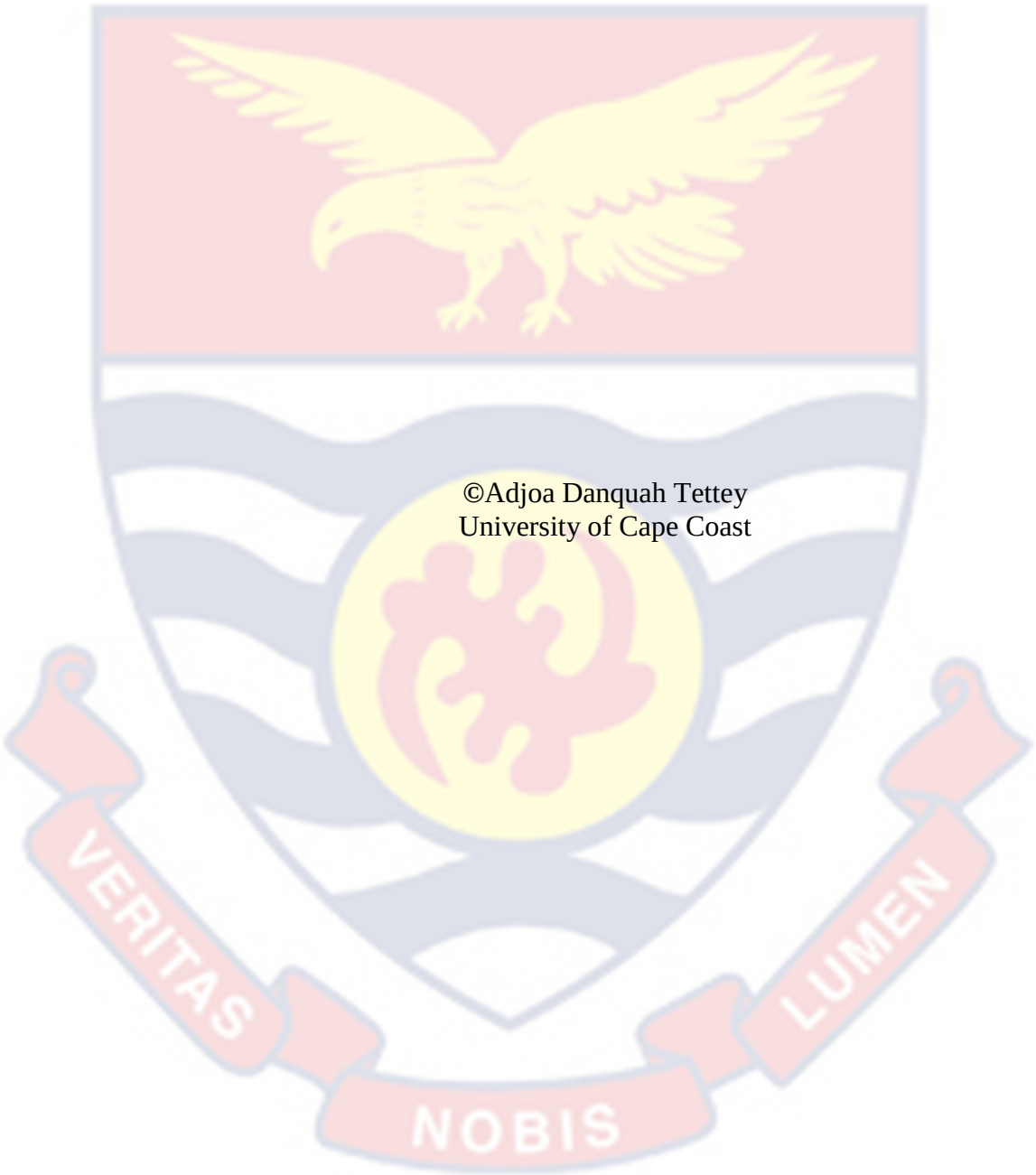
UNIVERSITY OF CAPE COAST

GENETIC ASSOCIATION BETWEEN HLA-G 14bp POLYMORPHISM AND MALARIA DISEASE SEVERITY AND ANAEMIA AMONG IRON AND NON-IRON FORTIFIED GHANAIAN CHILDREN

TETTEY DANQUAH ADJOA

2023



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BY

TETTEY DANQUAH ADJOA

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

OCTOBER 2023

DECLARATION

Candidate's Declaration

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

Candidate's Signature: ..... Date: .....

Name: Tettey Danquah Adjoa

Supervisors' Declaration

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

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Name: Rev. Dr. Benjamin Amoani

Supervisor's Signature: ..... Date: .....

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Supervisor's Signature: ..... Date: .....

Name: Prof. Samuel Victor Nuvor

ABSTRACT

Malaria cases were reported in 241,000,000 people worldwide in 2020, with 96% occurring in Africa. Out of this number, 80% of the total deaths were children. Due to host and plasmodium interactions, clinical features might vary greatly. HLA-G is important for the outcome of plasmodium infection because it may induce a tolerogenic environment that allows parasites to evade an anti-malarial response. One of the most prevalent nutritional deficiencies is anaemia. According to certain research's findings, having anaemia caused by a lack of iron may be protective against contracting malaria, whereas having availability of iron can increase the risk of malaria-related illness and death. This is a cross sectional study and archival samples from a study done by Zlotkin et al, 2013 in the Tain and Wenchi Municipalities in the Bono region were used. Children between 6 and 36 months who can tolerate semi-solid foods and living in Tain and Wenchi were involved in the study while children who tested positive for malaria but have started antimalaria treatment or have other known medical conditions were excluded. A total of 432 samples were used and categorized into negative control, uncomplicated and complicated malaria. Genomic DNA was amplified with PCR, and 14bp+/- alleles counted. Statistical package for social sciences (SPSS), Hardy-Weinberg equilibrium, chi-square test, univariate, and multivariate regression models were used to analyse data acquired. The study revealed that the presence of HLA-G 14bp+/- and 14bp-/- variants were associated with malaria disease severity among the iron fortified children but not in the non-iron supplemented children. Also, HLA-G 14bp+/- was associated with the development of anaemia among iron supplemented children.

ACKNOWLEDGMENTS

The greatest thanks go to Jehovah for providing me with life and stamina to finish this. I'm quite appreciative of the assistance, guidance, training and counsel of my supervisors, Rev. Dr. Benjamin Amoani, Prof. Coutin and Prof. Nuvor. I appreciate the lab (MBB and DBS labs, UCC) personnel who provided me with so much assistance. To my colleagues who were there for me while also working on their various projects, I am most grateful to you. Finally, I acknowledge all other individuals and organizations who helped make this work a success.

DEDICATION

I devote this thesis to Jehovah God and the two men who have had the most impact on my life, my father Mr. Annor Tettey who didn't live to see me graduate and my dear husband Solomon Turkson.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
BET	Ethidium bromide.
dNTP	deoxyribonucleoside triphosphate
HLA-G	Human Leukocyte Antigen-G
Bp	Base pair
Ta	Hybridization temperature.
Tm	Melting temperature
UV	Ultraviolet

GLOSSARY

Splicing: Mechanism for removing introns (non-coding sequences) from a pre-messenger RNA, which thus becomes a messenger RNA.

Exon: sequence coding for a gene.

Electrophoresis: Technique for separating DNA, RNA, or proteins according to their molecular masses, their shapes, their loads under the effect of an electric field.

Glycoprotein: Aprotein linked covalently to several groups of oligosaccharides.

Isoforms: Proteins of different shapes but coming from the same gene. The process involved in the formation of isoforms is alternative splicing.

Malaria: A potentially fatal infection transmitted by a parasite of the genus plasmodium.

PCR: molecular biology technique used to exponentially amplify a sequence DNA target double- stranded by extension primers due to a DNA polymerase thermostable.

Polymorphism: refers to the coexistence of several alleles for a given gene or locus.

CHAPTER ONE

INTRODUCTION

Human leukocyte antigen (HLA), frequently also known as the major histocompatibility complex (MHC), is a glycoprotein that is central for initiating the adaptive immune response and for distinguishing between self and non-self. (Carosella et al., 2008). The HLA-G gene, being part of HLA class I, performs a significant role in controlling immune responses which can be advantageous or damaging. Negatively, HLA-G can help parasites avoid anti-malarial immunity by creating a tolerogenic environment as it acts as an immunosuppressive molecule that interacts with specific immune cells which has the effect of inhibiting the activity of these cells (Carosella et al., 2008). Positively, it protects the fetus throughout pregnancy by being expressed on the trophoblast of placental cells.

According to studies, iron deficiency anemia may prevent malaria infection, however, supplementing with iron may increase malaria mortality and morbidity. In 2016, WHO therefore recommended iron supplement in children with or without the threat of anaemia in regions where malaria is prevalent. This research seeks to investigate the probable link between HLA-G genetic variants (14bp deletion/ insertion) and malaria-related characteristics and anemia among iron and non-iron fortified Ghanaian children.

Background to the Study

Malaria is an infection that is caused by the parasite Plasmodium, and it is transmitted from person to person via the bite of an infected female Anopheles mosquito. Five plasmodium parasites affect human being: *Plasmodium knowlesi, Plasmodium vivax, Plasmodium ovale, and Plasmodium falciparum*. (Loy et al., 2017).

After transmission and infection, all these parasites can induce acute febrile illness symptoms, but *P. falciparum* is documented to cause extremely serious and menacing types of malaria and accountable for over 80% of malaria death worldwide (World Malaria Report 2022). There is a connection between host genetic characteristics, host-parasite interactions, parasite genetic diversity, and/or environmental factors and the molecular foundation of sensitivity and resistance to Plasmodium parasite infections (Mergani et al., 2010).

The glycoprotein HLA, which is also known as MHC, is essential to begin the adaptive immune response and is vital in distinguishing self from non-self. It is structured into three areas or classes and found on the short arm of human chromosome 6. These classes are referred to as HLA-I, HLA-II, and HLA-III. The HLA-G molecule is an integral component of the HLA-I molecules (Carosella et al., 2008).

There have been claims that HLA-G is an essential immunological regulator with the potential to alter immunological competent cells like natural killer (NK) cells, CD41 or CD81 T lymphocytes, and dendritic cells (Provatopoulou Xeni et al., 2012.). It has a negative immunological effect by inhibiting the activity of cytotoxic T lymphocytes (CTLs), natural killer cells

(NK cells), and antigen-presenting cells (APCs), all of which are essential cells in the process of establishing a cytotoxic antitumor immune response. However, this gene is known to protect the foetus throughout pregnancy by being expressed primarily in trophoblastic placental cells (Carosella et al., 2008).

There are eight exons in the HLA-G gene. These exons are responsible for encoding the information for the signal sequence, as well as the extracellular, transmembrane, and cytoplasmic domains (Park et al., 2004). Polymorphisms in the HLA-G gene are classified according to one of two categories: either the 5' Upstream regulatory regions (URR) or the 3' Untranslated regions (UTR)(Castelli et al., 2007). Polymorphisms in the 5'-URR region of the HLA-G gene could influence the regulation of the HLA-G gene, which would therefore have consequences for protein expression and disease susceptibility (Dias et al., Oliveira et al., 2018). Exon 8 has a 14bp+/- polymorphism in the 3'UTR of HLA-G and relates to the stability and splicing patterns of HLA-G mRNA isoforms (Park et al., 2004). Inflammatory diseases have been investigated using HLA-G 3' UTR polymorphisms. Eclampsia with other pregnancy problems has also been linked to these SNPs (Ferreira et al., 2017). The major transcripts of the HLA-G gene can undergo alternative splicing, which results in the formation of seven isoforms, four of which are membrane-bound (HLA-G1, -G2, -G3, and -G4) and three of which are soluble (HLA-G5, -G6, and -G7) (LeMaoult et al., 2005).

Nine distinct HLA-G protein variations are translated by 28 alleles. Out of this number 23 match to coding sequence alterations, reducing HLA-G polymorphism. HLA-G gene expression is likewise affected by a

polymorphism in noncoding areas, which has functional implications (Hviid et al., 2006). Peripheral blood mononuclear cells' (PBMCs') expression of soluble HLA-G (sHLA-G) and IL-10, as well as the amount and stability of HLA-G mRNA, are all affected by polymorphisms in the HLA-G gene's 3'untranslated and 5'upstream regulatory regions, particularly the 14-bp+/- polymorphism. HLA-G alleles are known to be linked to recurring spontaneous abortion, sarcoidosis, Vulgaris, inflammation of the bowel, and pemphigus in humans (Glas et al., 2007; Xue et al., 2007).

Limited evidence on the potential connection of HLA-G and parasite illnesses exist, however, high HLA-G molecule concentrations in the bloodstream have been discovered to aid Leishmania parasites in evading cell-mediated immune reactions (Donaghy et al., 2007a). Additional evidence suggests that HLA-G overexpression favors Toxoplasma congenital spread (Robert-Gangneux et al., 2011b). Studies have shown that anaemia due to iron deficiency might protect against contracting malaria, whereas iron supplementation can cause increased risk of malaria-related illness and death. (Nyakeriga et al., 2004; Zlotkin et al., 2013).

Currently, no research in Ghana has explored the potential correlation of HLA-G 14bp gene variants and malaria-associated characteristics among iron and non-iron fortified children in Ghana.

Problem Statement

The goal of the Global technological strategy (GTS) for malaria is to reduce the number of new cases and fatalities from malaria to at least 75% by 2025 and 90% by 2030 (World Malaria Report 2020). Despite these objectives, WHO reported 627,000 malaria fatalities and more than 241

million cases of malaria in 2020. The most affected continent is Africa recording about 95% of all cases of malaria with 96% of deaths globally. Children younger than five made up 80% of the total mortalities, demonstrating that they are by far the group at the greatest risk of developing malaria. (World Malaria Report 2020).

HLA-G, a tolerogenic molecule, is involved in the phenomenon of maternal-fetal immunological tolerance. Research has shown that the gene is expressed during several infectious illnesses that cause immunological evasion. (Ober et al., 1998.). However, the clinical variability of malaria can be influenced by population diversity as well as environmental factors. It is critical to comprehend the underlying complicated molecular foundation of malaria in specific contexts with similar geographical features. However, no research in Ghana has examined the genetic relationship between HLA-G 14bp-/+ polymorphism and *P. falciparum* infection and anemia in both iron and non-iron fortified children.

Hypothesis

I hypothesized that, the presence of HLA-G 14bp polymorphism will have a negative effect on malaria disease susceptibility and severity among Ghanaian children under 5years**.**

Aims and objectives.

Aim

The primary goal of this study is to explore the prevalence of HLA- G 14bp-/+ polymorphism and its association with *Plasmodium falciparum* malaria susceptibility and severity and anemia among iron and non-iron fortified Ghanaian children.

Specific Objectives

1. To determine the frequency of 14 bp insertion/deletion polymorphism among iron and non-iron fortified Ghanaian children.
2. To determine the association between HLA-G 14bp polymorphism and malaria severity among iron fortified Ghanaian children.
3. To assess the association between HLA-G 14bp polymorphism and malaria disease severity in noniron fortified Ghanaian children.
4. To assess the relationship among HLA-G 14bp and anemia among iron and non-iron fortified Ghanaian children

Significance of the Study

This work will establish the relationship between HLA-G polymorphisms and malaria disease as well as anemia among iron and non-iron fortified Ghanaian children. The findings from the study will help understand the role of host genetic and host immune response contribution to the pathogenesis and help with the management of malaria disease among children. Development of vaccines and drugs to combat the *P. falciparum* parasite will be aided by knowledge of the genetic link between the host and the infectious parasite.

Additionally, it will contribute to the GTSM's goal of decreasing malaria case incidence and mortality rates to at least 75% by 2025 and 90% by 2030. Finally, this study will contribute to achieving SDG 3, Good health, and well-being.

Delimitation

Kintampo North Municipality was the focus of the study. The Municipal Hospital serves as a referral and treatment center for people living with P. *falciparum* in the Municipality thereby justifying the selection.

Limitation

The main limitation of this study was that the integrity of samples was affected since archival samples were used. Also, frequent power outage during laboratory analysis demanded the repetition of the work severally because the results were interrupted.

Organization of the Study

The thesis is broken down into five (5) chapters with the initial one describing the context for the research. Chapter 2 examines appropriate works of literature reviewed discussing in detail the strains of Plasmodium and the diseases they cause, the epidemiology, and laboratory diagnostic techniques of *P. falciparum* infection. Chapter 3 presents the research methods where the study design, study site, sample collection, analysis, and identification of HLA-G 14bp+/- polymorphisms are reported. Chapter four reviews and documents the study's findings. The fifth chapter focuses on the study's summary, conclusion, and suggestions.

