UNIVERSITY OF CAPE COAST

# HOST GENETIC VARIANTS THAT PROTECT AGAINST CLINICAL MALARIA MAY INFLUENCE ASYMPTOMATIC MALARIA PARASITE CARRIAGE

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BY

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

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# DECLARATION

**Candidate's Declaration**

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

Candidate's Signature …….. Date ……..

Name: CECIL KWAME MFUM ASA–ATIEMO

**Supervisors' Declaration**

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

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

Name:

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

Name:

## ABSTRACT

The outcome of a *Plasmodium falciparum* infection is influenced by host genetic factors. The relationship between host genetics and severe malaria has been studied more intensely than the relationship between host genetics and the more common mild and asymptomatic malarias. With a global change of focus from malaria control to elimination, there is the obvious need to understand and target all forms of the disease, particularly asymptomatic malaria, with its high burden and transmission potential. The Candidate Gene Approach was used to test the hypothesis that symptomatic Malaria-Protection Associated Single Nucleotide Polymorphisms (SNPs) were associated with protection against asymptomatic parasite density. Five SNPs, Toll-Like Receptor 4 (*TLR* 4)-Asp299Gly (A>G), Nitric Oxide Synthase (*NOS2*)-954 G>C, Interleukin 10 (*IL 10*)-592 A>C, Mannose Binding Lectin (*MBL2*) G230A and Interferon-gamma (*IFN-γ*)+874 T>A. These SNPs, with "pro-parasitic" and antiparasitic effects in symptomatic malaria, were genotyped by Restriction Fragment Length Polymorphism and sanger sequencing and tested for associations with asymptomatic *P. falciparum* parasite density, as detected by Photo-Induced Electron Transfer Polymerase Chain Reaction, in school-going children in Simiw, Ghana. *IFN-γ*+874 T>A was associated with a lower asymptomatic parasite density while *MBL2* G230A was monomorphic in the population. According to these results, a host genetic influence on asymptomatic malaria parasite density does exist, partly overlapping that of clinical malaria infections. Parasitic immunity among the malarias may share a similar, yet complex immunogenetics despite their differences in symptomatic severity.

**KEYWORDS**

Asymptomatic Malaria

Candidate Gene Approach

Genotyping

Host Genetics

Parasite Density

Single Nucleotide Polymorphism

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## DEDICATION

*For the love of science; the search for truths we cannot know.*

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## LIST OF ACRONYMS

ADCI - Antibody Dependent Cellular Inhibition

AIC - Akaike Information Criterion

AS-PCR - Allele-Specific PCR

BIC - Bayesian Information Criterion

CI - Confidence Interval

DALY - Disability-Adjusted Life Years

DBS - Dried Blood Spots

eNOS - endothelial NOS

$F_{IS}$ - Wright's Inbreeding Coefficient

G6PD - Glucose-6-Phosphate Dehydrogenase

GAS - Genetic Association Study

GPI - Glycosylphosphatidylinositol

GWAS - Genome-Wide Association Study

*HbC* - Haemoglobin C allele

*HbS* - Haemoglobin S allele

HF-PCR - High-Fidelity PCR

HLA - Human Leukocyte Antigen

HWE - Hardy-Weinberg Equilibrium

IFN-γ - Interferon-gamma

Ig - Immunoglobulin

IL - Interleukin

iNOS - inducible NOS

iRBC - Infected Red Blood Cell

kDa - kilodaltons

LRR - Leucine-Rich Repeat

MAF - Minor Allele Frequency

MAp19 - Mannose-binding lectin Associated protein 19

MASP - MBL-Associated Serine Proteases

MBL - Mannose Binding Lectin

MHC - Major Histocompatibility Complex

MPA - Malaria-Protection Associated

MSA - Malaria-Severity Associated

n - Number of participants

NAI - Naturally Acquired Immunity

NCBI - National Center for Biotechnology Information

NEB - New England Biolabs

NF-κB - Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NK - Natural Killer

nNOS - neuronal NOS

NO - Nitric Oxide

NOS - Nitric Oxide Synthase

OR - Odd Ratio

p/µl - parasites/µl

PAM - Pregnancy-Associated Malaria

PCR - Polymerase Chain Reaction

PET - Photon-induced Electron Transfer

*Pf*IL - *P. falciparum* Infection Level

RBC - Red Blood Cell

RFLP - Restriction Fragment Length Polymorphism

SE – Standard Error

SNP - Single Nucleotide Polymorphism

TAE - Tris-Acetate-Ethylenediaminetetraacetic

TLR - Toll-Like Receptor

TNF-α - Tumour Necrosis Factor-alpha

WHO - World Health Organisation

# CHAPTER ONE

## INTRODUCTION

This chapter provides a comprehensive overview of the present research study with a background to the field of malaria host genetics, the relevant research gap, as well as the aim and objectives of the present study. The chapter closes with the research hypothesis that was considered for the study and the relevance for the study findings to the scientific community and the world.

As a chapter layout, this thesis begins with a study introduction, proceeds with a review of the essential literature, briefly reports the applied methods, presents the results with an engaging discussion and closes with a conclusion on the major findings.

### Background

Malaria is a vector-borne and potentially life-threatening infectious disease caused by *Plasmodium spp*. *Plasmodium falciparum* is the species with the most public health-relevance, while *Anopheles spp*. is the most implicated vector. Malaria is common in Sub-Saharan Africa, Asia and other impoverished settings, with pregnant women and children being at the highest risk of death and injury.

A malaria infection can progress into one of three phenotypes/ outcomes: severe, mild and asymptomatic malarias. Severe malaria presents with life-threatening symptoms. It is common in children less than five years old, pregnant women and adults with immune naivety to the etiological agent. Mild malaria presents with milder symptoms. It is common in older children and adults in endemic areas. Asymptomatic malaria, as suggestive of the name,

does not present with typical malaria symptoms, such as fever. There is, however, some body of literature that argues that asymptomatic infections have unapparent clinical signs and may be prone to subsequent clinical malarial states (Chen et al., 2016). Clearly, a topical understanding of this phenotype is lacking.

The current congruent public health mandate to eliminate malaria is unachievable without addressing the asymptomatic parasite pool. Although symptomatic parasite carriers are more infectious to the vector, they are outnumbered (Coura, Suárez-Mutis, & Ladeia-Andrade, 2006; Nsobya et al., 2004) and outlasted by asymptomatic parasite carriers (Ashley & White, 2014). This makes asymptomatic malaria infections a greater source of infection transmission and persistence in endemic areas (Chaumeau et al., 2019); which should not be overlooked.

The malaria natural history (progression of malaria to one of the three phenotypes) depends on parasite genetics and virulence, host genetics and immune response and the environment (i.e., climate, housing and vector population and dynamics). Although a host's immune response is essentially a product of immunogenetics, there are other determinants, such as age, history of immune exposure, nutritional status, sleep and stress, pregnancy and comorbidities (i.e., acquired immunodeficiencies and coinfections). Put together, genetically-based resistance and cell- and antibody-mediated resistances contribute to host survivability of malaria.

Host genetics, from cytogenetics to immunogenetics, are thus a separate factor in the malaria natural history to be considered. Host genetic determinants include gender, race, primary immunodeficiencies and congenital mutations immuno- and non-immuno-genes. Commonly elucidated is the associated

malaria-protection and -susceptibility afforded by sickle cell trait, thalassemia, ABO blood groups and cytokines, some of which are physiological elements not directly involved in the immune system.

