Identifying the Parasite Targets Mediating Resistance to Severe Malaria

By

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Thesis

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Abstract

Abstract of Identifying the Parasite Targets Mediating Resistance to Severe Malaria, by Rim Fawaz, ScM, Brown University, February, 2025

Malaria continues to be a global burden and one of the leading causes of child mortalities in endemic Sub-Saharan Africa. Malaria largely affects populations of low socioeconomic backgrounds who do not have consistent access to treatment. A vaccine is much needed to stop the spread of the disease, especially with resistance to treatment and control measures on the rise. Previous vaccine efforts have not yielded high efficacy or promising results, particularly for severe malaria, which has a large fatality rate of 30%. There is growing evidence that the epidemiology of severe malaria is unique, with patients naturally acquiring immunity after only one or two episodes of severe malaria, and this protection is distinguished from responses that control parasitemia. New vaccine targets are much needed to address this gap. Here, we use a whole proteome differential screening using serum from children pre and post-susceptible to severe malarial anemia, before and after they develop resistance to severe malaria and the acute vs convalescent phase of cerebral malaria. After three rounds of biopanning, we generated a diverse list of genes uniquely expressed by children post-susceptible to severe malarial anemia or after developing resistance. Our screens lead us to PfP113, a protein with limited sequence variation that plays a role in anchoring Rh5 to the merozoite surface. We show that antibodies to PfP113 inhibit parasite growth. From our resistant screen, we selected a conserved protein that is still unknown but has interesting properties worth further investigating. Our approach allowed us to discover new antigen targets uniquely expressed after an episode of severe malarial anemia, after developing resistance to severe malaria anemia, and during cerebral malaria. Thus we expand on the limited repertoire of antigen targets for a blood-stage vaccine, specifically against severe malaria.

Chapter 1: Introduction

1.1 Global Impact and Burden

Malaria is a debilitating disease at the core of the global health issue and a leading cause of mortality in many developing countries. According to the latest World Health Organization (WHO) estimates, there were 249 million cases of malaria worldwide and 608,000 deaths. Around 80% of these cases are children under the age of 5(1). This is an increase from 2019, which saw around 400,000 deaths worldwide. It is worth noting that COVID exacerbated the death toll. Most cases of severe malaria and fatalities are endemic to sub-Saharan Africa, with 95% of malaria-related fatalities occurring in the region (2). However, malaria also occurs in tropical regions such as Central and South America, as well as parts of Southeast Asia (Figure 1.1), milder forms are more common in these areas (3). Symptoms of malaria vary drastically, with milder symptoms being fever, headache, chills, and mild anemia. Severe cases can result in severe anemia, difficulty breathing, cerebral malaria and other health complications (4).

Malaria is caused by the bite of a female Anopheles mosquito carrying a *plasmodium* protozoan parasite. Six plasmodium parasite species can affect humans: *P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi* and *P. ovale wallikeri (5). P. falciparum* and *P. vivax* are the two most common species, with *P. vivax* prevalent in the subtropical regions affected and is associated with milder and uncomplicated forms of the disease (6). *P. falciparum* is the most prevalent species associated with more severe symptoms and fatalities (7). It is the strain that causes most cases in endemic areas of Africa and will be the focus of this thesis.

It is important to note that malaria is treatable and largely preventable. Early eradication efforts to combat the disease focused primarily on mosquito control, such as widescale use of insecticides, draining wetlands and increased availability of antimalarials (8). While this reduced the incidence, the death rate has not significantly decreased since 2015 (9). This is because malaria currently predominantly affects countries that have not thrived socioeconomically, and the population affected does not have consistent access to treatment. In addition, the disease has been able to rapidly spread and develop resistance to many preventative and treatment measures (10).

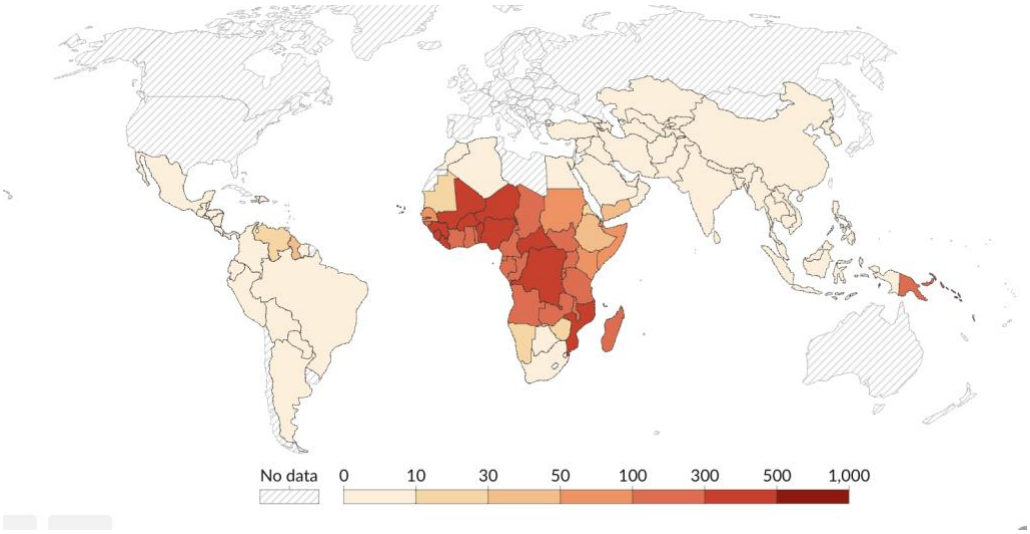

Figure 1.1 Map showing the worldwide incidence of malaria in 2021 *(11).*

1.2 Parasite Life Cycle

The life cycle of the parasite consists of a series of cyclical events. The first stages are known as the pre-erythrocytic stage also seen as the asymptomatic stage. It starts when a female anopheles mosquito that carries a sporozoite, the infective form of the plasmodium parasite, located in its salivary glands, bites a human host and releases the sporozoite. Once the sporozoite enters a

human, it infects hepatocytes, where, for the next two weeks it us undergoing massive rounds of asexual reproduction, producing merozoites (12). It keeps reproducing until the cell is packed and it ruptures. Once the liver cell bursts, tens of thousands of merozoites are released into the bloodstream, where they infect red blood cells (13).

The release of merozoites begins the erythrocytic stage, where symptoms start to manifest clinically. When the merozoites are released into the bloodstream, they infect the red blood cells (RBCs). Within 48 hours, they undergo rounds of asexual production. During this process, the cell takes a ring form known as the trophozoite, a key stage where the parasite is feeding and growing (14) and the stage where we start to see symptoms manifest clinically. The trophozoite then takes on a schizont form (15). Similar to the pre-erythrocytic stage, the merozoites continue reproducing until the RBC is packed and bursts, where around 25 merozoites are released into the bloodstream. These merozoites infect more and more red blood cells with each one eventually rupturing (16). As a result, there is a decrease in RBCs and the infected person becomes anemic. In addition to an overall decline in oxygen delivery, the infected RBCs develop a sticky and rigid texture where they stick in the periphery of your vasculature, resulting in clogs. These clogs cause blockages where fresh blood is no longer able to move to tissues downstream and thus these tissues no longer receive oxygen (17).