Chapter Summary

Despite all efforts made to reduce malaria mortality and morbidity, pregnant women, and children below the age of 5 are by far the group at the greatest risk of developing the disease. HLA-G 14bp polymorphism is documented to be associated with malaria disease susceptibility and severity because of its tolerogenic effect, acting as an immunosuppressant and allowing the malaria parasite to evade the body's immunity. Studies have also shown that, iron deficiency anemia may prevent malaria infection, however, supplementing with iron may increase malaria mortality and morbidity. This research seeks to investigate the probable link between HLA-G genetic variants (14bp deletion/ insertion) and malaria-related characteristics and anemia among iron and non-iron fortified Ghanaian children.

CHAPTER TWO

LITERATURE REVIEW

Introduction

Knowledge about the etiology of malaria and the emergence of severe or moderate clinical outcomes may result from research into the role of immune components and host iron fortification. Meanwhile, substantial evidence indicates HLA-G contributes to both protective and pathogenic situations, therefore research into HLA-G polymorphism and iron fortification begins with the best intentions of improving malaria understanding. Additionally, this review will discuss the significance of HLA-G, its connection to malaria and anemia, the roles that iron fortification plays in the pathogenesis of malaria, and how other variables impact the expression of HLA-G. The consequences of parasite immunosuppression on other infections and a brief discussion of the immune evasion methods used by Plasmodium species will also be discussed. Due to the large information available on the parasite, plasmodium will be used as a model system to evaluate the erythrocytic cycles of the pathogens in its host and finally discuss recognized variations and parallels in host immune responses during infection.

Epidemiology of Malaria

Malaria is part of the very common vector-borne infections, with an increasing death rate within the past ten years. (Narain & Nath, 2018, Dasgupta, 2018). According to the WHO, 627,000 malaria fatalities and over 241 million cases of malaria were reported in 2020. There has been malaria since the 18th century and the word "malaria" was described as "poor air." (Gitta & Kilian, 2020a). Despite attempts to lessen the burden of illness, about

40% of people worldwide are still at risk of this infection. (Escalante & Pacheco, 2019). These diseases are known as vector-borne diseases (VBD), and they are spread through the bite of an infected arthropod species like an Anopheles mosquito. In Africa, the three species of Anopheles that are most prevalent are Anopheles gambiae *sensu stricto*, *Anopheles funestus*, and *Anopheles arabiensis*. (Dasgupta, 2018a).

Diverse protozoan genus plasmodium (Apicomplexa: Plasmodiidae) has an impact on several vertebrate hosts, including primates. As a result of this diversity, a disease relating to several parasites and vector species has emerged in many ecosystems across the world (Muehlenbein et al., 2015; Escalante & Pacheco, 2019). The four species of Plasmodium most frequently seen infecting individuals are *P. falciparum, P. malariae, P. ovale, and P. vivax*. *P. falciparum* and *P. vivax* are the two most prevalent malarial infections that cause illness and mortality. (Escalante & Pacheco, 2019; Loy et al., 2018; Muehlenbein et al., 2015).

Host genetics, environmental factors, and parasite virulence are some of the complicated causes of malaria (Mackinnon et al., 2005a). The host genetic factors have an impact on the severity of *P. falciparum* infection outcomes, such as complicated malaria anemia, cerebral malaria, etc. (Driss et al., 2011a).

Forms of malaria and causative agents

Plasmodium parasites are the protozoan organisms that cause malaria. In Africa, *P. falciparum* is extensively dispersed compared to *P. vivax, P. ovale*, and *P. malariae* infections, which are less prevalent and geologically confined (Howes et al., 2015; Roucher et al., 2014). This genus is home to

over 200 unique species of parasites, and those parasites can infect a broad variety of hosts, such as mammals, birds, amphibians, and reptiles (30 of the 53 total species parasitize primates) (Z. Luo et al., 2015). There are currently 6 major Plasmodium species known to be human pathogenic, each of which causes a different type of malaria, including *P. Knowlesi* (zoonotic), *P. vivax* (tertian), *P. ovale* (tertian), and *P. cynomolgi* (Milner, 2018) (Rosenthal et al., 2019; Garrido-Cardenas et al., 2019).

Anopheles gambiae, *Anopheles funestus*, and *Anopheles arabiensis* are the three species of Anopheles mosquitoes most seen in Africa (Z. Luo et al., 2015). In contrast, this parasite undergoes ten or more morphological changes throughout the mosquito-human life cycle, replicates in quantities between one and ten thousand cells, and has a population size ranging from one to over 106 organisms, just like the other five species that infect people (Antinori et al., 2012; Karlsson et al., 2014)

Factors that facilitate malaria transmission, development, and severity

Transmission

The period it takes for malaria parasites to advance influences how quickly the disease spreads. According to Johnston et al. (2014), the lifespan, development, and biting rates of mosquitoes are influenced by temperature, hence rising temperatures are expected to increase the rate of malaria transmission (Dasgupta, 2018b). According to Paaijmans et al. (2010), The ideal range of water temperatures for the hatching of *Anopheles gambiae* eggs is between 24°C and 30°C. (Paaijmans et al., 2010; Impoinvil et al., 2008). The transition from larvae to pupae is influenced by temperature as well; temperatures between 22°C and 26°C is best for the quickest transformation

(Cohen et al., 2008). Further research by Cohen et al. revealed the best temperature choice for parasite growth to be between 25°C and 30°C, *P. falciparum* may live at temperatures as low as 18°C and as high as 40°C. The development of a parasite takes approximately 12 days at a temperature of 25°C, whereas it takes more than 30 days at 20°C. (O'Meara et al., 2008). Since less time is needed for parasite growth at substantially higher temperatures, the likelihood of infection spreading is increased (Ikemoto, 2008).

Rainfall frequently creates stagnant water which is essential for mosquito breeding. (Thomson et al., 2005). As a result, one of the effects that is most impacted by climate is malaria. Extreme rains can synchronize parasite and vector host-seeking, increasing the spread of malaria and death (Ermert et al., 2012). The optimal temperature for the spread of malaria was documented as 25°C by Mordecai et al. (2013) utilizing thermal reaction roles (Mordecai et al., 2013).

However, Mustaffa et al. (2017) suggested that 15°C and 18°C were minimally acceptable temperatures for *P. falciparum* and *P. vivax* survival, respectively. (Mustaffa et al., 2017). Under laboratory circumstances, Paaijmans et al. (2010), discovered that breeding water is typically 4-6°C warmer than the surrounding air, which promotes faster larval development and population growth rates (Paaijmans et al., 2010). It is also well acknowledged that socioeconomic factors play a part in the spread of malaria. This is because lower rates of malaria transmission have been related with higher levels of socioeconomic status. (De Silva & Marshall, 2012).

The risk of malaria is also decreased in urban areas with better socioeconomic conditions (de Beaudrap et al., 2011), whereas a higher prevalence of malaria is caused by lower socioeconomic conditions (Somi et al., 2008). Improved living conditions, which are frequently linked to high financial status, further reduce the incidence of malaria (Dasgupta, 2018; Tusting et al., 2013; Narain & Nath, 2018).

Since breeding areas are more polluted as urbanization increases, there are often fewer vectors and lower transmission rates, which lowers the spread of malaria (D. L. Smith et al., 2004; Dasgupta, 2018; Alirol et al., 2010). Additionally, urban inhabitants have easier access to healthcare (Noor et al.2003).

Insecticide-treated bed nets (ITNs) and indoor pesticide spraying are two methods that are applied most frequently to inhibit the spread of malaria through the bites of mosquitoes. These techniques are more than 50% helpful (Flaxman et al., 2010). These actions, however, are frequently related to financial states since those from low - income often cannot afford to take these preventative steps.

Life cycle of *Plasmodium falciparum*

The Plasmodium parasite can reproduce both sexually and asexually throughout its life cycle, with the female Anopheles mosquito serving as a definitive host while humans play the role of intermediate hosts (Hodson et al., 2015). In efforts to progress through the several developmental stages and long enough to reproduce within this intricate life cycle, Plasmodium parasites must infect and reside in a diversity of cell types (Hodson et al., 2015).

The main method by which *P. falciparum* spreads infection is through asexual intraerythrocytic developmental cycles (IDCs), which last for around 48 hours each and result in 8–30 new parasites (Thomson-Luque et al., 2021). The "ring stages" of the parasite that causes falciparum malaria are young forms of the parasite that circulate inside each IDC and express parasite antigens on the surface of the host cell. This helps the parasites adhere better to the vascular endothelium and prevents the spleen from clearing them out. (Thomson-Luque et al., 2021).

All Plasmodium species share some characteristics throughout their life cycles. A portion of the blood-circulating parasites (merozoites) in all species mature into gametocytes, which are then consumed by the mosquito vector (Escalante et al., 2015). The process starts with midgut gametogenesis and ends with the release of sporozoites by the vector's salivary glands to permit the spread to the hosts during a blood meal (Steere et al., 2016). Mosquitoes may use their proboscis to penetrate human skin in a matter of seconds and draw blood (Djokic et al., 2021).

Plasmodium starts its asexual life cycle in the host by infecting hepatocytes with sporozoites that have been injected into the dermis by a mosquito (Gowda & Wu, 2018). A single cycle of liver replication in P. *vivax*/P. *ovale* and up to 30,000 in *P. falciparum* released approximately 10,000 merozoites (Antinori et al., 2012). To ensure nutrient supply from haemoglobin digestion along with the uptake of blood serum, the elimination of poisonous waste, defence against the host immunological reaction, and the development of the life cycle, the fatally distinguished and metabolically reduced host cell undergoes a complex reorganization in the erythrocytic

schizogony that takes place within the human host's erythrocytes (Kilian et al., 2013). However, after a few weeks (to months or years) have passed after the first infection, *P. vivax* and *P. ovale* generate a dormant liver stage called a hypnozoite, which then becomes active and causes a recurrence of the disease (infection of the RBC). Therefore, in the treatment of malaria in a patient with any of these two parasites, it is vital to eliminate any latent stages that are absent from *P. falciparum* or *P. Malariae* (Escalante & Pacheco, 2019a).

These parasite species do, however, differ significantly from one another in relation to their life cycles. For example, a *P. vivax* infection will create gametocytes faster than a *P. falciparum* infection will, but the gametocytes produced by *P. falciparum* infections are more infectious. (Bousema & Drakeley, 2011a). These variations may have an impact on how they are transmitted and how various treatment methods change their survival(Escalante & Pacheco, 2019a; Schneider & Escalante, 2013). There may only be one Plasmodium variant in certain regions that malaria is prevalent, whereas all four may coexist in other areas. *P. vivax* may survive in temperate zones, in contrast to other man parasites, which are often limited to tropical and subtropical regions (Escalante & Pacheco, 2019). *P. falciparum* and *P. vivax* may have comparable geographic disseminations in several tropical and subtropical regions.

As with any perfectly effective parasite, evolution in malaria has been found to result in a smooth transition from a mosquito to a human, with no impact on the vector or host.

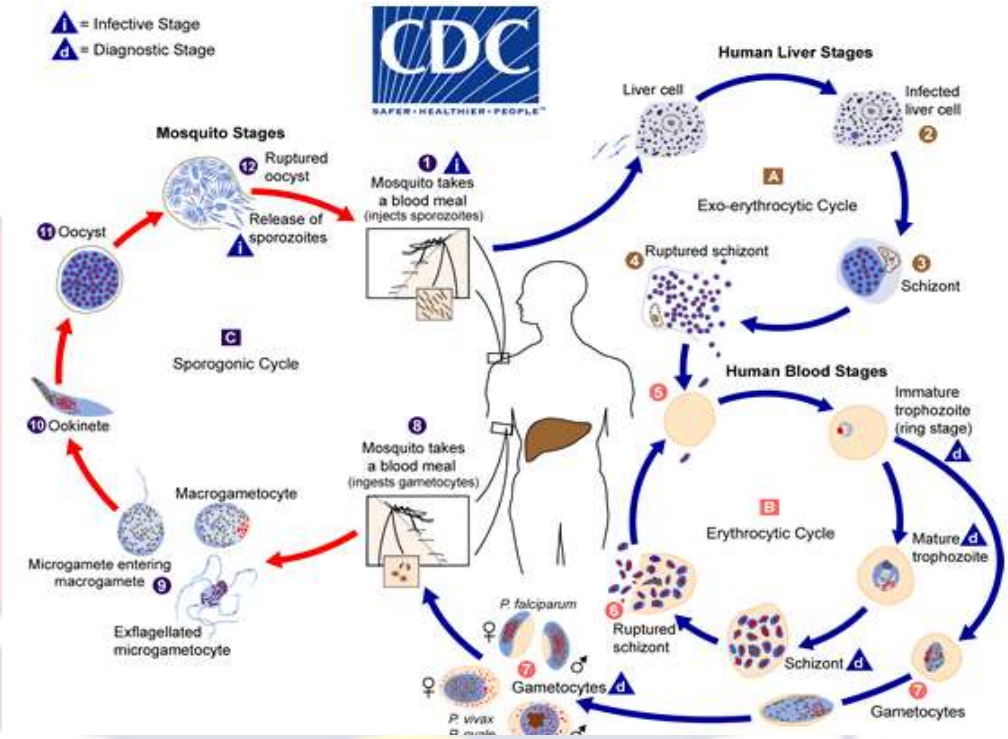

***Figure 1*: The natural history of malaria**

Malaria Pathogenesis

Human malaria pathogenesis is a complex story with several characters, settings, and possible clinical outcomes. Pathogenesis is the process by which a disease develops.

In uncomplicated malaria, *P. falciparum* infection can cause several different clinical outcomes, such as an asymptomatic state, fever, and other vague symptoms. Most patients who present themselves with non-specific symptoms such as fever, rigors, and chills do not require hospitalization. (Lindblade et al., 2013). However, the patient might die or need to be hospitalized due to the severe symptoms brought on by the parasite's replication, the host's erythrocyte's remodeling, the relations among the host and the parasite, the cytoadherence, and the resetting, which include cerebral malaria, severe anemia, jaundice, shock, multiple organ dysfunction

syndromes (MODS), acute renal damage, acute pulmonary edema, and coma. (Mustaffa et al., 2017)

Falciparum malaria creates Maurer's clefts, a type of secretory organelle that extends over the cellular boundaries of the parasite and into the cytoplasm of the host. This is done to increase host-parasite contact and sequestration, as well as to impede splenic clearance of infected erythrocytes. (Gitta & Kilian, 2020). In non-falciparum malaria infections, this mechanism has also been connected to unusually severe clinical symptoms (Wassmer et al., 2015). *P. malariae*, on the other hand, can result in a persistent infection that can linger in the blood for years if left untreated(Escalante & Pacheco, 2019).

Similar to Sinton Mulligan's clefts, *Knowles,* the parasite that causes zoonotic malaria in Southeast Asia, create them in the erythrocyte cytoplasm. The parasite produces protein inside the erythrocyte, and it appears that these clefts are essential for absorbing and harbouring this protein (Asare et al., 2018). The parasite can successfully infect erythrocytes and cause a rearrangement that causes Ziemann stippling. However, *P. malariae* infection does not result in severe malaria (intraerythrocytic dots like Schüffner dots) during the infection. (Edison et al., 2011).

Fatih et al. stated it further in an in vitro experiment. Inducible human endothelium receptors for intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-2 (VCAM-2) and host erythrocyte binding to these receptors may be produced by *P. Knowles* (VCAM) (Fatih et al., 2012). The sequestration of *P. Knowlesi*-infected erythrocytes by P. falciparum-like endothelium was not observed in more recent studies,

however; instead, it was discovered that intravascular haemolysis might perform a key part in the emergence of severe symptoms (Barber et al., 2018). Older parasites circulate and parasitemia decreases in each 48-hour asexual replication cycle, according to studies by Portugal et al (2017). These findings lend credence to the hypothesis that parasites can isolate themselves from circulation by adhering to endothelial cells, evading splenic clearance, and rapidly worsening clinical symptoms and parasitemia. (Portugal et al., 2017).

Additionally, the biology of the parasite interacts with human pathophysiological mechanisms causes disease in humans. Factors and corollaries include the genetic variety of key proteins in parasites, coinfections, comorbidities, treatment delays, human polymorphisms, and environmental factors that complicate the host-parasite relation (Gonçalves et al., 2017)

Immunity against malaria

The greatest widespread malaria parasite in Africa, *P. falciparum*, produce a varied variety of clinical signs, from moderately severe, life-threatening malaria to asymptomatic infections (Abel et al., 2018; World Malaria Report 2016). One crucial element influencing the result of a *P. falciparum* infection is the immunological response of the infected host(A. M. Abel et al., 2018). Those who have had only minor prior exposure to *P. falciparum*, experience a robust, pro-inflammatory reaction upon infection, which results in fever and aids in the progression of malaria symptoms (A. Abel et al., 2018a)

Since vertebrates are required to have an internal life cycle, both parasites can avoid detection by the immune system of their hosts throughout

the majority of their asexual reproductive phases. At this point in time, the host immune response can unfortunately only be directed towards the changed surface of the infected erythrocyte. (Allred, 1995). Different immune reactions involving IL10, and Interferon-y may result from changes throughout infancy (Sylvester et al., 2018).