Evidence points to the evolution of population-, geographic-, and environment-specific host defences against *P. falciparum* infections (Tishkoff et al., 2001). Population specificity points to the involvement of host factors, including genetics (Modiano et al., 1998; Modiano et al., 1999; Modiano et al., 1996). Most studies have investigated the influence of host genetics on severe and uncomplicated malarias, with a few studies focusing on asymptomatic malaria and its parasitaemia. Studies investigating asymptomatic malaria host genetics will be relevant in advancing our understanding of asymptomatic malaria and ultimately tackling the menace. Thus, the present study investigated the volatility of parasite density against the backdrop of malaria-relevant host genetic variants, hereby identified as "SNPs of Interest". These parasite characteristics are relevant for their indication of the antiparasitic effects of the immune system. In order to test the hypothesis, later described, specific host genetic variants in the following genes of the innate immune system: Toll-Like Receptor 4, Nitric Oxide Synthase, Interleukin 10, Mannose Binding Lectin and Interferon-gamma were selected for their association with protection or worsening of the clinical state in malaria cases (De Mendonça, Goncalves, & Barral-Netto, 2012).

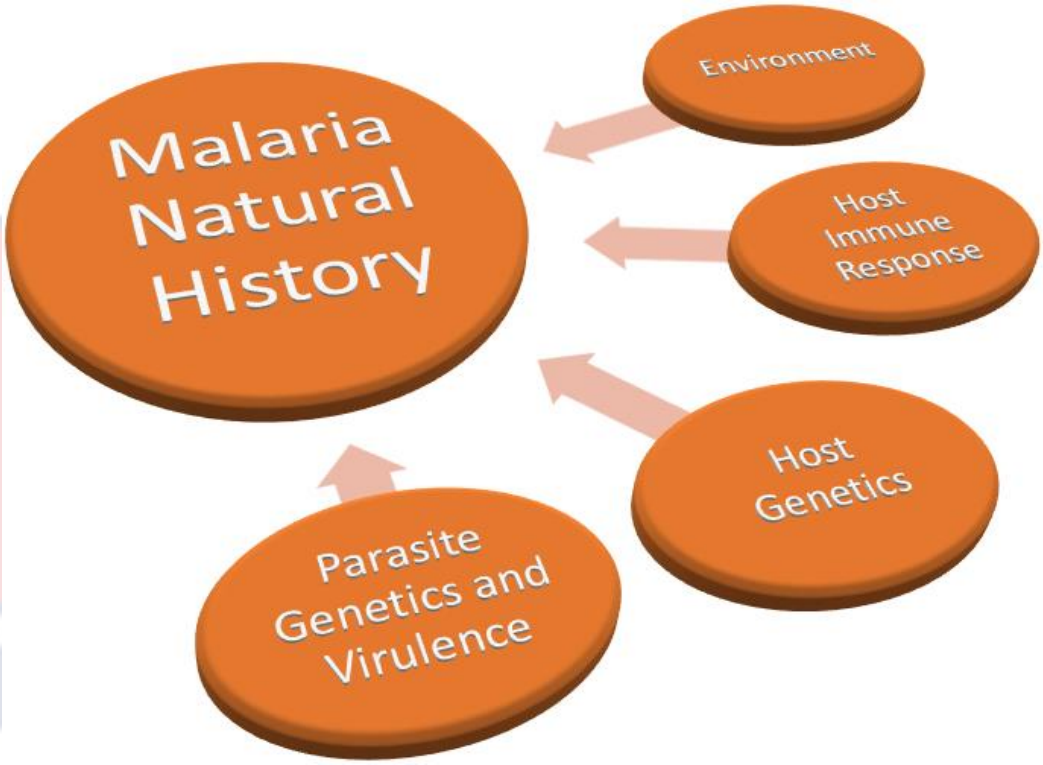



Figure 1 Factors that determine the natural history of malaria.

**Problem Statement**

Symptomatic malarias are a major cause of morbidity and mortality in children less than five years old. Pregnant women constitute another risk group. Pregnancy-Associated Malaria (PAM) can lead to maternal anaemia, abortion, preterm delivery, stillbirth and low birthweight. These emphasise the need and urgency to eliminate malaria. Being a disease of poverty, the public health problem is further compounded by the inadequate accessibility and affordability of quality health services in impoverished communities where malaria is endemic. Although asymptomatic malaria infections do not present a clinical challenge, the migration of symptomless parasite carriers threatens malaria-free areas with disease re-emergence.

In order to achieve malaria elimination, there is a focus on both disease prevention and transmission reduction (Griffin et al., 2010; malERA, 2017; Shim, Feng, & Castillo-Chavez, 2012). Asymptomatic malaria is a major hurdle in transmission reduction, because it is a "silent driver of transmission" (Galatas, Bassat, & Mayor, 2016). That is, since such infections are asymptomatic, they do not trigger treatment-seeking behaviour in the host, and therefore escape medical attention and malaria control programs that use fever-based surveillance systems (Zhao et al., 2018). Therefore, asymptomatic infections can perpetuate the *Plasmodium* life cycle in communities through the constant production of gametocytes, which are needed for host-to-vector transmission. This is especially relevant in places with seasonal malaria, where a large asymptomatic parasite pool is maintained during the dry season, seeding transmission in the wet season, when vector populations rise.

On the bright side, relatively effective host defences and reduced parasite virulence, characteristics of asymptomatic malaria infections, protect the host against morbidity and mortality (Rodriguez-Barraquer et al., 2018). Despite this apparent benefit to the host, research has shown unapparent detrimental health consequences of asymptomatic malaria to the host (Chen et al., 2016). It is therefore unfortunate that school-age children have been identified as a major pool of asymptomatic infections (Coalson et al., 2016).

Tackling asymptomatic malaria requires an understanding of symptomless parasite carriage and related host genetic factors. This can be partly accomplished with host genetic studies. Past research in malaria host genetics had, however, been focused on symptomatic malaria because of its ruinous effects on the vulnerable in society, particularly women and children,

leaving limited data on asymptomatic malaria, with its loss in malignancy and a rise in diagnostic challenge. This has changed as contemporary research has revealed the role of asymptomatic malaria infections in malaria transmission and endemicity. Tackling asymptomatic malaria is a progressive key to improving the health (especially of women and children), education, productivity by elimination of malaria in the modern world (Prusty et al., 2021).

**Aim**

The aim of this study was to identify associations, if any, between selected SNPs relevant to malaria pathology and asymptomatic *P. falciparum* density in school-going children in Simiw, Ghana.

**Objectives**

The aim was accomplished by:

1. Assessing *P. falciparum* infections in malaria asymptomatic school-going children in Simiw.
2. Determining the allelic frequencies of Toll-Like Receptor 4 (*TLR* 4) Asp299Gly (A>G), Nitric Oxide Synthase (*NOS2*)-954 G>C, Interleukin 10 (*IL 10*)-592 A>C, Mannose Binding Lectin (*MBL2*) G230A and Interferon-gamma (*IFN-γ*)+874 T>A among malaria asymptomatic school-going children in Simiw.
3. Determining the association between Single Nucleotide Polymorphisms (SNPs) of Interest and parasite density in malaria asymptomatic school-going children in Simiw.

