UNIVERSITY OF GHANA
MEDICAL SCHOOL
DEPARTMENT OF MEDICAL BIOCHEMISTRY

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The Dean
School of Graduate Studies
Univertsity of Ghana
Legon

Dear Sir,

CORRECTED THESIS FOR FINAL SUBMISSION – BENJAMIN AGRAH (10637893)

I have gone through the final thesis of the above named MPhil student. I can certify that he has effected all the corrrection in his thesis as instructed by his examiners for final submission.

Thank you.

Yours faithfully,

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COMMENTS	STUDENT'S RESPONSE TO COMMENTS
The background is good except that the introduction is written without a paragraph.	The background information has been re-written and sectioned into six (6) paragraphs. Also, the entire thesis has been sectioned into sub-headings.
The problem statement under investigation is clear albeit few grammatical errors.	All grammatical errors in the problem statement have been corrected.
Most factual statements were without citations, also summarize malaria diagnostic sub-topics in the literature review.	All factual statements have been cited accordingly and the malaria diagnostic sub-topics have been summarized into one sub-heading
The candidate worked on archived samples of which parasitaemia were already established. This section must be re-written.	Sub-section 3.7.1 has been re-written to acknowledge the fact that microscopy was previously done before the commencement of this study.
Categorize results according to specific objectives	All results have been sectioned logically according to the specific objectives of the study and all figures have been cross-checked.
The description of demographics should come before the demographics table.	The description of demographics of the study has been changed to precede the table of demographics.
The discussion should have sub-headings.	The discussion section has been categorized into four sub-headings
Provide more information on Capillary electrophoresis and add why the use of ELISA was warranted in the study.	Detailed information on capillary electrophoresis and how it is used to separate the various HB fractions (Sub-headings 2.13). Also, a detailed information on ELISA and why it was used in this study has been added.
Some cited references are not in the final reference section. The candidate is advised to go through the reference section and fix incomplete references as well.	All citations have been included in the final reference and all incomplete references have been fixed.
Student should clarify which health facilities were chosen for the study. Also clarify if two sets of age-specific population were used.	The names of the health facilities have been included and can be found in sub-heading 3.1. The age range for this study was 2-89 years and there was no age matched control group.
Why didn't the candidate read any microscopy slide?	The microscopy slides were already read by two WHO-certified malaria microscopists and any disagreement in reading was re- examined by a third microscopist. Moreover, the microscopy slides were not readily accessible.

Signed:

Nii Ayite Aryee (Principal Supervisor)

Linda Eva Amoah (Co-Supervisor)

ANTIBODY RESPONSES GENERATED AGAINST *P. FALCIPARUM* AND *A. GAMBIAE* ANTIGENS IN SUSPECTED MALARIA PATIENTS WITH VARIANT HAEMOGLOBIN GENOTYPES

BY

BENJAMIN AGRAH

(10637893)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF

MPHIL IN MEDICAL BIOCHEMISTRY DEGREE

JULY, 2021

DECLARATION

I performed the experimental work described in this thesis at the Department of Immunology of the Noguchi Memorial Institute for Medical Research and the SickleGenAfrica Lab at the University of Ghana Medical School, Korle Bu, under the supervision of Dr. Nii Ayite Aryee and Dr. Linda Eva Amoah.

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DEDICATION

I dedicate this work to my family, especially my hardworking mum, for her immense contribution to my education and her ceaseless prayers for me.

ACKNOWLEDGMENT

My deepest gratitude goes to God Almighty for sustaining me through the challenging moments I faced. Special thanks go to my supervisors, Dr. Linda Amoah of the Immunology Department, Noguchi Memorial Institute for Medical Research (NMIMR) for her unfailing scholarly advice and the substantial financial support she provided for this study and Dr. Nii Ayite Aryee of the Department of Medical Biochemistry, College of Health Sciences, University of Ghana, for his time, guidance, and support

My heartfelt thanks go to Dr. Festus Acquah of the Department of Immunology (NMIMR) for leading me through the optimization of my ELISA and the analysis of my research.

A word of gratitude also goes to Professor Ben Gyan, Head of the Department of Immunology at NMIMR, for enabling me to work in the department. I would also like to thank the rest of the staff of the Immunology Department for their contribution.

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To Dr. William Kudzi, Kelvin, and Joseph, I say a big thank you for your contributions in the course of my work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS.

Ab	Antibody
CDC	Centre For Disease Control and Prevention
DNA	Deoxyribonucleic Acid
EBA	Erythrocyte-binding Antigen
ELISA	Enzyme-Linked Immunosorbent Assay
GHS	Ghana Health Service
GLURP	Glutamate Rich Protein
gSG6-P1	gambiae Salivary Gland
Hb	Haemoglobin
HO	Heme Oxygenase
HRPO	Horseradish Peroxidase
IFN	Interferon
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgE	Immunoglobulin E
IL	Interleukin
iRBCs	Infected Red Blood Cells
NMCP	National Malaria Control Program
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cell
Pf	*Plasmodium falciparum*
PfEMP	Plasmodium *falciparum* Erythrocyte Membrane Protein

PfHRP	Plasmodium *falciparum* Histidine Rich Protein
pLDH	Parasite Lactate Dehydrogenase
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
SPSS	Statistical Package for Social Science
TH	T Helper Cell
TH1	T Helper 1
TH2	T Helper 2
TGF	Tumor Growth Factor
TNF	Tumor Necrosis Factor
WHO	World Health Organization

ABSTRACT

HbAS and HbAC are known to be protective against *P. falciparum* infection, but it is unclear how this protection is conferred in malaria symptomatic patients in Ghana. Theoretically, HbAS and HbAC protect against *P. falciparum* malaria by improving naturally acquired immunity to the parasite. Also, Immunoglobulin G (IgG) has played a significant role in blood parasite clearance in individuals infected with *P. falciparum*, suggesting the protective mechanism of the acquired immunity. This study investigated haemoglobin genotypes and their effects on IgG levels in symptomatic malaria. This research was a nested archival cross-sectional study that enrolled 600 symptomatic malaria patients aged between 2 to 89 from the ten regions in Ghana. Parasite species and density were determined, followed by haemoglobin phenotyping for malaria microscopy-positive patients. An indirect enzyme-linked immunosorbent assay (ELISA) was performed on all the samples to examine the differential effects of exposure to Anopheles mosquitoes (gSG6-P1), sexual stage malaria parasites (Pfs230), and asexual stage malaria parasites (EBA 175 3R). The haemoglobin variants observed among malaria microscopy-positive patients in eight regions of Ghana were HbAA, HbAC, HbSC, HbAS, HbSS, HbCC, and HbAF.

In conclusion, there were significant differences in the total concentration of anti-EBA 175 3R and anti-gSG6-P1 antibodies in malaria negative and positive microscopy samples. Although a significant association was established between the concentrations of IgGs measured against the various antigens in different haemoglobin variants in malaria microscopy positive samples, it was clear that the number of participants with IgG against gSG6-P1 antigen was significantly greater in HbAA participants than in the other haemoglobin variants. Yet the same assessment could not be made for the sexual stage antigen (Pfs230) and the erythrocyte binding antigen (EBA 175 3R). In general, no

significant relationships were established between the influence of age, gender, and haemoglobin variants on the anti-Pfs230, anti-EBA 175 3R, and anti-gSG6- P1 antibodies.

CHAPTER 1

INTRODUCTION

1.1 Background

Malaria poses a significant health challenge in many countries worldwide, particularly in the WHO African region. In 2018, an estimated 228 million malaria cases occurred worldwide, while 213 million were recorded in the World Health Organization (WHO) African Region (WHO, 2019). Malaria is an infectious disease caused by a *Plasmodium* genus unicellular haemosporozoan parasite, four of which cause disease in humans, namely *P. vivax, P. malariae, P. ovale, and P. falciparum.*

It has recently been shown that *Plasmodium knowlesi and P. cynomolgi,* identified as simian malaria parasites, also cause life-threatening human malaria (Cox-Singh, 2008; Luchavez, 2008; Ta, 2014). These parasites are transmitted to humans through the infective bite of a female Anopheline mosquito, which serves as the vector. Nevertheless, *P. falciparum* infection is primarily associated with the most severe forms of the disease, such as cerebral malaria and severe malaria anemia (Miller,1994).

P. falciparum is the most prevalent malaria parasite in the WHO African Region, accounting for 99.7 % of estimated malaria cases recorded in 2018 (WHO, 2019). The global estimated malaria deaths documented by the WHO were 405,000 in 2018 (WHO, 2019). While high-risk groups include persons with less evolved immune systems, children under five years of age rarely exposed to infection in endemic areas are also at risk (Miller, 1994). *P. falciparum* infections can cause a wide range of diseases. There is some non-sterile clinical immunity after repeated parasite exposure (Bull, 1998).

Recent research shows that development has slowed after an unparalleled period of success in global malaria prevention. In 2020, between January and March, Ghana recorded approximately 1,001,070 malaria cases. And out of this number, 21,201 were children under five years old, and 28,764 were pregnant women (Shretta, 2020). Several efforts have been taken to eradicate it, but it continues to spread over the globe, even in previously exterminated places.

The most challenging aspect of malaria control is the emergence of drug-resistant variants of the parasite, particularly *P. falciparum* (Akanbi, 2014). In endemic areas, characteristics offering partial resistance to infection or illness progression are selected, and this has had and continues to have a widespread impact on human population genetics (Amoah, 2018). It is commonly known that haemoglobin (Hb) variants, particularly the sickle cell trait (HbAS), have been validated for several genetic polymorphisms that confer relative resistance to malaria (Aidoo, 2002). Likewise, alpha-thalassaemia protects from severe malaria in Papua New Guinea (Allen, 1997).

Among the mechanisms involved are resistance to parasite invasion, reduced intracellular proliferation, altered neoantigen production, and increased vulnerability to oxidative stress (Wilkinson, 1997). In Gabon, HbAS was linked to a significant proportion of polyclonal *P. falciparum* infections, indicating a high multiplicity of infection (Lassana Konaté, 1999) but not in Senegal. The protective effect of HbAS has been connected to some modification of the immune response against malaria, but new findings demonstrate that it involves rapidly acquired immunity to the parasite; nonetheless, the mechanisms of protection remain unknown.

1.1.1 Problem Statement

Malaria is a major public health concern, with a high incidence of mortality in Ghana (Shretta, 2020). There is a fascinating co-evolutionary process between the *Plasmodium* species and humans. Malaria-affected populations have a variety of haemoglobin variations that may work together to diminish *Plasmodium* virulence in the human body, resulting from distinct geographical originating effects. In the search for immunological surrogates of immunity against malaria, a plethora of research has focused on antibody levels without determining the phenotype of haemoglobin associated, which is undoubtedly a critical parameter in measuring and monitoring immunity to malaria. There is a need to know whether the haemoglobin variants affect antibody production in malaria microscopy-positive individuals across Ghana. Although the stories of HbS and HbC have provided fascinating insights into evolutionary biology, a thorough knowledge of their malaria-protective mechanisms is required before these lessons can be applied. This situation can be considered among the reasons why malaria vaccine development has been a challenging task for scientists

1.1.2. Justification

In this study, it will be essential to elucidate the influence of haemoglobin variants on immune response, thus, adding more insight into malaria surveillance in Ghana and the development of a more potent drug regime or vaccine to curb this menace. Haemoglobin variants are relatively common in Ghana, many of which have been described (Danquah, 2010). Several antibodies identified as key to malaria protection and alleviating symptoms of the disease lack rigorous information on the influence of haemoglobin variants on IgG levels in malaria symptomatic patients across Ghana. This research investigates the relationship between antibodies against

malaria antigens, EBA-175 (Region III), Pfs 230, and gSG6 –P1 and haemoglobin genotype in malaria-infected persons. This information will equip researchers and health workers to design and administer vaccines and drugs based on an individual's haemoglobin phenotype leading to an individual-centered treatment, thus pharmacogenomics in malaria treatments in Ghana.

1.1.2 Research Hypothesis

The levels of IgG elicited against malaria parasites and vector antigens in suspected malaria patients with varying haemoglobin genotypes differ.

1.1.3 Aim of Study

The study investigated IgG responses to three malaria antigens, EBA 175 3R, Pfs230, and gSG6-P1, and the effects of haemoglobin variants on IgG levels in malaria microscopy-positive symptomatic patients across Ghana.

1.1.4 Specific Objectives

1. To determine and compare IgG responses to two malaria parasite antigens (EBA 175 3R, Pfs230) and one mosquito vector antigen, gSG6-P1, in plasma samples collected from suspected malaria patients across Ghana.
2. To determine the different haemoglobin genotypes in microscopy-positive samples across Ghana
3. To determine the seroprevalence of two malaria parasite antigens (EBA 175 3R, Pfs230, and one mosquito vector antigen (gSG6-P1) in microscopy positive and negative samples collected across Ghana.
4. To elucidate the relationship between haemoglobin variants and malaria anti-IgG levels in microscopy-positive samples across Ghana

CHAPTER 2
LITERATURE REVIEW

2.1 The Burden of Malaria

Malaria remains an overwhelming problem in developing countries, affecting the economy and productivity in endemic areas. Globally, 228 million malaria cases were reported in 2018, compared to 251 million cases in 2010 and 231 million in 2017 (WHO, 2019). Also, 93 % of these cases occurred in the WHO African region, followed by 3.4 % for the WHO South-East Asia region and 2.1 % for the WHO Eastern Mediterranean region (WHO, 2019). About 19 countries in sub-Saharan Africa and India accounted for nearly 85 % of the global burden of malaria (WHO, 2019). Between 2010 and 2018, the global malaria incidence rate dropped from 71 to 57 cases per 1000 people at risk (WHO, 2019). However, the rate of change slowed dramatically from 2014 to 2018, dropping to 57 in 2014 and remaining at similar levels through 2018 (WHO, 2019). *P. falciparum* is the most common malaria parasite in the WHO African Region, accounting for 99.7% of malaria cases estimated in 2018 (WHO, 2019).

Globally, malaria deaths in 2018 were estimated to be 405,000 compared with 2017 and 2010, which were 416,000 and 585,000, respectively (WHO, 2019). Further, children under five years old, the most vulnerable group, accounted for 67 % of malaria deaths worldwide (WHO, 2019). Africa saw the most significant absolute decrease in deaths from malaria, from 533,000 to 380,000 from 2010- 2018 (WHO, 2019). However, despite progress, the mortality rate has decreased since 2016; though the disease burden of 155 million in high-impact countries saw significant decreases from 2010 to 2018, a higher number of cases occurred in 2018 compared to previous years (WHO, 2019). In Africa, Ghana and Nigeria reported the highest absolute increases in malaria cases in 2018 compared with 2017 (WHO, 2019).

The 2018 malaria burden was similar to that of 2017 in all other countries, except Uganda and India, where reductions of 1.5 and 2.6 million have been reported (WHO, 2019). But in Ghana, there was a notable increase (8 %) in malaria cases in Ghana (WHO, 2019). Finally, the economic burden of malaria in sub-Saharan Africa is unmistakable; much of the world's poorest countries are in sub-Saharan Africa, where malaria is endemic (Sachs, 2002). The worldwide distribution of per-capita gross domestic product reveals a robust association between malaria and poverty (Asante, 2003). In contrast, malaria-endemic countries often have lower levels of economic growth than non-endemic countries (Asante, 2003). The effect of malaria on real GDP growth in Ghana is negative and decreases by 0.41 % for every 1 % increase in malaria morbidity (Asante, 2003).

2.2 The life cycle of *Plasmodium falciparum*

During a blood meal, infected female *Anopheles spp*. mosquitoes inject sporozoites into the bloodstream, causing infections in humans. This is accompanied by the release of merozoites into the bloodstream, which initiates the multiplication stage of asexual parasites (see figure 1). The length of an infection in the blood system is highly variable: several infections are cleared early, while others remain for several months (Felger, 2012). A fraction of merozoites form sexual gametocytes, the only parasite forms capable of transmitting from humans to mosquitoes. In the bone marrow, immature gametocytes (those in stages I – IV) are sequestered, and only mature gametocytes (stage V) circulate in the peripheral blood. In peripheral blood, the number of mature gametocytes is usually less than 100 gametocytes per µl of blood (Drakeley, 2006), and the vast majority are present at submicroscopic densities. After mosquito ingestion, each gametocyte forms one female macrogamete or a maximum of eight male microgametes. Gamete fusion in the mosquito midgut produces a zygote that grows into a motile ookinete that can infiltrate the midgut wall and form oocysts.

The oocysts grow over time and burst to release sporozoites that migrate to the salivary gland of the mosquito, from which they may infect humans during the next meal (Baidjoe, 2013).

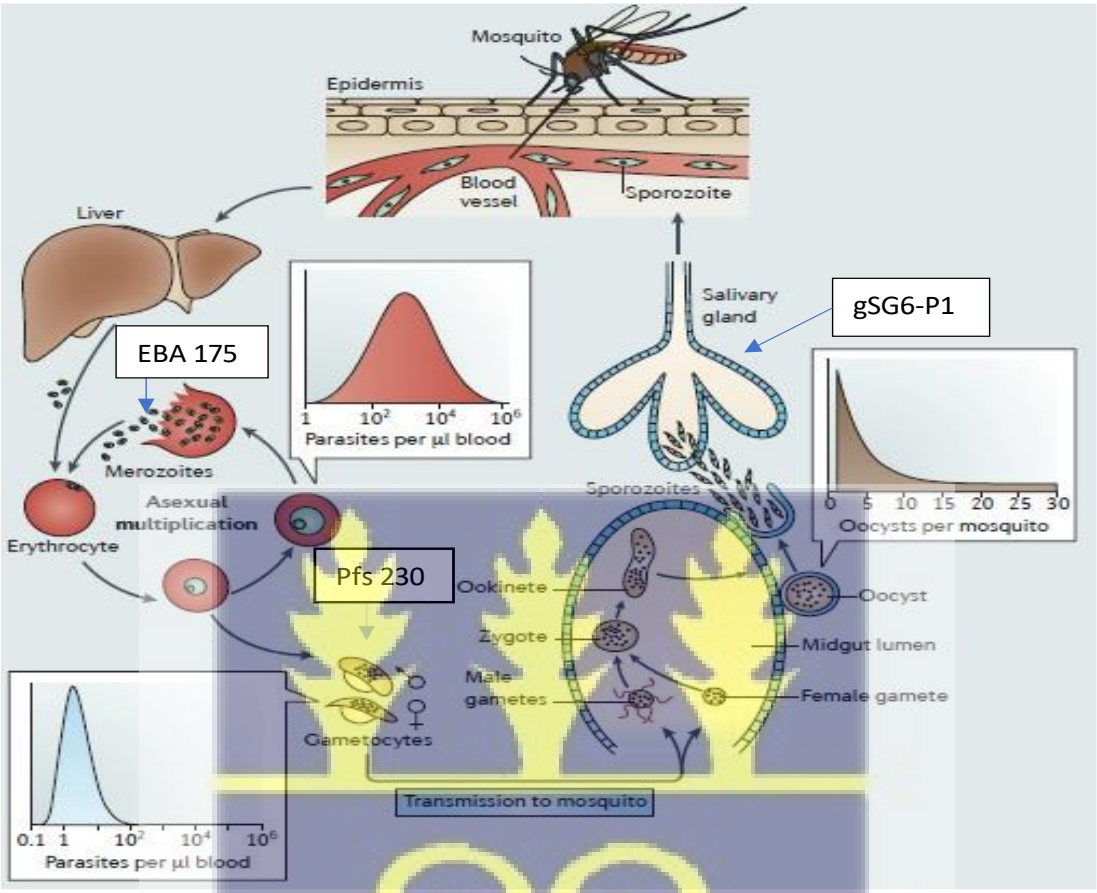

Figure 1: Life Cycle of *Plasmodium falciparum* (Bousema et al.,2014)

2. 3 Case definition of clinical malaria

Clinical disease caused by the parasite is due to the asexual replication of parasites in the blood stage, so much attention has been paid to the erythrocytic stage to identify antigens intended for defensive immune responses. Clinical symptoms of *P. falciparum* infection include chills, fatigue, headache, nausea, vomiting, and diarrhea. The primary complications of *P. falciparum* malaria in children include cerebral malaria, extreme anemia, seizures, and respiratory distress. Clinical malaria description is generally based on microscopic identification of malaria.

