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EXPOSURE TO MOSQUITO BITES AND STAGE-SPECIFIC ANTIBODY RESPONSES AGAINST *PLASMODIUM FALCIPARUM* IN TWO COMMUNITIES IN SOUTHERN GHANA

SEBASTIAN SHINE KWAPONG

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BY

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A Thesis submitted to the Department of Microbiology and Immunology of the School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfillment of the requirements for the award of Master of Philosophy degree in Infection and Immunity.

JUNE 2024

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature ……..... Date ……..

Name: ……...

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised per the guidelines on supervision of the thesis laid down by the University of Cape Coast.

Supervisor's Signature ……..... Date ……....

Name: ……

Co-Supervisor's Signature ..... Date.....

Name: .....

ABSTRACT

Immunity is gained through repetitive exposure to malaria, which differs greatly based on the severity of the infection and environmental factors. In today's research, a measure of *Anopheles gambiae* bite exposure has become fundamental for malaria endemic area identification, malaria risk prediction, and determination of the dynamics of malaria prevalence and reoccurrence in communities. This study aimed at investigating whether increased exposure to *Anopheles* mosquito bites in afebrile individuals enhances gametocyte production and subsequently antibody responses against *Plasmodium falciparum* gametocytes. Participants aged between 3 and 70 years were recruited from two communities, Simiw and Obom. Whole blood (1 ml) was collected from participants in Simiw (December 2019) and Obom (January 2020). Aliquots of blood were used to prepare dried blood spots for *P falciparum* identification by PCR. IgG antibodies against gSG6-P1 and *P. falciparum* antigens were quantified from plasma using indirect ELISA. Antibodies (IgG) against gSG6-P1 for individuals with the age groups 10-15 years for both Simiw and Obom showed a significant difference ($p<0.0001$) with participants in Simiw recording higher median (95%CI) antibody concentration (2.980 (3.077-3.662) ng/ml) than those in Obom (2.088 (2.176-2.602) ng/ml). Individuals from Obom within the age group 10-15 years had higher anti-Pfs230 antibody levels (6591 (1210551-4984924) ng/ml) compared to individuals from Simiw with age group 0-9 years (4707 (5874-10446) ng/ml) ($p=0.0008$). The higher exposure of participants from simw to mosquito bites were mostly from uninfected mosquitoes as anti-*Pf*CSP IgG antibody titres were found to be relatively lower in Simiw compared to Obom.

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DEDICATION

To my Parents, Elsie Kpongbosu and Lawrence Gameti Kwapong (of blessed memory), and my Siblings Odilia Jemima Kwapong and Sylvia-Vera Peggy Kwapong.

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CHAPTER ONE

INTRODUCTION

This chapter explores the background information on malaria, identifies the problem, the purpose of the study, and the research objectives. It also introduces the study hypothesis and justifies the necessity of the study. Lastly, it takes into account any constraints on its implementation, application, and study area.

Background

Considering the decline in malaria cases between 2010 and 2017 globally with estimated cases of 239 million and 219 million respectively, malaria remains a public health concern with reference to its endemicity in sub-Saharan Africa. According to the 2019 World Malaria Report, World Health Organization (WHO) Africa Region recorded 93% of malaria cases (213 million) and was considered the largest contributor to malaria morbidity in 2018, followed by the WHO South-East Asia Region which recorded 3.4% and then the WHO Eastern Mediterranean Region which accounted for 2.1% (WHO, 2019). Although research has helped provide ways of preventing, controlling, and treatment of malaria by the use of treated bed nets, malaria vector control, and the use of effective anti-malarial drugs, *Plasmodium* species especially *Plasmodium falciparum* still thrives regardless of all the aforementioned efforts (Doolan & Dobano, 2009; Rogers & Vijay, 2021).

Since 2005, malaria interventions put in place have resulted in a decline in malaria-related mortality in Ghana (WHO, 2015). Malaria elimination in Ghana, however, may not be feasible in the immediate future due to high levels of asymptomatic *Plasmodium* carriage, which leads to the continued production

of transmissible sexual stage parasites known as gametocytes (Owusu, et al., 2016; Tadesse, et al., 2015; Dinko, et al., 2013). Prior to treatment, WHO recommends using microscopy or an RDT to identify any probable cases of malaria. Accurate diagnosis aids in the treatment of febrile diseases and ensures that antimalarial medications are only administered when necessary (WHO, 2017). Asymptomatic asexual *P. falciparum* parasite carriage, as well as gametocyte carriage, are frequently present at submicroscopic densities and are not accompanied by any clinical manifestations, therefore infected persons barely seek medical attention (Ayanful-Torgby, et al., 2018).

Anopheles gambiae salivary gland protein-6 peptide 1 (gSG6-P1) is one of the salivary proteins of the *Anopheles* mosquito and antibodies directed against gSG6-P1 have proven to be associated with an individual's exposure to *Anopheles gambiae*, *Anopheles funestus*, and *Anopheles arabiensis*. Antibodies against gSG6-P1 are said to be possible serological biomarkers in determining different stages of *Anopheles* mosquito bite exposure in populations where malaria infection is low (Kassam, et al., 2021) yet, there is still the existence of malaria-transmitting vectors (Stone, et al., 2012). Frequent individual exposure to *Anopheles* mosquito bite is essential for immunity development against malaria since antibodies against gSG6-P1 antigen have a shorter lifespan in the absence of exposure (Drame, et al., 2010; Drame, et al., 2015).

A growing amount of research supports the creation of antibodies by malignant and healthy non-B cells, including those found in proximal tubuli cells and epithelial cells (Tijani, et al., 2021). Antibodies are thought to be an effective by-product of the immune system and are typically produced by B cells/plasma cells. Naturally acquired antibodies against pathogens can perform

their effector roles by simple binding (steric hindrance), complement activation, cellular cytotoxicity, and opsonic phagocytosis, neutralizing the parasite (Tijani, et al., 2021). Gametocytes that are picked up from malaria infected individuals respond to their surroundings in the mosquito lumen, and following fertilisation, they develop into zygotes. *Pf*s230 and *Pf*s48/45 are members of the 6-cystein protein family that are expressed on gametocyte surfaces and are crucial for fertilisation (Tijani, et al., 2021). Most malaria endemic communities have naturally occurring antibodies against *Pf*s230 and *Pf*s48/45 antigens, even in people who have only had minimal exposure to malaria, according to studies (Bousema JT, 2007; Tijani, et al., 2021). Additionally, these antibodies are ingested by mosquitoes as they consume blood meals, and they can exercise their transmission-blocking effects by preventing fertilisation, a crucial phase in the transmission of malaria (Tijani, et al., 2021). Previous studies conducted in Gambia and Burkina Faso proved that people residing in regions where malaria is intermittent naturally develop antibodies against various parasite phases (Drakeley, et al., 2004; Nebie, et al., 2008). Antibodies developed against the circumsporozoite protein (CSP), a sporozoite stage antigen, have been shown to be comparatively transient and vary considerably with infection severity. Studies also indicated that antibodies acquired against numerous asexual stage antigens like microneme proteins erythrocyte binding antigen 175 (EBA-175) and 140 (EBA-140), merozoite surface antigens 1 and 3 (MSP1 and MSP3 respectively) are noted for protecting children against clinical malaria (Amoah, et al., 2018; Drakeley, et al., 2004; Nebie, et al., 2008). *Plasmodium*'s gametocytes, which are specialised and permanently

differentiable *Plasmodium* forms, are what transmit the parasite from the human host to the *Anopheles* mosquito (Tijani, et al., 2021).

Asymptomatic and symptomatic *Plasmodium* carriers may harbour gametocytes which when picked up by a female *Anopheles* mosquito, will lead to the transmission of malaria infection (Ayanful-Torgby, et al., 2018). On the surface of these gametocytes is *Plasmodium falciparum* sexual-stage surface antigen (Pfs230), an ookinete surface antigen that is exposed to the human immune system as the zygotes transit to ookinetes. Anti-Pfs230 antibodies initiate the destruction of gametes and eventually inhibit the parasite from undergoing the sporogonic cycle in the mosquito hence preventing the transmission of malaria (Duffy, 2021).

After being exposed to malaria repeatedly, the body develops immunity that differs greatly based on the rate of spread and the environment. Nonetheless, this does not necessarily prevent infection nor eliminate parasites completely though antibodies are key in the control of blood stage parasite invasion (Bousema, et al., 2014; Behet, et al., 2014). Following repetitive contact with individuals living in a malaria endemic region to *Plasmodium falciparum*, partial immunity against clinical malaria develops. This immunity is partly IgG antibody-mediated as purified immunoglobulins obtained from immune individuals to infected persons led to a decline in parasitaemia and clinical symptoms (Cohen, 1961). Although immunity to malaria results in the reduction of disease development and its progression, it does not provide complete protection but rather gives room for persons with partial immunity to harbour parasites without presenting symptoms of clinical disease (Amoah, et al., 2018). Despite the potentially serious and abrupt start of malaria cases, more

than 75% of cases found during communal studies are asymptomatic. Some of these develop into symptomatic malaria cases a few days after their early discovery, but the majority of cases can last for several months and stay asymptomatic at various parasite densities during continuous surveillance (Bousema, et al., 2014). Nonetheless, individuals who are found to harbour *Plasmodium falciparum* but are asymptomatic often live in high malaria transmission communities and tend to carry multiple clones of parasites (Amoah, et al., 2018).

Problem Statement

During the recent decade, breakthroughs in genomic and proteomic technologies have aided mosquito salivary extract research, which has resulted in salivary transcriptomes for various mosquito species, including *A. gambiae*. Salivary protein reactions with the mammalian defense mechanism, in particular, have piqued curiosity. Indeed, the human immune system recognizes some of these salivary constituents and elicits specialized reactions. Lyme illness, Chagas illness, leishmaniasis, and, most lately, human African trypanosomiasis have all been linked to particular saliva antibodies (Ab). Remarkably, studies have described salivary proteins from *Anopheles* species as having an immunomodulatory role, and not much is understood about the human host reaction to *Anopheles* saliva (Cornelie, et al., 2007).

During a mosquito bite, mosquito saliva containing *Plasmodium falciparum* sporozoites progress to establish an infection, however, the influence of uninfected mosquito saliva on the progression of asymptomatic infection in the human host is not known. It has been suggested that saliva from arthropods such as sand flies, ticks, and mosquitoes can increase illness of arthropod-borne

pathogens (Styer, et al., 2011). Research indicates that arboviruses transmitted by mosquito bites result in an enhanced infection in animal hosts relative to illness with the same viruses by needle inoculation (Styer, et al., 2011).

Purpose of the Study

To investigate whether increased exposure to *Anopheles* mosquito bites in afebrile individuals enhances antibody responses against different life stages of *Plasmodium falciparum* (sporozoite, asexual, and gametocytes).

Research Objectives

1. To assess antibody responses against *Anopheles gambiae* salivary gland protein-6 peptide 1 (gSG6-P1) and selected *Plasmodium falciparum* antigens (*Pf*EBA-175, *Pf*CSP and *Pf*s230)
2. To determine *Plasmodium falciparum* stage-specific IgG levels in individuals with similar mosquito exposure.
3. To determine if the antibody responses to gSG6-P1 and the selected *Plasmodium falciparum* antigens (*Pf*EBA-175, *Pf*CSP, and *Pf*s230) are influenced by the presence of an active infection.

Hypothesis

This study hypothesized that repeated exposure of people living in malaria endemic communities to uninfected *Anopheles gambiae* mosquito bites increases gametocyte production and ultimately exposure to *Plasmodium falciparum* gametocytes.