**Research Hypothesis**

This study investigated the relationship between SNPs associated with clinical malaria and asymptomatic *P. falciparum* carriage/ density (not asymptomatic malaria phenotype). I hypothesised that the protection of symptomatic Malaria-Protection Associated (MPA) SNPs against severe and mild malarias may, by extension, reduce parasite density within an asymptomatic malaria case. This was contrasted with symptomatic Malaria-Severity Associated (MSA) SNPs. Evidence suggests that the mechanisms of malaria immunity may be common in all disease states of malaria infection (Antwi-Baffour et al., 2018; Dobbs, et al., 2020; Dobbs et al., 2017; Warimwe et al., 2013).

**Relevance**

The high degree of malaria transmission contributed by the asymptomatic parasite reservoir maps it out as essential in the malaria elimination process. According to mathematical models, the inclusion of asymptomatic parasite carriers in community-wide drug treatments will help reduce transmission (Bousema, Okell, Felger, & Drakeley, 2014; Okell et al., 2011). It is therefore paramount that all contributing factors, including host genetic factors, to the asymptomatic carriage of parasites are identified. The unexpected negative results of this study, by virtue of elimination, prompt a shift of focus to other candidate genetic variants. Once associated host genetic variants are identified, functional studies can be considered.

Genetic Association Studies (GASs) have been described as *in natura* observational studies of host immunity that complement *in vitro* and *in vivo* findings (Mangano & Modiano, 2014). Excitedly, such functional studies could

help properly define the asymptomatic malaria phenotype. A better understanding of the molecular and cellular events of the asymptomatic malaria phenotype, will also be advisory in translational medicine and the development of public health strategies against the asymptomatic malaria pool. This can be accomplished through the design and development of therapies and vaccines and affect health policies; and ultimately, the lives of the participants of this study.

Nature's mechanics of malaria protection on the host's genetic stage are nearly paralleled by man's efforts against natural selection. Quantitatively, the protection associated with *HbAS* (91%), *HbCC* (73%), and homozygous α-thalassemia (37%) are comparable to that of malaria control efforts, such as Intermittent Preventive Antimalarial Therapy in infants (38%) (Aponte et al., 2009) and in children (87% to 69%) (Dicko et al., 2011; Konaté et al., 2011), and treated bed nets (45%) (Lengeler, 2004). Translational medicine based on host genetic research is more likely to close that gap. In the past, malaria control programs, usually focused on clinical malaria, have led to a 17%-25% reduction in all-cause mortality in children (Binka et al., 1996; D'Alessandro et al., 1995). If history is to be learned from, the public health benefits of malaria elimination, via the tackling of asymptomatic malaria, may be insurmountable.

**Summary**

In summary, malaria host genetics is a relevant research area with many unanswered questions concerning the asymptomatic malaria state. The present study sought to answer the question of the influence of a host's genetics in the case of an asymptomatic infection.

# CHAPTER TWO

# LITERATURE REVIEW

## Introduction

Every good study begins with a thorough review of the existing literature. The context for this particular review was set with an overview of the malaria infection and its three disease states. This was followed with an intertextual and dialogic examination and review of the possible aetiologies of asymptomatic malaria, malaria host genetics and the SNPs of Interest.

## Malaria Disease

Malaria is a microbial disease transmitted through the bite of a female *Anopheles* mosquito, blood transfusion, organ transplantation and needle stick injuries. Approximately, 200 *Plasmodium* species cause malaria in primates, rodents, birds and reptiles. Five of these species cause infections in humans. Of these, *P. falciparum* infection has the highest rate of mortality and causes most clinical cases in the world. After parasites are injected into the host by the vector, the most relevant mode of transmission, they infect the liver and Red Blood Cells (RBCs). The asexual parasites infect, reproduce and rupture RBCs in a blood-stage infection cycle, releasing their metabolites, which cause anaemia and fever (Warrell & Gilles, 2017). Fever, anaemia and other symptoms of an infection may occur 11 to 14 days after the bite. Breaking away from the blood-stage infection cycle, a proportion of asexual parasites commit to gametocyte (sexual stage) development (Eichner et al., 2001), for onward transmission to the vector; the *Plasmodium* life cycle begins anew.

**An Endemic Problem**

The global malaria burden was reported to be 247 million cases in 2021; an increase of 2 million cases from the previous year (WHO, 2022). This rise was reported in the WHO African Region. Although a disruption of health services during the COVID-19 pandemic might explain the surge, malaria deaths stood roughly unchanged at approximately 600,000 (14.8 per 100,000 people at risk). Seventy-six percent (76%) of these deaths were of children under 5 years, while approximately 32% of all pregnancies were recorded with PAM (WHO, 2022).

Another useful metric of malaria burden is the Disability-Adjusted Life Years (DALY); the loss of years of healthy living because of death or disability. The DALY of malaria can range from 251.09 DALYs in Zimbabwe (Gunda, Chimbari, & Mukaratirwa, 2016) to 365,900 years in Ethiopia (Girum, Shumbej, & Shewangizaw, 2019). Additionally, according to macroeconomic studies, high malaria endemicity reduces a country's economic growth by a percent every year (Malaney, Spielman, & Sachs, 2004).

Over the years, several efforts have been made to control this Apicomplexan disease. These include surveillance, investment in research and development and the distribution of diagnostics, as well as anti-malaria and -vector therapies. However, there have been hindrances to malaria control and elimination goals, including parasite and vector drug resistances, host and vector behavioural factors, climate change and the many limitations of the developing world. Adding on, almost excitedly, asymptomatic malaria presents an invisible challenge to malaria control and elimination. The arms race continues.

**Three States of Malaria Disease**

Malaria infections could result in either symptomatic or asymptomatic disease states. Symptomatic malarias could manifest as either complicated (severe anaemia, cerebral and placental) or uncomplicated malarias.

**Symptomatic Malarias**

Less than three percent (<3%) of all malaria cases progress to the complicated disease state (The Malaria Genomic Epidemiology Network, 2008). Severe malaria involves a myriad of symptoms: fever, anaemia (less than five (<5) g/dl of haemoglobin or <10% packed cell volume), hyperparasitaemia (>250,000 parasites/µl or more than five percent (>5%) of infected red blood cells, Acute Respiratory Distress Syndrome, jaundice, renal damages and hyperlactatemia. Cerebral malaria, in particular, can lead to a loss of consciousness, convulsions, increased intracranial pressure, encephalopathy, hemiplegia, coma and death. Severe malaria may also be accompanied by secondary bacterial sepsis and Disseminated Intravascular Coagulation.

Uncomplicated/ mild malaria presents with fever and non-specific malaria symptoms. The milder symptoms of uncomplicated malaria include cyclic fever, chills, headaches, anaemia, vomiting, diarrhoea, jaundice, splenomegaly and a reduced appetite. Symptoms are usually accompanied by a parasitaemia between 1,000 to 50,000 parasites/µl (p/µl) and a haemoglobin level greater than eight (>8) g/dl. Uncomplicated malaria occurs in semi-immune people, as opposed to severe malaria which occurs in children less than 5 years old because they lack a sufficiently developed specific immunity from a history of exposure, primigravidae women who lack parity-specific immunity and immune-naïve people from non-endemic countries.