Parasitemia, fever, and febrile temperature > 37.5°C. Malaria fever is caused by the release of parasite toxins when schizont-infected red blood cells rupture to release merozoites (Kwiatkowski, 1989). Thus, malaria is said to be directly caused by the rapturing of the red blood cells that contribute to febrile temperatures. Using febrile fever > 37.5 °C and parasitemia at various rates results in a highly susceptible and precise case description of malaria (Armstrong, 1994). However, individuals in highly endemic areas may have parasites without fever or other symptoms associated with malaria.

2. 3 Signs and symptoms

Asexual erythrocytic or blood-stage parasites are responsible for all clinical symptoms associated with malaria (CDC, 2019). The time between the contagious bite and the appearance of clinical signs is around 9 to 14 days (Garrison, 2015). Malaria typically causes recurrent attacks or paroxysms, each of which has three stages: chills, fever, and sweating. Along with chills, a person is likely to have headaches, malaise, exhaustion, muscle aches, and sometimes nausea, vomiting, and diarrhea. The body's temperature increases within an hour, and the skin feels hot and dry (Bartoloni, 2012). And, as the body's temperature falls, a drenching sweat begins. The person, feeling tired and weak, is likely to fall asleep (Bartoloni, 2012). Typical paroxysm, however, is present in only a minority of cases, especially in *P. falciparum* malaria (Hall, 1977). Clinical attacks may be triggered by overwork, emotional shock, pregnancy, delivery, illness, surgical operation, or general anesthesia. The onset can be sudden and severe, particularly in children and unprotected non-immune individuals, with death following in a matter of time (Felger, 2012). Malaria symptoms can be classified into two categories: uncomplicated and severe malaria. Uncomplicated malaria is diagnosed when symptoms are present, but no clinical or laboratory signs indicate a severe infection or dysfunction of vital organs. Individuals with uncomplicated malaria can develop severe malaria if the illness is not treated or their immunity

to the disease is insufficient or non-existent. Uncomplicated malaria symptoms usually last 6-10 hours and come in cycles every other day, while some parasite strains might cause a longer cycle or mixed symptoms. Symptoms are often flu-like and may be undiagnosed or misdiagnosed in areas where malaria is less common. Symptoms of uncomplicated malaria include cold, hot, and sweating: the sensation of cold, shivering, fever, headaches, and vomiting (seizures sometimes occur in young children), sweats followed by a return to normal temperature, with tiredness (CDC, 2019). Severe malaria is defined by clinical or laboratory evidence of vital organ dysfunction. This form can be fatal if left unchecked. Symptoms of severe malaria include fever and chills, impaired consciousness, and prostration (CDC, 2019).

2. 4 Haemoglobin Variants

Haemoglobin is an erythrocyte protein that comprises four polypeptide globin chains looped around a haem molecule. It is responsible for bringing oxygen from the lungs and carbon dioxide from the tissues to the lungs. The globin chains are encoded on chromosome 11 and chromosome 16 by their respective genes and are known to have multiple alleles (Weatherall, 2011). Many of these alleles suffer point mutations in the DNA sequence, which leads to single amino acid substitution in the moiety of globin, resulting in the production of haemoglobin variants. The abnormal haemoglobin genotype occurs when an affected person inherits mutated globin genes from both parents. Abnormal haemoglobin genotypes are inherited in an autosomal codominant pattern and occur through different combinations. Several hundred unusual haemoglobin genotypes have been found; however, only a few are typical and cause significant public health problems in many parts of the world (Weatherall, 2011). More than 1,000 human haemoglobin variants have been discovered with single amino acid substitutions that contribute to physiological consequences of varying severity (Giardine, 2013).

HbS is the most prevalent pathological haemoglobin mutation leading to global substitution (Weatherall, 2011). HbS is Africa's most prevalent pathological form of haemoglobin, while HbD and HbE are among Indian and Southeast Asian populations (Walker, 2015). HbA2 fractions and fetal haemoglobin (HbF) may be increased in thalassemia, a disorder that affects the synthesis of the haemoglobin alpha or beta-globin chains. Beta-thalassemia can also occur when HbS and HbE are present. A combination sickle/beta-thalassemia phenotype occurs most commonly in the geographical region of the Mediterranean Sea (Walker, 2015).

The sickle haemoglobin mutation is a structural variant of normal adult haemoglobin A that results from a single-point mutation in which the amino acid, glutamic acid is substituted by valine at position 6 of the haemoglobin A β- globin chain (Orkin, 2019). Carriers or heterozygous (AS) individuals inherit the HbS allele from one parent and HbA allele from the other but are typically asymptomatic, while homozygous (SS) individuals inheriting HbS alleles from both parents have the genotype of the disease and suffer from sickle cell anemia, which often leads to acute and chronic complications (Williams., 2011). Due to its haemolytic influence, the homozygous (SS) sickle haemoglobin type is called sickle cell anemia (SCA), which is generally the most frequently inherited genetic condition in the world and the most common form of sickle cell disease. The most severe types of sickle cell disease (SCD) include people with HbSS and HbSβ ° (Creary, 2007; Rees, 2010). Haemoglobin variants are likely to be relatively common in Africa, and several hundred have been described (Schaefer, 2016).

Haemoglobin mutations can cause a wide range of phenotypic outcomes, including protein instability, cell lysis anemia, and microvascular circulation occlusion leading to ischemia, infarction, and chronic haemolytic anemia (Kanter, 2013; Bissé, 2017). In a study carried out on haemoglobin variants among patients attending Ho Teaching Hospital in Ghana, the prevalence of HbAS, HbA, HbSC, HbS, HbAC, HbSF, HbC, and HbAF among the study population for three years was estimated at 40.3%, 37.3%, 7.9%, 6.2%, 5.2%, 2.6%, and 0.3%, respectively (Awaitey, 2020).

2. 4. 1 Malaria and Haemoglobin variants

While existing treatments have lessened morbidity in some cases, we need to increase our fundamental understanding of *P. falciparum* malaria pathogenesis to discover molecular and cellular targets for next-generation therapeutic and preventive strategies. Due to the complex tangle of parasite virulence factors, host susceptibility features, and innate and adaptive immune responses that affect the development of various malaria syndromes, mechanisms of *falciparum* malaria pathogenesis remain unknown (Bejon, 2008; O'Meara, 2008; Amaratunga., 2011). The risk of severe falciparum malaria in Sub-Saharan African children is reduced by 90% and 70% by heterozygous haemoglobin (HbAS) and homozygous haemoglobin C, respectively (Taylor, 2012). These structural haemoglobin variants do not protect against *P. falciparum* infection, suggesting that they interfere with the specific molecular mechanisms responsible for the morbidity of *falciparum* malaria (Taylor, 2012). By isolating these pathogenic processes and resolving the Gordian knot of malaria pathogenesis, haemoglobinopathies offer an attractive "natural experiment" to identify molecular correlations of clinical morbidity. These correlations may be capable of being exploited by future parasiticidal, adjunctive, or preventive therapies to yield targets for a new "Alexandrian solution" to the global *P. falciparum* malaria problem.

2. 4. 2 Erythrocytes and *Plasmodium falciparum*

Erythrocytes are critical to the spread of malaria parasites. After inoculation in humans by a mosquito and a brief, clinically silent incubation in the liver, *P. falciparum* parasites enter the erythrocytic stage of their life cycle (Taylor, 2013). Parasites successively enter and break away from their host RBCs throughout this time, causing malaria signs and symptoms; while developing within RBC, the parasite transfers proteins to the RBC surface that mediate binding to extracellular host receptors, and it enables the parasite to sequester in the placenta, brain, and virtually any other organ. RBC variants are produced from some of the most common human genetic polymorphisms; their widespread prevalence has been assumed to result from their evolutionary selection by severe, life-threatening *falciparum* malaria (Taylor, 2013). Clinical data for several common haemoglobin disorders strongly supports this natural selection, and major haemoglobinopathies result from molecular lesions that either reduce the production of α or β globins or encode single amino acid substitutions in β-globin (in HbS, HbC, and haemoglobin E) (Weatherall., 2000). The most severe haemoglobinopathies, HbSS homozygosity, and thalassemia major are typically incompatible with early childhood life without sophisticated medical care. Other haemoglobin traits such as HbAS, HbAC, HbCC, HbAE, HbEE, and minor thalassemias are associated with essentially average life span and far less directly attributable morbidity. Remarkably, these simple polymorphisms provide dramatic levels of protection against complex diseases: for HbAS, replacing glycine with the amino acid at position 6 in only one of two β-globin chains reduces the risk of severe *falciparum* malaria in children by approximately 90 % (Taylor, 2013).

The current comprehension of *P. falciparum* malaria pathogenesis suggests four general hypotheses for investigating the nature of malaria protection by haemoglobinopathies

1. Restriction of RBC invasion or intraerythrocytic parasite growth.
2. Interference with parasite-derived mediators of pathogenesis.
3. Modulation of innate host responses.
4. Enhancement of the host's adaptive immune clearance of parasite-infected RBCs (iRBCs).

2. 5 Malaria immunity

2. 5. 1 Innate immune response

The innate immune system provides non-specific protection through monocytes, macrophages, dendritic cells, natural killer cells, eosinophils, neutrophils, mast cells, complement, and acute phase proteins. This also requires physical defenses such as epithelial layers and antimicrobials on these surfaces (Taylor, 2013). Neutrophils, mononuclear phagocytes, and natural killer cells play a role in malaria infections in innate immunity. Natural killer cells are involved in erythrocytes infected with *P. falciparum* lyses in vitro. They contain cytokines such as interferon (IFN-y) that cause macrophages to phagocytose invasive foreign particles (Orago, 1991; Artavanis-Tsakonas, 2002). In addition, innate immune mechanisms by natural killer cells lead to the stimulation of IFN, which limits the initial phase of parasite replication; this has also been demonstrated in studies done on murine malaria (Good, 1999; Fell, 1998).

2. 6 Acquired immunity

2. 6. 1 Humoral immune response

Malaria infection induces strong humoral immune responses through the development of high concentrations of immunoglobulins (Ig), especially IgG and IgM, as well as IgE. The extent of defense against malaria infection in humans and mice has been shown to be correlated with the level of antibodies against asexual blood stage antigens (Piper, 1999; Hirunpetcharat, 1998; Astagneau, 1995). The role of antibodies in malaria immunity is evident in the protection provided to neonates and infants by malaria-specific antibodies that mothers acquire (Sabchareon, 1991; McGregor, 1964). Passive transfer of monoclonal antibodies to *plasmodium* parasite antigens provided protection to naive mice (Spencer, 1998; Narum, 2000). Although various immunoglobulins protect people from malaria, IgG is the most important. The use of purified immunoglobulin from sera of African adults in clinical trials to treat some sick children substantially decreased clinical symptoms and parasitemia (Bouharoun-Tayoun, 1990). It has been identified that immunoglobulin G (IgG) is the main component of defense against the asexual blood stage of *P. falciparum* (Druilhe, 1994).

2. 6. 2 Mechanism of antibody response

Antibodies are believed to mediate malaria defense through a series of mechanisms. In vivo studies indicate that one mechanism can confer clinical immunity to malaria by antibody interrupting parasite multiplication (McGregor, 1964; Sabchareon, 1991). Antibodies to blood-stage merozoite antigens can block or make parasite invasion of erythrocytes susceptible to phagocytosis, leading to a reduction in parasitemia (Blackman, 1994; Holder, 1992). Other mechanisms are the clearance of infected erythrocytes from circulation by antibodies binding to their surface through Fc receptors and their removal from the body.

Some parasites induce antibodies that form clumps or rosettes that the immune system recognizes and eliminates from circulation by opsonization or phagocytosis (Treutiger, 1992). Cell-dependent antibody-mediated cytotoxicity of parasites may be induced by cytophilic antibodies and by inhibition of parasites by effector cells such as neutrophils and monocytes (Bouharoun-Tayoun, 1990). Parasite agglutination and indirect effects such as antibody-dependent cellular inhibition (Oeuvray, 1994) are other immune mechanisms that protect against *P. falciparum* malaria. Malaria parasites also set up systems for immune evasion. These processes include antigenic variability since many different parasite antigens are introduced to the immune system. Others include modulation of the host immune response that could lead to pathological changes, polymorphism of the parasite protein, and competition between protective and non-protective reactions (Troye-Blomberg, 1984).

2. 6. 3 Cell-mediated immunity

Though antibody plays a significant role in malaria immunity, T-cells and cytokines are also involved in immune regulation and effector phases of anti-malaria immunity via T helper cells (Weidanz, 1988). Cell-mediated immune responses to malaria protect against erythrocytic and erythrocytic parasites (Troye-Blomberg, 1984). The importance of cytokines in conferring protection immunity to malaria infection in animal models has been documented (Kobayash, 1996; Shear., 1990; Stevenson, 1990). Several studies have identified cellular processes such as lymphocyte proliferation, IFNy development, activation, and killing of macrophages when peripheral blood mononuclear cells (PBMCs) from immune individuals have been stimulated with malaria antigens in vitro (Ballet, 1985; Brown, 1986; Ockenhouse., 1984).

Similar studies were conducted on non-immune individuals infected with *P. falciparum, and* decreased cellular recognition of plasmodial antigens was reported (Ho, 1995). T-cells that control the production of antibodies are also involved in inflammation and cytokine regulation; separating TH-cells into their ThI and Th2 subsets may have significant biological and immunological consequences for susceptibility or resistance to diseases or infections (Troye-Blomberg., 1984). Thus, different subsets of Th cells play different roles in inflammation or anti-inflammation in malaria infection.

2. 6. 4 Mechanisms of cellular immune response

Studies have shown that ThI and Th2 cells are responsible for regulating immune-mediated antibodies and cell-mediated immunity. This is evident from a study in which T-cells regulated cytokine-induced antibody development through ThI and Th2 (Weidanz, 1988). ThI cells secrete cytokines such as interleukin-2 (IL-2), interferon (IFN) y, tumor growth factor (TGF), and tumor necrosis factor α (TNF α). These cytokines activate macrophages that aid with IgG antibodies, facilitate inflammatory reactions leading to tissue injury, and mediate delayed hypersensitivity reactions (Abbas, 1996). Th2 cells secrete cytokines like IL-4, IL-5, IL-6, IL-10, and IL-13. These cytokines stimulate the proliferation of mast cells and eosinophils and aid B-cells during infection (Abbas, 1996). These cells cross-regulate the differentiation and activity of each other via the cytokines they generate. Studies have shown that Th2-secreted cytokines regulate functional activity and the development of ThI secreted cytokines in *P. falciparum* infection (Ho, 1995). ThI secreted cytokines regulate the production of Th2 cytokines, while Th2 cells also secrete cytokines that control the production of ThI cells (Troye-Blomberg., 1984). For example, IFNγ secreted by ThI cells inhibits the development and spread of Th2 cells, while IL-4

and IL-10 secreted by Th2 cells inhibit the development of ThI cells. Cytokines such as IL-1, IL-6, IFN-γ, and TNF-α have been shown to defend by inducing macrophages and neutrophils to destroy parasites (Kumaratilake, 1992; Taylor-Robinson, 1993; Troye-Blomberg ., 1999).

2. 7 Haemoglobin variants restrict *P. falciparum* growth in RBCs

Numerous investigations into the invasion and growth of *P. falciparum* in RBCs containing variant haemoglobin quickly followed the development of in vitro cultivation systems (Trager., 1976; Haynes., 1976). Reductions in RBC invasion have been reported for a variety of haemoglobinopathies, including α-thalassemia trait (Bunyaratvej, 1992), HbH disease (Ifediba, 1985; Chotivanich, 2002), HbEE (Bunyaratvej, 1992; Chotivanich, 2002) HbAE (Chotivanich, 2002) and the compound heterozygous β-thalassemia/HbE disorder (Chotivanich, 2002; Bunyaratvej, 1992; Brockelman, 1987); reductions in the intraerythrocytic growth or maturation of parasites have been reported for HbH disease (Ifediba, 1985; Brockelman, 1987),β-thalassemia minor (Brockelman, 1987) HbSS (Pasvol, 1978; Pasvol., 1980), HbAS (Pasvol, 1978)HbCC (Friedman, 1979; Fairhurst, 2003; Olson, 1986). A recent study proposes a novel mechanism for inhibiting *P. falciparum* growth in HbS-containing RBCs. Both HbAS and HbSS RBCs display host microRNA (miRNA) profiles that are distinct from those of HbAA RBCs (Chen, 1998; Sangokoya, 2010).

2. 8 Haemoglobin variants interference with mechanisms of *P. falciparum* Malaria

Two major pathogenic iRBC phenotypes were described: those mediating binding of iRBCs to endothelial receptors (Baruch, 1996) and those mediating binding of iRBCs to uninfected RBCs (Carlson, 1990; Kaul, 1991). Both adherence phenotypes are conferred by *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) expression (Su, 1995; Baruch, 1996; Smith, 1995).

Other pathogenic mechanisms that could be associated with the disease include the production of cytokines in response to *P. falciparum* glycosylphosphatidylinositol (PfGPI) (Schofield, 1993) and parasite-derived uric acid (Lopera-Mesa, 2012) direct haemolysis due to parasite egress from RBCs. Several studies indicate that the deterioration of cytoadherence interactions partly controls haemoglobinopathy malaria defense. Further analysis of this phenomenon showed that HbAC and HbCC significantly interfered with the binding of iRBCs to human microvascular endothelial cells (HMVECs) under static and physiological flow conditions (Fairhurst., 2005). Subsequent investigations also found significant reductions in the binding of HbAS iRBCs (Cholera, 2008), HbF-containing iRBCs (Amaratunga., 2011), and α-thalassemic iRBCs (Krause, 2012) to human microvascular endothelial cells. Recent research confirms this malaria-protective candidate mechanism; researchers investigated the iRBC's protein-trafficking network and showed that the parasite remodels the actin cytoskeleton of the RBC to export parasite-derived proteins to the iRBC surface knobs (Cyrklaff, 2011). This actin cytoskeleton is disrupted in HbSC and HbCC iRBCs, and the export of parasite proteins to surface knobs is likely to inhibit actin polymerization by hemichromes (Cyrklaff, 2011). These oxidized, denatured haemoglobin accumulate in RBCs containing HbS and HbC, thus providing a possible connection between haemoglobin instability and abnormal PfEMP1/knob display (Cyrklaff, 2011).

2. 9 Haemoglobin variants influence the Innate host defense responses to *P. falciparum*.

The impact of aberrant host responses on the pathogenesis of malaria, especially severe *falciparum* malaria, is increasingly recognized (Schofield., 2005; Hunt, 2003; Clark, 2004). The innate host defense response encompasses myriad stereotypical pathways that are activated by

microorganisms and orchestrated to mitigate insults while minimizing collateral toxicity (Takeuchi, 2010). Typically initiated by recognizing pathogen-associated molecular patterns (PAMPs) by leukocyte-associated Toll-like Receptors (TLRs), these responses then progress through:

1. A pro-inflammatory phase is marked by the release of cytokines, activation of endothelial cells, and recruitment of circulating and locally resident immune effector cells.
2. A counter-regulatory phase in which tissue-protective molecules such as erythropoietin (Villa, 2003; Siren, 2001), heme oxygenase-1 (HO-1) (Wagener, 2001; Kapturczak, 2004), and angiopoietin-1 (Kim, 2011) are deployed to limit inflammatory damage.
3. A repair phase is mediated by vascular- and tissue-specific stem cells (Koh, 2009; Erbayraktar, 2009).

Such phases emerge from the host and pathogenic factors that jointly regulate specific responses that are pro-inflammatory and counter regulatory.

In *P. falciparum* malaria, these innate immune responses are potently triggered by PfGPI (Krishnegowda, 2005) and haemozoin (Parroche, 2007; Jaramillo, 2004) activation of TLRs on leukocytes, as well as by microvascular inflammation induced by PfEMP1-mediated binding of iRBCs to the endothelium (Tripathi, 2009). Severe malaria has been associated with increased angiopoietin-2, decreased angiopoietin-1, and decreased levels of endothelial nitric oxide, and counter-regulatory molecules including HO-1 and erythropoietin have been identified in human studies (Taylor, 2013). The association of HO-1 levels with severe malaria was variable in Gambian children and HO-1 promoter polymorphisms which confer higher constitutive levels of

HO-1 was associated with an increased risk of severe malaria (Taylor, 2013). Such results, obtained mainly from HbAA patients, indicate that HO-1 can be either protective or deleterious over a wide range of in vivo rates (Taylor, 2013). Given the lack of studies on haemoglobinopathies and innate host defenses, a separate line of nitric oxide (NO) and extreme malaria investigations highlight the significance of interactions between iRBC and endothelium in serious malaria pathogenesis. NO also exhibits anti-inflammatory behavior by reducing the expression of host receptors used by iRBCs to bind microvascular endothelial cells (De-Caterina, 1995). Thus, haemoglobinopathies and increased NO production are associated with in vivo defense against severe malaria and decreased binding of iRBCs to in vitro endothelium.

