Investigation of antibody-mediated immune mechanisms during submicroscopic infections of pregnancy-associated malaria in pregnant women from Colombia

By

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Abstract

Malaria has been a threat to humans for centuries and continues to be a global health problem. Repeated exposure to malaria in childhood can result in protective immunity that reduces parasite invasion of red blood cells and the sequestration of infected red blood cells (iRBC) in the tissues. However, pregnancy offers a new site of attack for the parasite, the placenta. In the placenta, iRBCs bind to chondroitin sulfate A (CSA) and sequester here via variable surface antigens (VSAs) that are only expressed during pregnancy. One known VSA is VAR2CSA, a large 350 kD protein. In high transmission areas of sub-Saharan Africa, multiple instances of pregnancy-associated malaria (PAM) can result in acquired antibodies that block iRBC binding to CSA. Thus, these antibodies may be protective against adverse birth outcomes, which include low birthweight, preterm birth, and maternal anemia.

Previously, we conducted a longitudinal study in Colombia, where both *Plasmodium falciparum* and *P. vivax* co-circulate, which followed pregnant women from early pregnancy to delivery. This included a cohort of women who had at least one submicroscopic infection of PAM during pregnancy, which cannot be detected with microscopy diagnostic methods but were instead detected retrospectively with qPCR. In this subset of infected women, we observed a protective association between antibody inhibition against iRBC binding to CSA *in vitro* and maternal hemoglobin at delivery, but the mechanisms behind this association are unknown. To develop effective treatments against PAM, the human host's own defense mechanisms must be well understood. In this thesis, we aimed to elucidate potential protective mechanisms of the humoral immune system against adverse pregnancy outcomes, with a focus on protection from maternal anemia. We first assessed IgG and IgM-VAR2CSA levels against recombinant VAR2CSA and found variable levels within the cohort, but these levels were not associated with

improved pregnancy outcomes. We also assessed total VSA_{PAM} IgG by testing for antibody staining in flow cytometry against whole iRBCs selected to express VSA(s) that adhere to CSA, but we observed minimal activity in this assay. But, as we previously observed functional activity in this cohort, we then turned to other measurements of antibody-mediated functional activity.

Cytophilic antibodies acquired during PAM may mediate classical complement activation through fixation of the first component, C1q, and recruit phagocytes through opsonic phagocytosis. We observed functional activity in sera collected from the SMI cohort in both assays, and both were negatively associated with infant birthweight. This may indicate a dysregulated immune response which could contribute to placental pathology, but further investigation is required. Additionally, IgM-VAR2CSA and C1q fixation levels were highly associated to each other in early pregnancy and at delivery, which suggests IgM may fix C1q to a greater extent than IgG antibodies. No further associations between the humoral response and maternal anemia were observed, which may be due to several factors including study setting, cohort size, and the assays used for measurements of humoral immunity. Further investigation should focus on understanding the connections between humoral immunity and adverse pregnancy outcomes and elucidating mechanisms such that protective responses can be elicited in future therapies and vaccines against PAM.

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List of Abbreviations (in order of appearance)

CSP – circumsporozoite protein

RBC – red blood cell

MSP1 – merozoite surface protein 1

AMA1 – apical membrane antigen-1

RON – rhoptry neck protein

PvRBP – *P. vivax* Reticulocyte Binding Protein

PfPR – *P. falciparum* parasite rate

API – annual parasite incidence

iRBC – infected red blood cell

SMI – submicroscopic infection

ACT – artemisinin-combination therapy

SP – sulphadoxine-pyrimethamine

PAM – pregnancy-associated malaria

IPTp – intermittent preventative treatment

ITN – insecticide-treated bed net

VSA – variable surface antigen

*Pf*EMP1 – *P. falciparum* erythrocyte membrane protein 1

STEVOR – subtelomeric variable open reading frame

RIFIN – repetitive interspersed family

LBW – low birthweight

PTB – preterm birth

CSA – chondroitin sulfate A

DBL – Duffy-binding like

nRBC – non-infected red blood cell

PS – phosphatidylserine

Th – T helper

IBA – inhibition of binding assay

Ang-1 – angiopoietin-1

Ang-2 – angiopoietin-2

Hb – hemoglobin

ELISA – enzyme-linked immunosorbent assay

BSA – bovine serum albumin

PBS – phosphate buffered saline

PBST – Tween 20 in 1X PBS

HRP – horseradish peroxidase

TMB – tetramethylbenzidine substrate

OD – optical density

AU – arbitrary units

FBS – fetal bovine serum

DAPI - 4,6-Diamidino-2-Phenylindole

MFI – median fluorescent intensity

EtBr – ethidium bromide

PI – phagocytosis index

$eRBCs_i$ – nRBCs exposed to iRBCs in a transwell plate

$eRBCS_n$ – nRBCs exposed to nRBCs in a transwell plate

RPMI – Roswell Park Memorial Institute media

RPMC – RPMI complete media

EV – extracellular vesicle

Chapter 1 - Introduction

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1.1 Malaria

1.1.1 Brief history

Malaria has posed one of the greatest threats to human existence for centuries and continues to be a pressing global health problem. In the 20th century, the parasite claimed 150-300 millions lives, accounting for approximately 2-5% of all human deaths during this time (Carter & Mendis, 2002). Malarial antigen has been detected as far back as 3200 BC in skin samples of ancient Egyptians (Miller et al., 1994). Written accounts of malaria are found throughout history, including records authored by philosophers Aristotle and Plato (Medicine, 2004). Before the discovery of the parasite, observations of fever and enlargement of the spleen were noted in patients, until Charles Louis Alphonse Lavern noticed small, crescent-shaped bodies in the blood of a febrile solider under a microscope (Medicine, 2004). After examining 192 patients also suffering from fever, he noted these shapes in 148 of these patients.

Later, Sir Ronald Ross also detected the parasite in the stomach tissue of an anopheline mosquito that had recently taken a blood meal from an infected patient, confirming that the parasite could be transferred to humans by a vector (Medicine, 2004). The various species of malaria were originally designated by the pattern of fevers observed in patients. *Plasmodium vivax* causes a tertian fever, or fever that appears every three days (Carter & Mendis, 2002). This fever pattern is also seen in *P. ovale*, as opposed to *P. malariae* and *P. falciparum* that cause quartan and subtertian fevers, respectively (Carter & Mendis, 2002). Today, *P. falciparum* and *P. vivax* are the dominant species of malaria infecting humans and are found in many parts of the world.

1.1.2. Lifecycle

Malaria relies on two hosts for survival – the human intermediate host, where asexual reproduction occurs, and the mosquito definitive host, where sexual reproduction occurs. This lifecycle is shown in Figure 1.1 (Cowman et al., 2016). The complexity of the lifecycle is due in part to the parasites ability to respond to selective pressures and adapt to different host environments (reviewed in (Aly et al., 2009)).

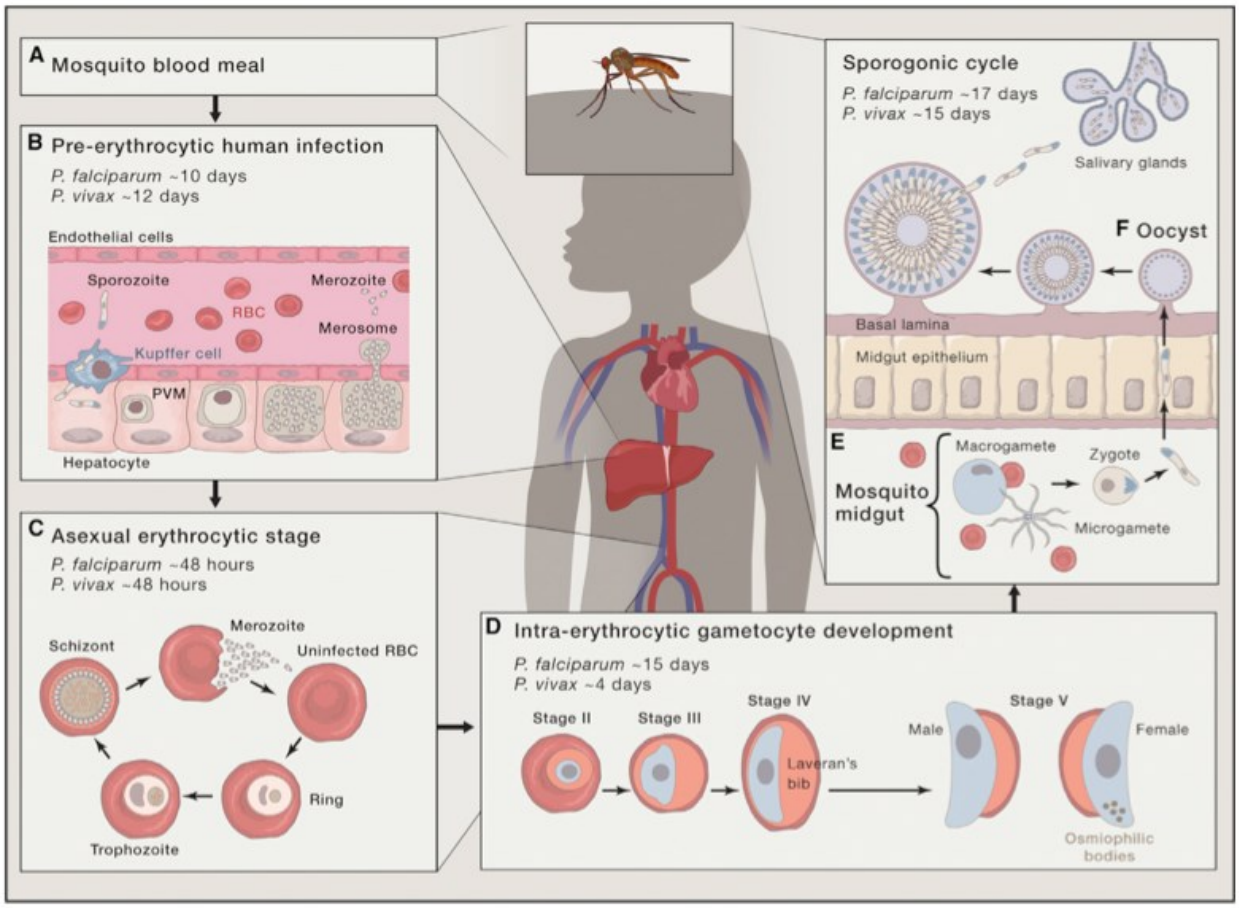

Figure 1.1 The complete lifecycle of *P. falciparum*. The lifecycle begins with infection of the human host during a blood meal (A), followed by the pre-erythrocytic stage with the invasion of hepatocytes and release of merozoites (B). Next, the asexual erythrocytic 48-hour cycle begins with invasion of RBCs by merozoites, asexual replication, and release of new merozoites for further invasion (C). During the asexual cycle, some parasites will switch to gametogenesis (D), and these gametocytes will transfer from the human host to a mosquito during a blood meal, followed by invasion of the mosquito midgut (E) (Cowman et al., 2016).

The sexual reproduction portion of the malaria lifecycle begins after a female *Anopheles* mosquito ingests gametocytes during a blood meal from an infected human host. Gametes will develop in the mosquito midgut, followed by the formation of a zygote. After meiosis, the zygote grows into an ookinete, which is motile and capable of passing through the mosquito midgut epithelium (reviewed in (Aly et al., 2009; Cowman et al., 2016)). The ookinete then attaches to the basil lamina the other side of the epithelium and transforms into an anchored oocyst (Adini & Warburg, 1999). After a series of mitotic divisions, lobes form within the oocyst plasma

membrane called sporoblasts. Circumsporozoite protein (CSP) is necessary in the development of sporoblasts, which then develop into sporozoites (Menard et al., 1997). Following oocyst rupture, sporozoites gain motility and invade the mosquito's salivary glands.

Transmission to the human host occurs when the mosquito takes a blood meal and sporozoites in the salivary glands are injected into a subcutaneous blood vessel or into a pool of blood caused by surface damage to the skin during the bite (Simonetti, 1996). Six species of *Plasmodium* can infect the human host, which are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. After the blood meal, the sporozoites travel to the liver via the bloodstream, where they infect hepatocytes by traversing endothelial cells and Kupffer cells (Tavares et al., 2013). Here, a parasitophorous vacuole membrane forms within the hepatocyte and malarial genes responsible for the liver stage of the lifecycle are expressed (reviewed in (Aly et al., 2009)). CSP, which covers the outside of the sporozoite, continues to play an important role and is required for invasion of the hepatocytes, among other important functions including sporozoite formation, development, and motility (reviewed in (Aly et al., 2009)).

After 2-10 days in the liver, thousands of merozoites will develop within the hepatocyte, which are released into circulation and quickly invade red blood cells (RBCs), relying on the merozoite surface protein 1 (MSP1) to bind RBCs and mediate invasion (reviewed in (Cowman et al., 2016)). This triggers the deformation of the RBC, allowing for the RBC membrane to wrap around the merozoite. The merozoite then reorientates such that the apical end faces the RBC (reviewed in (Cowman et al., 2016)). The attachment is strengthened by apical membrane antigen-1 (AMA1) and the rhoptry neck protein (RON) complex (reviewed in (Besteiro et al.,

2011)). The merozoite pushes into the RBC and a parasitophorous vacuole forms, fusing behind the merozoite to trap the merozoite within the RBC (reviewed in (Cowman et al., 2016)).

Next, in a *P. falciparum* infection, a 48-hour asexual lifecycle begins known as schizogony, where merozoites develop into the ring stage which then mature into trophozoites. As trophozoites, the parasite will digest hemoglobin within the RBC for nutrition and produce the by-product heme, which is highly toxic and cannot be digested (Goldberg et al., 1990). To neutralize heme, it is converted to hemozoin, a malarial pigment that can be seen under the microscope as dark brown bodies during the trophozoite stage (Goldberg et al., 1991). Trophozoites then undergo DNA replication and nuclear division, forming clusters of merozoites called schizonts. After RBC rupture, daughter merozoites are released to infect new RBCs. During this process, some parasites will undergo sexual development into gametocytes. This is stimulated by cues in the parasite's environment that indicate that the host may no longer be a suitable environment, including treatment with antimalarials or unfavourably high parasitemia (reviewed in (Cowman et al., 2016)). Gametocytes will enter peripheral circulation, where they can be ingested by a mosquito when taking a blood meal, beginning the sexual reproduction portion of the lifecycle again.

Plasmodium species have additional adaptations for surviving in the human host. Instead of forming schizonts, *P. vivax* and *P. ovale* can enter the dormant hypnozoite stage, which allows the parasite to survive for extended periods in the host's liver (reviewed in (Venugopal et al., 2020)). The parasites may stay in this stage for weeks, months, or even years, followed by a relapse (Hulden & Hulden, 2011). In the erythrocytic stage, *P. vivax* exclusively invades

reticulocytes (immature erythrocytes), mediated by proteins belonging to the *P. vivax* Reticulocyte Binding Protein (PvRBP) family (reviewed in (Chan et al., 2020)).

1.1.3. Global burden and transmission of malaria

Despite decades of research and scientific advancements, malaria remains the deadliest human parasitic disease. Globally, 87 countries are considered endemic for malaria (WHO, 2020). In 2019, a decrease in cases was reported, compared to statistics from 2000. Case numbers have decreased from 238 million in 2000 to 229 million in 2019 (WHO, 2020). The bulk of cases in 2019 were found in the African Region, which accounts for an estimated 215 million cases or 94% of total worldwide cases (WHO, 2020). Deaths attributable to malaria have also reduced since 2000, decreasing from 736,000 deaths per year in 2000 to 409,000 in 2019 (WHO, 2020). This includes a decrease in deaths in children under five years of age, from 84% of deaths attributable to malaria in 2000, to 67% in 2019 (WHO, 2020). Despite these decreases in cases since 2000, malaria eradication has since plateaued, with reports beginning in 2017 stating that numerous countries were off-track to reach their eradication goals (WHO, 2017). Today, eradication is further challenged by additional threats to global health, including the COVID-19 pandemic (WHO, 2020).

P. falciparum accounts for the majority of malaria cases globally. In 2019, *P. falciparum* made up >99% of cases in the African Region, 53% of cases in the South-East Asia Region, 73% of cases in the Eastern Mediterranean region, and 68% of cases in the Western Pacific Region (WHO, 2020). In contrast, *P. vivax* is the predominant species in South America, accounting for

approximately 76% of cases here, while *P. falciparum* and mixed infections together accounted for approximately 24% of malaria cases in this region (WHO, 2020). Figures 1.2 and 1.3 show the *P. falciparum* and *P. vivax* 2017 global incidence rates, respectively.

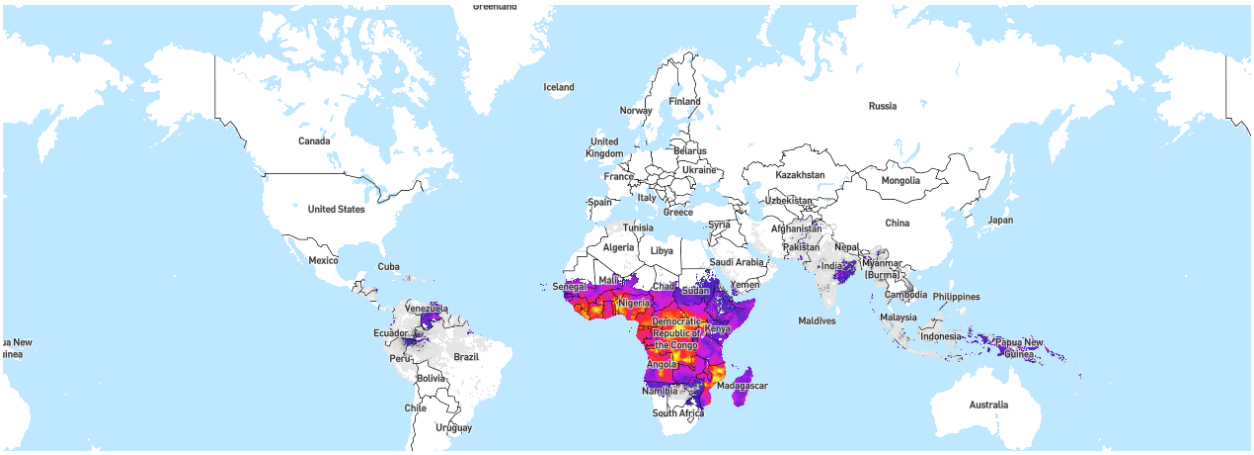

Figure 1.2. Global incidence of *P. falciparum* in 2017. Malaria Atlas Project.

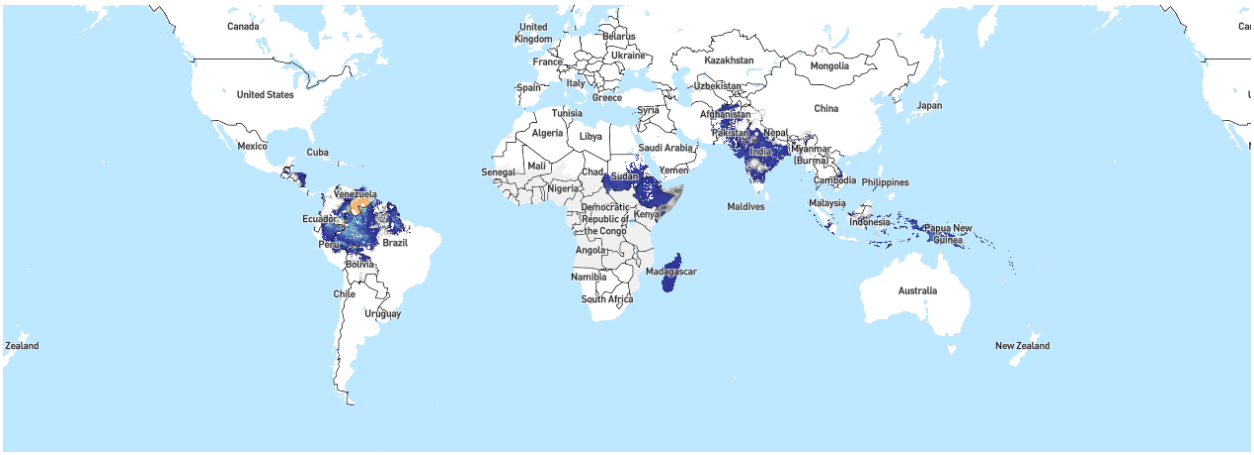

Figure 1.3. Global incidence of *P. vivax* in 2017. MAP.

Malaria transmission can be defined as high (≥ 35% *P. falciparum* parasite rate (PfPR) or ≥ 450 cases per 1000 individuals in a population annual parasite incidence (API)), moderate (10-

35% PfPR or 250-450 API) or low (1-10% PfPR or 100-250 API) (WHO, 2018). Transmission is categorized as stable when little change in incidence occurs over years; for example, stable transmission is observed in areas of the sub-Saharan African region (WHO, 2019). In contrast, unstable transmission occurs when large variations in malaria incidence is observed between seasons and years (WHO, 2019). Transmission is influenced by several factors and affects the rates of malaria cases globally. Transmission rates may change with changes in the prevalence of parasite strains in an area, vector behaviour, migration, climate, and economic development and changes in infrastructure (reviewed in (Castro, 2017)). Transmission rates may decline when efforts to reduce poverty are increased and are effective (WHO, 2020). The WHO 2019 malaria report stated that from 2000 to 2019, no area that had previously been declared malaria free reported instances of new transmission (WHO, 2020).

1.1.4. Clinical presentation of malaria

During a malaria infection, symptoms arise from the obligate rupturing of infected RBCs (iRBCs), leading to fever, headache, and sweating (reviewed in (Trampuz et al., 2003)). Other symptoms can include vomiting, diarrhea, and jaundice (reviewed in (Laishram et al., 2012)). Recovery is likely with prompt treatment of the disease with antimalarials. If a patient living in an endemic area survives, this and additional exposures to malaria may give rise to immunity, potentially reducing the severity of symptoms in future infections (reviewed in (Doolan et al., 2009)). Thus, children under five years of age living in high transmission areas are most at risk of developing severe symptoms and of death, but may only experience asymptomatic infections by adulthood (reviewed in (Doolan et al., 2009)). If a malaria infection is left untreated, the

disease may progress to severe malaria, which is almost exclusively caused by the most virulent species, *P. falciparum*. Severe malaria symptoms include anemia, acute renal failure, and cerebral malaria (reviewed in (Trampuz et al., 2003)). Individuals over 65 years of age, lacking previous immunity, or who are pregnant are at increased risk for developing severe malaria, which may be exacerbated by delayed treatment, co-infection or a co-existing medical conditions (reviewed in (Trampuz et al., 2003)).

Asymptomatic malaria is typically defined as the absence of symptoms despite parasitemia detected in a patient's blood smear (reviewed in (Kimenyi et al., 2019)). This is further complicated by the presence of submicroscopic infections (SMIs), where parasites cannot be detected using the typical microscopy methods, but instead require genetic amplification techniques to detect parasite DNA in the blood (reviewed in (Kimenyi et al., 2019)). Thus, submicroscopic infections often go undetected and untreated. This hinders the assessments of global malaria burden and the development of treatment strategies.

1.1.5. Treatment of malaria

To treat malaria, various compounds have been used throughout history, notably quinine, mepacrine, and chloroquine (reviewed in (Butler et al., 2010)). Quinine, found in cinchona tree bark, became popular in the 1800s, and in 1932 an alternative version, mepacrine, was produced. Chloroquine was later developed by American scientists, based off of another antimalarial drug called Sonotquine, which was developed in Germany (reviewed in (Butler et al., 2010)). Unfortunately, resistance to chloroquine has since widely spread and significantly decreased the

efficacy of the drug worldwide. Its replacement, artemisinin, was developed by Chinese scientists from herbs and is highly effective (reviewed in (Butler et al., 2010)). Today, in efforts to prevent resistance to artemisinin, WHO recommends artemisinin-combination therapy (ACT) in most cases of *P. falciparum*, where artemisinin is combined with other antimalarials, including sulphadoxine-pyrimethamine (SP) (WHO, 2021). ACTs are also used to treat *P. vivax*, but chloroquine may still be used in areas where *P. vivax* has not developed resistance (Baird et al., 2016).

Pregnancy offers a new challenge for malaria treatment. Artemisinin is not widely accepted to be safe for use in pregnancy-associated malaria (PAM), as demonstrated in animal studies where artemisinins were shown to be embryotoxic and teratogenic (reviewed in (Clark, 2009)). Other treatment options are available, but quick diagnosis and treatment are essential for preventing poor pregnancy outcomes. Malaria prevention is often included in regular antenatal care in areas of high transmission. The current prophylactic course in PAM is intermittent preventative treatment with a single dose of SP per month (IPTp-SP) (WHO, 2021). In addition to antimalarials, it is also recommended that pregnant women sleep under insecticide-treated bed nets (ITNs) to prevent infectious mosquito bites at night. Folate consumption may also be restricted, as it is essential for the growth of the parasite, but this must be balanced with the mother's folate nutritional needs for a healthy pregnancy (reviewed in (Nzila et al., 2014)).

1.1.6 Humoral immunity in uncomplicated malaria

In uncomplicated *P. falciparum* malaria (infection without additional complications, including cerebral, severe, and pregnancy-associated malaria), the parasite expresses variant surface antigens (VSAs) on the surface of iRBCs to attach and sequester in capillaries and venules in various locations of the body (Miller, 1969). These VSAs belong to three known gene families, *P. falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1), Subtelomeric Variable Open Reading frame (STEVOR) proteins, and repetitive interspersed family (RIFIN) proteins (reviewed in (Lee et al., 2019)). Adherence to capillaries can affect blood flow and contributes to patient morbidity (Spitz, 1946). The clearance function of the spleen acts as a selective pressure for the parasite to bind to tissues and sequester, avoiding this clearance (David et al., 1983). In response, antibodies against VSAs can develop during infection to block sequestration, with levels usually correlating with age and reflecting infection history in endemic areas (reviewed in (Doolan et al., 2009)). There is some understanding of the specificity of different antibody isotypes and subclasses that contribute to the overall immune response. The IgG subclasses can be divided into having cytophilic or non-cytophilic activity. IgG2 and IgG4 are both non-cytophilic and thought to be non-protective in response to a malaria infection (Bouharoun-Tayoun & Druilhe, 1992; Ndungu et al., 2002). IgG1 and IgG3 are cytophilic subclasses and can interact with Fc receptors on other immune cells, including monocytes and macrophages to promote opsonic phagocytosis (Tebo et al., 2002). Leoratti et al showed levels (and even avidity) of IgG1 and IgG3 were significantly higher in individuals with a history of more than one malaria infection (Leoratti et al., 2008).

1.2 Pregnancy-associated malaria

1.2.1. Burden of PAM

In Africa, pregnant women are especially vulnerable to malaria compared to non-pregnant adults (McGregor, 1984) and it was previously reported that globally there were nearly 125 million pregnancies at risk for complications from PAM (Dellicour et al., 2010). In 2019, 12 million pregnant women in sub-Saharan Africa were at risk of PAM (WHO, 2020). PAM is responsible for poor fetal and maternal outcomes including spontaneous abortion, low birthweight infants (LBW, <2,500 g), premature births (PTBs, <37 weeks), and accounts for nearly 200,000 newborn deaths each year (Moore et al., 2017; Steketee et al., 2001). In 2019, it is estimated that instances of PAM were responsible for 822,000 low birthweight infants (WHO, 2020). PAM also contributes significantly to severe maternal anemia (Brabin & Rogerson, 2001), leading to approximately 10,000 maternal deaths each year (Guyatt & Snow, 2001). During a *P. falciparum* infection in high transmission settings, primigravid women have an especially high risk of poor pregnancy outcomes, but this risk decreases with gravidity and with increased exposure to VSAs expressed by the parasite specifically in pregnancy (reviewed in (Ataide et al., 2014)).

1.2.2. Placental sequestration

Sequestration of iRBCs in the placenta is mediated by VSAs expressed on the surface of iRBCs, namely VAR2CSA, which is a member of the *Pf*EMP1 family (Salanti et al., 2003). This

expression of VAR2CSA is believed to be limited to pregnancy, as VAR2CSA specifically binds chondroitin sulfate A (CSA) on the surface of the syncytiotrophoblast and in the intervillous spaces of the placenta (Salanti et al., 2003). VAR2CSA is a large 350 kD transmembrane protein made up of six Duffy-binding-like (DBL) domains (Srivastava et al., 2011). The attachment to CSA is mediated by the core domain, where two binding channels for CSA are located (Ma et al., 2021). Figure 1.4 shows the arrangement of the individual domains and the binding channels of CSA (Ma et al., 2021). The binding of CSA and thus sequestration in the placenta contributes to poor pregnancy outcomes (reviewed in (Steketee et al., 2001)), reduces clearance of iRBCs by the spleen (David et al., 1983; Fried & Duffy, 1996), and promotes dangerous inflammation in the placenta (Kabyemela et al., 2008; Rogerson SJ, 2007; Sharma & Shukla, 2017). Elevated levels of IL-10 are a predictor for placental malaria infection and placental inflammation and has also correlated with instances of maternal anemia (Hountohotegbe et al., 2020; Kabyemela et al., 2008).

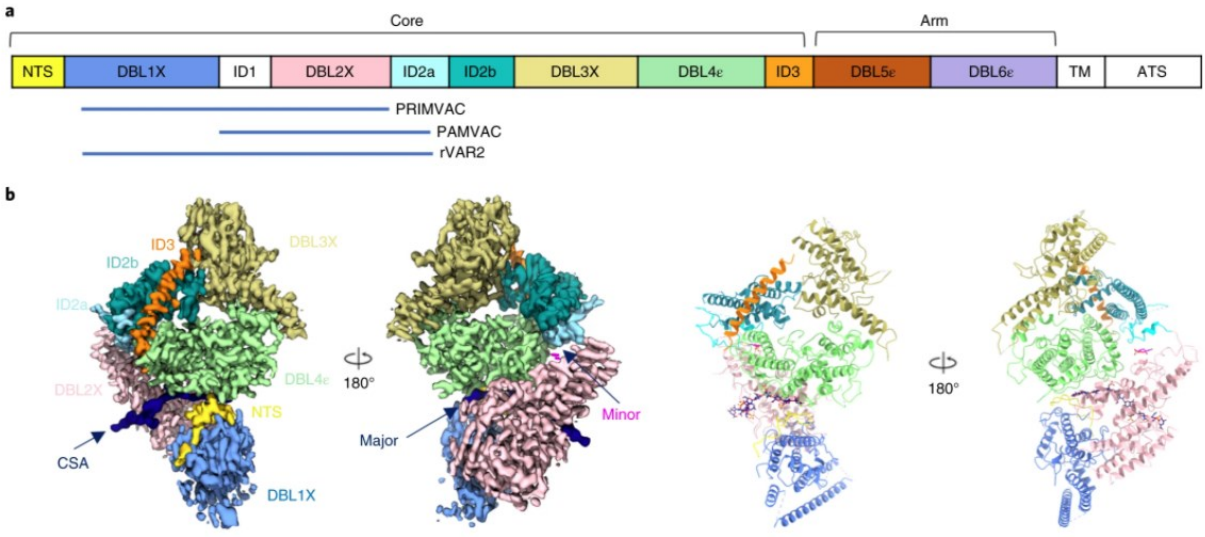

Figure 1.4. The structure of VAR2CSA and binding site of CSA. VAR2CSA (NF54 strain shown here) is made up of various sub-domains, shown in (A) with the targets of the PAM vaccines PRIMVAC and PAMVAC highlighted. Two views of the core structure of VAR2CSA (B) shown as cryo-electron microscopy structures with CSA binding site shown (left) and atomic models (right), which match the domain coloring in (A). Modified from (Ma et al., 2021).

1.2.3. Humoral immunity in PAM

In PAM, pregnant women can acquire antibodies against unique VSA expressed in pregnancy (VSA_{PAM}) as gravidity increases. VSA_{PAM} antibodies can disrupt the adherence of iRBCs to CSA in the placenta and prevent parasite sequestration here (Fried & Duffy, 1996; Ricke et al., 2000). VAR2SA antibodies are associated with improved pregnancy outcomes (Salanti et al., 2004) and as a result, are the focus of vaccines to prevent PAM (Mordmuller et al., 2019; Sirima et al., 2020). Other antibody-mediated immune responses such as opsonic phagocytosis also have potential to reduce placental parasitemia and promote iRBC clearance by the spleen (Ataide et al., 2011; Feng et al., 2009).

As multigravid women are more likely to have protective antibodies than primigravid women and thus experience better pregnancy outcomes, this suggests that VSA_{PAM} antibodies are maintained into the next pregnancy. Staalsoe et al found that by six months postpartum, levels of VSA_{PAM} IgG levels had decreased, but levels were boosted in women by the second trimester of their next pregnancy (Staalsoe et al., 2001). However, follow-up of women from one pregnancy into the next is challenging and this study only included six women. In Thailand, evidence of antibody boosting during pregnancy showed that the humoral response could be maintained and expanded (Fowkes et al., 2012). Pregnant women were followed from enrollment to delivery and regular testing of antibody levels was performed, showing substantial fluctuation in antibody levels between blood samples collected from pregnant women enrolled in the study. Compared to uninfected controls who showed little level change, these fluctuations suggested

parasites boosted antibody responses throughout pregnancy. Based on modeling, VAR2CSA antibodies are estimated to persist over many years (Fowkes et al., 2012).

The IgG1 and IgG3 subclasses are the most likely responders of the humoral response in PAM (Elliott et al., 2005; Nasr et al., 2013; Tornyigah et al., 2021). In Cameroon, levels of VSA_{PAM} IgG1 and IgG3 were significantly increased in pregnant women compared to unexposed controls, whereas levels of VSA_{PAM} IgG2 and IgG4 were not significantly higher (Megnekou et al., 2005). Levels of cytophilic antibodies also correlated with VSA_{PAM} total IgG from Malawian donors (Elliott et al., 2005). Furthermore, there was a significant correlation between the levels of VSA_{PAM} IgG1 and IgG3 and the ability of serum to block parasite adhesion to CSA ($r = -0.65$ and $r = -0.4$ respectively), suggesting that these subclasses of VSA_{PAM} antibodies are protective (Elliott et al., 2005).

In Benin, IgG1 and IgG3 were also found to be the dominant subclass among pregnant women, while low levels of IgG2 and IgG4 were observed (Tornyigah et al., 2021). Lower levels of IgG3 were associated with lower birthweight infants, compared to IgG3 levels of women who delivered infants of a healthy birthweight (Tornyigah et al., 2021). Despite overall low levels of IgG4 in the study, IgG4 levels were higher in women with a history of SMIs during pregnancy (Tornyigah et al., 2021). This may reflect a regulatory immune response to SMIs, as regulatory cytokines IL-10 and TGF-ß were also elevated (Tornyigah et al., 2021). A similar immune response is triggered in chronic parasite infections outside of the PAM setting (reviewed in (McSorley & Maizels, 2012)); thus, SMIs in PAM may promote this type of immune response. Notably, from study enrollment to delivery, overall total IgG decreased (Tornyigah et al., 2021).