**Pathophysiology**

As with any infectious disease, a pathophysiology exists to explain clinical malaria presentations. The destruction of parasitized and non-parasitized RBCs causes anaemia (White, 2022), while the rupture of parasitized RBCs releases parasites' toxic by-products, which cause anaemia and fever (Warrell & Gilles, 2017). *Plasmodium* toxins trigger the release of Tumour Necrosis Factor-alpha (TNF-α) and IL 1 from monocytes and macrophages, which, in turn, trigger the release of IL 6 and IL 8 and the expression of host adhesion molecules involved in cytoadhesion. Parasite sequestration in the brain, placenta and lungs can cause occlusion, characteristic in PAM and malaria cases with brain and lung involvement. The ratio of pro-inflammatory to anti-inflammatory responses in the host has also been implicated in malaria immunopathology (Achidi et al., 2013; Kurtzhals et al., 1999; Othoro et al., 1999; Ouma et al., 2008). For instance, high levels of pro-inflammatory cytokines, IL 1, IL 1β, IL 6, IL 10, IL 12 and TNF-α, have been correlated with malaria severity (Lyke et al., 2004).

**Host Immune Response**

Survivors of symptomatic malaria infections exhibit two defence strategies: (a) Infection Resistance and (b) Disease Tolerance.

Infection resistance or immune resistance is the host's ability to control parasite load through effective immune responses. A rise in pro-inflammatory cytokines and antibody responses are, thus, relevant in infection resistance (Ibitokou et al., 2014), although uncontrolled responses have been associated with immunopathology. Antiparasitic immunity, the driver of infection resistance, appears to be exposure-independent and correlates with the maturity

of the host's immune system in high and moderate transmission areas (Prusty et al., 2021). Therefore, older children and adults tend to have submicroscopic parasitaemia, usually with milder or no clinical presentations. Antiparasitic immunity is measured as an inverse of parasite burden. At this point, it is worth noting that asymptomatic malaria can occur at any age (Lindblade, Steinhardt, Samuels, Kachur, & Slutsker, 2013).

Disease tolerance, or simply tolerance, is the ability of the host to protect tissues and organs from parasite virulence and immunopathology without influencing parasite load or fitness (Vinhaes et al., 2021). Tolerance may be determined by both host and parasite factors (Galatas et al., 2016; Laishram et al., 2012), as opposed to infection resistance, which is only determined by host factors. One such host factor is the modulation of pro-inflammatory responses (Ademolue, Aniweh, Kusi, & Awandare, 2017; Vallejo et al., 2018). Antidisease immunity, the driver of disease tolerance, is exposure-dependent (Gonçalves et al., 2014). Exposure-dependent immunity to severe malaria is typically established after survival of the first or second clinical malaria episodes (Gupta, Snow, Donnelly, Marsh, & Newbold, 1999). As such, children in malarious regions acquire protection against severe malaria from the ages of two to five years and exhibit a lowered frequency of clinical malaria incidences by adolescence (Filipe, Riley, Drakeley, Sutherland, & Ghani, 2007). Tolerance is measured as a slope of host fitness against infection intensity (Galatas et al., 2016). Though antiparasitic and antidisease immunities have different effectors, mechanisms and outcomes, they are not mutually exclusive (Galatas et al., 2016; Prusty et al., 2021).

With that said, survivors of asymptomatic malaria exhibit one immunity, premunition. Premunition is an exposure-dependent non sterilising/ partial immunity to malaria that appears to provide both antidisease and antiparasite immunities to the host (Galatas et al., 2016; Lindblade et al., 2013). As such, it is likely that the establishment of asymptomatic malaria is dependent on both host and parasite factors. It is interesting to wonder about the youngest age at which one could develop asymptomatic malaria, considering antiparasitic immunity is maturity-dependent while anti-disease immunity is not. Whatever the answer, malaria asymptomatic children usually have a higher parasitaemia than asymptomatic adults, possibly because adults have a more capable antiparasitic immunity (as expected). To resound, the phenomenon, that is asymptomatic malaria, is not fully understood.

**The Invisible Challenge with Malaria**

With such severity of symptoms, clinical malaria can cause losses in productivity, injury and death. Over the years, public health efforts have been made to control and eliminate malaria, however, these have not been without challenges. Distinct and unavoidable, is the challenge of silent malaria infections, the focus of the present study. Although their asexual parasites do not present a clinical risk to infected individuals, asymptomatic infections create a near-perpetual source of sexual parasites, sustaining parasite transmission beyond the limits of season and weapons of Public Health.

## Asymptomatic Malaria

### Starts with Definition

There is no standardised definition for an asymptomatic malaria infection. However, it can be described as an infection without fever or acute malaria symptoms at any parasitaemia in the absence of antimalarial treatment (Lindblade et al., 2013). WHO defines it as the presence of blood parasitaemia without accompanying malaria symptoms (WHO, 2016). These definitions, unfortunately and inadequately, do not exclude pre-symptomatic (in the incubatory period) (Bousema et al., 2014) and intermittently symptomatic chronic infections (Hamainza et al., 2014) not perfectly controlled by the host's immune system (Chen et al., 2016). That being said, asymptomatic malaria may yet still represent a form of disease tolerance to symptomatic disease or a mode of entry into symptomatic malaria, as thought by others (Degefa, Zeynudin, Zemene, Emana, & Yewhalaw, 2016). A query to ponder is the highest parasitaemia that can be reported for the phenotype. An interesting group that complicates this discussion is infected infants less than 6 months old, who are asymptomatic with a low parasitaemia (<100 p/µl) afforded by maternal antibodies transferred *in utero* (Dobbs & Dent, 2016).

### Continues with Transmission

Although asymptomatic infections are more likely to have low parasite densities and less likely to infect mosquitoes than symptomatic infections (Bousema et al., 2012; Schneider; et al., 2007), they make a significant and sustained contribution to the overall transmission of *Plasmodium* (Alves et al., 2005) because of their higher prevalence, protractedness, higher quality of gametocytes (Hallett et al., 2006; Nwakanma et al., 2008; Paul, Bonnet, Boudin,

Tchuinkam, & Robert, 2007) and clinical silence. The percentage contribution of asymptomatic malaria to malaria transmission ranges from ~20% to 90% (Bretscher, Maire, Felger, Owusu-Agyei, & Smith, 2015; Buchwald et al., 2019; Chen et al., 2016; Lin, Saunders, & Meshnick, 2014; Lin Ouédraogo et al., 2016; Lindblade et al., 2013; Okell et al., 2012; Tadesse et al., 2018). It is interesting to note that the risk of gametocyte carriage increased with afebrility (Degefa et al., 2016; Sowunmi, Fateye, Adedeji, Fehintola, & Happi, 2004; von Seidlein et al., 2001).