2. 10 Haemoglobin variants enhance the adaptive immune responses to *P. falciparum*

Field studies evidence suggests an association between several haemoglobinopathies, adaptive immunity, and malaria defense, while investigations of these relationships are complicated by the lack of accurate correlates of immune response (Taylor, 2013). The ability of polyclonal IgGs from malaria-immune adults to clear parasitemia in children with malaria indicates a central role for antibodies in malaria immunity (Cohen, 1961). Antibody-mediated phagocytosis of iRBCs is an essential mechanism of effectors in malaria defense; research with normal RBCs has shown that monocytes prefer iRBCs to phagocytosis compared to uninfected RBCs and that this preference is potentiated by binding IgG to iRBCs (Celada, 1982). In addition, hyper-immune sera polyclonal IgG binds more avidly to both α-thalassemic iRBCs (Luzzi, 1991; Williams., 2002) and β-thalassemic iRBCs (Luzzi, 1991) compared to non-thalassemic iRBCs, indicating that this mechanism can ideally clear iRBCs with haemoglobin variants. While these data suggest that Haemoglobinopathies functionally enhance the clearance of iRBCs, the precise mechanism of this enhancement remains unclear. There is no evidence of this role in the clearance of parasites by other

haemoglobinopathies and the correlation between this mechanism and the protection from the clinical disease have not been specifically investigated. Attenuating host-cell injury, coupled with mechanisms of parasite growth restriction in haemoglobinopathic iRBCs, may be involved in prolonging the asymptomatic phase of parasitemia. This delay in developing symptoms may provide more time for erythrocytic stage antigens and RBC senescence markers to be exposed to the immune system, thus enhancing both the acquisition and maintenance of adaptive and memory-based immune responses that ultimately protect individuals from developing the deadliest complications of *P. falciparum* infection.

2.11 Malaria Diagnostics

Malaria presents a diagnostic challenge to laboratories in most countries. The urgency and importance of obtaining results quickly from examining blood samples from patients with suspected acute malaria render some of the more sensitive methods for malaria diagnosis impractical for routine laboratory use (Moody, 2002). Changing patterns of known morphological appearances of malaria species, possibly due to drug pressure, strain variation, or approaches to blood collection, have created diagnostic problems that cannot easily be resolved merely by reference to an atlas of parasitology (Moody, 2002). Thus, one of the primary interventions of the Global Malaria Control Strategy by the WHO is prompt and accurate diagnosis of the disease (WHO,1999)

2.12 Malaria serology

Malaria endemicity has been assessed using antibody prevalence determined by immunofluorescence (Voller & O'Neill, 1971 & Druilhe et al., 1986). Still, its use was limited by reliance on cultured parasites, expensive fluorescence microscopes, and the subjective nature of slide reading. Subsequently, the measurement of antimalarial antibodies by ELISA was shown to be a potentially helpful epidemiological tool (Esposito, 1988).

The resistance of *P. falciparum* threatens malaria elimination efforts to anti- malarial drugs by increasing and widespread mosquito vector resistance to insecticides and by the lack of an effective vaccine conferring strong protective immunity to infection. In malaria-endemic areas, human populations develop natural immunity against *P. falciparum* which can lead to premonition (Crompton, 2010; Lu, 2017; Ranson, 2016). This acquired protective immunity takes years to establish following recurrent *Plasmodium* parasite exposure, is relatively short-lived, and is only partially effective. It can efficiently control malaria parasite infection leading to a decline in clinical malaria since low parasitemia mainly persists in the presence of circulating antibodies (Abs). Specific Abs, such as immunoglobulin G, primarily mediate protective immunity. Immune responses are complex traits, and vaccine development necessitates extensive information on human populations' processes and determinants that modulate immune responses. The effect of age, genetic factors, pathogen co-infection, and nutritional status have been more intensively explored and are recognized to influence anti-*Plasmodium* Ab responses and to have some association with malaria clinical protection. Antibodies are essential to the acquisition of defense against malaria. During the developmental phases of *P. falciparum*, the parasites transmit specific proteins or antigens on their surfaces. Antibodies to these antigens provide protection through several mechanisms (Groux, 1990). Some of the antigens found on *P. falciparum* include Erythrocyte binding antigen175 (EBA-175), Gamete surface antigen, Pfs230, Merozoite surface

protein 3 (MSP3), the Circumsporozoite Surface Protein (CSP), *P. falciparum* Erythrocyte Membrane Protein (*Pf*EMPl), Apical Merozoite Antigen 1 (AMA1), Liver stage antigen (LSA), and the Glutamate Rich Protein (GLURP). In recent immuno-epidemiological studies, several antigens have been identified as possible targets for anti-malaria defense. Which include GLURP, MSP1-19, and MSP3, which are currently in phase I clinical trials CSP (Hoffman, 1992), LSA, and *Pf*EMPl (Bull., 2016) have also been studied in connection with protection.

2.12.1 Asexual Stage Antigen - PfEBA-175

PfEBA-175 has been shown to play a crucial role during the fast cascade of interactions between the parasite and host molecules before the merozoites ultimately invade the erythrocyte (Chen, 2013). PfEBA-175 binds to glycophorin A on the surface of erythrocytes, and this interaction depends on Salic acid on the receptor (Sim, 1994). Antibodies induced against diverse antigenic components of the erythrocytic parasite are essential mediators of anti-disease immunity (Amoah, 2018). Antibodies specific to EBA 175 RIII-V are associated with protection from malaria in symptomatic cases (Amoah, 2018). In the absence of infection, however, a few investigations, notably by Wipasa et al., found that antibody and memory B cell responses to malaria antigens remained constant throughout time (Wipasa, 2010). This antigen was chosen because it's a potential vaccine candidate and uses different invasion pathways. During the asexual erythrocytic phase of the *P. falciparum* life cycle, the parasite population grows exponentially, resulting in clinical symptoms. The rapidity with which merozoites invade erythrocytes suggests that particular receptor-ligand interactions between host and parasite molecules are involved (Duraisingh, 2003) And because erythrocyte invasion is an obligate part of the parasite's lifecycle, blocking invasion should prevent parasite growth, and this has become

an attractive target for vaccine development. *P. falciparum* invades erythrocytes by using multiple receptor-ligand interactions defined as invasion pathways (Camus, 1985; Doolan, 1999). The first *P. falciparum* ligand identified to bind to erythrocytes with high affinity was erythrocyte-binding antigen 175 (Camus, 1985). Before the merozoite completely invades the erythrocyte, PfEBA-175 has been demonstrated to play an essential part in the quick cascade of interactions between parasites and host molecules (Aikawa, 1978). PfEBA-175 binds to glycophorin A on the surface of erythrocytes, and this interaction depends on sialic acid on the receptor (Sim, 1994). The binding region of PfEBA-175 to glycophorin A involves the cysteine-rich region II consisting of two halves called F1 and F2 domains. Antibodies raised against the F2 domain of PfEBA-175 have been shown to partially inhibit the invasion of P. falciparum merozoites into human erythrocytes (Narum, 2000). Antibodies recognize PfEBA-175 RII in individuals with naturally acquired immunity (Okenu, 2000). In addition, antibody levels are associated with protection from malaria (Okenu, 2000; McCarra, 2011; Richards, 2008), although this association is not observed in groups with a low incidence of disease (Osier, 2014)

2.12.2 Sexual Stage Antigen - Pfs230

Pfs230 is a surface antigen of *P. falciparum* that has shown transmission-blocking activity. Transmission-blocking immunity to P. falciparum in malaria-immune individuals was associated with antibodies to the gametocyte surface protein Pfs230 (Healer, 1999). Their results confirm Pfs230 as a possible candidate for inclusion as a malaria transmission-blocking vaccine. A previous study by (Eksi., 2002) demonstrated that targeted disruption of Pfs230 resulted in the production of truncated proteins that were not retained on the surface of the gametocyte or gamete (Eksi, 2006). Pfs230 disruptants successfully emerge from RBCs and male gamete sex flagellate-producing microgametes (Eksi, 2006). However, ex-flagellating Pfs230- minus males could not

Interact with RBCs and form exflagellation centers. Oocyst production and mosquito infectivity were also significantly reduced, 96–92% and 76–71%, respectively (Eksi, 2006). Their results suggest that Pfs230 is a surface molecule in males that mediates RBC binding and plays an essential role in oocyst development, a critical step in malaria transmission (Eksi, 2006). In a recent study conducted by (Farrance, 2011), they successfully produced a region of the Pfs230 antigen in their plant-based transient-expression system and evaluated the role of the protein in an animal model. In rabbits with high titers of transmission-blocking antibodies, administration of 230CMB with > 90 % purity causes robust immunological responses, resulting in a greater than 99 % reduction in oocyst counts in the presence of complement, as assessed by a conventional membrane feeding assay (Farrance, 2011). Additionally, sera from people with a strong Pfs230 response have been demonstrated to have transmission-reducing immunity. Several studies looking at naturally acquired immune responses to Pfs230 have now been conducted across several sites to expand our understanding of naturally acquired immunity to sexual stage antigens. To understand the dynamics of sexual stage immunity, traditional indices of parasite exposure, such as age, transmission setting, malaria transmission season, and parasite prevalence, have been analyzed. However, the reported relationships between the parameters above and the seroprevalence of antibodies to sexual-stage antigens are inconsistent. Immune responses to Pfs230 were studied in ten research spanning 15 study sites in Africa. The range of seroprevalence estimates was quite broad, ranging from 6% reported by Stone et al. in Soumousso and Dande villages, Burkina Faso, to 72% reported by (Amoah, 2018). Immunological responses to numerous malaria parasite antigens should be studied, and persons with haemoglobinopathies should be included in these studies. As a result, this study will shed further information on how changes in haemoglobin morphologies in symptomatic malaria patients affect natural malaria protection (IgG levels).

2.12.3 Salivary Gland antigen- gSG6-P1

Human antibodies recognizing the Anopheles gambiae salivary protein gSG6 P1 were associated with recent exposure to Anopheles bites in tropical Africa. These observations suggested that anti-gSG6-P1 antibody levels might serve as an entomological proxy to estimate anopheles biting intensity by reflecting recent exposure to anopheles bites (Pollard, 2016). In the case of malaria, a recent study has shown that human IgG response to the gSG6-P1 peptide represented a specific biomarker of mosquito bites' exposure, which will aid malaria surveillance. This will better understand the influence of haemoglobin variants on IgG levels in symptomatic malaria in Ghana, thus correlating the IgG levels to haemoglobin variants across Ghana. So far, there's minimal information on the influence of haemoglobin variants such as HbAS and HbAC on Pfs230, EBA 175 3R, and gGS6-P1 levels in symptomatic patients across Ghana.

2.13 Capillary electrophoresis

The Capillarys haemoglobin (E) kit is designed for the separation of the normal haemoglobins (A, A2, and F) in human blood samples, and for the detection of the significant haemoglobin variants (S, C, E, and D), by capillary electrophoresis in alkaline buffer (pH 9.4) with the SEBIA capillarys two flex-piercing instruments. The capillarys haemoglobin (E) kit is designed for laboratory use. The capillarys two flex-piercing instruments is an automated analyzer that performs a complete haemoglobin profile for the quantitative analysis of the normal haemoglobin fractions A, A2, and F and the detection of significant haemoglobin variants S, C, E, and D. The assay is performed on the haemolysate of whole blood samples collected in tubes containing K_2EDTA or K_3EDTA as an anticoagulant

Principle of the test

Haemoglobin is a complex molecule composed of two pairs of polypeptide chains. Each chain is linked to the haem, a tetrapyrrolic nucleus (porphyrin) that chelates an iron atom. The haem part is common to all haemoglobin and their variants. The type of haemoglobin is determined by the protein part called globin. Polypeptide chains α, ß, δ and γ constitute the normal human haemoglobins:

• haemoglobin A ..... = α2 ß2

• haemoglobin A2 ..... = α2 δ2

• fetal haemoglobin F ..... = α2 γ2

The α-chain is common to these three haemoglobins. The haemoglobin spatial structure and other molecular properties (like all proteins) depend on the amino acids' nature and sequence constituting the chains. Substitution of amino acids by mutation is responsible for forming haemoglobin variants with different surface charges and electrophoretic mobilities, which also depend on the pH and ionic strength of the buffer.

The resulting qualitative (or structural) abnormalities are called haemoglobinopathies (Landers, 1995; Livingstone, 1986; Schneider, 1978). Decreased synthesis of one of the haemoglobin chains leads to quantitative (or regulation) abnormalities called thalassemias. Haemoglobin electrophoresis is a well-established technique routinely used in clinical laboratories for screening samples for haemoglobin abnormalities (Bardakdjian-Michau, 2003; Fairbanks, 1980; Galacteros, 1986; Hempe, 1997; Oda, 1997). The capillarys two flex-piercing instruments have been developed to provide complete automation of this testing with fast separation and reasonable resolution. The methodology can be considered an intermediary between classical zone electrophoresis and liquid chromatography (Krauss, 1986; Maier-Redelsberger, 1989). The capillarys two flex-piercing instrument uses the principle of capillary electrophoresis in free

solution. This technique separates charged molecules by electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electroosmotic flow (Huisman, 1977). The capillary's two flex-piercing instruments have capillaries functioning in parallel, allowing eight simultaneous analyses for haemoglobin quantification from whole blood samples. A sample dilution with haemolysing solution is prepared and injected by aspiration at the anodic end of the capillary. A high voltage protein separation is then performed, and the haemoglobins are directly detected at 415 nm at the cathodic end of the capillary. Before each run, the capillaries are washed with a wash solution and prepared for the subsequent analysis with buffer. The haemoglobins, separated in silica capillaries, are directly and detected explicitly at an absorbance wavelength of 415 nm, which is specific to haemoglobins. The resulting electropherograms are evaluated visually for pattern abnormalities.

Direct detection provides accurate relative quantification of individual haemoglobin fractions, with particular interest, such as A2 haemoglobin for ß thalassemia diagnostic. In addition, the high resolution of this procedure should allow the identification of haemoglobin variants, in particular, to differentiate haemoglobins S from D and E from C. The haemoglobin A2 quantification can also be performed when haemoglobin E is present. By using an alkaline pH buffer, normal and abnormal (or variant), haemoglobins are detected in the following order, from cathode to anode: δA'2 (A2 variant), C, A2/O-Arab, E, S, D, G-Philadelphia, F, A, Hope, Bart's, J, N-Baltimore, and H.

The carbonic anhydrase is not visualized on the haemoglobin electrophoretic patterns; this permits the identification of haemoglobin A2 variants in this migration zone.

Figure 2: Capillary electrophoresis system; CAPILLARYS 2 FLEX PIERCING

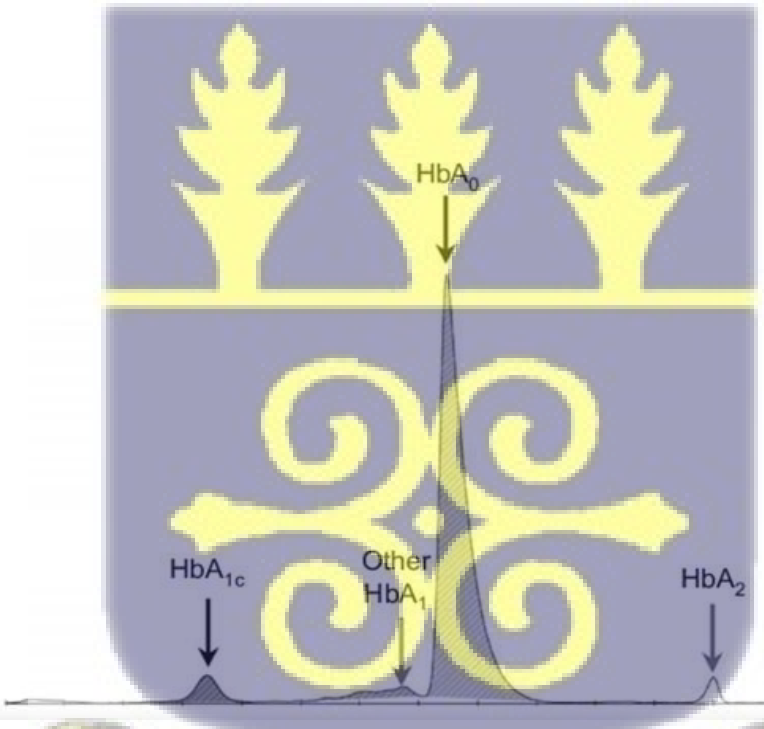

Figure 3: A typical separation profile obtained with the Capillarys 2 Flex Piercing analyzer.

2.14 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a common laboratory technique used to measure an analyte's concentration (usually antibodies or antigens) in a solution. ELISA begins with a coating step, where the first layer- either an antigen or antibody is adsorbed (passive attachment of a liquid to a solid surface, creating a thin film) to a solid surface, usually a polystyrene multiwell plate. The coating is followed by blocking and detection steps. When there is interaction, this can be detected by a conjugate (an anti-human Ig coupled to an enzyme linked to a substrate), which reduces the substrate to produce a visible color change. The colored end product correlates to the amount of analyte in the original sample.

ELISA is a very useful tool in malaria research, where it has been used to diagnose tertian malaria (Ming, 1987) and for determining *Plasmodium falciparum* antigens (Spencer et al., 1979). There are three different types of ELISA based on the detection method. They are Direct, Indirect, and Sandwich/Capture assays. The difference between Direct ELISA and Indirect ELISA is that Direct ELISA involves labeling the detection antibody with an enzyme or an alternative signaling molecule such as a fluorophore. In contrast, Indirect detection involves an additional probe (a secondary antibody) labeled with a detectable tag. This secondary antibody produces a measurable signal tag by binding to the detection (primary) antibodies. Direct ELISA is faster than Indirect ELISA but is less sensitive and cannot provide the amplification gained from using a secondary antibody. With Sandwich ELISA, the antigen is quantified between two layers of antibodies (i.e., a capture and detection antibody). To detect antigen, wells of microtiter plates are coated with capture antibodies (usually monoclonal or polyclonal antibodies) followed by incubation with the test solutions containing the antigen. Afterward, an antigen-specific antibody conjugated to an enzyme is applied as the detection antibody. This is a direct sandwich ELISA. But there is also Indirect Sandwich ELISA where an antigen-specific detection antibody is used to sandwich the incubated antigen before introducing the enzyme-linked secondary antibody for detection.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study type and description of study sites

This is a nested study to compare IgG levels of malaria parasite and vector antigens in two populations and determine the frequency of different haemoglobin variants in the microscopy-positive malaria population.

Ten health facilities in Ghana's previous ten regions were chosen randomly. The facility size was calculated using Probability proportional to size estimates (PPSE) based on the total monthly number of confirmed malaria cases in outpatient departments (OPDs) in 2016. Three district/municipal hospitals and seven health centers were selected from each of the ten Regions(New Abirem Hospital, Shai-Osukodu Hospital, Abura Dunkwa District Hospital, Praso Health Centre, Gyedu Health Centre, Kalba Health Centre, Paga Health Centre, Lambussi Polyclinic, Kpetoe Health Centre and Agona Nkwanta Health Centre). The research included any patient suspected of uncomplicated malaria at the designated health.

3.1.1 Subjects/study population

- Archived packed cells and plasma collected from suspected malaria patients seeking treatment in all the participating health facilities that have been consented for reuse

3.2 Inclusion Criteria

- All samples used in this study were archived samples collected within 2018-2019 and aged 2 to 89 years.

3.3 Exclusion Criteria

- Archived samples containing any other species of *Plasmodium* aside *Falciparum* was excluded from the study

3.4 Sample size determination

The sample size was determined using the binomial model to estimate the confidence interval (CI) (Vallejo, 2013). The sample size with a 95% CI and precision level of 5% was estimated according to the formulae below: The sample size was calculated with the formula below.

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

Where N = sample size, Z = statistic corresponding to the level of confidence, P = expected prevalence and e = precision.

- With a level of confidence at 95% corresponding to Z = 1.96
- The proposed prevalence of malaria diagnosis by microscopy is 25.2 % (Kweku, 2017)
- Precision set at 5%

The minimum number of samples based on the above calculation was 288; thus, 300 microscopy-positive archived samples and 300 microscopy-negative archived samples were used for the study.

3.5 Ethical Consideration

Ethical clearance was obtained from the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR) and the Ethical Committee of the College of Health Sciences of the University of Ghana.