Most parasites are eliminated by protective humoral, cellular, and innate immune responses in immunocompetent individuals, but in circumstances of immunological deficit, the infection can be lethal, particularly if not properly diagnosed and treated (Gitta & Kilian, 2020a). When parasites infect hosts, a struggle takes place over whether the victim will survive or die.

Wale et al. (2018) discovered three immune response components that had varied impacts on parasites and disease by applying the rat malaria parasite as a model system (Patgaonkar et al., 2018). They assert that to reduce the number of erythrocytes that are available for invasion, early in the infection, the host employs a technique that causes RBCs to die. The elimination of both infected and uninfected RBCs, which causes anaemia, was linked with a reduction in total RBCs. The host appeared to gain from this immunopathological process by reducing the Plasmodium load (Djokic et al., 2021).

A potent pro-inflammatory response is downregulated all through malaria development (Perez-Mazliah et al., 2015). Previous studies conducted on both humans and animals have shown that severe cases of malaria lead to an increase in the synthesis of molecules that inhibit other molecules, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4).

Compared to kids with simple malaria, kids with severe malaria exhibited different CD4+ T cell frequencies. Increased CD39+ and GrzB+CD4+ T cell percentages were found in children with simple malaria, while increased CTLA-4+ and PD-1+CD4+ T cell percentages were found in children with complicated malaria. This finding suggests that distinct regulatory mechanisms are activated and may affect the clinical presentation of severe malaria (Abel et al., 2018). Furthermore, CD4+ T cells are significant for the adaptive immune response to plasmodia and, although they may offer defense, they can have adverse impacts and result in serious impediments (Okonkwo et al., 2015). The thought that acute malaria causes qualitative changes in the T cell response is supported by several data. (Portugal et al., 2014).

Clinical features of malaria in the acute stage and severe stage

Several factors, including ecological considerations, the severity of the parasite, and host traits like age or genetic background, influence the variation in the host's response to infection. In fact, human clinical disorders are extremely rare when compared to the global network of human-mosquito encounters (Driss et al., 2011b)). The parasitaemia in asymptomatic children is frequently low and sub-microscopic, but parasitaemia is frequently greater in cases of severe malaria (Andrade et al., 2020). Following a limited clinical incident of malaria in malaria endemic zones, protection from severe malaria is quickly acquired, and years of contact causes largely asymptomatic sequelae in adolescence and adulthood, demonstrating that gradual natural immunity to malaria can be gained through exposure over time (Tran et al., 2013). There is a substantial financial impact on public health from both asymptomatic

infection and mild malaria attack, which are the most prevalent clinical manifestations of malaria. For an infection to be considered asymptomatic, parasitaemia must be detected in the blood but no other clinical signs must be present. (Noor et al., 2003.).

Plasmodium species often go through their erythrocytic cycle during the rainy season, when mosquitoes are most active. In addition to the typical symptoms of fever, chills (despite the hot outside temperature), headache, etc., a patient with malaria may also have coughing, fast breathing and heartbeat, weariness, and general malaise. Mali, where symptoms of malaria are only correlated with the transmission season has recorded cases of infection persistence during asymptomatic malaria (Andrade et al., 2020). This spectrum of malarial symptoms is linked to parasite load and various phases of a growing protective response that gets stronger with parasite exposure and host aging (Weiss et al., 2010). Higher parasitaemia is often linked to a poorer prognosis, even though it is unclear whether parasite and host variables affect the clinical outcome of *P. falciparum* infections (Almelli et al., 2014). As mentioned by Armiot et al., HLA-G protein is overexpressed in many disorders, and this overexpression may affect how the immune system responds to certain diseases. protein (HLA-G) is overexpressed in numerous diseases, according to Armiot et al., and this overexpression may influence how the immune system reacts to diseases ((Driss et al., 2011b).

Clinical diagnosis of malaria

There is a possibility that malaria was documented in the medical records of certain ancient societies, including China, Egypt, India, Mesopotamia, and Greece. (Bruce-Chwatt & Obe, 1981). When no diagnostic

laboratory is available or the patient is diagnosing themselves at home, a clinical diagnosis is made based only on the patient's symptoms (Bisoffi et al., 2012). Self-diagnosis and self-medication, however, are mistake-prone practices that can cause diagnostic errors and mistreatment since other tropical illnesses have symptoms that are comparable to those of malaria (Oladosu & Oyibo, 2013). Therefore, any clinical suggestion of malaria should be assessed at a health facility.

Since the late 1800s, parasites in peripheral blood have been detected using light microscopy of stained blood smears. Microscopy may be quite sensitive and specific when done properly, yet it is also reasonably inexpensive. The accuracy of microscopic diagnosis is frequently put at risk by subpar tools, unqualified personnel, a severe workload, and limited supplies of electricity (Gitta & Kilian, 2020a). Therefore, a thick blood smear examination under a light microscope is still required for laboratory confirmation of malaria (Escalante & Pacheco, 2019). However, when parasitaemia is low, as it is in people who don't show any symptoms, microscopy has limited sensitivity (Wu et al., 2015). Additionally, in underdeveloped regions or where transmission of malaria is no more considered a danger, it might be challenging to maintain qualified microscopists (Escalante & Pacheco, 2019).

Rapid Diagnostic Test (RDT) developed on monoclonal antibody immunochromatographic recognition of specific parasite proteins were manufactured in response to these problems. (Zimmerman & Howes, 2015). The circulating antigen in an active infection is identified by one-time immunochromatographic antigen-based diagnostics. Tests can be much more

sensitive and specific than optical microscopy, with significant sensitivity loss at very low parasite quantities (Zimmerman & Howes, 2015). RDTs requires no training, no specialized facilities or equipment, and no electricity, and their results are reproducible and easily accessible (Nkumama et al., 2017). There are still technical limitations, including handling problems that cause antibody denaturation and samples from patients who had low parasitaemia when they had malaria (such as *P. vivax* and *P. malariae*), which reduce sensitivity of the test.

There are further molecular techniques, including reverse transcription-polymerase chain reaction (RT-PCR), loop-mediated isothermal amplification, conventional PCR, and quantitative reverse transcription-PCR (qRT-PCR). These methods concentrate on mitochondrial genes, 18S rRNA, and repetitive sequences. (Espié et al., 2015). The commonality across these strategies is that they focus on many copies of the parasite genome, increasing their sensitivity when parasitaemia is low. To fully comprehend the comparative contribution of this resource to the eradication goal in several transmission circumstances, further studies employing sensitive molecular techniques is necessary (Tibayrenc & Ayala, 1991).

It is uncertain which elements of the disease can and should be evaluated to precisely follow evolving epidemiology given the great diversity of clinical presentations of malaria (Nkumama et al., 2017). An accurate diagnosis (detection) permits investigation into how risk factors influence incidence and case distribution. (Conway, 2007). Furthermore, the accuracy of diagnostic testing is affected by parasite density. The detection threshold for microscopy by an expert and well-trained technician is 15 parasites per L of

blood(Guerin et al., 2002), However, RDTs can detect as few as 200 parasites per L by picking up on circulating parasite antigens. Many pregnant women who experience malaria symptoms have parasite densities that are above the detection criteria, therefore such testing may be adequate for their care (Kyabayinze et al., 2016).

Furthermore, parasite density influences diagnostic testing accuracy. When conducted by a skilled and well-equipped expert, the detection threshold for parasites in blood is 15 parasites per L of blood(Guerin et al., 2002). Although this limit can be as low as 200 parasites per L for RDTs that find circulating parasite antigens. These diagnostic procedures may be sufficient since pregnant women with malaria symptoms commonly have parasite densities exceeding detection thresholds (Kyabayinze et al., 2016).

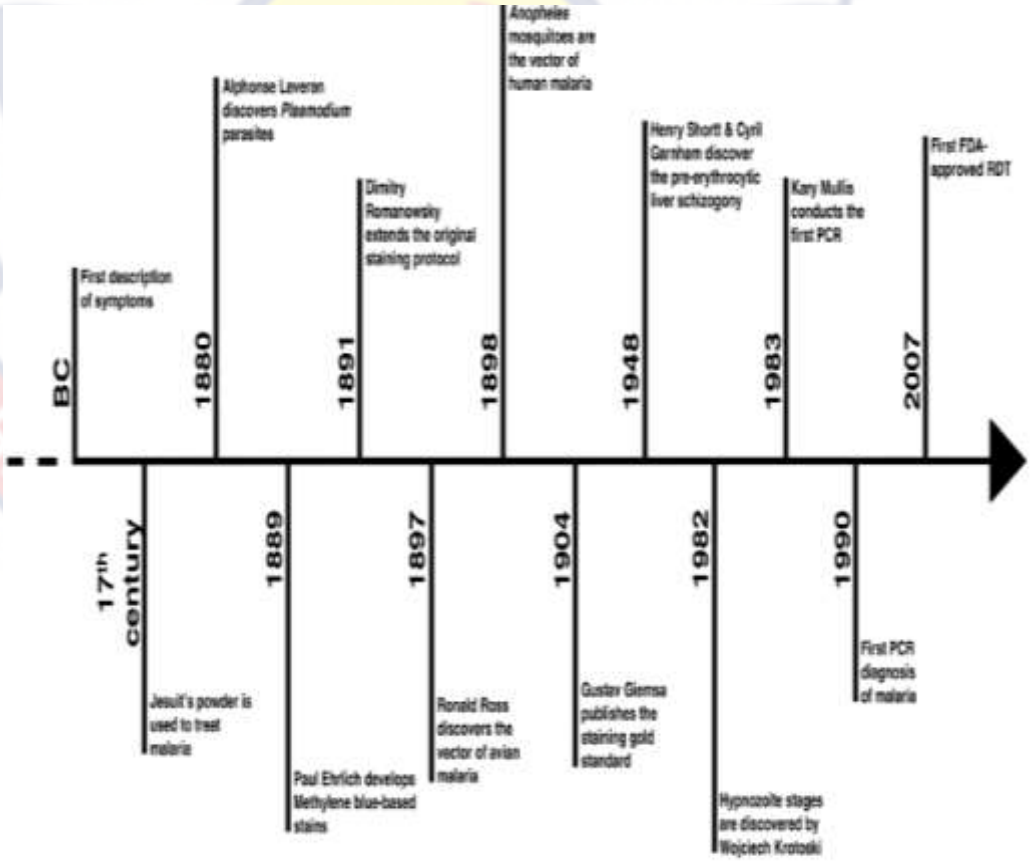

***Figure 2*: History of malaria diagnosis**

Epidemiology

Malaria produced by the *P. falciparum* parasite is among the main contributors to mortality and morbidity in tropical settings. (le Port et al., 2013). A staggering 40% of the earth's populace, spread across 104 countries, is today in danger from the devastation of malaria. According to the world malaria report, in 2021, there were around 247 million instances of malaria, and nearly 619,000 people lost their lives because of the disease. The majority of those who lost their lives were young children in Africa.

However, according to research, as compared to projections from 2000, malaria cases have significantly decreased globally (Nonvignon et al., 2016). Despite these incredible advancements, Sub-Saharan African (SSA) continue to face a serious public health threat from malaria., where it was responsible for 96% of all global fatalities and 95% of all cases in 2021 with children under the age of 5 years recording about 80% of the total death. In 2021, Ghana recorded 2.2% of the total malaria cases and 2% of the total malaria deaths.

Malaria vector reproduction and transmission, and consequently malaria transmission, require a balance between temperature and precipitation is essential. Although it is generally known that climatic conditions affect malaria transmission (Talisuna et al., 2007), it is less clear how exposure to climatic factors affects malaria mortality (Dasgupta, 2018b). Because over 85% of parasite-positive individuals in certain endemic locations are asymptomatic, these people need to be closely monitored (Dasgupta, 2018b). The extent of local malaria transmission or the amount of endemicity also affects malaria epidemiology, which varies by place (Tchum et al., 2021).

WHO projected that between 2016 and 2030, investments in the eradication of malaria may cost up to $101.8 and lower the international burden of malaria by 50% (Newby et al., 2016; Rabinovich et al., 2017). Previous research has offered estimates of the financial burden that malaria places on Ghanaian households as well as the country's overall economy. According to Asante and Asenso-Okyere, a malaria episode costs households US$1579, and a 1 percent rise in malaria morbidity has a 0.41 percent impact on economic growth (Newby et al., 2016). Abotsi et al. (2012) published that, malaria episode can cost a household anything from US$10.20 to US$4662 (Komla Abotsi et al., 2012). Furthermore, Sicuri et al. (2016) reported that malaria treatment cost families in Ghana ranges from US$5.70 (uncomplicated malaria) to US$48.73 (severe) (Nonvignon et al., 2016).

As the malaria burden decreases, a growing emphasis has been placed on low-density chronic illnesses (Nkumama et al., 2017). According to recent studies, new-borns, and school-age children (Mathanga et al., 2015; Pinchoff et al., 2016), pregnant women, and nonpregnant adults bear a significant burden (Nkumama et al., 2017). Chronic infections can be microscopic or sub-microscopic, and while various research has shown that the form is transmissible to mosquitoes, it could possibly act as a forgotten reserve for ongoing transmission, although the proof for this is not clear. (Gonçalves et al., 2016). Understanding malaria epidemiological patterns, then, demands knowledge of incidence rates and clinical outcomes in terms of population demographics.

HLA-G Polymorphisms among the human infected host

Recent research reports describe polymorphisms that are closely linked to *P. falciparum* malaria susceptibility and resistance. Genes involved in host immunity, such as the HLA gene, cytokine genes, complement regulatory genes, and endothelium receptor genes, contained the great majority of the polymorphisms found in these investigations. Though they are associated with the severity of malaria, these polymorphisms do not result in host genetic illness.

Since the 1980s, research gathered using molecular genetics methodologies has unquestionably provided proof of polymorphisms linked to malaria and their complicated interactions. Some gene variants and polymorphisms in human hosts provide survival benefits and have increased in frequency owing to natural selection throughout a lifetime (Driss et al., 2011c). This has offered information on a two-pronged method for maintaining genetic variety, involving natural selection and polymorphism co-adaptation among the malaria parasite and its human host (Driss et al., 2011d)Human genetic factors have an important impact on malaria risk. The selection of polymorphisms linked to better survival by malaria has had a significant effect on the human genome (Ndila et al., 2018). These polymorphisms comprise the typical RBC variations, sickle cell trait and α+ -thalassemia. Other SNPs, on the other hand, have the potential to confer malaria protection.

Polymorphisms in the MHC region on chromosome 6p21.3, which encodes HLA antigens, have received a lot of attention since the 1970s (d'Almeida et al., 2019a)). In 1987, HLA-G was described for the first time

(Geraghty et al., 1987). HLA-E and F are nonclassical HLA molecules (Geraghty et al., 1987)). Compared to the HLA-A, -B, and -C consensus sequences, it differs by about 86% along its eight exons and seven introns. (Amiot et al., 2014a). HLA-G varies from standard class I molecules in that it possesses a short cytoplasmic tail of six amino acids due to a premature stop codon in exon 6 (De Almeida et al., 2018). A minimum of nine HLA-G 3′UTR variation sites has polymorphic rates in the global populaces, includes the 14bp polymorphism (rs371194629), +3003 C/G (rs1707), +3010 G/C (rs1710), +3027 C/A (rs17179101), +3035 C/T (rs17179108), and +3142 C/G (rs1063320) (rs1233331). Functional research done on the 14bp, +3142C/G, and 3187A/G sequences (Veit & Chies, 2009). The presence/absence of the 14bp fragment (14bp+/-), sited among regions +2961 and +2974, was the main polymorphic site found at the HLA-G 3′UTR and is the highly studied (Castelli et al., 2014). HLA-G is strongly associated with maternal-foetal tolerance and is mostly expressed on the placental extra villous cytotrophoblast cells. In non-pathological settings, it is limited on organs like the thymus, cornea, pancreas, and hematopoietic cells (Calderaro et al., 2013). HLA-G molecules are distinct from typical HLA class I (-A, -B, and -C) molecules due to their low protein variability, restricted tissue expression under non-pathological situations, and immune response cell regulation.