Justification

Mosquito saliva contains a variety of immunomodulating proteins with anti-inflammatory and immunosuppressive activities, as well as anti-

haemostatic, anti-platelet aggregation, and vasodilator compounds (Ribeiro, 1989; Schleicher, et al., 2018; Titus, 1990). These saliva constituents serve primarily to assist blood feeding, nonetheless, they may also stimulate pathogen transmission. Saliva peptides can indirectly affect microbes by altering the milieu and promoting spread. (Ribeiro, 1989; Titus, 1990; Schleicher, et al., 2018). Maxadilan, a vasodilator, and immunomodulator found in sandfly's saliva, intensifies *Leishmania major* infection by suppressing both innate and adaptive immunological functions as well as the stimulation of host hematopoiesis. Other salivary peptides of vectors can interact positively with diseases to affect the spread (Schleicher, et al., 2018; McDowell, 2015). Salp15, an immunosuppressive tick saliva protein, binds to Borrelia burgdorferi's surface and prevents complement and antibody-mediated death (Schleicher, et al., 2018; McDowell, 2015). Proteins critical for *Plasmodium* survival within the vector, such as saglin, have been studied in mosquito salivary glands. Furthermore, recent research also discovered that immunizing against AgTRIO, a readily available SG protein unique to *Anopheles*, can reduce the amount of pathogen in the patient after mosquito-borne infection (Dragovic, et al., 2018; Schleicher, et al., 2018).

The gold standard for measuring malaria transmission intensity is using entomological inoculation rate (EIR), however, the insufficiencies associated with the traditional estimation of EIR using infected mosquitoes have led to the identification of some serological markers of exposure to malaria parasites as alternative and more effective measures of malaria transmission intensity in both high and low transmission settings (Kassam, et al., 2021; Stone, et al., 2012). According studies, estimates of anti-AMA1, anti-MSP119, and anti-

MSP2 antibody seroconversion rates from modelled cross-sectional data correspond favourably with conventional EIR estimates in regions with consistent medium-to-high transmission. (Bousema, et al., 2010). Since antibodies degrade more slowly than parasite clearance rates, sero-epidemiological methods for estimating transmission have an advantage over those used to estimate parasite prevalence (Kusi, et al., 2016). Hence the reason for quantifying antibody levels against sporozoite and blood stage antigens of *Plasmodium falciparum*.

Limitations

The study did not obtain any information relating to the mosquito vector from study sites making it difficult to validate the effect of mosquitoes on malaria transmission. The study did not look at human behaviour of participants from the two communities.

Organisation of the study

There are five chapters in this study. The background of the study is covered in its entirety in the first chapter. The study's second chapter pays attention to the literature review which talks about malaria impact on humans and host immune responses to malaria, mosquito saliva, and antigens of interest. The third chapter (research method) describes the details of how the study was designed and performed. The results obtained, the statistical analysis, and the discussion of the key findings form the core of the fourth chapter. The last chapter (chapter five) includes a work summary, conclusion, and recommendation for further studies.

Chapter Summary

This chapter served as an introduction to the study, outlining the problem, providing background information on malaria, and demonstrating the necessity of the investigation. We also include the research hypothesis, the aim, and the specific objectives that served as a guide for this research.

CHAPTER TWO

LITERATURE REVIEW

Chapter Introduction

This chapter examines the literary support of the study. We made an effort to follow the funnel style of review, moving from the big picture to the details. We also delved into the biology and life cycle of the *Plasmodium falciparum* as well as the impact of malaria on humans. We also reviewed the host's immune response to malaria. Finally, we reviewed mosquito saliva and its immunogenicity and immune response to the antigens of interest in the study.

Malaria: Disease Burden

Among the most frequent mosquito-borne diseases, malaria is considered the most prevalent worldwide. The public interest on malaria stems from its adverse effects on health and economic growth globally (WHO, 2018). Even though there was a decline in malaria cases by 20 million between 2010 and 2017 globally with estimated malaria cases of 239 million and 219 million respectively, it is still a major public health concern with reference to its endemicity in sub-Saharan Africa (WHO, 2018). Despite the combined resources of research and increasing international aid which aim to help eradicate malaria, it is still a canker to public health. That is, with an estimated mortality of 655,000 in 2010 (WHO, 2011) with children under age 5 years standing a higher risk, hence accounted for 61% (266,000) of the total malaria deaths globally (WHO, 2018). Estimated malaria cases and deaths by WHO Region in 2010 placed the African Region on top with estimated cases of 174 million and 596,000 estimated mortality (WHO, 2011).

Climate factors like rainfall patterns and temperature can influence the percentage of mosquitoes and their ability to survive, which in turn can influence the spread of malaria. Malaria spread varies seasonally, peaking during and after the wet season in many endemic areas. When the environment and other factors promote the spread in regions in which residents possess little to no immunity to malaria, an epidemic of the disease may develop. This could also be the result of persons with poor immunity migrating to regions where malaria is widely transmitted in search of greener pastures or refuge (WHO, 2019).

Parasite Life Cycle

According to studies conducted by Vuk et al., (2008) & Soulard et al., (2015), cited by Talakpo et al., (2019), the life cycle of *Plasmodium* is complex such that it occurs in two phases, namely the sexual stage which happens in the vector (*Anopheles* mosquito) and asexual stage which happens in vertebrates including human (Talakpo, et al., 2019). Humans are thought to contract malaria when an infected female *Anopheles* mosquito bites and injects the salivary gland-based sporozoites into their epidermis. These sporozoites are embedded in mosquito saliva, which helps the mosquito to take in blood (Talakpo, et al., 2019). For a successful infection establishment, it is essential for the *Plasmodium* parasite to employ motility to pilot through both the host skin and the hepatocytes (Schleicher, et al., 2018). To initiate malaria infection, the sporozoites move from the skin enter the human blood, and invade the hepatocytes after an hour and a half of blood circulation (Talakpo, et al., 2019) where they replicate asexually within a period to ten to fourteen days showing no symptoms. The sporozoites then mature into schizonts and rapture to release

merozoites which proceed to invade erythrocytes. Some of the merozoites, on the other hand, differentiate to form sexual erythrocytic stages called gametocytes (male and female) which circulate in the blood and are later picked up by the female *Anopheles* mosquito during the intake of blood. Once in the mosquito, the male gamete fuses with the female gamete to form an ookinete which enters the midgut and after a while, matures into an oocyst which eventually breaks to discharge sporozoites that later invade the mosquito salivary glands (Mawson, 2013).

Malaria Immunity

According to Bruce-Chwatt (1980), malaria immunity can be termed as the state of resistance to the steps that come into play in destroying the *Plasmodium* species or limiting their multiplication. Immunity to malaria comes in two forms: Natural immunity, which is an inherent property of the host where there is an immediate inhibitory response to the parasite inoculation which is independent of the previous infection. Acquired immunity, develops as a result of an enhancement of the defence mechanism the host mounts after a previous infection (Doolan & Dobano, 2009).

According to Orish et al., (2021), malaria immunity is referred to as a state of tolerance to the *Plasmodium* parasite obtained through recurrent events relating to the clearance and restraint of parasite replication. Malaria acquired immunity commences as disease immunity has successfully inhibited symptoms manifestation within a given threshold of parasitaemia not disputing the fact that disease immunity can fail beyond a given threshold of parasite load (Orish, et al., 2021). People living in malaria endemic communities are repeatedly exposed to the bites of malaria infected mosquitoes from childhood all through

to adulthood and disease symptoms manifestation is greatly associated with children. When a child is born in a malaria endemic community, he/she inherits passive-acquired immunity from maternal immunoglobulin G (IgG) which wanes with age thereby increasing the risk of malaria morbidity and mortality. Nonetheless, through frequent exposure to infectious bites, the child develops active immunity which peaks around puberty. In people living in malaria prevalent communities, asymptomatic infection is primarily demonstrated by patients with malaria immunity hence conferring some form of protection against malaria, even though this form of immunity is not sterile and can wane if malaria exposure is halted for about 3-4 years (Orish, et al., 2021).

Malaria immunity commences with an innate immune response comprising macrophages, dendritic cells, and natural killer cells (NK cells) leading to the release of pro-inflammatory cytokines that bring about inflammation resulting in parasite development inhibition. To prevent the actions of the pro-inflammatory cytokines that may result in severe malaria and mortality, the anti-inflammatory cytokines come to play. This is preceded by the stimulation of CD4 cells whose subsequent clonal expansion produces T helper cell type 1 (Th1) which leads to cell-mediated immunity and T helper cell type 2 (Th2) which also leads to antibody-mediated immunity. Th2 CD4 cells stimulate B cells to generate antibodies, particularly IgG which plays fundamental roles in immunity against parasites such as *Plasmodium falciparum* (Orish, et al., 2021). IgG is the key antibody responsible for the triggering of the cascade of immune responses involving opsonisation and subsequent phagocytosis, complement-mediated cell destruction. It is also involved in antibody-dependent cell-mediated destruction of parasitized red

blood cells that could result from the point at which antibodies attach to merozoites and parasitized red blood cells (Orish, et al., 2021). According to research, elevated stages of circulating IgG showed a correlation with lower malaria infection possibilities in some malaria endemic communities (Orish, et al., 2021). Research has made it known that Th1 CD4 cells aid macrophages in parasitized RBCs destruction, hence, promoting cell-mediated immunity (Orish, et al., 2021).

Antigens such as *Pf*CSP, *Pf*EBA-175 and *Pf*s230 expressed by *Plasmodium* species are rational antibody targets for vaccine development to inhibit sporozoite infection of the hepatocyte, inhibit parasite adhesion to the erythrocytes and prevent sexual stage sequestration and maturation of early gametocyte stage respectively (Chandley, et al., 2023).

A number of *Plasmodium falciparum* antigens have high prevalence and antibody levels which offer potential as a technique for estimating exposure to malaria. One way to monitor the spread and eradication of *P. falciparum* malaria and help with vaccine development is to measure antibody levels to antigens of the parasite (Ondigo, et al., 2020). Hence, the choice for selecting *Pf*CSP, *Pf*EBA-175, *Pf*s230 and gSG6-P1 for the study.

Mosquito Saliva and its Immunogenic Properties

Mosquito saliva is composed of physiologically active constituents that have the ability to inhibit blood coagulation, activate complement, and the ability to impede bite injury. The ability of some of these salivary constituents to trigger and alter immune mechanisms in the vertebrate host and subsequently alter the course of disease spread by vectors is an exciting development (Londono-Renteria, et al., 2015). In addition to the molecules in the mosquito

saliva that act as vasodilators, there are a series of proteins that work as immunomodulatory proteins (Schleicher, et al., 2018). Hematophagous arthropods' salivary proteins help them subsist on blood by reducing inflammatory and hemostatic responses and modifying the immunological response of their mammalian hosts (Palosuo, et al., 1997; Remoue, et al., 2007). Aside from facilitating feeding, salivary proteins could in a way influence the transmission of microbes by modifying the host microenvironment and enhancing disease transmission. Maxadilan, a vasodilator, and immunomodulator found in sandfly saliva is one of several examples that aggravates *Leishmania major* infection. Studies conducted in 2008 by Schuijt et al., showed that Salp15 found in the saliva of ticks prevents the complement-mediated killing of *Borrelia burgdorferi* (Schleicher, et al., 2018).

Antibody Response to Mosquito Saliva

It has been suggested that measuring the antibody (Ab) response to vector saliva in human populations is a useful method for determining how exposed a host is to vector bites and how likely they are to get a vector-borne illness. (Remoue, et al., 2005; Schwartz, et al., 1990). Studies have shown that some of the salivary proteins such as *Anopheles gambiae* salivary gland protein-6 (gSG6) can also stimulate particular antibody responses which might signify an unfailing marker of vertebrate exposure to vector bites obtained from arthropod vectors, like *Aedes, Culex, Anopheles gambiae* (Palosuo, et al., 1997; Remoue, et al., 2007; Brummer-Korvenkontio, et al., 1997). However, the entire saliva was unable to provide useful biomarker due to possibility of cross-reactivity with salivary epitopes of other hematophagous arthropods, a lack of repeatability across batches of saliva, and insufficient production required for

large-scale investigations (Drame, et al., 2010; Poinsignon, et al., 2008). For these reasons, a more appropriate biomarker for *Anopheles* bites have been found and verified as a particular, antigenic, simple to produce, and highly conserved peptide between gSG6-P1 (Poinsignon, et al., 2008).