3.6 MATERIALS

3.6.1 Reagents

Deep well plates, plastic seals, plate reader: Bio-Rad Model 680 Microplate Reader, plate washer, Tween-20, absolute ethanol, sulphuric acid were all obtained from Sigma-Aldrich (U.S.A), MAXISORP NUNC-immune plates from Thermo Scientific, Anti-Human Immunoglobulin G (H+L) HRPO conjugate from Life Technology, Phosphate buffered saline from Oxoid, (England) and dried skimmed milk from Marvel, (Ireland), Haemolysing solution, Capillarys 2 flex piercing instrument.

3.7 METHODS

3.7.1 Examination of malaria Parasite by Microscopy

The archived samples used for this research were previously analyzed for the presence of malaria parasites by two independent WHO-certified malaria microscopists; any disagreement on smear (thick and thin) readings was resolved by re-examination by a third microscopist's assessment. The assessment by the third microscopists was considered to be the final decision.

3.7.2 Indirect ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to determine the natural levels of antibodies of the Salivary Gland peptide (gSG6-P1), asexual stage antigen (PfEBA-175 Region III), and sexual stage antigen (Pfs230). NUNC 96-well streptavidin-coated ELISA plates were coated with 100 µl of 1 µg/ml of Mosquito Salivary Gland antigen in phosphate-buffered saline (PBS, pH 7.4). Also, NUNC 96-well ELISA plates were coated with 20 µl of 1 µg/ml of asexual stage antigen in phosphate-buffered saline (PBS, pH 7.4) and 100 µl of 1 µg/ml of sexual stage antigens in carbonate buffer (0.05 M carbonate/bicarbonate buffer, pH 9.2) and kept overnight at four °C. The plates were washed four times with wash buffer (1X PBS and 0.0 5% Tween- 20). Each washing step involved emptying the contents of the wells of unbound materials by flipping the plate, adding 250 µl of wash buffer, and incubating for 1 min. The wash buffer is then discarded. Unbound regions in the wells were then blocked by incubating with 150 µl of blocking buffer (3% skimmed milk in wash buffer) for 1 hr. The plates were then washed twice with wash buffer as before and incubated for 1 hour with 100 µl per well of 1:200 dilutions (samples) and 1% skimmed milk for dissolving negative and positive controls consisting of pools of previously sampled high titer plasma. Plates were washed as before, and wells incubated with 50 µl of peroxidase-conjugated antibodies (1:3000 dilution of goat antihuman IgG-HRPO) for 1 hr. The plates were washed and incubated with incubated peroxidase substrate TMB (3,3',5,5'-Tetramethylbenzidin) for 30 min, and the enzyme The reactions were stopped by adding 50 µl of 0.2 mM sulphuric acid. Care was taken to ensure that all plates were incubated with a substrate for relatively the same time. The optical densities of the contents of the wells were then read using the Biotek ELISA plate reader at 450 nm.

The reader determined the optical densities of the contents of each well using the Beer-Lambert Law. Briefly, the distribution of log10-transformed OD values was fitted as the sum of two Gaussian distributions using maximum likelihood methods; one of the two distributions represents the seronegative population, and the other represents the seropositive population. For each antigen, the mean log10-OD of the Gaussian corresponding to the seronegative population plus log10-three standard deviations was calculated, back-transformed, and used as the cut-off for seropositivity. Seroprevalence was calculated as the proportion of samples with OD above this cut-off.

3.7.3 Haemoglobin Phenotype determination

Capillary electrophoresis was performed on symptomatic samples; Samples were brought to thaw at room temperature. The samples were then arranged in sample tubes in racks according to sample ID order. A working list was prepared per the sample ID order. Then, 200 µL of the haemolysing solution was pipetted into Eppendorf tubes labeled according to the sample ID order. The samples were vortexed, and 25 µL of the sample was pipetted into the Eppendorf tubes containing the haemolysing solution. The mixture was then vortexed for 5 seconds. The dilution segment with the capillary two flex piercing equipment was placed on the sample rack. 100 µL of the haemolysate was pipetted into the dilution segment from 1 to 8. The sample rack was slithered into the Capillarys 2 Flex-Piercing instrument to start the analysis.

3.8 Data analysis

The ADAMSEL software was used to convert OD values obtained for the samples into weighted concentration values. Log-transformed antibody data were analyzed using SPSS (version 16.0) and GraphPad Prism (GraphPad Software, version 5.01). A Mann-Whitney U test (a nonparametric test used to replace an unpaired T-test) was used to compare means between positive and negative malaria microscopy patients. Kruskal Wallis Variational Test was performed to compare the different IgGs of the various haemoglobin variants.

CHAPTER 4

RESULTS

A total of 600 archived samples from malaria suspected patients in the ten regions of Ghana were used in this study. Out of the samples collected, 344 were from females, whiles 256 were from males. The average age across the ten regions was 21.84 years, while the minimum and maximum ages recorded were 1 year and 88 years, respectively, as shown in **Table 1**

Table 1: Demographic Characteristics of the Study Participants Grouped Regionally.

Parameter	A (60)	BA (60)	C (60)	E (60)	GA (60)	N (60)	UE (60)	UW (60)	V (60)	W (60)	TOTAL
SEX											
MALE	25	33	27	23	26	29	20	24	25	24	256
FEMALE	35	27	33	37	34	31	40	36	35	36	344
AGE (YRS)											
MEAN	20.57	19.40	23.98	19.75	27.75	22.85	22.90	21.15	21.80	18.27	21.84
SEM	2.66	2.50	3.10	2.55	3.58	2.95	2.96	2.73	2.81	2.36	2.82
MINIMUM	2	2	2	3	3	1	1	1	1	1	1.70
MAXIMUM	83	72	83	78	77	81	82	88	85	72	80.40

SEM, standard error of the mean; A, Ashanti; BA, Brong Ahafo; C, Central; E, Eastern; GA, Greater Accra; N, Northern; UE, Upper East; UW, Upper West; V, Volta; W, Western.

Objective 1: TO DETERMINE AND COMPARE IGG LEVELS IN THREE MALARIA ANTIGENS IN MALARIA PATIENTS.

Table 2: Comparison of the IgG Levels for gSG6-P1 in Malaria Positive and Negative Individuals at Each Region Using Mann Whitney U Test

Region	Min	Max	Mean ± SD	95% CI	*P*
Ashanti					0.1421
Neg	443.60	5995.00	1513.00±1189.00	1069.00-1958.00	
Pos	0.00	2760.00	1503.00±598.80	1279.00-1726.00	
Brong Ahafo					**0.0013**
Neg	750.10	3573.00	2822.00±6260.00	484.50-5159.00	
Pos	455.60	2409.00	1141.00±475.30	963.40-1318.00	
Central					0.8087
Neg	607.10	4739.00	1709.00±946.20	1356.00±2063.00	
Pos	0.00	2690.00	1511.00±610.40	1283.00-1739.00	
Eastern					0.2359
Neg	0.00	3364.00	1461.00±661.60	1214.00-1708.00	
Pos	0.00	3364.00	1296.00±748.10	1016.00-1575.00	
Greater Accra					0.1910
Neg	678.70	5866.00	1652.00±1150.00	1223.00-2081.00	
Pos	0.00	2295.00	1147.00-533.10	947.40-1346.00	
Northern					0.5325
Neg	459.20	3979.00	1384.00±772.80	1096.00-1673.00	
Pos	619.30	3852.00	1506.00±864.50	1183.00-1828.00	
Upper East					0.0850
Neg	0.00	3074.00	1398±729.10	1126.00-1670.00	
Pos	415.40	2340.00	1129.00±436.10	966.20-1292.00	
Upper West					**0.0319**
Neg	954.80	4643.00	2328.00±917.10	1985.00-2670.00	
Pos	0.00	5114.00	1872.00±1176.00	1433.00-2311.00	
Volta					**0.0009**
Neg	0.00	2947.00	1237.00±622.90	1004.00-1469.00	
Pos	781.20	5697.00	2270.00±1415.00	1741.00-2798.00	
Western					**0.0446**
Neg	754.60	3983.00	1990.00±771.80	1702.00-2278.00	
Pos	627.50	4936.00	1686.00±1057.00	1292.00-2081.00	

Pos; Positive malaria microscopy sample, Neg; Negative malaria microscopy samples, Min; Minimum, Max; Maximum, Mean ± SD; Mean ± Standard Deviation, 95%CI; 95% Confidence Interval, *P*<0.05 implies statistically significant.

A Mann-Whitney U test (a non-parametric test that is used to replace an unpaired T-test) was used to compare the IgG levels for gSG6-P1 in the positive and negative samples collected from each

Region. The minimum, maximum, and mean standard deviations of positive and negative malaria samples from each region were compared. For instance, the minimum levels of the IgG for gSG6-P1 for malaria negative and positive samples from the Ashanti region were 443.60 and 0.00, respectively. An example of the maximum concentration of IgG levels for gSG6-P1 observed were 4739.00 and 2690.00 in microscopy negative and positive samples, respectively, from the Central Region. The Brong Ahafo, Upper West, and Western regions observed significantly ($p<0.05$) high levels of the IgG for gSG6-P1 for malaria negative samples when the means were compared to that of the positive malaria microscopy samples. However, samples from the Volta region recorded an average of significantly higher IgG levels for gSG6-P1 for malaria microscopy positives than for malaria microscopy negatives. The mean comparison of the IgG levels for gSG6-P1 for all other regions insignificantly indicated that the majority of the regions recorded higher levels of the IgG in malaria microscopy negative samples than positive samples. **(Table 2)**

Table 3: Comparison of the IgG Levels for Pfs-230 in Malaria Positive and Negative Individuals at Each Region Using Mann Whitney U Test.

Region	Min	Max	Mean ± SD	95% CI	P-value
Ashanti					**<0.0001**
Neg	0.00	19705.00	3741.00±4154.00	2190.00±5293.00	
Pos	4023.00	31670.00	9617.00±6495.00	7192.00±12043.00	
Brong Ahafo					**<0.0001**
Neg	1608.00	10083.00	3746.00±1810.00	3070.00-4422.00	
Pos	550.70	6838.00	1607.00±1205.00	1157.00-2057.00	
Central					0.1159
Neg	725.40	6550.00	2605.00±1596.00	2009.00-3201.00	
Pos	664.20	7236.00	2079.00±1444.00	1540.00±2618.00	
Eastern					0.0433
Neg	0.00	12252.00	2538.00±2744.00	1513.00-3562.00	
Pos	0.00	5227.00	1400.00±1037.00	1013.00-1788.00	
Greater Accra					**0.0307**
Neg	0.00	4538.00	1279.00±1083.00	874.90-1684.00	
Pos	0.00	4099.00	1604.00±820.40	1298.00-1911.00	
Northern					0.4147
Neg	874.6	10579.00	2963.00±1824.00	2882.00-3644.00	
Pos	0.00	34074.00	4146.00±7222.00	1449.00-6842.00	
Upper East					0.4829
Neg	0.00	19817.00	3742.00±3870.00	2297.00-5187.00	
Pos	0.00	8597.00	2876.00±2047.00	2112.00-3640.00	
Upper West					0.9124
Neg	964.40	6395.00	5246.00±2079.00	994.90-9497.00	
Pos	1128.00	7104.00	3098.00±1585.00	2506.00-3690.00	
Volta					0.5793
Neg	927.50	15022.00	3463.00±3344.00	2214.00-4712.00	
Pos	1205.00	8961.00	2701.00±1595.00	2105.00-3296.00	
Western					0.7523
Neg	0.00	15504.00	3429.00±3064.00	2285.00-4573.00	
Pos	0.00	6753.00	2900.00±1753.00	2245.00-3555.00	

Pos; Positive malaria microscopy sample, Neg; Negative malaria microscopy samples, Min; Minimum, Max; Maximum, mean±SD; Mean ± Standard Deviation, 95%CI; 95% Confidence Interval, $P<0.05$ implies statistically significant.

Table 3 Compares the IgG levels for Pfs-230 in malaria-positive and negative samples in each region using the Mann- Whitney U Test. The minimum, maximum and

mean±standard deviation of the IgG levels for Pfs-230 positive and negative malaria samples from each region was compared. For example, the minimum levels of the IgG for Pfs-230 for malaria negative and positive samples from the Upper West region were 964.40 and 1128.00, respectively. Regarding the maximum concentrations, the IgG levels for Pfs-230 observed in microscopy negative and positive samples from the Northern region were 10579.00 and 34074.00, respectively. On average, there was a significant rise in the IgG levels for the malaria negative samples (3746.00±1810.00) compared to that of the malaria microscopy positive samples (1607.00±1205.00) in the Brong Ahafo Region. On the other hand, the levels of IgG for Pfs-230 in the malaria microscopy positive samples from the Ashanti region (9617.00±6495.00) and the Greater Accra Region (1604.00±820.40) were significantly higher than the IgG levels for Pfs-230 in negative malaria microscopy samples in the two regions (3741.00±4154.00 and 1279.00±1083.00 respectively). No significant relationships were established between levels of IgG for Pfs-230 in the rest of the regions. However, it is worth noting that although the concentrations observed in the other regions were significant, most of these regions had higher levels of IgG for Pfs-230 in the malaria microscopy negative samples.

Table 4: Comparison of the IgG Levels for EBA 175 3R in Malaria Positive and Negative Individuals at Each Region Using Mann Whitney U Test.

Parameter	Min	Max	Mean ± SD	95% CI	P-value
Ashanti					0.0745
Neg	0.00	106964.00	19521.00±24655.00	10314.00-28727.00	
Pos	0.00	82864.00	11866.00±19180.00	4704.00-19028.00	
Brong Ahafo					**0.0358**
Neg	3009.00	77800.00	16520.00±20035.00	9039.00±24002.00	
Pos	1899.00	75545.00	16520.00±13546.00	4610.00±14727.00	
Central					0.797
Neg	860.70	53898.00	9412.00±10392	5531.00±13293.00	
Pos	1308.00	37808.00	10309.00±1756.00	6717.00-13901.00	
Eastern					0.8545
Neg	1193.00	216558.00	18653.00±40921.00	3372.00-33933.00	
Pos	860.70	53898.00	9412.00±1897.00	553100±13293.00	
Greater Accra					**0.001**
Neg	0.00	41847.00	11384.00±10993.00	7279.00-15488.00	
Pos	0.00	17293.00	4397.00±863.00	2629.00-6165.00	
Northern					**<0.0001**
Neg	0.00	8433172.00	851966.00±2004095.00	103655.00- 1600338	
Pos	1354.00	118048.00	10354.00±21383.00	2370.00±18339.00	
Upper East					0.4581
Neg	0.00	62019.00	10711.00±14635.00	5246.00±16176.00	
Pos	909.80	38258.00	9595.00±9522.00	6039.00-13150.00	
Upper West					0.3898
Neg	6226.00	115033.00	16736.00±20696.00	9008.00±24464.00	
Pos	0.00	241151.00	33640.00±56509.00	12539.00±54741.00	
Volta					0.6650
Neg	0.000	335362.00	30211.00±61626.00	7200.00±53223.00	
Pos	2727.00	210490.00	20693.00±38125.00	6456.00±34929.00	
Western					0.4125
Neg	0.00	132626.00	16737.00±30062.00	5511.00-27962.00	
Pos	1393.00	247994.00	14825.00±44724.00	-1875.00-31526.00	

Pos; Positive malaria microscopy sample, Neg; Negative malaria microscopy samples, Min; Minimum, Max; Maximum, mean ± SD; Mean ± Standard Deviation, 95%CI; 95% Confidence Interval, $P<0.05$ implies statistically significant.

Table 4: Compares the IgG levels for EBA 175 3R in malaria microscopy positive and negative samples at each region using the Mann-Whitney U test. The minimum,

maximum and mean±standard deviation of the levels of IgG for EBA 175 3R positive and negative malaria microscopy samples from each region were compared with a $P<0.05$, indicating a statistically significant comparison. The minimum concentrations observed in the negative and positive samples from the Eastern region were 1193.00 and 860.70, respectively. The maximum concentrations of IgG levels for EBA 175 3R in negative and positive samples in the same region were 216558.00 and 53898.00. Notably, the comparison of the IgG levels for EBA 175 3R for all regions was insignificant statistically except in the Brong Ahafo, Northern, and Central Region of Ghana. The IgG levels for EBA 175 3R in the Greater Accra Region were 11384.00±10993.00 and 4397.00±863.00 for the microscopy negative and positive samples, respectively.

Table 5: Comparison of the Levels for IgG gSG6-P1, Pfs230, and EBA 175 3R in Malaria Microscopy Positive and Negative Individuals Using the Mann Whitney U Test.

Parameter	Microscopy	Min	Max	Mean ± SD	95% CI	P-value
gSG6-P1						**0.0316**
	Neg	0.00	35730.00	1749.00±2169.00	1503.00-1996.00	
	Pos	0.00	5697.00	1506.00±907.00	1403.00-1609.00	
Pfs230						0.1285
	Neg	0.00	6395.00	3275.00±4516.00	2726.00-3788.00	
	Pos	0.00	34074.00	3203.00±231.60	2747.00-3658.00	
EBA 175 3R						**<0.0001**
	Neg	0.00	8433172.00	100188.00±673345.00	23684.00-176693.00	
	Pos	0.00	247994.00	13506.00±28925.00	10214.00-16798.00	

Pos; Positive malaria microscopy sample, Neg; Negative malaria microscopy samples, Min; Minimum, Max; Maximum, Mean ± SD; Mean ± Standard Deviation, 95%CI; 95% Confidence Interval, $P<0.05$ implies statistically significant.

Table 5 shows a nationwide comparison of the levels of IgG for gSG6-P1, Pfs230, and EBA 175 3R antigens in malaria microscopy positive and negative samples using the Mann-Whitney U test.

The difference between the levels of IgG for Pfs230 was insignificant when the positive and negative samples were compared. Yet it should be noted that averagely, the levels of IgG for Pfs230 in negative malaria microscopy (3275.00±4516.00) samples were higher than in positive malaria microscopy samples (3275.00±4516.00). The levels of IgG for gSG6-P1 and EBA 175 3R were significantly higher in malaria microscopy negative samples (1749.00±2169.00 and 100188.00±673345.00, respectively) compared to average IgG levels for the same antigens in positive malaria microscopy samples (1506.00±907.00 and 13506.00±28925.00 respectively).

Objective 2: TO DETERMINE THE DIFFERENT HAEMOGLOBIN GENOTYPES IN MALARIA MICROSCOPY POSITIVE PATIENTS

Figure 4: Regional Distribution of Individuals with Haemoglobin Variants

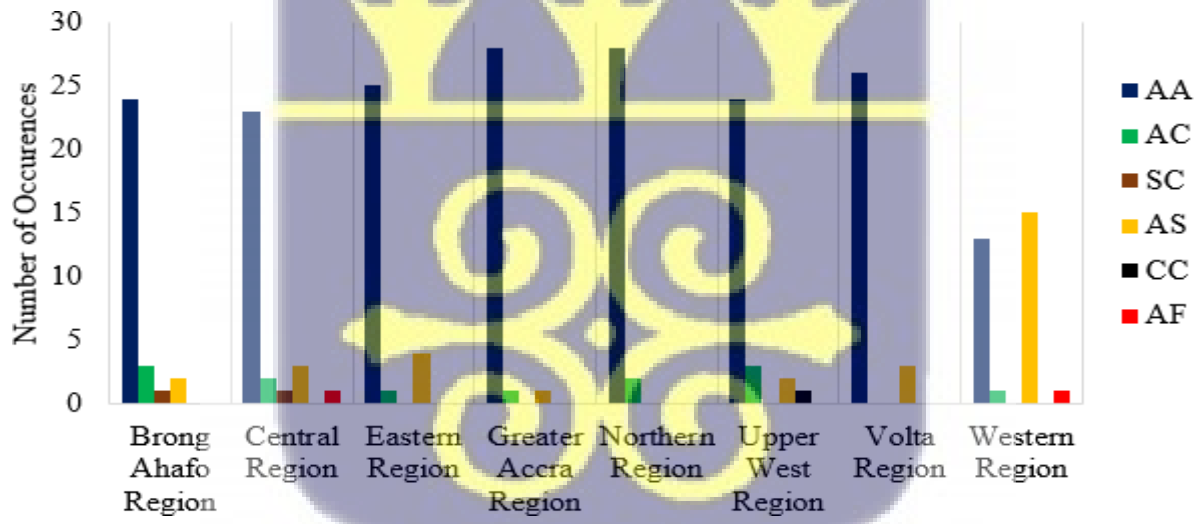

Figure 4 shows the haemoglobin variants observed among the malaria microscopy-positive samples from eight regions. In all regions except the Western region, the dominant haemoglobin variant observed from the samples was the HbAA. However, the dominant haemoglobin variant in the Western region was the HbAS. Notwithstanding, HbAC was the second most

dominant haemoglobin variant observed across the regions comparatively. The least observed haemoglobin variant observed was the HbCC.