**Ends with "Morbidities"**

Despite the name, asymptomatic infections may be associated with some unapparent morbidities, at least indirectly, unless treatment is given. Asymptomatic malaria has been diagnosed alongside non-typhoidal *Salmonella* and Kaposi Sarcoma-associated Herpesvirus infections (Biggs et al., 2014; Mackenzie et al., 2010; Wakeham et al., 2013). Asymptomatic malaria has also been associated with worsening Nodding Syndrome (Kariuki et al., 2011; Ogwang et al., 2018), reduced effectiveness of measles and Bacillus Calmette-Guérin vaccinations (Kizito et al., 2013; Lule et al., 2015), cognitive impairment and reduced academic performance (Nankabirwa et al., 2013; Vitor-Silva, Reyes-Lecca, Pinheiro, & Lacerda, 2009). If a definition allows it, asymptomatic malaria has also been associated with subsequent recurrent symptomatic malaria episodes (Omonuwa & Omonuva, 2002; Prusty et al., 2021). Obviously, these associated comorbidities have serious health consequences. As such, Cheaveau et al. (2019) described asymptomatic malaria as a misnomer, recommending a reclassification under "subclinical" malaria.

Chen et al. (2016) also had the view that asymptomatic infections are not truly devoid of symptoms and should be renamed as “chronic malaria infections”.

**Further Thoughts**

A host will not present with the symptoms of an infection when the infecting microbe is non-pathogenic or pathogenic and in an incubatory or dormant stage. *P. falciparum* parasite is not benign and studies have positively correlated parasite density with symptom severity, in some respects (Smith, Killeen, Lengeler, & Tanner, 2004). It is therefore understandable that a very low parasite load, especially in a host without sterilising immunity, usually accompanies a seeming-lack of “classic” malaria symptoms. However, technically, some pathogenesis at the lowest parasite densities should be expected. I propose case and case-control studies to investigate correlations between low parasite densities and “morbidities” of asymptomatic malaria, alongside *in vitro* studies to ascertain possible cytopathic effects, sequestration and inflammation at these low parasite densities. The findings of these suggested studies may very well warrant a reclassification of malaria outcomes from a symptom-wise class system to a parasitaemia-symptom-wise class system. This theory does not account for asymptomatic malaria with higher parasite densities; of course, the current classification has been called to question.

## Aetiologies

### Host Factors

Premunition, also known as Naturally Acquired Immunity (NAI), is probably the main cause of the asymptomatic malaria state (Cheaveau et al., 2019). NAI is not strain-specific but depends on the transmission setting as individuals with persistent asymptomatic infections are usually exposed to more infective bites than symptomatic individuals (Cheaveau et al., 2019). Additionally, in areas of high endemicity, there is an early age-development of NAI. Premunition may develop slowly because of the immune-evasive strategies of parasites, such as antigenic variation (Obi, Okangba, Nwanebu, Ndubuisi, & Orji, 2010).

Premunition usually presents with low parasitaemia and no clinical disease. It is mediated by protective antibodies to various *Plasmodium* stages that may employ Antibody Dependent Cellular Inhibition (ADCI) against intraerythrocytic parasite forms (Obi et al., 2010). Antibodies play a role in malaria defence because of the lack of Human Leukocyte Antigens (HLAs) on infected Red Blood Cells (iRBCs). ADCI has been suggested to reversibly impede the development of parasites, possibly resulting in the low parasite densities of most asymptomatic infections (Galatas et al., 2016). ADCI against merozoites is only triggered at a certain merozoite density, explaining why ADCI cannot completely sterilise parasites, being inactivated once the merozoite density falls below a threshold. Antibodies that mediate premunition also cooperate with immune effector cells, such as monocytes and polymorphonuclear cells. Children with premunition, yet no circulating antibodies, may possess antitoxic immunity (Obi et al., 2010).

The question of the role of cytokines in the asymptomatic malaria phenotype is unsettled. Asymptomatic infections have been largely associated with a decrease in pro-inflammatory responses and an increase in anti-inflammatory responses, relative to the immunoregulatory cytokine IL 10 (Ibitokou et al., 2014; Kimenyi, Wamae, & Ochola-Oyier, 2019; Wilson et al., 2010). That being said, asymptomatic malaria has also been associated with a suppressed immunoregulatory response, on one hand (De Jong et al., 2017; Wammes et al., 2013), and a balanced ratio of pro-inflammatory to anti-inflammatory responses on the other hand (Frimpong et al., 2020). Finally, asymptomatic infections have also been characterised by immunosuppressive limited T cell activation and regulation (Frimpong, Kusi, Tornyigah, Ofori, & Ndifon, 2018).

**Parasite Factors**

Parasite biology and physiology may also factor in the development of asymptomatic malaria. Parasites may establish asymptomatic infections by delaying ring stage development over weeks to months; entering dormancy or quiescence during the $G_0$ phase (Nyarko & Claessens, 2021). Although the parasite multiplication rate in asymptomatic infections has not been measured, it was three times higher in complicated malaria cases than uncomplicated cases in Thailand (Chotivanich et al., 2000), but not significantly different in Mali and Kenya (Deans et al., 2006). Deans et al. (2006) suggested that the pathophysiology of malaria may differ between sub-Saharan Africa and Asia. However, if a pattern is to be recognised, parasites in asymptomatic infections might have a relatively lower rate of replication.

The epigenetic environment of the parasite may, by virtue of febrility, also play a role in the establishment of low-density mild and asymptomatic infections, and the switch between chronic to acute infections (Merrick et al., 2012). *Plasmodium* transcriptional regulatory protein, *P. falciparum* Sirtuin 2a, which is responsible for the epigenetic regulation of *var* gene switching (Duraisingh et al., 2005), may be influenced by the high temperature and $NAD^+$/NADH changes of febrile malaria to modulate *Plasmodium* rDNA transcription (Oakley, Gerald, McCutchan, Aravind, & Kumar, 2011), thereby influencing ribosome synthesis, as well as, parasite growth and replication (Mancio-Silva, Lopez-Rubio, Claes, & Scherf, 2013). This may be related to the increase in risk of gametocyte carriage with afebrility (Degefa et al., 2016; Sowunmi et al., 2004; von Seidlein et al., 2001).

Finally, asymptomatic malaria infections may also be unique to certain parasite strains (Claessens et al., 2014; Galatas et al., 2016; Rottmann et al., 2006). For instance, drug-resistant parasite strains are more likely to be found in asymptomatic infections (Dokunmu et al., 2019; Ikegbunam et al., 2019; Ojurongbe, Tijani, Fawole, Adeyeba, & Kun, 2011; Tukwasibwe et al., 2014).

**Transmission Setting and History**

Although not an aetiology, it is worth noting the influence of transmission setting and history on the epidemiology of asymptomatic malaria cases. The prevalence of asymptomatic infections in high transmission settings, where acquired immunity develops rapidly to control parasitaemia, ranges from 20% to 97% (Lindblade et al., 2013) and exists as less than 10% in low transmission settings (Laishram et al., 2012). In low transmission areas, asymptomatic carriers make up approximately 60% of the malaria infected

populations (Sturrock et al., 2013); a greater proportion than that in high transmission areas.

Certain malaria elimination interventions may also provide a selective pressure for less virulent and drug-resistant strains that are associated with asymptomatic malaria (Galatas et al., 2016). For instance, a high proportion of asymptomatic infections in recently endemic areas (less than eight years ago) with residual malaria transmission may implicate a persisting immunity in the development of asymptomatic malaria. In corresponding fashion, there is a lower prevalence of asymptomatic infections in places where transmission was interrupted more than seven years ago (Galatas et al., 2016). In addition, low immune-stimulation by less virulent parasites and rapidly waning immunity in low transmission zones have also been suggested to be a cause of low-density persistent infection in such areas (Crompton et al., 2014). A better understanding of asymptomatic malaria is therefore necessary to properly consider the long-term consequences of public health intervention strategies.