During pregnancy, HLA-G is an immuno-modulatory molecule that interacts with immune cells of both innate and adaptive responses. As a result of these interactions, HLA-G plays an essential role in the development of maternofoetal tolerance (Sylvester et al., 2018a; Bahri et al., 2006). Non-classical HLA class I antigens like HLA-G differ from conventional class I

molecules in tissue delivery, protein isoform diversity, and polymorphism (D'Almeida et al., 2019b). During viral infections, infected cells can over-express HLA-G to produce a tolerogenic condition that permits the pathogen to avoid the immune system (Poras et al., 2017). Similar effect has been described in many cancers (Menter & Tzankov, 2018). HLA-G plays has no effect on antigen presentation, inhibits CD8 + T and NK cell cytotoxic activity, inhibits CD4 + T cell production and antigen presentation, inhibits B cell function, and induces T regulatory cell lines via direct binding primarily to the ILT-2 and ILT-4 leucocyte receptors, which can also interact with classical HLA molecules but have a higher affinity for HLA-G (Menter & Tzankov, 2018).

According to other studies, host-parasite interactions during infection result in a wide range of clinical manifestations, which are explained in part by the type and extent of the anti-malarial acquired immune response (Roucher et al., 2014). Due to its immunosuppressive properties, the HLA-G molecule seems to be a promising answer for such immune response modulation targeted on Plasmodium falciparum (Nkumama et al., 2017b). Furthermore, malarial immunity is dependent on the availability of specific, yet enough IgG3 subclass antibodies that recognize merozoite surface antigens and reduce the severity of P. *falciparum* infection in children under the age of five to

decrease the burden of anemia (Sinka et al., 2010)

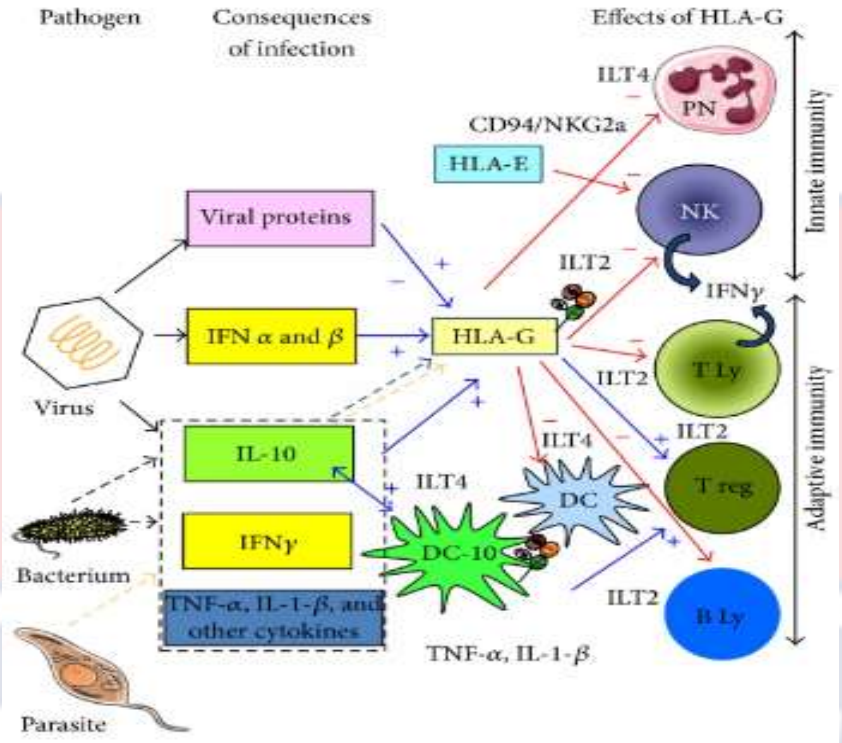

Figure 3: **Relations among polymorphisms and risk for severe malaria**

The role of polymorphism of HLA-G regulatory region in infection

There aren't much published clinical data for parasitic diseases, except for one study that looked at the defensive effects of HLA-G polymorphism in malaria, and those that are mostly concerned with plasma concentrations of sHLA-G (Sabbagh et al, 2013). Malaria infections, including asymptomatic, CM, and SMA, have been shown to vary widely in severity among individuals and populations. Malaria severity has been associated with a variety of genetic diseases or traits caused by gene mutations ((Verra et al., 2009)). Epistatic interactions between HbAS, thalassemia, HbE, and other hereditary hemoglobin disorders give evidence of heterozygote immunity to malaria. (Driss et al., 2011d)

Donaghy et al. (2007) discovered that soluble HLA-G points rose in 35% of patients with HIV-seronegative visceral leishmaniasis (Leishmania infantum) (VL) and 57% of patients with Leishmania infantum infection (Donaghy et al., 2007b). However, when patients with HIV infection and VL were compared to persons with HIV infection alone, the proportion of HLA-G-positive patients and the mean sHLA-G value were both suggestively lower (Donaghy et al., 2007b). Amiot et al. claim that a general tolerogenic environment may be to blame for a rise in sHLA-G levels in HIV-infected patients with VL, increasing the tenacity of Leishmania and decreasing the life expectancy of HIV-infected patients and another immunological indicator of effective therapy is sHLA-G (Amiot et al., 2014a).

HLA-G may also act as an immunomodulator, which helps evade foetal damage but may cause the mother to transmit Toxoplasma gondii to the foetus (Robert-Gangneux et al., 2011a). Toxoplasmosis-infected pregnant women's amniotic fluid includes significant levels of sHLA-G. When a foetus is infected from birth, the levels of this protein are at their peak. However, in a small group of patients, every foetus was delivered alive, which is consistent with proper inflammatory response downregulation (Amiot et al., 2014a).

Alleles, for example, are not always appropriately reassembled from single-mutation reports because they are produced by individuals infected by distinct parasite lineages with varying mutation patterns (Escalante et al., 2015). In contrast, the role of host genetics in malaria growth has remained widely explored over the last twenty years and is considered to contribute for 25% of overall diversity in malaria occurrence (Mackinnon et al., 2005b).

These immunoregulatory features of HLA-G have emerged as a reaction to a wide variety of pathological and physiological situations. These reactions, depending on the nature of the underlying sickness, may be detrimental to the body's health (de Almeida et al., 2018a). In addition, the presence of 14-bp is connected to alternative splicing of the HLA-G main transcript, which results in a more stable mRNA.

However, the increased stability does not appear to compensate for the decreased production of HLA-G that is brought on by the 14-bp insertion (Sylvester et al., 2018). Generally, the 14bp+/+ is linked with lower HLA-G production, whereas the 14bp-/- has been associated with increased HLA-G production (Veit & Chies, 2009). Numerous studies have linked 14bp alleles to disease morbidity and susceptibility. Despite the impact of 14bp on gene expression, the findings remain deemed inconclusive (de Almeida et al., 2018a).

Many studies identified the 14bp insertion to be the allele linked with illness susceptibility, whilst others found the 1-bp deletion to be the allele linked with susceptibility, and sometimes none were found to be related when the diseases were categorised as having either an increased or decreased HLA-G expression (Moreau et al., 2008; Zidi et al., 2015, Amodio et al., 2016). According to D'Almeida et al., (2019) et al., the highly expressed immunological checkpoint molecule HLA-G promotes immune control escape (D'Almeida et al., 2019b). HLA-G does interact with ILT2 (expressed by T and B lymphocytes, NK cells, monocytes/macrophages, and dendritic cells) and ILT4 (expressed by monocytes/macrophages, neutrophils, and dendritic cells (Alaoui et al., 2018).

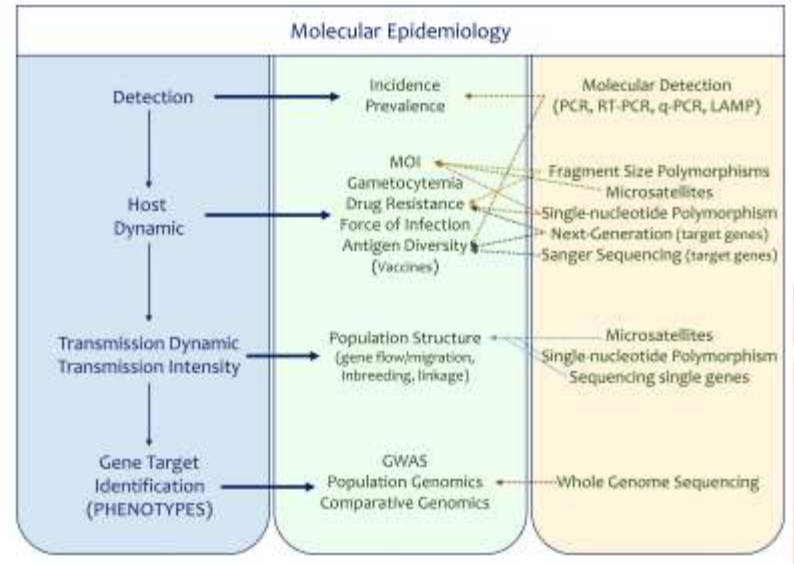

Figure 4: **Molecular epidemiology**

The Role of Asexual Replication and Host Erythrocyte Reorganization in the Development of Severe Complications

The erythrocyte, also known as red blood cell (RBC), plays a critical role in the metabolism and survival of the organism by constantly supplying oxygen to an organism's cells and tissues in developmental, physiological, and regenerative settings (Bresnick et al., 2018). To take these possibilities into consideration, it will be necessary to conduct a more in-depth examination into the underlying biology of erythrocytic infection.

The process of invasion is a lengthy and inefficient one that may only infect a very small percentage of the erythrocytes that were intended to become infected. Upon invasion, parasite antigens are released leading to the detection of several parasite-encoded erythrocyte-adhesive proteins in increased amounts in plasma. According to Stoute et al. (2003), one obvious hallmark is the increased destruction of uninfected erythrocytes. They will probably adhere to uninfected erythrocytes, causing IgG or complement binding and eliminating the erythrocytes from circulation (Stoute et al., 2003).

Additional binding mechanism referred to as rosetting, is linked to severe malaria in children in Africa, by means of the IRBC adhering to uninfected RBCs (Rowe et al., 2009). Some host components have been identified, such as complement receptor 1 (CR1), heparan sulfate (HS), blood type antigens, and, more lately, host serum proteins such as immunoglobulin M (IgM) IgM and α2M (Tonkin-Hill et al., 2018).

Polymorphisms in CR1, thalassemia, and RBC, such as the AS trait, have been demonstrated to offer protection from severe malaria (Andrade et al., 2020). PfEMP1 also has a strong affinity for blood type A and B antigens, which have been related to severe malaria. (Moll et al., 2015). Several investigations have found IgM to be part of the serum proteins implicated in rosette formation attachment (Bandoh et al., 2022).

Effect of host genetics on malaria predisposition and severity

At this stage, the parasite's ability to digest haemoglobin, develop channels to import nutrients, move waste products, transport produced proteins from around the cell, and, lastly, successfully disintegrate the cell membrane during egress are all important to the parasite's success (Groom et al., 1990).

Many well-known genetic changes in erythrocyte membrane proteins have been connected to reduced merozoite invasion. In the promoter region of the Duffy antigen receptor for chemokines gene, for example, Africans have a reasonably common single nucleotide polymorphism (SNP) (DARC or Duffy; rs2814778) (Lelliott et al., 2015b; Tournamille, 2005). A ligand for the *P. vivax* merozoite is Duffy. Duffy-binding protein is obligatory for erythrocyte merozoite invasion (John Barnwell et al., 1995.). As a result, Duffy-negative

persons are immune to Plasmodium vivax infection, and *P. vivax* infection is essentially non-existing in places in Africa where this SNP is prevalent (Gething et al., 2010.).

The erythrocyte surface band 3-GYPA complex was then found to be necessary for invasion by *P. falciparum* and the rodent malaria pathogen, *P. yoelii* (Miller et al., 207). Alterations in the erythrocyte glycophorins GYPB and GYPC are common in malaria-prevalent regions, and a current study has established that both proteins play an equal role in parasite invasion.

Complement receptor 1 (CR1), which is also produced by erythrocytes, has been demonstrated to be a receptor for the *P. falciparum* merozoite protein PfRh4 and to inhibit *P. falciparum* merozoite invasion (Spadafora et al., 2010). This might help to explain why persons with CR1-deficient mutations have higher malaria resistance (Cockburn et al., 2003).

To find new host binding proteins for merozoite proteins implicated in invasion, two high- throughput screening techniques have recently been established (Cécile Crosnier, 2011). Compared to parasitized HbAA erythrocytes, P. *falciparum* parasitic HbAS, HbSS, HbAC, HbC, and alpha thalassaemic erythrocytes do not adhere to human microvascular endothelial cells as readily in vitro (Kilian et al., 2015). Despite the presence of normal levels of knob-associated histidine-rich protein, PfEMP-1 levels on the exterior of parasitized HbAC and HbCC erythrocytes were shown to be reduced (Fairhurst et al., 2003). Mechanism of action explanations ranges from a mild membrane stiffness to direct meddling with band 3/parasite binding, loss or reduction of band 3 complex-forming merozoite ligands such as GYPA and HLA-G, and reduced band 3 complex-forming merozoite

ligands such as GYPA and HLA-G. 3 mobility (Goel et al., 2003; Smythe et al., 2005). Overall, erythrocyte defects can protect the host from malaria infection by preventing parasite invasion. This is especially evident when the mutations impair a specific parasite-erythrocyte binding interaction (Lelliott et al., 2015).

According to a new molecular study, HbAC and HbAS cells exhibit inadequate parasite protein trafficking through the parasitophorous vacuole, resulting in aberrant Maurer's clefts produced by parasites growing there (Cortés et al., 2005; Mcgilvray et al., 2000). This appears to contradict SAO's proven protective effect during severe malaria. However, the precise consequences of increased CD36 affinity in defining SAO malaria resistance remain uncertain. Erythrocytes go through a range of age-related physiological changes that eventually lead to macrophage phagocytosis and recycling. Cellular shrinkage, an increase in density and stiffness, and modifications to the proteins on the erythrocyte surface are all signs of senescence. The literature frequently mentions two primary models for erythrocyte senescence. The first theory, known as "band 3 senescence," proposes that a build-up of haemachromes (by-products of haemoglobin breakdown) induces crosslinking amid the cytoplasmic domains of band 3 proteins and the development of aggregates (Lutz et al., 1987).

IgG antibodies serve an important role in building antimalarial protection by lowering parasitaemia to levels beneath those that induce malaria. Between the IgG subclasses, cytophilic IgG1 and IgG3 are thought to be the most important (Lelliott et al., 2015). These subclasses are assumed to have the ability to directly defuse parasites by preventing parasite invasion or

growth in erythrocytes, or indirectly through a mechanism relating to cooperation between parasite-opsonizing antibodies and monocytes through binding to the Fc receptor IIA, which results in the secretion of unidentified soluble factors that inhibit parasite growth (Katherine E. Wright, 2014). Practical investigation on the role of this polymorphism have not been conducted; it is however worth mentioning that this variation site is positioned in the identified (AU)-rich motif sequence that regulates HLA-G mRNA degradation, indicating that this SNP may be active in modifying HLA-G expression.

Furthermore, there was no previously observed link among the +3196 G allele and malaria clinical and parasitological features, implying that this SNP may contribute to HLA-G expression regulation. Similarly, neither previously observed link between the +3196 G allele and clinical and parasitological phenotypes associated with malaria (Sabbagh et al., 1989).

Erythrocyte abnormalities that change the cytoadherence properties of infected cells may aid to reduce blood vessel obstruction, coagulation, and inflammation in addition to boosting immune cell identification. There is considerable evidence that these illnesses and disorders are connected to this protective mechanism, but more study on other erythrocyte abnormalities linked to malaria resistance is needed.

Prevalence mutation among children with malaria

In malaria-endemic regions, malaria episodes generate significant levels of childhood anaemia and undernutrition, but severe malaria, comprising severe malarial anaemia, cerebral malaria, and respiratory distress, is linked to high acute mortality (Ndila et al., 2018).

Morbidity and death are notably prevalent among African children and pregnant mothers (Gitta & Kilian, 2020). Many control measures, for example, were developed with the idea that acquired immunity makes an individual non-infectious, hence asymptomatic infections in semi-immune persons therefore play no significant role in transmission (Escalante & Pacheco, 2019). Detecting and measuring gametocytes is required for determining differential patient infectiveness (e.g., those with clinical symptoms vs asymptomatic). (Kroner et al., 2007).