Table 6: Kruskal Wallis Variational Test Between the Haemoglobin Variants and the Concentration of IgG's measured in Microscopy Positive Samples.

Parameter	HB Variants	Min	Max	Mean ± SD	95% CI	P-value
gSG6-P1						0.7376
	AA	0.00	5696.60	1555.10±987.06	1414.20-1696.00	
	AC	592.37	2930.90	1667.0±840.75	1159.00-2175.10	
	AS	506.41	3653.40	1505.40±862.29	1183.40-1827.40	
Pfs230						0.6199
	AA	0.00	34074.00	2491.60±3232.20	2030.30-2952.90	
	AC	0.00	3803.50	1718.70±835.39	1213.90-2223.50	
	AS	0.00	6214.40	2841.70±1681.40	1853.90-3109.60	
EBA 175 3R						0.1934
	AA	0.00	247994.00	15159.00±34125.00	10276.00-20043.00	
	AC	0.00	20673.00	5312.00±5429.40	2031.10-8592.90	
	AS	860.71	111745.00	12133.00±20610.00	4437.10-19829.00	

Min; Minimum, Max; Maximum, Mean ± SD; Mean ± Standard Deviation, 95%CI; 95% Confidence Interval, $P<0.05$ implies statistically significant.

Table 6 Compares the haemoglobin variants (HbAA, HbAC, and HbAS) to the IgG levels for the antigens. It was observed that none of the IgG measured for the antigens in the aforementioned haemoglobin variants was significantly higher. That notwithstanding, it could be observed that the IgG levels for all antigens were higher in HbAA samples, followed by HbAS and then HbAC.

Objectives 3 and 4: TO DETERMINE THE SEROPREVALENCE OF THREE MALARIA ANTIGENS IN SYMPTOMATIC MALARIA PATIENTS AND THE RELATIONSHIP BETWEEN HAEMOGLOBIN VARIANTS AND IgG LEVELS

Figure 5: Seroprevalence of IgG Antibodies for gSG6-P1, Pfs 230, and EBA 175 3R antigens in Microscopy Positive and Negative Samples.

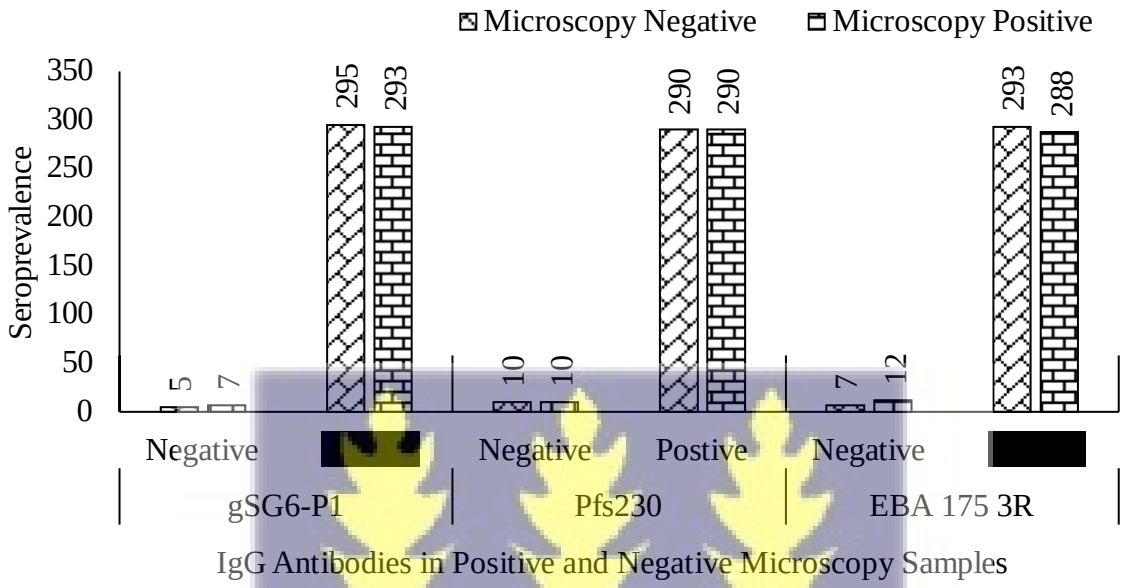

Figure 5 above shows the IgG seroprevalence for the various antigens in microscopy positive and negative samples. The antibody presence was high in both microscopy positive and negative samples regardless of the antigen measured. For example, the positive IgG recorded for EBA 175 3R antigens were 97.7 % and 96.0 % in microscopy-positive and negative samples. Whiles, samples without IgG recorded for the same antigen were 2.3 % and 4.0 % in microscopy positive and negative samples, respectively.

Table 7: Seroprevalence of IgG Antibodies for gSG6-P1, Pfs230, and EBA 175 3R antigens in Microscopy Positive and Negative Samples Stratified Across Gender, Age, and HB Phenotype.

Microscopy Positive									
Parameter	**gSG6-P1**		***P***	**Pfs230**		***P***	**EBA 175 3R**		***P***
	NEG	POS		NEG	POS		NEG	POS	
Gender			0.720			>0.999			0.082
Male	4 (57.1 %)	144 (49.1 %)		5 (50.0 %)	143 (49.3 %)		9 (75.0 %)	139 (48.3 %)	
Female	3(42.9 %)	149 (50.9 %)		5 (50.0 %)	147 (50.7 %)		3 (25.0 %)	149 (51.7 %)	
Age			0.478			0.391			0.621
<21 yrs.	6 (85.7 %)	200 (68.3 %)		5 (50.0 %)	201 (69.3 %)		8 (66.7 %)	198 (68.8 %)	
21-40 yrs.	0 (0.0 %)	49 (16.7 %)		3 (30.0 %)	46 (15.9 %)		3 (25.0 %)	46 (16.0 %)	
>40yrs.	1 (14.3 %)	44 (15.0 %)		2 (20.0 %)	43 (14.8 %)		1 (8.3 %)	44 (15.3 %)	
HB Phenotype			**0.001**			0.966			0.832
AA	5 (83.3 %)	186 (79.8 %)		6 (75.0 %)	185 (80.1 %)		6 (85.7 %)	185 (79.2 %)	
AC	0 (0.0 %)	13 (5.6 %)		1 (12.5 %)	12 (5.2 %)		1 (14.3 %)	12 (5.2 %)	
SC	0 (0.0 %)	2 (0.9 %)		0 (0.0 %)	2 (0.9 %)		0 (0.0 %)	2 (0.9 %)	
AS	0 (0.0 %)	30 (12.9 %)		1 (12.5 %)	29 (12.6 %)		0 (0.0 %)	30 (12.9 %)	
CC	0 (0.0 %)	1 (0.4 %)		0 (0.0 %)	1 (0.4 %)		0 (0.0 %)	1 (0.4 %)	
AF	1 (16.7 %)	1 (0.4 %)		0 (0.0 %)	2 (0.9 %)		0 (0.0 %)	2 (0.9 %)	
Microscopy Negative									
Parameter	**gSG6-P1**		***P***	**Pfs230**		***P***	**EBA 175 3R**		***P***
	NEG	POS		NEG	POS		NEG	POS	
Gender			0.657			0.751			>0.999
Male	1 (20.0 %)	107 (36.3 %)		4 (40.0 %)	104 (35.9 %)		2 (28.6 %)	106 (36.2 %)	
Female	4 (80.0 %)	188 (63.7 %)		6 (60.0 %)	186 (64.1 %)		5 (71.4 %)	187 (63.8 %)	
Age			0.937			0.706			0.471
<21 yrs.	2 (40.0 %)	142 (48.1 %)		6 (60.0 %)	138 (47.6 %)		4 (57.1 %)	140 (47.8 %)	
21-40 yrs.	2 (40.0 %)	102 (34.6 %)		3 (30.0 %)	101 (34.8 %)		1 (14.3 %)	103 (35.2 %)	
>40yrs.	1 (20.0 %)	51 (17.3 %)		1 (10.0 %)	51 (17.6 %)		2 (28.6 %)	50 (17.1 %)	

P<0.05 implies statistical significance. POS -POSITIVE, NEG -NEGATIVE, *P* – p-value

Table 7 above shows the seroprevalence of the IgG antibodies for gSG6-P1, Pfs230, and EBA 175 3R antigens in microscopy positive and negative samples stratified across gender, age, and HB phenotype. The positive microscopy samples established no significant association among the parameters and the antibodies for the three antigens except for the Hb phenotype in gSG6-P1 antibodies. Thus, HbAA was significantly higher for gSG6-P1 positive antibodies 186(79.8 %) than all other phenotypes in positive microscopy samples. Also, samples without gSG6-P1 antibodies were significantly higher in Hb AA samples 5 (83.3 %) than all other phenotypes. Generally, among the males and females, antibodies for the antigens were higher in the females than in the males in the positive microscopy samples. Although these were statistically insignificant. On the negative microscopy samples, a similar pattern was observed in the presence of antibodies for the antigens among the male and female participants. These were also statistically insignificant. Age-wise, the most seroprevalent antibodies were observed among participants aged <21years for all antigens in the negative microscopy samples.

CHAPTER 5

DISCUSSION, CONCLUSION, AND RECOMMENDATION

5.1 DISCUSSION

As part of the millennium development goals, malaria, an ancient human pathogen troubling about half of the world's population for eons, needs eradication. This plan has caused an increase in scientific research aimed at developing a vaccine for the disease or possible targeted treatment for specific individuals. This study sought to add to the pool of scientific data available by looking at the effects of haemoglobin variants on the immune response elicited by people with clinically symptomatic malaria and the seroprevalence of these immunoglobulins in the ten regions of Ghana. The most prevalent Hb phenotypes identified were HbAA (79.9 %), HbAS (12.6 %), and HbAC (5.4 %), with none of the other variants identified exceeding 1%. This study of haemoglobin variant distribution agrees with some earlier studies in Ghana but disagrees with the findings from another study in Ghana, where HbAS dominated (Awaitey, 2020).

An. gambiae Salivary Gland Protein-6 peptide 1 (gSG6-P1)

Recent research has shown that measuring antibody (Ab) responses to vector saliva in human populations could be a valuable biomarker for determining human exposure to vector bites and, consequently, the risk of transmission of vector-borne diseases. (Sadia-Kacou, 2019; Sagna, 2013). Therefore, the seroprevalence, the levels of concentrations, and distribution of *An. gambiae* Salivary Gland Protein-6 peptide one antibodies (anti-gSG6-P1) determined in this study were not farfetched. Of the samples analyzed, 98 % of the participants produced IgG response to gSG6-P1 salivary peptide. This finding was higher than the 50-60 % of IgG

responses to gSG6-P1 in an earlier study conducted in Ghana (Badu, 2015) but similar to the IgG responses to the gSG6-P1 peptide in studies from Burkina Faso and Cameroon (Cheteug, 2020; Faso, 2018). The generally high levels of anti- gSG6-P1 antibodies recorded in this study agree with previous deductions that the level of vector exposure contributes to a decrease or increase in the concentration of the anti- gSG6-P1 antibodies above the detectable threshold. Therefore, people with limited or no exposure to the vector will have very low or no IgG to the gSG6-P1 salivary peptide, and this could be a result of their geographical location, usage of treated insecticide nets, sewage disposal practices, season, and other socio-demographical factors (Cheteug, 2020; Traoré, 2019). This data consequently confirms that the antibody response to the gSG6-P1 peptide is a pertinent marker to assess human exposure to Anopheles mosquitoes.

Moreover, the seroprevalence of anti-gSG6-P1 antibodies was insignificantly higher in microscopy, negative 98.3 % and positive individuals 97.7 %. This study was dissimilar to a previous Cameroonian survey where infected individuals had higher levels of antibodies than uninfected individuals (Cheteug, 2020). Further disparity with previous studies linked infection to a rise in anti-gSG6-P1 antibodies (Badu, 2012; Traoré, 2019). Perhaps the presence of the IgG in negative malaria microscopy individuals results from the general persistent exposure to the mosquito vector by participants in this study.

Furthermore, an observation of the sero-distribution of gSG6-P1 antibodies among male and female participants in both malaria-positive and negative microscopy individuals from this study was insignificant. This confirms earlier findings that antibody responses to gSG6-P1 peptides are not gender-specific (Cheteug, 2020; Londono-Renteria, 2015; Ya-umphan, 2017). No significant IgG responses to gSG6-P1 peptides were observed within age groups. This

study disagrees with an earlier study in Southeast Asia, where aging directly correlated with the increase in IgG responses to the salivary peptide. Thus, further disagreeing with the assertion by the earlier study, which reported that the anti-gSG6-P1 IgG response with age is generally consistent with the gradual acquisition of immunity against Anopheles mosquito saliva (Ya-umphan, 2017). However, this study's finding agrees with the statement that children have higher responses to salivary gSG6-p1 proteins. In contrast, adults had diminished antibody responses, suggesting desensitization of the immune response to the salivary proteins (Badu, 2012) since most of the IgG recorded in this study were from those below the age of 20 years. Additionally, the phenotypic distribution of haemoglobin in microscopy-positive individuals from this study showed a significantly greater number of participants with antibodies against the gSG6-P1 were people with the haemoglobin AA phenotype (79.8 %), followed by haemoglobin AS (12.9 %) and then AC (5.6 %).

***Plasmodium falciparum* surface protein (Pfs 230)**

A serological assessment of the distribution, prevalence, and concentration levels of immunoglobulins against the Pfs230 antigens in clinically malaria-positive and negative microscopy samples indicated that out of the participants recruited, 96.7 % of them had detectable levels of the antigens. Nevertheless, the levels in malaria-negative microscopy samples (3275.00±4516.00) were more significant than those observed in malaria-positive microscopy samples (3203.00±231.60). A regional comparison of the levels of concentration of IgG against Pfs230 reported most regions had no comparative difference in anti-Pfs230 IgG concentrations between the positive and negative individuals. The seroprevalence of 96.7 % of this sexual stage antibody observed in this study was more significant than the 28.6% reported from Burkina Faso (Ouédraogo, 2011). The high prevalence of anti-Pfs230 antibodies

expressed in this study could result from submicroscopic gametocytes in the participants. Thus, the increase in anti-Pfs230 IgG was expected as antibody responses to gametocyte antigens have been suggested to be influenced more by recent exposure (Acquah, 2020; Amoah, 2018), and participants in this study, considering both microscopically reported positive and negatives samples may have had recent gametocytes infection which was not detected by microscopy. Perhaps a molecular approach for detecting submicroscopic gametocytaemia in both the microscopically infected and uninfected participants would have provided information on why most of the participants, regardless of the infection status by microscopy, presented with the antibodies (Bousema, 2006).

Although no significant associations were established between the number of participants with detectable levels of Pfs230 antigens in malaria microscopy positive and negative samples across gender, age, or haemoglobin phenotype, it is interesting to note that female participants aged below 21 years and haemoglobin AA phenotype recorded higher numbers of participants with antigen present at a detectable level. The age-antibody response pattern presented in this study disagrees with the findings presented by Stone et al. in 2018, which stipulated those antibodies against the sexual stage-specific antigens positively correlated with age (Stone, 2018). That notwithstanding, the age-sexual stage antibody responses presented in previous studies have been inconclusive as no consensus has been drawn in previous studies on the association of the sexual stage immunity to the presence of infection (Bousema, 2010; Bousema, 2006; Ouédraogo, 2011). Sexual stage antibody responses in participants < 20 years are likely to result from high gametocyte exposure in this age group, especially among children. In participants >21 years, gametocyte exposure may be the lowest. Still, sexual stage commitment during infections may be relatively increased, and antibody responses to sexual

stage antigens may become more long-lived, reflecting the maturation of the immune response (Ouédraogo, 2011).

This study further compared the concentration of anti-Pfs230 antibodies measured in the participants with different haemoglobin types. The concentration levels for immunoglobulins against Pfs230 were insignificantly higher among the HbAS participants (2841.70±1681.40) than in the other phenotypes measured. The insignificant association between the haemoglobin phenotypes and the sexual stage-specific antibody Pfs230, the finding of this study could be ground-breaking since a possible controlled cohort study between these haemoglobin variants and immunity against the sexual stage antigen could provide insight into the dynamics of host immune responses. Nevertheless, the concentration of anti-Pfs230 being greater in haemoglobin AS than in AA and AC could suggest that AS individuals may have a lower propensity to contribute to communal malaria transmission patterns (Bougouma, 2014) since the influence of haemoglobin variants (AS and AC) have been implicated in the gametocyte density and the chronicity of infection (Gonçalves, 2017). Perhaps the function of the gamete surface antigen (Pfs230) essential in the formation of oocyst is blocked in by the IgG for Pfs230, thus contributing to the reduced infectivity among haemoglobin AS participants controlling subsequent studies could provide more detailed insight (Eksi, 2006).

Erythrocyte binding antigen 175 Region 3 (EBA-175 3R)

The assessment of the erythrocytic stage antibody against EBA 175 3R antigens to establish its seroprevalence, distribution, and concentration levels among participants taking into account the influence of their haemoglobin variants, revealed that the total number of participants with detectable levels of anti-EBA 175 3R antibodies present was 96.8 %. These high antibody levels reported in this study correlate with the 85.6 % seroprevalence reported

from Obom by Amoah *et al.* in the Greater Accra Region of Ghana but were definitively higher than 58.0 % presented from Asutsuare in the same study (Amoah, 2018). The differences in antibody concentration from the earlier study were attributed to the low prevalence of malaria in Asutsuare compared to Obom. Conceivably, it is not without merit to speculate that high seroprevalence in this study, irrespective of microscopy results, could be due to undetectable parasitic density at the submicroscopic level. However, it is prudent to note that females, participants ages below 21 years, and the haemoglobin AA Phenotype recorded higher anti-EBA 175 3R antibodies.

Interestingly, a comparison of the levels of concentration of antibodies for EBA-175 3R to different haemoglobin variants in microscopy positive samples indicated that antibodies against the EBA 175 3R antigens in HbAA samples (15159.00±34125.00) were higher than in HbAC (5312.00±5429.40) and HbAS (12133.00±20610.00). Concerning age, the results presented from this study compared to that of an earlier study in Nigeria were incongruent. The Nigerian study significantly reported a positive correlation between age and the IgG antibodies for EBA 175 3R. A controlled study considering age and immunity development may provide deeper insights into possible vaccine production targeting the erythrocytic stage of the parasite. Perchance, the high endemicity of malaria, the predominance of HbAA, and the season of sample collection contributed to the significantly increased anti-EBA 175 3R antibodies in the Nigerian study. The similarities of this to that of the earlier ones contributed to the commonalities in the findings. Moreover, although studies have reported that anti-EBA 175 IgG confers immunity to malaria infection, a study in Senegal stated that immunity conferred by the IgG in different Hb variants depends on the IgG subclass (Bwire, 2019; Nafady, 2014; Sarr, 2006).

Influence of haemoglobinopathies on malaria

Finally, the influence of haemoglobinopathies on reducing the morbidity and mortality of malaria has been associated with the ability of these variants of haemoglobin to reduce parasitic invasion into RBCs (Bunyaratvej, 1992; Chotivanich, 2002; Ifediba, 1995; Taylor, 2013), decreasing in intraerythrocytic growth (Fairhurst, 2003; Pasvol, 1978; Taylor, 2013), enhancing sickling of red blood cells at low oxygen tension (Luzzatto, 1970; Roth, 1978), producing micro-RNA that inhibits the enzymatic activities of the parasites (Chen, 2008; LaMonte, 2012; Sangokoya, 2010), and interfering with the intrinsic pathogenic pathway of the parasite by down-regulating the effect *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) thus impairing rosetting and co-adherence of infected RBCs to the endothelial membranes (Cholera, 2008). However, the involvement of immunoglobulins in thwarting malaria activity has been inconclusive, as some studies have reported higher seroreactivity to specific antigens while others have not (Taylor, 2013). Therefore, the lack of evidence to support the claim that haemoglobinopathies influence the IgG antibody response at different stages of *falciparum* malaria is unsurprising.