Sometimes, the parasites decide to sexually reproduce where they differentiate into male and female gametocytes. If they both get taken up by another anopheles mosquito, they unite and form a fertilized egg. This egg grows in the stomach of the mosquito and moves up until it develops into a sporozoite and remains in the salivary glands (Figure 1.2).

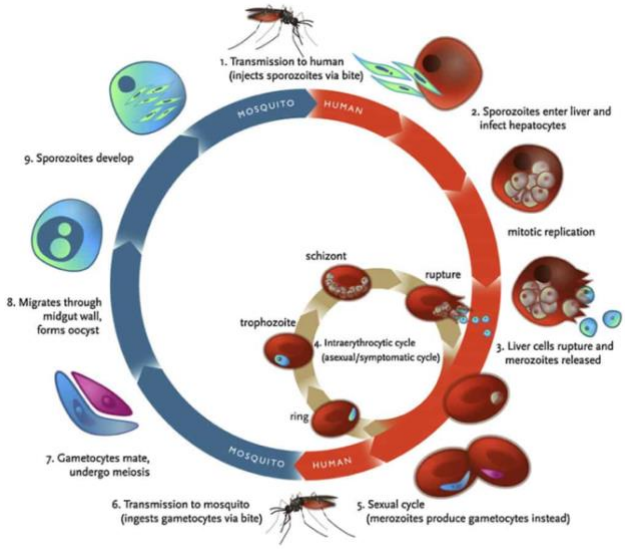

Figure 1.2 Life cycle of the Plasmodium parasite*. Female anopheles mosquito ingests plasmodium parasite gametocytes from a human, where the gametocytes produce a sporozoite. The mosquito carrying the sporozoite releases it into the human host, where it travels and infects the hepatocytes. They asexually replicate for 2 weeks until the liver cells burst, and thousands of merozoites are released into the blood. Merozoites begin infecting red blood cells and asexually reproducing and releasing daughter merozoites infecting more red blood cells and starting the clinical stage of malaria. The parasite can differentiate into male and female gametocytes where it gets taken up by an anopheles mosquito (18).*

1.3 Treatment and Disease Prevention

Despite the complex life cycle and the nature of the parasite, malaria is preventable and curable. Preventative measures for malaria rely on vector control the WHO highly recommends insecticide-treated nets (ITNs), the most common and effective method of control, or indoor residual spraying (IRS) (18). The insecticides that are used in the treatment of bed nets kill and repel mosquitos, thus reducing the overall number that come and feed on the human host. These methods have been critical in the decline of malaria incidence. When an entire community has access to these ITNs, the overall number of mosquitos in the region will decrease, as such, these methods have been critical in the decline of malaria incidence over the past two decades (19). This decrease is coupled with the introduction of long-lasting insecticidal nets: ITNs that contain pyrethroids (a type of insecticide) and can last years without retreatment. This is opposed to ITNs not containing pyrethroids, which have to be retreated every 6-12 months, posing a significant risk to their

effective implementation (20). While pyrethroids provide a solution to this issue, there has been a rise in resistance to pyrethroids, significantly impacting their effectiveness (21). To combat this, ITNs now also have piperonyl butoxide-PBO, which can reverse pyrethroid resistance (22). ITNs have contributed significantly to the decline of malaria incidence and are critical for its prevention, however, consistent universal distribution in affected areas is needed for their effectiveness. Malaria is already a disease with a lack of funding, and with the current economic situation declining worldwide as a result of COVID, it is expected that funding for ITNs will decrease over the next few years (23). Therefore, new measures need to be found to combat malaria.

When it comes to treating the disease, antimalarial drugs are the first line of defense. Most drugs target the disease at its erythrocytic stage, the stage where we start to see clinical symptoms manifest (24). Initially, chloroquine was the antimalarial drug most commonly used. If the infection of malaria is uncomplicated and caused by a strain sensitive to chloroquine, the drug is very effective (25). Chloroquine works by inhibiting parasite growth by targeting the parasite acid vesicles, resulting in an increase in the internal pH. This kills the parasite and prevents the spread of infection. Chloroquine is beneficial in that it has a long half-life, is tolerated well by patients, and has proven successful in preventing reinfection (26). However, as stated before, the drug works on strains sensitive to it, and unfortunately, many parasites across all strains have quickly developed resistance to it (27). Moreover, chloroquine is not effective against cases of severe malaria. As such, it is no longer a recommended form of treatment in areas endemic to *falciparum* malaria (28).

In 1972 Artemisinin was discovered by Tu Youyou. It is a drug derived from a Chinese herb called Artemisia annua (29). Clinical trials were published by 1979, showing that this herb could help clear malaria parasites. Since its discovery, artemisinin derivatives have been combined with a related longer acting drug for the treatment of malaria, which is known as Artemisinin-based combination therapies (ACTs) (30). ACTs are currently the most effective and first line of defense for the treatment of malaria, having been recommended as such by the WHO since 2001 and replacing chloroquine. ACTs are beneficial as they are potent and fast-acting, quickly decreasing parasitemia (31). In addition, they are generally well tolerated. The WHO highly discourages the use of artemisinin without being in combination with other drugs as it could increase the rate of resistance (32), and in recent years, we have started to see a rise in resistance to *falciparum* malaria, causing alarm (33). Coupled with the fact that there have been nine reports of non-travel-related cases of *falciparum* malaria, a vaccine is needed now more than ever to stop the spread of the disease or at least alleviate the life-threatening symptoms.

1.4 Vaccine Development

Malaria is a disease controlled by a parasite that is constantly and rapidly evolving and developing resistance to preventative and standard treatments. Even at high efficacy, preventive measures and standard treatment of care have not yielded long-term results, with the mortality rate remaining the same over the past decade. A vaccine is, therefore, much needed to stop the spread of the severe symptoms once and for all. Over the years, there have been many efforts to develop a vaccine, but these have not yielded optimal results.

Currently, there are two WHO-approved vaccines: RTS, S/AS01 (Mosquirix), R21/Matrix-M. Both of these target the pre-erythrocytic liver stage, specifically the circumsporozoite protein, found on the surface of sporozoites. The vaccine induces antibodies that target the circumsporozoite and prevent them from infecting liver cells (34). By targeting the cycle at the first stage, the idea is that the infection will not be able to develop to the symptomatic erythrocytic stage. These vaccines have fragments of the circumsporozoite protein that is linked to a protein found in hepatitis B (35). This protein is known to self-assemble into virus-like particles. This linkage triggers the immune response and thus induces a stronger response (36). The main difference between the two vaccines is the composition, and the adjuvants used. At present one has not been determined to be more effective than the other. Both require three doses and a booster dose given 12 months after the third. The efficacy rate of RTS,S is considered quite low, with only 30% for severe malaria (37). R21 is more recent and seems to have high efficacy against seasonal uncomplicated malaria, at around 75%, further trials are needed to determine whether it is effective for severe malaria (38).

There is ongoing research targeting the different stages of the parasite life cycle as potential targets for a vaccine (Figure 1.3), however, targeting a specific stage has not proven effective. Sequence variation is a common problem where the parasite can evolve and respond accordingly, and blood-stage vaccines have currently been unable to produce high enough antibody titers for an adequate immune response (39). Moreover, ongoing research into a vaccine has been limited to only a few antigen candidates. Due to the issues surrounding specific life cycle stages, the Kurtis Laboratory sought to identify new vaccine targets by looking into naturally acquired immunity. It has been shown that exposure to episodes of malaria allows a patient to naturally develop immunity (40).