Anopheles Gambiae Salivary Gland Protein-6 Peptide 1 (gSG6-P1)

Studies conducted by Kuadkitkan et al., (2010) & Ribeiro et al., (2007) cited by Londono-Renteria, et al., (2015), advocate that in regions with minimal malaria peril, the IgG response against gSG6-P1 is a trustworthy substitute to correctly evaluate the efficacy of malaria control strategies and monitoring programs relating to malaria vector exposure. A helpful surrogate predictor of human-vector interaction with American *Anopheles* species is the artificial peptide gSG6-P1 (Londono-Renteria, et al., 2015). The use of antibody responses against salivary proteins (for example gSG6-P1) as a marker of exposure to *Anopheles* bites was previously revealed by the early epidemiological studies among people living in malaria endemic areas (Drame, et al., 2010; Poinsignon, et al., 2010). Certainly, distinct IgG reactions to this gSG6-P1 peptide appeared to provide a precise assessment of very moderate and minimal levels of *Anopheles gambiae* exposure and bites obtained from *Anopheles funestus*, Africa's next most common malaria transmitter (Poinsignon, et al., 2009; Poinsignon, et al., 2010).

Plasmodium falciparum Circumsporozoite Protein (*Pf*CSP)

Plasmodium falciparum circumsporozoite protein is a glycosylphosphatidylinositol (GPI) anchored membrane protein which serves as a covering of the surface of the sporozoite (Ghosh & Jacobs-Lorena, 2009). *Pf*CSP is found in all *Plasmodium* species, and in as much as there are amino

acid sequence variations across species, the overall domain structure is well conserved (Zhao, et al., 2016). The shedding of *Pf*CSP comes about when the sporozoite glides on a solid surface leading to the creation of a *Pf*CSP trail. *Pf*CSP is crucial in sporozoite differentiation and oocyst development. The *Pf*CSP is an essential factor in the deposition of the inner membrane and microtubule network formation associated with the oocyst's outer membrane (Ghosh & Jacobs-Lorena, 2009). The *Pf*CSP comprises three important regions namely, (i) the NH2-terminal region comprising the ligand-binding domain, proteolytic site, multiple human HLA-restricted epitopes and region I; (ii) a centrally located repeated region; (iii) the COOH-terminus containing a type I thrombospondin repeated region (Noe, et al., 2014). In the *Plasmodium* parasite life cycle, *Pf*CSP is known to help in sporozoites formation in the mosquito midgut, sporozoites release from the oocyst, salivary gland invasion, sporozoites attachment to the hepatocytes, and the invasion of hepatocytes by sporozoites (Zhao, et al., 2016).

Antibody response to sporozoites mainly targets the central repeated region which is the immune-dominant region. Studies conducted by Tam et al., (1990) in mouse models, revealed that there was higher antibody titre to the central repeated region of *Pf*CSP indicating immune protection against malaria (Noe, et al., 2014). *Pf*CSP is said to be the most clinically advanced malaria vaccine candidate, presenting significant but non-sterile immune protection against malaria in humans (Harupa, et al., 2014). Antibodies against sporozoites might end up promoting their antibody-dependent cellular phagocytosis by the monocyte cell line THP-1 (Aitken & Mahanty, 2020). The antibody against *Pf*CSP tends to block the motility of sporozoites and prevents the invasion of

hepatocytes (Harupa, et al., 2014). Several research investigations carried out in various epidemiological settings have shown that the levels and sero-prevalence of sporozoite antibodies, especially those against the repetitive epitope of *Pf*CSP, are a predictor of *Plasmodium* parasite exposure over time and are a good indicator of malaria transmission intensity (Pereira, et al., 2018).

Plasmodium falciparum Erythrocyte Binding Antigen -175.5R (*Pf*EBA-175.5R)

A family of genes known as erythrocyte binding ligand (EBL) that encodes proteins implicated in the unique host cell receptor recognition of *Plasmodium knowlesi* and *Plasmodium vivax* Duffy-binding proteins includes *Plasmodium falciparum* Erythrocyte Binding Antigen-175.5R (*Pf*EBA-175.5R). Erythrocyte Binding Antigen-175.5R (*Pf*EBA-175.5R) is released from micronemes and aggregates at the apical merozoite surface. (Barua, et al., 2019). *Pf*EBA-175.5R closely engages with erythrocyte surface by sialic acid-dependent epitope on glycophorin A and is considered as the dominant ligand that mediates parasite invasion of erythrocytes through glycophorin A pathway (Amoah, et al., 2018; Adams, et al., 2001). *Pf*EBA-175.5R's extracellular domain is defined by two cys-rich regions, an amino Duffy-binding-like (DBL) region and a C-cys region close to a transmembrane region (Adams, et al., 2001). Research has shown that, in as much as the C-cys domain has no identified function, it is more highly preserved compared to the DBL domains. The ligand region in the erythrocyte binding protein (DBL-EBP), an EBL component, known as the DBL region, controls erythrocyte binding process. *Pf*EBA-175.5R possesses a tandem DBL region that, in mature proteins, happens to serve as a single region; the F2 region by itself could facilitate

attachment to erythrocytes. (Adams, et al., 2001). Antibodies developed against region III and region IV of *Pf*EBA-175.5R have been associated with protection from malaria (Barua, et al., 2019).

Plasmodium falciparum Surface Antigen (*Pf*s230)

The intraerythrocytic gametocyte of *Plasmodium falciparum* expresses the ookinete surface antigen, *Pf*s230, that is revealed on the surface of extracellular gametes in the midgut of mosquitoes during the reproductive stage of the parasite's life cycle (Acquah, et al., 2017). Antibodies directed to the sexual stage antigen, *Pf*s230 can end up inhibiting early gametocyte stage sequestration and maturation or could also act against the gametes in the midgut of the female anopheles after a blood meal (Aitken & Mahanty, 2020) hence serves as malaria transmission blocking. Naturally acquired antibodies in the human host recognize antigens expressed on the immature gametocytes' surface but are not able to recognize those on the surface of the mature gametocytes. Even though the mechanism by which these antigen recognitions occur is unclear, it is presumed to be similar to that of antibodies recognizing asexual antigens expressed on the surface of infected erythrocytes (Aitken & Mahanty, 2020). According to studies conducted, naturally acquired antibodies that have transmission-blocking properties specifically target gametocyte antigens *Pf*s230 hence, prevent fertilization in the female *Anopheles* mosquito midgut by inhibiting the *Pf*s230 role (Stone, et al., 2018). Studies have shown that antibodies against *Pf*s230 are partially reliant on complement for transmission-blocking activity and likely consequences in complement-mediated lysis of parasites (Duffy, 2021).

Chapter Summary

Through citations to scientific literature, we examined a few malaria-related topics in this chapter. To understand the impact of malaria, we first looked at historical and contemporary descriptions of the illness's impact on humans. The parasite's life cycle and biological makeup were also briefly discussed. Following a brief overview of malaria antibody and vaccine development, we looked at the immunological responses to the disease in the human host with a focus on adaptive and innate immune responses. Lastly, we reviewed the malaria parasite antigens (gSG6-P1, *Pf*CSP, *Pf*EBA, and *Pf*s230) that were used for this research work.

CHAPTER THREE

RESEARCH METHODS

Chapter Introduction

We go into detail in this chapter about the design and implementation of the study's laboratory analysis and sampling phases. We also provide a synopsis of the data handling and analysis procedures.

Study Design

A pilot study was carried out where samples were taken from two different communities in the Southern part of Ghana. This study design was chosen to obtain the required preliminary data and research skills for a future larger study.

Study Site

The study was carried out in two communities, Simiw and Obom, where *Plasmodium falciparum* is the major causative agent for malaria. Simiw is a farming community situated about 2 kilometers behind the Ankaful maximum prisons. It is under the authority of Komenda Edina Eguafo Abrim District (KEEA) in the Central Region of Ghana and it is adjacent to the Cape Coast municipality. Obom is a farming rural community situated in the Ga South district in the Greater Accra Region and has been classified in the past as having a high asymptomatic parasite prevalence (Acquah, et al., 2020). The wet season, which peaks between May and August, and the dry season, which starts in November and lasts until April, both coincide with the recurrent nature of malaria spread in Simiw and Obom (Amoah, et al., 2018).

Study Population

Participants from Simiw and Obom communities had no symptoms of clinical malaria and were between 3 and 70 years of age.

Sampling Method

In this study, a simple randomized sampling method was employed.

Sample Size Determination

$$n = \frac{Z^2 N(1-P)}{d^2}$$

Where n=Sample size with an estimated population

Z= Z statistic for a level of confidence [confidence level at 95% (1.96)]

P= Estimated prevalence

N= Population size

d= margin of error at 5% (0.05) (Daniel, 1999)

Prevalence by PCR for Obom is 71.4% (Abagna, et al., 2022)

$$n = \frac{1.92^2 x\ 0.714(1-0.714)}{0.05^2}$$

n = 301.1 ≈ 301

Prevalence by PCR for Simiw is 26.6% (Obboh, et al., 2020)

$$n = \frac{1.92^2 x\ 0.266(1-0.266)}{0.05^2}$$

n = 287.89 ≈ 288

The minimum number of samples required for this study was 301 in Obom and 288 in Simiw, however, a larger number of samples was used because of the possibility of reducing the margin of error and the reporting of false-positive or false-negative results when a large number of samples is used for a study. The total number of samples used for the study was 292 in Simiw and 349 in Obom.

Inclusion Criteria

Malaria RDT-positive participants without signs and symptoms of complicated malaria were included in the study.

Exclusion Criteria

Participants who were unable to walk to the sample collection point and people who were non-residents in any of the study communities were excluded.

Ethical Consideration

Ethical clearance was obtained from the Institutional Review Boards of Noguchi Memorial Institute for Medical Research with reference number #024/14-15 and 089/14-15. All experiments were carried out strictly in accordance with the ethics and regulations of the Institutional Review Board of Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. All research participants provided written consent. Parental consent was obtained from parents and guardians for participants below 18 years, while children aged between 12 and 17 years provided assent.

Laboratory Procedure

Sample Collection

The participant was made to sit in front of the phlebotomist and a tourniquet was applied above the site for phlebotomy. The skin was cleaned with 70% ethanol and 1 ml of blood was drawn through the vein. The blood was transferred into a microcentrifuge tube containing ACD anticoagulant labelled with the participant ID, and a spot plaster was placed on the site to prevent blood infection.

Haemoglobin Level Assessment

The Urit-12 haemoglobin meter (URIT Medical Electronic Co. Ltd., China) was calibrated by inserting the chip in the haemoglobin strip container into its appropriate pot located at the side of the meter and the meter was switched on by pressing the power button. The appropriate haemoglobin strip was inserted into the meter with the circular patch on top of the green light. A drop of whole blood (15 µl) from the syringe was dropped at the circular patch of the strip after an indicator showing a drop appeared on the meter screen. The haemoglobin level was recorded against the participant ID in the field notebook.

Whole Blood Blot

Four separate spots of about 50 µL of blood on the Whatman #3 filter paper (GE Healthcare, WI, USA) labelled with the participant ID and date. The spotted Whatman filter paper was placed on the filter paper stand and left to dry. The dried Whatman filter paper was placed in a zip-lock bag containing silica gel and sealed.

***Plasmodium falciparum* Density Estimation**

WHO guidelines were used to process the blood films (WHO, 2016). At the time of recruitment, each patient had blood taken and thin and thick blood films were made and allowed to dry. The thin film was fixed by dipping the thin part into a small container containing methanol for 2 seconds, carefully avoiding contact between the thick film and methanol. The slides were placed on a drying rack for 2 minutes to air dry the methanol and placed on a staining rack. Approximately 3 ml of 10% Giemsa solution was gently poured on the slides and was left for 10 minutes for the blood films to stain. The slides were later flushed with water gently to remove the stain and the slides were placed on a drying rack to air dry. *Plasmodium falciparum* density was estimated in

accordance with the WHO guidelines for malaria parasite counting (WHO, 2016). The thick and thin blood slides were afterward examined at high magnification (100x) under oil immersion to detect parasite density. The number of parasites per 200 white blood cells (WBC) was determined, assuming an average normal WBC level of 8000/µl for thick film and RBC level of 5,000,000/µl for thin film.