According to the statistics on malaria deaths compiled by the IHME, infants face a significant threat of dying from the disease. The probability of malaria mortality among children increases beyond a temperature of 19.3°C (1.3°C lesser than the optimum for all-age mortality). In Africa, the ideal temperature for malaria mortality in children is found to be 28.2°C which is 0.2°C lower than the all-age death rate. The researcher showed that malaria mortality is still correlated with temperature in this age range. While increases in the 3-month and 6-month SPI both increase malaria mortality, increases in the 99th percentile of precipitation increases malarial mortality in children by 9.7 and 12.8 percent, respectively. These rises are somewhat lesser than the estimates for malaria mortality across all ages. These polymorphisms do not cause host genetic disease, although they are linked to malaria severity. However, in age-matched persons with essentially identical parasite exposures, diverse clinical results are associated with parasites that cause severe malaria with increased or reduced parasitaemia, or parasites that promote uncomplicated vs. severe malaria, without permitting for significant conclusions about the role of parasite transcription in disease outcome.

Nonetheless, differing clinical results have been associated with parasites triggering severe malaria with greater or lower parasitaemia favouring non-severe against severe malaria in age co-ordinated patients with seemingly equal parasite contacts (Milner et al., 2012), or parasites that promote non-severe against severe malaria (Tonkin-Hill et al., 2018), without allowing for significant inferences on the part of parasite transcription in the outcome of the disease (Djokic et al., 2021)

Role of HLA-G in determining the outcome of *P. falciparum* infection

Significant proof of correlations in multiple independent research, including the HLA-B53 and HLA-DRB1 04 allele groups, support the significance of HLA in severe malaria clinical presentation (Catamo et al., 2015). According to Hill et al. (1992), this relationship is strengthened by the likely role of encoded antigens in the HLA-restricted immune response to malaria. The current study's phenotypes associated with HLA polymorphisms are constant with the premise that HLA polymorphisms are undoubtedly implicated in the regulation of relations between the host immune system and parasite (Catamo et al., 2015). Escalante and Pacheco's (2019) findings suggest the contribution of HLA-G in the diversity of response to *P. falciparum* infection. HLA-G polymorphisms were revealed to be related to asymptomatic infection via two parasitological phenotypes: the severity of *P. falciparum* infection and the mean level of parasite density (MLDP) (Escalante & Pacheco, 2019).

Effect of iron fortification and other co-morbidities on malaria development among children

In regions where malaria is common, severe anaemia is commonly blamed on malaria as the primary cause of the condition. Anaemia is a significant contributor to both morbidity and mortality rates in these regions (Ndila et al., 2018). Anaemia has been linked to infections including bacteraemia, hookworm, and HIV, according to a new thorough study in Malawian children. (Calis et al., 2016a).

Additionally, dietary deficiencies and genetic diseases were linked, indicating that anemia might have a variety of causes. In addition, *P. falciparum* parasitemia was substantially connected to severe anemia in parts of periodic transmission but not in holoendemic areas. This may be because it is difficult to determine the cumulative impact of malaria due to the multiplicity of infections that occur in holoendemic areas. (Haldar & Mohandas, 2009a). Even though the patient has malaria parasites, it is important to consider the possibility that the anemia is being caused by something else. The relationships between severe anaemia and nutritional deficiency can be changed by malaria. (Bassat et al., 2008).

Numerous data imply that co-infections with worms or bacteria enhance correlations with severe malarial anaemia in both murine models and human populations (Hartgers et al., 2009a). This is probably due to the way that co-infections alter the cytokine balance. According to several reports, helminthic infections aggravate the inflammatory nature of the immunological response to malaria. (Hartgers et al., 2009b). For instance, in research from Mali, it was discovered that filarial infections modify the *P falciparum*

specific IL-12p70/IFN- secretion pathways (which are IL10 dependent and are believed to be crucial for malaria resistance). When bacteraemia is present, the death rates of severe malaria increase. Gram-positive and gram-negative bacteria are both presents, and as in the previous instance, co-infection likely upsurges the inflammatory nature of the immune response to malaria (Hartgers et al., 2009a).

Effect of iron status on malaria susceptibility

The effect of iron on malaria has been discussed for over a decade, particularly in sub-Saharan Africa (Gwamaka et al., 2012a). Comprehending the impact of iron on malaria risk is critical for developing and executing iron deficiency (ID) control strategies in SSA(Gwamaka et al., 2012b). In children, ID anaemia is related to poor cognitive and motor development, decreased growth velocity, and anorexia (Nancy F. Sheard, 2006). In places with a high prevalence of anaemia, international standards prescribe iron and folic acid supplementation in children under 2years.

As a result, iron supplementation programs are being developed as a principal approach for averting ID and anaemia in pregnant women and children (Gwamaka et al., 2012b). Even though the negative impacts of ID suggest antagonistic intervention, the safety of widespread regular iron supplementation remains unknown. Randomly children chosen in a Tanzanian district where malaria is prevalent, who took iron supplements experienced a 15% rise in all-cause mortality (Sunil Sazawal PhD, 2006). Subsequent investigations have found no risk related to iron supplementation programs (Iannotti et al., 2006a). Meanwhile, Kabyemela et al., (2008), recently discovered that maternal ID is related to a 5.5-fold lower occurrence of

placental malaria (Kabyemela et al., 2008). Furthermore, little research has investigated the effect of the iron level in altering malaria risk in children who are not supplemented.

Malaria infection contributes to the progression and severity of anaemia by activating immunological mechanisms that, among other things, involve the destruction of parasitized RBC and dyserythropoietic dysregulation (Iannotti et al., 2006b). Its relationship to intake is less understood. Other research has shown that there may be further risks linked with malaria infection and iron supplementation in children, increasing public awareness of this association. J Berger, (2000) discovered no statistically increased risk in the iron supplementation groups (J Berger, 2000), however (Sunil Sazawal PhD, 2006) reported a higher frequency of malaria-related adverse events. Sunil Sazawal et al (2006) discovered that iron treatment groups (iron folic acid and iron folic acid zinc) had a 16% higher risk of grave adverse outcomes because of clinical malaria than placebo groups. This group exhibited a higher prevalence of infections associated with clinical signs of cerebral malaria and a malaria-positive blood film. Sunil Sazawal et al. (2006) observed that iron + folic acid treatment increased mortality from cerebral malaria by 70% (Sunil Sazawal PhD, 2006).

Mutation studies with N-ethyl-N-nitrosourea (ENU) revealed that the transferrin receptor 1 (Tfrc) gene is implicated in malaria susceptibility (Lelliott et al., 2015a). Transferrin receptors are necessary for haem synthesis in erythropoiesis, and their absence causes low iron levels in erythroblasts, resulting in microcytosis (Joanne E. Levy, 1999). Poor iron bioavailability in the host body is often related to improved Plasmodium resistance, as

demonstrated by a lower frequency of malaria in children with iron deficiency anaemia, (IDA) (Gwamaka et al., 2012a), whereas iron supplementation increases malaria risk (Goheen et al., 2016). Interestingly, having a low number of transferrin receptors, the Transferrin reception (Tfrc) mutant discovered by Lelliott et al. (2015) had usual haemoglobin levels and higher malaria susceptibility, indicating a different mechanism of action than IDA (Lelliott et al., 2015a).

To conclude, iron deficiency is typically induced by starvation instead of genetic alterations in human populations, therefore these findings are not exactly comparable. In spite of this, mouse models with Tfrc mutations give an ideal platform for future research into the connection between iron levels and Plasmodium in a regulated setting, which is something that human populations cannot provide.

Malaria vulnerability in infants born to women with placental malaria

Babies born to moms with placental malaria (PM) appear to experience their first malaria infection more quickly. (le Port et al., 2011). Immune tolerance (IT) is a phenomenon that could be due to changes in the neonatal immune system development that involve the synthesis of at least certain cytokines in cord blood (Natama et al., 2018). It is known that children born to PM women are at high risk of developing non-malarial fever (Rachas et al., 2012). Moreso, a study recently found that infants delivered to PM women have an elevated probability of the first malaria episode but not future ones (Bouaziz et al., 2018). Intriguingly, a different study discovered that moms with PM, regardless of their sHLA-G level, have a higher risk of giving birth to a child with a high level of sHLA-G during the first two years of life

(D'Almeida et al., 2017). Children born to PM moms' cord blood exhibit an immune response that is characterized by inducible parasite antigen-specific regulatory T-cells that can block T1-type T cell activation (Natama et al., 2018). It is also known that infants born to women with PM are more susceptible to non-malaria fever supporting the concept that immunological tolerance can exceed a precise parasite and that PM might be a biomarker of diverse immunosuppressive phenomena (d'Almeida et al., 2019b).

Substantial evidence emphasizes the part of HLA-G in the destruction of immune responses and immune escape or tolerance (Garcia et al., 2013a). Considerable evidence suggests that HLA-G has a function in immune response suppression and immunological escape or tolerance (Garcia et al., 2013a). A high amount of HLA-G expression is defined as an immunological checkpoint molecule that favours immune evasion. Indeed, HLA-G interacts with T and B lymphocytes, natural killer (NK) cells, monocytes/macrophages, and dendritic cells, as well as ILT4, which is expressed by monocytes, and macrophages, neutrophils, and dendritic cells (Alaoui et al., 2018).

Furthermore, the function of immune cell populations can be controlled by the binding of HLA-G proteins to their inhibitory receptors and stimulating the differentiation of CD4+ (Morandi et al., 2016) (Sabbagh, Courtin, Milet, Massaro, Castelli, 2013d) and CD8+ T cells into distinct subsets of regulatory cells that produce IL-10 and TGF-β and promoting the development of CD4+ and CD8+ T cells into diverse populations of regulatory cells that secrete IL-10 and TGF- β (Pankratz et al., 2014.)(Castelli et al., 2014; de Almeida et al., 2018a). The immunological consequences mentioned

above may occur as a result of PM altering both the mother's and the fetus's HLA-G production.

HLA-G expression upsurges following in vitro infection of primary human trophoblast cells with Toxoplasma gondii, according to Amiot et al., (2014), most likely due to the parasite-induced production of proinflammatory cytokines (Amiot et al., 2014). By establishing a tolerogenic environment, it's likely that HLA-G contributes to the prevention of *P. falciparum* infection very early in life, during the establishment of immunity, or even during fetal life. Research has indeed shown that *P. falciparum* placental infection makes newborns more susceptible to infection in the first few months of life (le Port et al., 2013)

Effect of age on malaria susceptibility and severity

Infants born to mothers with elevated sHLA-G levels are more likely to get malaria than other children, according to some nonexclusive processes (Escalante & Pacheco, 2019). A variety of nonexclusive processes might explain the higher malaria vulnerability of children born to mothers with elevated sHLA-G levels (Escalante & Pacheco, 2019). After early infancy, parasitaemia prevalence and the chance of clinical, even acute illness induced by infection reduce significantly. Young children have rapidly developed "anti-infection immunity," which increases the likelihood and degree of morbidity related to a certain parasite density (Sabbagh et al., 2013b). Infection immunity is never entirely established, and most people are asymptomatic carriers (Escalante & Pacheco, 2019e).

Naturally acquired immunity is dependent on the existence of cytophilic IgG antibodies with varying specificities and perhaps divergent

useful properties. (Sabbagh et al., 2013b). Additionally, children between the ages of 5 and 16 are increasingly being recognized as having an increased risk of developing uncomplicated malaria. They had a greater number of episodes with progressively decreased transmission than their younger counterparts under the age of five (Sabbagh et al., 2013). Maximum parasite frequency in asymptomatic infections has also increased in older children, suggesting a slowdown in the rate at which parasite control is acquired (Espié et al., 2015). Prevention strategies that traditionally targeted children under the age of five must now be extended to older age groups.

A recent study supports the premise that adults are also becoming a demographic that needs to be studied (Nkumama et al., 2017c). Adults are apparently at a higher risk of acquiring clinical malaria episodes, most likely due to declining antimalarial immunity because of diminished parasite exposure (Murungi et al., 2013).

According to Gabonese research, the incidence of microscopically confirmed parasitemia in symptomatic persons presenting to hospitals grew from 19 to 42 percent over 20 years, and these magnitudes may be higher with more effective diagnostic technologies (Nkumama et al., 2017; Koepfli et al., 2015). Adults may also increase the risk to people around them by maintaining low-density chronic illnesses even when the rate of transmission decreases (Trape et al., 2014). It is also vital to note that household surveys for parasitaemia, which significantly contribute to approximations of the malaria burden, sometimes only include children (Nkumama et al., 2017). As a result, determining the exact burden of malaria in older kids and adults is unusual,

and it is unknown how this is evolving over time as transmission declines (Nkumama et al., 2017).

The regional grouping of diseases becomes increasingly obvious when transmission decreases and the percentages of people with asymptomatic or clinical infections decline. Numerous malaria indices, such as occurrence, prevalence, serological markers of exposure, and mosquito density, have been used to identify these places, also known as malaria hotspots. Depending on the amount of study, the size of a hotspot might range from a few homes to entire subnational areas; nonetheless, they normally become more noticeable as transmission decreases (Trape et al., 2014). Identifying hotspots might theoretically aid in improved resource allocation to reduce residual transmission. Hotspot recognition is not universal, and longitudinal studies suggest that it may be dynamic in both place and time, complicating identification and targeting (Bousema & Drakeley, 2011b). From a conceptual sense, targeted malaria control inside hotspots is an enticing approach, but its efficacy has yet to be proved (Bousema et al., 2016).

Current study

Several researchers have examined the medical importance of malaria illness using various approaches, according to the previous literature analysis. They were, however, primarily limited in scope, and none of them discovered a correlation between HLA-G 14bp polymorphism and malaria and anaemia. Severity differed between Ghanaian youngsters who were iron-fortified and those who were not. Some of them investigated the impact of iron on malaria severity (Calis et al., 2016b; Haldar & Mohandas, 2009b). Others were only interested in HLA- G's role in the development and severity of malaria (M. Luo et al., 2013; Naji et al., 2014). Further different studies have attempted

various approaches to examine the impact of host genetics on malaria susceptibility in children under the age of five (Ceesay et al., 2008). Given the present drive and excitement for malaria eradication in Africa, this analysis compares newly published modelled burden patterns to empirical investigations and finds that much more work has to be done. This study examines new obstacles to control in locations where it has been successful as well as issues in regions where it has been limited, indicating research requirements.

HLA-G gene polymorphism has not been researched as well as iron fortification, particularly in the context of malaria. All these investigations revealed that HLA-G expression may contribute to an immune response that influences the result of viral infection. Concerning parasitic infections, all known research has concentrated on sHLA-G levels rather than gene polymorphisms.

The outcomes of this research, which are frequently conflicting, was interpreted with caution due to methodological limitations including a small sample size, cohort heterogeneity, selection bias, phenotypic heterogeneity, or a lack of corrections for gene-environment interactions.

In this study, we will look at the probable connection between HLA-G 14bp and malaria infection severity and anaemia in iron-fortified and non-iron-fortified Ghanaian children in the Tain and Wenchi municipalities in the Bono region for the first time. Because the level of HLA-G expression is likely to be influenced by 30 UTR variations via post-transcriptional mechanisms (D'Almeida et al., 2019), we will concentrate the research on the role of 14bp insertion and deletion polymorphic sites, iron fortification, and

their relationship to malaria-related phenotypes. All these points highlighted the need of understanding the immune response and its potential genetic influence over symptomatic and asymptomatic individuals. In light of this, our agreement with a potential function for HLA-G must be taken as a first insight and explored. This study will present a proof of an association between HLA-G polymorphisms and malaria infection and anaemia in iron and non-iron fortified children in Ghana, which may have prognostic significance for the outcome of parasite infection, even though additional research into the potential role of the HLA-G molecule in the control of *P. falciparum* infection is currently underway (Dahl et al., 2015). The study carried out a thorough review of literature, followed by a meta-analysis of all association studies that have already been published for the 14-bp polymorphism in several disorders, stratified in accordance with children with complicated, uncomplicated and no malaria who received iron supplements, as well as children with complicated, uncomplicated and no malaria who did not receive iron supplements.

Chapter Summary

Research into the function of immune components and host iron fortification may help us understand the aetiology of malaria and the emergence of severe or mild clinical consequences. Meanwhile, a growing body of evidence suggests that HLA-G influences both protective and pathogenic circumstances; as a result, research into HLA-G polymorphism and iron fortification is underway with the hope of advancing our understanding of malaria. This chapter reviewed previous articles on the importance of HLA-G, its relationship to malaria and anaemia, the functions that iron fortification plays in the pathogenesis of malaria, and how other factors affect the expression of HLA-G.