Building on this, the Kurtis lab followed children in Tanzania from birth until the age of 4, where they monitored them before and after episodes of malaria, specifically before and after they developed resistance to malaria or 'susceptible' vs 'resistant'. The lab used a whole proteome differential screening (WPDS) (41) that identified antigens that are detected by antibodies from 'resistant' but not 'susceptible' stages. They used a cDNA library against *P. falciparum* 3D7 strains. This led to the identification of Schizont Egress Antigen-1 (PfSEA-1) and Plasmodium falciparum Glutamic Acid-Rich Protein (PfGARP). PfSEA-1 is a 244 kDA parasite antigen that is expressed in the schizont-infected RBCs. The lab showed that PfSEA arrested schizont rupture and thus decreased parasite replication (42). PfGARP, the other antigen identified by WPDS, is 80kDa and found on the exofacial surface of erythrocytes infected by trophozoite stage parasites. They demonstrated that antibodies that recognize and attack PfGARP kill trophozoite-infected erythrocytes through programmed cell death (43). This suggests that PfSEA and PfGARP could be good vaccine targets and could work with other vaccines targeting parasite invasion of hepatocytes or the invasion and egress from erythrocytes.

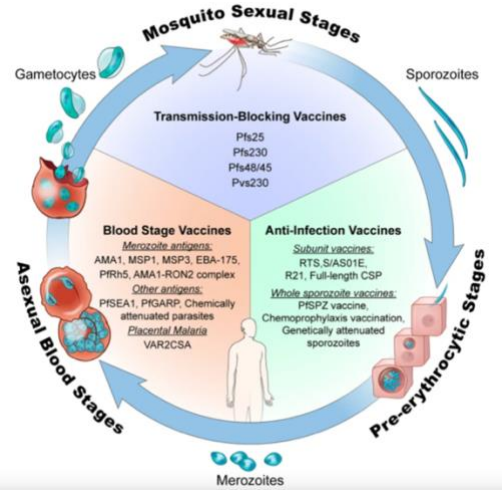

Figure 1.3 Current vaccine targets at the different stages of the parasite's life *(44). Diagram displaying the current vaccine and vaccine candidates being studied and at what stage of the plasmodium parasite life cycle they target.*

1.5 Severe Malaria

There is increasing evidence that the epidemiology of severe *falciparum* malaria is unique. Severe malaria (SM) represents the most complicated form of malaria with a fatality rate of 30%. It is defined by one or more of the following: hyperparasetimia over 10%, seizures followed by coma persisting for 30 minutes, severe anemia with hematocrit less than 15%, renal dysfunction, and acidosis (45).

Previously, *Gupta et al.* have suggested that children can have one or two episodes of severe malaria but not a third time, thus naturally acquiring immunity to non-cerebral severe malaria after only one or two exposures (46). This was a hospitalization study where they looked at children aged between 1 month and 10 years from three different communities in Kenya and two communities in The Gambia. They developed a mathematical model to analyze exposure to malaria dependent on age, rate of immunity, and the period of protection seen in infants, most likely a result of maternal antibodies. *Gupta et al.* found that a significant amount of immunity against non-cerebral severe malaria is acquired after one or two episodes. This suggested that naturally acquired immunity to severe malaria develops much more quickly than previously thought: the common belief was that naturally acquired immunity is a slow process occurring over years of exposure. They also found that this rapidly acquired immunity occurs across various diseases and transmission intensities. This study had big implications as it encouraged more research into naturally acquired immunity to malaria and serves as the scientific premise of this thesis as it suggests that immunity to severe malaria is independent of immunity acquired primarily to parasitemia or uncomplicated malaria.

Another longitudinal study by *Duffy et al.* analyzed the relationship between parasite density and the incidence of SM (47). The study included 882 children from holoendemic regions in Tanzania who were followed from birth up until the age of 2. They found that most high parasite-density infections were not associated with SM, and nearly all the children who did develop SM did not have more than one or two episodes, validating the study done by *Gupta et al.* Overall, the study by *Duffy et al.* suggests that a high parasitemia or high parasite density burden was not sufficient to cause SM and thus further suggests that SM has a unique epidemiology.

Despite these studies, the vaccine pipeline is very limited, with little focus on SM specifically, instead relying on the idea that protection against parasitemia will prevent the disease from reaching SM, something that epidemiologic data does not seem to confirm. We believe that antibodies likely mediate the naturally acquired protection to SM, and more importantly, this protection can be distinguished from responses that simply control parasitemia. With this, we sought to identify antigens that target SM. To do this, we did three new WPDS: pre and post-susceptible to severe falciparum malarial (this will be referred to as the susceptible screen throughout the thesis), resistant vs susceptible to severe *falciparum* malaria (referred to as the resistant screen), and before and during cerebral malaria (CM) (referred to as the CM screen). The screen from pre and post-susceptible used serum from severe malarial anemia longitudinal cohort where kids' serum was taken the month before severe malarial anemia vs the months after severe malarial anemia. The resistant vs susceptible screen investigated serum from kids after they naturally acquired immunity to SM. The CM screen corresponds to serum from kids before and during the acute phase vs convalescent phase of CM. Unlike the previous screens used to identify PfGARP and PfSEA, these SM screens are not defined by susceptibility to parasitemia. We

hypothesize that the antigen targets will be different to malaria defined by parasitemia. The goal of this study is to identify new antigen targets of human antibodies that protect against SM and expand on the currently limited repertoire of vaccine candidates, particularly at the blood-stage level. We will develop selected proteins into vaccines and determine their ability to kill and inhibit parasite growth. Thus, these antigen targets could be used to develop or work in synergy with current vaccines or vaccine targets to protect against *P. falciparum* severe malaria.

Chapter 2: Methods

2.1 Differential screening and phage display

We used a previously constructed *P. falciparum* blood-stage cDNA expression library for our differential biopanning experiments using 3D7 blood-stage strain. For differential screening and phage display, we used the Novagen T7Select System Manual. Three different screens were done: pre- and post-susceptible to SM, resistant vs susceptible to SM, and before and during cerebral malaria (CM). For the susceptible screen, seven children between the ages of 2-7 were enrolled, and the same children served as their own control (Table 2.1). For the resistant screen, eight children between the ages of 5-6 were included, with different people being used for the resistant vs susceptible defined at baseline serum (Table 2.2). The CM serum came from a partner research lab.