Less is known about the roles of IgE and IgM in PAM. In placentas collected from women in Malawi, there was a negative correlation between placental parasitemia and deposition of IgE in fetal blood vessels (Maeno et al., 1993). There are mixed observations for IgM in immunity during PAM. This isotype was detected in placental blood samples in PAM (Maeno et al., 1993), but several studies observed low levels or no difference in IgM levels between blood samples collected from infected pregnant women and non-infected controls (Elliott et al., 2005; Leoratti et al., 2008; Serra-Casas et al., 2010). In addition, IgM can bind non-specifically to iRBCs and block the subsequent binding of VSA_{PAM} IgG in, leading to decreased phagocytosis of iRBCs (Barfod et al., 2011). Further investigation is required to characterize this isotype.

1.2.4. Modulators of VSA_{PAM} antibodies

IPTp treatment during pregnancy has the potential to prevent the acquisition of protective antibodies to PAM, as parasitemia may be reduced or cleared to the point that plasma cells will not produce antibodies against VSA_{PAM} targets. This may lead to increased risk of morbidity and mortality in subsequent pregnancies. In Cameroon, a decrease in VSA_{PAM} antibodies was observed in the second trimester in women on chemoprophylaxis (Staalsoe et al., 2001). An inverse correlation was also noted between VSA_{PAM} IgG levels at delivery and placental parasitemia among women who had placental malaria. In Ghana, antibody levels against a crude antigen from whole blood-stage parasites were decreased at delivery in women treated with IPTp (Stephens et al., 2017).

An HIV co-infection may also affect the development and maintenance of VSA_{PAM} antibodies. In Kenya, plasma samples were collected from primigravid and multigravid women, and separated into HIV positive and negative groups (Keen et al., 2007). In samples from the HIV positive multigravid women, opsonic phagocytosis activity against iRBCs was significantly reduced compared to samples from the HIV negative women. Similar results were observed in studies from Malawi (Ataide et al., 2010; Jaworowski et al., 2009).

1.2.5. Submicroscopic PAM

SMIs are frequently observed in pregnancy and risk being undetected and untreated if only the typical microscopy detection methods are available in an area (reviewed in (Kattenberg et al., 2011). A study in Benin found that younger mothers with a history of infection prior to pregnancy have a greater risk of developing both SMI and microscopic infections of *P. falciparum* during pregnancy (Hounkonnou et al., 2020). SMIs have been shown to have mixed associations with pregnancy outcomes. Specifically with maternal anemia, there is both evidence for an association between SMI (Cohee et al., 2014; Cottrell et al., 2015; Mayor et al., 2012) and microscopic infection history (Unger et al., 2019) and maternal anemia. In Colombia, a study previously conducted by our laboratory revealed a protective association between antibodies that could block adhesion of iRBCs to CSA *in vitro* collected from pregnant women who developed SMIs at some point in pregnancy and maternal anemia (Gavina et al., 2018). The mechanisms of protection from maternal anemia by these blocking antibodies are unknown.

1.3 Anemia

1.3.1. Causes of anemia in pregnancy

During the course of a healthy pregnancy, the maternal vasculature must vasodilate approximately 5 weeks into pregnancy and total blood volume and red cell mass are significantly increased (Chapman et al., 1998). This hemodilution creates a normal physiological anemia (reviewed in (Sanghavi & Rutherford, 2014)). Anemia is typically defined as a hemoglobin level less than 11 g/dL, with mild and severe anemia for pregnant women defined at 10-10.9 g/dL and less than 7 g/dL, respectively (WHO, 2011). Anemia severity in pregnancy may be propagated by deficiencies in iron, vitamin B2, and folate (reviewed in (Agbozo et al., 2020)). Iron deficiency in particular is prevalent in developing companies and a significant contributor to maternal anemia (reviewed in (Abu-Ouf & Jan, 2015)). Iron deficiency severity may be affected by malnutrition, blood loss due to injury or disease, and socio-economic status (reviewed in (Abu-Ouf & Jan, 2015)). In more severe cases, a mother may develop hemolytic anemia leading to multi-organ failure, aplastic anemia due to bone marrow dysfunction, or sickle cell anemia. Other causes of anemia in pregnancy include micronutrient deficiencies, genetic factors, and chronic inflammation (reviewed in (Cao & O'Brien, 2013)).

1.3.2. Causes of anemia in uncomplicated malaria

Anemia is a common consequence of malaria infection, with severity increasing in settings with restricted access to medical care and treatment, high prevalence of nutritional

deficiencies, and high probability of infection with other pathogens, particularly helminths (reviewed in (Ghosh & Ghosh, 2007; White, 2018)). Key predictors of anemia in an endemic area are malaria transmission intensity and biting patterns of mosquitoes (reviewed in (White, 2018)). In high transmission settings, young children with little immunity to malaria often exhibit the most severe anemia, especially when combined with malnutrition (Ehrhardt et al., 2006), but children who survive to adulthood will likely be asymptomatic in subsequent infections and have low risk of developing severe anemia as adults (reviewed in (White, 2018)). In low transmission settings, the risk of developing anemia due to malaria infection is similar among age groups (reviewed in (White, 2018)).

During a *P. falciparum* infection, there are several pathways that can lead to anemia. First, obligate red cell invasion and destruction occurs as the parasite asexually replicates in the host RBCs. However, a more significant contributor to red cell loss is the reduction in non-parasitized RBCs (nRBCs) (reviewed in (Totino et al., 2016)). Slowed rates of erythropoiesis due to a malaria infection can result in an overall older red cell population that is more susceptible to lysis (Looareesuwan et al., 1991). Also, the stability of the red cell membrane is altered during a malaria infection such that red cell deformability is reduced, which has been correlated with lower hemoglobin concentrations (Dondorp et al., 1999; Looareesuwan et al., 1991). This leads to increased RBC clearance by the spleen, as splenic thresholds for clearance are lowered during a malaria infection as the spleen enlarges (Looareesuwan et al., 1987). The deposition of malarial antigens on non-infected RBCs (nRBCs) may also be a contributor to anemia, leading to hemolysis of nRBCs (Omodeo-Sale et al., 2005) and phagocytosis of erythroid precursor cells (Layez et al., 2005). Finally, the expression of phosphatidylserine (PS) on the surface of nRBCs

may be targeted by antibody-independent phagocytosis (McGilvray et al., 2000), as the phagocytic receptor CD36 can recognize PS and induce engulfment (Greenberg et al., 2006).

Common to most pro-inflammatory disease states, cytokine dysregulation can significantly contribute to both acute and chronic inflammation, which also contributes to anemia. During malaria infections, pro-inflammatory markers such as IFNγ, TNFα, and IL-6 are elevated (reviewed in (Ghosh & Ghosh, 2007; White, 2018)). Notably, a reduced level of IL-10 and IL-12, both anti-inflammatory cytokines, are often observed, especially in severe anemia, which points to dysregulation of the pro-inflammatory immune response (Perkins et al., 2000). Elevated pro-inflammatory markers can inhibit erythropoiesis, leading to bone marrow dysfunction (reviewed in (Perkins et al., 2011)). Bone marrow dysfunction due to a malaria infection is correlated with fatal anemia in mice, where an underproduction of the important regulatory cytokine IL-12 was also observed (Mohan & Stevenson, 1998). IL-12 is also a hemopoietic growth factor that is believed to be a key regulator in response to malaria in mice. In B6 mice infected with *P. chabaudi*, low levels of IL-12 were associated with significantly lower rates of bone marrow and splenic erythropoiesis, along with severe anemia (Mohan & Stevenson, 1998).

1.3.3. Causes of anemia in PAM

Pregnancy adds another layer of complexity when determining the role of malaria in the development of anemia. As the body is already experiencing hemodilution, severe acute anemia due to a malaria infection may result in maternal morbidity and mortality as the red cell

population becomes significantly compromised. Some mechanisms of anemia are shared between uncomplicated malaria and PAM, such as obligate hemolysis as the parasite continues its lifecycle, which can trigger a pro-inflammatory response in the placenta. iRBC sequestration in the placenta is known to recruit immune cells to the placenta, including monocytes (Ordi et al., 1998; Rogerson et al., 2003). As a profound loss of nRBCs is known to occur in uncomplicated malaria (Jakeman et al., 1999), it is likely that this also occurs in PAM, though the mechanisms specific to PAM are yet to be revealed. Figure 1.5 outlines several mechanisms that may lead to malaria-attributable maternal anemia.

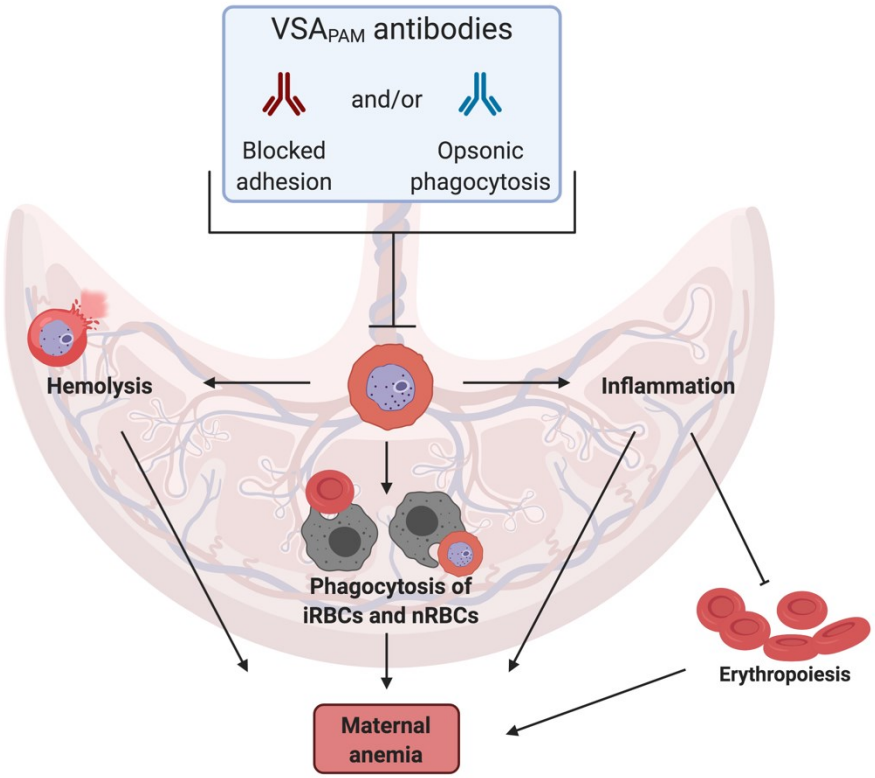

Figure 1.5. Potential mechanisms of antibody-mediated protection from maternal anemia in PAM. At the trophozoite stage, the parasite expresses the surface antigen VAR2CSA, which adheres to chondroitin sulfate A in the placenta. Parasite material is detected by TLRs present on cells in the intervillous space after obligate hemolysis of infected cells during the lifecycle, activating a Th1 (pro-inflammatory) response. Increased inflammation, along with several other factors, can contribute to the clearance of uninfected RBCs, increasing the risk of maternal anemia. Inflammation can also lead to dysregulated erythropoiesis in the bone marrow. However, after multiple instances of PAM, protective VSA_{PAM} antibodies may be acquired that can prevent these mechanisms, including blocking antibodies, opsonizing antibodies, or antibodies that may have multiple functions (created with BioRender.com).

Several unique features of pregnancy may contribute to maternal anemia. As good nutritional health is important for normal pregnancy, a deficiency caused or exacerbated by malaria can contribute to poor pregnancy outcomes. Iron or folate deficiencies may be further exacerbated by the utilization of hemoglobin by the parasite and the body's response by locking iron stores in immune cells, leading to hypoferrimia (reviewed in (Ghosh & Ghosh, 2007)). Folic acid is often administered at the beginning of pregnancy to assist the mother in meeting the increasing demands of the growing fetus and placenta. However, these supplements of folic acid may interfere with the effectiveness of IPTp by stimulating parasite growth (Moya-Alvarez et al., 2018). Fortunately, recent findings from a study in Benin showed that higher folate levels did not increase the risk of malaria or increase parasite density, but supplementation in pregnancy correlated with protection from anemia (Moya-Alvarez et al., 2018).

In pregnancy, the elevation of pro-inflammatory mediators due to infection can contribute to poor pregnancy outcomes, including maternal anemia, as the pregnancy requires a regulatory immune phenotype to avoid rejection of the fetus. In PAM, an inflammatory immune response begins with the detection of malaria parasite products by several Toll-like receptors (TLRs) on trophoblasts in the placenta (Abrahams & Mor, 2005). Detection by TLR2 (Krishnegowda et al., 2005), TLR4 (Krishnegowda et al., 2005), and TLR9 (Parroche et al., 2007) during PAM can lead to increased release of a number of pro-inflammatory intercellular messengers, promoting a T helper (Th) 1 response. This is appropriate for defense against malaria (reviewed in (Wegmann et al., 1993)), but may risk the maintenance of a healthy pregnancy (reviewed in (Robertson et al., 1994)). Tolerance to the developing fetus must be maintained with T helper (Th) 2 cytokines, which have been detected in both murine pregnancies (Lin et al., 1993) and in the human

placenta (de Moraes-Pinto et al., 1997). A transition to a Th1 state can risk the health of the mother and fetus.

In PAM, elevated TNFα, IFNγ, and IL-2 levels were elevated in the placentas of infected pregnant women, while levels of Th2 cytokines TGF-β, IL-4, and IL-6 were similar between infected and uninfected groups (Fried, Muga, et al., 1998). In primigravid women, low birthweight was associated with significantly higher levels of IFNγ and TNFα, and increased TNFα levels were associated with maternal anemia (Fried, Muga, et al., 1998). Moormann et al also detected increased TNFα and decreased TGF-β levels in infected placentas, compared to uninfected controls (Moormann et al., 1999). These cytokines can also be produced by phagocytes that engulf hemozoin pigment in the placenta and amongst fibrin deposits in the intervillous space (Walter et al., 1982). Both pro-inflammatory cytokines TNFα and IL-8 were produced by macrophages containing hemozoin, and these cytokine secretions were associated with intrauterine growth restriction (Moormann et al., 1999). Taken together, this shift in cytokine production to a pro-inflammatory response could contribute to dangerous inflammation in the placenta, contributing to maternal anemia, as illustrated in Figure 1.5.

1.4 Associations between VSA_{PAM} antibodies and maternal anemia

1.4.1 Summary of previous literature

The role of protective VSA_{PAM} antibodies must be characterised and fully understood, as these antibodies should be elicited during vaccine development for PAM. There is limited knowledge on associations between VSA_{PAM} antibodies and maternal anemia, with mixed evidence on whether these antibodies can protect women from anemia, as shown in Table 1.1.

Table 1.1 Summary of studies on VSA$_{PAM}$ antibodies and maternal anemia

Citation	Setting	Assays used	Time of blood collection	Anemia-related outcomes
No association between VSA$_{PAM}$ antibodies and maternal anemia				
Duffy & Fried, 2003	Kenya	IBA	Delivery	VSA$_{PAM}$ blocking antibodies were not associated with maternal hemoglobin at delivery
Ndam et al., 2015	Benin	ELISA, IBA	Delivery	VSA$_{PAM}$ blocking antibodies were not associated with maternal anemia at delivery
Ataide et al., 2010	Malawi	Flow, opsonic phagocytosis	Late third trimester	VSA$_{PAM}$ antibodies from primigravid women were not associated with maternal hemoglobin
Ataide et al., 2011	Malawi	Flow, opsonic phagocytosis	Late third trimester	VSA$_{PAM}$ antibodies from secundigravid women were not associated with maternal hemoglobin
Mayor et al., 2013	Mozambique	ELISA, Flow	Delivery	VSA$_{PAM}$ antibodies were not associated with maternal anemia
Ndam et al., 2006	Senegal	ELISA	First to second trimester and delivery	Seroreactive antibodies against VAR2CSA subdomains were not associated with maternal anemia
Serra-Casas et al., 2010	Mozambique	Flow	Delivery	VSA$_{PAM}$ antibodies were not associated with maternal anemia
Aitken et al., 2010	Malawi	Flow	28-34 weeks gestation	VSA$_{PAM}$ antibodies were not associated with maternal hemoglobin
Fried et al., 2018	Mali	Multiplex	Second trimester	Seroreactive antibodies against VAR2CSA subdomains were not correlated with a reduced risk of maternal anemia
Mixed association between VSA$_{PAM}$ antibodies and maternal anemia				
Lloyd et al., 2018	Cameroon	Multiplex	Delivery	Seroreactive antibodies against VAR2CSA were associated with lower hematocrit levels and increased prevalence of anemia in women negative for placental infection
Staalsoe et al., 2004	Kenya	Flow	Delivery	Levels of VSA$_{PAM}$ antibodies correlated with hemoglobin in women with chronic placental infection but not acute or past infection
Association between VSA$_{PAM}$ antibodies and maternal anemia				
Feng et al., 2009	Malawi	Opsonic phagocytosis	14-20 weeks gestation	Two-fold increase in opsonic phagocytosis index was associated with reduced odds of maternal anemia
Jaworowski et al, 2009	Malawi	Opsonic phagocytosis	Third trimester	VSA$_{PAM}$ antibodies from anemic women displayed significantly lower opsonizing activity compared to non-anemic women
Chandrasiri et al., 2016	Malawi	Flow, opsonic phagocytosis	Before 20 weeks gestation	10% increase in opsonic phagocytosis index was associated with maternal hemoglobin increase of 0.4 g/L at 36 weeks gestation
Sander et al., 2011	Cameroon	Detection of *var2csa* copy number	First, second, and third trimester	In Ngali II, increasing *var2csa* copy number, which correlated with antibodies against DBL4ε, was associated with higher maternal hemoglobin
Chandrasiri et al., 2014	Sudan	Flow, opsonic phagocytosis, cytokine profiles	Second and third trimester	DBL5ε antibodies and maternal hemoglobin were negatively correlated with pro-inflammatory cytokines; opsonizing antibodies were positively associated with hemoglobin
Gavina et al., 2018	Colombia	ELISA, IBA	First and second trimester	VSA$_{PAM}$ blocking antibodies were positively associated with maternal hemoglobin

1.4.2. Studies with no association between VSA_{PAM} antibodies and maternal anemia

In the first study to relate measures of VSA_{PAM} antibodies to birth outcomes, plasma from Kenyan pregnant mothers was tested in the inhibition of binding assay (IBA) with placental isolates and correlated to infant birthweight, gestational age, and maternal hemoglobin at delivery (Duffy & Fried, 2003). Plasma that blocked >35% of parasite binding to CSA was considered positive for blocking antibodies. Such antibodies were only observed in 1 of 47 primigravid women, while in secondigravid women, anti-adhesion activity was greater and associated with both increased infant birthweight and gestational age. However, there was no relationship between anti-adhesion antibodies and maternal hemoglobin levels (Duffy & Fried, 2003).

Similar associations were also observed in a study in Benin, where antibody levels were measured against the recombinant proteins DBL1-DBL2X, DBL5ε, and DBL6ε by ELISA, and functional activity was assessed by IBA (Ndam et al., 2015). Increased anti-adhesion activity was associated with a decreased risk of placental infection at delivery and low birthweight, but there was no association between antibody levels or anti-adhesion activity and maternal anemia at delivery (Ndam et al., 2015).

In primigravid women from Malawi, serum samples were collected in the late third trimester and tested for reactivity to CS2 parasites by flow cytometry and opsonic phagocytosis (Ataide et al., 2010). No association was observed with maternal anemia or infant birthweight and VSA_{PAM} antibodies. The study was continued with third trimester samples from

secundigravid women (Ataide et al., 2011). Again, no association was observed between maternal anemia and VSA_{PAM} IgG or opsonic activity, but a positive correlation between opsonic phagocytosis and infant birthweight was observed.

Other studies compared maternal hemoglobin measurements to IgG levels but not to antibody function. In Mozambique, serum samples collected at delivery were tested by ELISA for reactivity against several malarial antigens expressed in uncomplicated malaria and during PAM, including DBL5ε and DBL6ε, along with reactivity to two placental isolates (Mayor et al., 2013). VSA_{PAM} IgG against one isolate was associated with increased birthweight and gestational age of infants, but no association was found with maternal anemia. In Senegal, IgG was measured against the VAR2CSA subdomains DBL1X, DBL5ε, and DBL6ε by ELISA and compared with maternal anemia at enrollment and delivery in samples from primigravid, secondigravid, and multigravid women (Tuikue Ndam et al., 2006). IgG levels against DBL5ε and DBL6ε at enrollment and delivery were parity-dependent, and high IgG levels correlated with past malaria infection rather than acute PAM. In this study, there was no association between the levels of antibodies against these VAR2CSA domains and maternal anemia or birthweight at any measured time point during pregnancy. Similarly, in a study from Mozambique, the levels of antibody that stained the native VSA on CS2 parasites in flow cytometry did not associate with maternal anemia at delivery (Serra-Casas et al., 2010). These antibodies did not correlate with other birth outcomes either, including preterm birth or low birthweight.

Two longitudinal studies reported VSA_{PAM} antibody and hemoglobin levels throughout pregnancy, and both failed to identify a correlation between antibody levels at enrollment and pregnancy outcomes. The first study reported IgG levels in pregnant Malawian women from serum collected at various points in pregnancy including study enrollment, 24-28 gestational weeks, and 1-6 months postpartum (Aitken et al., 2010). There was a non-significant downward trend in antibody levels against VSA-expressing parasites CS2 and HCS3-VSA from enrollment to one month postpartum, but IgG remained detectable 6 months after delivery in 72% of women. Despite the persistence of the VSA_{PAM} antibodies, they were not associated with an increase in maternal hemoglobin levels. Fried et al published findings from Mali with plasma samples collected at enrollment, 30-32 gestational weeks, and at delivery (Fried et al., 2018). A multiplex bead assay was used to determine seropositivity to many VAR2CSA subdomains, including DBL2X, DBL4ε, and ID1-ID2a, but notably not the full-length protein. Increased antibody levels against any of the DBL domains were not significantly associated with any pregnancy outcome.

1.4.3. Mixed association between VSA_{PAM} antibodies and maternal anemia

In Cameroon, two groups of women, one positive and one negative for placental malaria, both had overall low antibody levels to full-length VAR2CSA at delivery (Lloyd et al., 2018). Among women positive for placental malaria, higher seropositivity against VAR2CSA was associated with a reduced risk of placental parasitemia and low birthweight infants, but not maternal anemia at delivery. Interestingly, in women negative for placental malaria, the presence of VAR2CSA antibodies was associated with lower hematocrits at delivery and increased

prevalence of maternal anemia. This is hypothesized to be the product of a malaria infection that was cleared early in pregnancy, with the presence of antibodies marking this past infection. But, the maternal hematocrit and prevalence of anemia were only slightly different between women with and without VAR2CSA antibodies, thus this effect may not be biologically relevant.

With samples from a large cohort of pregnant women from Kilifi, VSA_{PAM} IgG levels were measured by flow cytometry against various *P. falciparum* placental isolates and compared to hemoglobin levels (Staalsoe, Shulman, Bulmer, et al., 2004). Increased severity of maternal anemia was correlated with low IgG levels in chronic cases of PAM (placentas infected with parasites and hemozoin pigment present) and the level of VSA_{PAM} antibodies in these women was a strong predictor of maternal hemoglobin levels. However, no correlation was observed in women with acute or past placental infection.

1.4.4. Studies that inversely associate VSA_{PAM} antibodies to maternal anemia

The findings from six studies suggest that VSA_{PAM} antibodies can reduce the severity of maternal anemia. In Malawi, two studies investigated the function of VSA_{PAM} antibodies by testing for opsonic phagocytosis. The first study revealed that a two-fold increase in the phagocytosis index was associated with a significant, 70% reduction in the odds of developing maternal anemia at delivery (Feng et al., 2009). Another study investigated third trimester serum samples and found that VSA_{PAM} IgG reactivity in flow correlated with activity in the opsonic phagocytosis assay ($r = 0.60$), but serum from anemic women displayed significantly lower opsonic activity (Jaworowski et al., 2009). Notably, co-infection with HIV reduced opsonic

activity, but did not affect inhibition of binding levels or total IgG against VSA_{PAM} (Jaworowski et al., 2009).

In a later study from Malawi, antibody levels and function correlated to maternal hemoglobin at 36 weeks gestation, infant birthweight, gestational age, infant length, and placental histology at delivery (Chandrasiri et al., 2016). There was a significant positive association between IgG reactivity against CS2 parasites and maternal hemoglobin levels; a 10% increase in reactivity in flow corresponded to a hemoglobin increase of 0.5 g/L. Similarly, a 10% increase in the phagocytosis index also corresponded to a hemoglobin increase of 0.4 g/L. Collectively, these studies support the hypothesis that opsonic phagocytosis could be an important effector mechanism of protection from maternal anemia.

VSA_{PAM} antibody-mediated protection from anemia may relate to the number of *var2csa* genes per *P. falciparum* genome in the parasites that infect pregnant women (Sander et al., 2011). Parasites with more than one *var2csa* gene are more common in PAM, compared to the non-pregnant population, and may provide a selective advantage over parasites with only one *var2csa* gene. In the Cameroonian village of Ngali II, increasing *var2csa* copy numbers in the parasites infecting pregnant women were associated with higher hemoglobin levels at delivery, but not with birthweight. However, in another region, Yaoundé, this relationship was reversed, with a negative correlation observed between *var2csa* copy number and birthweight. In Ngali II, IgG against DBL4ε, but not the full-length VAR2CSA, correlated positively with gene copy number. The authors hypothesized that parasites with multiple copies of *var2csa* may elicit

immunity to specific DBL domains of VAR2CSA, and the protection observed might be due to a strong immune response that develops in women infected with these parasites.

Along with level and functional assays, Chandrasiri et al examined cytokine profiles of pregnant women from Sudan (Chandrasiri et al., 2014). Cytokine profiles were measured with a multiplex bead array that detected IL-1β, IL-6, IL-8, IL-10, IL-12, and TNFα. IFNγ was also measured by ELISA. Distinct cytokine profiles were observed in women with severe PAM compared to uninfected controls, with IFNγ, IL-6, and IL-10 significantly elevated. Parasite density was positively associated with levels of pro-inflammatory cytokines IL-6 and IL-8. Women with severe cases had low levels of IgG against VSA_{PAM} tested in flow, compared to uninfected controls. However, levels of antibodies against DBL5ε measured by ELISA were significantly greater than controls and negatively correlated with IL-1β, IL-6, and IL-8. Hemoglobin levels in these women were also negatively associated with IL-1β, IL-6, IL-8, and TNFα. Consistent with the studies from Malawi, opsonizing activity was positively associated with maternal hemoglobin at study enrollment. This study revealed associations between cytokine levels and VSA_{PAM} antibodies. An influx of pro-inflammatory cytokines could increase the risk of maternal anemia, but the presence of antibodies may influence cytokine profiles.

Only one study from outside Africa identified a positive correlation between VSA_{PAM} antibodies and hemoglobin levels. This study, conducted by our laboratory, was based in Colombia, and examined a cohort of women with submicroscopic PAM (Gavina et al., 2018). While IgG-VAR2CSA antibody levels measured at study enrollment did not correlate with

hemoglobin levels, the activity of these sera in the IBA was significantly associated with higher maternal hemoglobin levels at delivery (Figure 1.6).

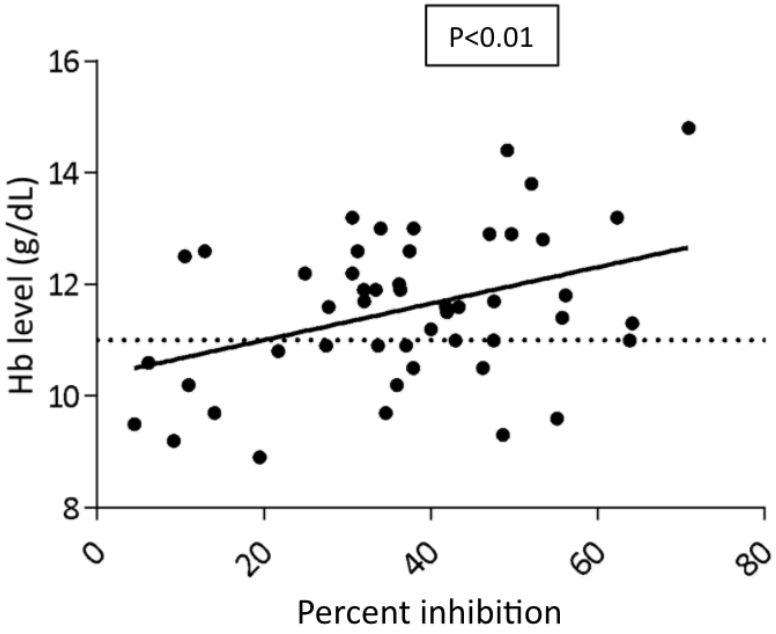

Figure 1.6. Blocking inhibition mediated by antibodies is positively associated with maternal hemoglobin levels at delivery. Linear regression analysis was performed to determine the association between inhibitory antibodies and maternal hemoglobin at delivery. Horizontal dotted line shows threshold for anemia (11 g/dL). Modified from (Gavina et al., 2018).

1.4.5. Potential mechanisms of protection

Though evidence for a specific role for VSA_{PAM} antibodies in protection against maternal anemia is currently inconclusive, we can speculate on putative mechanisms of protection to help design future research (Figure 1.5). We hypothesize that protective antibodies could directly target the iRBCs in the placental environment, either through opsonization or by physically blocking adhesion to CSA. Based on the group of studies that found an association with improved outcomes, VSA_{PAM} antibodies that mediate opsonic phagocytosis of iRBCs may be more likely to be protective against maternal anemia than antibodies that inhibit parasite binding to the placenta, suggesting these antibodies may have distinct effector mechanisms. But, antibodies with dual functions may also exist.

Antibody binding to iRBCs can lead to different downstream effects. Clearing the iRBC from the placenta either by preventing sequestration and/or recruiting immune cells would reduce the hemolysis and the associated inflammatory response. Erythropoiesis is also susceptible to a dysregulated pro-inflammatory response, a common cause of anemia in uncomplicated malaria and PAM (reviewed in (Ghosh & Ghosh, 2007)). VSA_{PAM} antibodies can potentially contribute to these responses by targeting immune cells to iRBCs and reducing cytokine dysregulation. As observed by Chandrasiri et al, pro-inflammatory cytokines IL-1β, IL-6, and IL-8 negatively correlated with antibody levels against DBL5ε in pregnant women with severe malaria (Chandrasiri et al., 2014). In this study, opsonizing antibody activity and cytokine profiles were not compared directly, but maternal hemoglobin levels increased with increasing levels of opsonizing antibodies (Chandrasiri et al., 2016).

In another study, cytokine secretions were measured after opsonic phagocytosis of CS2 parasites had occurred, using serum from Malawian pregnant women. These results were compared to antibody-independent phagocytosis and a shift in the cytokine profile was observed, with an increase in production of IL-1β and TNFα when antibodies were present (Zhou et al., 2012). Opsonized iRBC activated the inflammasome and the production of IL-1β by macrophages, but this was also observed with unopsonized iRBC. Thus, the secretion of pro-inflammatory mediators likely occurs in the placenta with and without sufficient levels of VSA_{PAM} antibodies. Perhaps the labeling of iRBCs with VSA_{PAM} antibodies leads to Th1-like immune response to effectively clear parasites without leading to dysregulated inflammation and inhibition of erythropoiesis.

Data is emerging on the role of the complement system in uncomplicated malaria. Detection of the classical complement pathway was observed in children against merozoite proteins and was an indicator of protective immunity (Reiling et al., 2019). In uncomplicated malaria, both IgG and IgM isotypes were shown to fix complement components in the classical cascade (Boyle et al., 2019). When IgM and IgG isolated from patients were tested for fixation of C1q, the beginning component of the cascade, and downstream components including C3b and C5b-C9, IgG fixed C1q two times greater than IgM, but levels of downstream components were nine times higher in tests with IgM (Boyle et al., 2019).

Less is known in the PAM setting, with several studies suggesting the dysregulated activation of the complement system may drive pathogenesis (reviewed in (McDonald et al., 2015)). In healthy pregnancy, tight regulation of the complement system is necessary for both

responding to infection and controlling this immune response (Derzsy et al., 2010). Complement components and regulators are synthesized by the placenta, including C3 and C4 secreted by the trophoblast cells (Bulla et al., 2009), and C1q secreted by the endothelial cells (Bulla et al., 2008). But, when dysregulation or mutation in complement regulatory proteins occurs, these components can lead to poor pregnancy outcomes including preeclampsia, spontaneous abortion, and hemolysis (reviewed in (Chighizola et al., 2020)). In placental malaria, increased levels of the complement component C5a was associated with placental malaria and fetal growth restriction in humans and a mouse model (Conroy et al., 2013). C5a was also negatively correlated with angiopoietin-1 (Ang-1), which is involved in the formation of fetal vessels, and positively correlated with angiopoietin-2 (Ang-2), which opposes Ang-1 and activates vascular remodeling. The researchers hypothesized this interference with vascular development altered fetal growth (Conroy et al., 2013).

1.5 Thesis objectives

Our previous observation of a positive association between the blocking activity of VSA_{PAM} antibodies and maternal hemoglobin levels (Gavina et al., 2018) supports the hypothesis that VSA_{PAM} antibodies may have protective functions against poor pregnancy outcomes, notably maternal anemia. Protection from maternal anemia may be through antibody-mediated immune responses including blocking, opsonic phagocytosis, and the recruitment of complement. However, a recent meta-analysis revealed that measures of humoral immunity are a marker of exposure, rather than a mediator of protection in PAM (Cutts et al., 2020). Further research is required to continue the characterisation of VSA_{PAM} antibodies to determine if they

impact pregnancy outcomes in a biologically relevant manner. If these antibodies are protective, the mechanisms behind this protection must be elucidated. The study from Colombia also highlights the importance of including submicroscopic infections in the investigation of immune responses during PAM. My thesis aims to continue the characterisation of serum samples collected from a cohort of pregnant women who experienced at least one SMI at some point in pregnancy, and test for both antibody level and function against VSAs expressed in PAM, notably VAR2CSA. This was accomplished by dividing my research into two objectives:

i) To measure the levels of IgG and IgM-VAR2CSA antibodies present in serum collected from pregnant women who experienced at least one SMI of *P. falciparum*, *P. vivax,* and/or mixed infection and correlate these findings to pregnancy outcomes.

ii) To measure antibody-mediated functional responses to VAR2CSA and iRBCs by measuring opsonic phagocytosis and the activation of the classical complement cascade and correlate these findings to pregnancy outcomes.