Plasma Separation

The remaining 1ml of the whole blood in the microcentrifuge tube was placed in the mini centrifuge and another sample was used as a balance, and the mini centrifuge was turned on to spin for 2 minutes. The plasma was separated using a pipette and transferred into a 1.5ml microcentrifuge tube labelled with the participant ID and date and stored at -20^{o}C.

Enzyme-Linked Immunosorbent Assay (ELISA)

Total IgG antibody levels of *Anopheles gambiae* salivary gland protein-6 peptide 1 (gGS6-P1), circumsporozoite protein (*Pf*CSP), erythrocyte binding antigen (*Pf*EBA-175.5R) and *Plasmodium falciparum* asexual antigen (*Pf*s230) were quantified using indirect enzyme-linked immunosorbent assay (ELISA).

Determination of IgG Levels by Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The *Anopheles gambiae* salivary gland protein-6 peptide 1 (gSG6-P1) antigen was diluted to a final concentration in phosphate buffered saline (1X PBS) with pH 7.4, and vortexed to obtain a uniform mixture. Nunc Maxisorp 96 well ELISA plates were coated at 1 µg/well with recombinant antigens gSG6-P1, *Pf*CSP, 20 µg/well *Pf*EBA-175.5R in phosphate buffered saline (PBS, pH 7.2) and 1 µg/well *Pf*s230 in carbonate buffer (pH 9.0). The first two

wells were coated with purified polyclonal IgG (BP055, The Binding Site) as the standard at a dilution concentration of 1/10000 of a 2-fold serial dilution for 6 additional concentrations and incubated overnight at a temperature of 4℃. The plates were washed three times with 250 µl PBS containing 0.05% tween 20 (PBS-T) after which, the plates were hit face down on several layers of tissue paper on the bench to remove excess liquid and bubbles. The wells were then blocked with 3% skimmed milk at a volume of 200 µl to prevent nonspecific binding to the unbound regions, incubated at 37℃ for 1 hour, and washed 3 times repeatedly with 250 µl PBS-tween. Serum was diluted in 1% skimmed milk using a 1:200 dilution concentration. 100 µl of the diluted serum, positive samples acquired from a pool of seropositive persons, and negative control samples acquired from a pool of seronegative persons were added to the well in duplicates and incubated at 37℃ for 1 hour. The plates were washed three times with 250 µl PBS-tween, and the plates were hit face down on several layers of tissue paper on the bench to remove excess liquid and bubbles. Conjugated goat anti-human IgG-HRP diluted in 1% skimmed milk in a dilution concentration of 1:3000 was added at a volume of 100 µl/well and incubated at 37℃ for 1 hour. The plates were washed three times afterward with 250 µl PBS-tween, and the plates were hit face down on several layers of tissue paper on the bench to remove excess liquid and bubbles. After washing, the colour development was observed after adding peroxidase substrate 3,3’,5,5’-tetramethylbenzidine (TMB) 100 µl/well and incubated for 10 minutes at 37℃. The enzymatic reaction was halted by adding 100 µl of 0.2 mM sulfuric acid (H2SO4) per well and their optical density (ODs) were read at 450 nm using a Multiskan FC plate reader (Thermo Scientific, USA).

Tween-20/Chelex DNA Extraction

Two punches of indexed blood blot filter paper were made to a size of 5-6 mm into a sterile 1.5 ml microfuge tube. 1 ml of 1X Phosphate Buffered Saline supplemented with 0.5% Tween (PBST) was added, vortexed, and incubated overnight at room temperature on a plate shaker. The following day, the tubes containing the dry blood spot (DBS) and PBST mixture were vortexed briefly and spun for 2 minutes at a speed of 14,000 rpm, decanted, and padded to remove the reddish PBST supernatant. 1 ml of ice-cold 1X PBS was added to the tube containing the filter paper, vortexed, and incubated at 4℃ for 30 minutes. The tube containing 1X PBS mixture spun at a speed of 14,000 rpm for 2 minutes and the supernatant was aspirated. 50 µl of 20% chelex and 100 µl of nuclease free water was added and incubated at 95℃ for 10 minutes on a heat block and vortexed at an interval of 2 minutes. The tubes were spun for 8 minutes at a speed of 14,000 rpm. To avoid the chelex, the supernatant (DNA) was carefully transferred into a sterile labelled 0.5ml microfuge tube and stored at a temperature of -20℃.

***Plasmodium* Species Identification**

The unique regions of *P. falciparum*, *P. ovale* and *P. malariae* 18s rRNA gene from genomic DNA extracted from samples was amplified using nested PCR based on the protocol described by Amoah et al., 2019 with minimal alterations. In the primary reaction, thus nest 1, 80 nM of genus specific primers rPLU5 (forward) and rPLU6 (reverse) were used in a total reaction volume of 15 µl comprising 3 µl DNA template, 1X PCR buffer, 166 nM dNTPs, 2.5 nM MgCL2, and 1 U of OneTaq DNA polymerase. In the secondary reaction which is nest 2, the species-specific primers rFAL1/rFAL2 (133.33 nM),

rOVA1/rOVA2 (333.33), and rMAL1/rMAL2 (333.33 nM) were each used in different 15 µl reactions for *P. falciparum, P. ovale* and *P. malariae* identification respectively. 0.5 µl of nest 1 product was used as the template for the secondary reaction (nest 2). An initial denaturation cycling condition of a temperature of 95℃ was run for both the primary and secondary reactions for 5 minutes followed by 35 cycles of cyclic denaturation process at a temperature of 94℃ for 30 seconds, primer annealing at 55℃ (for nest 1) and 58℃ (for nest 2) for 30 seconds, and extension at 68^0C for 1 minute 40 seconds for nest 1 and 30 seconds for nest 2 excluding P. *Ovale* and a finishing extension at 68℃ for 5 minutes. The PCR products of the nest 2 reaction were resolved on a 2% agarose gel pre-stained with ethidium bromide, which was subsequently visualized using the UV setting on a Vilber gel documentation system (Vilber, Collegien, France).

Data Analysis

The four-parameter fitting application ADAMSEL FPL (Ed Remarque) was used to convert the optical density (OD) values obtained from the ELISA plate reader (Thermo Scientific Multiskan FC) into weighted concentrations (wConc). Plasma samples were assigned seropositive for antibodies against an antigen (PfCSP, gSG6-P1, Pfs230proC, and PfEBA-175.5R) if the concentration of the antibodies in the sample was greater than the average plus two times the standard deviation of that for the negative control sample (pooled malarial-naïve plasma).

Graphs were plotted using GraphPad Prism software version 9.0.2 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel (Microsoft Crop., Redmond, WA, USA). The data was categorized into study sites, gender,

age groups, and diagnostic tests used. Kruskal-Wallis test and Dunn's multiple comparison post-hoc tests were used to compute statistical significance between median values of quantitative data between the various age groups. The antibody concentrations of gSG6-P1, *Pf*CSP, *Pf*EBA-175, and *Pf*s230 were log 10 transformed. Correlations between IgG responses against *Pf*CSP and gSG6-P1was determined using Spearman's rank correlation. The concentration of gSG6-P1 was categorized into moderate (1-1.999 ng/ml), high (2-4.999 ng/ml), and extreme (5-5.999 ng/mL) mosquito exposure, and 0-0.999 ng/mL was considered as negative for mosquito exposure (Kassam, et al., 2021). The association between *Plasmodium falciparum* carriage for *Pf*CSP, gSG6-P1, *Pf*s230, and *Pf*EBA-175.5R was also computed using GraphPad Prism version 9.0.2 statistical significance was computed at $p<0.05$.

Chapter Summary

We went over the specifics of the study's design and implementation in this chapter, including both the sampling and laboratory analysis phases. The procedures followed in handling and analysing the data were also outlined.

CHAPTER FOUR

RESULTS AND DISCUSSION

Chapter Introduction

There are two main sections to this chapter. To test the study hypothesis, the first section which is the results explains the outcomes as well as the statistical analysis and techniques used on the data. It also includes detailed graphics that show the outcomes of the different tests that were performed. The outcomes are discussed in the second section in the context of earlier research on the topic. It also makes an effort to explain the outcomes by concluding the data and pertinent literature.

Results

Participants' Demographic and Clinical Data

This study recruited 641 afebrile participants in both Simiw and Obom communities in the Central and Greater Accra Regions of Ghana respectively. A total of 292 participants were recruited in the Obom community with a median (IQR) age of 14 (10-23) years of which 55.1% (161/292) were females accounting for the majority. A total of 349 were recruited in the Simiw community with a median (IQR) age of 14 (11-32) years and 67.9% (237/349) were females. The median haemoglobin levels of participants in Obom were higher compared with that of Simiw (Kruskal-Wallis statistic=12.2 and 11.2 respectively, p=0.0001; Table 1).

Furthermore, the median temperature of participants in Obom was higher than that of Simiw (Kruskal-Wallis statistic = 36.8, p=0.0001; Table 1). Individuals who tested positive for *P. falciparum* by PCR in Simiw (50%) were higher compared with Obom (39.38%) even though participants showed no clinical

malaria symptoms. The microscopy positive at both Simiw (35.9%) and Obom (30.5%) were lower compared with microscopy negative (64.1%) and (69.5%) respectively.

Table 1: Demographic and clinical data for the study participants

		Simiw	Obom	P value
Number of Participants (N)		348	292	
Age (years)		14 (11-32)	14 (10-23)	P=0.0366
Sex	Males	112 (32.1%)	131 (44.9%)	
	Females	236 (67.8%)	161 (55.1%)	
HB (g/dL)		11.2 (10.3-12.5)	12.2 (11.1-13.85)	P=0.0001
Temperature (°C)		36.6 (36.2-36.8)	36.8 (36.45-36.9)	P=0.0001
Microscopy Diagnosis n/N (%)	Positive	125/348 (35.9%)	89/292 (30.5%)	P=0.3200
	Negative	223/348 (64.1%)	203/292 (69.52%)	P=0.7804
P. falciparum PCR Positive (%)		174/348 (50%)	115/292 (39.38%)	

Seroprevalence of IgG antibody levels of gSG6-P1, *Pf*CSP, *Pf*EBA-175, *Pf*s230 in both communities

A highly significant difference (p<0.0001; Table 2) was seen in the seroprevalence of IgG antibody responses of gSG6-P1 in Simiw (81% (282/348)) and Obom (65.5% (190/292). The differences in the seroprevalence for *Pf*CSP, *Pf*EBA-175, and *Pf*s230 were insignificant for both Simiw and Obom.

Table 2: Seroprevalence of IgG antibody levels of gSG6-P1, *Pf*CSP, *Pf*EBA-175, *Pf*s230 in both communities

	Simiw n/N (%)	Obom n/N (%)	P value
gSG6-P1 IgG	282/348 (81)	190/292 (65.5)	<0.0001
*Pf*CSP IgG	234/348 (67.2)	219/292 (75)	0.7044
*Pf*EBA-175 IgG	304/348 (87.4)	266/292 (91.1)	0.8613
*Pf*s230 IgG	275/348 (79)	244/292 (83.6)	0.9572

Antibody Response to gSG6-P1 Antigen

Total IgG levels unique to gSG6-P1 were measured to assess *Anopheles gambiae* mosquito bite exposure using indirect ELISA. Kruskal-Wallis and Dunn's multiple comparison post-hoc test was used to compute statistical significance between the communities and the various age groups. Individuals from Simiw recorded higher significant median IgG stages of gSG6-P1 compared to individuals from Obom (p<0.0001; Figure 1A). The antibody response of each participant was categorised into either of the Three (3) age groups used for the study; 0-9 years, 10-15 years, and 16 years and above. There

was a significant difference between total IgG against gSG6-P1 for the age categories 0-9 years and 10-15 years in Simiw (p=0.0008) with the age category, 10-15 years having a higher median (95%CI) antibody concentration of 2.980 ng/ml (3.077-3.662) than age group 0-9 years 2.130 ng/ml (1.946-2.463). There was also a statistically significant difference between total IgG against gSG6-P1 for the age groups 0-9 years and 16 years and above in Simiw (p=0.0483; Figure 1B) with the age group 16 years and above having a higher median anti-gSG6-P1 (2.544 (2.887-3.611) ng/ml) compared to the age group 0-9 years (2.130 (1.946-2.463) ng/ml).