Variables	Severe Malaria (SM)	non-SM	p Value
Count (n=)	7	391	/
Age by median (range, years old)	2 (2-6)	4 (2-7)	0.02
Sex (% female)	28.57	50.13	0.3
baseline parasitemia (per 200 WBC[1])	398.86	123.02	0.2
baseline parasitemia (per uL whole blood)	5.983	1.587	0.3
median parasite density overtime (per uL whole blood)	11.111	9.799	0.3

Table 1.1 Characteristics of serum for susceptible screen. *Table shows different factors measured for the pre vs post-susceptible screen. 7 children were enrolled and used as their control between the pre and post-susceptible stages. [1]WBC denotes white blood cells.*

Variable		Resistant		Susceptible		P value
		median or %	IQR[3]	median or %	IQR	
# of participants		8		8		
Sex (% female)		50		37.5		1
Age		6	5 - 6	3.5	2.5 - 5	0.019
Parasite density (PDs[1]) by qPCR[2] at baseline		398	169-1466	345	29.7-1182	0.79
PDs by microscopy at baseline		46.5	11.5-82.0	33	0.0-124	0.79
# of mild/severe malaria	0 – 100 weeks	1.5	0.5-5.0	3	2.0-5.0	0.37
	101 – 157 weeks	0	0.0-1.5	0.5	0.0-1.0	1
	Whole period	2.5	0.5-6.0	4	2.5-5.0	0.49
# of Treatment	0 – 100 weeks	1	0.0-1.5	2	0.5-3.5	0.33
	101 – 157 weeks	0	0.0-0.0	0	0.0-0.0	1
	Whole period	1	0.0-1.5	2	0.5-4.0	0.36
# of *Pf* positive by qPCR	0 – 100 weeks	21.5	20.5-22.0	21	20.0-22.0	0.71
	101 – 157 weeks	**4.5**	**3.5-5.0**	**8**	**7.5-8.0**	**<0.001**
	Whole period	**25**	**24.5-27.5**	**28**	**28.0-30.0**	**0.007**
PDs by qPCR (parasites/µL)	0 – 100 weeks	268	36.4-1222	423	43.3-1440	0.16
	101 – 157 weeks	**15.4**	**0.0-109**	**772**	**126-1612**	**<0.001**
	Whole period	**131**	**13.3-693**	**500**	**55.4-1522**	**<0.001**

Table 1.2 Characteristics of serum selected for resistant screen. *Table shows different factors measured for the resistant vs susceptible screen. 8 children were enrolled with serum from resistant vs susceptible coming from different children. [1]PDs refers to parasite density as measured by parasites/µl number of parasites. [2]qPCR refers to real-time polymerase chain reaction. [3]IQR stands for inter quartile range.*

Four rounds of positive selection using serum from post-susceptible-SM were done, followed by a negative selection from pre-susceptible serum. For our resistant screen, four rounds of positive selection using serum from resistant individuals were used, followed by a negative selection from

susceptible serum, and for the CM screen, four rounds of positive selection using serum from individuals during CM were used, followed by a negative selection from those before CM.

Biopanning was done based on the T7 Phage Display system (Figure 2.1). For our Whole Proteome differential Screening (WPDS) we used Immulon 4HBX ELISA plates from Thermo Fisher and added 50µl of 1:100 dilution of serum from pooled children who were susceptible to severe malaria. The plate was plastic wrapped and kept at 4°C overnight to allow for the binding of antibodies to the plate. The next day serum was unbound and washed 10 times with 1x TBST (10mM Tris HCL, 150mM NaCl, 0.05% Tween 20) at 1-minute intervals. Wells were then blocked with 300µl of 2% BSA in TBST for 2 hours at room temperature. After blocking for 1 hour, wells were washed 10 times with TBST. Phage at 1 x 10^8 was added for 2 hours at room temperature. Wells were washed 10 times with TBST. The bound phage was eluted with 50µl of 5M NaCl and kept for 20 minutes at room temperature over a gentle shaker. Bound phage was taken through pipetting. Eluted 50µl phage was grown with 10mL of BLT5240 bacteria at an optical density (OD) of 0.8 on shaker until lysis was observed. If bacterial lysis was not observed within 3 hours, we diluted 1:2 with fresh media. Lysis was confirmed by observing thread like structure by the naked eye and measuring the OD at 600nm. Phage was isolated by centrifuge at 10000g for 10 minutes at 4°C. The supernatant was collected and filtered through a 0.2µl filter and stored at 4°C.

To plate our serum, we serially diluted the eluted phage and took 0.1mL of the diluted phage in 0.2mL of BLT5240 bacteria, with an OD of 1, and added with 3mL of top agar (50°C). We then poured it evenly on top of an agar plate containing carb antibiotics. Phage was observed through the naked eye (Figure 2.2). The phage encodes parasite DNA that encodes parasite protein and

through biopanning, we are selecting for antibodies. These phage have parasite protein on the surface and the parasite gene on the inside. Each phage was individually counted and titer was calculated according to the following formula: number of plaques/phage x dilution factor x 10. pfu/mL was calculated with the formula: number of plaques counted/volume used x dilution factor. Phage and antibody dilution were calculated using the formula: n1v1=n2-v2.

For biopanning amplification 50µl of phage from the previous round was bound and the steps above were repeated. Four rounds of positive selection biopanning were done.

Negative selection:

For negative selection, 50µl of serum was bound on the well overnight. The next day the well was washed 10 times with TBST and then blocked with 300µl of 2% BSA in TBST for 1 hour at room temperature. The well was then washed 10 times with TBST. Phage from 4th round of biopanning was diluted at 50,000/50µl. 50µl of phage at 50,000 was added to bound serum for 2 hours. The serum was transferred to the next well and kept for 2 hours for binding. Transfer was repeated until well 5, with 2 hours incubation each time. Unbound phage from well 5 was taken and serially diluted. Same as positive selection, 0.1mL of diluted phage was added with 0.2mL of bacteria (OD 1) and 3mL of top agar and evenly spread onto an agar plate. Plates were kept at room temperature overnight. Phage were counted as previously described.

2.2 PCR and sequencing

98 phage from the plate were selected from the negative selection plates and were individually placed in 50µl of water on a 98-well PCR plate. They were denatured at 99°C for 10 minutes 2µl

of each sample was used for PCR. PCR product was purified using the PCR clean-up kit from zymogen, ZR-96 DNA Clean-Up Kit (C#D4017).

Gel electrophoresis was done to confirm DNA was present in our samples (Figure 2.3). This was done by using 50mL of Tris-acetate EDTA (TAE) and adding 0.5g of agarose. The solution was placed in the microwave for 1 minute 30 seconds for the agarose to dissolve and a colorless solution was observed. 1.5µl of cybergrin was then added and the solution was poured onto a gel tray. Once gel had solidified we added 3µl of ladder at 1Kb and 100-10Kb on opposite ends of the wells and 2µl of PCR product was added into the remaining wells. TAE was poured until the gel was covered and the box was filled. We ran at 110V for 20 minutes and then took out the gel for imaging.

2.3 Bioinformatics

Purified PCR samples were sent to Eurofins for sequencing. Sequences were then analyzed by blasting nucleotides on the NCBI database (blast.ncbi.nlm.nih.gov) and using the mega blast feature, only looking for matches that were highly similar to 3D7. The resulting gene characteristics were analyzed by searching them on PlasmoDb (plasmodb.org).

2.4 Plasmid Transfection

Selected proteins were produced by Atum.bio and were transformed into plasmid. The plasmid was purified using the Qiagen kit (C# 1054578) in preparation for transfection. Plasmid was

transfected in immortalized human kidney cells (HEK293**),** a mammalian expression system. This was done using the Expi293™ Expression System.

2.5 Nickel Column Chromatography

Our selected proteins were purified through nickel column chromatography. Once the column was packed we washed it with 2cv (1cv = 5mL) of dH_2O at a flow rate of 3mL/minute. It was then primed with 25mM of Imidazole at 2cv. Samples were then loaded at a flow rate of 1mL/minute. To elute the sample at different concentrations, we first added 100mM imidazole and collected 10mL. We repeated this at 200mM and 500mM, collecting 10mL each time.