**Microscopic and Submicroscopic Asymptomatic Malaria Infections**

Malaria researchers categorise malaria cases under microscopic or submicroscopic because of the diagnostic relevance of optical microscopy in Public Health and epidemiology. This has become a trend in the field permitting the comparison of findings and its application by public health offices.

Asymptomatic malaria may present with either microscopic or submicroscopic parasite densities. Microscopic malaria infections are malaria infections that can be detected by microscopy and PCR, while submicroscopic malaria infections can be detected by PCR but not microscopy. The greater

sensitivity of PCR over microscopy is leveraged to detect the lower parasite densities of submicroscopic infections.

Most asymptomatic infections are submicroscopic (Bousema et al., 2014). The mean parasite density of asymptomatic infections is approximately five parasites/µl (~5 p/µl), below the 30-500 p/µl detection limit of microscopy (Imwong et al., 2016). In high transmission settings (>75% microscopic parasite prevalence), submicroscopic infections are estimated at 20%, and 70% to 80% in low transmission settings (<10% microscopic parasite prevalence) (Lindblade et al., 2013). Despite the difference in proportions of submicroscopic infections in both settings, submicroscopic infections in high transmission zones outnumber those in low transmission zones.

Asymptomatic infections in high transmission settings may have higher parasite densities than those in low transmission settings (Slater et al., 2019). Intriguingly, the rate of asymptomatic microscopic infections tends to be significantly higher in children than adults, indicating an age-dependent factor (Baliraine et al., 2009; Franks et al., 2001; Igbeneghu, Odaibo, Olaleye, & Practice, 2011; John et al., 2005).

**Malaria Host Genetics**

The heritability of susceptibility and resistance to infectious diseases has been known for a long time. Surprisingly, this heritability might be higher than the heritability of non-infectious diseases, which usually have evident genetic bases. A study of adoptees in Denmark, over 75 years ago, reported that offspring of people who died of infectious diseases had a 5.8-fold increased risk of also dying of an infection (Sørensen, Nielsen, Andersen, & Teasdale, 1988).

This value was higher than the 1.2-fold and 5.2-fold increased risk of death from cancer and cardiovascular diseases respectively, in similar circumstances. Death from cancer and cardiovascular disease may be influenced by the environment to a greater extent than the host's genetic background. Other studies have also reported this relationship between host genetics and infections. Jepson et al. (1997) reported that monozygotic twins were more likely to experience fever during a malaria infection than dizygotic twins. Sjöberg et al. (1992) reported that monozygotic twins had much similar anti-Ring Erythrocyte Surface Antigen titres than dizygotic twins, age- and sex-matched siblings and unrelated people in similar transmission settings. Ranque et al. (2005) also reported a sibling risk of 2.5 for cerebral malaria and 4.9 for malarial anaemia; "sibling risk" referred to both genetic and shared environmental factors.

Malaria has had the strongest selective pressure on the human genome. This is evident by the high frequencies of certain MPA hemoglobinopathies and polymorphisms in Africa (Kwiatkowski, 2005). Additional evidence that points to the relationship of malaria with host genetics is the geo-historical overlap of MPA alleles and malaria hotspots. With that observation, John Burdon Sanderson Haldane, the founder of population genetics, theorised the "malaria hypothesis". It describes how natural selection has increased the frequencies of MPA hemoglobinopathies and immunological polymorphisms in malarious regions. Haldane's hypothesis stipulated a "balanced polymorphism/ compensated polymorphism", where Haemoglobin S allele (*HbS*) is maintained in malaria endemic populations by a high frequency of the heterozygous *HbS* genotype, despite the clinical consequences of homozygous *HbS* genotype. Taylor, Cerami, and Fairhurst (2013) recognized this selective pressure of

malaria as a "natural experiment" that could help uncover the cellular and molecular mechanisms of malaria pathology and immunity. It is also interesting to note that host and vector immune systems exercise a selective pressure on approximately 10% of the parasite's genome (mostly consisting parasite membrane proteins) (Kidgell et al., 2006).

**Percentage Contribution**

Addictive host genetic factors account for approximately 25% of variation in incidence of uncomplicated *P. falciparum* malaria and over 33% of the variation in severe malaria (Kariuki & Williams, 2020). Two percent (2%) of the variance in severe malaria is accounted for by both *HbS* and α-thalassemia. The relatively small contribution of *HbS*, a vintage MPA allele, indicates that there are several unknown MPA alleles ("missing heritability"), possibly with addictive effects.

The Fulani group, an ethnic group with an iconic protection against malaria, has been shown to lack or have very low frequencies of classic MPA alleles, such as *HbS*, *HbC*, α-thalassemia, Glucose-6-Phosphate Dehydrogenase Deficiency A- (G6PDA-) and HLA-Bw53 (Modiano, Luoni, Sirima, Lanfrancotti, et al., 2001). This also suggests that there may be other genetic factors that influence malaria resistance (Mangano, 2008).

According to Mackinnon, Mwangi, Snow, Marsh, and Williams (2005), unidentified household factors explained an additional 29% of the total variation in severe malaria. With a similar percentage contribution to malaria as environmental factors, malaria host genetics also demands a spotlight in public health planning and community awareness.

Back on the genetic stage, investigations of the natural history of malaria are excitedly complicated by allele-allele interactions, allele-environment interactions, polygenicity and epigenetics, any of which may very well elucidate the missing heritability. For instance, positive and negative epistases have been reported for the associated-protection of some malaria-relevant alleles (Amenga-Etego, 2012; Goheen, Campino, & Cerami, 2017; Mmbando et al., 2015; Mpimbaza et al., 2018; Penman, Pybus, Weatherall, & Gupta, 2009; Wambua, Mwacharo, Uyoga, Macharia, & Williams, 2006; Williams, Mwangi, Wambua, Peto, et al., 2005).

a. b.