There was a statistically significant difference between the age category 0-9 years and 16 years and above in Obom (p=0.0038; Figure 1B) with the age group 16 years and above recording a higher median antibody concentration (2.408 (2.421-2.894) ng/ml) than age group 0-9 years (1.746 (1.760-2.302) ng/ml). There was no statistically significant difference between total IgG against gSG6-P1 for the age groups 10-15 years and 16 years and above in Simiw (p>0.999) and Obom (p>0.999). No statistically significant difference was observed between total IgG against gSG6-P1 for the age category 0-9 years and 10-15 years in Obom (p=0.154; Figure 1B).

The total IgG against gSG6-P1 for the age groups 10-15 years across both communities indicated a statistically significant difference (p<0.0001; Figure 1B) with participants in Simiw recording higher median (95%CI) antibody concentration (2.980 (3.077-3.662) ng/ml) than those in Obom (2.088 (2.176-2.602) ng/ml). There was no statistically significant difference against gSG6-P1 between the age groups 16 years and above across both communities (p=0.48; Figure 1B).

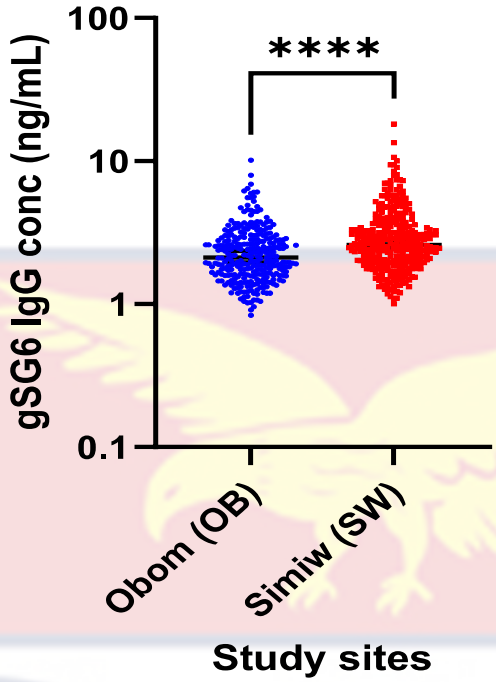


Figure 1A: gSG6-P1 IgG antibody responses for both study sites

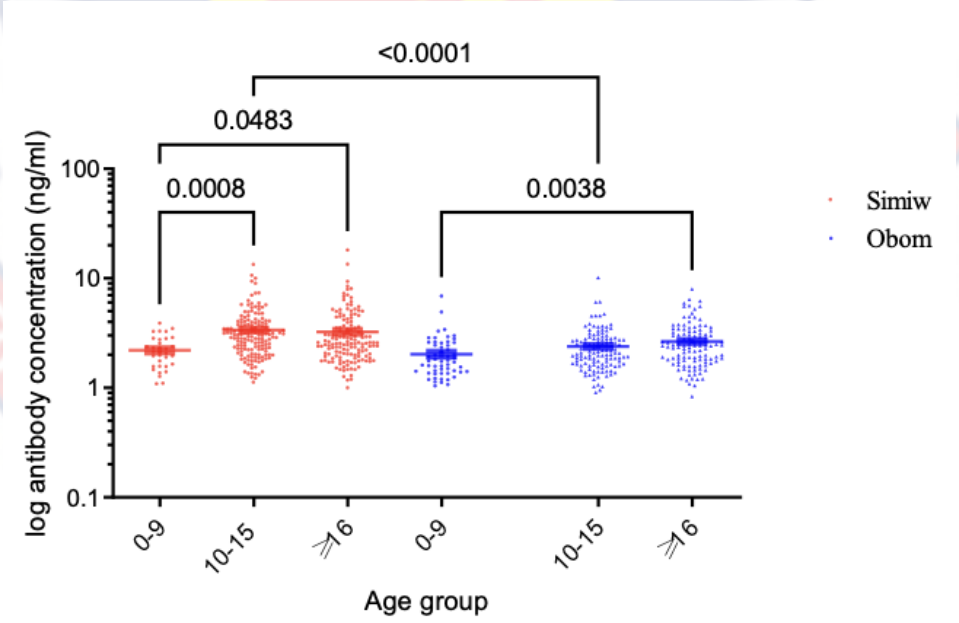


Figure 1B: Antibody Response to gSG6-P1 Antigen.

The absence of bars (p values) indicates the absence of statistical significance. gSG6-P1 antibody concentrations were measured across various age groups in years.

Antibody Response to *Pf*CSP Antigen

Indirect ELISA was done to determine the concentration of *Pf*CSP antibodies present in the participant's plasma and their protective capacity in terms of vaccine development. Log transformation was done on the graph to cluster data that was widely apart. Individuals from Obom recorded significantly higher *Pf*CSP IgG levels median concentrations (27781 (23674-33921) ng/ml) than individuals from Simiw (18330 (15984-22924) ng/ml; P=0.002; Figure. 2A). Although the statistical difference between total IgG stages against *Pf*CSP for the different age groups in Simiw (p=0.999) and Obom (p=0.999) was insignificant, individuals with the age group, 0-9 years in Obom had higher median *Pf*CSP IgG antibody levels (24006 (25177-53412) ng/ml) compared to their counterpart in Simiw (178822 (19162-42046) ng/ml). The median *Pf*CSP IgG antibody levels for individuals with age category 10-15 years (29804 (32334-46705) ng/ml) from Obom was higher than their counterparts from Simiw (20028 (24947-33834) ng/ml), the same was also observed for individuals with the age group, 16 years and above in Obom (31752 (32019-51972) ng/ml) and (21333 (25482-35951) ng/ml) for individuals in Simiw (P=0.999; Figure 2B).

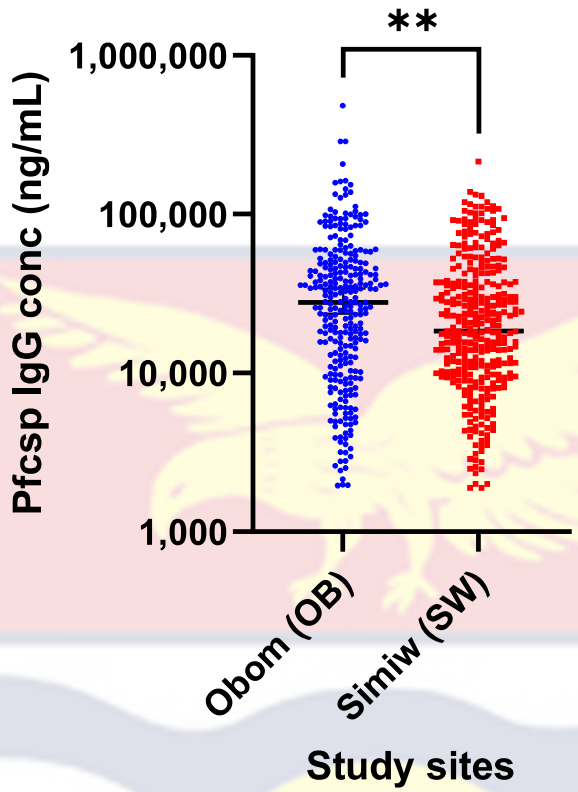


Figure 2A: IgG antibody levels against *Pf*CSP response for both study sites

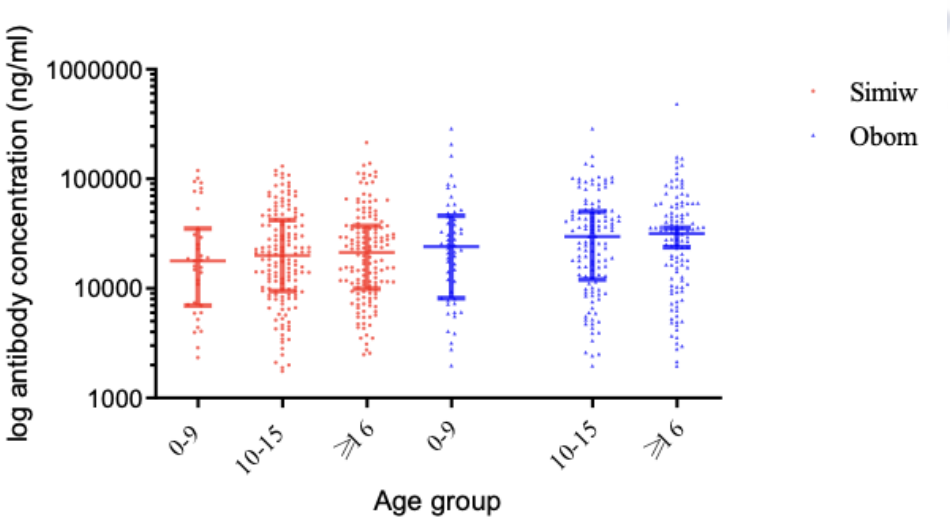


Figure 2B: Antibody Response to *Pf*CSP Antigen.
The absence of bars (p values) indicates the absence of statistical significance. PfCSP antibody concentrations were measured across various age groups in years.

Antibody Response to *Pf*EBA-175.5R Antigen

The statistical significance between *Pf*EBA-175 antibody responses of persons from Simiw and Obom was indifferent (p=0.924; Figure 3A). Antibody responses for *Pf*EBA-175.5R antigen between the age category, 0-9 years and 10-15 years of participants in Simiw showed a statistically significant difference (p=0.0005) with participants with age groups, 10-15 years (3.000(3.095-3.676) ng/ml) recording the highest median (95%CI) antibody concentration as compared with those with age groups, 0-9 years (2.130 (1.946-2.463) ng/ml). The anti-*Pf*EBA-175.5R antibody response for participants of age groups, 0-9 years and 16 years and above is statistically significant (p=0.0262) with participants from age 16 years and above recording the highest median (95%CI) antibody concentration (2.565 (2.925-3.646) ng/ml) compared to those with age groups, 0-9 years (2.130 (1.946-2.463) ng/ml). But the difference between total IgG against *Pf*EBA-175.5R for participants with the age groups, 10-15 years and 16 years and above was statistically insignificant (p>0.99; Figure 3B).

In Obom, individuals in the age group 16 years and above, had a higher *Pf*EBA-175.5R IgG antibody (median (95%CI) ng/ml) (2.130 (1.946-2.463) ng/ml)) than individuals with the age category 0-9 years (1.746 (1.760-2.302) ng/ml) (p=0.0039; Figure 3B). But the difference between total IgG against *Pf*EBA-175.5R for the age category, 0-9 years and 10-15 years; 10-15 years and 16 years and above were found to be statistically insignificant.