2.6 Western blot

Western Blots were done on our purified proteins and transfected plasmid for confirmation.

The reagents used were as follows:

- 1x Running Buffer made from BioRead 10x Tris/Glycine/SDS (C#1610732) and diluted with dH_2O.
- 1x Transfer Buffer diltued using 10x Transfer, Methanol and dH_2O.
- TBST-T was made using dH_2O, 10X TBS and Tween20.

Purified nickel column samples were boiled for 10 minutes at 100°C. BioRad Mini-PROTEAN TGX precast gels (C#456-1083) were used for the SDS-PAGE. Between 5-15µl of samples were loaded at concentrations between 100-500mM, and 3.5µl of ladder was used. 1x Running buffer was added, and the gel was run for 19 minutes at 250V. The gel was then transferred onto a membrane and sandwiched with filter paper and foam. 1x Transfer buffer was added to the cassette

and was run for 1 hour at 100V. The membrane was then transferred onto a tray with 5% milk in TBS-T for blocking for 30 minutes. Milk was dumped out, and the membrane was washed by adding dH_2O onto the tray and placing it on the shaker. Washing was done three times at 5-minute intervals. After washing, Anti-6X His tag® antibody (C#ab18184) in TBS-T was added to the membrane overnight. The membrane was then washed as described previously, and BCIP/NBT Substrate (C#B5655) was used to develop the membrane visually.

2.7 Mice Immunization

Five Balb/c mice from each group were given intraperitoneal (IP) injections. The mice were immunized biweekly for 8 weeks, receiving recombinant P113 protein with TiterMax® Gold Adjuvant (C#T2684) or PBS and Adjuvant if in the control group. At the end of the 8 weeks, the mice were sacrificed, and their serum was collected (Figure 2.4).

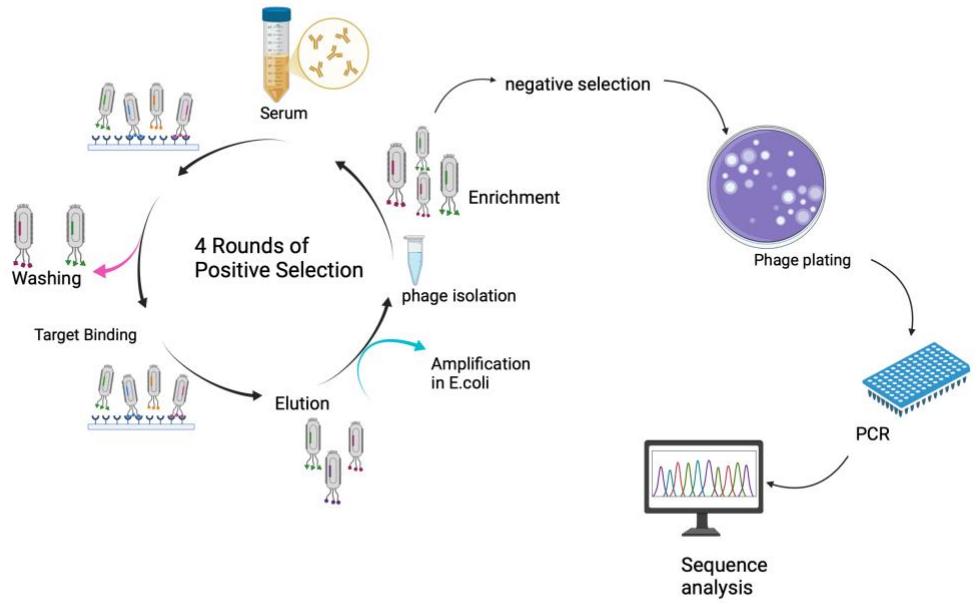

Figure 2.1 Schematic displaying biopanning method. *Method is based on T7 select phage display system. Serum is plated on well of an ELISA plate where the phage binds. Washing is done to remove unbound phage. After further target binding and washing, the phage is eluted. One round of negative selection is done, following the same steps. After amplification, it is plated on plates treated with carb antibiotics. PCR is done on plaques, which is sent for sequence analysis.*

Figure 2.2 Plated phage from resistant screen. *Image of phage plated after 4 rounds of biopanning. The plaques represent antigen targets. Each phage encodes a parasite DNA that encodes parasite protein. The phages have parasite protein on the surface and the parasite gene on the inside.*

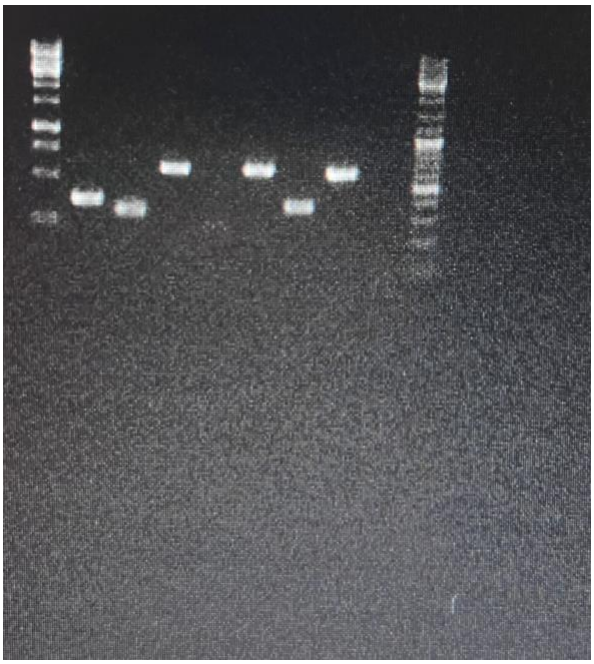

Figure 2.3 Gel electrophoresis image of PCR samples from resistant screen. *Electrophoresis was done on purified PCR product to confirm the presence of DNA before sequencing, with each band serving as confirmation.*

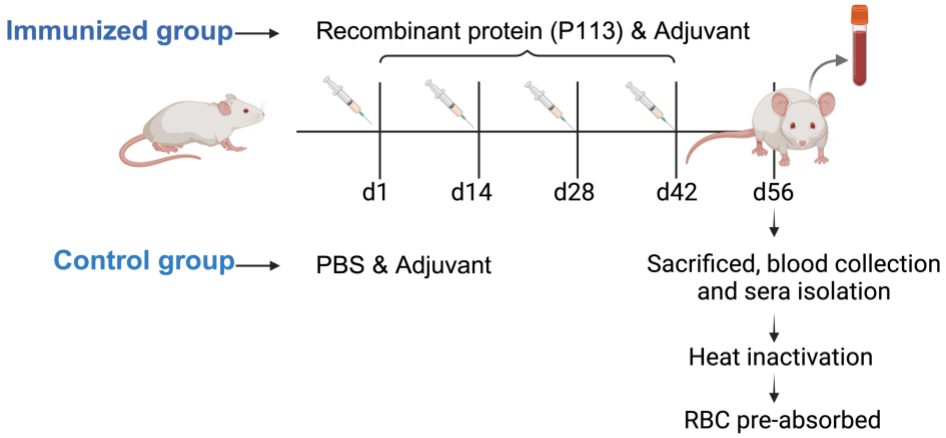

Figure 2.4 Schedule of mice immunization (48). *Mice were immunized biweekly for 8 weeks with recombinant PfP113 Protein with TiterMax Gold adjuvant or PBS & Adjuvant. After 8 weeks the mice were sacrificed, and serum was collected.*

Chapter 3: Results

3.1 Pre and post-susceptible screen

We used a whole proteome differential screening where we generated a list of parasite genes uniquely recognized by antibodies expressed by children post-susceptible to severe malarial anemia but not before (Table 2.1). We found that the third round of biopanning (BP3) produced the most diverse list of proteins, whereas by BP2 we had not amplified for all the genes and by BP4 we were enriching for a select few. To visualize and validate our amplification through subsequent biopanning rounds, we calculated the titer concentrations of the NaCl/Crude eluted samples. We saw an increase after every round, with BP4 showing the biggest increase, thus confirming our amplification throughout the rounds (Figure 3.1). The increase in amplification was consistent across all three of our screens.