Chapter 2 – Materials and Methods

2.1. Study design

2.1.1. SMI cohort

The experiments in this thesis characterised serum samples collected from a cohort of 45 pregnant women, described in Table 2.1, who were previously followed longitudinally by our team. This cohort was part of a larger cohort of 187 women, which included 135 women who did not develop a microscopic or submicroscopic infection of malaria during pregnancy and seven women who developed microscopic infections of malaria during pregnancy. All women were followed from study enrollment, typically in the late first trimester to early second trimester, until delivery. Recruitment for the study occurred at the antenatal care clinic, where women were treated for a malaria infection if they tested positive by microscopy. The women in the SMI cohort developed at least one SMI of *P. falciparum, P. vivax*, and/or a mixed infection at some point in pregnancy (Table 2.2). Serum was collected at study enrollment, up to three time points before delivery, and at delivery peripherally from mothers and from the placenta. Due to limited serum availibity at each collection point, some individual serum samples were excluded from the experiments. Additionally, serum collected in between study enrollment and delivery were excluded from characterisation in this thesis.

Table 2.1. Characteristics of pregnant women who developed submicroscopic malaria infections at one or more points in pregnancy

Parameter	Value of Parameter
Median age (year)	21
Median height (cm)	157
Median weight (kg)	53
Primigravid (no. of subjects [%])	18 (40)
Secundigravid (no. of subjects [%])	11 (24.4)
Multigravid (no. of subjects [%])	16 (35.6)
Median gestational age (weeks)	21
Median Hb level (g/dL)	11.6
Use of bed nets (no. [%])	25 (55.6)
Mean weight of newborn (g ± SD)	3220 ± 510
Low birth weight[a] babies (no.)	5
Mean gestational age[b] (weeks ± SD)	38.5 ± 2.1
Preterm births[c] (no.)	5
SGA births (no.)	5
Maternal anemia[d] (no.)	16
Median APGAR score[e] (range)	8.5 (5-9)

[a]Defined as <2,500g
[b]Determined by ultrasound
[c]Defined as <37 weeks.
[d]Defined as Hb level of <11 g/dL
[e]The Apgar index was determined after the first minute of birth

Table 2.2. Infection types among the SMI cohort detected by qPCR.

Infection type	Number of infections among SMI cohort
P. falciparum	21
P. vivax	16
Mixed infection	8

2.1.2. Study site

The longitudinal study was performed in the municipality of Puerto Libertador in the Department of Córdoba (7°53′17″N 75°40′18″W). During the time of the study in 2013, the population was 44,964, which increased to 49,179 by 2016. Both *P. falciparum* and *P. vivax* co-circulate in this region at a ratio of 2:1 and the mean parasite index in Puerto Libertador during 2000-2016 was 13.29 per 1,000 inhabitants (Carmona-Fonseca et al., 2017). The intensity of transmission in this region is low and stable (Rodriguez et al., 2011).

2.1.3. Data collection

Demographics for all participants in the study were collected, including information on age, weight, height, malaria infection history, bed net usage, medical history, history of pregnancy, and gestational age at enrollment (determined by ultrasound). Hemoglobin (Hb) measurements were taken using a HemoCue Hb 20+ System using the manufacturer's standard protocol. Pregnant women also attended antenatal care. Women were excluded from the study if they resided outside of the area of Puerto Libertador or suffered from renal, heart, or respiratory disease. Morbidities including sexually transmitted infections, behavioural disorders, seizures, jaundice, edema, and chronic disease were also excluded. Additionally, if women had received antimalarial treatment two weeks prior to study enrollment, they were excluded from the study. If serum samples were not collected from mothers at study enrollment or delivery, they were excluded from the study.

2.1.4. Ethics

For the longitudinal study in Colombia, all participants consented voluntarily and were able to withdraw from the study at any time. For participants under the age of 18, a parent/legal guardian provided informed consent on their behalf. Ethical approval was obtained from the Health Research Ethics Board at the University of Alberta in Canada (Pro00041720) and the Comité de Ética of Instituto de Investigaciones Médicas of Universidad de Antioquia in Colombia (009-2013, 002-2015, 009-2016).

For the Ugandan serum pools that were used as controls, sera were collected from both primigravid and multigravid women (protocol HDREC368). Additionally, serum from multigravid women was also collected from Uganda in 2019 and ethical approval was obtained from the Institutional Review Boards at the School of Public health, Makerere University College of Health Sciences (The Higher Degrees, Research and Ethics Committee – HDREC), the Uganda National Council of Science and Technology (UNCST) and the Health Research Ethics Board at the University of Alberta, Canada.

For routine culture of laboratory strains, donor O+ blood was regularly collected every 2-3 weeks. Ethical approval was obtained from the Health Research Ethics Board at the University of Alberta in Canada (Pro00001841). Blood was collected by a trained research phlebotomist, who processed, labelled, and stored the blood at 4ºC in media for up to three weeks. After this, the blood was destroyed. The names or any identifying information of the donors were not on any labeling of blood and donors could also request their sample be destroyed at any point.

Typically, blood was collected from the same donor on a regular basis (>6 months), with 25-40 mL of whole blood collected at each donation, which was then washed, and packed RBCs were stored at 4ºC. Individuals who had been on antimalarial drugs within the last 60 days were excluded from blood draws.

2.2 Parasite cultures

The following culture protocols are based on methods previously described (Trager & Jensen, 1976).

2.2.1. Establishing parasite cultures from a frozen ampoule

Laboratory CS2 FCR3 parasites were received from MR4 and stored in a liquid nitrogen tank. To thaw an ampoule, parasites stored in cryogenic tubes were first removed from the tank and thawed in a water bath set at 37ºC for 1-2 minutes. The parasites were then transferred to a 15 mL conical tube. A volume of approximately 0.1 x total blood volume of 12% sodium chloride was added dropwise to the conical, while shaking to combine with the parasites. After a five-minute incubation at room temperature, 10x the blood volume of 1.8% sodium chloride was also added to the mixture with gently shaking. Then, the mixture was centrifuged at 448 g for five minutes. After aspirating the supernatant with a clean 200 µL pipette tip, the pellet was washed in 10 mL of Gibco Roswell Park Memorial Institute (RPMI) media, followed by centrifugation. Then, the supernatant was aspirated, and 10 mL of RPMI complete (RPMC) media was used to transfer the pellet to the Petri dish. Approximately 300 uL of fresh, washed

O+ blood was added to the Petri dish to bring the hematocrit up to 3% (RBC volume: total volume of liquid in dish).

To determine parasitemia for any of the following culture protocols, Petri dishes are first set at an angle such that RBCs collect in a line across the plate. Then, 3.5 μL of this line is collected with a pipette and transferred to a glass slide and resuspended by gently pipetting up and down. A thick smear is made by taking another glass slide, angling it against the slide with the resuspended parasites, and gently dragging the suspension across the slide to create a smear. Then, the smear is dipped in methanol to fix cells in place and transferred to a conical containing 10% Giemsa stain diluted in MiliQ water. The slide incubates for 10-15 minutes at room temperature before the cells are fully stained and ready to be counted under a microscope. Parasitemia is defined as the number of infected RBCs divided by total RBCs. Following any culture protocol described below, the chamber containing the petri dishes was flooded with nitrogen gas (5% carbon dioxide, 1% oxygen, nitrogen balance) for one minute, then returned to the incubator set at 37^{o}C.

2.2.2 Changing culture media

Parasite media was changed daily, except the day after a subculture/split (discussed in section 2.5.3.). To change the culture media, Petri dishes were tipped slightly at an angle such that media was collected on the side of the dish without disturbing the RBCs that settled at the bottom of the dish. Then, 7 mL of media were removed from the dish using a sterile pipette. If the culture was re-suspended to the point that RBCs would also be aspirated during a media

change, the culture was spun down at 448 g for 5 minutes and 7 mL of the supernatant were then aspirated off. After media was removed, 7 mL of fresh media were added to the dish using a new pipette. The media and RBC suspension were re-suspended with gentle mixing.

2.2.3 Sub-cultivation of parasites

When the parasitemia reached 10% or higher, the culture was split to 2-3% parasitemia if it was not being used within 48 hours for experiments or an alternative purpose. To split the culture, the parasitemia was first calculated by creating a thin smear and staining with Giemsa to detect the parasites within the RBCs. The parasitemia was calculated based on a count of 200 RBCs, which was typically 1-2 fields of view on the microscope. Once parasitemia was calculated, the following formula was used to calculate the total volume of iRBC to add to the new culture:

$$V = 3 \times \text{target parasitemia} \times 100 / \text{current parasitemia}$$

Once the total volume of both iRBC and fresh nRBC was at the appropriate parasitemia, the culture was re-suspended with a sterile pipette, transferred to a 15 mL conical and spun down at 448 g for five minutes. The supernatant was aspirated and 10 mL of RPMI was used to re-suspend the culture as the ‘wash’ step. After another centrifugation, the appropriate volume of iRBCs was resuspended in 10 mL of fresh RPMC media and added to the culture dish, followed by the appropriate amount of fresh O+ nRBCs. The suspension was gently swirled to mix the two populations of RBCs.

2.2.4 Sorbitol synchronization

To prevent asynchronous life stages in the cultures, plates were routinely synchronized with sorbitol at the ring stage. This allowed for higher yields when purifying late trophozoites/schizonts, as most parasites will be at the same stage (see Section 2.5.7). The synchronization solution was prepared with 5% D-sorbitol diluted in MilliQ water, followed by filter sterilization. Before synchronizing a culture, the parasitemia was calculated to ensure at least 5% of the parasites were in the ring stage, as trophozoites do not survive synchronization. Next, the plate was re-suspended and transferred to a 15 mL conical with a sterile pipette and spun down at 448 g for 5 minutes. The supernatant was aspirated and five times the pellet volume of 5% sorbitol was slowly added dropwise and mixed gently to saturate the pellet. The pellet is then incubated at 37^{o}C for five minutes. The total volume was then brought up to 10 mL by adding RPMI to the conical, then centrifuged at 448 g for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 10 mL of RPMC and transferred to a new culture dish. The appropriate amount of fresh O+ nRBC was then added to bring the hematocrit up to 3%.

2.2.5. Selection of VSA-expressing cultures

To ensure parasites were expressing VSA that bound CSA, routine selections were performed every 2-3 weeks. First, 10 mL of sterile CSA diluted to 100 μg/mL were coated on a polystyrene petri dish and incubated overnight at 4^{o}C. The next day, the dish was washed once with 10 mL of filter sterilized 1XPBS, then blocked with 15 mL of 3% BSA diluted in RPMI and

incubated for 1 hour at 37ºC. Then, the dish was washed three times with 1XPBS, swirling the liquid in the dish and then aspirating with a clean 200 µL filter tip each time. Parasites at >10% mid-late trophozoite/schizont stage were then added to the dish and incubated for 1 hour at 37ºC, gently swirling every 15 minutes to keep the RBCs suspended. After this, the dish was washed gently three times with 10 mL of 1X PBS to ensure all unbound RBCs were washed away. Then, 10 mL of sterile CSA solution diluted to 500 µg/mL in 1X PBS were added to the dish and incubated for 10 minutes at 37ºC. Next, the bound cells on the dish were resuspended and transferred to a 15 mL conical to be spun down at 448 g for 5 minutes. Finally, the new culture was set up by adding 10 mL of fresh RPMC and the appropriate amount of fresh nRBCs to bring the hematocrit up to 3%.

2.2.6 Cryopreservation of cultures

Cultures that were growing well and showed high expression of VAR2CSA were routinely preserved for future use. When a culture was over 5% in the ring stage, it was ready for cryopreservation. The culture was re-suspended in a 15 mL conical with a sterile pipette and spun down at 448 g for 5 minutes. After aspirating the supernatant, the culture was washed with 10 mL of RPMI and centrifuged again. The supernatant was then aspirated, and the total volume of the pellet was estimated. An equal volume of deep-freeze solution was added dropwise. After the solution was gently mixed, 0.5 mL aliquots of the culture were transferred into ampoules and properly labeled with culture name, date, and approximate parasitemia. The ampoules were transferred into the liquid nitrogen storage tank.

2.2.7 Purification of CS2 schizonts and late trophozoites

When the parasitemia of a culture was >10% late trophozoite/schizont stage, the expression of VAR2CSA was the highest; thus, the cultures were ready for experimentation. To begin the purification, the cultures were first re-suspended in a 15 mL conical with a sterile pipette and spun down at 448 g for 5 minutes, followed by a wash with 10 mL of RPMI. After another centrifugation, the pellet was re-suspended at 20% hematocrit with 2% FBS-1X PBS and stored on ice until the purification column was prepped. To prep the LD column, it was inserted into the VarioMacs magnet and 2 mL of 2% FBS-1X PBS was loaded into the column and allowed to run through. After this, the column was ready to be loaded with parasites. With a 1 mL pipette, the culture was re-suspended well and carefully loaded onto the column. After the culture had fully run through the column, the column was washed twice with 2 mL of 2% FBS-1X PBS, letting the wash fully run through on each time.

To elute the iRBCs from the column, the column is first removed from the magnet and placed on top of a new 15 mL conical. Then, 3 mL 2% FBS-1X PBS were loaded onto the top of the column and gently pushed through with a syringe top, eluting the iRBCs from the column. The elution is then spun down at 448 g for 5 minutes to prep the pellet for experimentation. To calculate the cell concentration, a 1/10 dilution of the purified culture was loaded onto a hemocytometer. Cell concentrations were typically 1 x 10^8 cell/mL or higher. To calculate the purity (iRBCs: nRBCs), a thin smear was made of the purified culture and stained with Giemsa. Purities were typically 90-99%.

2.2.8 THP-1 culture protocols

To make media for thawing new THP-1 ampoules from the nitrogen tank, media containing 20% FBS, 1640 1X RPMI (11875-093; Gibco), 1% Pen-Strep, and approximately 15 µL of HCl was made up. The same media composition was used for daily culturing, except the media was supplemented with 10% FBS instead. To thaw the THP-1 cells, the ampoule was placed in a 37^{o}C water bath for approximately two minutes, then thawed at room temperature. Immediately after thawing, ~5 mL of 2% FBS-1X PBS were added to the ampule and the mixture was transferred to a 15 mL conical. The suspension was spun down at 252 g for 5 minutes. The supernatant was aspirated, and the pellet was resuspended with 4 mL of THP-1 media containing 20% FBS. The suspension was transferred to a T25 flask and incubated in a CO_2 chamber at 37^{o}C.

Cell concentration was calculated by loading 10 µL of a mixture of equal volume THP-1 cell culture and Trypan Blue into a hemocytometer. When the cell concentration surpassed 1 x 10^6 cells/mL, the culture was split to 0.5 x 10^6 or 0.33 x 10^6 by adding fresh THP-1 media containing 10% FBS. Once the total culture volume was 10 mL or higher, the cells were transferred to a T75 flask. THP-1 cells were not used for experimentation until approximately 10 passages had occurred, then phagocytosis activity was tested with a simple opsonic phagocytosis experiment using a pool of hyperimmune serum as the primary antibody (see section 2.4.3.). Once strong opsonic phagocytosis was observed (20-50% phagocytosis), the THP-1 cells were used routinely for experimentation. Cells were used in experiments cautiously after the 20th passage, ensuring that opsonic phagocytosis is still observed with the positive control and is not

decreasing to levels observed with the negative control. THP-1 cells were not used after the 25th passage.

2.3 Recombinant proteins and controls used in experiments

2.3.1. Recombinant proteins used for serology assays

For all plate-based serology experiments presented in this thesis, recombinant VAR2CSA protein was kindly shared by Dr. Ali Salanti from the University of Copenhagen. The FCR3 allele of the full extracellular domain of VAR2CSA was expressed in Sf9 cells. For the C1q fixation assay, human C1q protein was used (EMD Millipore 204876-1MG).

2.3.2. Serum samples used for controls in serology and functional assays

The Ugandan serum pools used as controls were collected from primigravid and multigravid Ugandan women who had experienced one or multiple instances of PAM, respectively. For IgG enzyme-linked immunosorbent assays (ELISAs), the primigravid pool was used in several optimization experiments as a negative control and consisted of three individual serum samples (UPG 57, 113, 354). The multigravid pool, used as a positive control in optimizations, the IgG ELISA, and total IgG flow cytometry, consisted of four individual serum samples that had high levels of IgG-VAR2CSA antibodies (UMG 58, 174, 460, 502). Thus, this pool was labelled as hyperimmune. For IgM ELISAs, the positive control pool consisted of two individual serum samples collected from Ugandan multigravid women with high IgM-

VAR2CSA antibody levels (UMG 474, 15). For the opsonic phagocytosis assay, serum samples collected in the 2019 Ugandan study from two individual Ugandan multigravid women were pooled to make the positive control (UMG 40, UMG 20). All pools were made by vortexing and microcentrifuging each individual serum sample before pipetting, then combining equal volumes of each individual sample included in the pool, followed by vortexing the mixture well. This mixture was then divided into equal aliquots that were stored at -80ºC until usage, then at -20ºC while the aliquot was actively used in experiments until emptied.

For a negative control pool, serum samples were collected from individuals living in Medellín, where no species of *Plasmodium* circulate. For total IgG flow cytometry and the IgG, IgM, and C1q fixation ELISAs, six serum samples collected from both female and male donors were pooled (12 individual serum samples total) using the methods described above. For the opsonic phagocytosis assay, 10 serum samples collected from both female and male donors (20 samples total) were pooled.

To test for VAR2CSA expression by iRBCs in culture in the total IgG flow cytometry experiments (section 2.4.2), serum from a rabbit immunized with recombinant VAR2CSA (VAR2CSA Rab) was kindly shared with our laboratory by Dr. Ali Salanti. This serum sample was also used in some ELISA optimization experiments as a positive control for recombinant VAR2CSA binding. For a negative control for VAR2CSA expression in flow cytometry, serum from a non-immunized rabbit (Normal Rab) was used.

2.4. Serology assays

2.4.1. IgG-VAR2CSA ELISA

Levels of IgG against recombinant VAR2CSA protein were measured in ELISA using methods previously developed (Gnidehou et al., 2014). All test samples and controls were run in duplicate (two plate wells per sample) and averaged at the end of the experiment. Thermo Fisher Scientific polystyrene 96-well plates (catalogue no. 439454) were coated with 50 μL per well of VAR2CSA recombinant protein at a concentration of 0.5 μg/mL and incubated overnight at 4ºC. The following day, the plate wells were first filled with 275 μL per well of 4% bovine serum albumin (BSA, catalogue no. A7906; Sigma-Aldrich) diluted in 1X phosphate buffered saline (PBS), expelled, and then re-filled with 4% BSA-1XPBS and incubated at 37ºC for one hour. During this incubation, primary serum dilutions were prepared in 2% BSA-1XPBS at 1/1000 dilution for human samples and 1/500 for rabbit samples. After the plate was washed once with 0.01% Tween 20 in 1X PBS (PBST) and expelled, 50 μL of the pre-made serum dilutions were added to the respective duplicate wells and incubated for one hour at room temperature.

Next, the wells were washed four times with PBST, with liquid expelled after each wash, and 50 μL of a horseradish peroxidase (HRP) conjugated goat anti-human IgG (catalogue no. ab98624; Abcam) antibody was added at a 1/15,000 dilution and incubated for one hour at room temperature. During this time, an appropriate amount of tetramethylbenzidine substrate (TMB, catalogue no. T0440; Sigma-Aldrich) was aliquoted and allowed to come to room temperature on the bench. After four washes with PBST, 50 μL of TMB was added to each well and developed

for half an hour at room temperature. Acid (0.5N H_2SO_4) was then added to each well to stop the reaction, and the optical densities were assessed with a plate reader at 450 nm and 570 nm using the program YANOW 450_570 ELISA-HRP-v2.

On each plate, the hyperimmune pool of serum collected from Ugandan multigravid women was run in duplicate as a positive control, along with the unexposed Medellín pool as the negative control. Additionally, duplicate wells only containing the antigen coating plus the secondary antibody were run to measure any reactivity between the antigen and secondary antibody (background). The optical density (OD) of each for each sample was calculated by averaging the duplicate wells. Optical densities were then converted to arbitrary units (AU) using the following formula:

$$AU = (OD_{sample} - OD_{background}) / (OD_{hyperimmune\ pool} - OD_{background}) \times 100\ \%.$$

To determine seropositivity, the twelve individual unexposed serum samples collected from Medellín individuals were run in duplicate and optical densities were converted into AUs using the same formula above. AUs of the twelve samples were then averaged and two standard deviations were added to create the cut-off point, as shown in Figure 2.3.1 (10.8 AU). Seropositivity was defined as being above this cut-off.

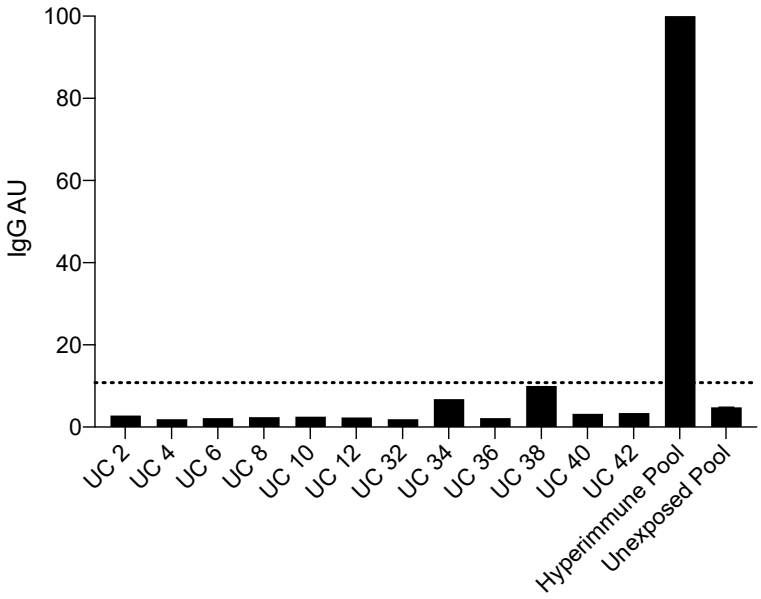

Figure 2.4.1. IgG-VAR2CSA seropositivity cut-off. The cut-off point (horizontal line = 10.8 AU) was calculated by averaging the individual reactivities of 12 unexposed Colombian samples, then converting ODs to AUs and adding two standard deviations. The individual unexposed samples measured were also pooled to make the unexposed pool.

2.4.2. IgM-VAR2CSA ELISA

The IgM ELISA was performed with a similar protocol as the IgG ELISA with the following modifications. Primary human serum samples were run at 1/500 instead of 1/1000 dilutions. The secondary antibody was a HRP goat anti-human IgM (catalogue no. AP114P; Millipore), which was run at a dilution of 1/7500. The cut-off point was determined with the same twelve serum samples from unexposed individuals in the IgG ELISA, using the same protocol to establish a cut-off point and seropositivity, as shown in Figure 2.3.2 (40.4 AU).

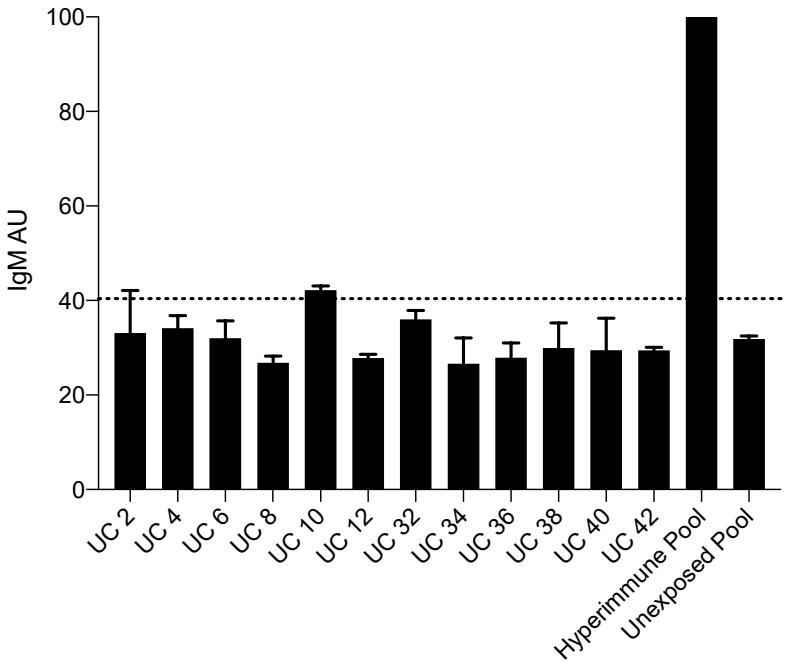

Figure 2.4.2. IgM-VAR2CSA seropositivity cut-off. The cut-off point (horizontal line, 40.4 AU) was calculated by averaging the individual reactivities of 12 unexposed samples, then converting to AUs and adding two standard deviations. The individual unexposed samples measured were also pooled.

2.4.3. Serum preparation for flow cytometry

For the total IgG flow cytometry and opsonic phagocytosis assays, serum was heat inactivated by incubating serum at 57^{o}C for 45 minutes (vortexing midway through the incubation) to de-activate any complement present in the serum. For the total IgG flow cytometry, serum was also pre-absorbed against packed AB+ RBCs to remove any antibodies against RBC surface antigens. Blood was collected from an AB+ donor and washed. Packed AB+ RBCs were pelleted in a microcentrifuge and an equal volume of serum and packed RBCs were incubated together on ice for one hour. The mixture was then spun down at 1008 g 5 minutes, and the serum was carefully extracted from the packed RBCs and stored at -20^{o}C for future experimentation.

2.4.4. Total VSA_{PAM} IgG flow cytometry

Purified late trophozoites/schizonts at a concentration of $1x10^8$ cells/mL or higher and a purity of 90-100% were used for experimentation (see section 2.5.6 for purification protocol). The purified parasites in 2% fetal bovine serum diluted in 1X PBS (2% FBS-1X PBS) were kept on ice until the experiment began. For experiments with human serum, 0.5 mL of parasites at a concentration of 0.5 x 10^6 cells/mL were aliquoted into microcentrifuge tubes for each sample tested (0.25 x 10^6 cells per tube). To test the expression of VAR2CSA in each experiment with rabbit serum (VAR2CSA Rab and Normal Rab), 0.5 mL of parasites at a concentration of 1.0 x 10^6 cells/mL were aliquoted into tubes (0.5 x 10^6 cells per tube). Sample tubes were spun down at 1008 g for 5 minutes and the supernatant was aspirated. Primary antibodies were added at dilutions of 1:20 for Colombian serum, 1:80 for Ugandan serum, and 1:40 for rabbit serum, using 2% FBS-1X PBS for dilutions. Primary antibodies were incubated with iRBCs for one hour on ice. Next, the tubes were spun down and washed once with 2% FBS-1X PBS for rabbit serum (twice for human serum) by resuspending the pellet in 0.5 mL of 2% FBS-1X PBS, centrifuging at 1008 g for 5 minutes, and removing the supernatant for each wash.

For testing rabbit serum, 200 μL of a Rb647 conjugated goat-anti rabbit secondary antibody (Life Technologies) at a dilution of 1/500 was added and incubated for one hour on ice. For human serum, 200 μL of a rabbit-anti human secondary IgG antibody (A0242; Dako) at a dilution of 1/500 was first added, incubated for one hour on ice, then washed twice with 2% FBS-1X PBS. Then, 200 μL of a tertiary Rb647 conjugated goat-anti rabbit antibody at a dilution of 1/250 was used and incubated for one hour on ice. For the last step, pellets were washed

(twice for rabbit serum; three times for human serum) then 350 µL of 4,6-Diamidino-2-Phenylindole (DAPI, catalogue no. D1306; University of Alberta Biochemistry Stores) at a concentration of 5 µg/mL in 2% FBS-1X PBS were added to resuspend the pellet and transfer it to a FACS tube for each sample. DAPI was incubated for half an hour at room temperature, then the tubes were transferred to ice until acquiring on the Fortessa X-20 flow cytometer. The V450 laser was used to measure the DAPI expression and the R670 laser was used to measure serum staining on the iRBCs. Median fluorescent intensities (MFIs) were recorded by the flow cytometer and analyzed in FlowJo.

For controls, the hyperimmune Ugandan multigravid pool (UMG_{test}) was run as the positive control. For the negative control, the pool of twelve unexposed individuals from Medellín was run. Typical flow plots and gating strategies for the human controls are shown in Figure 2.3.3. Additionally, VAR2CSA expression by iRBCs was assessed with the VAR2CSA Rab, with the Normal Rab as the negative control for VAR2CSA expression, as shown in Figure 2.3.4. iRBCs without primary serum but with the secondary and tertiary (when applicable) antibodies was also run on each experiment to account for any reactivity between iRBCs and the secondary and/or tertiary antibodies (background). Each SMI serum sample was tested twice on separate days for total VSA_{PAM} IgG and the two MFI readings for each sample were averaged.

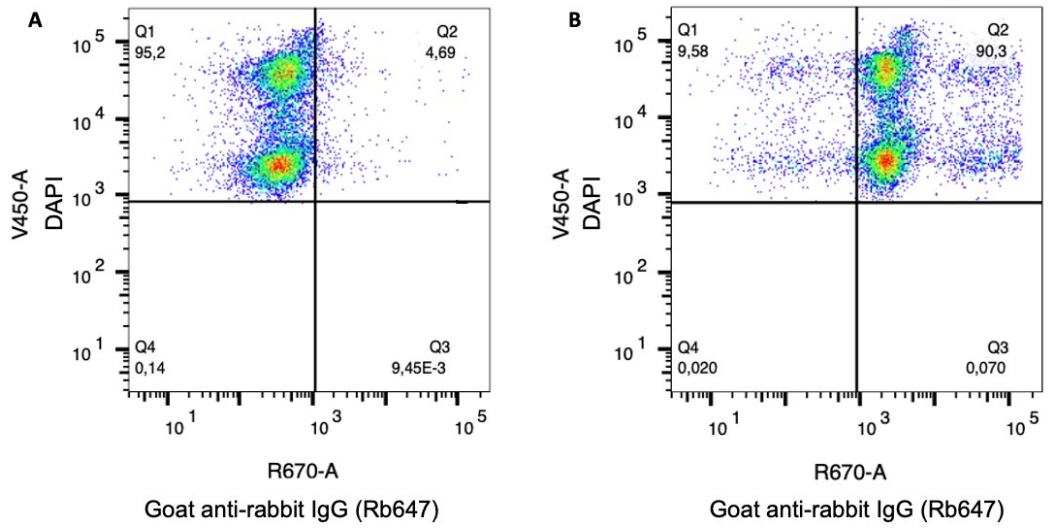

Figure 2.4.3. Typical flow plots for human serum control samples. Unexposed Medellín (A) and Ugandan multigravid (B) primary serum pools (followed by secondary rabbit-anti human IgG and tertiary goat anti-rabbit IgG) were run on each experiment as the negative and positive controls, respectively.

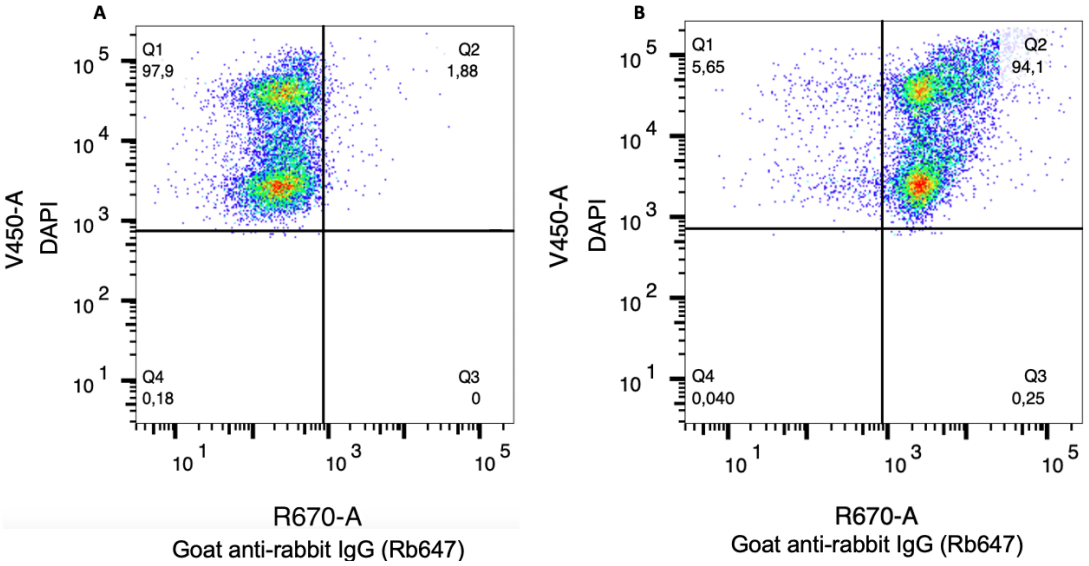

Figure 2.4.4. Typical flow plots for rabbit serum control samples. Normal Rab (A) and VAR2CSA Rab (B) sera were run on each experiment to assess VAR2CSA expression by iRBCs.

To determine seropositivity and control for day-to-day variations in the flow cytometry experiments, the twelve individual unexposed serum samples collected from Medellín donors were first run and MFIs were recorded before any testing was performed with the SMI serum samples. The reactivity of the hyperimmune UMG pool was also run ($UMG_{control}$) in this first experiment, along with the twelve unexposed Colombian serum samples pooled in the unexposed pool. A cut-off was created by averaging the individual reactivities of the twelve samples, plus adding two standard deviations, as seen in Figure 2.3.5 (182.3 MFI).

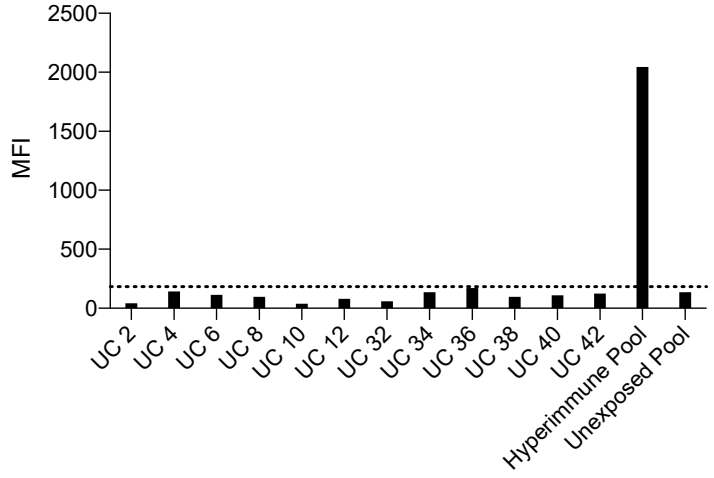

Figure 2.4.5. **Total IgG flow cytometry cut-off.** The cut-off point (horizontal line, 182.3 MFI) was calculated by averaging the individual MFIs of 12 unexposed samples and adding two standard deviations. The individual unexposed samples measured were also pooled to make the unexposed pool.

In subsequent experiments, the MFIs of individual SMI serum samples were adjusted with the following steps:

(1) Subtract background from each individual MFI value, including the positive and negative controls.