Between study sites, there was a statistically significant difference between antibody responses in participants aged 10-15 years with individuals from Simiw recording higher median antibody responses (3.00 (3.095-3.676)

ng/ml) compared to individuals from Obom (2.088 (2.171-2.598) ng/ml). (p>0.0001; Figure 3B)

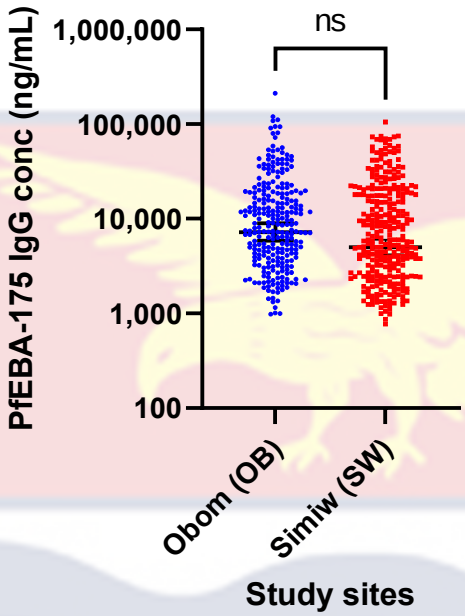


Figure 3A: IgG antibody levels against *Pf*EBA-175 responses for both study sites

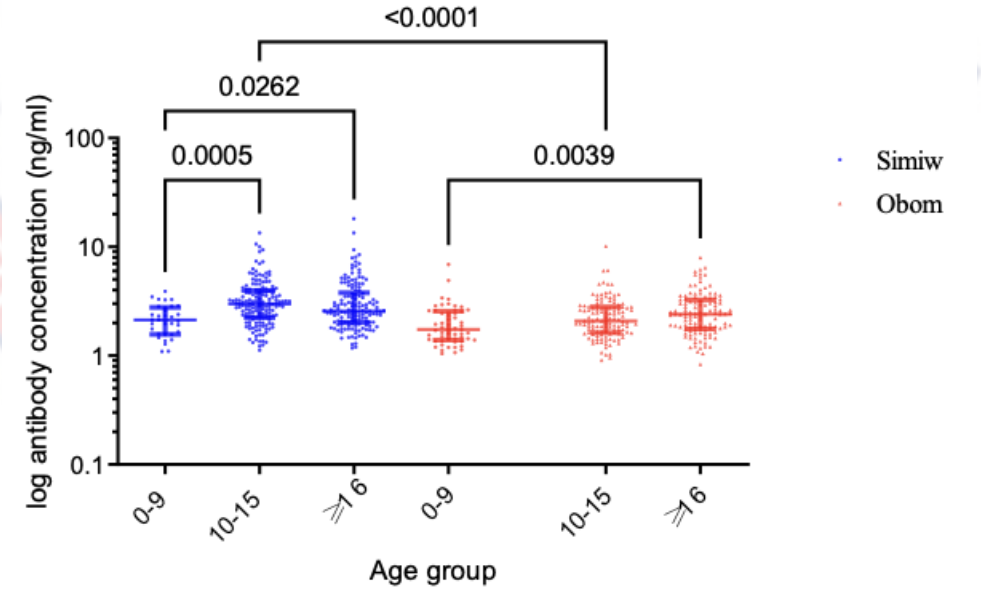


Figure 3B: Antibody Response to *Pf*EBA-1755R Antigen. The absence of bars (p values) indicates the absence of statistical significance. *Pf*EBA-175.5R antibody concentrations were measured across various age groups in years.

Antibody Response to *Pf*s230 Antigen

Antibody responses against gametocyte antigen *Pf*s230proC were quantified to determine gametocyte exposure. Significantly higher anti-*Pf*s230 IgG antibody responses were seen in individuals from Obom (5993 (5112-7296) ng/ml) compared to individuals in Simiw (4822 (4306-5328) ng/ml) (p=0.0006; Figure 4A). The IgG antibody response against *Pf*s230 for participants of age category 10-15 years showed a statistically significant difference with participants in Obom recording the highest median antibody concentration (6591 (1210551-4984924) ng/ml) than their colleagues in Simiw (4707 (5874-10446) ng/ml) (p=0.0243; Figure 4B).

In Simiw, antibody responses against *Pf*s230 were not statistically different between the various age groups (p>0.99; Figure 4B). A similar observation was made in Obom (p>0.99; Figure 4B). Between study sites, the anti-*Pf*s230 antibody response of participants aged 0-9 years showed no statistically significant difference (p>0.99 Figure 4B). The anti-*Pf*s230 antibody responses of the age group 16 years and above across study sites showed no statistically significant difference in their antibody response to *Pf*s230. (p=0.2017; Figure 4B).

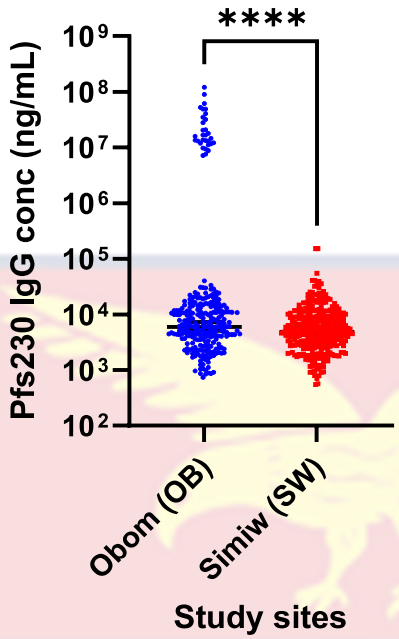


***Figure 4A*: IgG antibody responses against *Pf*s230 for both study sites**

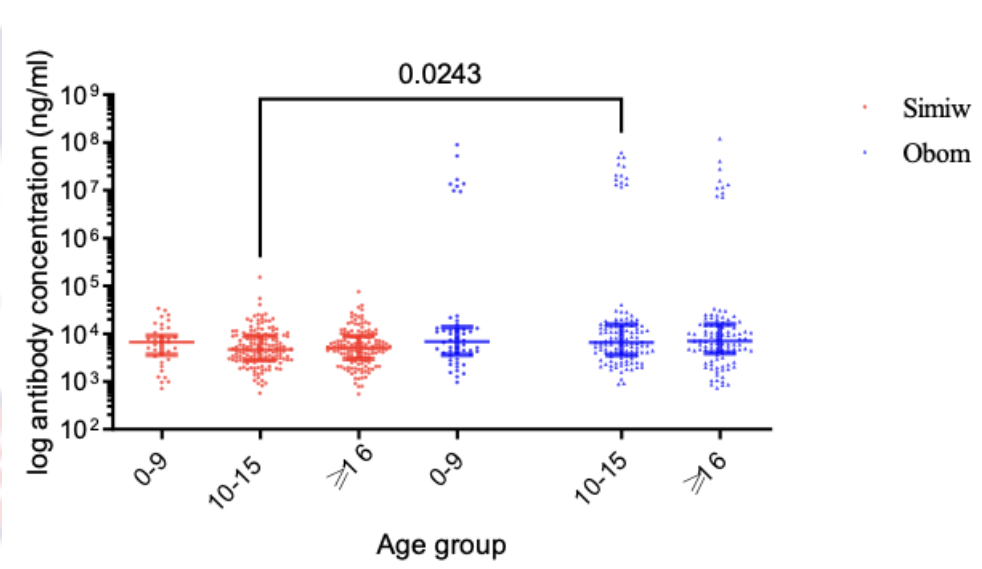


Figure 4B: Antibody Response to *Pf*s230 Antigen. The absence of bars (p values) indicates the absence of statistical significance. *Pf*s230 antibody concentrations were measured across various age groups in years.

Association Between Antibody Responses Against *Pf*CSP Antigen and gSG6-P1 Antigen

A nonparametric Spearman correlation was computed between *Pf*CSP and gSG6-P1 for the different age groups in all study sites. In Simiw, there was no correlation between antibody responses to *Pf*CSP and gSG6-P1 in participants aged 0-9 years (p=0.545), 10-15 years (p=0.138), and 16 years and above (p=0.298; Table 3).

In Obom however, a significantly weak and negative correlation was observed between antibody responses in only participants with the age groups 16 years and above (P=0.011; Table 3).

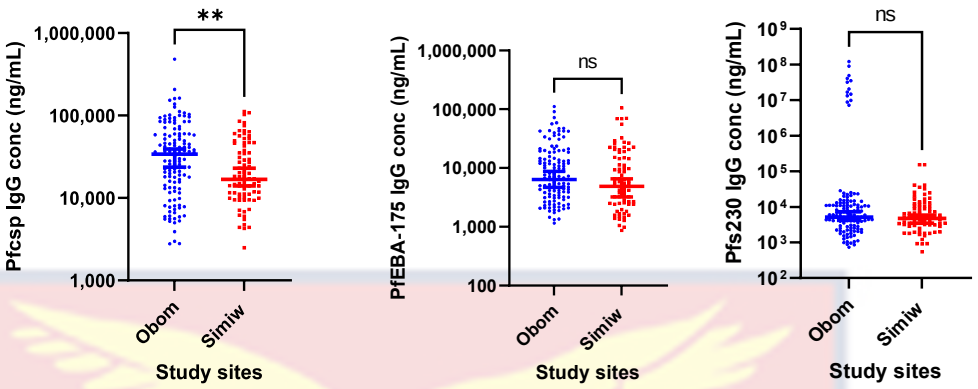
Table 3: Correlation between *Pf*CSP Antibody and gSG6-P1 Antibody indicating the association between *Pf*CSP and gSG6p1-P1 for both sites

	Simiw	Obom
Age (Years)	Spearman r (P value)	Spearman r (P value)
0-9	0.109 (0.545)	-0.100 (0.473)
10-15	-0.122 (0.138)	-0.120 (0.117)
≥16	0.088 (0.298)	-0.236 (0.011)

Association between salivary gland IgG levels and *Plasmodium falciparum* IgG levels

Antibody concentrations of *Pf*CSP, *Pf*EBA-175, and *Pf*s230 was assessed among individuals with similar mosquito exposure levels (gSG6-P1 IgG concentrations of 1-1.999 ng/ml, 2-4.999 ng/ml and >5 ng/ml) across Simiw and Obom. The categorization into the various gSG6-P1 IgG concentration is to see if at any level of gSG6-P1 IgG concentration, be it

moderate, high or extreme, there will an association between gSG6-P1 IgG and that of *Pf*CSP, *Pf*EBA-175 and *Pf*s230 at the various malaria developmental stages in humans. The data revealed that participants with moderate (1-1.999 ng/mL) IgG gSG6-P1 antibody levels at Simiw (Mann-Whitney U=4128, p=0.002; Figure 5) had considerably reduced levels of anti-*Pf*CSP IgG compared to Obom. However, neither participants from Simiw nor Obom with moderate gSG6-P1 IgG responses (1–1.999 ng/mL) had significantly different *Pf*s230 and *Pf*EBA-175 IgG antibody concentrations. Participants from Simiw with elevated gSG6-P1 IgG concentrations (2-4.999 ng/mL) recorded substantially less *Pf*s230 and *Pf*EBA-175 IgG concentrations compared to persons from Obom having the same gSG6-P1 IgG levels (Mann-Whitney U=11740, p=0.0004, and U=27103, p=0.046; Figure 6) respectively. However, persons from Simiw and Obom who had significant stages (2-4.999 ng/mL) of gSG6-P1 IgG antibodies had comparable IgG responses to *Pf*CSP. *Pf*s230 levels were considerably greater in persons from Obom with >5 ng/mL anti-gSG6-P1 IgG stages compared to persons from Simiw having the same gSG6-P1 IgG antibody stages (Mann-Whitney U=146.5, p=0.034; Figure 7).