From our list of genes, we found no overlap between this screen and the lab's prior parasitemia screen other than PfGARP (PF3D7_0113000). We selected surface protein PfP113 (PF3D7_1420700) for further investigation. PfP113 was selected as it represented 5.5% of our overall hits, is expressed on the surface of merozoites, displayed limited sequence variation (a common problem with blood-stage vaccines) (49), and is part of the Rh5 invasion complex. The Rh5 complex is a complex involved in the merozoite invasion of erythrocytes and consists of four proteins: PfRH5, PfCyRPA, PfRipr, and PfP113 (50). Due to its role in parasite invasion, the complex is being researched as a vaccine candidate with the hypothesis that if you block Rh5, the symptoms of malaria could be prevented (51). PfP113 is specifically implicated in anchoring Rh5 to the surface of merozoites (52). However, few literature and studies have focused on it.

To express PfP113 we transformed it into a plasmid and expressed it using the Expi293 expression system, transfecting in HEK293 cells. We purified PfP113 through nickel column chromatography and tangential flow filtration. We validated the presence of our protein through SDS-page and western blots. PfP113 displays a high molecular weight, most likely due to its acidic nature (Figure 3.2). Mice were immunized with recombinant plasmid PfP113 to generate polyclonal anti-PfP113 antibodies. Growth Inhibition Assay performed on synchronized 3D7 parasites showed that antibodies to PfP113 inhibit parasite growth (Figure 3.3).

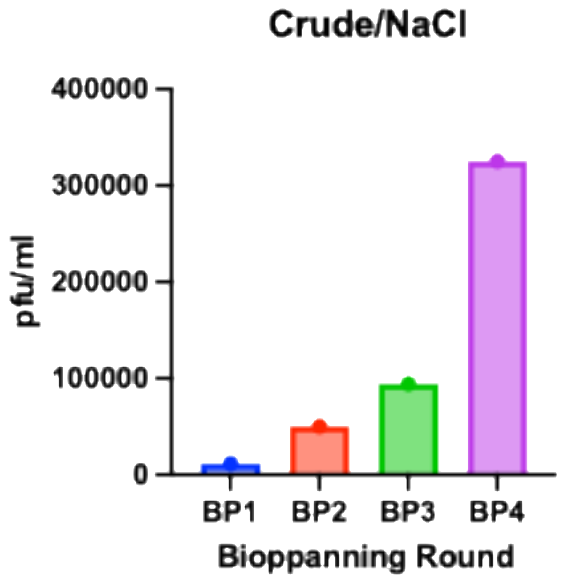

Figure 3.1 Amplification of biopanning. *Titer calculated for each biopanning round expressed in pfu/ml. Exponential increase is seen across the rounds validating amplification after each round of biopanning.*

Gene name	Gene Id
Plasmodium falciparum 3D7 AP2 domain transcription factor, putative (PF3D7_1139300), partial mRNA	PF3D7_1139300
Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function (PF3D7_1221300), partial mRNA	PF3D7_1221300
Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function (PF3D7_1228700), partial mRNA	PF3D7_1228700
Plasmodium falciparum 3D7 coronin (PF3D7_1251200), partial mRNA	PF3D7_1251200
Plasmodium falciparum 3D7 DNA/RNA-binding protein Alba 1 (PF3D7_0814200), partial mRNA	PF3D7_0814200
Plasmodium falciparum 3D7 DnaJ protein, putative (PF3D7_1473200), partial mRNA	PF3D7_1473200
Plasmodium falciparum 3D7 exportin-1, putative (PF3D7_0302900), partial mRNA	PF3D7_0302900
Plasmodium falciparum 3D7 glutamic acid-rich protein GARP (PF3D7_0113000), partial mRNA	PF3D7_0113000
Plasmodium falciparum 3D7 glutamine–tRNA ligase, putative (PF3D7_1331700), partial mRNA	PF3D7_1331700
Plasmodium falciparum 3D7 heat shock protein 70 (PF3D7_0917900), partial mRNA	PF3D7_0917900
Plasmodium falciparum 3D7 HECT-like E3 ubiquitin ligase, putative (PF3D7_0826100), partial mRNA	PF3D7_0826100
Plasmodium falciparum 3D7 heterochromatin protein 1 (PF3D7_1220900), partial mRNA	PF3D7_1220900
Plasmodium falciparum 3D7 nucleolar protein 5, putative (PF3D7_1008800), partial mRNA	PF3D7_1008800
Plasmodium falciparum 3D7 Plasmodium exported protein, unknown function (PF3D7_1252500), partial mRNA	PF3D7_1252500
Plasmodium falciparum 3D7 ring-exported protein 2 (PF3D7_0936000), partial mRNA	PF3D7_0936000
Plasmodium falciparum 3D7 sortilin (PF3D7_1451800), partial mRNA	PF3D7_1451800
Plasmodium falciparum 3D7 surface protein P113 (PF3D7_1420700), partial mRNA	PF3D7_1420700

Table 2.1 List of genes from susceptible screen. *Proteins were generated from biopanning 3 from the pre and post-susceptible severe malarial anemia where the proteins listed are uniquely recognized by those who are post-susceptible. PfP113 highlighted in yellow is the protein that was selected for further investigation. PfGARP, highlighted in orange, is the only protein to show up in severe malaria and parasitemia-based screens.*