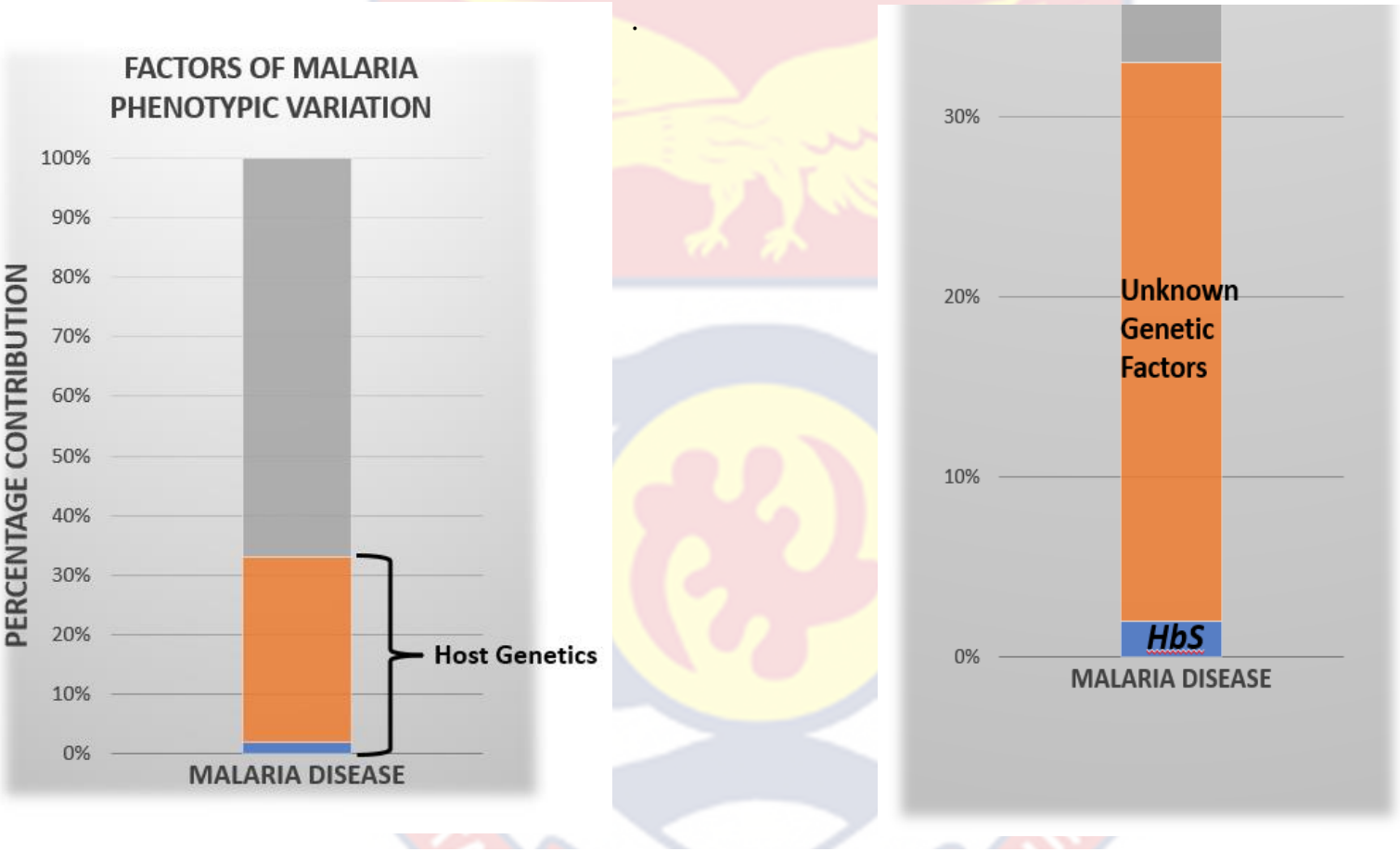



Figure 2a Host genetic factors contributing to malaria disease outcome.

b. Zoomed-in view details in host genetic factors

(Kariuki & Williams, 2020)

**Host Genetics and Symptomatic Malaria**

Polymorphisms are genetic variations that occur in at least one percent (≥1%) of a population. Polymorphisms include point mutations (i.e., SNPs), insertions and deletions, which may or may not be associated with a disease or clinical complication. The distribution of polymorphisms is influenced by natural selection, migration and genetic drift among others.

There is evidence that susceptibility and resistance to malaria are influenced by variations in the host's genetic makeup. One SNP that has been associated with severe malaria susceptibility is *TNF-α* 308 G>A (Levesque et al., 2010). The risk of severe malaria with A and B blood types ranges from 1.26 to 2.95 (Fischer & Boone, 1998; Lell et al., 1999; Rowe et al., 2007). *TNF-α* 308 G>A and similar variations contribute to malaria morbidity and mortality. Perhaps fortunate, affected individuals become symptomatic and seek medical attention, or become injured or die off, thereby departing from the population's gene pool without progeny. Evidence from several studies suggest that genetic loci associated with malaria disease susceptibility are relevant to the morbidities a number of other parasitic diseases, autoimmune diseases and allergies; suggesting that parasites may have influenced the evolution of the host immune system and immunological disorders (Mangano & Modiano, 2014).

**Protective Alleles**

Symptomatic MPA alleles can be grouped under:

1. Cytogenetics (hemoglobinopathies, enzymopathies and structural modifications in erythrocytic membrane proteins).
2. Immunogenetics (polymorphisms in TLRs, cytokines, Ig receptors and NOS2).
3. Genetics of host's cytoadhesive-receptors (Intercellular Adhesion Molecule 1 *Killifi* and *CD36* polymorphisms).

Identified polymorphisms that are associated with protection against complicated malaria in different populations include sickle cell trait, G6PD deficiency, α- and β- thalassemias, blood group O, ovalocytosis (Genton et al., 1995), Swain-Langley 2 polymorphism (Cockburn et al., 2004), glycophorin variants, Heme Oxygenase-1 polymorphisms and polymorphisms in the receptors of IL 12 and IL 23. MPA variants also occur in the Major Histocompatibility Complexes (MHCs) and CD40 ligand of the adaptive immune system. A few of these variants are outlined in the ensuing sections. Interestingly, many severe MPA polymorphisms of erythrocytes are "loss-of-function" or reduced-expression mutations, while severe MPA polymorphisms of the immune system are "increase expression" mutations (Mendonça et al., 2012; Hedrick, 2011).

**Haematological Protective Alleles**

*Haemoglobin C* and *Haemoglobin S*

The malaria protection associated with Haemoglobin AS allele (*HbAS*) (rs334) was first reported in 1954. *HbAS* was associated with protection against severe (Ackerman et al., 2005; Aidoo et al., 2002; Jallow et al., 2009; May et al., 2007; Williams, Mwangi, Wambua, Alexander, et al., 2005) and uncomplicated malarias caused by *P. falciparum* (Clark et al., 2008; Crompton et al., 2008; Kreuels et al., 2010; Williams, Mwangi, Wambua, Alexander, et al., 2005). *HbAS* affords the host approximately 90% protection against severe malaria and 30% protection against uncomplicated malaria. *HbAS* reduced hospital admissions by 75% and delayed onset of clinical malaria by a month. Interestingly, studies have reported an age-dependent protective effect associated with *HbAS,* suggesting the involvement of acquired immunity.

Haemoglobin C allele (*HbC*) (rs33930165) is severe MPA, with homozygosity being more protective than heterozygosity (Agarwal et al., 2000; Mockenhaupt et al., 2004; Modiano, Luoni, Sirima, Simpore, et al., 2001), unlike *HbS*. The sufficiency of this protection of *HbC* is exemplified in the Dogon ethnic group of Mali, which is adequately protected against severe malaria, despite the low frequency of *HbS* in the population (Agarwal et al., 2000). *HbC* homozygotes and heterozygotes exhibit 80% and 20% respective reductions in severe malaria (Modiano, Luoni, Sirima, Simpore, et al., 2001; Taylor, Parobek, & Fairhurst, 2012). *HbC* was also associated with protection against mild *P. falciparum* malaria, possibly by reducing parasitaemia (Rihet, Flori, Tall, Traore, & Fumoux, 2004).

However, the findings of a meta-analysis of the protection of *HbC* against uncomplicated malaria was inconclusive (Taylor et al., 2012).

Incidentally, *HbS* and *HbC* have been shown to provide antidisease immunity against severe and uncomplicated malarias by proposed mechanisms that might also dictate other epidemiological parameters, such as infectiousness to the mosquito. The findings of Gouagna et al. (2010) and Gonçalves et al. (2017) provided the first demonstrations that human genetic variations may directly influence malaria transmission dynamics by an increased production of gametocytes.