A B C

Figure 5: IgG antibody response of *Pf*CSP, *Pf*EBA-175 and *Pf*s230 at moderate gSG6- P1 concentration

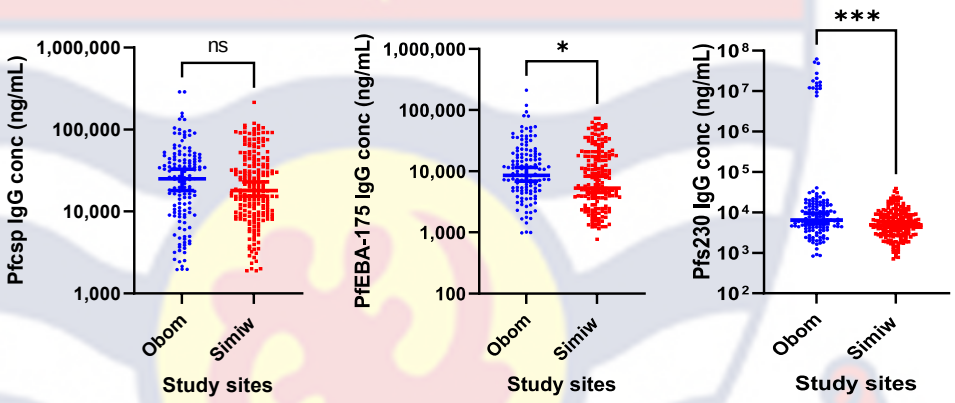


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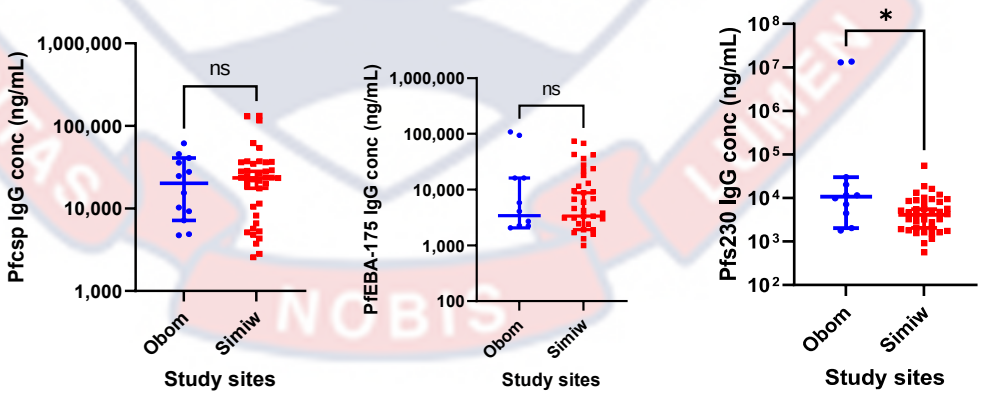
Figure 6: IgG antibody response of *Pf*CSP, *Pf*EBA-175 and *Pf*s230 at high gSG6-P1 concentration



A B C

Figure 7: IgG antibody response of *Pf*CSP, *Pf*EBA-175 and *Pf*s230 at extreme gSG6-P1 concentration

Anti-gSG6-P1 Antibodies Response in the Presence or Absence of Current Infection of *Plasmodium falciparum*

It was observed in Simiw community that, gSG6-P1 antibody levels between participants with current infection (PCR+) and participants with no active infection (PCR-) showed a statistically significant difference (p=0.0223). The difference between gSG6-P1 antibody levels of participants with active infection (PCR+) and participants without active infection (PCR-) in Obom community was found to be statistically insignificant (p>0.999; Figure 8).

In both study sites, there was a statistically significant difference between gSG6-P1 antibody responses for participants with active infection (p<0.0001) with participants in Simiw recording the highest median antibody response than participants in Obom. Also, the difference between participants with no infection was found to be statistically significant (p=0.0088; Figure 8) with participants in Simiw recording the highest median antibody response than participants in Obom.

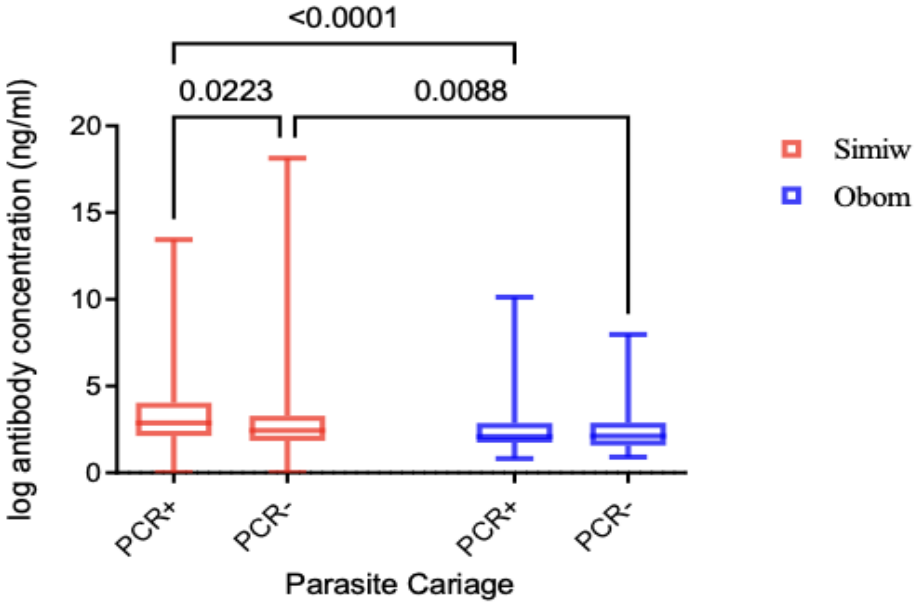


Figure 8: Anti-gSG6-P1 antibody responses in the presence (PCR+) or absence (PCR-) of current infection. The absence of bars (p values) connotes the absence of statistically significant differences.

Effects of *Pf*CSP Antibody Response in the Presence or Absence of Current Infection of *Plasmodium falciparum*

Participants of both sites (Simiw and Obom) were categorized into persons with active infection, thus parasites identified by polymerase chain reaction (PCR+), and those with no active infections, that is PCR-. It was seen that *Pf*CSP antibody levels between participants with active infection (PCR+) and participants with no active infection (PCR-) in the Simiw community showed no statistically significant difference ($p>0.9999$) and hence had similar anti-*Pf*CSP antibody levels. Similar to Obom, the difference between *Pf*CSP antibody levels of participants with active infection (PCR+) and participants without active infection (PCR-) was found to be statistically insignificant ($p>0.9999$). Between sites however, participants who were actively infected in Obom had significantly higher *Pf*CSP IgG antibody stages compared to participants in Simiw ($p=0.0139$), while those without active infection showed no statistical difference between median anti-*Pf*CSP antibody levels ($p=0.47$; Figure 9).

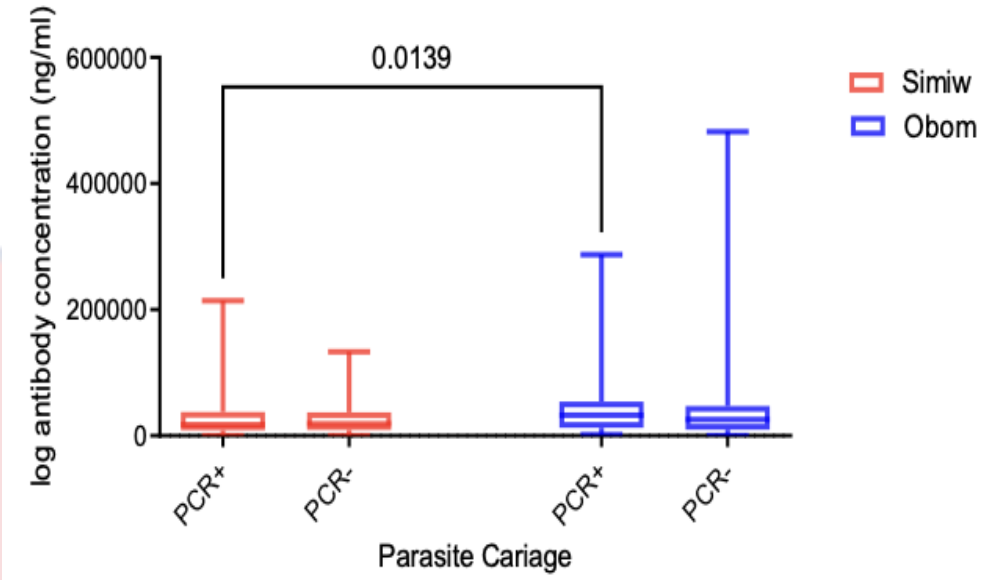


Figure 9: Anti-*Pf*CSP antibody responses in the presence (PCR+) or absence (PCR-) of current infection. The absence of bars (p values) connotes the absence of statistically significant differences.

Anti-*Pf*EBA-175.5R Antibodies Response in the Presence or Absence of Current Infection of *Plasmodium falciparum*

It was observed in Simiw community that, there was a significant difference (p=0.0075) in *Pf*EBA-175.5R antibodies levels between participants with active infection (PCR+) and participants with no active infection (PCR-). The difference between *Pf*EBA-175.5R antibodies levels of participants with active infection (PCR+) and participants without active infection (PCR-) in Obom community was found to be statistically insignificant (p>0.999**;** Figure 10).

For both sites, participants who were actively infected had a significant difference (p<0.0001) in *Pf*EBA-175.5R with individuals from Simiw recording a higher median antibody response (2.899 (3.089-3.687) ng/ml) than individuals

from Obom (2.085 (2.285-2.788) ng/ml). Also, the difference between participants with no active infection was found to be statistically significant (p=0.0064; Figure 10).

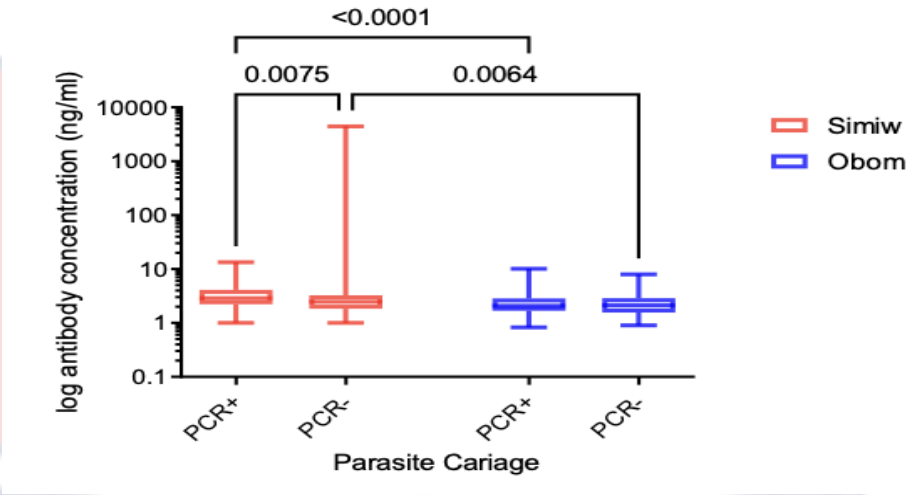


Figure 10: Anti-*Pf*EBA-175.5R antibody responses in the presence (PCR+) or absence (PCR-) of current infection. The absence of bars (p values) connotes the absence of statistically significant differences.

*Pf*s230 Antibodies Response in the Presence or Absence of Current Infection of *Plasmodium falciparum*

It was observed in the Simiw community that, *Pf*s230 antibody levels between participants with active infection (PCR+) and participants with no active infection (PCR-) showed no statistically significant difference (p>0.999). The difference between *Pf*s230 antibody levels of participants with active infection (PCR+) and participants without active infection (PCR-) in the Obom community was also found to be statistically insignificant (p>0.999). Between the sites, participants who were actively infected in Obom had a higher *Pf*s230 antibody level compared to those in Simiw (p=0.0203) and hence statistically significant. Also, the difference between participants with no active infection in

Obom was a higher median *Pf*s230 antibody response than those in Simiw hence, statistically significantly different (p=0.111; Figure11).

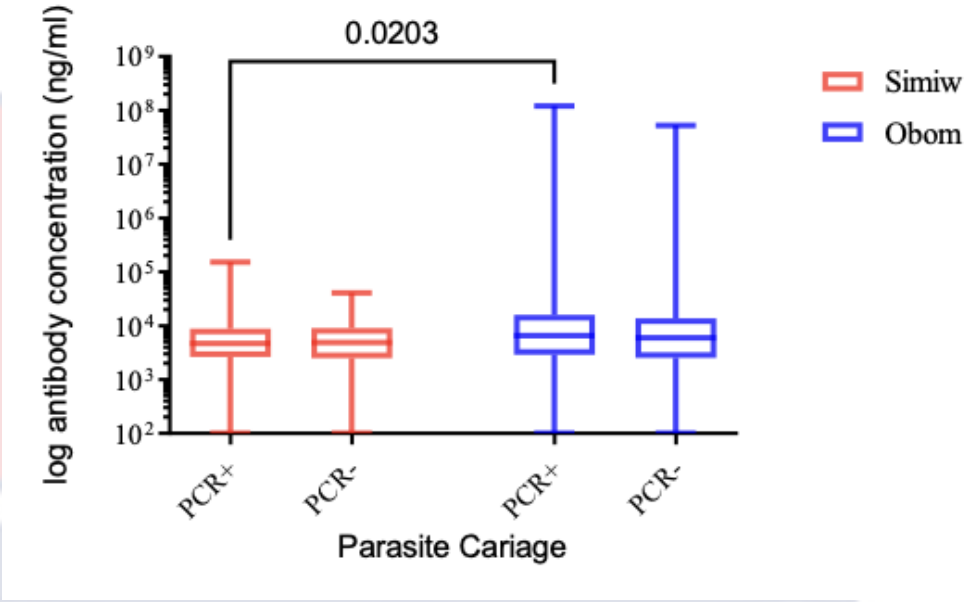


Figure 11: Anti-*Pf*s230 antibody responses in the presence (PCR+) or absence (PCR-) of current infection. The absence of bars (p values) connotes the absence of statistically significant differences.