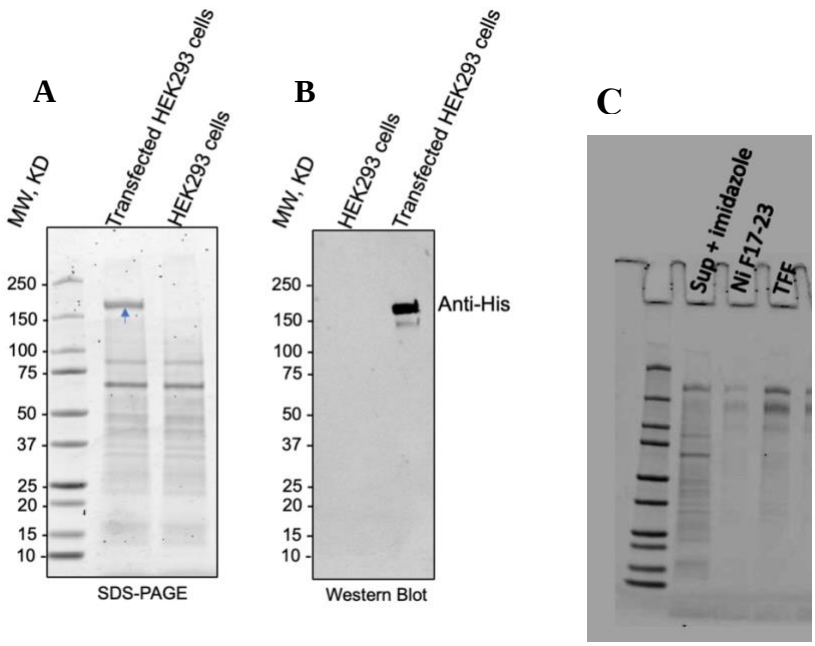

Figure 3.2 Purification and expression of PfP113. **A)**SDS-Page of unpurified culture supernatants from Transfected HEK293 cells with PfP113 expressing plasmid, contrasted with culture supernatant from HEK293 (negative control). **B)** Western blot of HEK293 transfected with Pf113 expressing plasmid and HEK293 (negative control) probed with anti-6x-His Antibody. **C)** SDS-Page of supernatant from HEK293 transfected with PfP113 expressing plasmid purified through nickel column chromatography and Tangential flow filtration (TFF).

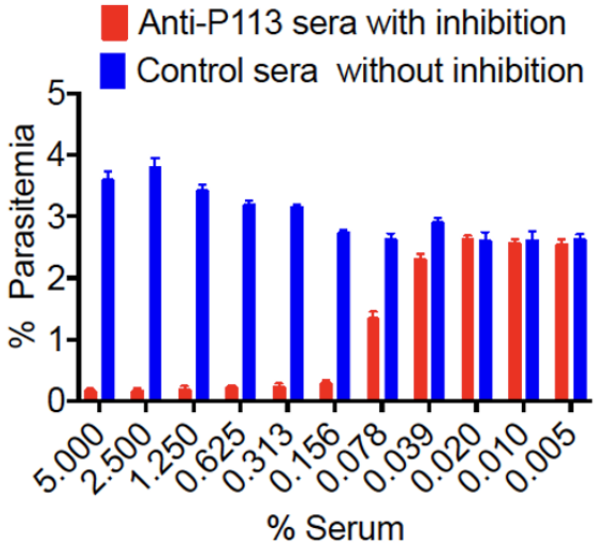

Figure 3.3 Antibodies to PfP113 inhibit parasite growth (48). *Growth Inhibition Assay performed on synchronized 3D7 parasites using polyclonal anti-PfP113 antibodies generated by recombinant protein immunization in mice. Control included adjuvant and PBS but no PfP113. Antibodies to PfP113 inhibit parasite growth as seen in the decrease in percent parasitemia as the percent serum increase.*

3.2 Susceptible vs Resistant and Cerebral Malaria Screen

With our WPDS, we identified a list of parasite genes that are uniquely recognized by antibodies expressed by children after developing resistance or naturally acquired immunity to malaria but not by children who are still susceptible (Table 2.2). BP3 generated the most diverse list. Our screen yielded genes that are currently being investigated as vaccine targets, such as Merozoite surface protein 1 (PF3D7_0930300) and Merozoite surface protein 4 (PF3D7_0207000), validating our WPDS method.

From our screen, we selected two genes, both currently unknown: PF3D7_0308300 and PF3D7_1409400. These two genes were selected because they showed up multiple times throughout our hits, their unknown nature makes them interesting to study, they are conserved, and they were a size that could be expressed and purified.

Our CM screen yielded a diverse list of genes (Table 2.3) that is currently under review for selection of genes for further investigation. We generated a list of genes that were common to both SM and CM (Table 2.4). This confirms CM as being a subset of SM, and selecting genes from this table could be beneficial for a vaccine target as it could help not only prevent SM but CM as well.

Gene name	Gene Id
Plasmodium falciparum 3D7 ring-exported protein 1	
Plasmodium falciparum 3D7 ring-infected erythrocyte surface antigen	PF3D7_0102200
Plasmodium falciparum 3D7 glutamic acid-rich protein GARP	PF3D7_0113000
Plasmodium falciparum 3D7 merozoite surface protein 4	PF3D7_0207000
Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function	PF3D7_0308300
Plasmodium falciparum 3D7 mature parasite-infected erythrocyte surface antigen	PF3D7_0500800
Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function	PF3D7_0604500
Plasmodium falciparum 3D7 erythrocyte binding antigen-175	PF3D7_0731500
Plasmodium falciparum 3D7 V-type proton ATPase subunit a, putative	PF3D7_0806800
Plasmodium falciparum 3D7 conserved Plasmodium membrane protein, unknown function	PF3D7_0824800
Plasmodium falciparum 3D7 heat shock protein 70	PF3D7_0917900
Plasmodium falciparum 3D7 patatin-like phospholipase, putative	PF3D7_0924000
Plasmodium falciparum 3D7 merozoite surface protein 1	PF3D7_0930300
Plasmodium falciparum 3D7 conserved protein, unknown function	PF3D7_1121100
Plasmodium falciparum 3D7 Plasmodium exported protein (PHISTc), unknown function	PF3D7_1148700
Plasmodium falciparum 3D7 conserved protein, unknown function (nuclear export mediator factor NEMF, putative)	PF3D7_1202600
Plasmodium falciparum 3D7 male development gene 1, partial mRNA	PF3D7_1216500
Plasmodium falciparum 3D7 acidic basic repeat antigen	PF3D7_1228600
Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function	PF3D7_1248700
Plasmodium falciparum 3D7 conserved protein, unknown function	PF3D7_1409400
Plasmodium falciparum 3D7 atypical protein kinase, ABC-1 family, putative	PF3D7_1414500
Plasmodium falciparum 3D7 Hsp70/Hsp90 organizing protein	PF3D7_1434300

Table 2.2 List of genes from resistant screen. *Genes generated from biopanning 3 from the susceptible vs resistant screen, where the proteins listed are uniquely recognized by children who have developed resistance to severe malaria but not those who are susceptible. The genes highlighted in yellow are selected for further investigation.*