CHAPTER THREE

MATERIAL AND METHODS

Introduction

This is a cross sectional study and archival samples from randomized control trial by Zlotkin et al, 2013 was used. Samples were taken from the Tain and Wenchi municipalities in the Bono East region of Ghana. Children with ages ranging from 6 - 35 months who tolerate solid food and have stayed in the two municipalities for a minimum of six months were recruited for the study. The study subjects were recruited in March and April 2010. They were randomized into two clusters: iron and non-iron groups. The iron group participants were given iron supplements whereas the non-iron group were given a placebo over five months and monitored for a month after discontinuing supplementation. Blood samples were collected as and when the study subjects reported being sick. Malaria parasites were detected using a rapid diagnostic test and Blood microscopy. This chapter describes in detail the study site and design, study participants, inclusion and exclusion criteria, ethical considerations, laboratory analysis, and how data obtained were analysed.

Study Area

The municipalities of Wenchi and Tain in Ghana's Bono region served as the site for this investigation. Nine communities located within the study site were used for the study. The Bono region is a transitional ecological zone between the forest and the savannah in central Ghana. There are 32,329 houses and a total population of about 140,000 in the 7162 km2 region. Most of the locals are subsistence farmers who raise both crops and livestock. The study

involved baseline sampling and a follow-up five-month post micronutrient powder (MNP) treatment. The population of Wenchi and Tain in 2010 (153 633) was typical of Ghana's general population, particularly in rural areas. 11, 215 children below 5 years accounted for nearly 0.3% of Ghana's population of pre-schoolers overall (11 215/533 000) in 2010.

Malaria prevalence among children who are 6-59 months old is high in this region. Transmission of malaria is elevated throughout the raining season (April-November). The prevalence of anaemia among pre-schoolers is 76.1% (95% CI, 73.9%-78.2%). Due to their proximity to the Kintampo Health Research Centre (KHRC), where samples could be stored and analysed, these municipalities were selected. The prevalence of anaemia among pre-schoolers is 76.1% (95% CI, 73.9%-78.2%).

Study Design and Setting

This was a cross sectional study using archival samples from a study done by Zlotkin et al in 2013. The Tain and Wenchi Municipalities in Ghana's Bono region served as the study's locations. There are 99 villages and 8,548 compounds in these two municipalities. The total populace in the Wenchi and Tain municipalities at the time samples were taken was 153,633 in 2010, a true representation of the overall populace in Ghana, and predominantly of rural areas. Children under five years made up 11,215 people in Ghana in 2010, or about 0.3% of the country's total number of children in preschool (3,533,000).

The Bono region has a significant malaria prevalence in children between 6 and 59 months. April - November marks the rainy season in this region and malaria transmission is high during this period. Also, anemia

incidence among the nursery-age population is 76.1% (95% CI,73.9%-78.2%). The two municipalities were chosen because of their closeness to KHRC, where samples could be stored and analyzed.

Sample Size

Children within the ages of 6 to 35 months who have lived within the Tain and Wenchi municipalities for at least six months were involved in the study. In the original work done by Zlotkin et al, a total of 1,958 children living in 1,552 clusters were recruited. A compound with one or more households was defined as a cluster. The Yamane formula was applied to determine the sample size (Israel, 1992) $n=N/1+N(e)^2$

N = the population size

'e'= the margin error (0.05)

n (minimum number of participants) = 1958

Thus, 332 respondents were used for each cluster, resulting in a total of 664 respondents.

However, due to time and financial constraints, 432 samples (244 from iron group and 188 from non- iron group) were analysed.

Participant Selection Criteria

Inclusion criteria

All children in the Tain and Wenchi municipalities of Ghana who are 6 to 35 months and eat quasi-solid meals (including or excluding breast milk) were involved in the work.

Exclusion criteria

The exclusion criteria included children with severe anemia (hemoglobin (Hb) level <7.0 g/dL), acute malnourishment (weight-for-length score<−3.0), those who had taken iron supplements within the past 6 months, and children with chronic illness (e.g., HIV, congenital abnormalities, etc.).

Study participants

For five months, all subjects were randomly given multi-micronutrients powder (MNP) daily with or without iron (12.5 mg) added to healthy diets. During the trial, anti-malaria, mosquito bed nets and medications were readily accessible.

Blood samples of children who had malaria during the 5-month period were taken. Also, samples of participants who did not have malaria during the period were taken after the fifth month. The inclusion criteria for severe malaria were participants with P. *falciparum* parasitemia, with a minimum of any of these ensuing symptoms: weakness, coma (score between 0 and 2 on the Blantyre coma scale). Children with anemia caused solely by asexual *P. falciparum* parasitemia and Hb levels below 5 g/dl were also included in this group. Uncomplicated malaria in children is defined as having an axillary temperature >37.5˚C, along with a blood smear showing *P. falciparum* positivity and Hb > 8g/dl. Healthy children who were aparasitemic were classified as negative control subjects.

Case definition for participants classified as complicated or uncomplicated malaria

A clinical state with an axillary temperature greater than 37.5°C, blood smear positive for *P. falciparum*, parasite count < 100,000, and hemoglobin > 8g/dl was designated as uncomplicated malaria.

Participants with complicated malaria were defined as those who had a positive *P. falciparum* blood smear and a parasite count of greater than 100,000. Additionally, they displayed at least one of the following symptoms: prostration, unrousable coma (Glasgow modified coma scale score between 0 and 2), or repetitive generalized convulsion. Children with P. falciparum parasitemia and hemoglobin levels less than 8 g/dl were considered anemic participants.

Ethical consideration

The following organizations approved this trial; the institutional ethics committee of the KHRC (KHRC/IEC/FEA/2009-2), the Food and Drugs Board of Ghana, the Ghana Health Service ethics committee, and the Hospital for Sick Children research ethics board, Toronto, Canada (REB File No.: 1000013476). were Specifics of the research explained to parents and guardians in the local language and an informed consent of participation obtained.

Data Collection

Participants who attended a health facility were tracked using individual study identity cards that were given to guardians during enrolment. A blood sample was obtained if a child was hospitalized to a medical facility or had a fever (axillary temperature >37.5°C or reported a fever during the

previous 48 hours) to diagnose malaria using RDT (Precheck Device, Orchid Biomedical Systems) and a parasite count via microscopy.

All children were physically assessed by a health professional after which baseline demographic data were taken and recorded. A phlebotomist collected about 2-5ml of venous blood into sterile EDTA tubes from participants and samples stored at -80 °C.

Laboratory analysis

Diagnosis of malaria

Initially, RDT kits were used to check for *P. falciparum* infection. Positive test findings were confirmed by investigation of thick- and thin-film blood smears. Positive blood samples were categorized according to the standard treatment guideline, thus, uncomplicated, and severe malaria. As defined by the standard treatment guideline, Uncomplicated malaria is the evidence of clinical features of malaria with confirmation with laboratory testing and no cerebral involvement or other organ dysfunction while severe malaria was categorised as the presence of clinical features of malaria and confirmed with laboratory testing with cerebral involvement and other organ dysfunction (severe anaemia, hypoglycaemia, renal failure, severe dehydration, and respiratory acidosis).

DNA Extraction

In this study, analysis of stored samples began with DNA extraction using the double salt precipitation method, genomic DNA was isolated from archival blood samples kept at -80°C. The details of the extraction protocol are described in Appendix I.

DNA amplification for HLA-G 14bp +/- Polymorphism

The Genomic DNA isolated was amplified using PCR. PCR is an art of molecular biology that allows to exponentially amplify a target double-stranded DNA sequence (present in small amounts in the sample). This reaction takes place in successive cycles in a thermal cycler. The amplification of HLA-G exon 8 was done using the forward primer 5′GTGATGGGCTGTTTAAA GTGTCACC -3′ and the reverse primer 5′-GGAAGGAATGCAGTTCA GCATGA-3′. The PCR amplifications were done following this protocol: 10x Taq buffer, 2.5 mM 2'-deoxyribonucleotide 5'-triphosphate (dNTP) mix, 3 µl of DNA, 7.13 µl of nuclease-free water,100 uM of each primer, 25 mM of MgCl2 and 1.25U/ uL Taq polymerase (Promega, Madison, CA) in a final volume of 15 µl. The PCR amplification will be done under the following conditions: 95°C for 5 min, 94°C for 60 s, at 61°C for 60 s, at 72°C for 1 min for 33 cycles, and a final extension at 72°C for 10 min. PCR product was then migrated in 3% agarose gel for 40min using a power of 100V. Direct counting was used to determine the total number of 14 bp insertion/deletion alleles.

Table 1: PCR reaction Master Mix Components

Reagents	Initial concentration	Final concentration	Volume (µL)
Free nuclease water(µL)	/	/	7.13
Taq buffer	10X	1X	1.5
MgCl2 (µL)	25mM	2.5mM	1.5
dNTP (µL)	25mM	0.2mM	0.12
Forward primer (µL)	10uM	0.5uM	0.75
Reverse primer (µL)	10uM	0.5uM	0.75
Taq polymerase (µL)	5U/ul	1.25U	0.25
DNA (µL)	/	/	3
Final volume (µL)	/	/	15

•Free nuclease water : Ultrapure water free of DNase and RNase

•Taq buffer with NH_4SO_4 (Promega): It is a buffer distributed by the supplier of Taq polymerase, allowing an optimal activity of DNA polymerase during amplification.

•$MgCl_2$ (Promega): Magnesium ions Mg^2 function as a cofactor needed for the functioning of the Taq polymerase by enabling the inclusion of dNTPs during polymerization. Also, magnesium ions present on the site activate the development of a phosphodiester bond between the end of a primer and the phosphate group of a dNTP. The Cl -, also allow for the maintenance of the ionic strength.

•dNTPs (Promega): dNTPs are deoxyribonucleoside triphosphates. It is designated generally as a mixture of dATP (deoxyadenosine triphosphate), dCTP (deoxycytidine triphosphate), dGTP (deoxyguanosine triphosphate), and TTP (thymidine triphosphate) which are the precursors of DNA monomers, and which are essential for the formation of complementary strand during elongation.

•Forward Primer: 5′GTGATGGGCTGTTTAAAGTGTCACC -3′

Reverse Primer: 5′-GGAAGGAATGCAGTTCAGCATGA-3′: These primers are short sequences of DNA complementary to the beginning of the sequence of the HLA-G gene, which will serve as the starting point for the synthesis of the complementary strand.

•Taq Polymerase (Promega): It is a thermostable DNA polymerase, isolated from the bacteria

The reaction mixture is subsequently dispensed into PCR tubes and DNA samples containing the sequence HLA-G, which will then be placed in a thermal cycler where the reaction of polymerization will take place according to a precise procedure:

PCR Program

Denaturation initial : 95°C for 5min.

Denaturation : 94°C for 1min.

Hybridization : 61°C for 1min.

Elongation : 72°C for 1min.

33X

Elongation final : 72°C for 10min

Hold at 4°C

Initial denaturation (5 min - 95 ° C): This step at 95 ° C will break the hydrogen bonds linking the two DNA strands. It will cause the effect of unhybridized DNA double- strands which will become single-stranded DNA. It also homogenizes the environment and distorts heat-sensitive enzymes that might be present in the solution.

Denaturation (1 min - 94 ° C): This step will unhybridized DNA double-strand which will become the DNA single-stranded.

33 cycles Hybridization (1 min - 61 ° C): This step will allow the specific binding of the forward and reverse primers upstream and downstream of the sequence of interest (HLA-G) thus starting the elongation phase.

Elongation (1 min - 72 ° C): This step allows Taq polymerase to synthesize a strand complementary to the DNA matrix to start of dNTPs stock present in the medium reaction and at a temperature of 72 ° C which is optimum for the functioning of the enzyme.

Final elongation (10 min - 72 ° C): This step corresponds to the extension of the last cycle. Its duration is longer to allow the complete synthesis of the last amplicons in a reaction medium depleted in reagents (dNTPs in particular).

After the reaction of PCR, a gel of agarose is carried out to verify by electrophoresis that the amplification worked well.

Migration of PCR Product

An agarose gel electrophoresis will allow us to verify that the PCR was successful. This technology allows separating DNA in the function of their molecular mass, their shapes, and their charges, under the effect of an electric field. 40ml of TAE 1X buffer was mixed with 0.8g of agarose in a conical flask to make a 2% agarose gel. In a microwave oven, the mixture is then heated to dissolve the agarose in the buffer. After the mixture cools down, 3µl of ethidium bromide (BET) is added to it. BET is a molecule that will fit between the bases of the double-stranded DNA and become fluorescent when exposed to UV rays. This particularly allows for the visualization of the DNA

during the revelation stage. The liquid gel is then poured into an electrophoresis mold with a comb which generate the deposit wells after solidification. At room temperature, the gel is then left to solidifies. The agarose, which is a polysaccharide, will form a matrix made up of pores when it is cooled, which will allow the separation of the DNA fragments. depending on their size during migration. After the gel solidification, the comb is removed and placed in an electrophoresis tank with 1X TAE buffer, which will facilitate DNA migration. The gel is placed so that the wells are oriented towards the negative pole (cathode) of the generator, to allow migration of the DNA (negatively charged) towards the positive pole (anode). On a piece of parafilm, 2µl of the molecular marker of weight 50bp is mixed with 1µl of X6 dye and deposited in the first well.

The molecular weight marker is used to determine the molecular weight of each fragment of DNA that has been amplified when in electrophoresis. 5µl of DNA sample was mixed with 1µl of X6 dye. 6µl of this mixture was loaded in each well and the gel was allowed to run for 45min with a voltage of 90V. Here, if the PCR worked a band should be observed at 210bp (Deletion) or 225bp (Insertion) or both 210bp and 225bp (Insertion/Deletion).

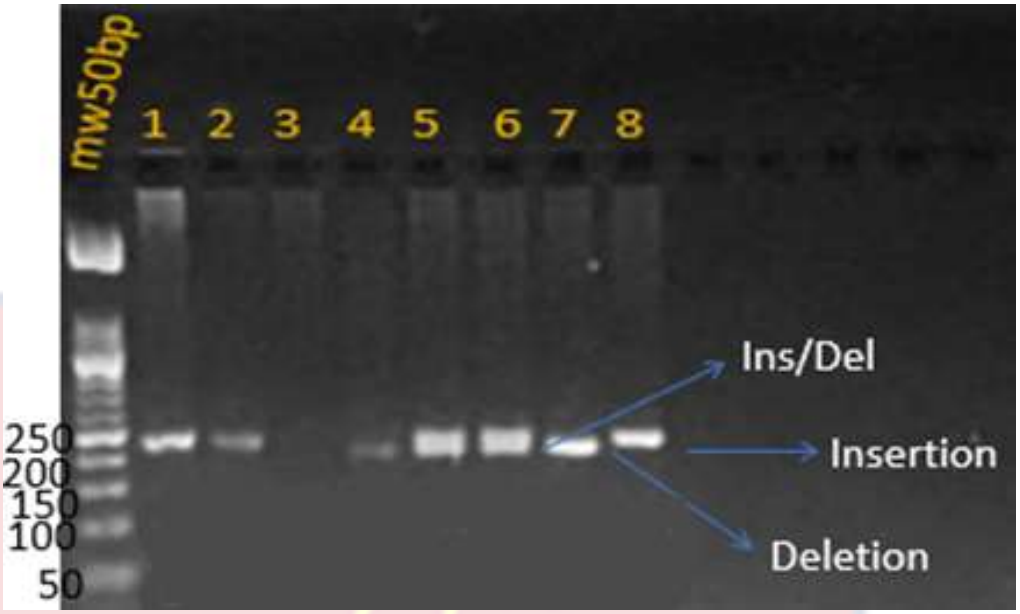

***Figure 5*: A gel electrograph showing the different 14bp bands.**

Wells 1,2 and 8= Insertion, Wells 4 and 7= Deletion, Wells 5 and 6=Insertion/Deletion, well 3 is negative control.

Analysis of Data

The data collected was input in Excel and analysed with SPSS version 21.

Hardy- Weinberg equilibrium (HWE)

This is used to test allelic frequency distribution. A genotypic frequency of a populace in HWE will conform to p2 + 2pq + q2 = 1, where p2 = freq (GG), 2pq = freq (GC), and q2 = freq (CC). To determine whether the population is in HWE the p and q values were calculated as; p = GG + ½GC, and q = CC + ½GC. Given that there are only two alleles in this situation, the frequency of the two must add up to 100%, which is to say; p + q = 1. Where, GG = wild type, GC = heterozygote mutant type and CC = homozygote mutant type.

The $\chi 2$ test was used to estimate the association between the polymorphism and the various clinical sub-groups. Also, univariate, and multivariate Poisson regression models were used to determine the

relationship between the gene variant with malaria severity and anaemia. Statistically a p-value <0.05 was considered significant.

Allele frequency calculation

The percentage of all copies of a gene that contain a specific gene variant is known as the allele frequency (allele). It can alternatively be expressed as the proportion of copies of one allele in a population's genetic location (locus) divided by the total number of copies of all alleles. It can be stated, for instance, as a percentage. Frequency of G (p) = 2(GG) + (GC) / 2 (GG + GC +CC) and frequency of C (q) = 2(CC) + (GC) / 2 (GG + GC +CC). Thus, 1- p = q.