(2) Divide the $UMG_{control}$ MFI reading by the UMG_{test} MFI reading on the experiment day to get the UMG factor (~0.75-1.15). Multiply all individual SMI MFI readings by this factor to adjust for day-to-day variation in the experiments. SMI sample is considered seropositive if it passes the cut-off point determined in the first experiment (182.3 MFI).

2.5. Functional assays

2.5.1. C1q fixation ELISA

The C1q fixation ELISA was modified from protocols previously published (Reiling et al., 2019) and the steps of this assay are shown in Figure 2.4.1. Briefly, 50 μL of recombinant VAR2CSA protein at 0.5 μg/mL were coated on 96-well plates and incubated overnight at 4ºC. The next day, the plate was washed three times with 0.05% PBST and then blocked with 4% BSA-1XPBS for two hours at 37ºC. During this time, primary serum dilutions at 1/100 were prepared in 2% BSA-1XPBS. After three washes, expelling the wash each time, 50 μL of the primary serum dilutions were added to respective wells in duplicate and incubated for two hours

at room temperature. During this incubation, a humidity chamber was prepared by placing damp paper towel in a sealed plastic chamber and left at room temperature.

Next, the plate was washed three times and 30 μL of recombinant C1q protein at 30 μg/mL were added to the plate and placed in the humidity chamber for one hour. After three washes, 50 μL of a mouse monoclonal anti-C1q antibody (MA1-83963; Invitrogen) diluted 1/2000 was added to each well and incubated for one hour in the humidity chamber. Another three washes were performed after this, and a HRP goat anti-mouse antibody (catalogue no. 170-6516; Bio-Rad) was added at a dilution of 1/3000 and incubated for an hour in the humidity chamber. Finally, TMB was aliquoted and warmed to room temperature on the bench, then 50 μL was added to each well and incubated at room temperature for an hour. The reaction was stopped with 50 μL per well of 0.5N H_2SO_4.

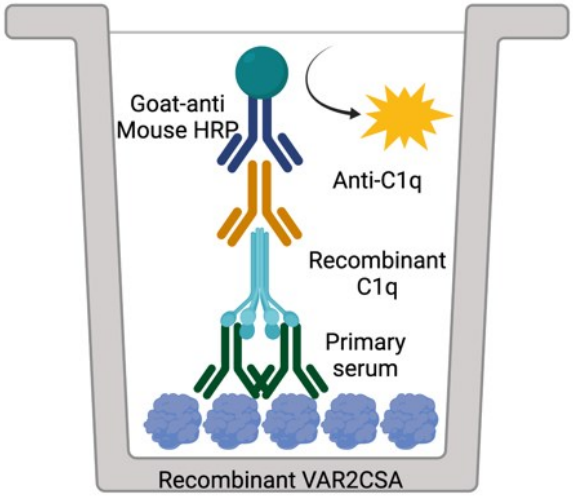

Figure 2.5.1. Layers of the C1q fixation ELISA. Recombinant VAR2CSA is first coated on the plate then incubated overnight. Next, primary antibody is added using the serum of interest. Then, recombinant C1q is added, followed by an anti-C1q antibody. The appropriate conjugated HRP is then added, followed by TMB, which reacts with acid. Made in BioRender.com

An individual serum sample collected from a pregnant Colombian woman with high C1q fixation levels was used as a positive control. The same unexposed pool used both in the flow cytometry and the IgG and IgM ELISAs was used as a negative control. For background, two wells were run with all layers of the ELISA except primary serum, to account for any C1q deposition directly onto the antigen (background). C1q was also coated directly on the plate at 1.0 μg/mL and detected with appropriate antibodies to ensure the secondary and tertiary antibodies were working correctly. AU and seropositivity were determined using the same formula above and the C1q fixation cut-off was created with the same twelve unexposed individual samples used in flow cytometry and the ELISAs, as shown in Figure 2.5.2 (27.5 AU).

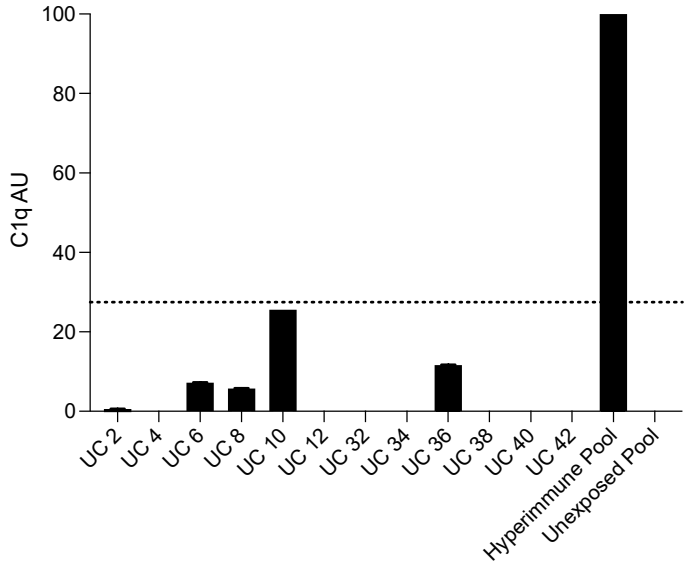

Figure 2.5.2. C1q fixation cut-off. The cut-off point (horizontal line, 27.5 AU) was calculated by averaging the individual reactivities of 12 unexposed samples, then converting to AUs and adding two standard deviations. The individual unexposed samples measured were also pooled to make the unexposed pool.

2.5.2. Opsonic phagocytosis assay

Purified late trophozoites/schizonts were kept on ice until the experiment began. First, the total iRBCs needed for the experiment was calculated by adding the number of iRBCs needed in each sample tube (2.5 x 10^6 cells per tube). Then, to obtain this number of cells at a concentration of 50 x 10^6 cells/mL, the total volume (V_t) was calculated with the formula below:

V_t = Number of cells needed / 50 x 10^6 cells/mL

To calculate the total volume of parasites needed from the original purification, the following formula was used:

V_{iRBCs} = (50 x 10^6 cells/mL) x (V_t) / Original cell concentration from purification (cells/mL)

To calculate the appropriate volume of 10 µg/mL ethidium bromide (V_{EtBr}) (BP1302-10; Fisher BioReagents), the following formula was used:

V_{EtBr} = (10 ug/mL) x (V_{iRBCs}) / 1000 µg/mL (EtBr stock concentration)

To bring the volume of EtBr and iRBCs up to the total volume (V_t), an appropriate amount 1X PBS was added. Then, iRBCs were incubated with EtBr for 30 minutes in the dark at room temperature, then washed once by spinning down the pellet at 700 g for 5 minutes, aspirating the supernatant, re-suspending with 500 µL THP-1 media, and centrifuging again. To bring cells up to the appropriate concentration for the primary antibody incubation, the following formula was used:

Number of cells = (V_t) x (50 x 10^6 cells/mL)

Volume added to pellet = Number of cells / 1.25 x 10^8 cells/mL (dilution factor)

(Dilution factor = number that will allow for 2.5 x 10^6 cells to be in each sample tube when 20 µL of EtBr-iRBC suspension is aliquoted)

Next, 20 µL of the cell suspension was aliquoted into individual microcentrifuge tubes for testing serum samples such that 2.5 x 10^6 cells are in each sample tube. To this, 3.0 µL of human serum, or 2.0 µL of rabbit serum, was added to each respective tube and incubated for 45 minutes at 37ºC. During this time, a 96-well round-bottomed plate was blocked with 2% FBS-1XPBS for one hour at 37ºC. Additionally, THP-1 cells were collected from culture flasks and counted using a hemocytometer. THP-1 cells were collected at 1.25 x 10^6 cells/mL and kept at 37ºC in a water bath for as long as possible until the phagocytosis step.

After the primary serum incubation, iRBCs were washed once with 0.5 mL of THP-1 media and spun down at 252 g for 3 minutes. The round-bottom plate was emptied and 50 µL of THP-1 media was added to each well. The iRBC pellet was resuspended with 100 µL of the THP-1 cell suspension previously collected from the culture flask, combining the two cell types. This suspension was transferred to the round-bottom plate and incubated for 40 minutes at 37ºC in a CO_2 incubation chamber. After this incubation, cell suspensions were transferred back to microcentrifuge tubes and spun down at 161 g for 5 minutes. The supernatant was aspirated and the iRBC/THP-1 pellet was re-suspended in 500 µL of an RBC lysis buffer (8.02g NH_4Cl, 0.84g $NaHCO_3$, 0.37g EDTA) at a 1/10 dilution, made in de-ionized H_2O. The suspensions were incubated for 10 minutes in the dark on an end-over-end mixer. Finally, the suspensions were pelleted at 161 g for 5 minutes and washed once with 0.5 mL of cold 2% FBS-1X PBS, followed by centrifugation at 161 g for 3 minutes. The pellets were re-suspended in cold 2% FBS-1X PBS and left on ice in the dark until acquiring. On the Fortessa X-20, the Y610 laser was used to detect EtBr staining the THP-1 cells.

To determine the phagocytic index (PI) of each sample, opsonic phagocytosis was first gated based off of the positive control or hyperimmune pool (% phagocytosis, range of phagocytosis between experiment days shown in Figure 2.4.3) and negative control (% phagocytosis, range of phagocytosis between experiment days shown in figure 2.4.4.). The PI of each sample was then calculated as a percentage of the opsonic phagocytosis observed with the hyperimmune pool with the following formula:

PI = (% phagocytosis of test sample) / (% phagocytosis of positive control) x 100

A cut-off point was also created to determine opsonic phagocytosis above background levels by measuring the opsonic phagocytosis of 20 individual Medellín samples, calculating the PI with the formula above, and averaging the 20 PI's and adding two standard deviations as shown in Figure 2.5.5 (24.1 PI).

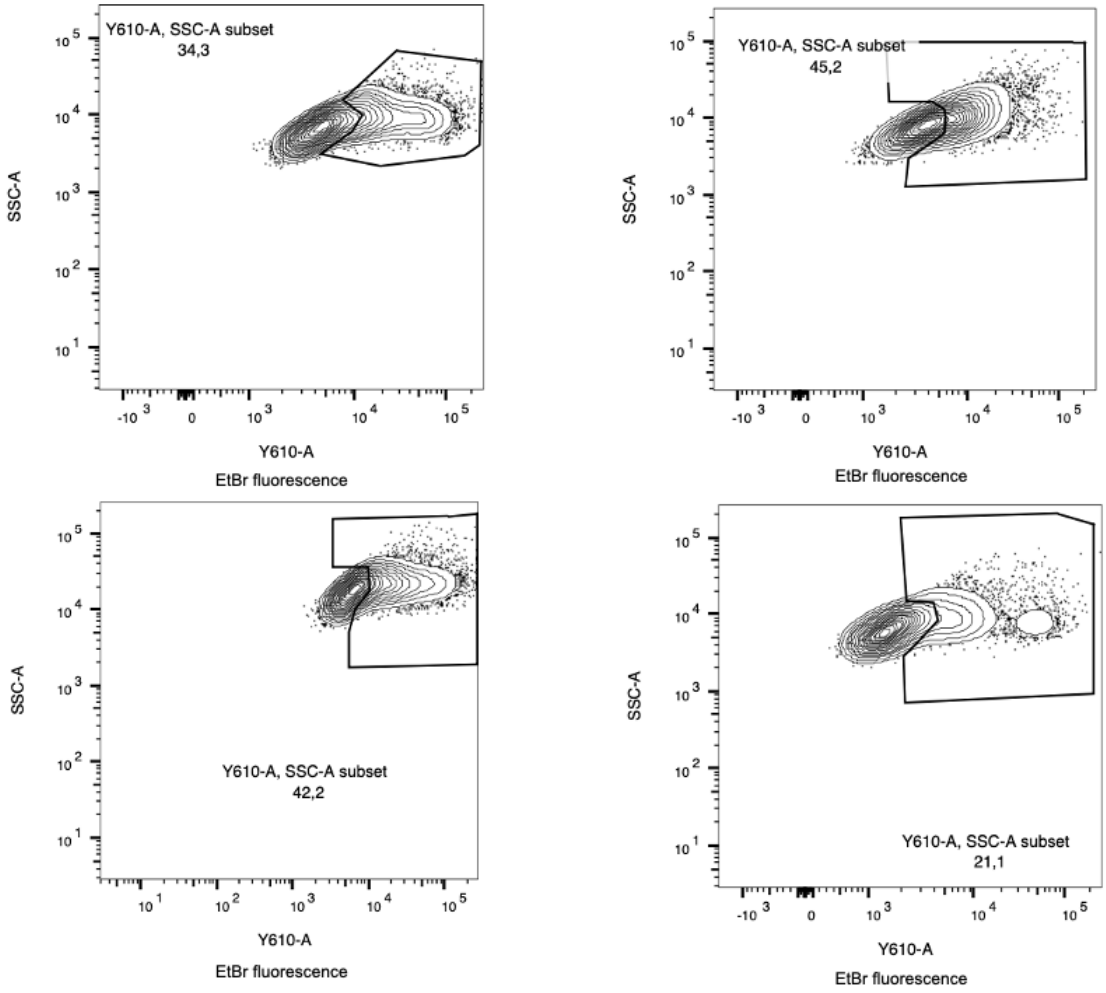

Figure 2.5.3. Range of opsonic phagocytosis observed with the hyperimmune pool between experiment days. Opsonic phagocytosis mediated by the hyperimmune Ugandan pool was measured on each experiment and % of THP-1 cells that engulfed iRBCs was gated (% phagocytosis).

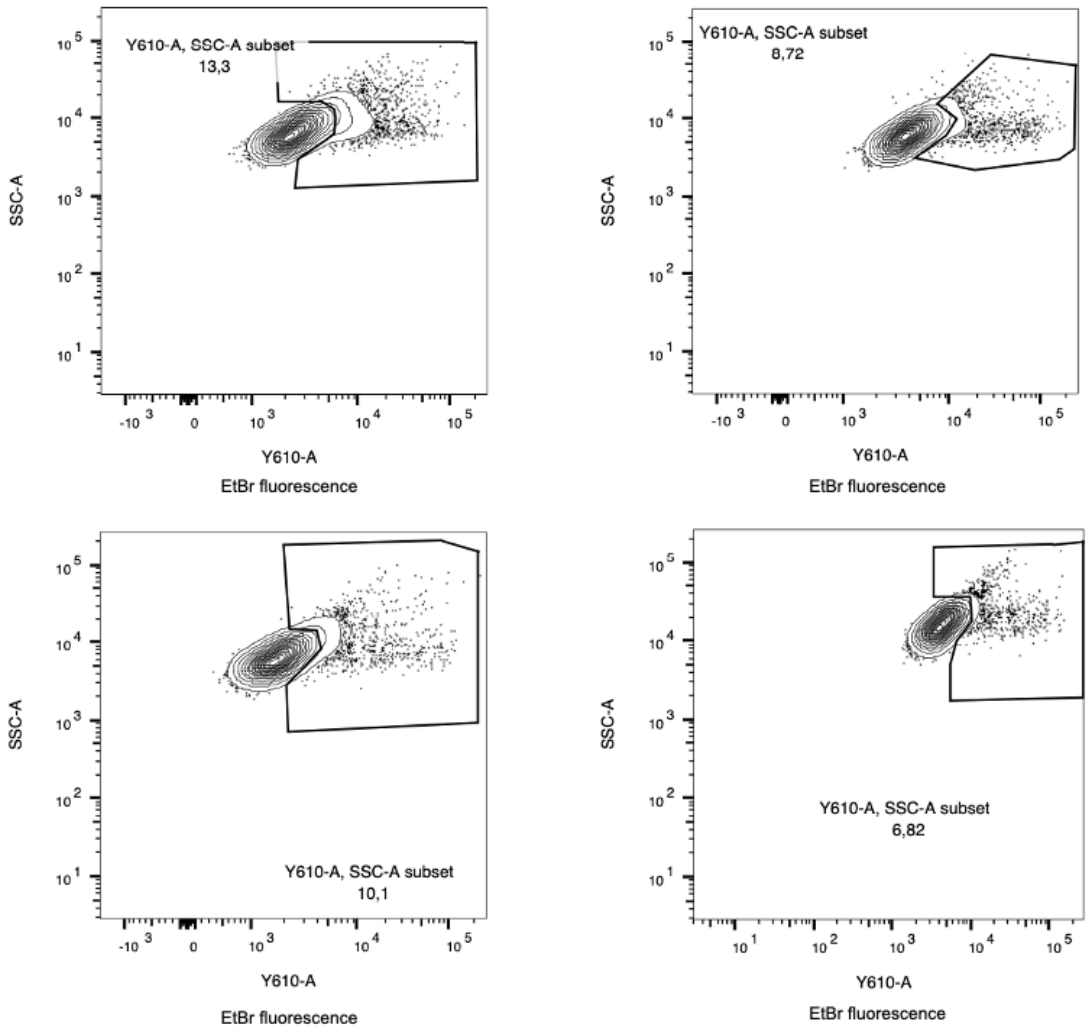

Figure 2.5.4. Range of opsonic phagocytosis observed with the negative control pool between experiment days. Opsonic phagocytosis mediated by the unexposed Colombian pool was measured on each experiment and % of THP-1 cells that engulfed iRBCs was gated (% phagocytosis).

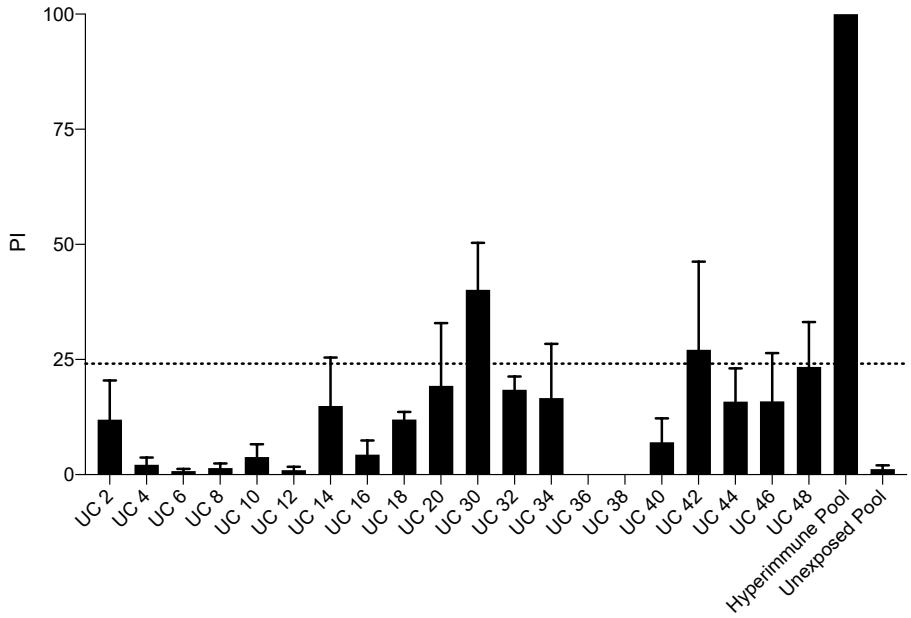

Figure 2.5.5. Opsonic phagocytosis cut-off. Cut-off (horizontal line, 24.1% phagocytosis) was calculated from the individual phagocytosis percentages (in comparison to a hyperimmune pool) of 20 unexposed Colombian samples. The reactivities of the unexposed samples were averaged and two standard deviations were added. Unexposed samples were pooled to make the unexposed pool.

2.5.3 Transwell preparation & DiD staining conditions

To expose fresh nRBCs collected from a donor to iRBCs grown in culture and easily isolate the two cell populations, tissue culture treated polycarbonate membrane polystyrene transwell plates were used (REF 3401; Corning Incorporated). First, after synchronization of the cell culture at the ring stage, the culture was spun down, and packed RBCs were placed into the upper compartment of the transwell plate. These RBCs contained a mixture of iRBCs and nRBCs grown together in culture, at parasitemia of 3-5% and a hematocrit of 3%. Fresh nRBCs were placed in the lower compartment at 3% hematocrit. For controls, the same arrangement was set up, but with fresh nRBCs on the top and bottom compartments. The arrangement of the RBC populations in the transwell plates is shown in Figure 2.4.6. The transwell plates were placed in the culture chamber, gassed for 1 minute, and incubated at 37°C. Due to the low parasitemia, cultures survived in the transwells for 48 hours without media changes.

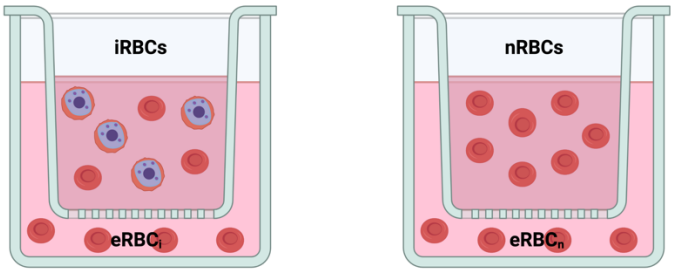

Figure 2.5.6. Transwell plate set-up. nRBCs collected from a donor were loaded into the bottom compartment of the transwell and exposed to iRBCs from culture in the top compartment of the transwell ($eRBCs_i$). For a control, fresh RBCs collected from a donor added to both the top (nRBCs) and bottom ($eRBCs_n$) compartments of the transwell. Made in BioRender.com

After 48 hours, cells from each compartment were smeared to ensure the parasites were viable in the top compartment and that there was no sign of cell damage or leaked iRBCs in the lower compartment. Each compartment was re-suspended into a 15 mL conical and spun down at 448 g for 5 minutes. Cell concentration was calculated for each compartment using a hemocytometer and purity for the iRBC chambers was calculated from the thin smear. The RBC populations were then ready for experimentation in the total IgG flow cytometry or opsonic phagocytosis assays.

To stain nRBCs, the lipid stain Vybrant DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate salt, V22997; Invitrogen) was used. In optimization experiments, the DiD stain was shown to be stable and did not leach out of RBCs after multiple washes and incubations. RBC pellets were stained with 40 µL DiD at a 1/10 dilution in 2% FBS-1X PBS, then incubated in the dark at 37ºC for an hour. After incubation, ~0.5 mL 2% FBS were added to the pellet and spun down at 1008 g for 5 minutes. The supernatant was aspirated, and the pellet was washed 2-3 more times with 2% FBS until the supernatant appeared to be without excess stain.

Chapter 3 – Serology characterisation of the SMI cohort

3.1 Introduction

To begin the characterisation of the SMI cohort and investigate associations with pregnancy outcomes, levels of IgG and IgM-VAR2CSA antibodies were measured. IgG are likely the primary antibody response in PAM (Elliott et al., 2005) and VSA_{PAM} antibody levels typically increase with gravidity in high transmission settings (Fried, Nosten, et al., 1998; O'Neil-Dunne et al., 2001; Staalsoe, Shulman, Bulmer, et al., 2004). Women with low levels of VSA_{PAM} IgG were previously shown to have both lower hemoglobin levels and give birth to infants with lower birthweights (Staalsoe, Shulman, Bulmer, et al., 2004), suggesting a protective effect of the VSA_{PAM} IgG. However, a meta-analysis revealed that VSA_{PAM} IgG are more likely to be markers of infection, rather than a source of protection (Cutts et al., 2020). Thus, we investigated the levels of IgG against recombinant full-length VAR2CSA, kindly shared by Dr. Ali Salanti, in the serum collected from the SMI cohort to determine if these levels were associated with infection history and/or pregnancy outcomes.

As the body of literature on the humoral response to PAM and associations with pregnancy outcomes focuses on *P. falciparum* infections in high transmission areas, primarily in sub-Saharan Africa, we were interested in how our observations would differ as our study was conducted in Colombia, where both *P. falciparum* and *P. vivax* co-circulate, and transmission is low, with the entomological inoculation rate recorded as 3.6 in 2009 (Naranjo-Diaz et al., 2013). Additionally, all the women in our cohort developed SMIs, not microscopic infections, which

often go untreated in rural areas. Thus, we were also interested in the relationship between VSA_{PAM} antibodies and adverse pregnancy outcomes when only SMIs occurred during pregnancy.

The measurement of IgG-VAR2CSA antibodies by ELISA was previously performed with a past aliquot of recombinant full-length VAR2CSA, and no association was observed between IgG-VAR2CSA levels and pregnancy outcomes (Gavina et al., 2018). However, we replicated these data, as we had a new aliquot of VAR2CSA (fresh aliquots of this protein are typically provided to our lab every 1-1.5 years). Additionally, the serum samples collected from the SMI cohort were stored at -80^{o}C for several years since those previous ELISA experiments were conducted and overall sera reactivity to VAR2CSA may have decreased. Thus, to properly compare results from the IgG ELISA to the IgM and C1q fixation ELISAs, the IgG ELISAs were repeated.

We hypothesized that variation in IgG levels within the SMI cohort may reflect differences in infection history (Fried et al., 2018), but not gravidity. Previously, our laboratory observed that elevated IgG-VAR2CSA levels were not associated with increasing gravidity (Gavina et al., 2018), which differs from what is typically observed in high transmission settings (Fried et al., 2018). This may be due to cross-reactivity between *P. vivax* and *P. falciparum* in Colombia, where prior infections with *P. vivax* outside of pregnancy contribute to VAR2CSA antibodies (Gnidehou et al., 2019). Thus, primigravid women may have IgG-VAR2CSA levels that do not differ significantly from secundigravid or multigravid women.

Less is known about the role of IgM in PAM. Several studies showed no significant difference in IgM levels between pregnant women and non-pregnant controls (Elliott et al., 2005; Leoratti et al., 2008) and IgM non-specifically competes and binds to IgG binding sites on VAR2CSA, potentially hindering a protective function of IgG antibodies (Barfod et al., 2011). Further research is needed to understand the role of IgM in PAM; thus, we included the measurement of IgM-VAR2CSA antibody levels by ELISA in our characterisation of the SMI cohort. Additionally, the associations between IgG and IgM levels were investigated at study enrollment and delivery. In response to *P. falciparum* and *P. vivax* infections, a meta-analysis revealed that IgG responses to VAR2CSA predominate but IgM responses are also elicited throughout the course of infection (reviewed in (Cutts et al., 2014)).

Finally, we tested for total IgG levels against VSA_{PAM} expressed by iRBCs grown in culture. These cultures were routinely selected for binding to CSA, thus we expected to see variable antibody staining among the cohort against the VSA expressed by the parasite. By observing staining with the VAR2CSA Rab, we confirmed VAR2CSA was highly expressed by iRBCs, but other VSAs may also be expressed. We hypothesized that we would observe similarities between the IgG-VAR2CSA ELISA and the flow cytometry assay, but that the flow cytometry assay would produce a more accurate representation of the humoral response to PAM, as whole iRBCs were used as an antibody target instead of recombinant protein coated on a plate. Thus, using flow cytometry to test for antibody staining against iRBCs better replicates the antibody response to placental parasites *in vivo*.

3.2 Evaluation of IgG-VAR2CSA antibody levels by ELISA

3.2.1. IgG-VAR2CSA levels in serum collected from mothers at study enrollment, peripherally from mothers at delivery, and placentas at delivery

We began serum characterisation of the SMI cohort by testing for IgG-VAR2CSA antibodies with ELISA. Due to limited serum availability, the full SMI cohort was not tested, but instead serum samples collected peripherally from 44 and 39 mothers were tested at enrollment and delivery, respectively, and 36 serum samples collected from the placenta at delivery. The cohort is described in Table 3.1. The variable levels of IgG-VAR2CSA antibodies at each collection point are shown in Figure 3.2.1. At enrollment, 53% of women (23/44) were seropositive for IgG-VAR2CSA antibodies. At delivery, 23% (9/39) of mothers were seropositive for IgG-VAR2CSA (Figure 3.2.2). IgG-VAR2CSA levels in serum collected peripherally from mothers were significantly lower at delivery than enrollment (A, Figure 3.2.2, paired t-test, $p<0.0001$). In serum collected from placentas at delivery, 50% (18/36) of serum samples tested positive for IgG-VAR2CSA. This differed significantly from IgG-VAR2CSA levels in serum collected peripherally from mothers at delivery (B, Figure 3.2.2, paired t-test, $p<0.0001$). When IgG-VAR2CSA levels were analysed by gravidity, no significant difference in levels was observed at any collection point between gravidities, which was previously observed (Gavina et al., 2018) (Figure 3.2.3 for serum collected at enrollment, Kruskal-Wallis test, $p=0.73$).

Table 3.1. Characteristics of the SMI cohort

Code	Age	Number of pregnancies	Infection at enrollment	Post-enrollment infection(s)	Infection at delivery	Infection in placenta	Pregnancy outcomes[1]
2	20	2	*P. vivax*	Mix	None	None	Healthy
4	16	1	None	Mix	None	None	Healthy
11	22	2	*P. vivax*	*P. vivax*	None	None	Anemic
12	20	1	None	*P. vivax,* mix	None	*P. falciparum*	LBW, PTB
16	20	2	None	None	*P. falciparum*	Mix	Anemic
23	21	3	None	None	Mix	None	Anemic
25	24	4	None	*P. vivax*	None	Mix	Healthy
27	36	3	None	None	*P. vivax*	None	Healthy
28	21	2	None	None	None	None	Healthy
45	15	1	*P. falciparum*	None	None	None	Healthy
51	37	6	*P. vivax*	*P. vivax*	*P. vivax*	None	Anemic
60	20	2	*P. vivax*	None	None	None	Healthy
61	39	13	None	*P. vivax*	None	None	PTB
88	31	4	None	Mix	None	None	Anemic, PTB
92	17	1	None	None	*P. vivax*	None	Anemic
108	22	3	None	None	*P. vivax*	None	Anemic
124	36	9	None	*P. falciparum*	None	None	Healthy
131	15	1	None	*P. falciparum*	None	None	Anemic
134	34	4	*P. vivax*	None	None	None	Healthy
141	25	4	*P. falciparum*	None	None	None	Healthy
146	16	1	None	None	*P. vivax*	None	Healthy
154	30	1	None	*Vivax*	None	None	Anemic
158	15	2	*P. falciparum*	None	None	None	Healthy
175	20	1	None	*P. falciparum*	None	*P. vivax*	LBW
188	34	3	None	None	None	None	Anemic, PTB
212	36	9	None	*P. vivax*	None	None	Healthy
231	21	1	None	*P. vivax*	None	None	Anemic
235	19	3	*P. vivax*	None	None	*P. falciparum*	Healthy
241	20	1	None	None	None	None	LBW, anemic
273	26	1	None	None	*P. falciparum*	Mix	Healthy
286	17	1	None	None	None	None	Anemic
290	32	4	None	*P. falciparum*	None	*P. falciparum*	Healthy
302	25	1	None	None	*P. falciparum*	None	Healthy
306	33	2	None	None	*P. falciparum*	None	Healthy
317	17	1	None	*P. falciparum*	None	None	Healthy
320	24	1	*P. falciparum*	None	*P. falciparum*	None	Healthy
321	19	1	None	None	None	None	LBW, PTB
322	19	1	None	None	*P. falciparum*	None	Healthy
326	15	1	None	*P. falciparum*	None	*P. falciparum*	Anemic, PTB
332	15	1	None	None	None	None	Healthy
338	14	1	None	None	Mix	None	PTB
339	25	2	None	*P. falciparum*	None	None	Anemic
349	28	4	None	*P. vivax*	None	None	Anemic
358	23	2	None	*P. falciparum*	None	None	Healthy
369	22	2	None	*P. falciparum*	None	None	LBW, PTB

[1]Outcomes include low birthweight (LBW), maternal anemia, and preterm birth (PTB)

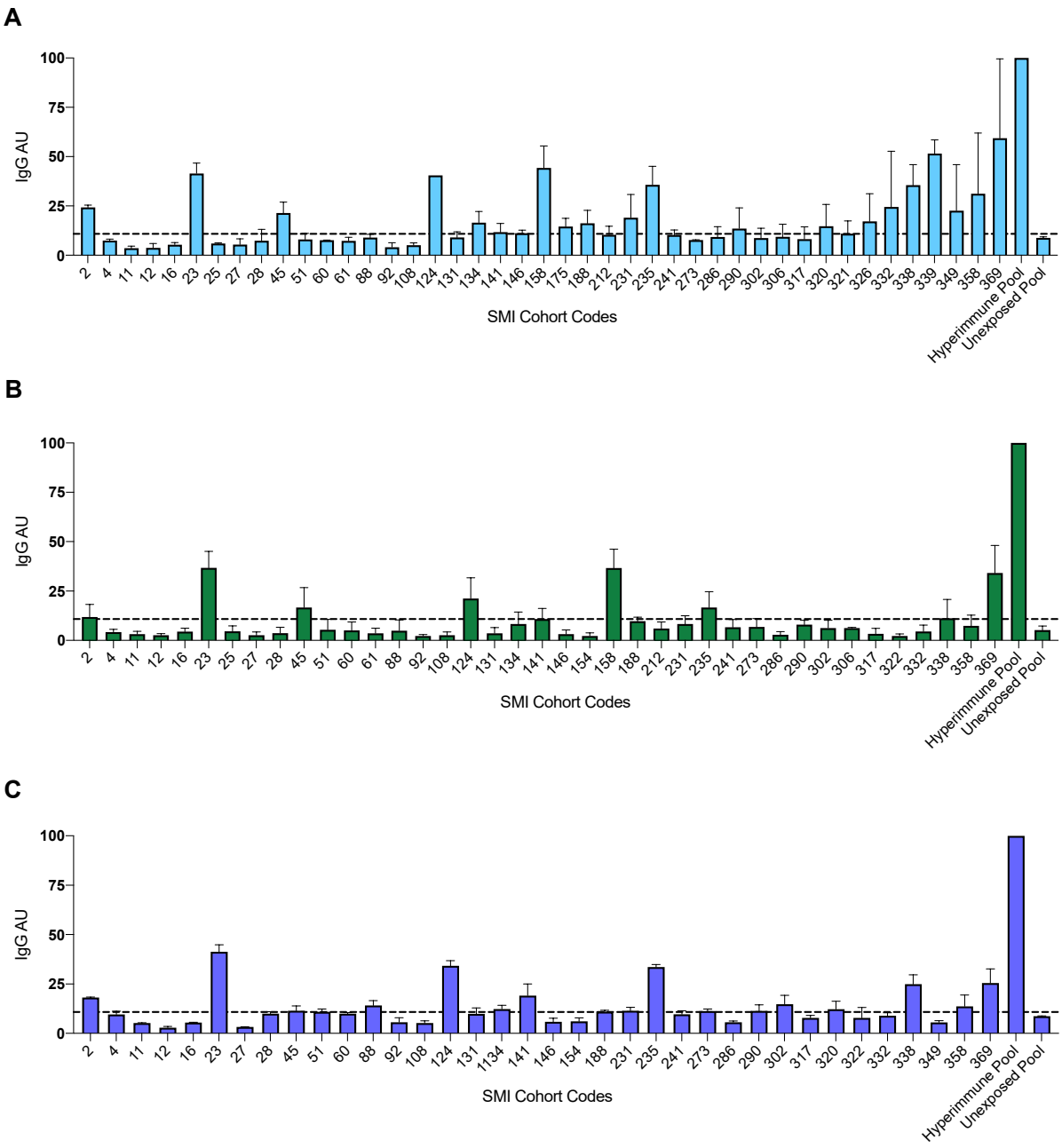

Figure 3.2.1. Variable IgG-VAR2CSA levels in serum collected from mothers at study enrollment (A), peripherally at delivery (B), and from placentas at delivery (C). Horizontal line indicates the IgG-VAR2CSA cut-off (10.8 AU). Error bars show standard deviation calculated from repeating the experiment twice on separate days.