Gene name	Gene Id
Plasmodium falciparum 3D7 myosin essential light chain ELC	PF3D7_1017500
Plasmodium falciparum 3D7 acyl-CoA synthetase	PF3D7_1200700
Plasmodium falciparum 3D7 myosin A	PF3D7_1342600
Plasmodium falciparum 3D7 U2 snRNP-associated SURP motif-containing protein, putative	PF3D7_1402700
Plasmodium falciparum 3D7 fructose-bisphosphate aldolase	PF3D7_1444800
Plasmodium falciparum 3D7 knob-associated histidine-rich protein	PF3D7_0202000
Plasmodium falciparum 3D7 merozoite surface protein 1	PF3D7_0930300
Plasmodium falciparum 3D7 photosensitized INA-labeled protein PHIL1	PF3D7_0109000
Plasmodium falciparum 3D7 peptidyl-prolyl cis-trans isomerase FKBP35	PF3D7_1247400
Plasmodium falciparum 3D7 18S ribosomal RNA	PF3D7_0725600
Plasmodium falciparum 3D7 merozoite surface protein 3	PF3D7_1035400
Plasmodium falciparum 3D7 rifin	PF3D7_1101300
Plasmodium falciparum 3D7 serine/threonine protein kinase, FIKK family	PF3D7_1039000
Plasmodium falciparum 3D7 alpha tubulin 2	PF3D7_0422300
Plasmodium falciparum 3D7 DNA/RNA-binding protein Alba 1	PF3D7_0814200
Plasmodium falciparum 3D7 glutamic acid-rich protein GARP	PF3D7_0113000
Plasmodium falciparum 3D7 karyopherin beta	PF3D7_0524000
Plasmodium falciparum 3D7 E3 ubiquitin-protein ligase, putative	PF3D7_0707700
Plasmodium falciparum 3D7 NYN domain-containing protein, putative	PF3D7_0406500
Plasmodium falciparum 3D7 cytoadherence linked asexual protein 3.1	PF3D7_0302500
Plasmodium falciparum 3D7 asparagine and aspartate rich protein 1	PF3D7_1233600
Plasmodium falciparum 3D7 conserved protein, unknown function (nuclear export mediator factor NEMF, putative)	PF3D7_1202600
Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function	PF3D7_1036900
Plasmodium falciparum 3D7 ornithine aminotransferase	PF3D7_0608800
Plasmodium falciparum 3D7 reticulocyte binding protein homologue 1	PF3D7_0402300
Plasmodium falciparum 3D7 CSTF domain-containing protein, putative	PF3D7_0933000
Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function	PF3D7_0323800
Plasmodium falciparum 3D7 prolyl 4-hydroxylase subunit alpha, putative	PF3D7_0829400

Table 2.3 List of genes from cerebral malaria screen. *Genes were generated from biopanning 3 from the cerebral malaria screen.*

Gene name	Gene Id
Plasmodium falciparum 3D7 glutamic acid-rich protein GARP	PF3D7_0113000
Plasmodium falciparum 3D7 merozoite surface protein 4	PF3D7_0207000
Plasmodium falciparum 3D7 Plasmodium exported protein, unknown function	PF3D7_0402400
Plasmodium falciparum 3D7 NYN domain-containing protein, putative	PF3D7_0406500
Plasmodium falciparum 3D7 karyopherin beta	PF3D7_0524000
Plasmodium falciparum 3D7 rhoptry-associated membrane antigen	PF3D7_0707300
Plasmodium falciparum 3D7 DNA/RNA-binding protein Alba 1	PF3D7_0814200
Plasmodium falciparum 3D7 heat shock protein 70	PF3D7_0917900
Plasmodium falciparum 3D7 ring-exported protein 1	PF3D7_0935900
Plasmodium falciparum 3D7 heterochromatin protein 1	PF3D7_1220900
Plasmodium falciparum 3D7 coronin	PF3D7_1251200
Plasmodium falciparum 3D7 M1-family alanyl aminopeptidase	PF3D7_1311800

Table 2.4 List of genes in common to severe malaria and cerebral malaria. *Table shows a list of genes that showed up in our severe malaria screens, susceptible and resistant, and the cerebral malaria screen across the biopanning rounds.*

Chapter 4: Discussion and Conclusions

Malaria continues to be a leading cause of child mortality worldwide, with a vaccine urgently needed to combat the spread of the disease. Current vaccines and candidates have not yielded high efficiency, particularly for severe malaria (SM). SM is the most complicated form of malaria, with a huge fatality rate upwards of 30%. It includes symptoms such as severe anemia, respiratory issues, and cerebral malaria. Previous studies have shown that patients acquire protection against severe malaria after only one or two episodes, and more importantly, this protection seems to be mediated by antibodies and distinguishable from responses that control parasitemia. Despite its unique epidemiology and large fatality rate, the focus on malaria research, particularly vaccine targets, has not specifically looked into SM. By addressing this gap in the research, we expand on the currently limited repertoire of targets being researched in SM. We believe that blood-stage vaccines remain the most attractive for the treatment of SM, as evidenced by adults who have robust protection against SM produce anti-blood stage antibodies, but the optimal targets have not yet been identified. Here, we used a whole proteome differential screening approach to identify specific genes at three different time points of SM: kids post-susceptible to SM, after naturally acquiring resistance to SM, and during cerebral malaria (CM). The goal is to identify new antigen targets that can be used to develop a blood-stage vaccine or to work in synergy with current targets or vaccines to improve their efficacy in SM.

From our post-susceptible screen, we selected PfP113, a protein that is part of the Rh5 complex. The complex is involved in the merozoite invasion of erythrocytes, where PfP113 plays a role in anchoring the complex to the merozoite. Its biggest benefit is that it displays limited sequence

variation. Sequence variation has been one of the biggest complications in developing a blood-stage vaccine as it allows the parasite to evolve and, thus, develop resistance. We successfully expressed and purified PfP113 and showed that antibodies to PfP113 inhibit parasite growth.

Further studies into PfP113 are still needed, including epitope mapping and further immunizations with and without adjuvant. An immunofluorescence assay will be done to analyze the endogenous location of PfP113. The biggest limitation of this screen is the small sample size, and a future study will be done by enrolling more children and replicating the screen to validate our findings. Another limitation is that the children in this screen are their own control and they are treated when presenting with illness, so we cannot account for the exact stage during which the antibodies are produced.

From our resistant vs susceptible screen, we generated a list of genes that are uniquely recognized by children who have naturally acquired immunity to SM, usually after one or two episodes, but are not recognized by those who are still susceptible to SM. Moreover, while the children are resistant to SM, they remain susceptible to uncomplicated malaria. We selected two unknown conserved proteins for further investigation, where we will express and purify them as we did PfP113 and then immunize them in mice. Like our post-susceptible screen, the biggest limitation of this screen is the small sample size. We are currently enrolling more children for this study to increase the sample size and replicate the screen and thus validate our findings. Another limitation is that the serum from those who are susceptible vs those who are resistant came from different people, and we cannot account for differences, such as some children being under bed nets. A larger study should help account for these slight differences, however.

We generated a similar list of genes using serum from children during the acute phase of CM vs the convalescent phase. We analyzed which genes overlapped between our CM screen and our post-susceptible and resistant screen. This overlap is significant as previous literature on the epidemiology of SM has focused on non-cerebral SM and as such we can confirm that CM is a subset of SM. Due to its large fatality rate, it is important that any treatment to SM involves CM and as such identifying the targets that overlap in both could help find the optimal blood-stage vaccine.

Most significantly, in all three of our screens, PfGARP (PF3D7_0113000), was the only protein that showed up in the severe malaria screens and the lab's previous parasitaemia-based screens. This implicates PfGARP as a potential antigen target for a vaccine and further implicates the role of PfGARP in both infection and disease. In addition, the lack of overlap between parasite genes recognized by children resistant to parasitemia vs severe malaria, outside of PfGARP, suggests that the epidemiology of severe malaria is unique and distinguished from responses that control parasitemia. It also emphasizes the need to address SM and that research against parasitemia will not yield optimal results for the treatment of SM.

Overall, our research validates the unique epidemiology of SM and addresses the much-needed gap in the treatment of malaria. Through our research, we expanded on the currently limited repertoire of antigen targets for blood-stage vaccines by identifying new antigen targets uniquely recognized in severe malaria. This comes at a time when the focus on malaria is increasingly less, and resistance to current treatment and preventative measures is on the rise. This research will

have wide implications on malaria as we show the need to address SM and have potentially identified new optimal blood-stage vaccine targets.

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