- At a significance level of 0.05, $\chi 2$ test was used to compare allelic frequency of the 14bp polymorphism in both the iron and non-iron groups at a.
- To ascertain the relationship between 14bp polymorphism and malaria disease severity in children, the $\chi 2$ test was used.
- The relationship between 14bp polymorphism and anaemia in children was analysed with multinominal regression model.

Chapter Summary

This ws a cross sectional study and archival samples from a randomized control trial by Zlotkin et al, 2013 was used. The Tain and Wenchi municipalities in the Bono East region of Ghana were the study sites and the study subjects were children between the ages of 6 - 35 months who could tolerate solid food and have stayed in the two municipalities for a minimum of six months. The study subjects were randomized into two clusters: iron and non-iron groups with the iron group being given iron

supplements and the non-iron group given a placebo. Blood samples were collected as and when the study subjects reported being sick. Malaria parasites were detected using a rapid diagnostic test kit and Blood microscopy. Genomic DNA was extracted from blood samples and amplified using a PCR. Amplicons were further migrated on an agarose gel and result counted under a UV light. Data obtained were analysed using statistical package for social sciences (SPSS) version 21. Allelic frequency distribution was tested according to the Hardy-Weinberg equilibrium. Chi-square test was used to estimate the association between the polymorphisms and the different clinical sub-groups. Also, univariate, and multivariate regression models was used to determine the relationship between the gene variants and morbidity/severity.

CHAPTER FOUR

RESULTS AND DISCUSSION

Introduction

The research primarily examined the prevalence of HLA- G 14bp-/+ polymorphism and its association with *P. falciparum* malaria severity and anemia among iron and noniron fortified Ghanaian children. Participants' clinical, sociodemographic, and blood histories were collected. HLA-G 14bp insertion and deletion, *P. falciparum* malaria, and hemoglobin levels were examined in blood samples. The findings are herein presented in the context of existing empirical findings. Demographic data was analyzed using SPSS version 21. The allelic frequency distribution was tested using the Hardy-Weinberg equilibrium, and the correlation between the gene variations and morbidity/severity was determined using univariate and multivariate regression models. The connection between the polymorphisms and the various clinical sub-groups was estimated using the $\chi 2$ test. To fulfill the study's objective, data obtained were analyzed under four categories: Demographic features of study participants in both iron and non-iron group, Association of polymorphism and malaria disease severity in both iron and non-iron group, Association of polymorphism on anemia among iron and non-iron group at baseline and endline and the impact of polymorphism on whether it is related with malaria severity. For all statistical analyses, computations of associations with P values less than 0.05 were regarded as statistically significant.

Results

Demographic features of study subjects in the iron group

For the molecular analysis, a total of 432 samples were used; of these, 244 respondents belonged to the iron group and 188 to the non-iron group. In the iron group, 138 respondents representing 56.6% were males while 106(43.4%) were females. The mean age in months was 19.01 ±8.43. Their infectious status was classified as negative, uncomplicated, and complicated malaria. 107, 85 and 52 respondents respectively. There was no statistical significance difference in the mean ages and infection statuses across the study groups ($p>0.05$). A total of 89 of the children were anemic at baseline and 135 were anemic at endline. At the endline, there was a significant ($p=0.025$) difference between the iron group and the non-iron group in terms of anemia status. A total of 99, 43 and 102 samples were recorded as insertion, deletion, and insertion deletion polymorphisms respectively.

Demographic characteristics of participants in the non-iron group

188 samples were recruited for the non-iron group. 98 respondents representing 52.1% were males while 90(47.9%) were females. The mean age in months was 18.83 ±7.97. Their infectious status was classified as negative, uncomplicated, and complicated malaria. 70, 71 and 47 respondents were recorded for the various infectious status respectively. There was no statistical significance in the difference in mean ages and infectious status across the study groups ($p>0.05$) A total of 65 of the children were anemic at baseline while 123 were not. At endline, 124 were anemic and 64 were not. With a P value of 0.025, the anemia status at the endline was shown to be statistically significant. A total of 59, 44 and 85 samples were recorded as insertion,

deletion, and insertion deletion polymorphisms respectively. WHO defines anaemia as: Hb less than 11 g/dL in children under the age 5 (Aggarwal et al., 2020).

Table 2: Socio-demographic Characteristics of iron and non-iron groups

Characteristics	Iron-Group (N=244)	Non-Iron Group (N=188)	P-Value
Gender			
Male	138 (56.6)	98 (52.1)	0.359[a]
Female	106 (43.4)	90 (47.9)	
Age (in months)	19.01 ±8.43	18.83±7.97	0.823 [b]
Infection Status			
Negative	107 (60.5)	70 (39.6)	0.365 [a]
Uncomplicated	85 (54.5)	71 (45.5)	
Complicated	52 (52.5)	47 (47.5)	
Anemia Status at Baseline			
Yes	89 (57.8)	65 (42.2)	0.683 [a]
No	155 (55.8)	123 (44.2)	
Anemia Status at Endline			
Yes	135 (52.1)	124 (47.9)	**0.025 [a]**
No	109 (63.0)	64 (37.0)	
Polymorphism			
Insertion	99 (62.7)	59 (37.4)	0.105 [a]
Deletion	43 (49.4)	44 (50.6)	
Insertion/Deletion	102 (54.6)	85 (45.5)	
Parasitemia	75693.15± 130358.3	79395.17± 104243.2	0.750 [b]

[a]P-value generated using T-test; [b] P-value generated using Chi-square test.

Allele frequency distribution of 14bp polymorphisms among iron group

The 14-bp polymorphism in the negative group with the highest frequency was insertion, with an allele frequency of 0.67, compared to deletion, with an allele frequency of 0.33. Insertion had the highest allele frequency (0.55 in the uncomplicated group) while deletion had the lowest (0.45). Deletion was the least allele frequency (0.38) recorded in the

complicated malaria group whereas the highest allele frequency (0.63) was insertion. The iron fortified group population recorded highest genotypic frequency of 41,6% for 14bp+/-(Insertion/deletion), followed by 14bp+/+(Insertion) with 40.8% and the lowest for 14bp-/- deletion). All the population were in HWE and there was significant distribution in the various 14bp variant among the iron group population (p=0.041).

Table 3: Allele frequency distribution of 14bp polymorphism among iron group

Type of SNP	Negative (N=107)	Uncomplicated (N=85)	Complicated (N=52)	Total	P-value
-/- (N=43)	17 (39.5)	20 (46.5)	6 (14.0)	43(17.6)	**0.041**
+/- (N=102)	37 (36.3)	37 (36.3)	28 (27.5)	102(41.6)	
+/+ (99)	53 (53.5)	28 (28.3)	19 (18.2)	100(40.8)	
Alleles					
+	0.67(143)	0.55(93)	0.63(66)	0.61(302)	
-	0.33(71)	0.45(77)	0.38(40)	0.38(188)	

+/+ = Insertion, +/- = Insertion/deletion, -/- = Deletion

Allele frequency distribution of 14bp polymorphism among non-iron group

The polymorphism in the negative group with the highest allele frequency was deletion, with an allele frequency of 0.44, as opposed to an SNP insertion allele frequency of 0.56. In the uncomplicated group, insertion had the highest allele frequency of 0.54 and deletion had the least with 0.46. Deletion was the least allele frequency (0.44) recorded in the complicated malaria group whereas the highest allele frequency (0.56) was insertion. The non-iron group's allele distribution, however, did not show any statistical significance (p= 0.939). The frequency of 14bp insertion and deletion in the non-iron group population were 0.55 and 0.45 respectively (Table 4.3).

Table 4: Allele frequency distribution of 14bp polymorphism among non-iron group

Type of SNP	Negative (N=70)	Uncomplicated (N=71)	Complicated (N=47)	Total	P-value
-/- (N=44)	18 (40.9)	17 (38.6)	9 (20.5)	44(23.4)	**0.939**
+/- (N=85)	31 (36.5)	31 (36.5)	23 (27.1)	85(45.2)	
+/+ (N=59)	21 (35.6)	23 (39.0)	15 (25.4)	59(31.4)	
Alleles					
+	0.56(73)	0.54(77)	0.56(53)	0.55(203)	
-	0.44(57)	0.46(65)	0.44(14)	0.45(163)	

+/+ = Insertion, +/- = Insertion/deletion, -/- = Deletion

Association of 14bp polymorphisms and malaria disease severity in iron fortified children

A multinominal logistic regression was used to analyse this objective. The logistic crude analysis performed on participants with uncomplicated malaria in the iron group indicated that, the presence of 14bp -/- was statistically significant(p=0.048) to cause uncomplicated malaria whereas 14bp+/- was statistically insignificant (0.053) to cause uncomplicated malaria. The model was however adjusted for age and sex, and this showed that both -/- and +/- were significant statistically hence having effect on one developing the severe form of malaria among children given iron supplement.

For participants with complicated infectious status: The logistic crude analysis performed on participants with complicated infectious status indicated that, participants with deletion polymorphism were not significantly related to malaria disease severity. However, the model was adjusted for age and sex but still there wasn't any significant association between 14bp -/- polymorphs and malaria disease severity. Therefore, the logistic crude and adjusted analysis indicated that Children with 14bp -/- variants had a less likelihood of getting

complicated malaria infection in the Iron group (P > 0.05). Therefore, no association existed between14bp -/- polymorphs and complicated malaria infection under the influence of iron fortification in the study group. The presence of +/- was statistically significant to cause complicated malaria in both instances when the crude and adjusted analysis were done (0.05). This means that, the presence of +/- influences one developing complicated malaria in the presence of iron fortification among children.

Table 5: Effect of 14bp polymorphism on malaria disease severity among iron group

	Crude Analysis		Adjusted Analysis	
Infection Status	**RRR (95% CI)**	**P-value**	**RRR**	**P-value**
Uncomplicated				
Insertion	Ref		Ref	
Deletion	2.23 (1.01, 4.92)	**0.048**	2.56 (1.13, 5.77)	**0.024**
Insertion/deletion	1.89 (0.99, 3.61)	0.053	2.09 (1.08, 4.05)	**0.029**
Complicated				
Insertion	Ref		Ref	
Deletion	1.04 (0.36, 3.04)	0.944	1.18 (0.40, 3.52)	0.767
Insertion/deletion	2.23 (1.08, 4.60)	**0.031**	2.51 (1.19, 5.30)	**0.016**

The model was adjusted for age and sex; Ref = Negative
RRR: Relative Risk Reduction
Multinomial logistic regression on the effect of Polymorphism type on Infection Status (N=244)

Association of 14bp polymorphisms and malaria disease severity in non-iron fortified children

In the non- iron group, a multinominal logistic regression analysis was performed. The logistic crude analysis performed on participants with uncomplicated malaria indicated that, individuals with 14bp -/- and +/- has no significant link to malaria disease severity (P > 0.05). After adjusting the

model for age and sex also showed that, again, both SNPs have no significant association with malaria disease severity (P > 0.05). Hence in the absence of iron, both 14bp -/- and +/- polymorphisms have no effect on causing Uncomplicated malaria.

The logistic crude analysis performed on participants with Complicated infectious status indicated that, participants with both 14bp -/- and +/- were not significantly associated to malaria disease severity. After the model was adjusted for age and sex, there was still no significant association between 14bp-/- and +/- polymorphisms and malaria disease severity in non-iron fortified individuals (Table 4.5).

Table 6: Effect of 14bp polymorphism on malaria disease severity among noniron group

	Crude Analysis		Adjusted Analysis	
Infection Status	**RRR (95% CI)**	**P-value**	**RRR**	**P-value**
Uncomplicated				
Insertion	Ref		Ref	
Deletion	0.86 (0.35, 2.10)	0.744	0.84 (0.34, 2.08)	0.711
Insertion/deletion	0.91 (0.42, 1.98)	0.818	0.89 (0.40, 1.94)	0.760
Complicated				
Insertion	Ref		Ref	
Deletion	0.70 (0.25, 1.98)	0.501	0.67 (0.23, 1.91)	0.451
Insertion/deletion	1.04 (0.44, 2.44)	0.931	1.01 (0.43, 2.39)	0.975

The model was adjusted for age and sex; Ref = Negative.
Multinomial logistic regression of the effect of Infection Status on Polymorphism (N=188)

Association of 14bp polymorphism on anemia among iron group at baseline and endline

In the iron group, a logistic crude analysis was performed which indicated that, participant with 14bp-/- variants at both baseline and endline had not statistically significance with anaemia. The model was adjusted for

age and sex and there was also no statistically significant relationship between participants with 14bp-/- variants and anaemia at both baseline and endline. Therefore, the 14bp-/- polymorphism was not associated with anaemia even after iron fortification.

Again, a logistic crude analysis performed indicated that, participant with 14bp+/- variants from the Iron group at baseline had no statistically significant relationship with anaemia (P> 0.05). After the model was adjusted for age and sex, there was still no statistically significant association between participants with 14bp+/- at baseline. However, at endline both logistic crude and adjusted analysis showed statistically significance with anaemia. This means 14bp+/- has an association with anaemia among children given micronutrient iron supplantation (Table 7).

Table 7: Effect of 14bp polymorphism on anemia among iron group at baseline and endline

	Baseline				Endline			
	Crude Analysis		Adjusted Analysis		Crude Analysis		Adjusted Analysis	
14bp SNP	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
Insertion	Ref		ref		ref		ref	
Deletion	1.50 (0.71, 3.17)	0.284	1.65 (0.74, 3.66)	0.221	0.81 (0.39, 1.66)	0.561	0.90 (0.43, 1.89)	0.789
Insertion/deletion	1.61 (0.90, 2.88)	0.109	1.68 (0.90, 3.14)	0.100	1.95 (1.11, 3.45)	**0.021**	2.14 (1.19, 3.84)	**0.011**

The model was adjusted for age and sex.

Logistic Regression of Effect of Polymorphism type on Anemia Status at baseline and endline (N=244)

Association of 14bp polymorphism on anemia among non-iron group at baseline and endline

A logistic crude analysis performed indicated that, at both endline and baseline both participants with 14bp -/- and +/- had no statistically significant relatedness with anaemia. After adjusting the model for age and sex there was still no statistically significant link between participants with both 14bp -/- and +/- variants and anaemia at baseline and endline (Table 8).

Table 8: Effect of 14bp polymorphism on anemia among noniron group at baseline and endline

	Baseline				Endline			
	Crude Analysis		Adjusted Analysis		Crude Analysis		Adjusted Analysis	
14bp SNP	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
Insertion	Ref		ref		ref		ref	
Deletion	1.58 (0.70, 3.57)	0.275	1.42 (0.61, 3.29)	0.414	0.99 (0.43, 2.26)	0.984	0.90 (0.39, 2.09)	0.810
Insertion/deletion	1.18 (0.59, 2.41)	0.650	1.08 (0.52, 2.25)	0.833	0.99 (0.49, 2.00)	0.978	0.93 (0.46, 1.90)	0.844

The model was adjusted for age and sex.

Logistic Regression of Effect of Polymorphism type on Anemia Status (Baseline) (N=188)

Effect of 14bp Polymorphism on whether malaria susceptible group will be complicated or not in both iron and non-iron groups

A logistic crude analysis performed indicated that, participant with both 14bp -/- and +/- variants from both the iron and non-Iron groups were not statistically related to malaria disease susceptibility. The model was adjusted for age and sex but again, there was no statistically significant association among participants with 14bp-/- and +/- variants with malaria progression to complicated or severe malaria disease.

Table 9: Effect of 14bp Polymorphism on malaria infection progression to complicated or severe malaria

	Iron group				Noniron group			
	Crude Analysis		Adjusted Analysis		Crude Analysis		Adjusted Analysis	
14bp SNP	OR (95% CI)	P-value	OR (95% CI)	P-value	aOR (95% CI)	P-value	aOR (95% CI)	P-value
Insertion	ref		ref		ref		ref	
Deletion	0.47 (0.16, 1.38)	0.170	0.41 (0.13, 1.24)	0.114	0.81(0.29, 2.29)	0.694	0.87 (0.30, 2.49)	0.792
Insertion/deletion	1.18 (0.55, 2.54)	0.678	1.17 (0.53, 2.57)	0.697	1.14 (0.49, 2.65)	0.765	1.22 (0.52, 2.91)	0.647

The model was adjusted for age and sex. Reference group= Uncomplicated case.