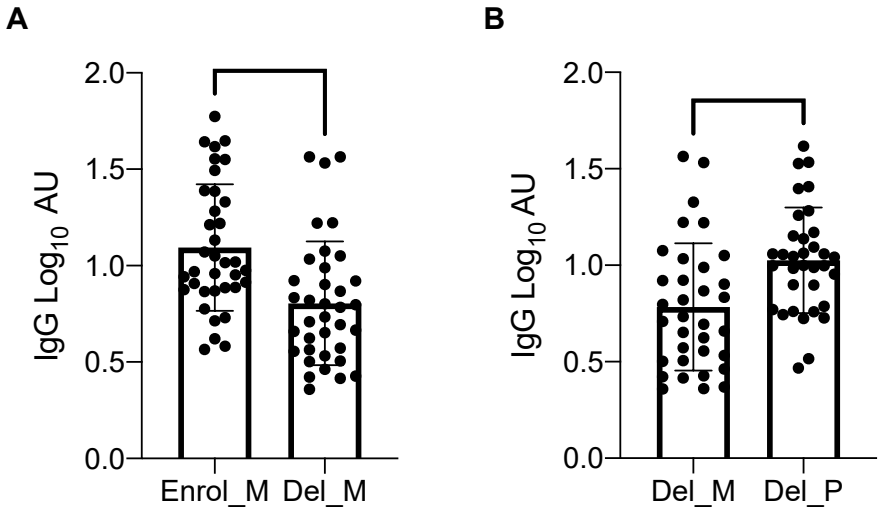

Figure 3.2.2. IgG-VAR2CSA levels differ significantly between collection points. Paired t-tests were performed to compare IgG-VAR2CSA levels in serum collected from mothers at enrollment (Enrol_M) and peripherally at delivery (Del_M) (A, n=36, p< 0.0001), and serum collected peripherally from mothers and from the placenta at delivery (Del_P) (B, n=34, p< 0.0001).

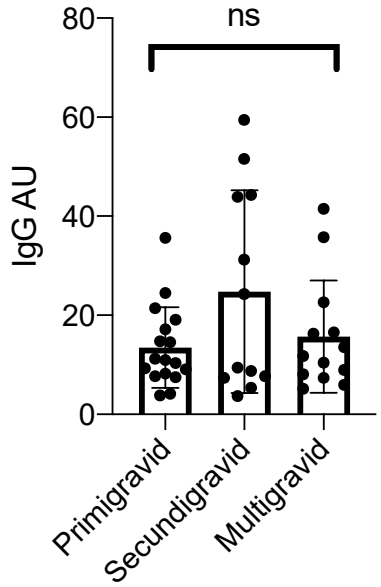

Figure 3.2.3. No association is present between IgG-VAR2CSA levels in serum collected at study enrollment and gravidity. Kruskal-Wallis test (p=0.73) was performed to test for associations between IgG-VAR2CSA levels in serum collected at study enrollment and gravidity.

We also investigated IgG-VAR2CSA levels in serum samples collected from women who tested negative for malaria infection by qPCR at study enrollment and later developed a SMI during pregnancy (n=28) to determine if antibody boosting occurred due to infection during pregnancy. No boosting was observed, but similarly to the whole cohort that contained women who tested positive for malaria at enrollment, IgG-VAR2CSA levels decreased significantly from study enrollment to delivery in serum collected peripherally from mothers, as shown in Figure 3.2.4 (Wilcoxon test, $p<0.0001$).

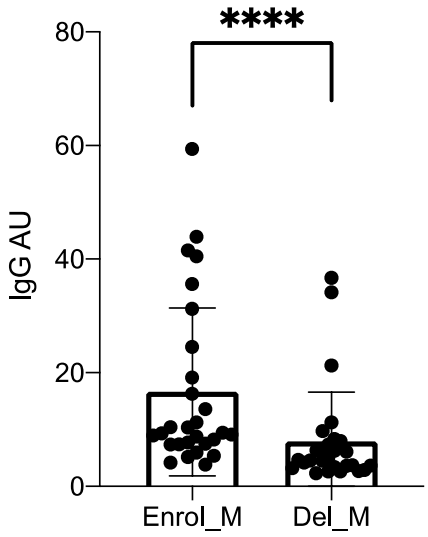

Figure 3.2.4. No boosting of IgG-VAR2CSA levels is observed from study enrollment to delivery among women who were negative for malaria infection at study enrollment but developed at least one SMI later in pregnancy. A Wilcoxon test was run to determine significance (n=28, $p<0.0001$) between IgG-VAR2CSA levels at study enrollment (Enrol_M) and delivery (Del_M) in serum samples collected peripherally from mothers.

Overall, the individual levels of IgG-VAR2CSA seroreactivity for each serum sample in this new analysis were significantly lower than IgG-VAR2CSA seroreactivity previously reported (Gavina et al., 2018) (shown in Figure 3.2.5 for serum collected at enrollment, Wilcoxon test, p=0.0001). However, when seropositivity was compared between the two data sets, the number of serums samples that were seropositive were not significantly different (X^2 (1, N = 41) = 0.049, p=0.82)).

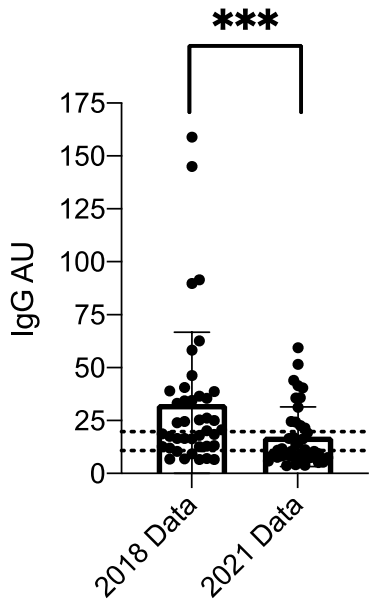

Figure 3.2.5. IgG-VAR2CSA seroreactivities significantly decreased from previous data set (2018) and current data set (2021). The IgG-VAR2CSA ELISA was repeated to compare current seroreactivities to a data set previously published (Gavina et al., 2018) and Wilcoxon test was run to determine significance (p=0.0001). Top horizontal line indicates the cut-off point for the 2018 data set (19.7 AU) and bottom horizontal line indicates the cut-off point for the 2021 data set (10.8 AU).

3.2.2. IgG-VAR2CSA levels and associations with pregnancy outcomes

To test if levels of IgG-VAR2CSA antibodies were associated with improved pregnancy outcomes, we compared levels to infant birthweight, maternal hemoglobin levels at delivery, and gestational age. In our previous analysis, we did not observe significant associations between IgG-VAR2CSA levels at enrollment and pregnancy outcomes (Gavina et al., 2018). Similarly, no significant associations were observed between pregnancy outcomes and IgG-VAR2CSA levels at any collection point (shown in Figure 3.2.6 for serum samples collected at enrollment, Figure 3.2.7 for serum samples collected peripherally from mothers at delivery, and Figure 3.2.8 for serum samples collected from placentas at delivery).

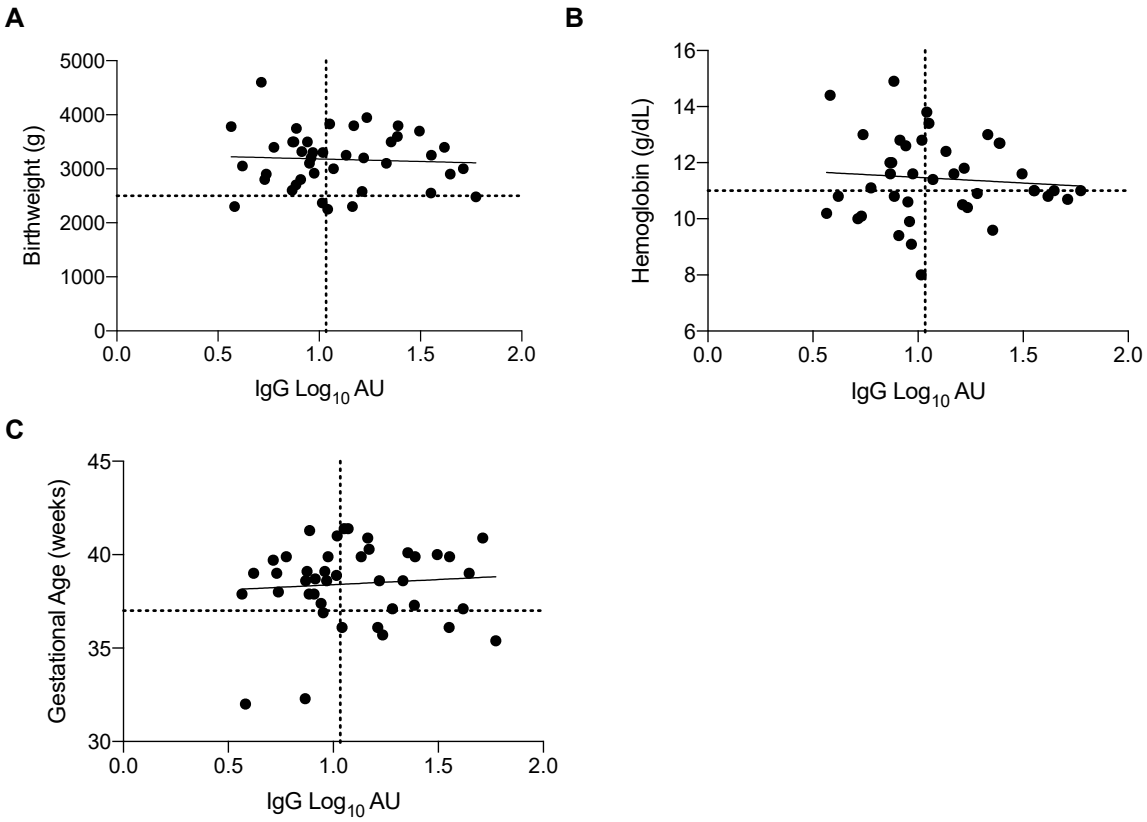

Figure 3.2.6. IgG-VAR2CSA levels in serum collected from mothers at study enrollment did not correlate with pregnancy outcomes at delivery. Pearson (A, B) and Spearman (C) correlations were performed to test for an association between serum VAR2CSA-specific IgG antibody levels and infant birthweight (A, p=0.73), maternal hemoglobin at delivery (B, p=0.58), and gestational age (C, p=0.61). Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for IgG seropositivity (10.8 AU).

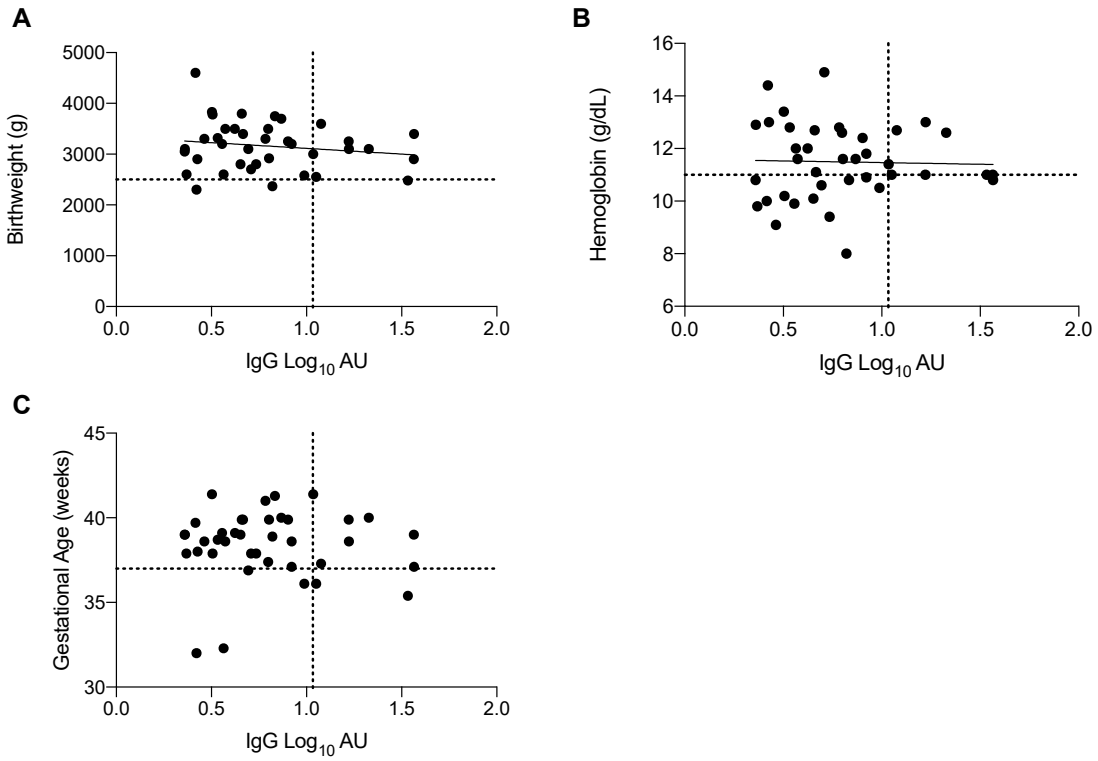

Figure 3.2.7. **IgG-VAR2CSA levels in serum collected peripherally from mothers at delivery did not correlate with pregnancy outcomes at delivery**. Pearson (A, B) and Spearman (C) correlations were performed to determine an association between serum VAR2CSA-specific IgG antibody levels and infant birthweight (A, p=0.34), maternal hemoglobin at delivery (B, p=0.86), and gestational age (C, p=0.98). Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for IgG seropositivity (10.8 AU).

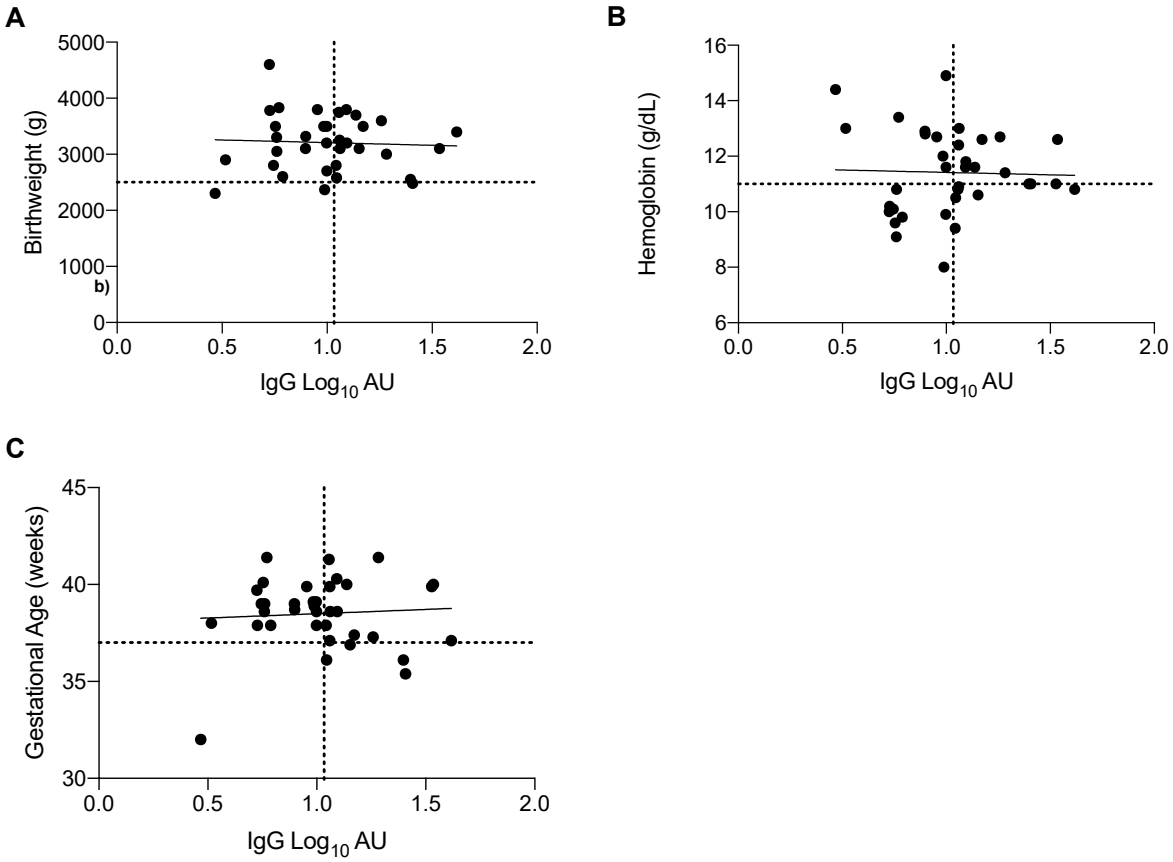

Figure 3.2.8. IgG-VAR2CSA levels in serum collected from placentas at delivery did not correlate with pregnancy outcomes at delivery. Pearson (A, B) and Spearman (C) correlations were performed to determine an association between serum VAR2CSA-specific IgG antibody levels and infant birthweight (A, p=0.78), maternal hemoglobin at delivery (B, p=0.86), and gestational age (C, p=0.71). Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for IgG seropositivity (10.8 AU).

3.3 Detection of total VSA_{PAM} IgG against iRBCs with flow cytometry

3.3.1. VSA_{PAM} IgG levels observed in serum collected from mothers at study enrollment

Total VSA_{PAM} IgG levels against VSA expressed by iRBCs were tested in flow cytometry. Serum samples collected at study enrollment from 44 mothers in the SMI cohort were tested. Only one serum sample was seropositive in this assay, as shown in Figure 3.3.1. This serum sample (number 92) was not seropositive in the IgG-VAR2CSA ELISA. During pregnancy, this individual was qPCR negative for malaria at study enrollment but became positive for a *P. vivax* infection at delivery. Since only one individual sample was seropositive by flow cytometry, associations with gravidity, pregnancy outcomes, and other serology assays were not investigated.

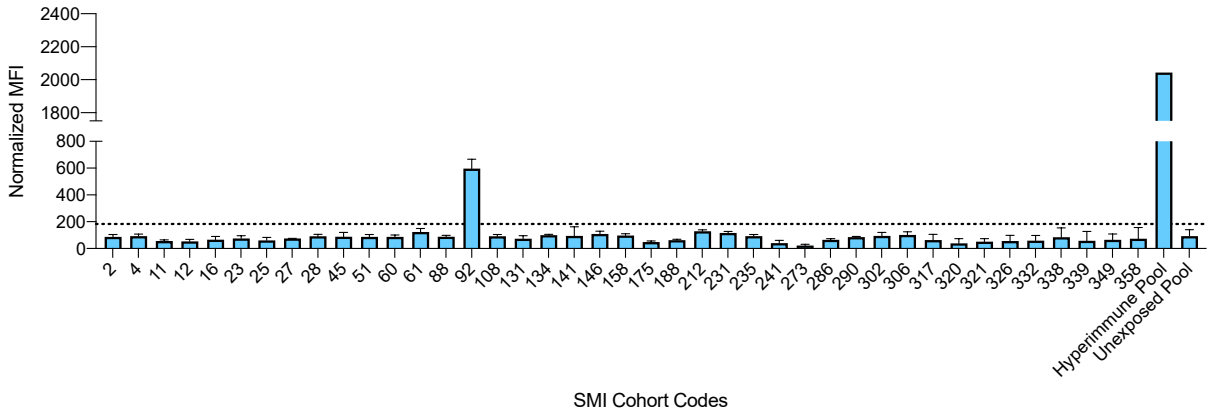

Figure 3.3.1. Total IgG levels in serum collected from mothers at enrollment are overall seronegative against whole iRBCs. Total IgG levels were measured in flow cytometry against whole iRBCs at the late trophozoite/schizont phase. Horizontal dotted line indicates the cut-off point for seropositivity (182.3 MFI). Error bars show standard deviation calculated based on experiments performed twice on separate days.

3.4 Evaluation of IgM-VAR2CSA antibody levels by ELISA

3.4.1. IgM-VAR2CSA levels in serum collected from mothers at study enrollment, peripherally from mothers at delivery, and placentas at delivery

Next, we assessed IgM-VAR2CSA levels within the SMI cohort with ELISA. The variable levels of IgM-VAR2CSA antibodies at each collection point are shown in Figure 3.4.1. At enrollment, 89% of women (39/44) were seropositive for IgM-VAR2CSA antibodies. At delivery, seropositivity significantly decreased to 31% (12/39) (A, Figure 3.4.1. paired t-test, $p<0.0001$). In serum collected from placentas at delivery, 31% (11/36) of serum samples tested positive for IgM-VAR2CSA. There was no significant difference in IgM-VAR2CSA levels between serum collected peripherally from the mother and from the placenta (B, Figure 3.4.2). When IgM-VAR2CSA levels were analysed by gravidity, no significant difference in levels was observed at any collection point between gravidities (shown in Figure 3.4.3 for serum collected at enrollment).

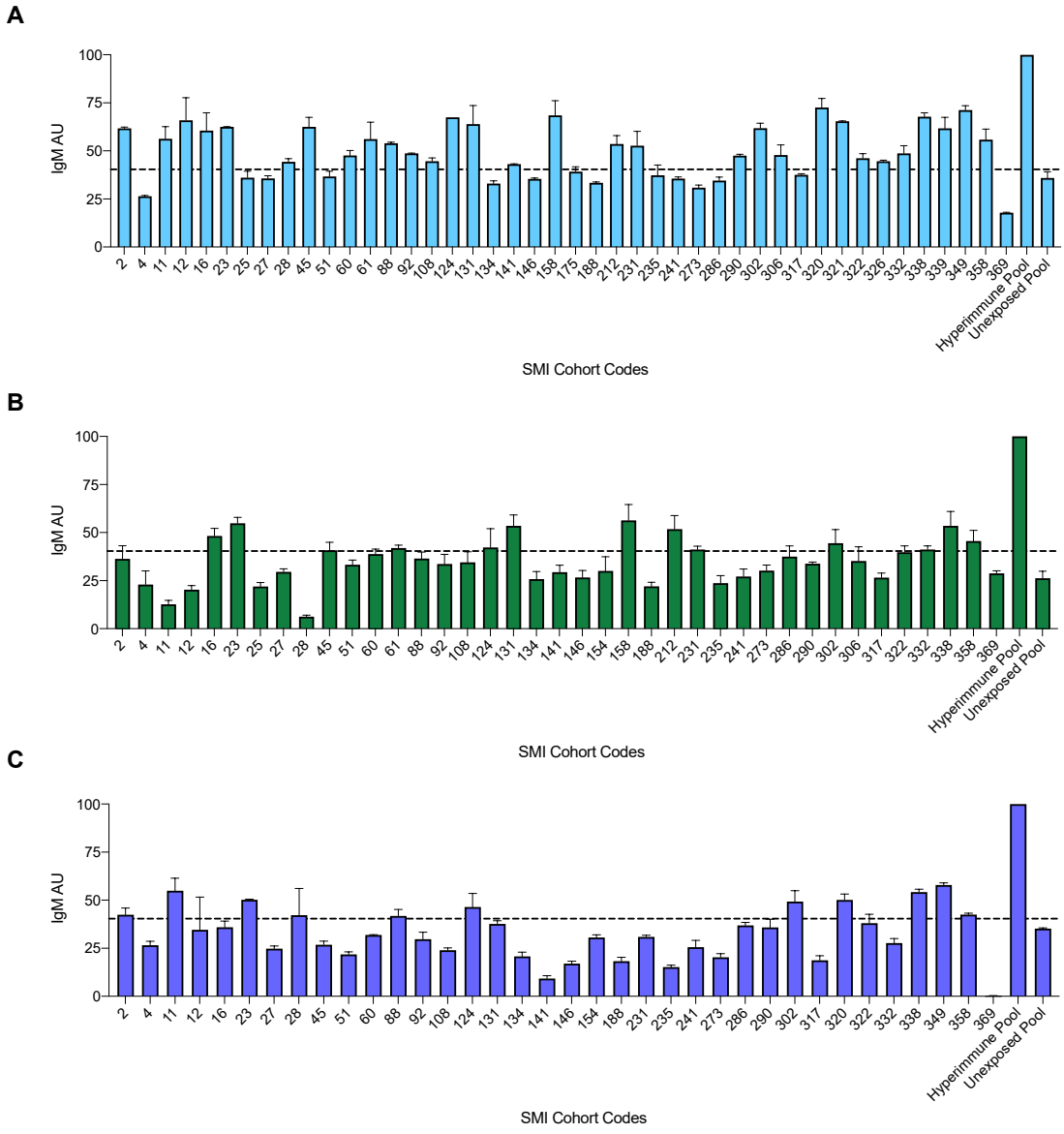

Figure 3.4.1. Variable IgM-VAR2CSA levels in serum collected from mothers at study enrollment (A), peripherally at delivery (B), and from placentas at delivery (C). Horizontal line indicates the IgM-VAR2CSA cut-off (40.4 AU). Error bars show standard deviation calculated from repeating the experiment twice on separate days.

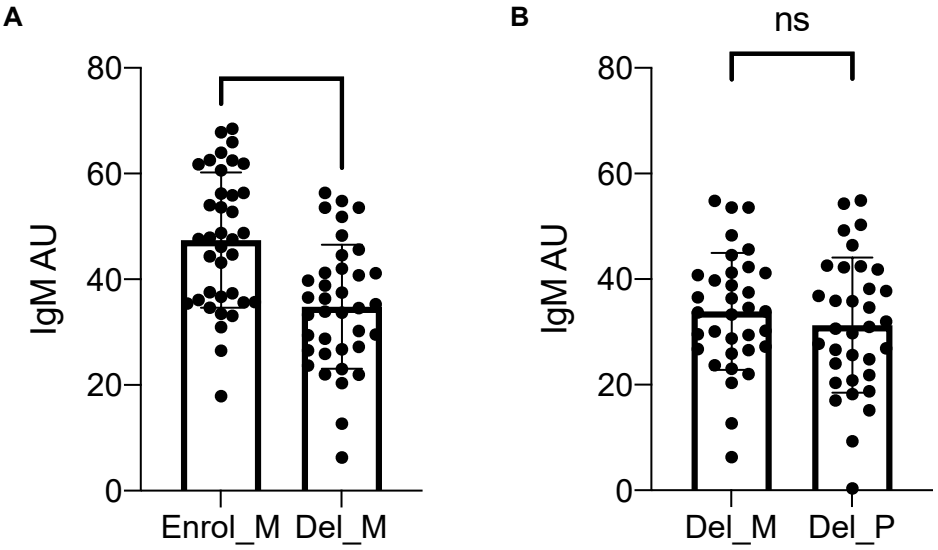

Figure 3.4.2. IgM-VAR2CSA levels differ significantly between serum collected peripherally from mothers at enrollment and delivery. Paired t-tests were performed to determine the significance in IgM-VAR2CSA levels in serum collected from mothers at enrollment and peripherally at delivery (Del_M) (A, n=36, p< 0.0001), and serum collected peripherally from mothers and from the placenta at delivery (Del_P) (B, n=34, p=0.26).

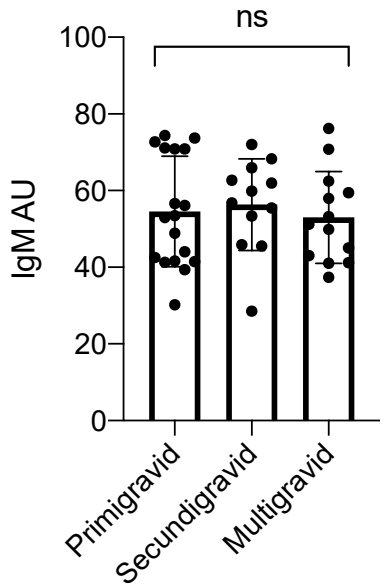

Figure 3.4.3. No association is present between IgM-VAR2CSA levels in serum collected at study enrollment and gravidity. Kruskal-Wallis test (p=0.61) was performed to determine association between IgM-VAR2CSA levels in serum collected at study enrollment and gravidity.

We also investigated IgM-VAR2CSA levels in serum samples collected from women who tested negative for malaria infection by qPCR at study enrollment and developed at least one SMI later in pregnancy (n=29) to determine if antibody boosting occurred due to infection during pregnancy. No boosting was observed, but IgM-VAR2CSA levels decreased significantly from study enrollment to delivery in serum collected peripherally from mothers, as shown in Figure 3.4.4 (Paired t-test, $p<0.0001$).

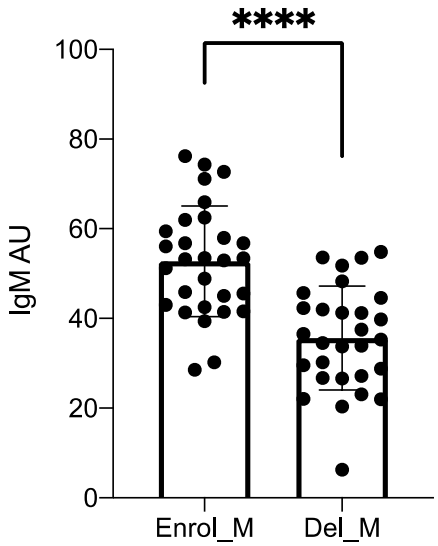

Figure 3.4.4. No boosting of IgM-VAR2CSA levels is observed from study enrollment to delivery among women who were negative for malaria infection at study enrollment but developed at least one SMI later in pregnancy. A paired t-test was run to determine significance (n=29, $p<0.0001$) between IgM-VAR2CSA levels at study enrollment (Enrol_M) and delivery (Del_M) in serum samples collected peripherally from mothers.

3.4.2. IgM-VAR2CSA levels and associations with pregnancy outcomes

To test if IgM-VAR2CSA antibodies were associated with improved pregnancy outcomes, we compared levels to infant birthweight, maternal hemoglobin levels at delivery, and gestational age. No significant association was observed between pregnancy outcomes and IgM-VAR2CSA levels at any collection point (shown in Figure 3.4.5 for serum samples collected at enrollment, Figure 3.4.6. for serum samples collected peripherally from mothers at delivery, and Figure 3.4.7. for serum samples collected from placentas at delivery).

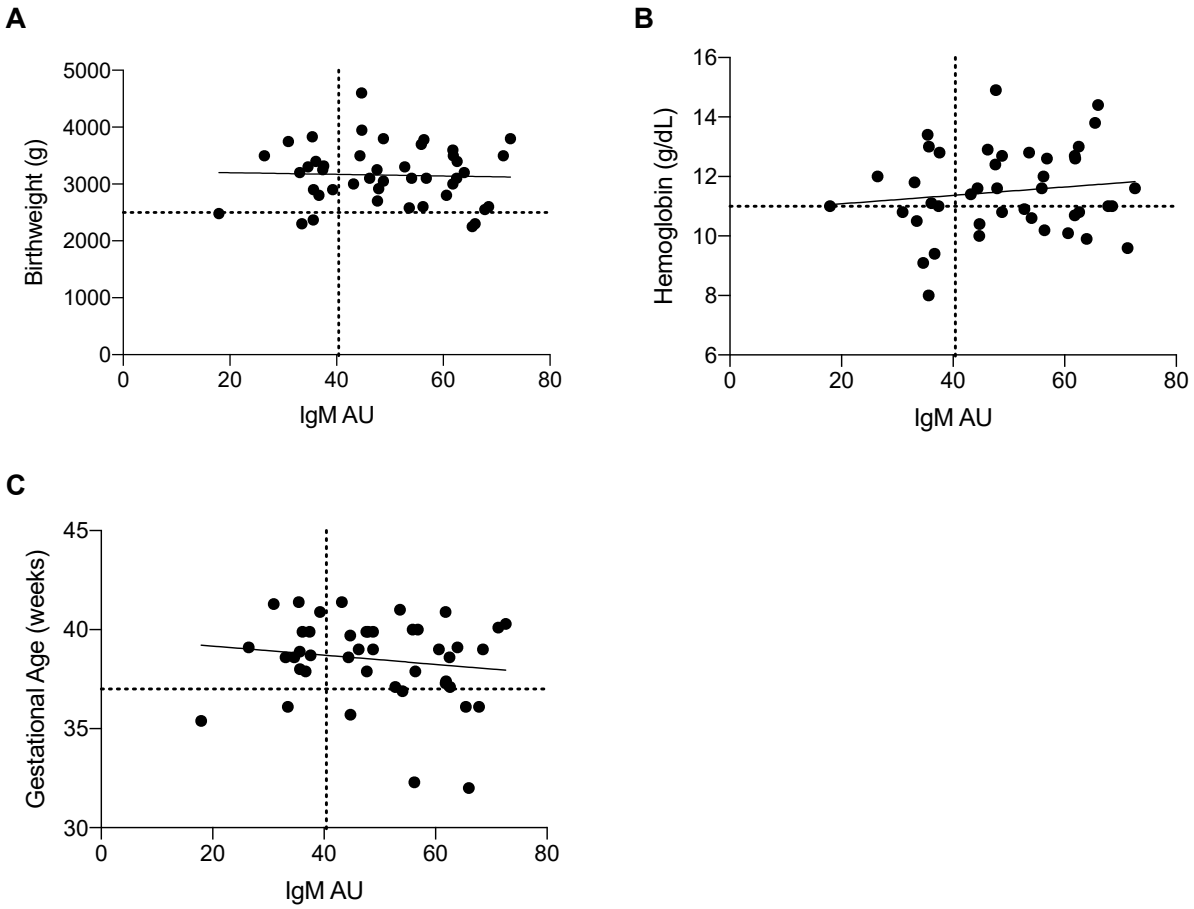

Figure 3.4.5. IgM-VAR2CSA levels in serum collected from mothers at study enrollment did not correlate with pregnancy outcomes at delivery. Pearson correlations were performed to test for an association between serum VAR2CSA-specific IgM antibody levels and infant birthweight (A, p=0.81), maternal hemoglobin at delivery (B, p=0.40), and gestational age (C, p=0.34). Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for IgM seropositivity (40.4 AU).