5.2 CONCLUSION

At the end of this study, the most dominant haemoglobin phenotype was Hb AA (79.9 %), followed by AS (12.6 %) and AC (5.4 %). Of the antibodies measured, there were significant differences in the total concentration of anti-EBA 175 3R and anti-gSG6-P1 antibodies in malaria negative and positive microscopy samples. Although no association was established between the concentrations of IgGs measured against the various antigens in different haemoglobin variants in malaria microscopy positive samples, it was clear that the number of

participants with IgG against gSG6- P1 antigen was significantly greater in Hb AA participants than in the other haemoglobin variants. Yet the same assessment could not be made for the sexual stage antigen (Pfs230) and the erythrocyte binding antigen (EBA 175 3R). In general, no significant relationships were established between the influence of age, gender, and haemoglobin variants on the anti-Pfs230, anti-EBA 175 3R, and anti-gSG6-P1 antibodies

5.3 RECOMMENDATION

The study was limited by the lack of a molecular approach to detecting the prevalence of malaria in negative microscopy individuals. Thus, ruling out the possibility of these participants having infections at the sub-microscopic level. Therefore, further study should be conducted considering molecular techniques to detect malaria parasites. Furthermore, a controlled longitudinal study that will consider the vector exposure level, haemoglobin density, and prevention of reinfection among the different haemoglobin variants may untie the gordian knot involved in malaria pathogenicity. Finally, a possible look at the specific subclasses of IgG of the antibodies produced in the different stages of malaria may be the key to creating a vaccine.

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APPENDICE

Appendix 1 ELISA PLATE FORMATS FOR ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std IgG 1000ng/ml	Std IgG 1000ng/ml	S_01Aa	S_01Ab	S_09Aa	S_09Ab	S_17Aa	S_17Ab	S_25Aa	S_25Ab	S_33Aa	S_33Ab
B	333.3 ng/ml	333.3 ng/ml	S_02Aa	S_02Ab	S_10Aa	S_10Ab	S_18Aa	S_18Ab	S_26Aa	S_26Ab	S_34Aa	S_34Ab
C	111.1 ng/ml	111.1 ng/ml	S_03Aa	S_03Ab	S_11Aa	S_11Ab	S_19Aa	S_19Ab	S_27Aa	S_27Ab	S_35Aa	S_35Ab
D	37.0 ng/ml	37.0 ng/ml	S_04Aa	S_04Ab	S_12Aa	S_12Ab	S_20Aa	S_20Ab	S_28Aa	S_28Ab	S_36Aa	S_36Ab
E	12.3 ng/ml	12.3 ng/ml	S_05Aa	S_05Ab	S_13Aa	S_13Ab	S_21Aa	S_21Ab	S_29Aa	S_29Ab	S_37Aa	S_37Ab
F	4.1 ng/ml	4.1 ng/ml	S_06Aa	S_06Ab	S_14Aa	S_14Ab	S_22Aa	S_22Ab	S_30Aa	S_30Ab	PC01	PC02
G	1.4 ng/ml	1.4 ng/ml	S_07Aa	S_07Ab	S_15Aa	S_15Ab	S_23Aa	S_23Ab	S_31Aa	S_31Ab	NC01	NC02
H	0.5 ng/ml	0.5 ng/ml	S_08Aa	S_08Ab	S_16Aa	S_16Ab	S_24Aa	S_24Ab	S_32Aa	S_32Ab	Blank01	Blank02

Appendix 3

Preparation of buffers and media

0.05 M carbonate/bicarbonate buffer, pH 9.2,

Sodium hydrogen carbonate (2.93 g) and sodium carbonate (1.59 g) in 1 liter of distilled water

and add 1ml of methyl red from (1%) stock solution

1X PBS

One tablet of PBS into 500ml of

distilled water. Stir until it dissolves

using a magnetic stirrer

Washing buffer: PBST (PBS + 0,1% Tween)

For every 1000ml of PBS, add 1ml of Tween and stir using a magnetic stirrer till it foams

Blocking buffer: 3 % milk powder in PBST

For every 100ml of PBST, add 3g of skimmed milk powder

Sample Incubation buffer: 1 % milk powder in PBST

For every 100ml of PBST, add 0.02g of sodium azide and 1g of skimmed milk powder. Stir to dissolve.

Preparation of standard for IgG

1) In tube 1, add 1.7ml of coating buffer.
2) In tubes 2 – 8, add 1 ml of coating buffer (carbonate or PBS).
3) Transfer 1.7 µl of the stock IgG solution into tube one and mix well.
4) Transfer 0.5 ml from tube one into tube 2, and mix well.
5) Transfer 0.5 ml from tube two into tube 3, and mix well. Follow the order of step 5 up to tube 8.

0.2M H2SO4

1) For every 500ml of distilled water, add 1.15ml of conc H2SO4

Appendix 4

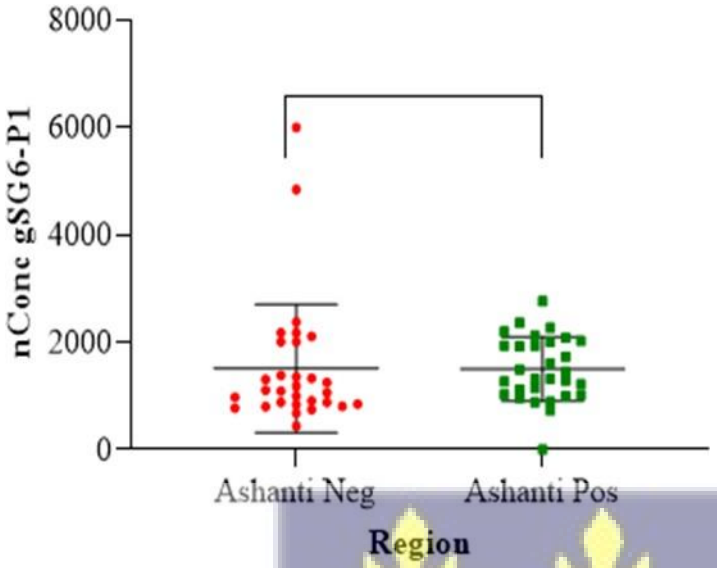

Fig 1: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Ashanti region.

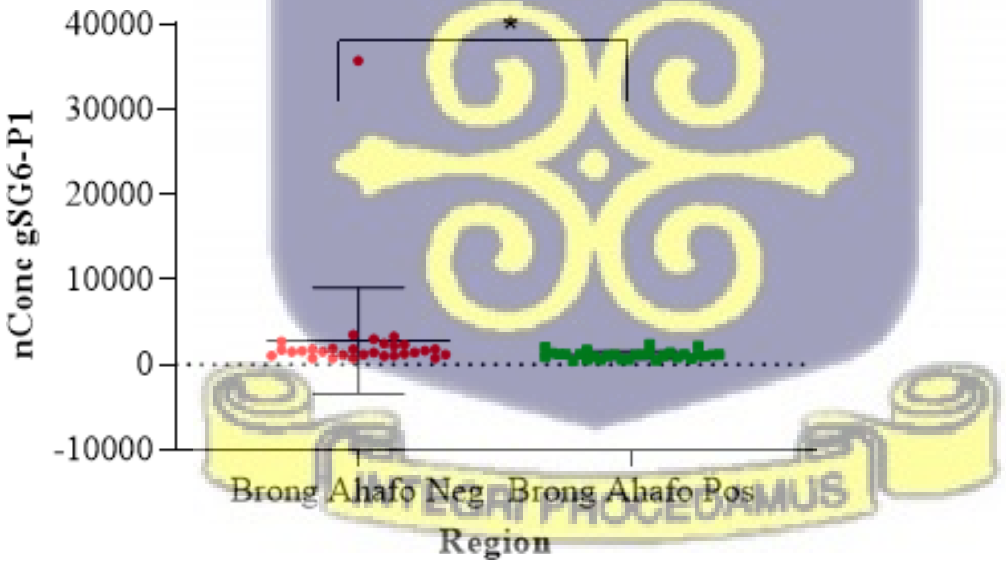

Fig 2: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals at the Brong Ahafo region.

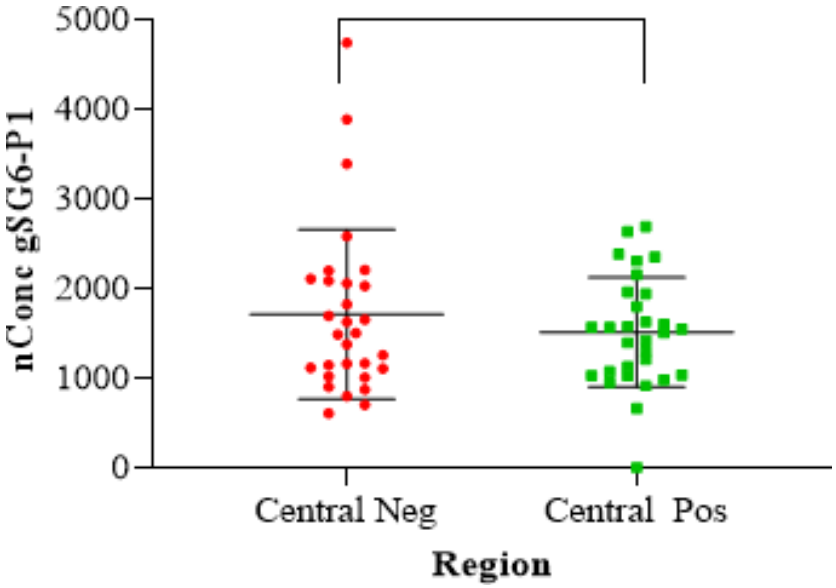

Fig 3: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Central region.

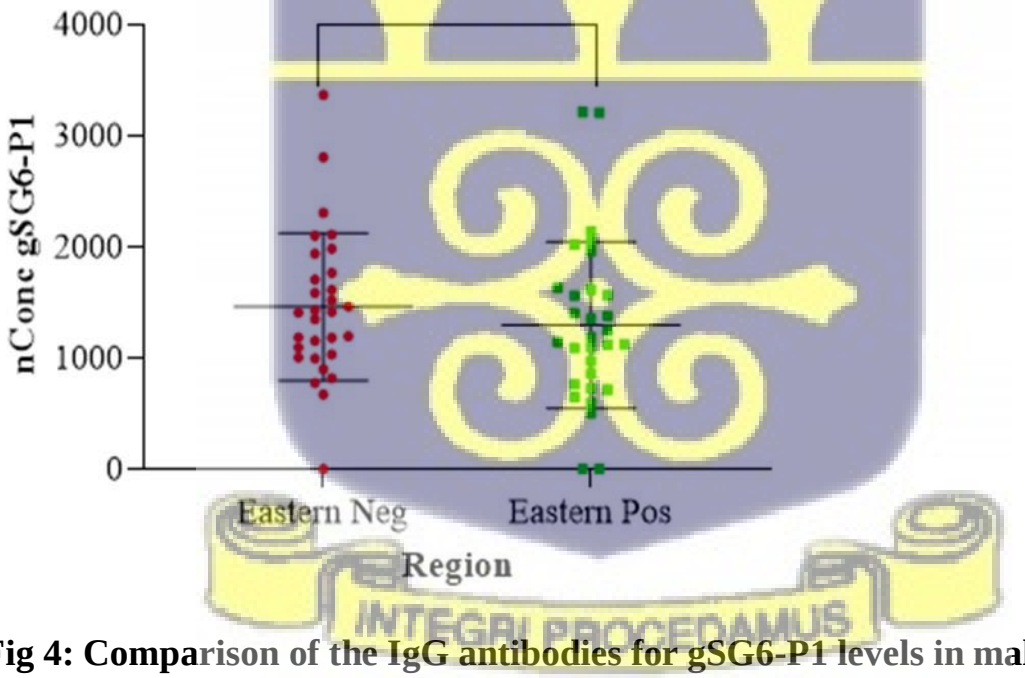

Fig 4: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Eastern region.

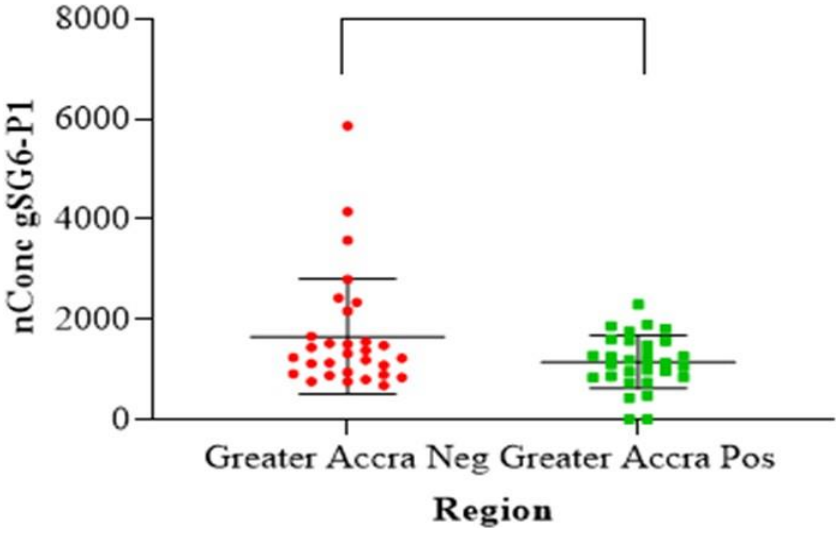

Fig 5: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Greater Accra region.

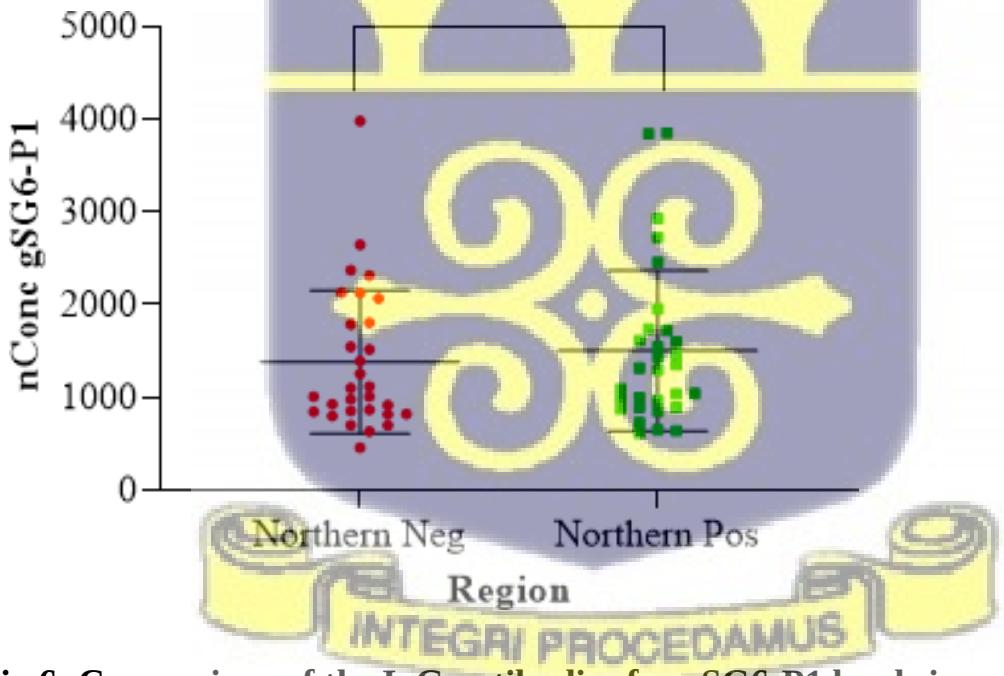

Fig 6: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Northern region.

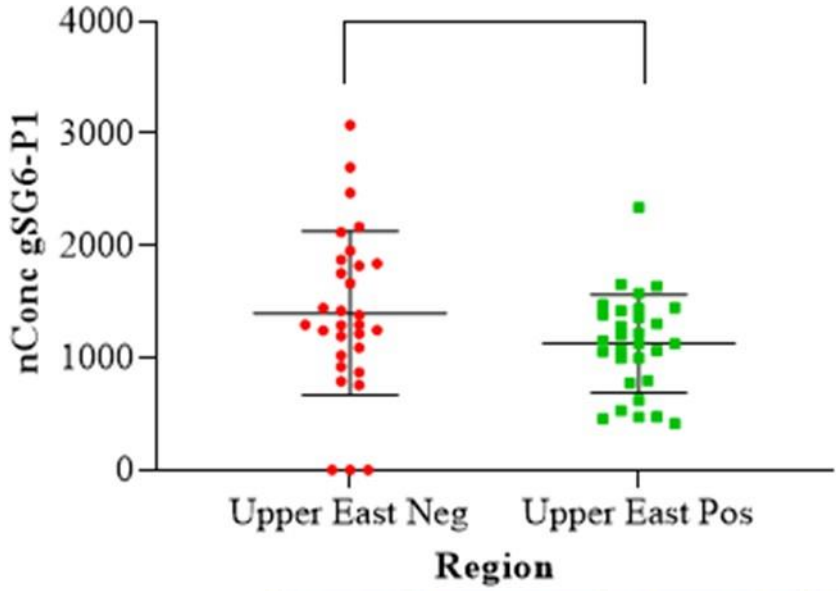

Fig 7: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Upper East region.

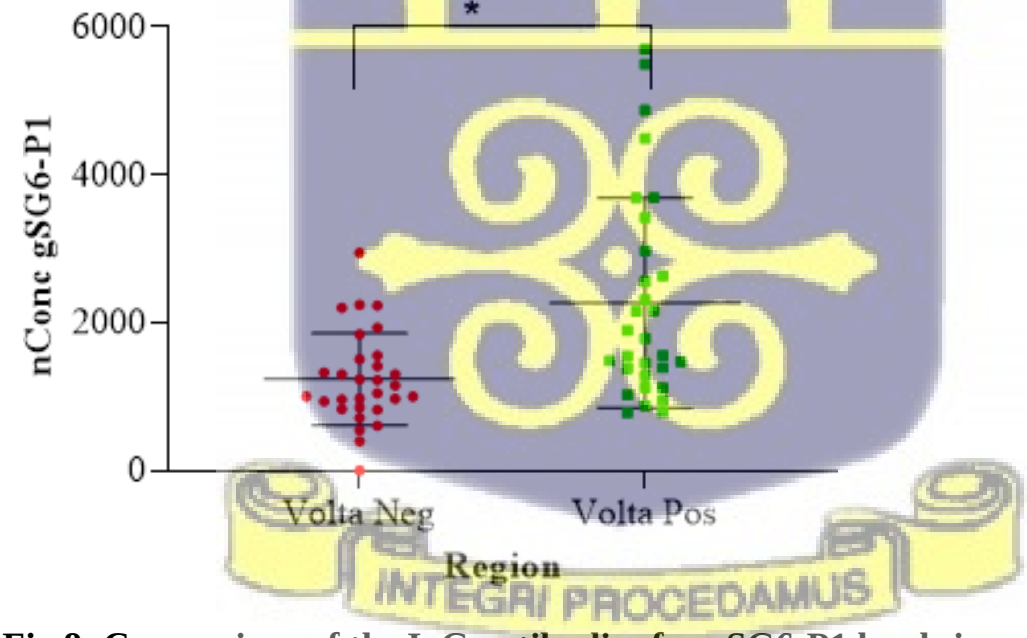

Fig 8: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals at the Volta Region.

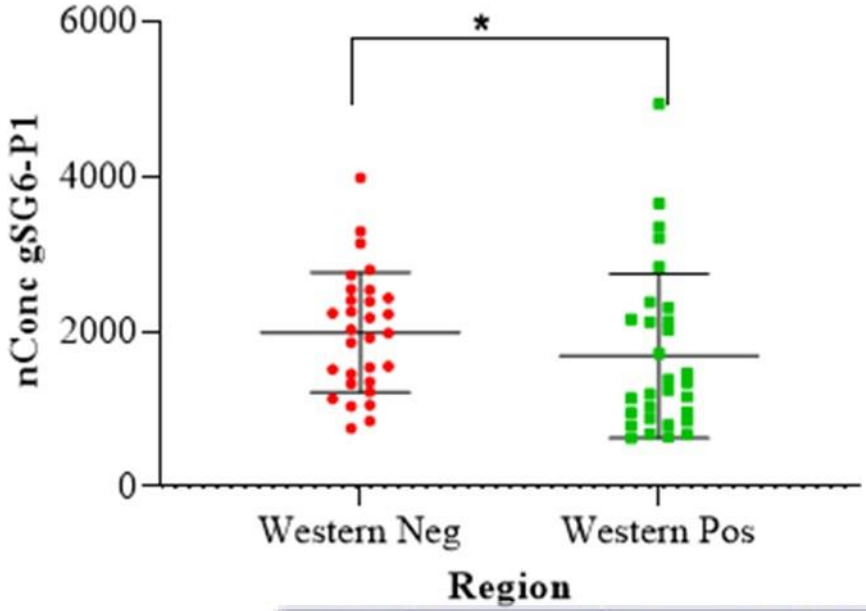

Fig 9: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Western Region.

