing risk of clinical malaria for A) parasite negative and B) parasite positive children at time of sampling

Kaplan-Meier survival curves showing the risk of malaria stratified by whether the children had low (blue), medium (red) or high (green) FH levels, Statistical significance was determined by the log rank test for the differences between all three groups