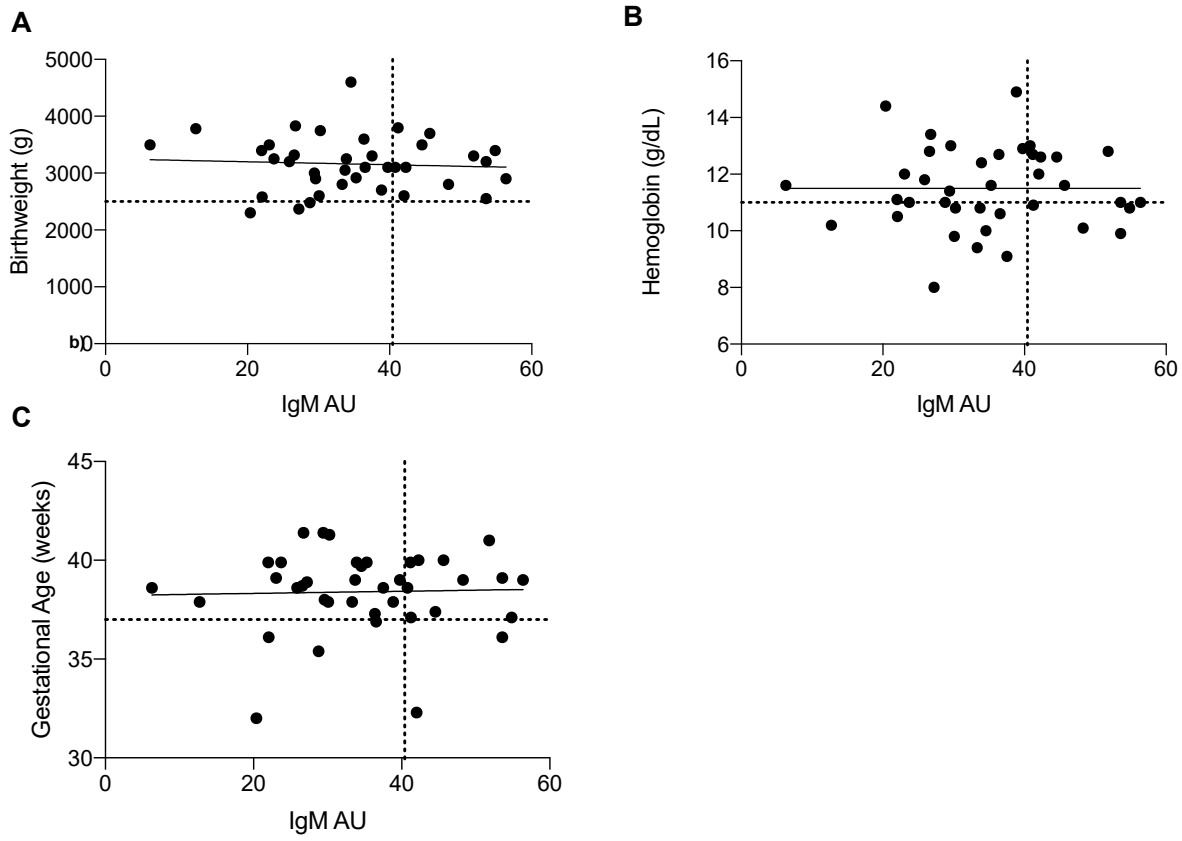

Figure 3.4.6. IgM-VAR2CSA levels in serum collected peripherally from mothers at delivery did not correlate with pregnancy outcomes at delivery. Pearson correlations were performed to test for an association between serum VAR2CSA-specific IgM antibody levels and infant birthweight (A, p=0.71), maternal hemoglobin at delivery (B, p=0.99), and gestational age (C, p=0.85). Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for IgM seropositivity (40.4 AU).

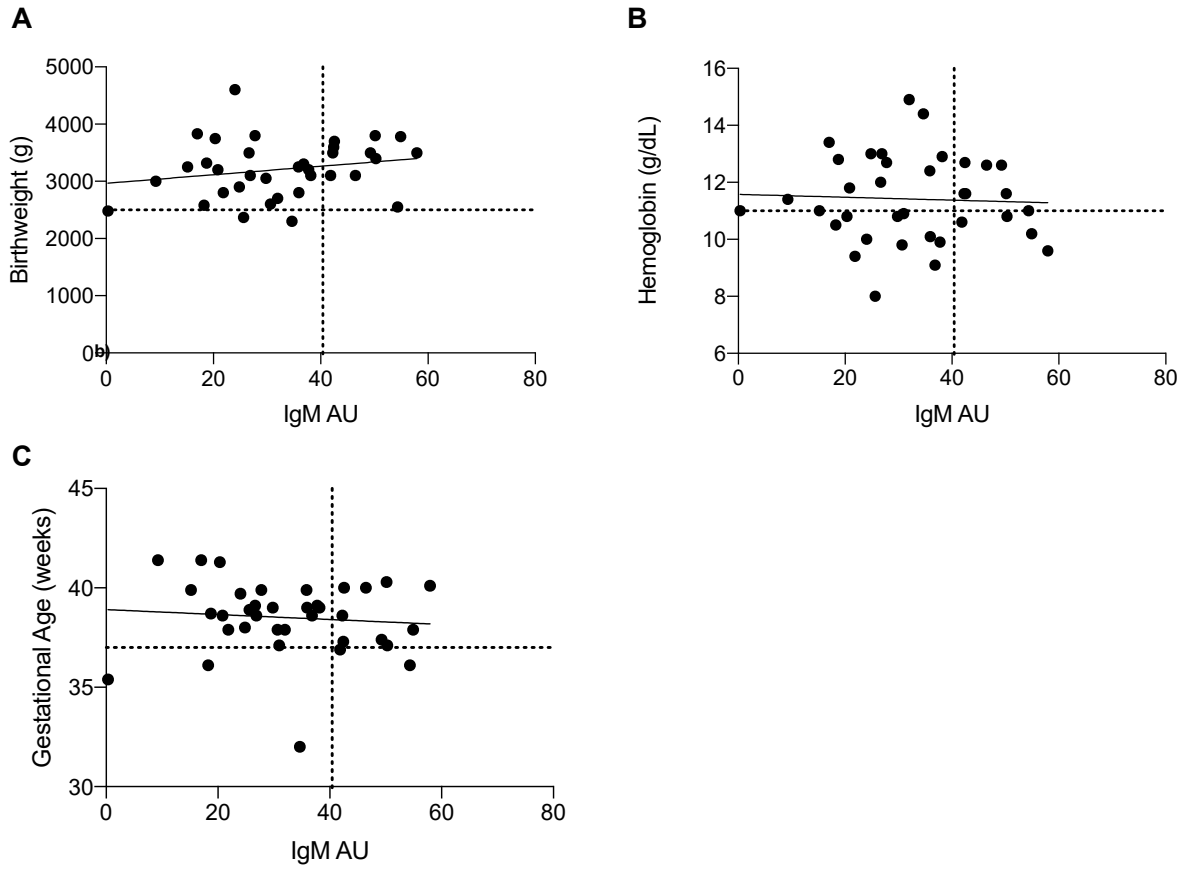

Figure 3.4.7. IgM-VAR2CSA levels in serum collected from placentas at delivery did not correlate with pregnancy outcomes at delivery. Pearson correlations were performed to test for an association between serum VAR2CSA-specific IgM antibody levels and infant birthweight (A, p=0.24), maternal hemoglobin at delivery (B, p=0.79), and gestational age (C, p=0.60). Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for IgM seropositivity (40.4 AU).

3.4.3. Comparison of IgG and IgM-VAR2CSA levels

Association between levels of IgG and IgM-VAR2CSA antibodies were investigated to determine if there was an association between IgG and IgM-VAR2CSA levels at each collection point. As shown in Figure 3.4.8, no significant association was observed between IgG and IgM-VAR2CSA levels at study enrollment or in serum collected from placentas at delivery, but a positive association was observed in serum collected peripherally from mothers at delivery.

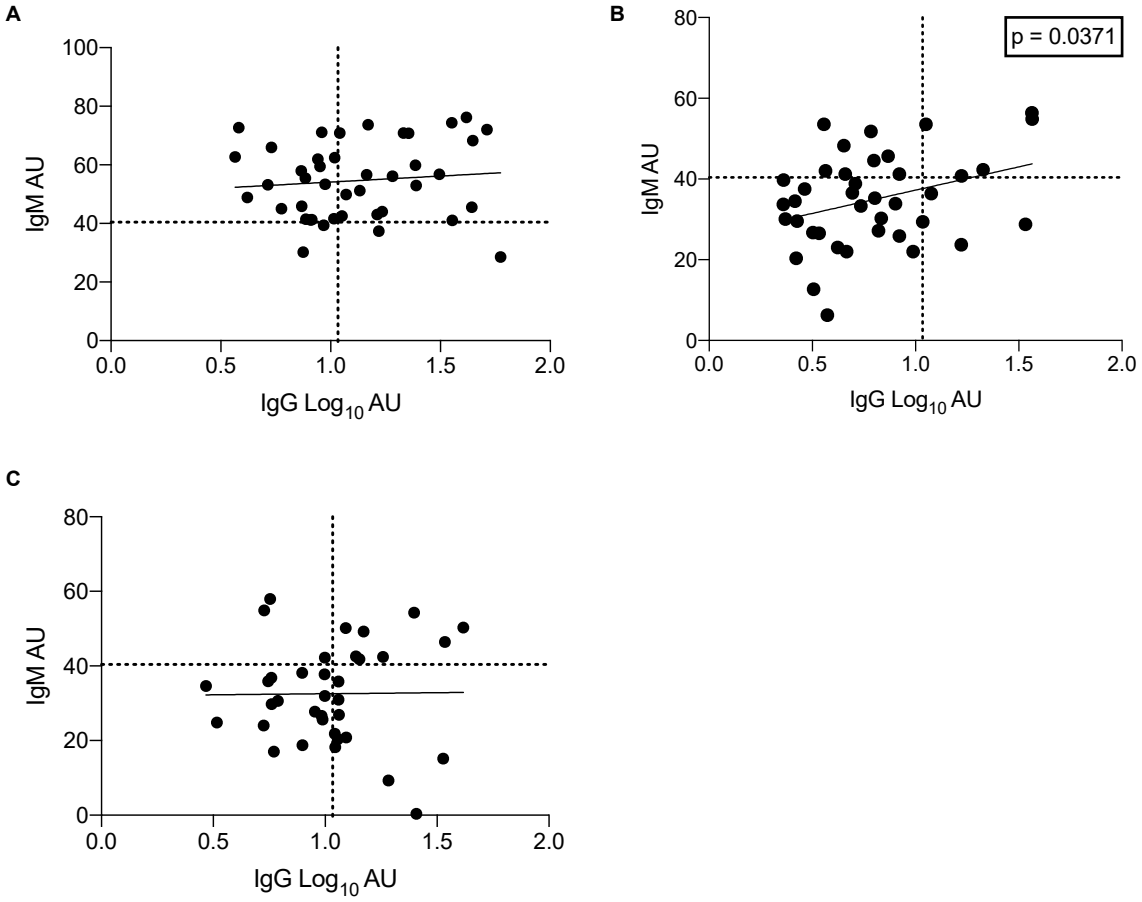

Figure 3.4.8. IgG-VAR2CSA levels correlate with IgM-VAR2CSA levels in serum collected peripherally from mothers at delivery, but not at study enrollment or in serum collected from placentas at delivery. Pearson correlations were performed to test for an association between IgG and IgM-VAR2CSA levels in serum collected from mothers at study enrollment (A, p=0.52), peripherally at delivery (B, p=0.037), and collected from placentas at delivery (C, p=0.95). Horizontal dotted lines indicate the cut-off point for IgM-VAR2CSA seropositivity (40.4 AU). Vertical dotted lines indicate the normalized cut-off point for IgG-VAR2CSA seropositivity (1.033 AU).

3.5 Chapter summary and brief discussion

Variable levels of both IgG and IgM-VAR2CSA levels were observed among the SMI cohort at the three collection points. Though individual sample seroreactivities were lower in this data set compared to the previous IgG-VAR2CSA data set (Gavina et al., 2018), there was no significiant difference in the proportion of seropositive samples. This may reflect a decrease in reactivity, as serum samples were stored for several years after the previous data set was recorded. But, this decrease in reactivity was proportional to the cut-off point, thus the new data set still accurately reported seropositivity. For future serology testing, it is important to note this decrease in seroreactivity if samples have been stored for several years.

In the new data set, IgG-VAR2CSA levels did not correlate with any pregnancy outcomes at any collection point, which was previously observed by our laboratory for IgG-VAR2CSA levels in serum samples collected from mothers at study enrollment (Gavina et al., 2018). Additionally, no boosting of IgG or IgM-VAR2CSA levels was observed among women who were originally negative for malaria infection at study enrollment but developed at least one SMI by delivery. This may be due to the nature of infections, as all the women in this cohort developed SMIs and were not smear positive by microscopy at any point in pregnancy. SMIs may only produce minimal changes in antibody levels during an infection, such that an association between antibody titres and a pregnancy outcome may not be observed.

Notably, the majority of women followed in this study did not suffer from an adverse pregnancy outcome, with 11% (5/45) delivering low birthweight infants, 36% (16/45)

developing anemia, and 16% (7/45) experiencing preterm births (Table 3.1). A SMI of *P. falciparum*, *P. vivax*, or a mixed infection may not have a clinical effect as detrimental as an infection detectable by microscopy, which would affect the association between antibody levels and pregnancy outcomes. Previous research is mixed on the association between SMIs and adverse pregnancy outcomes at delivery (Bardaji et al., 2017; Cohee et al., 2014; Cottrell et al., 2015), but more investigation is required to better understand the effect of SMIs outside of high transmission settings.

In the flow cytometry experiments, the overall low reactivity was unexpected, as it was hypothesized that the SMI cohort would show variable reactivity to iRBCs within the cohort and potentially correlate with the IgG-VAR2CSA ELISA data. These results may be due to low avidity antibodies present in the serum samples against VSA_{PAM} expressed by the iRBCs. As the assay requires 2-3 washes of pelleted iRBCs at each antibody staining step, the primary antibodies in the serum samples may have been washed away from the iRBCs, leaving minimal reactivity when the secondary and tertiary antibodies were added. Also, the iRBCs pellets often visibly decreased in size with each step of the assay, which may have resulted in loss of antibody-antigen complexes, also decreasing antibody staining. Finally, as this flow assay only assesses IgG binding using an IgG-specific secondary commercial antibody, IgM also present in the serum may interfere with IgG binding to whole iRBCs (Barfod et al., 2011).

In summary, IgG and IgM-VAR2CSA levels were not associated with infection history or with pregnancy outcomes and minimal staining of VSA_{PAM} antibodies was observed in flow cytometry. As we had already observed blocking activity by serum samples in this cohort

(Gavina et al., 2018), we continued the characterisation with assessments of antibody function, rather than levels, by testing the cohort for C1q fixation and activity in the opsonic phagocytosis assay.

Chapter 4 – Assessment of antibody-mediated effector mechanisms in serum collected from the SMI cohort

4.1 Introduction

In the previous chapter, we observed that both IgG and IgM-VAR2CSA titres were not associated with infection history nor were a correlate of protection in serum collected from the SMI cohort. However, our laboratory previously observed that the blocking activity in the inhibition of binding assay (IBA) of sera from this cohort positively associated with maternal hemoglobin levels at delivery (Gavina et al., 2018). In previous studies, this blocking activity was associated with higher infant birthweight at delivery and gestational age (Duffy & Fried, 2003; Ndam et al., 2015). Thus, we continued the characterisation of this cohort, turning to functional immune responses mediated by antibodies, rather than measurements of antibody levels.

First, we investigated the activation of the classical complement cascade by measuring fixation of the first component, C1q. This cascade begins when an antibody-antigen complex forms, either with IgG or IgM, and C1q is recruited (reviewed in (Noris & Remuzzi, 2013)). A cascade of cleavage and activation of various complement components occurs, producing proinflammatory anaphylatoxins (C3a and C5a), opsonins (C3b), and eventually resulting in the formation of the membrane attack complex (MAC), which inserts into the membrane of infected cells and lyses them (reviewed in (Noris & Remuzzi, 2013)). The other complement pathways are the alternative pathway, which begins with spontaneous lysis of the C3 component, and the lectin pathway, which begins when mannan-binding lectin (MBL) recognizes microbial

carbohydrates, leading to the cleavage of C2 and C4 (reviewed in (Noris & Remuzzi, 2013)). IgE antibodies cannot fix complement (reviewed in (Wills-Karp, 2007)), but IgA antibodies have been shown to activate the MBL pathway (Roos et al., 2001). However, IgA-C3 deposits are frequently observed in IgA nephropathy and used for diagnosis of this disease (Maillard et al., 2015).

In uncomplicated malaria in children, C1q fixation against merozoite antigens was associated with better health outcomes (Reiling et al., 2019). C1q fixation was also observed against sporozoite proteins and complement fixation was associated with protection against malaria in children (Kurtovic et al., 2018). Notably, the fixation of C1q was highly correlated with the formation of downstream complement products, including the MAC complex (Reiling et al., 2019). Thus, measuring the fixation of C1q is likely a reliable measurement of the activation of the complement system. Interestingly, both studies found that IgG seroreactivity was not necessarily predictive of the degree of complement fixation, suggesting that other factors may influence C1q fixation besides levels of antibody-antigen complexes (Kurtovic et al., 2018; Reiling et al., 2019).

C1q binding may be impacted by several factors that are important to consider, especially when working with the plate-based assay used to measure C1q fixation *in vitro*. The C1q component is made up of six globular heads with multiple heads needing to be engaged in order to activate the cascade (reviewed in (Kishore et al., 2004)). Thus, this could be accomplished with one IgM antibody, which has shown to be a more effective fixer of C1q than IgG when complexed with merozoite proteins (Boyle et al., 2019). C1q can also be fixed by IgG,

particularly the cytophilic subclasses IgG1 and IgG3 (reviewed in (Irani et al., 2015)). IgG3 antibodies show more flexibility in the hinge region for better complement binding to the antigen complex (Michaelsen et al., 1977). Less fixation is observed with IgG2 and IgG4, as differences in the C1q binding site (CH2 domain) decrease fixation abilities (reviewed in (Irani et al., 2015)). In our SMI cohort, we hypothesized that we would observe variable C1q fixation that may be independent of IgG seroreactivity.

The next antibody-mediated functional response we investigated was opsonic phagocytosis. This immune response may be an important function of VSA_{PAM} antibodies and a potential mediator of protection from poor pregnancy outcomes. Opsonic phagocytosis occurs when antibodies interact with phagocytes via the Fcγ receptors on these immune cells (reviewed in (Feng et al., 2021)). The cytophilic IgG1 and IgG3 subclasses activate opsonic phagocytosis to a greater extent than the non-cytophilic IgG2 and IgG4 subclasses, as these have a weaker affinity for Fcγ receptors (Keen et al., 2007). The activation of opsonic phagocytosis may also trigger a release of pro-inflammatory cytokines, enhancing the clearance of iRBCs (Osier et al., 2014). With the additional blocking function of VSA_{PAM} antibodies, together these responses may contribute to reducing iRBC placental sequestration, clearing iRBCs from the intervillous space, and preventing poor pregnancy outcomes.

In previous studies, protective associations were observed between opsonic phagocytosis activity and infant birthweight (Ataide et al., 2011) and maternal anemia (Chandrasiri et al., 2016; Chandrasiri et al., 2014; Feng et al., 2009; Jaworowski et al., 2009). These studies were conducted with pregnant women who experienced microscopic infections of PAM; thus, we

investigated if these protective associations were also observed with our cohort of women who only experienced SMIs during pregnancy. Overall, a low prevalence of adverse birth outcomes was observed in this cohort, which may reflect protection mediated by both blocking and opsonic antibodies.

The opsonic phagocytosis assay utilizes flow cytometry to measure engulfment of stained iRBCs in unstained human THP-1 monocytes. Despite low reactivity in the total IgG VSA_{PAM} flow cytometry assay, we hypothesized that we would observe variable opsonic phagocytosis activity in the cohort, as this flow cytometry assay measured a functional response, rather than antibody staining. Additionally, the opsonic phagocytosis assay only has three wash steps, rather than the repeated washes needed at each step of the total IgG VSA_{PAM} assay. Therefore, if the antibodies in this cohort had low avidity, they may be preserved throughout the assay until engulfment levels were measured on the flow cytometer.

Finally, we investigated a potential mechanism that may contribute to anemia in PAM. In uncomplicated malaria infections, the loss of nRBCs is a known cause of anemia. The loss of iRBCs, which occurs as the parasite continues its lifecycle, does not account for the total loss of RBCs (reviewed in (Akinosoglou et al., 2012)). In addition to the loss of iRBCs, non-infected RBCs (nRBCs) also decrease, potentially through bone marrow dysregulation or premature apoptosis (Jakeman et al., 1999; Totino et al., 2016). This has not yet been shown to occur in pregnancy but may contribute to the severity of maternal anemia.

The loss of nRBCs may be mediated by antigen transfer from iRBCs via the release of extracellular vesicles (EVs) (Babatunde et al., 2018). If parasite antigen attaches to nRBCs and is recognized by antibodies, the nRBC may become a target for antibody binding and antibody-mediated immune responses, such as the recruitment of phagocytes, resulting in the unnecessary loss of nRBCs. This has not been reported specifically in PAM. To address this, we used a transwell plate to expose iRBCs to nRBCs but kept the RBC populations separated by a filter. This allowed for the transfer of any extracellular vesicles, and cell populations could be easily isolated after exposure and used in further experiments (see Figure 2.4.6 in Chapter 2). After the incubation, nRBCs exposed to iRBCs ($eRBCs_i$) were used for total IgG flow cytometry and opsonic phagocytosis experiments with hyperimmune serum pools to test for antibody staining and/or engulfment of the eRBCs. As a control, nRBCs were also exposed to other nRBCs ($eRBCs_n$) in the transwell and tested in parallel.

4.2 The C1q fixation ELISA

4.2.1. C1q fixation levels in serum collected from the SMI cohort

The first functional mechanism we investigated was the fixation of C1q by immune complexes using ELISA. The C1q fixation assay was modified from protocols published previously (Reiling et al., 2019). With recombinant full-length VAR2CSA as the antigen target, we observed overall low ODs in this ELISA, compared to ODs typically observed in the IgG and IgM-VAR2CSA ELISAs. To confirm that a dynamic range of reactivity could still be observed in this assay despite the low ODs, we performed a titration with the hyperimmune UMG pool, with the UPG pool as the negative control (Figure 4.2.1). At four concentrations of primary serum, a dynamic range was still observed.

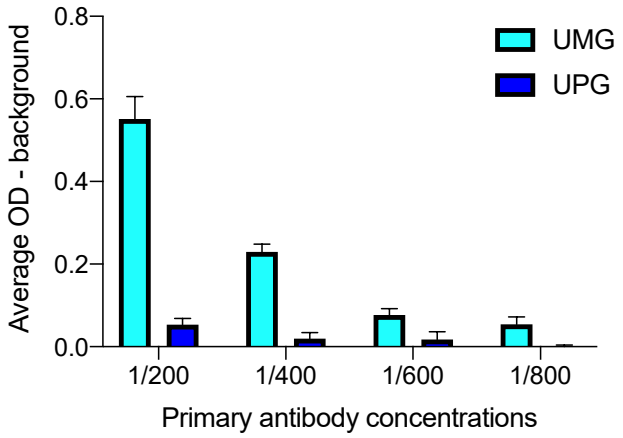

Figure 4.2.1. C1q fixation assay demonstrates the dynamic range between positive and negative controls, despite overall low OD measurements. The range of the C1q fixation assay was tested with serial dilutions of primary antibody from UMG and UPG serum. Error bars show standard deviation among two duplicate wells measured on the same day.

Due to limited serum availability, 44 serum samples collected peripherally from mothers an enrollment, 38 serum samples collected peripherally from mothers at delivery, and 36 serum samples collected from placentas at delivery were tested in the C1q fixation ELISA. The variable levels of C1q fixation at each collection point are shown in Figure 4.2.2. At enrollment, 67% of women (29/44) were above the cut-off for C1q fixation. At delivery, similar levels of C1q fixation were observed with 60% of samples being above the cut-off for C1q fixation (23/38) (Figure 4.2.2). C1q fixation at delivery was not significantly different from fixation at enrollment in samples collected from maternal peripheral blood (A, Figure 4.2.3). Finally, in serum collected from placentas at delivery, 39% (14/36) of serum samples were above the cut-off for C1q fixation, which differed significantly from C1q fixation levels in serum collected from peripheral blood at delivery (B, Figure 4.2.3, paired t-test, $p=0.03$). When C1q fixation levels were analysed by gravidity, no significant difference in levels was observed at any collection point between gravidities (shown in Figure 4.2.4 for serum collected at enrollment).

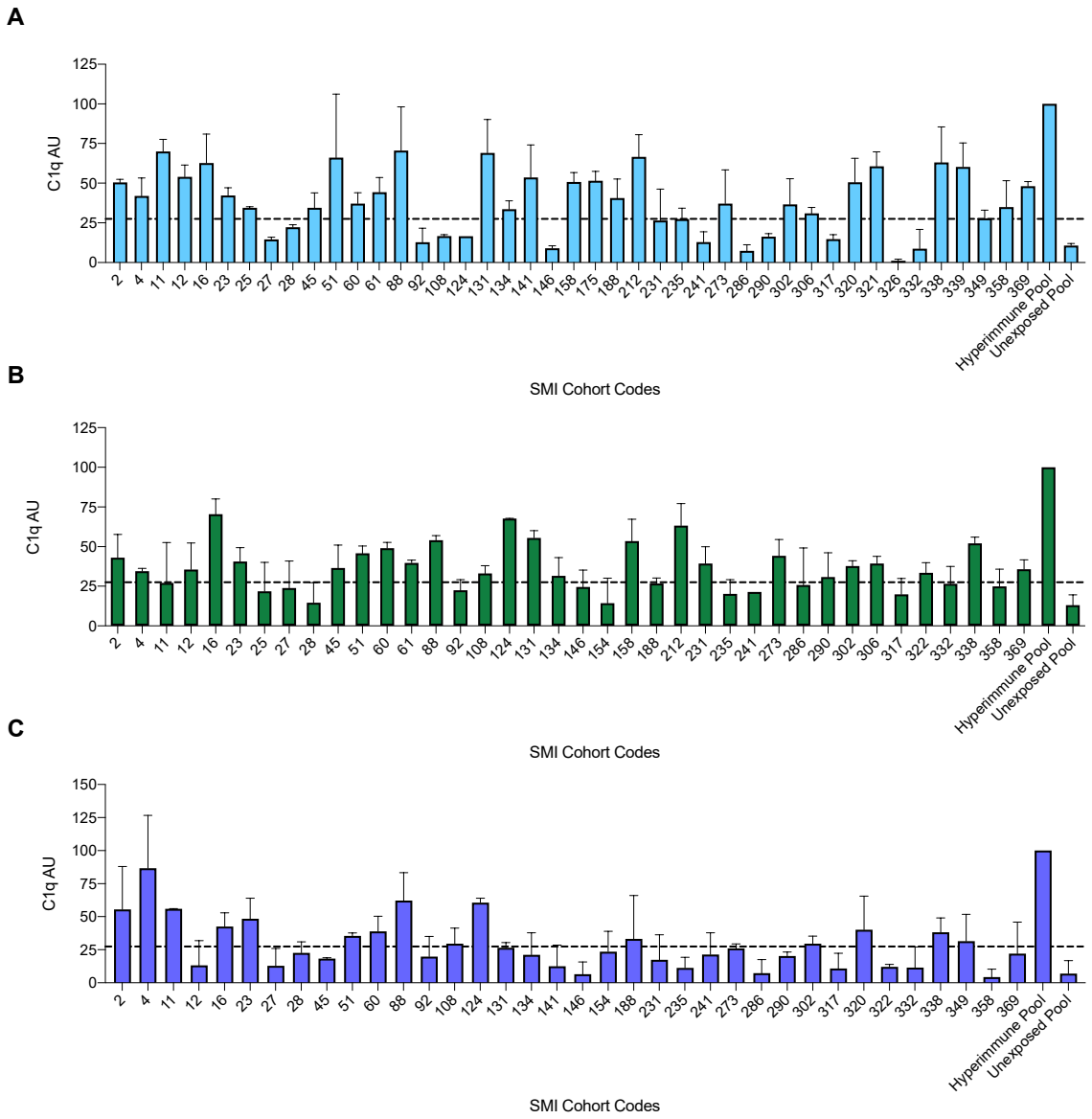

Figure 4.2.2. Variable C1q fixation in serum collected from mothers at study enrollment (A), peripherally at delivery (B), and from placentas at delivery (C). Horizontal line indicates the C1q fixation cut-off (27.5 AU). Error bars show standard deviation calculated from repeating the experiment twice on separate days.

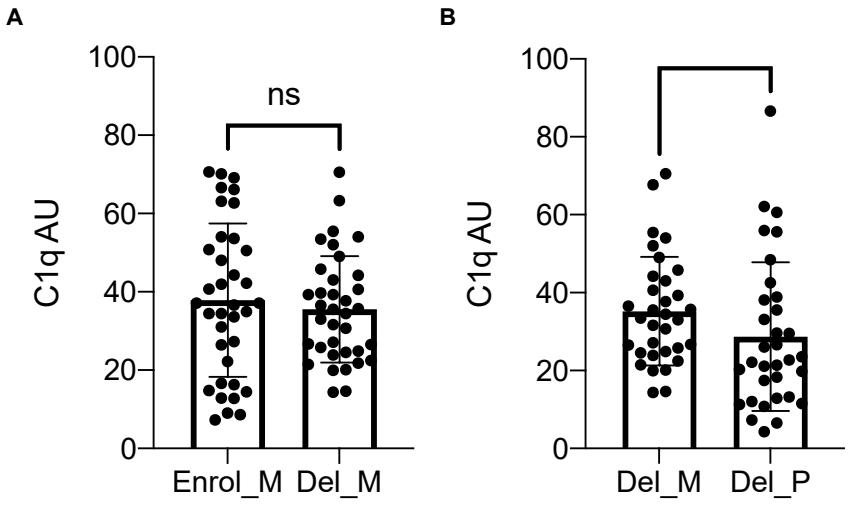

Figure 4.2.3. C1q fixation in serum collected from mothers peripherally and from placentas at delivery differ significantly, but not between serum collected peripherally at study enrollment and from mothers at delivery. Paired t-tests were performed to test for the significance in C1q fixation levels in serum collected peripherally from mothers at enrollment (Enrol_M) and delivery (Del_M) (A, n=36, p=0.32), and serum collected from mothers peripherally and from the placenta at delivery (Del_P) (B, n=34, p= 0.0302).

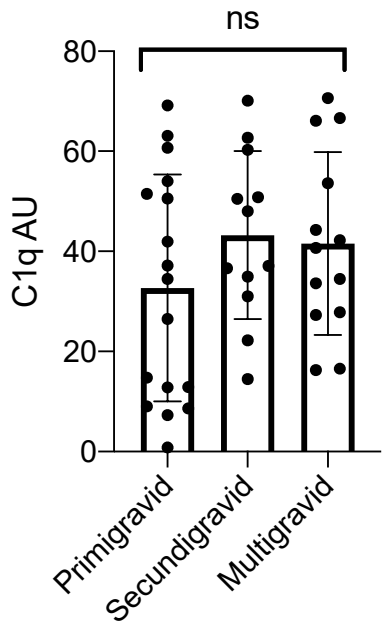

Figure 4.2.4. No association between C1q fixation in serum collected at study enrollment and gravidity. One-way ANOVA (p=0.21) was performed to test for an association between C1q fixation in serum collected at study enrollment and gravidity.

We also investigated C1q fixation levels in serum samples collected from women who tested negative for malaria infection by qPCR at study enrollment and developed a SMI later in pregnancy (n=28) to determine if fixation levels increased due to increased immune activation. No significant difference was observed between study enrollment and delivery, as shown in Figure 4.2.5 (Wilcoxon test, p=0.55).

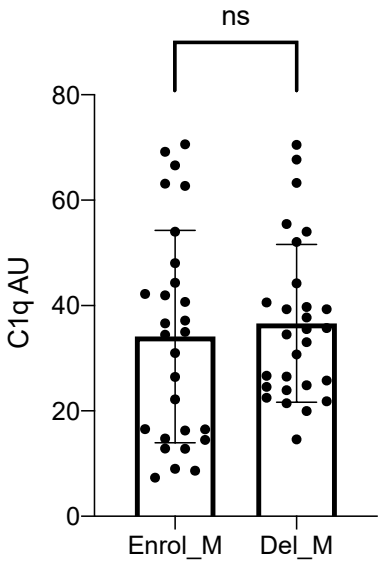

Figure 4.2.5. No increase in C1q fixation levels is observed from study enrollment to delivery among women who were negative for malaria infection at study enrollment but developed at least one SMI later in pregnancy. A Wilcoxon test was run to determine significance (n=28, p=0.55) between C1q fixation levels at study enrollment and delivery in serum samples collected peripherally from mothers.

4.2.2. C1q fixation and associations with pregnancy outcomes

To test if levels of C1q fixation were associated with pregnancy outcomes, we compared fixation levels to infant birthweight, maternal hemoglobin levels at delivery, and gestational age. At enrollment, C1q fixation was negatively associated with infant birthweight (A, B, Figure 4.2.6, Pearson correlation, p=0.0189). However, the significant association between C1q fixation levels and infant birthweight was lost when serum samples from mothers who only experienced *P. falciparum* infection during pregnancy were compared to birthweight (G, Figure 4.2.6). No association was observed at any other collection point for serum samples collected peripherally from mothers at delivery (Figure 4.2.7) and for serum samples collected from placentas at delivery (Figure 4.2.8).