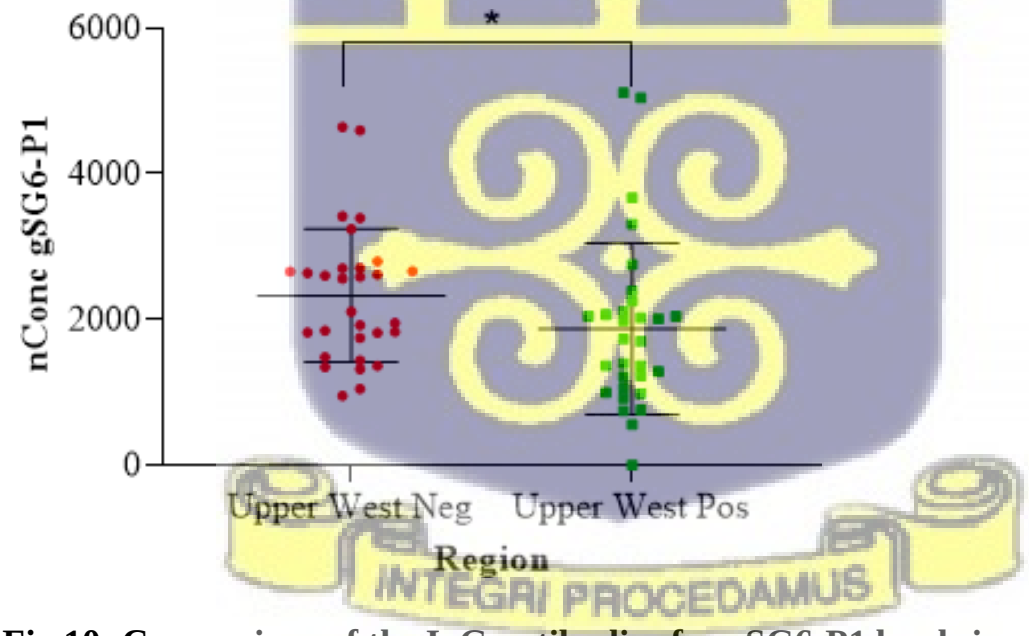

Fig 10: Comparison of the IgG antibodies for gSG6-P1 levels in malaria microscopy positive and negative individuals in the Upper West region

Appendix 5

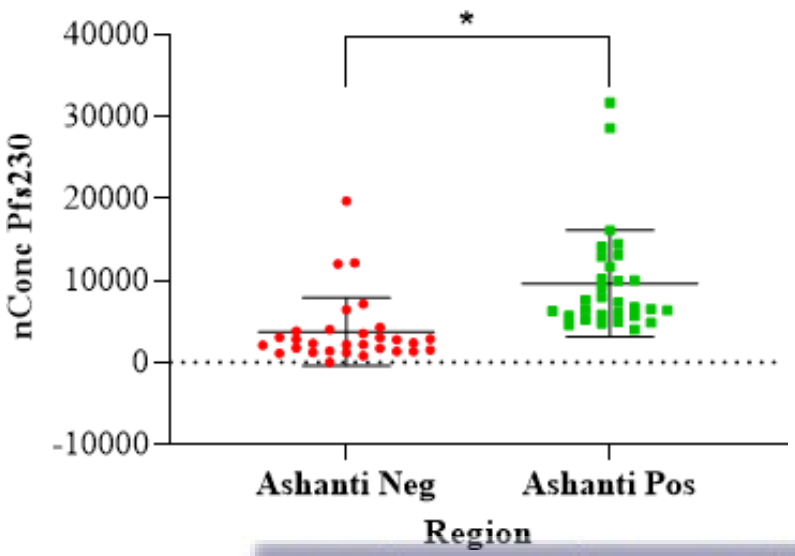

Fig 11: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Ashanti region.

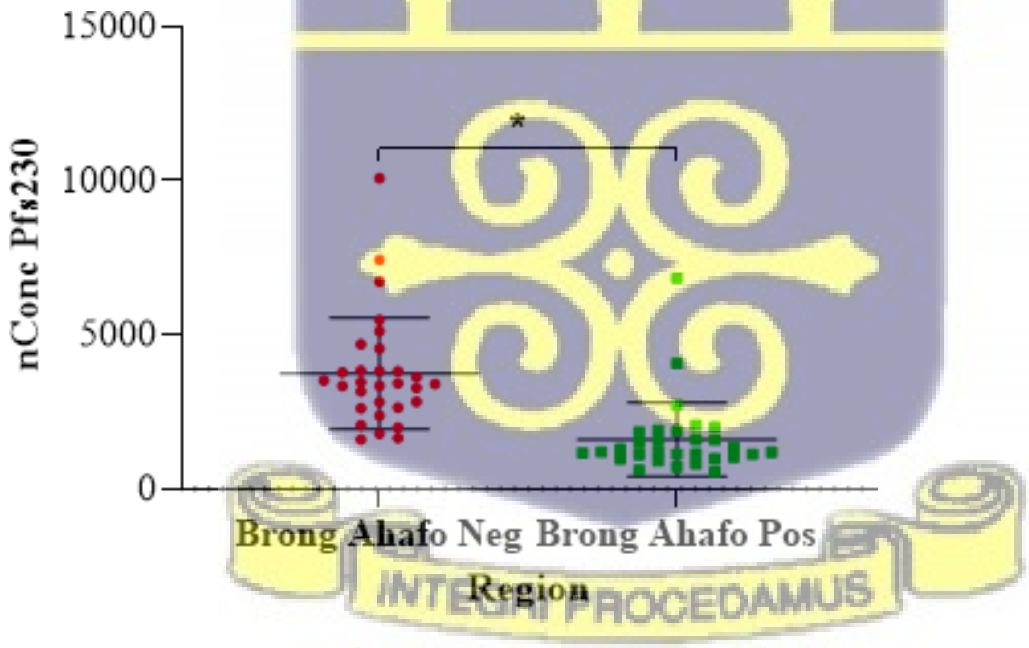

Fig 12: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals at the Brong Ahafo region.

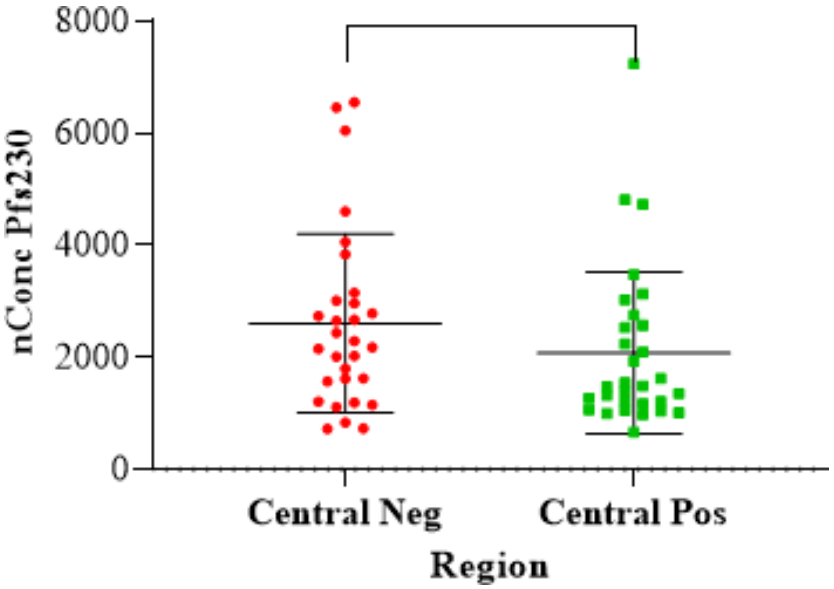

Fig 13: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Central region.

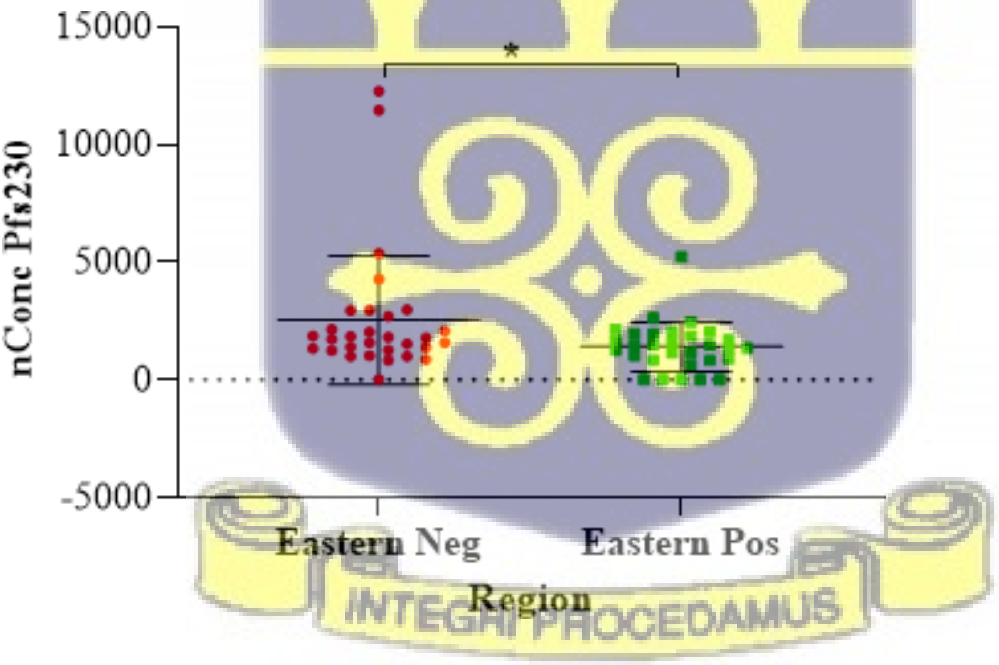

Fig 14: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Eastern region.

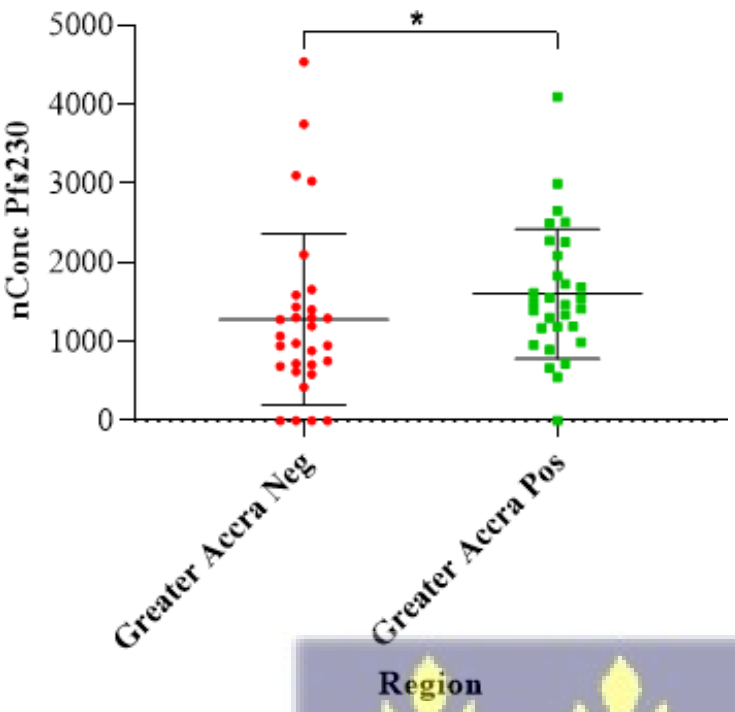

Fig 15: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Greater Accra region.

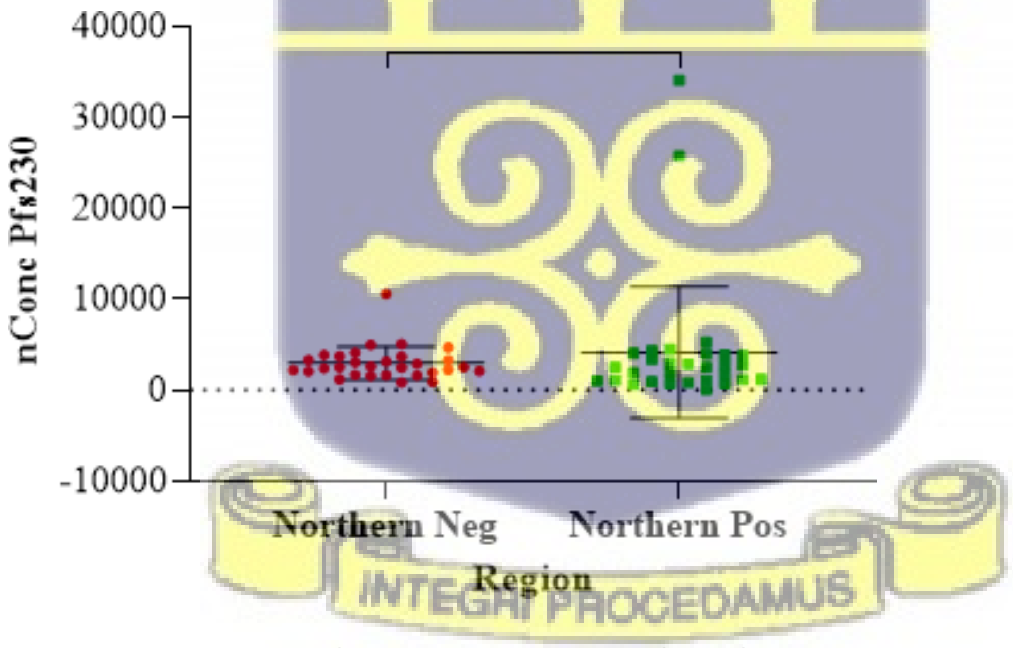

Fig 16: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Northern region

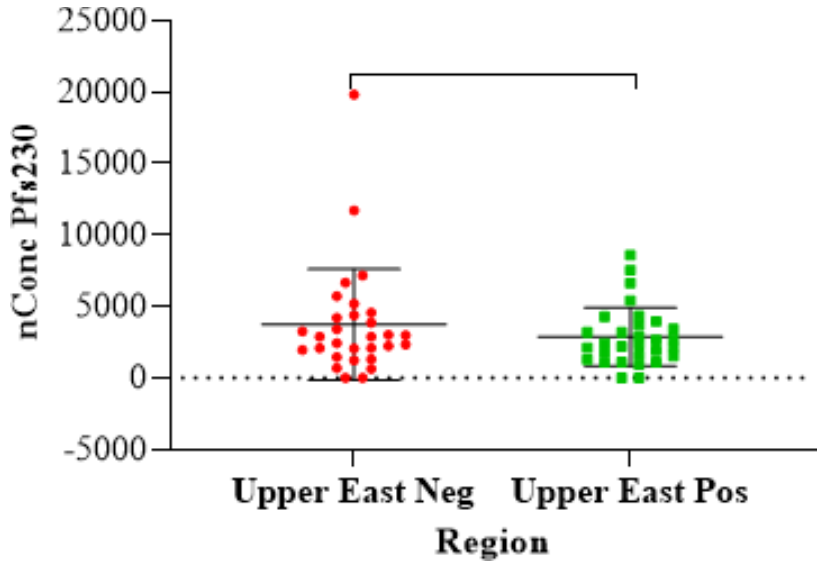

Fig 17: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Upper East region.

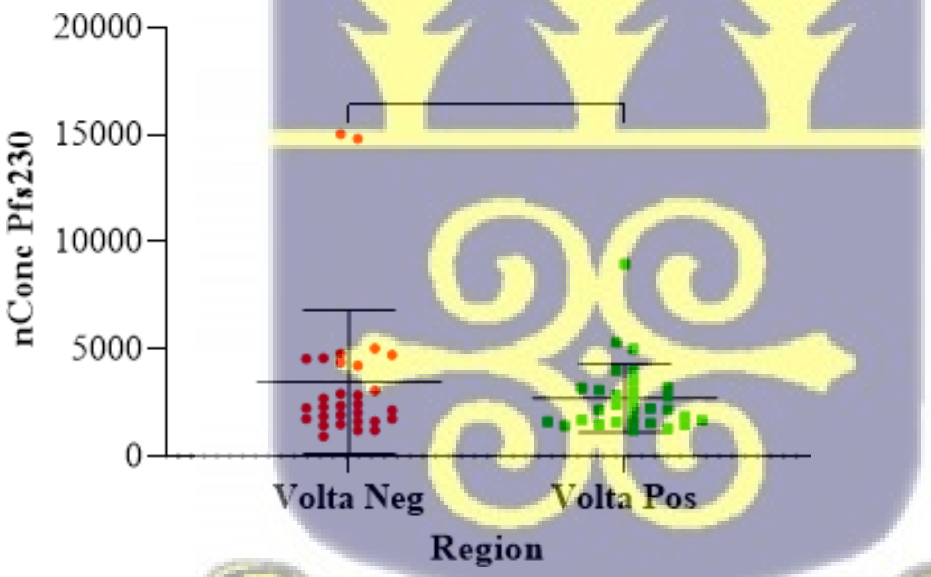

Fig 18: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Volta Region

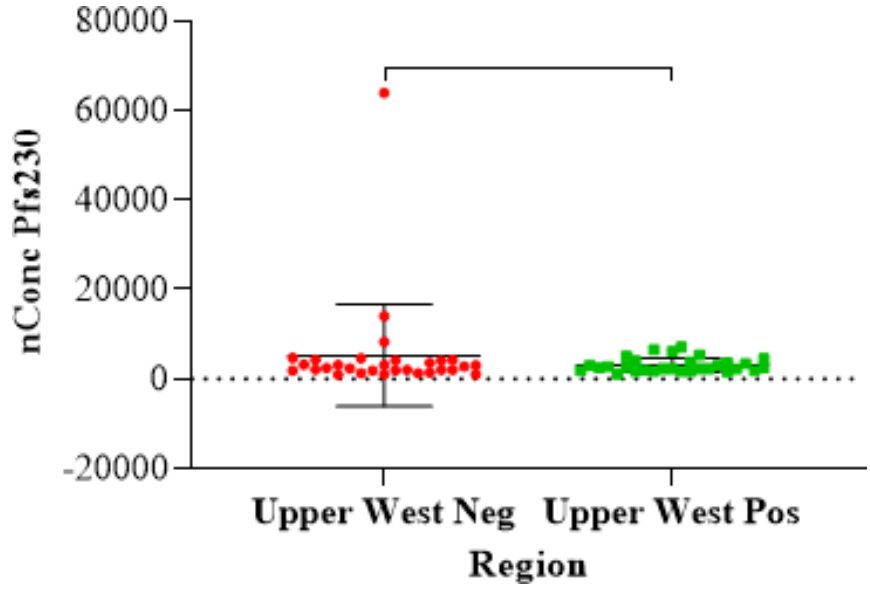

Fig 19: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Upper West region.

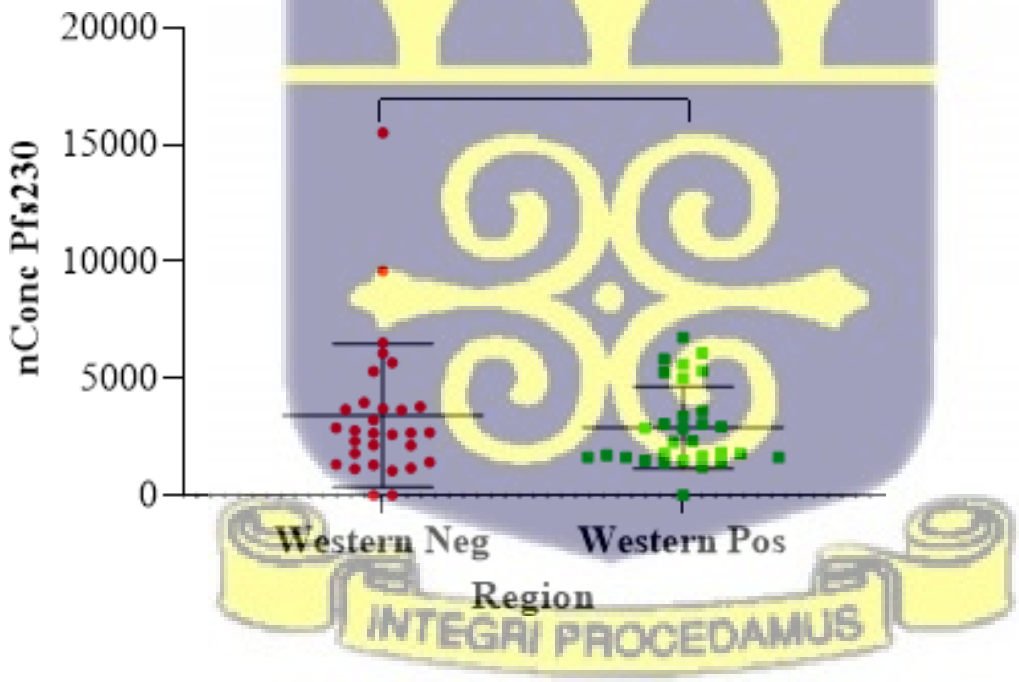

Fig 20: Comparison of the IgG antibodies for Pfs230 levels in malaria microscopy positive and negative individuals in the Western region.