The model was adjusted for age and sex. Reference group= Uncomplicated case

Discussion

The main cause of malaria is the parasite Plasmodium. Despite significant improvements in the prevention of malaria infection and significant declines in morbidity and mortality over the past few years, malaria remains a critical problem for public health in endemic areas. In 2020, the WHO estimated that there were approximately 241 million cases of malaria and 627,000 deaths from the disease. Africa is the most severely afflicted continent, accounting for 96% of all malaria deaths worldwide and 95% of all cases. 80% of the fatalities were in children under five, demonstrating that they are by far the group at the greatest risk of developing malaria. (World Malaria Report 2020). According to studies, iron deficiency anemia may prevent malaria infection, however, supplementing with iron may increase malaria mortality and morbidity. (Zlotkin et al., n.d.). In 2016, WHO therefore recommended iron supplementation in children with or without the threat of anemia in regions where malaria is prevalent.

This study determines the frequency of HLA-G 14bp-/+ polymorphism and its association with malaria disease severity and anaemia in iron- and non-iron-fortified Ghanaian children. As best as we can tell, no available data on HLA-G 14 bp polymorphism for Ghanaian children concerning malaria infection severity, susceptibility, or anaemia exists. We examined three polymorphisms in the HLA-G gene's 3'UTR (14 bp +/-, +/+ and -/-). These three polymorphisms are considered to alter the production of HLA-G proteins and have the potential to influence the amount of HLA-G protein synthesized. Since the 1980s (Piazza et al., 1980), the importance of polymorphisms encoding HLA

antigens has been stressed, and subsequent research has studied an association between HLA alleles and several pathologies, as summarized by Ghosh, 2008.

We compared the frequency of the 14 bp+/- polymorphism in Ghanaian children who received iron supplements and those who did not, and we discovered substantial variations in the frequencies of three SNPs among the iron-fortified children. In the negative group 14bp+/+ had the highest allele frequency while 14bp-/- had the least polymorphism frequency, followed by the uncomplicated group, and the complicated group having the lowest polymorphism frequency. Although no significant difference was identified among the non-iron-fortified group, the negative group had the highest polymorphism frequency of the three SNPs and alleles, with the complicated group having the lowest. Incomparable research, Martelli-Palomino et al., 2013, found that the 14 bp -/- and 14 bp +/-genotypes had higher soluble levels of HLA-G than the 14 bp +/+ genotype, with only the 14 bp -/- genotype reaching significance. The negative group demonstrated a slight increase in alleles prevalent for 14bp+/+ and 14bp -/- as compared to the other group. This finding is like that of Ben Fredj et al., 2016 and Persson et al., 2017, who found that the 14-bp+/+ allele (5'-ATTTGTTCATGCCT-3') is linked to low HLA-G expression and low synthesis of most mRNA isoforms for soluble and membrane-bound molecules. Among the mechanisms postulated to explain these findings, a 14-bp deletion resulted in the loss of at least two polymorphism sites in the HLA-G 3′-UTR, resulting in mRNA shortening and increased stability. Furthermore, the primary transcript's 92 bases remove an important potential target region that might bind to microRNAs and interfere with translation or diminish mRNA stability (Hviid et al., 2006; Martelli-Palomino et

al., 2013). The existence of a 14-bp+/+ is invariably correlated with the presence of +3142G and +3187A, two SNPs linked with low levels of HLA-G mRNA. As a result, the lower quantity correlated with 14-bp insertion may be attributed to the presence of these SNPs. Variations in this region have been linked to susceptibility to many illnesses, most notably infections (Donadi et al., 2011).

A multinominal logistic regression test was applied to examine the correlation among HLA-G 14bp polymorphism and the severity of malaria infection among the iron-fortified Ghanaian children. A significant correlation was found for 14bp-/- and 14bp+/- with malaria disease severity ($p<0.05$). This is in line with the results of the Castelli et al., 2007, who found that children with the HLA-G 3′ UTR-03 haplotype (containing the 14bp-/- allele) have a higher incidence of severe *P. falciparum* malaria episode. According to (de Almeida et al., 2018, Chen et al., 2008 and Rousseau et al., 2003, the presence of a 14-bp+/+ is related to lower mRNA expression compared to the 14bp-/- allele. However, at the protein level, 14bp+/+ allele homozygosity is related to reduced sHLA-G levels, whereas genotypes carry at least one copy of 14bp-/- (Chen et al., 2008). All these results indicate that HLA-G expression may contribute to the immune system microenvironment influencing the outcome of infection.

With regards to 14 bp+/- with uncomplicated outcomes in the iron group, there was no significant variation. (Kroner et al., 2007). A comparative study on the 14-bp+/- polymorphism of the HLA-G gene, detected no association between the 14-bp+/- polymorphism and the severity of malaria infection. Furthermore, Lee & Bae, (2015) reported that the 14 bp+/- polymorphism is not implicated with Multiple Sclerosis, rheumatoid arthritis, or

Crohn`s disease but rather linked to many other infections, suggesting that this polymorphism has disease functionality. HLA-G 14 bp+/- polymorphism might not be a common genetic factor in the development of diverse immunological diseases, and separate pathogenic processes may be involved in their emergence (Lee & Bae, 2015). However, among non-iron-fortified children, there was no significant difference in 14-bp-/- and 14bp+/- polymorphs between children with complicated infectious status, children with uncomplicated infectious status, and children who are negative ($P > 0.05$), which agrees with a similar work conducted by (Levesque et al., (1999). (Donadi et al., 2011) reported that HLA-G polymorphism has multiple immunomodulatory effects, including being favourable in embryo implantation and foetal survival but harmful in malignancies and infections. (Garcia et al., 2013b) discovered a very significant linkage disequilibrium between the 14 bp +/- and a variation in the HLA-G promoter region that may be the genuine causative variant. This result, which has never been documented previously, is critical since the functioning of the 14 bp+/- remained unclear till now. Since most prior research investigating genetic variations in malaria have relied on very large sample sizes in cross-sectional studies, at the expense of a less defined study cohort, the discovery in our study might be linked to the work's small sample size. The presence of severe anaemia makes this study stand out from others in a significant way. Our findings show that HLA-G production levels require other factors than predicted allelic effects on HLA-G expression. The phenomenon is undoubtedly complex and depends on the presence of specific cytokines, microRNAs, transcription factors, and nutrition. All these variables should be considered in future studies.

A logistic crude analysis was performed among participants with uncomplicated malaria in the iron group to examine the impact of HLA-G 14 bp on the development of anaemia during malaria pathogenesis. 14 bp-/- was discovered to be linked to simple malaria. ($p < 0.05$), whereas 4 bp+/- was not significantly different. Despite being published first, the findings agree with those of Marsh & Marsh, (2000), who found that the HLA-G-ILT2 interaction causes a decrease in bone marrow B cell proliferation, leading to acquired aplastic anaemia. Even though the mechanism is unclear, genetic, and environmental variables may have a role in the disease's genesis (Marsh & Marsh, 2000). All these findings underscore the need for researching the immune response and its possible genetic regulation among uncomplicated people who may develop anaemia. In that respect, our findings, which are consistent with a possible function for HLA-G, must be regarded as preliminary and should be investigated.

Chapter Summary

This chapter was split into two sections thus: results and discussion. Findings of data collected were presented in tables and the discussions related the findings to previous knowledge available. This study revealed that, the three HLA-G14bp single nucleotide polymorphisms were present among iron and non-iron fortified Ghanaian children. Allele frequencies for HLA-G 14bp -/- was 38% and 45% among iron and non-iron fortified Ghanaian children respectively. The study found that children with HLA-G 14bp+/- and 14bp-/- variants were associated with malaria disease severity among the iron fortified children but not in the non-iron supplemented children. Also, HLA-G 14bp+/- was associated with the development of anaemia among iron supplemented children.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

Introduction

The thesis is concluded in this chapter. It summarizes theoretical and empirical underpinnings of the study, indicating the scientific rationale behind the study. It also summarizes the scientific methods that were employed in this study. Again, this chapter summarizes the major determinations of our research and makes conclusions from the results of the research. This chapter finally gives recommendations based on the outcomes of our research and includes suggestions for potential research in the present area of research.

Summary of the Study

P. falciparum malaria is known to be the most serious and lethal form of malaria and is to blame for more than 80% of all fatalities globally (World Malaria Report 2019.). In 2020, about 241,000,000 malaria cases were reported worldwide and Ghana reported 2.4% (5,879,506) of the worldwide reported cases. Africa reported 96% of the total malaria mortality reported worldwide and out of this number 80% were children.

HLA-G, a glycoprotein that performs a vital role in initiating the adaptive immune response and essential for recognizing self and non-self. (Carosella et al., 2008) can be beneficial or detrimental as it plays its role. Negatively, by serving as an immunosuppressive molecule that interacts with particular immune cells, HLA-G creates a tolerogenic atmosphere that enables parasites to evade anti-malarial immunity and inhibits the activity of these cells. (Carosella et al., 2008). Positively, it protects the fetus throughout pregnancy by being expressed on the trophoblast of placental cells.

According to Dealmeida 2018, the 14bp+/+ has been associated with lower HLA-G production meaning less susceptibility to malaria and 14bp deletion is linked with higher HLA-G production hence high susceptibility. It is therefore hypothesized that, these mutations could potentially play a role in malaria resistance (Castro et al., 2000).

Studies suggest that while iron deficiency anemia may protect against malaria infection, the availability of iron may increase malaria morbidity and mortality. (Nyakeriga et al., 2004; Zlotkin et al., 2013). Thus, in places with high rates of malaria, WHO suggested iron supplementation in children who have anemia or are at risk for developing it in 2006. This research sought to investigate the prevalence of HLA- G 14bp-/+ polymorphism and its involvement with *Plasmodium falciparum* malaria severity and anaemia among iron and noniron fortified Ghanaian children.

The research reviewed published data and research findings across the globe. It covered major themes such as the etiology, biology, epidemiology, pathogenesis and pathophysiology of malaria and the importance of HLAG and its relationship with malaria and anaemia.

However, no research has examined the potential correlation of HLA-G 14bp gene variants and malaria-associated characteristics and anaemia among iron and non-iron fortified children in Ghana hence the basis for this study.

Archival samples were used for the study and a total of 432 samples were used of which 244 were iron fortified and 188 were not. Genomic DNA was extracted and amplified using a PCR to determine the presence of the polymorphism in each group. Version 21 of SPSS was used to analyze the

data. To evaluate the allelic frequency distribution, the Hardy-Weinberg equilibrium was applied. The connection between the polymorphisms and the various clinical sub-groups was estimated using the $\chi 2$ test.

Summary of the Findings

This research examined four objectives. The molecular study had 432 participants in total, of whom 244 were in the iron group and 188 were in the non-iron group. 138 respondents representing 56.6% were males while 106(43.4%) were females in the iron group with a mean age in months as 19.01 ±8.43. In the non-iron group, 98 respondents representing 52.1% were males while 90(47.9%) were females with a mean age in months was 18.83 ±7.97.

In the first objective, observations revealed that, in the iron group, HLA-G 14bp +/+ had the highest allele frequency while HLA-G 14bp -/- had the least. HLA-G 14bp+/+ still had the highest allele frequency in both the uncomplicated and complicated groups as HLA-G 14bp -/- had the least. The population in this group were all in HWE and statistically significant. In the non- iron group, HLA-G 14bp-/- had the highest allele frequency in the negative group. In both the uncomplicated and complicated groups, HLA-G 14bp +/+ had the highest allele frequency while HLA-G 14bp-/- had the least.

For the second objective that, the presence of HLA-G 14bp -/- was statistically significant to cause uncomplicated malaria whereas HLA-G 14bp +/- was statistically insignificant to cause uncomplicated malaria. Both HLA-G 14bp +/+ and HLA-G 14bp +/- indicated to be statistically significant to have an impact on getting uncomplicated malaria after the model was adjusted for age and sex. It was however observed that children with the HLA-G 14bp -

/- variant were less likely to develop complicated malaria because there was no relationship among HLA-G 14bp -/- and malaria under the impact of iron. HLA-G 14bp +/- was rather significant to cause complicated malaria in the presence of iron.

For the third objective, it was observed that, in the absence of iron, both HLA-G 14bp -/- and HLA-G 14bp +/- polymorphisms had no effect on malaria disease severity ($p > 0.05$).

Finally, in the last objective, it was detected that HLA-G 14bp -/- polymorphism was not associated with anaemia at both baseline and endline even after iron fortification. HLA-G 14bp+/- however was strongly associated with anaemia after iron fortification. For participants in the non-iron group, it was observed that at both baseline and endline, HLA-G 14bp +/- and HLA-G 14bp -/- had no association with anaemia.

Conclusion

In conclusion, this study revealed that, the three HLA-G14bp single nucleotide polymorphisms were present among iron and non-iron fortified Ghanaian children. Allele frequencies for HLA-G 14bp -/- was 38% and 45% among iron and non-iron fortified Ghanaian children respectively. The study found that children with HLA-G 14bp+/- and 14bp-/- variants were associated with malaria disease severity among the iron fortified children but not in the non-iron supplemented children. Also, HLA-G 14bp+/- was associated with the development of anaemia among iron supplemented children.

Recommendation

1. Further research, including those with severe malaria and anaemia, with a larger sample size in Ghana's southern zone, is required to pinpoint the precise influence of HLA-G promoter polymorphisms in the aetiology of severe malaria and anaemia.
2. New, more effective, and far less expensive public health strategies are needed to reduce severe disease and mortality from falciparum malaria in Tain and throughout Ghana.

Suggestions for Future Research

1. Given the HLA-G 14bp polymorphism of Tain and Wenchi populations, which exhibit association with malaria severity and anaemia, more research in diverse populations is critical to confirm our findings.
2. More children and adolescents must be included in future studies to fully understand the biological role and clinical significance of HLA-G in the presence of severe malaria.
3. Extensive research on the teratogenic effects of HLA-G due to its potential therapeutic usefulness as a diagnostic and prognostic biomarker as well as an immune checkpoint target for the treatment of malaria.

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APPENDIX

Appendix I: Protocol for DNA extraction using double salt precipitation method

To lyse red blood cells

1. Add 450µl of TKM1 buffer and 25µl of 0.7% Triton X-100 to 150µl of blood in an autoclaved 1.5ml Eppendorf tube
2. Vortex to mix well
3. Incubate the mixture at 37°C for 5min
4. Centrifuge at 8000rpmfor 3 min and discard the supernatant
5. Repeat steps 1-3 (2-3 times) decreasing the volumes of 0.7% Triton-X-100 by 5µl to completely lyse the RBCs (NB: Repeat steps until a clear colored supernatant and white unpigmented pallets are observed)

Step 1 repetition

	TKM1	Triton-X
1	450µl	25µl
2	450µl	20µl
3	450µl	15µl
4	450µl	10µl
5	450µl	25µl

To lyse the pallets of wbc

6. Add 150µl of TKM2 buffer and 20µl of 10% SDS
7. Incubate the mixture at 37°C for 5min
8. Add 50µl of 6M NaCl and vortex.
9. Centrifuge at 8000rpm for 5min
10. Transfer the supernatant into a new Eppendorf tube containing 150µl of isopropanol

11. Slowly invert each tube to mix and centrifuge at 8000rpm for 10min
12. Wash DNA pallets with 70% ethanol (500µl)
13. Centrifuge at 8000rpm for 5min
14. Carefully discard the supernatant
15. Air-dry the DNA and resuspend in 50µl of TE buffer

To determine the yield of isolated DNA, a few randomly selected samples were migrated on 2% agarose gel for 30min using a voltage of 90v to view the presence and quality of the DNA extracted.

Gel electrophoresis

A 2% Agarose gel was prepared by mixing 0.8g of agarose in 40mls of X1 TAE buffer. 3ul of ethidium bromide was added to the mixture and the gel was cast to solidify.

5ul of each raw DNA is mixed with 1ul of 6X loading buffer. 6ul of this mixture was deposited in each well. 2ul of 50bp molecular weight marker was mixed with 1ul of loading buffer. 3ul of this mixture was deposited in the first well. The gel was set to run for 40min at 90v.

Result of Gel electrophoresis

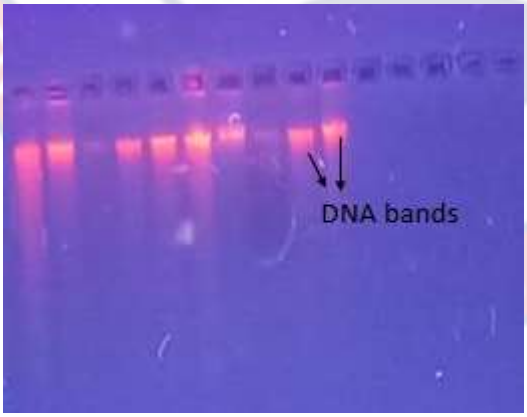

Figure 6: Gel showing DNA bands after electrophoresis.