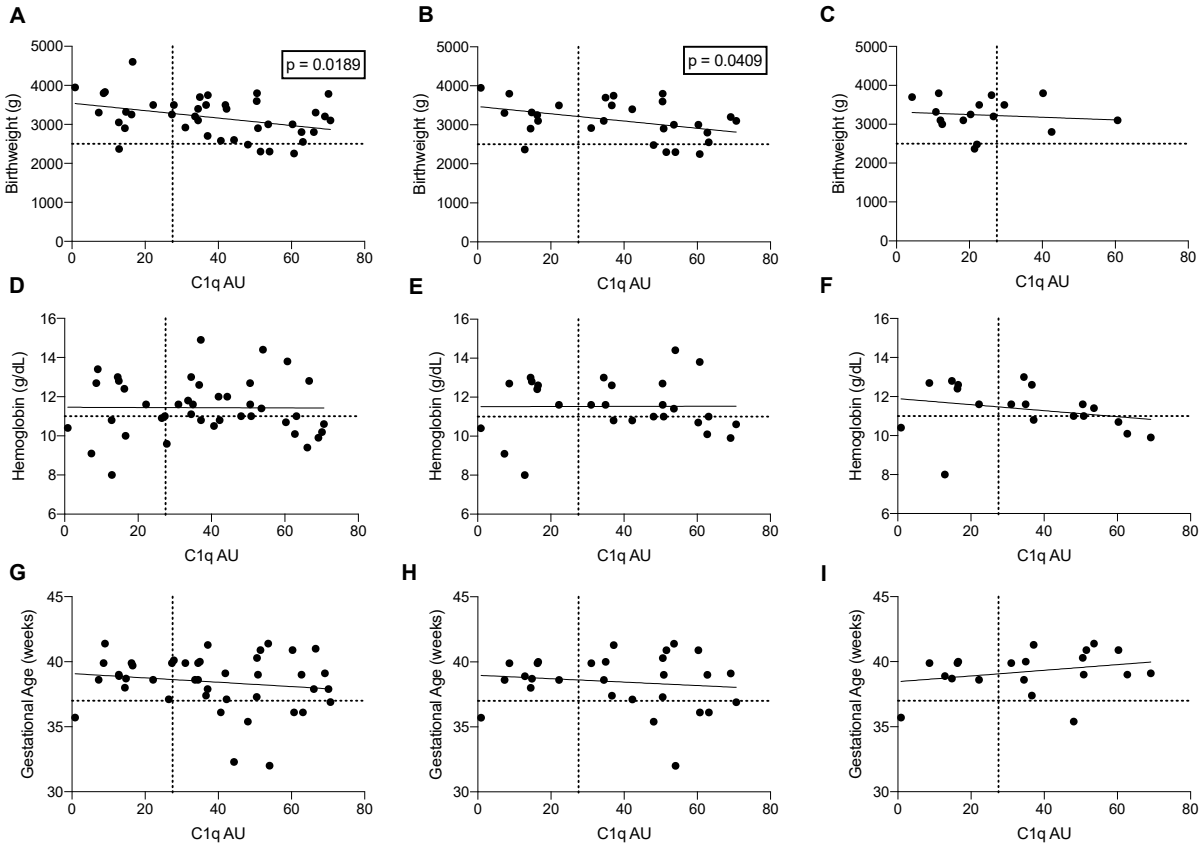

Figure 4.2.6. C1q fixation levels in serum collected at enrollment are negatively correlated with infant birthweight, but not with serum collected from mothers who only developed a *P. falciparum* infection at some point in pregnancy. Pearson correlations were performed to test for an association between C1q fixation by antibody-VAR2CSA complexes in serum collected peripherally at enrollment and infant birthweight (A, B, C), maternal hemoglobin at delivery (D, E, F), and gestational age (G, H, I) among mothers who developed a *P. falciparum*, *P. vivax*, and/or mixed infection (A, D, G), mothers who developed a *P. falciparum* and/or mixed infection (B, E, H), and mothers who only developed a *P. falciparum* infection at some point in pregnancy (C, F, I). Horizontal dotted lines indicate the threshold for low birthweight (A, B, C, 2,500 g), anemia (D, E, F, 11 g/dL), and pre-term birth (G, H, I, 37 weeks). Vertical dotted lines indicate the cut-off for C1q fixation (27.5 AU).

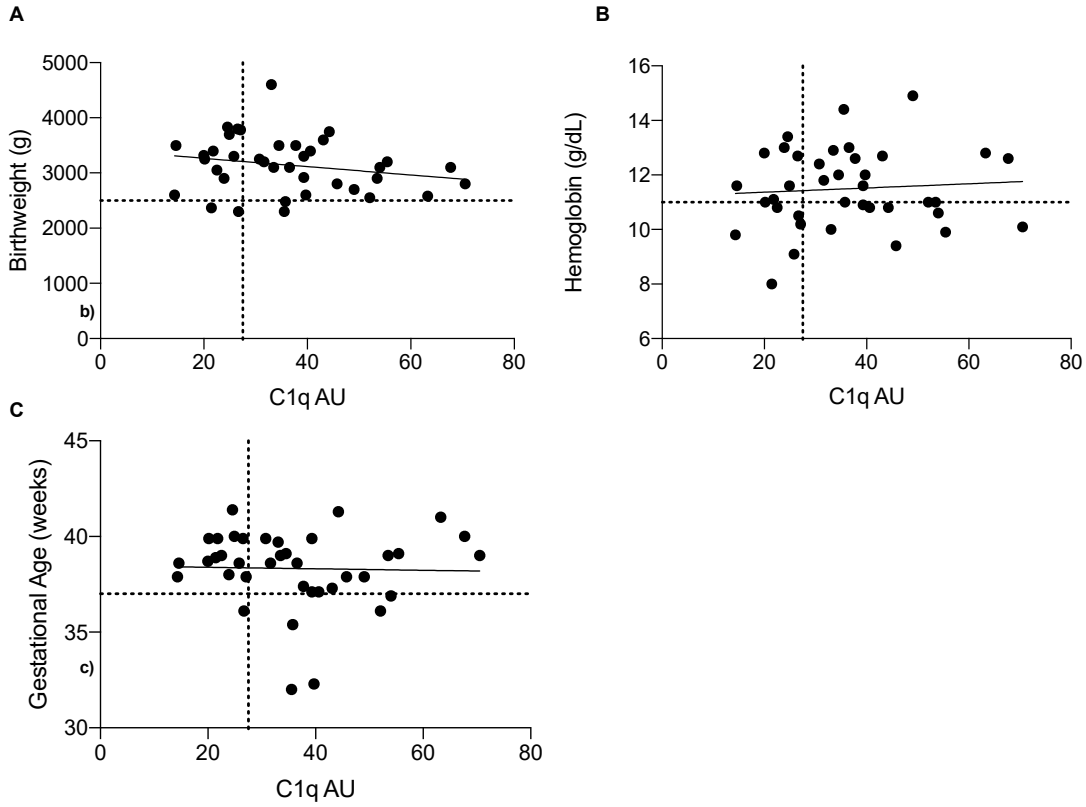

Figure 4.2.7. C1q fixation levels in serum collected from mothers peripherally at delivery are not correlated with pregnancy outcomes at delivery. Pearson correlations were performed to test for an association between C1q fixation by antibody-VAR2CSA complexes in serum collected peripherally at delivery and infant birthweight (A, p=0.19), maternal hemoglobin at delivery (B, p=0.65), and gestational age (C, p=0.87) among mothers with either *P. falciparum* or *P. vivax* mono-infection, or mixed infection at some point in pregnancy. Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for C1q fixation (27.5 AU).

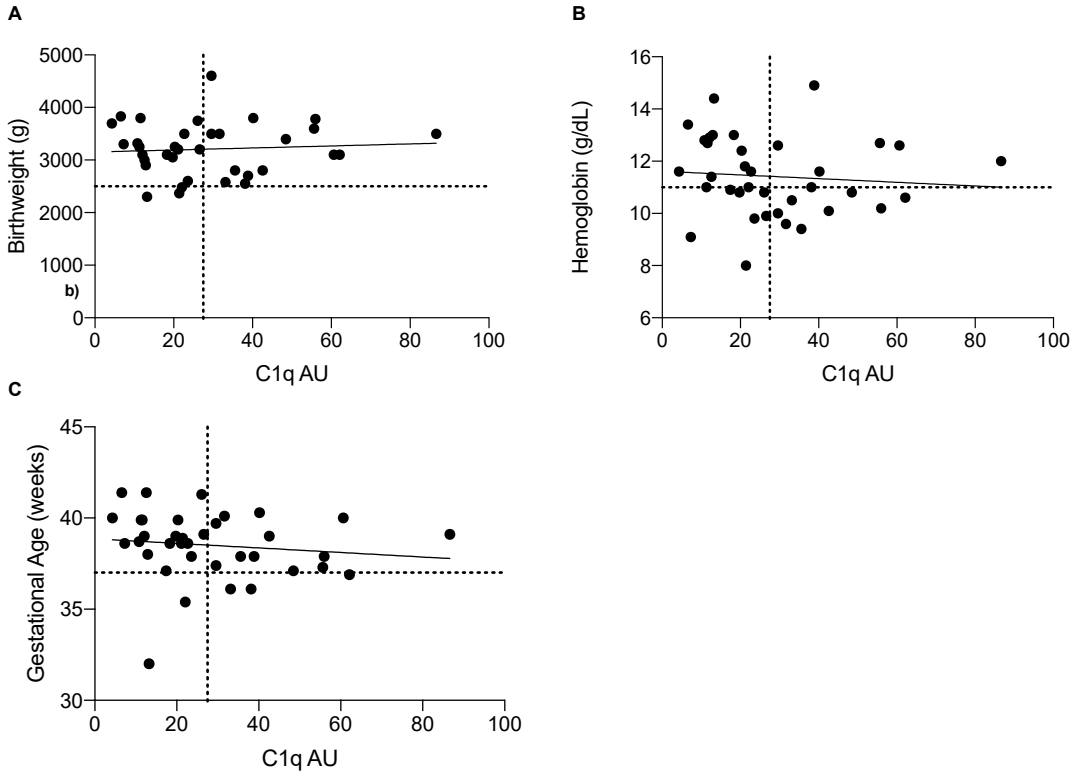

Figure 4.2.8. C1q fixation levels in serum collected from placentas at delivery are not correlated with pregnancy outcomes at delivery. Pearson correlations were performed to test for an association between C1q fixation by antibody-VAR2CSA complexes in serum collected from placentas at delivery and infant birthweight (A, p=0.68), maternal hemoglobin at delivery (B, p=0.62), and gestational age (C, p=0.46) among mothers with either *P. falciparum* or *P. vivax* mono-infection, or mixed infection at some point in pregnancy. Horizontal dotted lines indicate the threshold for low birthweight (A, 2,500 g), anemia (B, 11 g/dL), and pre-term birth (C, 37 weeks). Vertical dotted lines indicate the cut-off for C1q fixation (27.5 AU).

4.3. Opsonic phagocytosis

4.3.1 Opsonic phagocytosis activity observed in serum collected from mothers at study enrollment

The next functional mechanism we examined was opsonic phagocytosis, mediated by VSA_{PAM} antibodies in serum samples collected from the SMI cohort. A phagocytosis index (PI) was calculated for each serum sample tested in the SMI cohort (n=44), which was the percentage of the opsonic phagocytosis observed with the hyperimmune pool on that day of experiments. At enrollment, 68% of women (30/44) had a PI above the cut-off (Figure 4.3.1). When PIs were analysed by gravidity, no significant difference was observed (Figure 4.3.2).

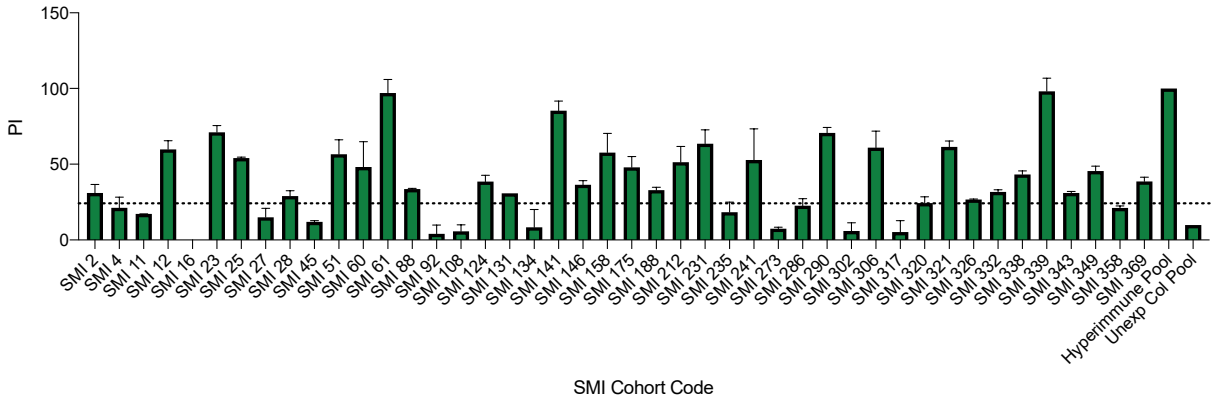

Figure 4.3.1. Opsonic phagocytosis of serum collected from mothers at study enrollment. PI was calculated based on phagocytosis observed with a hyperimmune pool (100% phagocytosis) on each day the experiment was run. Horizontal line shows cut-off for opsonic phagocytosis (PI = 24.1). Error bars are standard deviation calculated from repeating the experiment twice on separate days.

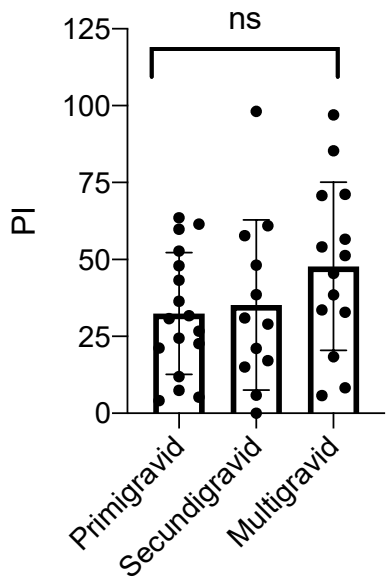

Figure 4.3.2. No association between opsonic phagocytosis in serum collected at study enrollment and gravidity. One-way ANOVA (p=0.54) was performed to test for associations between PI in serum collected at study enrollment and gravidity.

4.3.2. Opsonic phagocytosis and associations with pregnancy outcomes

To test if levels of opsonic phagocytosis were associated with pregnancy outcomes, we compared PI levels to infant birthweight, maternal hemoglobin levels at delivery, and gestational age. At enrollment, PI levels were negatively associated with infant birthweight (A, B, Figure 4.3.3, Pearson correlation, p=0.0040). No association was observed with the other pregnancy outcomes. Additionally, when serum samples collected at enrollment were analysed based on infection history throughout pregnancy, the significant association between PI levels and infant birthweight was lost when serum samples from mothers who only experienced a *P. falciparum* infection during pregnancy were compared to birthweight (C, Figure 4.3.3).

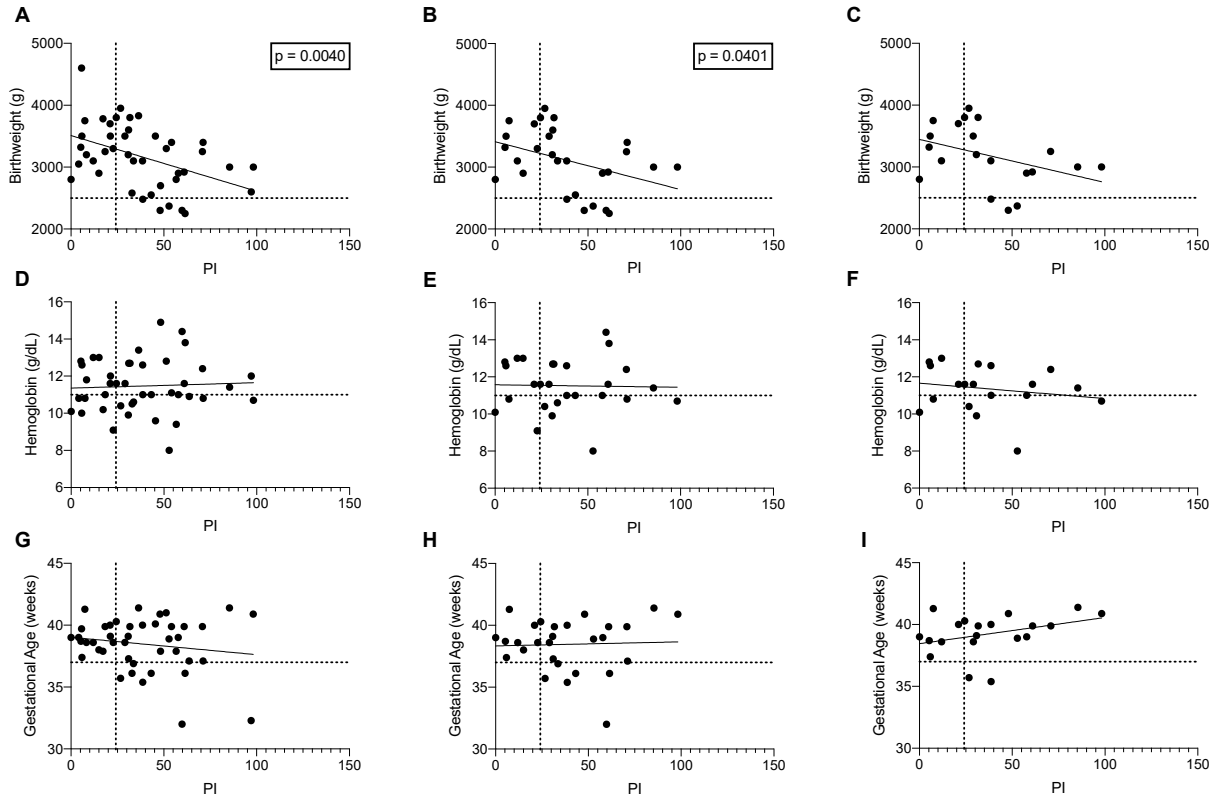

Figure 4.3.3. Opsonic phagocytosis levels are negatively correlated with infant birthweight in serum collected at study enrollment, but not for mothers who only developed a *P. falciparum* infection at some point in pregnancy. Pearson correlations were performed to test for an association between opsonic phagocytosis and infant birthweight (A, B, C) maternal hemoglobin at delivery (D, E, F), and gestational age (G, H, I) among mothers who developed a both a *P. falciparum*, *P. vivax*, and/or mixed infection (A, D, G), mothers who developed a *P. falciparum* and/or mixed infection (B, E, H), and mothers who only developed a *P. falciparum* infection at some point in pregnancy (C, F, I). Horizontal dotted lines indicate the threshold for low birthweight (A, B, C, 2,500 g), anemia (D, E, F, 11 g/dL), and pre-term birth (G, H, I, 37 weeks). Vertical dotted lines indicate the cut-off for opsonic phagocytosis (PI = 24.1).

4.4. Associations between serology and functional assays

4.4.1. Associations between the C1q fixation and IgG-VAR2CSA levels

In serum collected peripherally from mothers at delivery, C1q fixation and IgG-VAR2CSA levels were positively associated (Figure 4.4.1, Pearson correlation, p=0.0403). No significant association was observed at the other collection points.

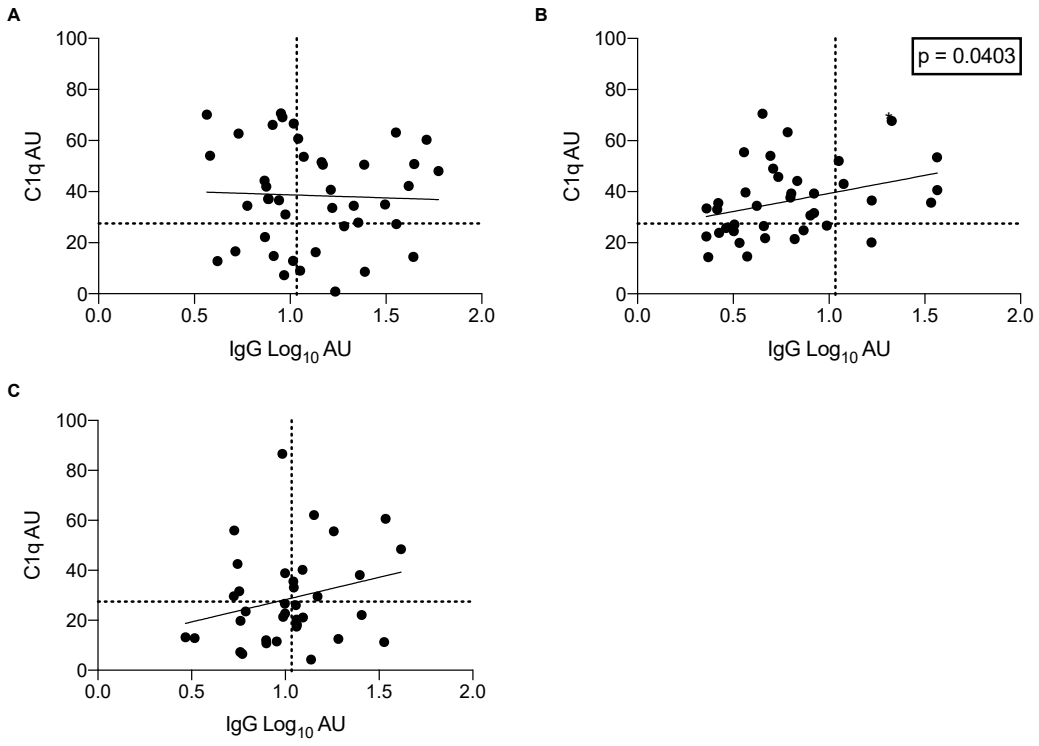

Figure 4.4.1. C1q fixation levels are significantly associated with IgG-VAR2CSA levels in serum collected peripherally from mothers at delivery, but not at study enrollment or in serum collected from placentas at delivery. Pearson correlations were performed to test for an association between C1q fixation and IgG-VAR2CSA levels in serum collected from mothers at study enrollment (A), peripherally at delivery (B), and collected from placentas at delivery (C). Horizontal dotted lines indicate the cut-off for C1q fixation (27.5 AU). Vertical dotted lines indicate the normalized cut-off for IgG-VAR2CSA seropositivity (1.033 AU).

4.4.2. Associations between the C1q fixation ELISA and IgM-VAR2CSA ELISA

At all three collection points, C1q fixation and IgM-VAR2CSA seroreactivity were significantly associated (Figure 4.4.2). This association was strongest in serum collected peripherally from mothers at delivery (Figure 4.4.2, Pearson correlation, $p<0.0001$).

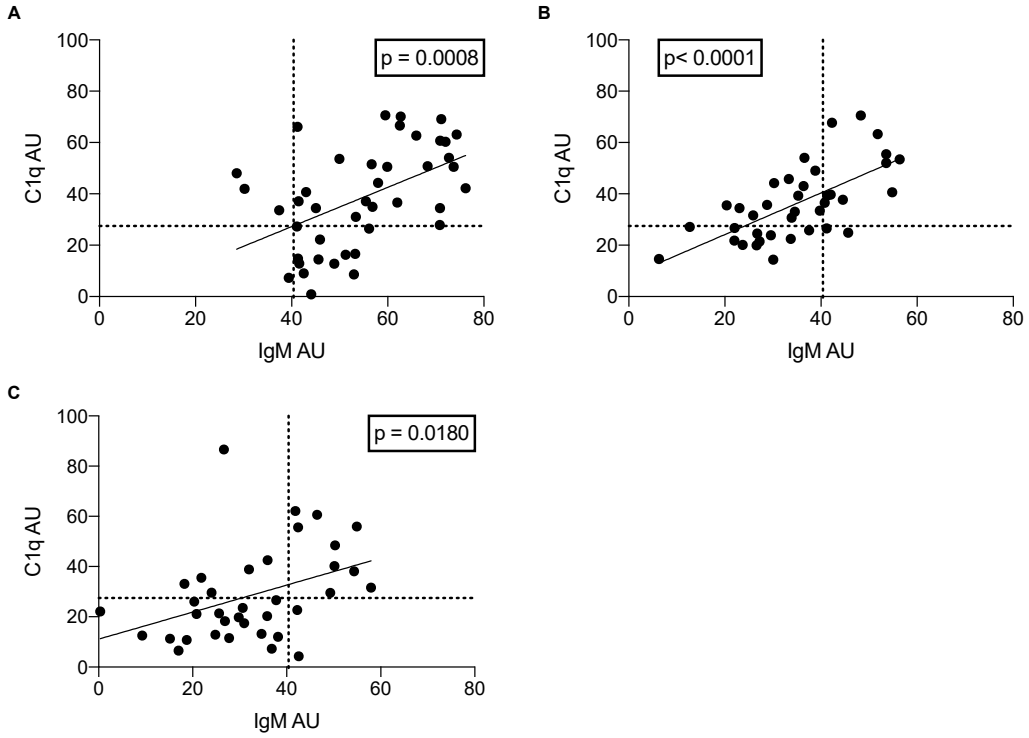

Figure 4.4.2. C1q fixation and IgM-VAR2CSA seroreactivity is positively associated at all three collection points. Pearson correlations were performed to calculate associations between IgM-VAR2CSA and C1q fixation levels for serum collected peripherally from mothers at study enrollment (A), delivery (B), and from placentas at delivery (C). Vertical lines indicate the cut-off for IgM seropositivity (40.4 AU) and horizontal lines indicate the cut-off for C1q fixation (27.5 AU).

4.4.3. Associations between the C1q fixation levels and opsonic phagocytosis

In serum collected peripherally from mothers at enrollment, C1q fixation and PI levels were not significant associated (Figure 4.4.3, Pearson correlation, p=0.0610).

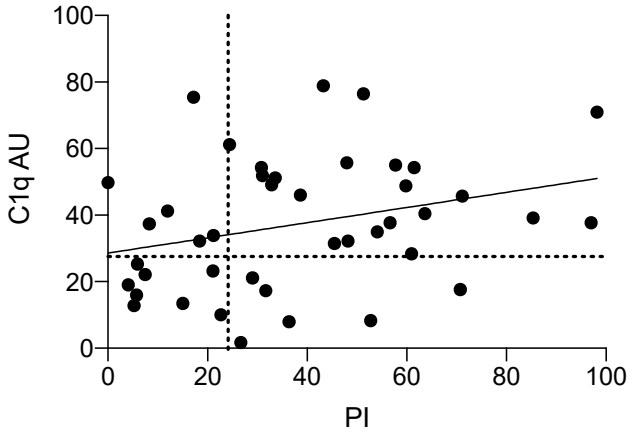

Figure 4.4.3. C1q fixation and PI levels at enrollment are not significantly associated. Pearson correlation was performed to calculate the association between C1q fixation and PI levels for serum collected peripherally from mothers at study enrollment. Horizontal line indicates the cut-off for C1q fixation (27.5 AU) and vertical line indicates the cut-off for PI (24.1 %).

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4.4.4. Associations between opsonic phagocytosis and IgG-VAR2CSA levels

In serum collected peripherally from mothers at enrollment, IgG-VAR2CSA and PI levels were not significantly associated (Figure 4.4.4, Spearman correlation, p=0.077).

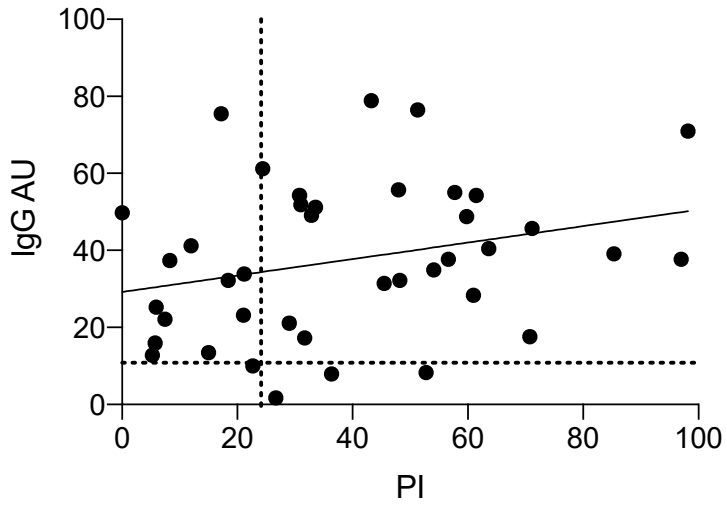

Figure 4.4.4. IgG-VAR2CSA and PI levels at enrollment are not significantly associated. Spearman correlation was performed to calculate the association between IgG-VAR2CSA and PI levels for serum collected peripherally from mothers at study enrollment. Horizontal line indicates the cut-off for IgG-VAR2CSA (10.8 AU) and vertical line indicates the cut-off for PI (24.1 %).

4.4.5. Associations between opsonic phagocytosis and IgM-VAR2CSA levels

In serum collected peripherally from mothers at enrollment, IgM-VAR2CSA and PI levels were not significantly associated (Figure 4.4.5, Pearson correlation, p=0.19).

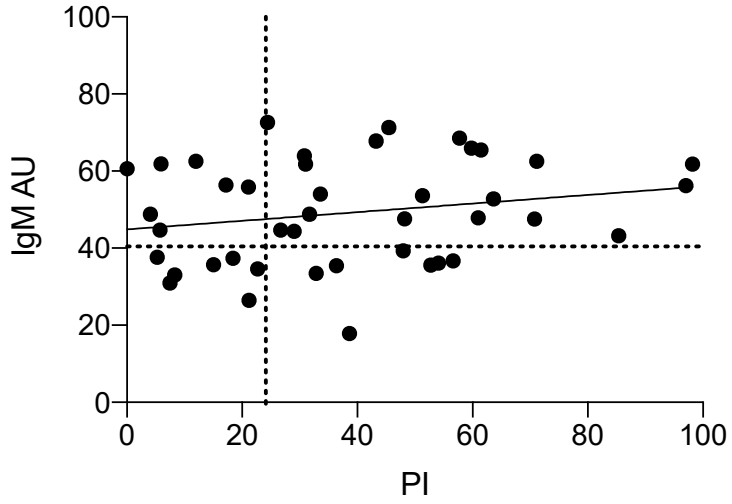

Figure 4.4.5. IgM-VAR2CSA and PI levels at enrollment are not significantly associated. Pearson correlation was performed to calculate the association between IgM-VAR2CSA and PI levels for serum collected peripherally from mothers at study enrollment. Horizontal line indicates the cut-off for IgM-VAR2CSA seropositivity (40.4 AU) and vertical line indicates the cut-off for PI (24.1 %).

4.4.6. Associations between opsonic phagocytosis and inhibition of binding levels

In serum collected peripherally from mothers at enrollment, previously published data on antibody binding inhibition (Gavina et al., 2018) and PI levels were not significantly associated (Figure 4.4.6, Pearson correlation, p=0.14).

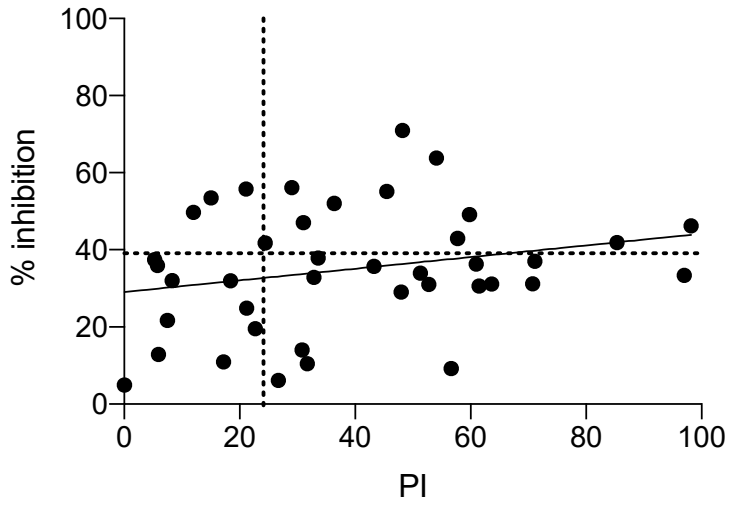

Figure 4.4.6. Binding inhibition and PI levels at enrollment are not significantly associated. Pearson correlation was performed to calculate the association between percent binding inhibition (% inhibition) and PI levels for serum collected peripherally from mothers at study enrollment. Horizontal line indicates the cut-off point for percent binding inhibition (39.1%) and vertical line indicates the cut-off point for PI (24.1 %).

4.5 Total VSA_{PAM} IgG staining and opsonic phagocytosis levels against nRBCs with a prior exposure to iRBCs using a transwell assay

After 48-hour exposure to iRBCs, $eRBCs_i$ were isolated and run in the total IgG flow cytometry and opsonic phagocytosis experiments, in parallel with $eRBCs_n$ that had been exposed to nRBCs. In addition, the top compartments of the transwells were also run in the experiments (iRBCs and nRBCs). For total IgG flow cytometry performed with VAR2CSA Rab and Normal Rab, no significant difference in antibody staining was observed between the cell populations in both the top and bottom compartments (Figure 4.5.1). For the iRBC population, this is not unexpected, as the parasitemia in the iRBC compartment is typically only 6-8% (iRBCs: nRBCs). Thus, though iRBCs were confirmed to be strongly expressing VAR2CSA in a sample of purified iRBCs at the trophozoite stage (95-100% iRBCs), high levels of staining with the VAR2CSA Rab will not be seen in the mixed iRBC/nRBC population in this assay, as iRBCs are diluted with nRBCs.

For the opsonic phagocytosis assay, the hyperimmune UMG pool was used to mediate engulfment, with UPG as the negative control (Figure 4.5.2). Similar levels of engulfment were observed between all cell populations, with a small shift observed in opsonic phagocytosis mediated by UMG serum in the nRBC cell population (B, Figure 4.5.2).

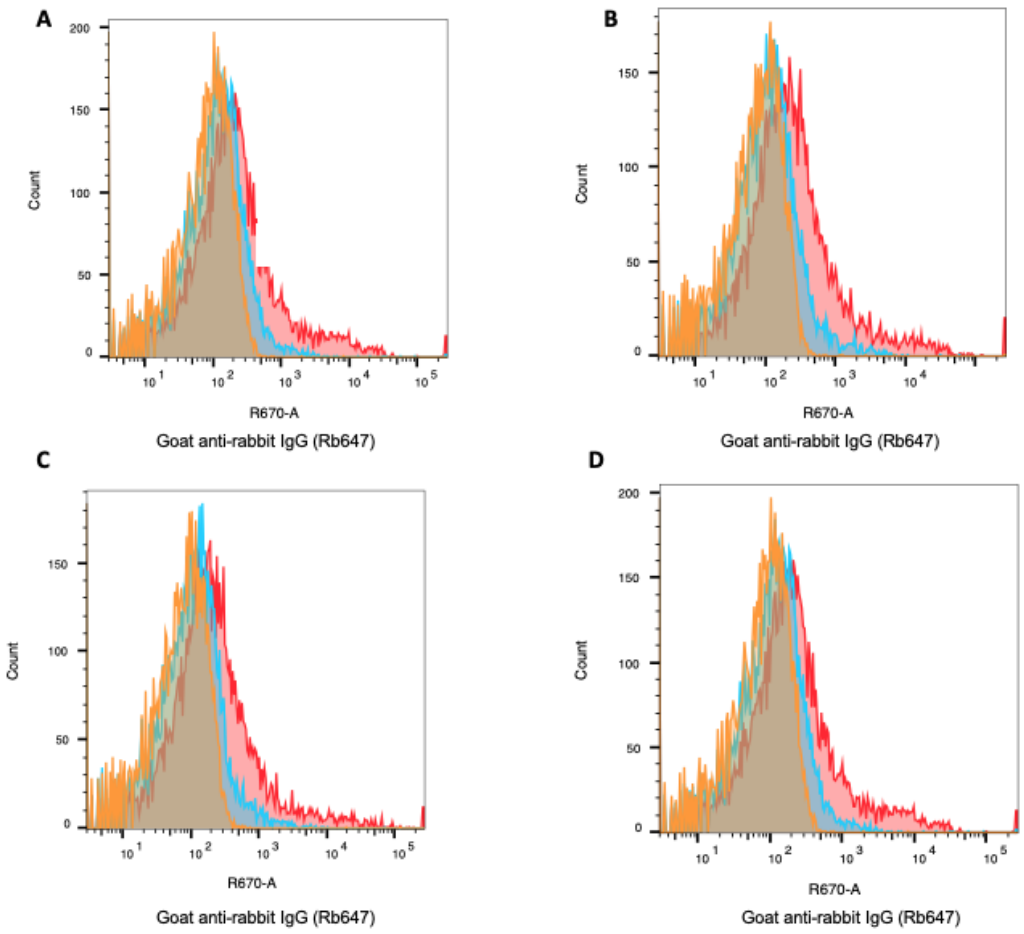

Figure 4.5.1. No difference in levels of antibody staining was observed between RBC populations in top and bottom compartments. Cell staining was measured with the R670-A laser for iRBCs in the top compartment (A), $eRBC_i$ populations in the bottom compartment (C), nRBCs in the top compartment (B), and $eRBC_n$ populations in the bottom compartment (D) stained with DAPI. Red peaks show VAR2CSA Rab staining, blue peaks show Normal Rab staining, and orange peaks show background staining (no primary antibody added).