Appendix 6

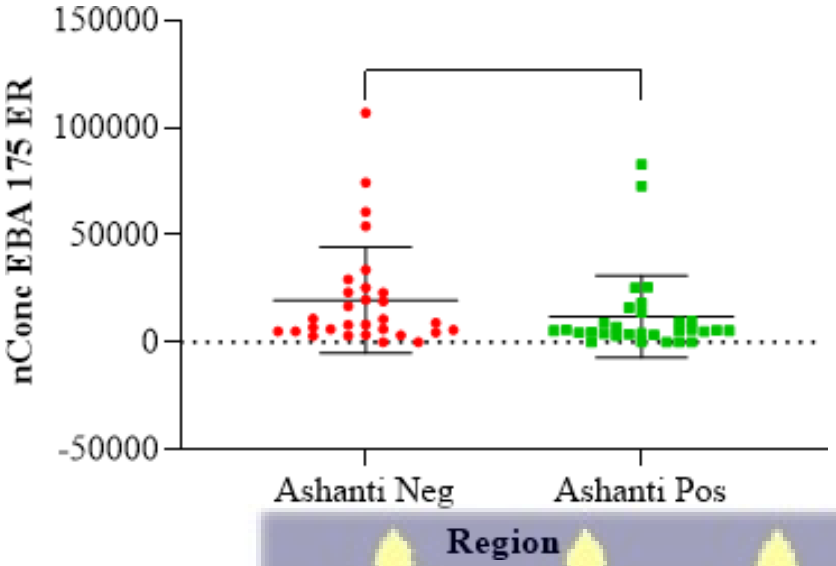

Fig 21: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Ashanti region.

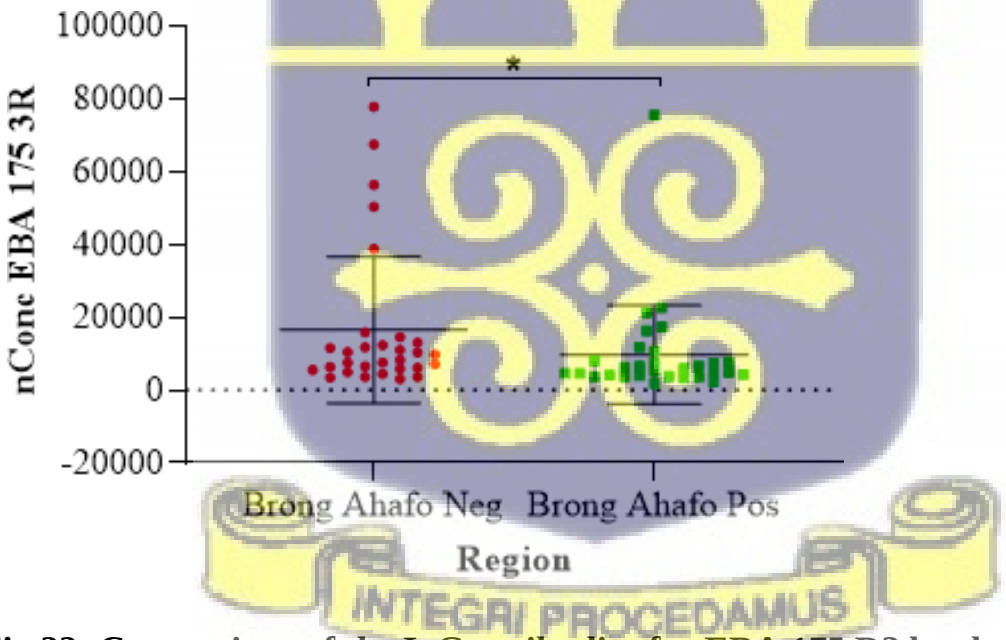

Fig 22: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals at the Brong Ahafo region.

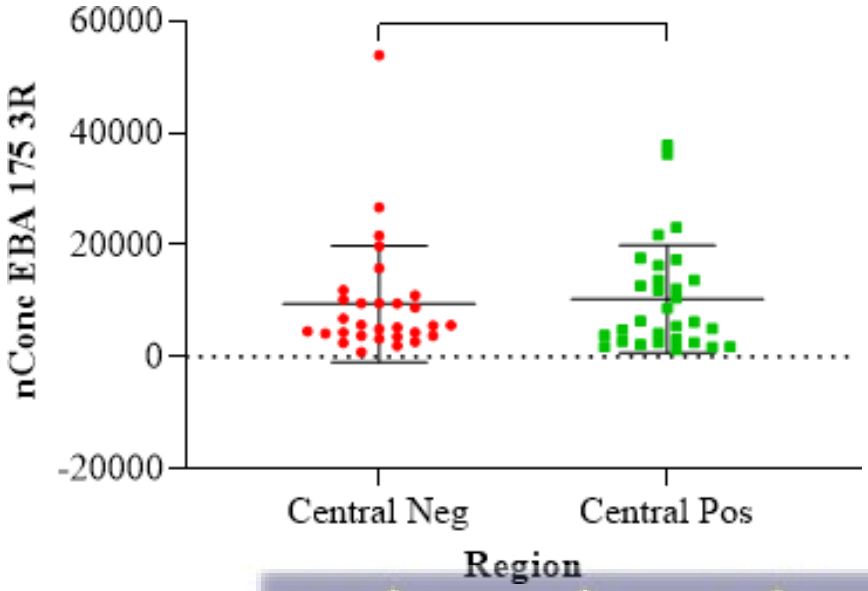

Fig 23: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Central region.

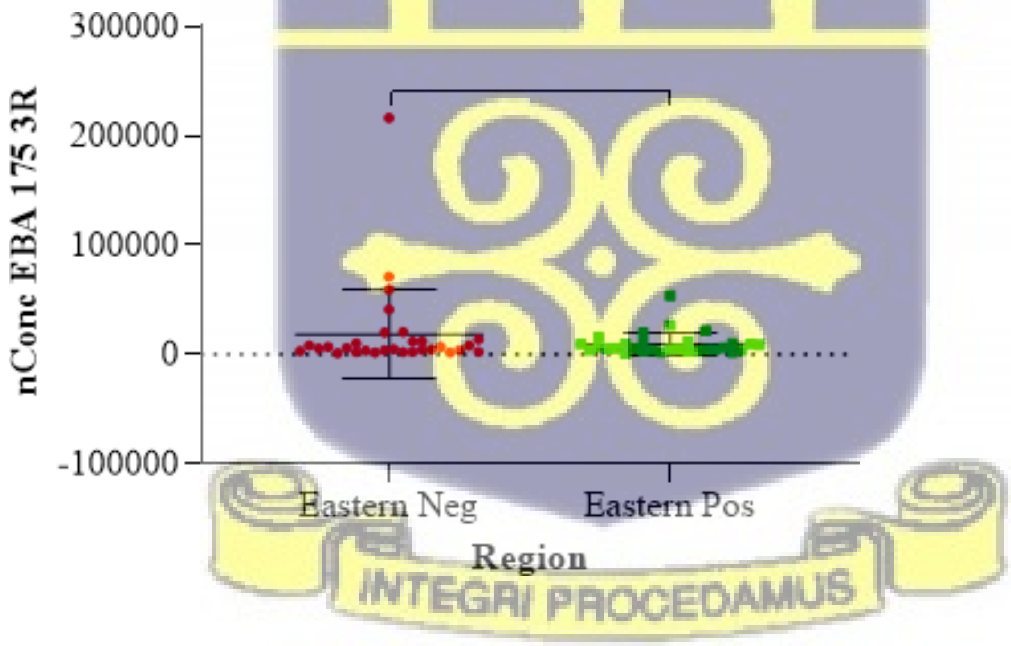

Fig 24: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Eastern region.

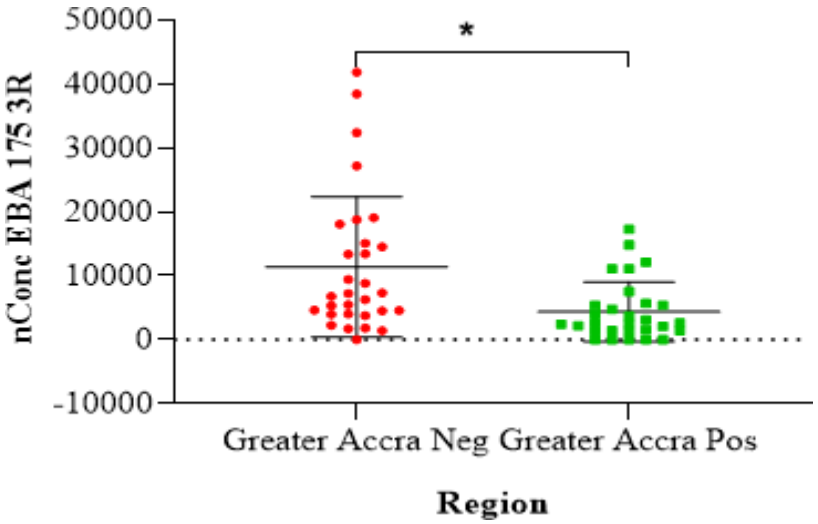

Fig 25: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Greater Accra region.

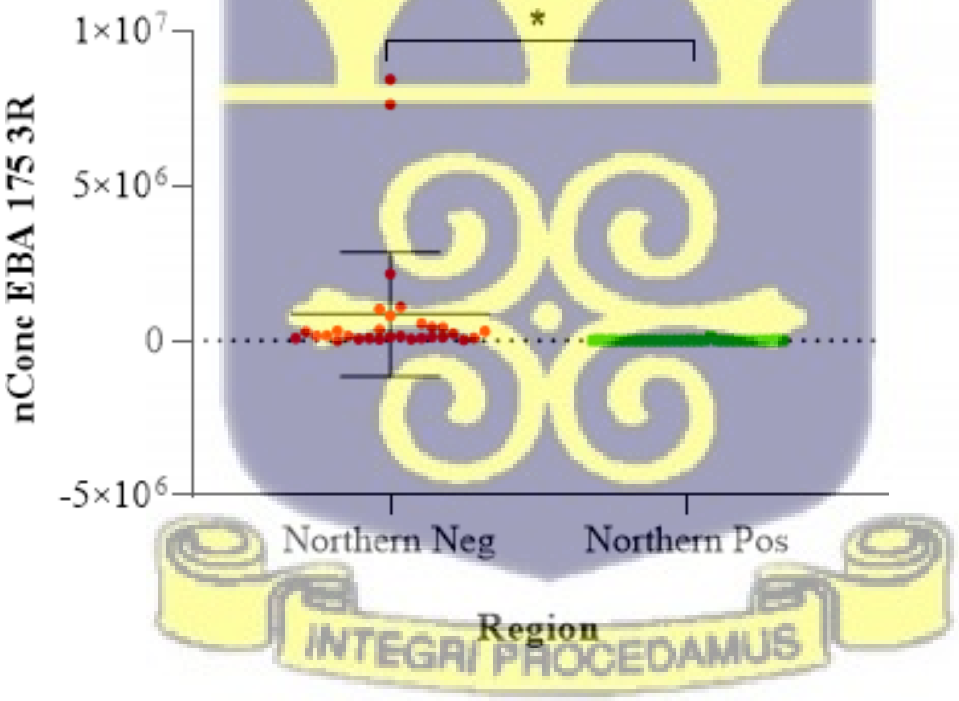

Fig 26: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Northern region.

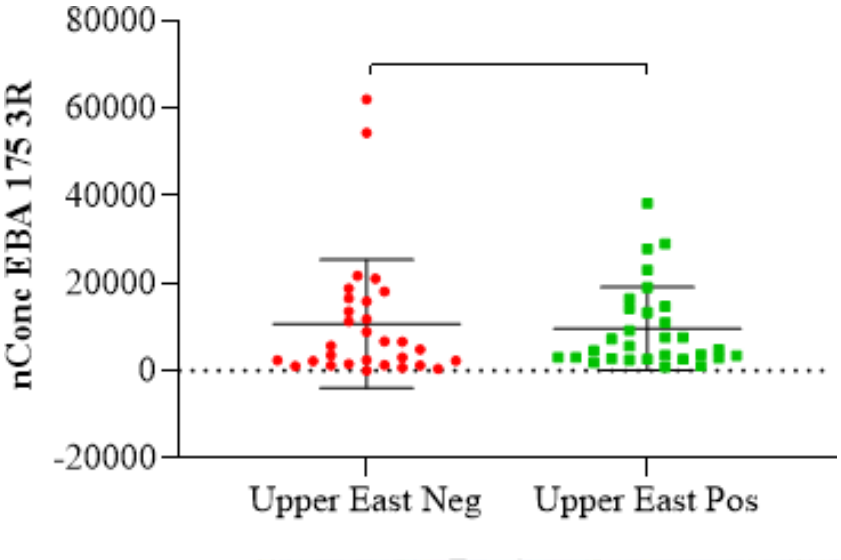

Fig 27: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Upper East region.

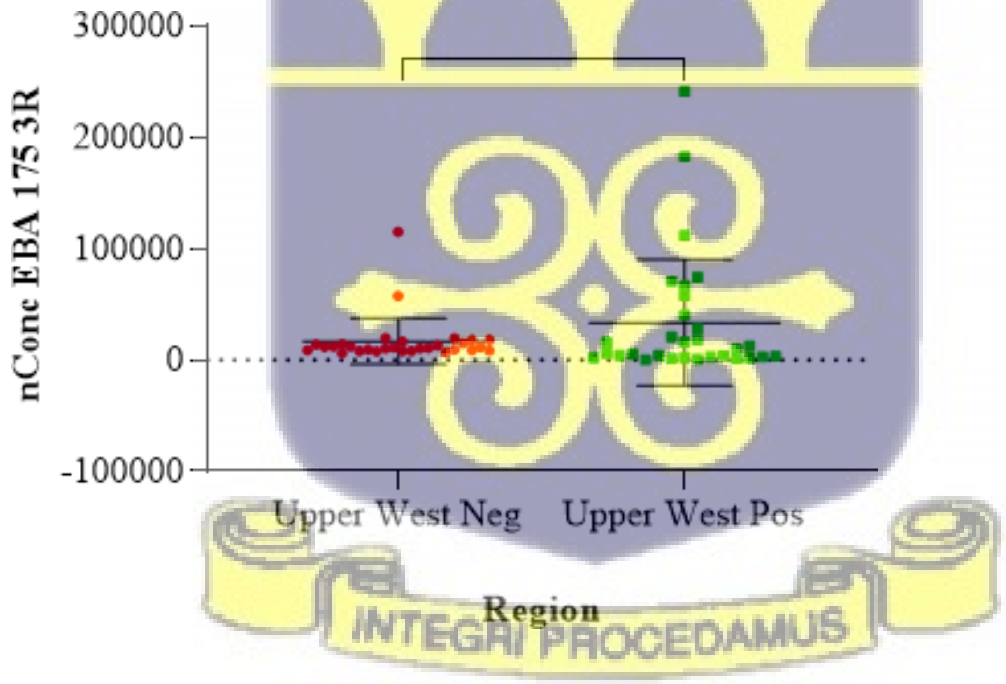

Fig 28: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Upper West region.

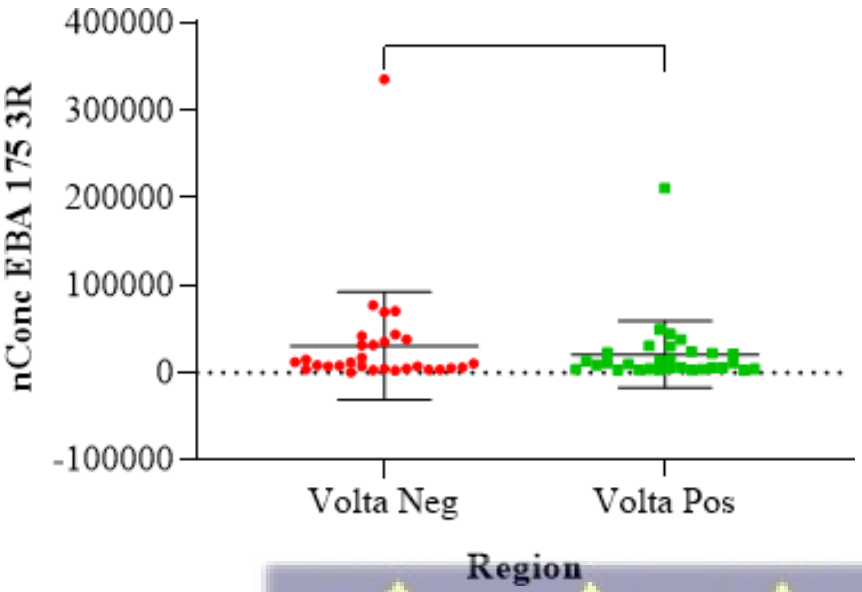

Fig 29: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals at the Volta Region.

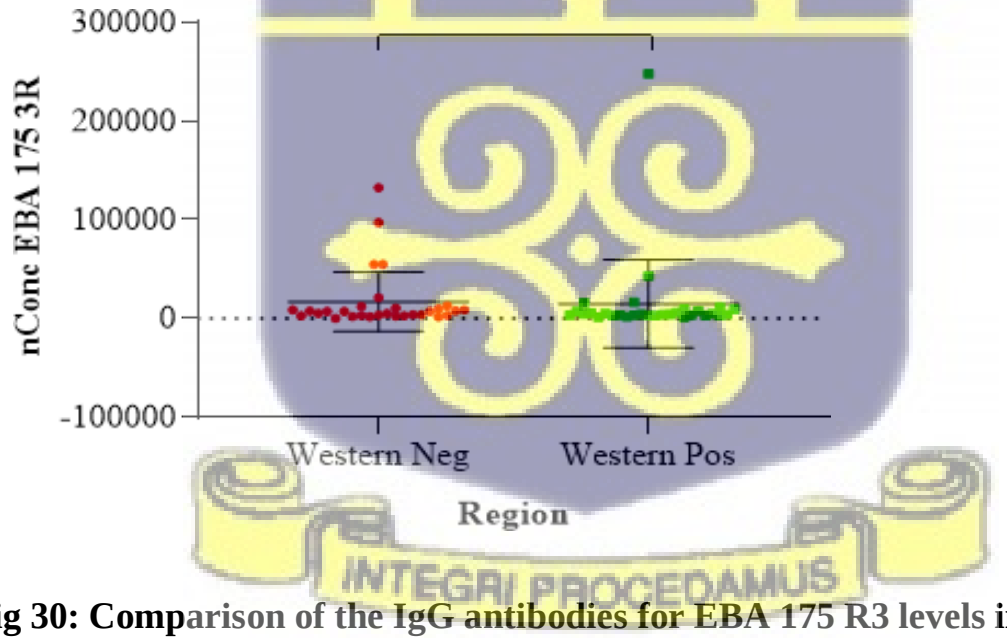

Fig 30: Comparison of the IgG antibodies for EBA 175 R3 levels in malaria microscopy positive and negative individuals in the Western region.

Appendix 5 **CLASSIFICATION OF HB PHENOTYPE**

Hb Phenotype	Hb A	Hb F	Hb S	Hb E	Hb A2	Hb C	Hb D	Hb H
AA	> 95				< 3.5			
AS	> 45	< 10	≤ 45		< 3.5			
AC								
SS		< 25	80 - 95		< 7			
SC		< 10	41 - 55		< 4	40 - 50		
SD		<10	≈ 40		< 10		≈ 50	
S/β+	15 - 45	< 25	45 - 75		< 6			
SS/S-HPFH		> 25	60 - 75		< 3.5			
S/HIGH A2		< 20	40 - 70		20 - 45			

Sickle Cell Syndromes

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