Discussion

With regard to the most important and complicated infections, such as malaria, efficient interventions and knowledge of host immunity are still lacking (Loiseau, et al., 2019). To reduce malaria incidence and track efforts toward eradication, it is crucial to estimate the possibility of malaria re-emergence in minimal malaria incidence communities and choose the maximum effective vector control treatments for those communities (Kassam, et al., 2021).

Measuring the entomological inoculation rate (EIR) is widely regarded as the gold standard method for evaluating malaria transmission, yet, it is highly challenged and has led to the discovery of some malaria parasite exposure biomarkers via antibodies quantification as a substitute and efficient procedures of malaria infection severity in both low and high malaria infection communities (Kassam, et al., 2021; Stone, et al., 2012). With current research, a measure of *Anopheles gambiae* bite exposure has become fundamental for malaria endemic area identification, malaria risk prediction, and determination of the dynamics of malaria prevalence and reoccurrence of infection in those communities (Stone, et al., 2012).

This study was designed to investigate whether increased exposure to *Anopheles* mosquito bites in afebrile individuals enhances antibody responses against different life stages of *Plasmodium falciparum* (sporozoites, asexual, and gametocytes). Research indicated that IgG antibodies against gSG6-P1 are a possible serological biomarker in determining different levels of exposure to *Anopheles* mosquito bites (Kassam, et al., 2021). The immunogenic nature of a number of mosquito salivary proteins leads to the stimulation of immune

responses such as specific antibody production that can be quantified (Fustec, et al., 2021).

The study showed a significantly high seroprevalence with high anti-gGS6-P1 IgG antibodies median concentration in Simiw (81%) compared to Obom (65.5%), and this may be due to a high possibility of elevated malaria infection in Simiw. The increased exposure in Simiw could result from the influence of environmental factors such as the warm moist climatic condition of the forest which favours mosquito development and survival (Srisuka, et al., 2022) and the wide surface area of stagnant water for mosquito breeding. Obom on the other hand, is more urban compared to Simiw, and as such individuals in Obom are more likely to take precautionary measures to protect themselves from getting exposed to mosquito bites (Awosolu, et al., 2021). Infectious bites lead to asexual parasite carriage making more people carry asexual parasites and as only a proportion of the asexual parasite carriers may have their asexual parasites developing into gametocytes (Acquah, et al., 2020), a lower prevalence of anti-*Pf*s230 compared to that of asexual antigen (*Pf*EBA-175) for both sites were observed. Also, asexual carriage comes about not just by infectious bites but persistent malaria infection (Bousema & Drakeley, 2011). The seroprevalence of *Pf*EBA-175 was higher than that of *Pf*CSP and this might have been developed from earlier parasite carriage and not from recent infectious bites thereby accounting for the higher increase in the prevalence of *Pf*EBA-175 compared to *Pf*CSP.

There were increased levels of IgG to gSG6-P1 among the age group, 16 years and above at both study sites. Antibody levels increase with age, indicating that IgG accumulated over time to a greater variety of parasite antigens, thereby

increasing antibody spectrum (Kyei-Baafour, et al., 2021; Stanisic, et al., 2015; Nebie, et al., 2008).

An increased level of gSG6-P1 IgG antibody was recorded among individuals in the 10-15 years age group compared to individuals in the 0-9 years age group in Simiw. The higher levels of gSG6-P1 IgG in the older children is likely because children within the 10-15 years age group most often help their parents with household chores outdoors at night, as well as have later bedtimes relative to children aged between 0-9 years. This reduces the time the older children sleep under a treated bed net and subsequently results in longer exposure time to *Anopheles* mosquito bites (Jaramillo-Underwood, et al., 2022).

According to previous studies, geographical transmission affects or causes changes in antibodies developed against *Pf*CSP antigens (Pereira, et al., 2018). This study showed significantly higher seroprevalence and IgG antibody levels against *Pf*CSP in Obom compared to individuals from Simiw. We also observed that individuals in Obom with the various age groups had higher median IgG antibody levels (27781 (23674-33921) ng/ml) against *Pf*CSP compared to their counterparts (18330 (15984-22924) ng/ml) in Simiw. This could be because the *Anopheles* mosquitoes in Obom are carriers of *Plasmodium falciparum* and hence, more capable of transmitting malaria than those in Simiw. However, this cannot be validated as mosquito population information was not obtained from both study sites. Studies have revealed that antibody responses against *Pf*CSP antigen of individuals in malaria endemic communities correspond with exposure and increase as age increases irrespective of blood stage infection (Pereira, et al., 2018). This study showed a significant difference between IgG antibody levels of gSG6-P1 and *Pf*CSP

among individuals in Obom with the age group, 16 years and above. Although there was no significant difference between total IgG levels for the various age groups for both sites, there was an increase in the median IgG antibody levels as the age increased for both Simiw and Obom.

Antibodies developed against *Pf*EBA-175 (5R) protect individuals from clinical malaria since antibodies produced against *P. falciparum* prevent the parasite from attacking the erythrocytes (Amoah, et al., 2018). It was observed from the study that *Pf*EBA-175 IgG antibody levels increased as the age increased in both Simiw and Obom. There was a statistical difference in the median IgG antibody concentration to *Pf*EBA-175 in individuals with the age group, 10-15 years from both sites with participants from Simiw having a higher *Pf*EBA-175 seroprevalence than individuals from Obom.

Gametocytogenesis is important for malarial transmission and it is a crucial part of the *Plasmodium* life cycle (sporogonic development). Nonetheless, gametocyte exposure may inhibit malaria transmission since antibodies developed against gametocyte antigens serve as transmission blocking for vaccine development. *Pf*s230 antibodies, for example, trigger immunological death through the complement-mediated destruction of gametes (Duffy, 2021). It was seen that participants from Obom recorded a higher median IgG antibody concentration against *Pf*s230 compared to participants from Simiw, even though they both had similar anti-*Pf*s230 IgG antibody seroprevalence. A similar observation was made for individuals with age category 10-15 years for both sites having similar seroprevalence but individuals from Obom had a higher median antibody concentration higher than individuals from Simiw. This observation could be because participants from

Obom were exposed to high gametocyte densities and served as reservoirs for *Plasmodium falciparum* capable of transmitting malaria.

The study evaluated similar levels of mosquito exposure in Simiw and Obom, and the findings revealed that Obom had higher exposure to *Plasmodium falciparum* sporozoites at a moderate concentration of gSGP1 IgG (concentration of 1–1.999 ng/ml), indicating a larger prevalence of uninfected mosquitoes in Simiw than Obom. Studies reported that relative to exposure to mosquitoes infected with *Plasmodium falciparum*, it has been hypothesized that contact with uninfected mosquito bites will result in the reduction of a load of liver stage hypnozoites and asexual stage parasites (Churcher, et al., 2017; Walk, et al., 2017). Mouse findings on the outcomes of mosquito saliva on the immune system indicated that mosquito saliva activates the immune response toward a Th2 response and downregulates the Th1 response (Schneider, 2008), however, studies conducted by Vogt et al. (2018) revealed hybrid upregulation of Th1 and Th2 responses (Vogt, et al., 2018). In this study, *Pf*EBA-175 IgG antibodies in Obom and Simiw did not significantly vary, indicating equivalent exposure to asexual parasites. It's interesting that people in Obom and Simiw with high gSGP1 IgG antibody levels (2–5 ng/mL) (high exposure to mosquito bites) also had high levels of *Pf*CSP IgG antibodies, indicating similar exposure to sporozoites. However, individuals from Obom showed higher levels of *Pf*EBA-175 IgG antibodies and asexual parasite exposure compared to individuals from Simiw, corroborating the finding that mosquito salivary components minimize the burden of asexual parasites.

The study compared antibody responses to the various antigens (gSG6-P1, *Pf*CSP, *Pf*EBA-175, *Pf*s230) in the presence or absence of active infection

of *Plasmodium falciparum* in Simiw and Obom. The results indicated that participants from Simiw had significantly higher median anti-gSG6-P1 antibody responses and lower median anti-CSP median antibody responses compared to their counterparts from Obom having significantly lower median anti-gSG6-P1 antibody responses and higher median anti-*Pf*CSP antibody responses in the presence of active infection. It was observed that there was a significant difference in *Pf*EBA-175 IgG antibody levels in both sites, with individuals from Simiw recording higher levels of *Plasmodium falciparum* asexual parasites exposure. Simiw however, had low sexual stage parasite density and exposure to sexual parasite *Pf*s230 compared to Obom. Hence this supports the fact that mosquito saliva upregulates the Th1 profile which stimulates IgG production that is vital for effectual anti-parasite immune response development (Lawaly, et al., 2012; Vogt, et al., 2018).

Chapter Summary

The study's results are presented in the first section of this chapter. For the results obtained from the laboratory analysis performed, we showed an association between salivary gland IgG levels and *Plasmodium falciparum* IgG levels, antibody responses to gSG6-P1, and the selected *Plasmodium falciparum* antigens, seroprevalence of IgG antibody levels the antigens of interest, and antibody responses to the various antigens in the presence or absence of current *Plasmodium falciparum* infection via ELISA and PCR. The chapter's second section discussed the findings in relation to other studies.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

Chapter Introduction

It also provides a summary of all work conducted in the research. It also offers recommendations for how to improve the research and increase its standard of validity.

Summary

This study sought to investigate whether increased exposure to *Anopheles* mosquito bites in afebrile individuals enhances antibody responses against different life stages of *Plasmodium falciparum* thus sporozoite, asexual, and gametocytes. *Anopheles Gambiae* Salivary Gland Protein-6 Peptide 1 (gSG6-P1), *Plasmodium falciparum* Circumsporozoite Protein (*Pf*CSP), *Plasmodium falciparum* Erythrocyte Binding Antigen (*Pf*EBA), and *Plasmodium falciparum* Surface Antigen (*Pf*s230) were selected as the antigens of interest to conduct this study. Our initial hypothesis for the study was that Repeated exposure of people living in malaria endemic communities to uninfected *Anopheles gambiae* mosquito bites increases gametocyte production and ultimately exposure to *Plasmodium falciparum* gametocytes. Through a variety of laboratory techniques, the study established that at comparable mosquito exposure levels in both communities, Obom had a higher *Plasmodium falciparum* sporozoites exposure at a moderate concentration of gSG6-P1 IgG, suggesting a higher population of uninfected mosquitoes in Simiw than in Obom. Unfortunately, it was difficult to validate the effect of mosquitoes on malaria transmission because the study did not obtain any data from the study site regarding the mosquito vector.

Conclusion and Recommendation

Regardless of the higher exposure of participants from Simiw to mosquito bites, the majority of the bites were obtained from uninfected mosquitoes and the reason is that total IgG antibody titres for asexual stage antigens (*Pf*CSP, *Pf*EBA-175) and sexual stage antigen *Pf*s230 were found to be relatively lower in Simiw compared to Obom. Hence Obom harbored a high number of asymptomatic malaria infectious individuals thereby making malaria transmission dynamics highly complex.

The gSG6-P1 results obtained do not conform to studies that have reported human behavior and its risk of exposure to mosquito bites and an example is a study performed by Guglielmo et al., (2021). However, we believe that this trend of having varying levels of gSG6-P1 antibodies in different age groups signifies different levels of exposure, and there could be an association with human behavior, especially their outdoor exposure levels, amongst other things which could predispose them to mosquito bites. Different ages have varying exposure to mosquito bites, and this study showed that.

Moving forward, we recommend that more research should be done in this area on analysis of human behavior and its corresponding risk of exposure to mosquitoes within their natural environment.

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