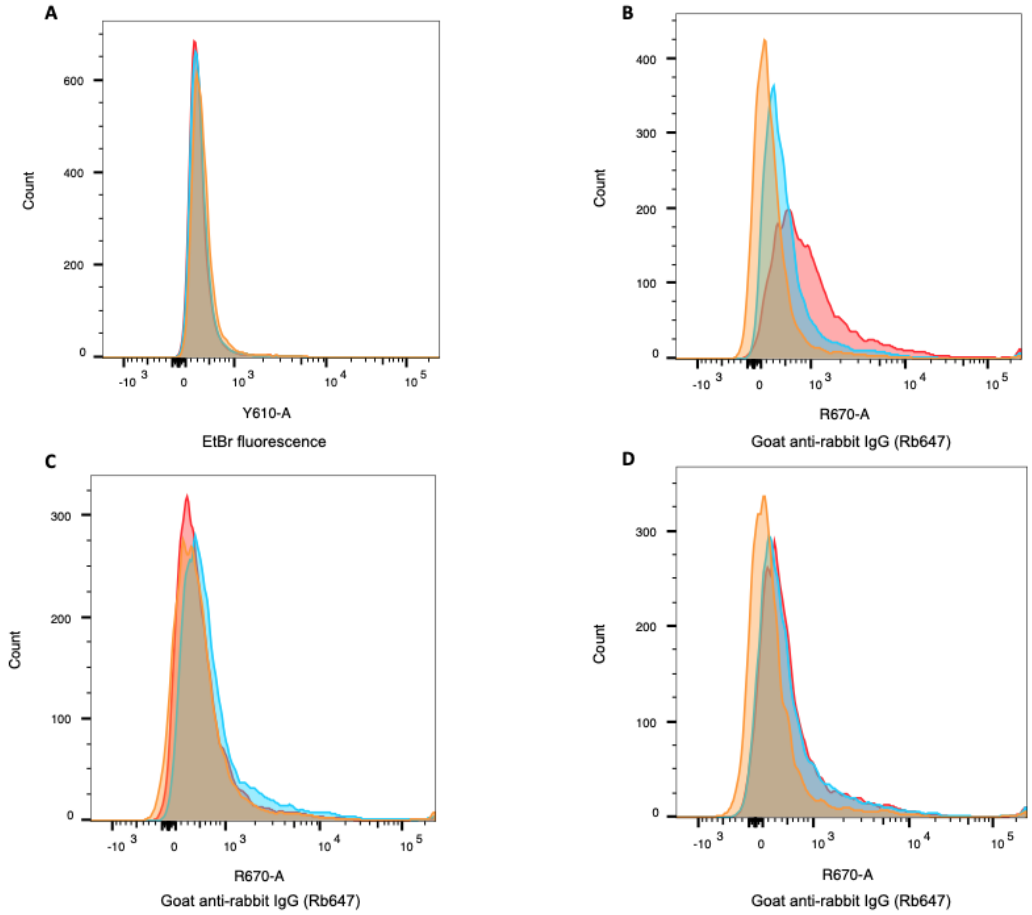

Figure 4.5.2. Minimal differences in levels of opsonic phagocytosis were observed between RBC populations in top and bottom compartments. Cell staining was measured with the Y610-A laser for the iRBC in the top compartment sample stained with EtBr (A) or the R670-A laser for the $eRBC_i$ populations in the bottom compartment (C), $eRBC_n$ populations in the bottom compartment (D), and the nRBC sample in the top compartment (B) stained with DiD. Red peaks show engulfment mediated by the UMG serum pool, blue peaks show engulfment mediated by the UPG serum pool, and orange peaks show engulfment mediated without antibody (background phagocytosis).

4.6 Summary and brief discussion

Variable levels of both C1q fixation and opsonic phagocytosis were observed in serum collected from the SMI cohort. No protective associations between these antibody-mediated effector mechanisms and pregnancy outcomes were observed, in contrast to our previous observation that antibody blocking activity in the IBA correlated with hemoglobin levels at delivery (Gavina et al., 2018). Instead, both C1q fixation and opsonic phagocytosis activity at enrollment were negatively associated with infant birthweight at delivery.

When considering the association between C1q fixation and infant birthweight, previous research on complement and PAM can be considered. A dysregulated complement response is known to have a role in promoting inflammation in the placenta, resulting in fetal growth restriction in PAM (Conroy et al., 2013; McDonald et al., 2015). This is driven by increased levels of the complement component C5a, which affects angiogenesis in the placenta (Conroy et al., 2013). Since fixation of C1q strongly associates with downstream complement products (Reiling et al., 2019), the C1q fixation assay could also be predictive of a harmful complement response that may result in lower birthweight. To confirm this, the formation of these downstream products, including C5a, should be investigated in this cohort by using an anti-C5a antibody and fresh serum as the source of complement in the complement assay.

Interestingly, C1q fixation and IgM-VAR2CSA seroreactivity were positively associated at all three collection points. IgM binds complement at greater levels than IgG in uncomplicated malaria, potentially due to its pentameric shape which has increased binding sites for the C1q

globular heads (Boyle et al., 2019). Additionally, in plate-based assays where antibody binding to both the antigen and C1q may be restricted, IgM antibodies may be able to overcome steric hindrance to a greater extent than IgG due to the additional C1q binding sites. Thus, IgM-VAR2CSA seroreactivity would be expected to be predictive of C1q fixation, rather than IgG-VAR2CSA seroreactivity. Previous research using the same assay showed that IgG seroreactivity was not predictive of the degree of C1q fixation (Reiling et al., 2019). *In vivo*, the IgM and C1q immune responses may also occur closer temporally than the IgG response, as the IgM isotype is the first antibody to be produced during a humoral response (Janeway CA, 2001) and complement is considered a front-line defence; though not solely an innate responder, it is also an effector of the adaptive response (reviewed in (Dunkelberger & Song, 2010)).

An unexpected result of these experiments was the negative association between opsonic phagocytosis activity in serum collected from mothers at enrollment and infant birthweight at delivery. Although this has not been reported in PAM, it may be explained by the following. An increased level of opsonic phagocytosis at study enrollment may reflect an elevated inflammatory state in the placenta, which may not resolve by delivery and thus result in a poor birth outcome. Monocyte and macrophage infiltration is known to occur in placental malaria as part of the inflammatory response (Ordi et al., 1998). In Malawi, detection of monocytes isolated from placentas containing parasite pigment were associated with low birthweight and maternal anemia (Rogerson et al., 2003). In Sudan, these infiltrates were detected in mothers with and without placenta malaria, but no significant difference in birthweight between the two groups was observed (Salih et al., 2011). However, neither of these studies assessed opsonic phagocytosis. It was hypothesized that opsonic phagocytosis may increase the specificity of the

phagocyte response, but perhaps in our cohort antibodies elicited by SMIs could not mediate opsonic phagocytosis in a protective manner as reported in studies conducted in Africa (Chandrasiri et al., 2016; Feng et al., 2009). A dysregulated complement response may also contribute to this, as the classical complement cascade produces opsonins, promoting a harmful inflammatory response rather than a targeted clearance of iRBCs from the placenta.

When investigating if nRBCs can become antibody targets with prior exposure to iRBCs, no change in staining or opsonic phagocytosis was observed between eRBC cell populations exposed to iRBCs or nRBCs. This may be due to a lack of extracellular vesicle transfer during the 48-hour exposure, or inability of the antibodies to stain antigen transferred from extracellular vesicles or mediate opsonic phagocytosis of these nRBCs. Interestingly, the degree of staining by the rabbit serum in the flow cytometry experiment was similar for all four RBC populations, suggesting that there is some general reactivity of VAR2CSA Rab serum to human RBCs. This may be an important point to note when using animal models, such as rabbits, for PAM vaccine development. Further investigation into nRBC loss in PAM is required, but this assay did not further elucidate if nRBC loss occurs in PAM because of antigen transfer from iRBCs.

In summary, these results reveal interesting associations between antibody-mediated mechanisms and pregnancy outcomes and highlight the importance of measuring both antibody levels and function. However, the link between antibody-mediated mechanisms and protection from maternal anemia was not further elucidated by these results. This may be due to a variety of factors discussed in the following section, but more research is required in this area, especially to expand the knowledge of SMIs in PAM observed in settings outside of Africa.

Chapter 5 – Discussion

Portions of this chapter have been published:

Wiebe MC, Yanow SK. 2020. Do antibodies to malaria surface antigens play a role in protecting mothers from maternal anemia? *Front Immunol* 11: 609957.

5.1 – Summary of the antibody-mediated immune response detected in serum samples collected from the SMI cohort

The experiments in this thesis characterized both serology and functional measures in serum collected from a cohort of pregnant women who experienced at least one SMI in pregnancy. At study enrollment (maternal serum) and delivery (maternal and placental serum), we measured levels of IgG, IgM, and C1q fixation against recombinant VAR2CSA. At study enrollment, we also measured total IgG against VSA expressed by iRBCs and opsonic phagocytosis. This adds to our knowledge on the humoral immune in mothers in Colombia that experienced SMIs of PAM.

At study enrollment (approximately first to second trimester), various levels of both IgG and IgM antibodies were measured within the cohort. Typically, in high transmission areas of Africa, IgG levels are higher in multigravid women who have experienced multiple instances of PAM, thus have acquired specific antibodies against VSA_{PAM} (Mayor et al., 2011). But, we did not observe a significant difference in IgG levels amongst gravidities, potentially due to cross-

reactivity between the co-circulating *P. falciparum* and *P. vivax* in Colombia (Gnidehou et al., 2019). By delivery, both IgG and IgM levels decreased, which may reflect insufficient boosting of IgG throughout pregnancy by the SMIs, despite that several women had multiple infections throughout pregnancy. A previous study found that IgG and IgM levels against parasite lysate were higher in maternal and cord samples from women positive for placental infection, but only microscopic infections were detected in that study (Mayor et al., 2018).

When compared, IgG and IgM were not significantly associated at any collection point, which suggests that in this setting the levels of the two isotypes may be independent of each other. In a typical course of infection, a class switch from IgM to IgG is expected, and IgG is the primary responder in PAM (Nasr et al., 2013). However, in uncomplicated malaria in children and adults, both IgG and IgM levels against a merozoite antigen remained stable throughout infection (Boyle et al., 2019). More investigation is required into the dynamics of IgM during PAM, specifically when only SMIs occur during pregnancy.

Total IgG against any VSA_{PAM} expressed on the surface of iRBCs was also measured, as it was hypothesized that these results would be more indicative of the *in vivo* IgG response to PAM, as seroreactivity against the native antigen is tested, instead of the recombinant protein. VAR2CSA is a known *Pf*EMP1 expressed in PAM (Salanti et al., 2003), but other VSAs that are capable of binding to CSA may be expressed. This assay captures any IgG staining to iRBCs that have been selected to express binding ligands to CSA. However, minimal reactivity was observed in this assay, with only one serum sample being seropositive for IgG.

This was an unexpected result, but as we already observed seroreactivity by ELISA and functional activity at study enrollment in this cohort (Gavina et al., 2018), we hypothesized the low seroreactivity in flow may be due to the avidity of the antibodies, or due to the assay itself, as many washes are performed after the antibodies are incubated with iRBCs. A previous study found that reactivity measured by flow cytometry was consistent with VAR2CSA seropositivity by ELISA, but samples were collected from areas of intense transmission and the prevalence of SMIs were not measured (Doritchamou et al., 2019).

We continued the characterisation by testing the antibody-mediated effector mechanisms C1q fixation and opsonic phagocytosis and observed variable levels of activity. Over half of the serum samples had an AU above the cut-off for C1q fixation (67%), and this positivity did not decline significantly by delivery (60%). Similarly, 68% of serum samples were above the cut-off for opsonic phagocytosis. This emphasizes the importance of using multiple types of assays when characterizing a cohort, as low reactivity in one assay may not be predictive of activity in others.

The classical complement cascade plays a unique role in immunity as a bridge between the innate and adaptative system. IgG seroreactivity was not predictive of C1q fixation, but IgM seroreactivity was highly associated with C1q fixation at all three collection points. This may be due the distribution of IgG subclasses in serum samples, as the cytophilic IgG1 and IgG3 subclasses can fix complement, but IgG2 and IgG4 show minimal to no complement fixation (reviewed in (Irani et al., 2015)). Thus, the measurement of total IgG may not be predictive of C1q fixation if serum samples have higher proportions of the non-cytophilic subclasses. The

subclass composition in this cohort is currently being investigated by a collaborator. The stability in C1q fixation from enrollment to delivery may be due to increases in IgG1 and IgG3 populations by delivery, even if total IgG levels decrease by delivery. As IgM does not have subclasses and contains multiple Fc binding sites for C1q, it is expected that this would be more predictive of C1q fixation than IgG.

In addition to fixing complement, antibodies in this cohort also mediated opsonic phagocytosis. This function has been highlighted as a potentially important antibody-mediated immune response in PAM (Chandrasiri et al., 2016; Feng et al., 2009; Lambert et al., 2014), and similarly to C1q fixation, most likely mediated by the IgG1 and IgG3 subclasses (Keen et al., 2007). Interestingly, there were no associations between the data from the opsonic phagocytosis assay and any other assay, including the IBA data which was previously reported (Gavina et al., 2018). This suggests that antibodies that have strong activity in one pathway may not be equally as active in another pathway, pointing to a division of functions in an individual's antibody repertoire, rather than dual-functioned antibodies. Additionally, activity in the opsonic phagocytosis assay was not associated with increased maternal hemoglobin at delivery, whereas activity in the IBA was (Gavina et al., 2018). Thus, not all functions of VSA_{PAM} antibodies may provide protection from the same adverse outcome since the only association between opsonic phagocytosis and outcomes was a negative association with infant birthweight.

Both C1q fixation and opsonic phagocytosis activity were negatively associated with infant birthweight at delivery. The association with C1q fixation may reflect a dysregulated activation of complement, which is known to contribute to pathology during pregnancy in some

settings (Conroy et al., 2013; McDonald et al., 2015). Further investigation is required to confirm this for the SMI cohort by testing for downstream components including the C3 convertase, C5a, and the C5b-C9 complex (MAC complex). This would also confirm that measures of C1q fixation are predictive of the formation of downstream complement components. A previous study in Sudan that tested for the C5b-C9 complex in maternal and cord blood did not find an association between levels of this component and birthweight, though approximately 80% of placentas in this study had no signs of malaria infection (Alim et al., 2015). Antibody-mediated complement activation remains an interesting aspect of the maternal immune response to PAM, and further investigations should include adding complement components to the opsonic phagocytosis assay to test if complement opsonins, such as C3b, may enhance the immune response and change associations with pregnancy outcomes.

The negative association between opsonic phagocytosis activity and infant birthweight was not expected and has not been reported previously in PAM. Immune cells including monocytes are known to infiltrate the placenta during PAM and produce a number of proinflammatory cytokines, contributing to placental inflammation (Ordi et al., 1998). Previously, the studies on the infiltration of mononuclear cells reported mixed associations with low infant birthweight (Menendez et al., 2000; Salih et al., 2011); but these studies did not assess opsonic phagocytosis specifically, so the effects of the antibody response after monocyte infiltration on infant birthweight remain unclear. Additionally, though opsonic phagocytosis was observed *in vitro* in our cohort, this measurement may not reflect the immune response *in vivo*. Though associated with birthweight in this cohort, the ability of antibodies to mediate opsonic phagocytosis *in vitro* may not be a direct cause of the adverse pregnancy outcome.

5.2 – Investigation of a potential mechanism of maternal anemia involving antibody-mediated immune responses

Though the mechanisms of anemia in uncomplicated malaria are well-understood (reviewed in (White, 2018)), these mechanisms are yet to be definitively shown in PAM. Specifically, the mechanisms behind the loss of nRBCs in PAM remain unknown. As we previously observed a positive association between antibody function in the IBA and maternal hemoglobin at delivery (Gavina et al., 2018), we further investigated a specific mechanism that could account for nRBC clearance by antibody-mediated mechanisms.

EV release by iRBCs is of growing interest in the malaria field, as these EVs may have multiple biological functions. Previous studies found that significantly more EVs were produced in a culture of iRBCs compared to nRBCs, and that these EVs contained human argonaute 2, which could bind parasite miRNAs and downregulate the expression of PfEMP1s (Wang et al., 2017). EVs have also been shown to contribute to inflammation in rodent malaria (reviewed in (Babatunde et al., 2020)). Knowledge on the roles of EVs in PAM is very limited, so we investigated a potential mechanism involving EVs that connects antibody-mediated immune responses to maternal anemia. We hypothesized that antigen transfer via EV release could occur between iRBCs and nRBCs, depositing the antigen on nRBCs and creating a target for antibody binding and effector mechanisms, including opsonic phagocytosis. This could potentially account for the clearance of nRBCs in PAM.

By using a transwell plate to separate cell populations, we measured IgG antibody staining and opsonic phagocytosis of uninfected RBCs that had been exposed to iRBCs (eRBCs, bottom compartments). For these tests, we used serum samples with known reactivity to VAR2CSA, including hyperimmune pools collected from multigravid women and rabbits immunized with VAR2CSA. Thus, since these serum samples were used for the primary serum samples, VAR2CSA was the antigen target of interest that may be transported by the EVs. We measured IgG staining and opsonic phagocytosis of four RBC populations, both iRBCs and nRBCs in the top compartments of transwells, and the respective eRBCs in the bottom compartments. Between these four populations, no shifts in reactivity were observed in either assay, except for a small shift in UMG-mediated opsonic phagocytosis in the nRBC population (top compartment). This may be due to enhanced staining of nRBCs in the top compartment, thus an apparent enhanced engulfment of stained nRBCs by monocytes.

In this transwell assay, antibodies do not appear to preferentially stain or target for phagocytosis the eRBCs exposed to iRBCs. This suggests that antibodies may not contribute to pathology in PAM by removing nRBCs and propagating maternal anemia but may indeed have protective functions. Future investigation could include changing conditions of the transwell assay to enhance EV release, such as altering the incubation conditions in the transwell to ensure parasites in iRBCs are viable and functioning normally. We incubated cells in the transwells for 48 hours to capture all stages of the *P. falciparum* asexual lifecycle, but perhaps this incubation could be focused on one stage of the lifecycle to minimize the time cells are incubated in the transwell. Additionally, confirmation of EV release should be performed by measuring these

particles in flow cytometry. This assay can also be adapted for testing different cargo in the EVs, including other VSAs or parasite material.

In summary, we did not further elucidate a connection between humoral immunity and protection from maternal anemia but determined that in this cohort, maternal hemoglobin is not associated with measures of IgG or IgM levels, C1q fixation, or opsonic phagocytosis. We observed a negative association between C1q fixation and opsonic phagocytosis and infant birthweight, but further investigation is needed to determine the mechanisms behind this. Additionally, we observed that antibodies do not target nRBCs that were exposed to iRBCs, thus the antibody immune responses are not likely to exacerbate maternal anemia in PAM.

5.3 – Why was an association between maternal hemoglobin and antibody levels or function not observed?

In this thesis, no association was found between maternal hemoglobin levels at delivery and any measures of antibody level or function. Previous literature on associations between antibodies and maternal hemoglobin is mixed. A recent meta-analysis suggested that VSA_{PAM} antibodies are more likely to be markers of infection, instead of a source of protection against adverse outcomes, including maternal anemia (Cutts et al., 2020). However, additional analyses of the primary data provided by the authors revealed heterogeneous associations of VSA_{PAM} antibodies with anemia depending on several variables, such as gravidity, study design and the type of assay used to characterize antibodies. These variables may also explain why we did not

observe an association between antibody levels or function and maternal hemoglobin in our study cohort in this thesis.

5.2.1 – Study setting

The level of malaria transmission in the study region influences the frequency of exposure to parasites and the diversity of strains. In turn, this could impact the magnitude and breadth of antibodies to VSA expressed in PAM. Lloyd et al collected samples from Yaoundé, a low transmission area with an entomological inoculation rate of approximately one infectious bite per person per month (Lloyd et al., 2018). In this study, just over half of women with a placental infection had antibodies to VAR2CSA at delivery, and this frequency was only 26.9% among women who had experienced four or more pregnancies. In our study in Colombia with low to moderate transmission, only 11% of the SMI cohort developed a placental infection detected in placental serum with qPCR, and of these women only 22% (2/9) had IgG-VAR2CSA antibodies at delivery in serum collected peripherally from mothers. Without sufficient exposure during pregnancy, a correlation between antibodies and pregnancy outcomes may not be detected. Based on these few studies, it is unclear how transmission intensity impacts associations between VSA_{PAM} antibodies and anemia, especially when SMIs occur in pregnancy.

Additionally, it may be more difficult to detect an association between antibodies and pregnancy outcomes when only SMIs occur during pregnancy, as opposed to microscopic infections. In a setting with intense seasonal transmission, Fried et al reported a positive correlation between infection and antibody levels against several VAR2CSA subdomains, but no

association between VSA_{PAM} antibodies and anemia was detected (Fried et al., 2018). In this setting, SMIs were reported with a frequency between 19.8% and 25.4% in women who were blood smear negative at enrolment. Thus, SMIs may boost antibody responses without contributing to poor pregnancy outcomes, such that an association between VSA_{PAM} antibody levels and outcomes may not be observed. In Colombia, SMIs were associated with damage detected in the placenta but not adverse pregnancy outcomes (Arango et al., 2013). In our longitudinal study, SMIs were not associated with maternal hemoglobin at delivery (Gavina et al., 2018). Thus, an association between antibody levels and maternal hemoglobin may not be likely in SMI pregnancies.

It is also important to note the prevalence of anemia in a study setting, as the fraction of malaria-attributable anemia may be low, masking a protective effect of VSA_{PAM} antibodies. In a previous study in Malawi that did not observe a protective association between VSA_{PAM} antibodies tested in flow cytometry or opsonic phagocytosis and maternal anemia, 62% of women were anemic and the mean hemoglobin level was 10.6 g/dL, likely reflecting non-malarial causes of anemia (Ataide et al., 2011). In Kenya, Staalsoe et al also reported overall low hemoglobin values, with 15.6% of women having particularly low hemoglobin concentrations under 7 g/dL (Staalsoe, Shulman, Bulmer, et al., 2004). Ndam et al also reported 62% of women were anemic at enrollment and 45% at delivery in a cohort from Benin, with only 16% and 12% of women positive for malaria infection at enrollment and delivery, respectively (Ndam et al., 2015).

Several factors besides malaria could influence maternal anemia, including micronutrient deficiency, co-infections, and quality of prenatal care (reviewed in (Ghosh & Ghosh, 2007; White, 2018)). Interestingly, in the cohort investigated by Chandrasiri et al where there was a protective association between VSA_{PAM} antibodies and maternal anemia, women were given supplements (including iron) and the median hemoglobin was 11 g/dL (Chandrasiri et al., 2016). Perhaps this supplementation controlled for malaria-independent causes of anemia, revealing an association between the VSA_{PAM} antibodies and malaria-attributable anemia. In our study, 35% of mothers were anemic at delivery, but SMIs were not associated with the development of maternal anemia (Gavina et al., 2018). If SMIs were not causing maternal anemia at delivery and this is instead due to non-malarial causes, this would affect the association between maternal anemia and VSA_{PAM} antibodies that are present during pregnancy.

5.2.2 – Study design

The design of the study and pattern of sample collection could impact an association between antibodies in the collected samples and pregnancy outcomes. For example, VSA_{PAM} antibodies detected earlier in pregnancy (collected at the first to early second trimester) may indicate a prior exposure to VSA_{PAM} in a previous pregnancy; thus, a pregnant woman may begin pregnancy with some immunity that may be boosted during subsequent pregnancies and be protective from poor outcomes. Staalsoe et al found that by six months postpartum, titres of VSA_{PAM} IgG decreased, but levels were boosted in women by the second trimester of their next pregnancy and PAM infection (Staalsoe et al., 2001). Modeling studies estimate that VAR2CSA antibodies can persist over many years (Fowkes et al., 2012).

Of previous studies that reported an association between VSA_{PAM} antibodies and maternal hemoglobin levels, 4 out of 6 assessed antibody levels and/or function at the first to second trimester (Chandrasiri et al., 2016; Chandrasiri et al., 2014; Feng et al., 2009; Gavina et al., 2018). In contrast, most studies that did not find a protective association with maternal anemia collected samples later in pregnancy at the third trimester and at delivery (Duffy & Fried, 2003; Lloyd et al., 2018; Mayor et al., 2013; Ndam et al., 2015; Serra-Casas et al., 2010; Tuikue Ndam et al., 2006). If these VSA_{PAM} antibodies developed because of infection late in pregnancy, increased antibody levels may reflect recent non-protective boosting from infection and obscure a relationship between antibody and protection from anemia. Thus, VSA_{PAM} antibodies detected later in pregnancy may be markers of infection, rather than a source of protection, as noted by Cutts et al (2020).

For these reasons, we assessed antibody levels and function at both study enrollment (first to second trimester) and delivery in serum collected peripherally from mothers, in addition to serum collected from the placenta at delivery to gain a fuller picture of antibody dynamics at the beginning and end of pregnancy. We previously reported a positive association between antibody binding inhibition in serum collected at enrollment and maternal hemoglobin, and similarly, the negative association between opsonic phagocytosis in infant birthweight was also observed in serum collected at enrollment. Though a protective association was not reported in this thesis, this reflects the importance of testing for antibody function at several time points in pregnancy, as no associations were observed with serum collected at delivery. Further investigation could include measuring antibody levels and function in time points between study enrollment and pregnancy.

Another consideration of study design is the gravidity of women included. Gravidity can greatly impact antibody levels, persistence, and functional activity. In high transmission settings, VSA_{PAM} antibodies develop with each successive infection in pregnancy and multigravid women are more likely to have antibodies that correlate with better pregnancy outcomes than primigravid women, supporting that these antibodies are maintained into the next pregnancy (reviewed in (Fried & Duffy, 2017)). Fried et al reported that VSA_{PAM} antibodies from primigravid women are short-lived compared to those from secundigravid and multigravid women (Fried et al., 2018). In a study that only included samples collected from primigravid women in the late third trimester, no protective association between VSA_{PAM} antibodies and anemia was observed (Ataide et al., 2010). The same cohort was followed into the second pregnancy, and again there was no association between maternal hemoglobin and VSA_{PAM} IgG or opsonic phagocytosis activity (Ataide et al., 2011). Thus, protective qualities of VSA_{PAM} antibodies might not fully develop until infections in multiple pregnancies have occurred.

In Malawi, recognition of iRBCs by VSA_{PAM} antibodies collected from samples in the late third trimester from secundigravid and multigravid women, but not primigravid women, was associated with a reduced odds of anemia (Aitken et al., 2010; Cutts et al., 2020). In our study, 44% of mothers were primigravid, 22% were secundigravid, and 33% were multigravid in the SMI cohort. A protective association between antibody blocking inhibition and maternal anemia was detected (Gavina et al., 2018), but perhaps in this study setting a larger cohort of multigravid women is needed to reveal protective associations between maternal anemia and the measurements of VSA_{PAM} antibodies investigated in this thesis. Notably, our study cohort of SMI women was small (n=45) and future studies should aim to recruit more women, though

when specifically investigating SMIs it may be difficult to confirm SMIs in rural areas instead of testing retrospectively in laboratories.

Finally, antimalarial treatment history within a cohort should be considered in study design. IPTp with 1500/75 mg of sulphadoxine and pyrimethamine (SP) during antenatal care visits is recommended by WHO in most areas in sub-Saharan Africa to reduce placental malaria (WHO, 2004). This treatment has the potential to affect the acquisition of VSA_{PAM} antibodies, as parasitemia is prevented or cleared to the point that plasma cells will not produce antibodies against VSA_{PAM} targets. In a previous study where no association was observed between VSA_{PAM} antibodies and maternal anemia at delivery, women received two doses of SP during pregnancy (Ndam et al., 2015). Aitken et al reported lower levels of VSA_{PAM} antibodies associated with IPTp treatment (Aitken et al., 2010). Similar observations were made in other studies from Kenya (Staalsoe, Shulman, Dorman, et al., 2004) and Ghana (Stephens et al., 2017) where reductions in antibody levels were associated with IPTp treatment (though pregnancy outcomes were not reported). In contrast, Serra-Casas et al did not observe an effect of IPTp on VSA_{PAM} antibody levels (Serra-Casas et al., 2010).

Thus, the effects of IPTp on VSA_{PAM} antibodies in relation to anemia are unclear. But, it is unlikely that IPTp treatment contributes to the severity of maternal anemia itself, as a controlled or lowered parasitemia should benefit the health of the mother. An IPTp treatment regime was not available in the communities where mothers from the SMI cohort resided, but even if this treatment was available in cases of malaria infection, SMIs go undetected. As a low prevalence of adverse birth outcomes was observed in this cohort, this suggests that natural

immunity to SMIs or low parasite virulence resulted in resolution of infections without IPTp treatment (Gavina et al., 2018), thus it is unclear what the effects of IPTp treatment would have been on the associations between antibodies and maternal anemia.

5.2.3 – Assay selection

The characterisation of a cohort can be performed with assays that quantify antibody levels and function. ELISA assesses levels of specific antibody isotypes, such as the IgG and IgM, and flow cytometry can assess IgG staining of whole iRBCs selected to express PAM-specific VSAs. In this thesis, we observed variable IgG and IgM levels within the cohort, but no associations with pregnancy outcomes were observed. Previous studies also did not detect an association between maternal anemia and levels of VSA_{PAM} antibodies using ELISA, flow cytometry, and multiplex bead assays (Aitken et al., 2010; Ataide et al., 2010; Ataide et al., 2011; Fried et al., 2018; Mayor et al., 2013; Ndam et al., 2015; Serra-Casas et al., 2010; Tuikue Ndam et al., 2006). Thus, we also investigated associations between pregnancy outcomes and functional activities of VSA_{PAM} antibodies.

Of studies that did observe a protective association between antibodies and maternal anemia, opsonic phagocytosis was a commonly used assay. Chandrasiri et al showed that antibodies with opsonic phagocytic activity were associated with reduced odds of severe malaria (severe anemia, cerebral malaria, hypoglycemia, and/or multi-organ failure), rather than antibody levels measured by ELISA (Chandrasiri et al., 2014). Similarly, Feng et al observed a correlation between a reduced odds ratio of maternal anemia and VSA_{PAM} antibody activity both by flow

cytometry and opsonic phagocytosis, but the relationship between phagocytic activity was stronger than VSA_{PAM} seroreactivity in flow (Feng et al., 2009). Interestingly, in one study, VSA_{PAM} IgG measured by flow correlated more strongly with opsonic activity than anti-adhesion activity in the IBA (Jaworowski et al., 2009).

Thus, we investigated the opsonic phagocytosis activity in our Colombian cohort, but instead of a positive association between this and maternal hemoglobin, we observed a negative association between opsonic phagocytosis in serum collected at enrollment and infant birthweight. Taken together with our previous observations (Gavina et al., 2018), this suggests that separate functions of VSA_{PAM} antibodies have differing effects on adverse birth outcomes. This is of importance in PAM vaccine design, as producing an immune response with a vaccine that mediates iRBC blocking in the placenta may be more important than other immune responses, including opsonic phagocytosis. Further characterisation could be performed with assays that measure other immunological responses, including the activation of natural killer (NK) cells and detection of downstream complement components, to fully understand a mother's immune response and best recapitulate this with a vaccine.

5.3 – Outstanding questions and knowledge gaps in the antibody response in PAM

Several gaps are highlighted by the work in this thesis. First, much of our knowledge of the immune response to malaria is studied in uncomplicated malaria, and many known mechanisms have yet to be specifically shown in PAM. This is especially true for anemia, as

these mechanisms still need to be fully elucidated for PAM, investigating both how a malaria infection may contribute to maternal anemia by affecting the placenta and the body systemically.

Second, more research on PAM is needed in areas outside of Africa to create a body of literature that reflects diverse settings, transmission rates, and *Plasmodium* species. Specifically, these studies also need to include the detection of SMIs, as this thesis demonstrated that many of the trends in antibody acquisition observed in Africa were not observed in Colombia, such as gravidity-dependent acquisition of VSA_{PAM} antibodies. This will aid in understanding different mechanisms in acquiring VSA_{PAM} antibodies, which may differ by geographic location.

Third, the connections between humoral immunity and adverse pregnancy outcomes still need to be addressed. Much of the literature on associations between VSA_{PAM} antibodies and outcomes focuses on the detection of these associations, but not the detailed mechanisms of how potential protection is mediated. Overall, the clearing and phagocytosis of iRBCs mediated by antibodies in the placenta is expected to contribute to both the health of the mother and fetus, but especially in the case of maternal anemia, these mechanisms still need to be proven. This could be performed with more assays that specifically test a potential antibody function that mediates anemia, such as the transwell assay used in this thesis to test targeted destruction of nRBCs.

5.4 – Concluding remarks

My work contributed to the characterisation of the SMI cohort by assessing both antibody levels and function. I first replicated previous data on IgG-VAR2CSA levels (Gavina et al.,

2018) and found that though these levels had significantly decreased from the first data set, the number of seropositive samples was not significantly different. I then continued the serological characterisation by measuring IgM-VAR2CSA at three collection points and evaluated trends in these data and associations with pregnancy outcomes. I optimized the IgM ELISA and added this to our laboratory's repertoire of assays. Another assay I optimized for our laboratory was the C1q fixation assay, which I used to test for complement recruitment to Ig from serum samples. In addition to these plate-based assay, I also used flow cytometry to test for IgG staining of whole iRBCs and opsonic phagocytosis. All these assays work well for serological characterisation and can be used to characterize serum in cohorts in future studies.

In conclusion, my research has explored humoral immune responses in serum collected from a cohort of women who experienced at least one SMI in pregnancy. As the bulk of literature on PAM focuses on microscopic infections, this research highlights the importance of investigating SMIs and understanding how these infections may affect pregnancy outcomes. The field of PAM is diverse as women from all around the world are exposed to malaria and the more investigation into each unique study setting will aid in finding effective treatments and vaccines to contribute to the fight against PAM.

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