**Alpha-Thalassemia**

Alpha-thalassemia is the most prevalent human genetic disorder. Homozygote and heterozygote α-thalassemia reduces the odds of severe malaria in children by 0.37 and 0.17, respectively (Goheen et al., 2017). Studies have also reported an age-dependent protective effect with this association (Veenemans et al., 2011), suggesting the involvement of acquired immunity. Alpha-thalassemia may actually protect against certain *Plasmodium* parasite clones (Vafa, Troye-Blomberg, Anchang, Garcia, & Migot-Nabias, 2008). Studies into the protective influence of α-thalassemia on uncomplicated malaria have however yielded conflicting results (Enevold et al., 2008; Lin et al., 2010; Rosanas-Urgell et al., 2012; Wambua, Mwangi, et al., 2006; Williams et al., 1996).

### Blood Group O

The relationship between ABO blood group variants and malaria outcome was suggested in 1957. Blood group O (rs8176719) has been associated with protection from severe malaria (Jallow et al., 2009; Rowe et al., 2007; Timmann et al., 2012), possibly by reducing rosetting (Carlson & Wahlgren, 1992). Blood group O provides a 66% reduction in the odds of severe malaria (Rowe et al., 2007). Strangely, blood group O is not present at high frequencies in malaria endemic areas, possibly because of a balancing selection; it is associated with other diseases of poverty, such as cholera and *Escherichia coli* infections (Rowe et al., 2007).

### Enzymatic Protective Allele

### Glucose-6-Phosphate Dehydrogenase

Glucose-6-Phosphate Dehydrogenase (G6PD) is the most common human erythro-enzymopathy. G6PD has a global distribution pattern similar to that of malaria. Most studies into the influence of G6PD on severe malaria have yielded conflicting results (Guindo et al., 2011; Manjurano et al., 2012; Ruwende et al., 1995; Toure et al., 2012). However, G6PD $A^-$ heterozygosity in females may protect against all malaria disease states, including asymptomatic malaria (Mombo et al., 2003), because of the complex erythrocytic environment created for parasites (Usanga & Luzzatto, 1985).

## Immunological Protective Allele

### Human Leukocyte Antigen Alleles

Although classical malaria-resistance alleles exist in low frequencies in the Fulani, the presence of certain Human Leukocyte Antigen (HLA) class I alleles in the population has been reported (Modiano, Luoni, Sirima, Lanfrancotti, et al., 2001). West African children who carried *class I HLA-Bw53* and *class II DRB1*1302-DQB1*0501* variants within their HLA genes were significantly protected against cerebral malaria and severe anaemia; approximately 15% reduction in number of cases (Hill et al., 1991). (*HLA DRB1*1302-DQB1*0501* encodes a variant of the HLA class II β chain.) da Silva Santos et al. (2012) also identified an association between a novel allele within the MHC class III region, Cytotoxic T-Lymphocyte-Associated protein 4 gene (rs2242665), and a reduced risk of mild malaria. Yet still, evidence suggests non-HLA loci genes are a greater genetic determinant of malaria immune response than HLA loci genes (Jepson et al., 1997; Mangano, 2008).

### Host Genetics and Asymptomatic Malaria

Asymptomatic malaria infections have been linked to several parasite and host factors by many studies. Altogether, it appears that a combination of malaria-resistant alleles, as opposed to any single one, may null the symptoms of malaria and create an asymptomatic disease state (Prusty et al., 2021). Of the few studies on asymptomatic malaria host genetics, the findings are unclear, as reviewed.

**Haemoglobin S and Haemoglobin C**

According to a systematic review by Taylor et al. (2012), protection against asymptomatic parasitaemia could not be demonstrated for *HbAS, HbAC* and *HbCC*. This is collaborated by Acquah et al. (2020). However, Migot-Nabias et al. (2006) found that unrelated asymptomatically infected children were protected against mild malaria attacks by sickle cell trait without controlling parasite levels in the transmission season. It would seem the antiparasitic protection of sickle cell trait against severe and uncomplicated malarias, an "erythrocytic immunity", if you will, does not extend to the asymptomatic phenotype; although antidisease protection does. This illustrates how sickle cell trait may sustain the asymptomatic phenotype and without controlling asymptomatic parasitaemia. Lawaly et al. (2010), did however, demonstrated that the sickle cell mutation was associated with the prevalence of gametocyte carriage in asymptomatic *P. falciparum*-infected individuals, but not in symptomatic *P. falciparum*- or *Plasmodium vivax*-infected individuals. Perhaps, an exception exists for sexual stage parasites. A tangled web indeed.

**Blood Group O**

Unlike in the case of symptomatic malaria, blood group O increases susceptibility to asymptomatic malaria parasitaemia (Alemu & Mama, 2018; Ofosu, Dotsey, & Debrekyei, 2017). Blood group O may not protect against higher parasitaemia in asymptomatic cases, because rosetting is not involved in the pathophysiology (Migot-Nabias et al., 2006).

### Glucose-6-Phosphate Dehydrogenase and Alpha-Thalassemia

Virtually all studies report in unison the protection of G6PD against asymptomatic malaria and parasitaemia (Amoah, Opong, Ayanful-Torgby, Abankwa, & Acquah, 2016; Mombo et al., 2003; Ouattara et al., 2014). For instance, Mombo et al. (2003) exhorted that G6PA$^{-}$ protected against all forms of the malaria infection outcome. On the other hand, α-thalassemia was not significantly association with asymptomatic malaria prevalence and parasitaemia (Fowkes et al., 2008; Lawaly et al., 2010; Shekalaghe et al., 2009; Wambua, Mwangi, et al., 2006).

## SNPs of Interest

### A Bird's-Eye View

The following SNPs were of interest in the present study: *TLR 4*-Asp299Gly (A>G), *NOS2*-954 G>C, *IL 10*-592 A>C, *MBL2* G230A and *IFN-γ*+874 T>A. The SNPs occur in the promoter regions of their respective genes, with the exception of *MBL2* G230A and *TLR 4*-Asp299Gly, which occur in exons. Of the SNPs that occur in the promoter region, only *IFN-γ*+874 T>A occurs downstream from the transcription start site. All the SNPs do not directly cause pathologies. However, they have antiparasitic influences in malaria infections (De Mendonça et al., 2012). *IL 10*-592 A>C accomplishes this indirectly by modulating the functions of other immune effectors. *IFN-γ*+874 T>A has both antiparasitic and immunopathological effects with reference to a threshold concentration. Occurring in genes of the innate immune system, *NOS2*-954 G>C and *IL 10*-592 A>C protect against symptomatic malaria by increased protein production, while *TLR 4*-Asp299Gly (A>G), *MBL2*

G230A and *IFN-γ*+874 T>A increase susceptibility to symptomatic malaria by decreased protein production and receptor affinity (Table 2).

In the ensuing review of the SNPs of Interest, few studies may stick out in disagreement with others. This is not unusual with candidate gene studies. Such results may however, point to the influence of obvious differences in sample sizes, population dynamics, geographical locations, endemicities and phenotype-definitions (Kwiatkowski, 2000), as well as, epigenetics factors (Gupta et al., 2017). It is also possible that the influence of host genetics to *Plasmodium* become less important in areas with differing epidemiologies (Boutlis et al., 2003).

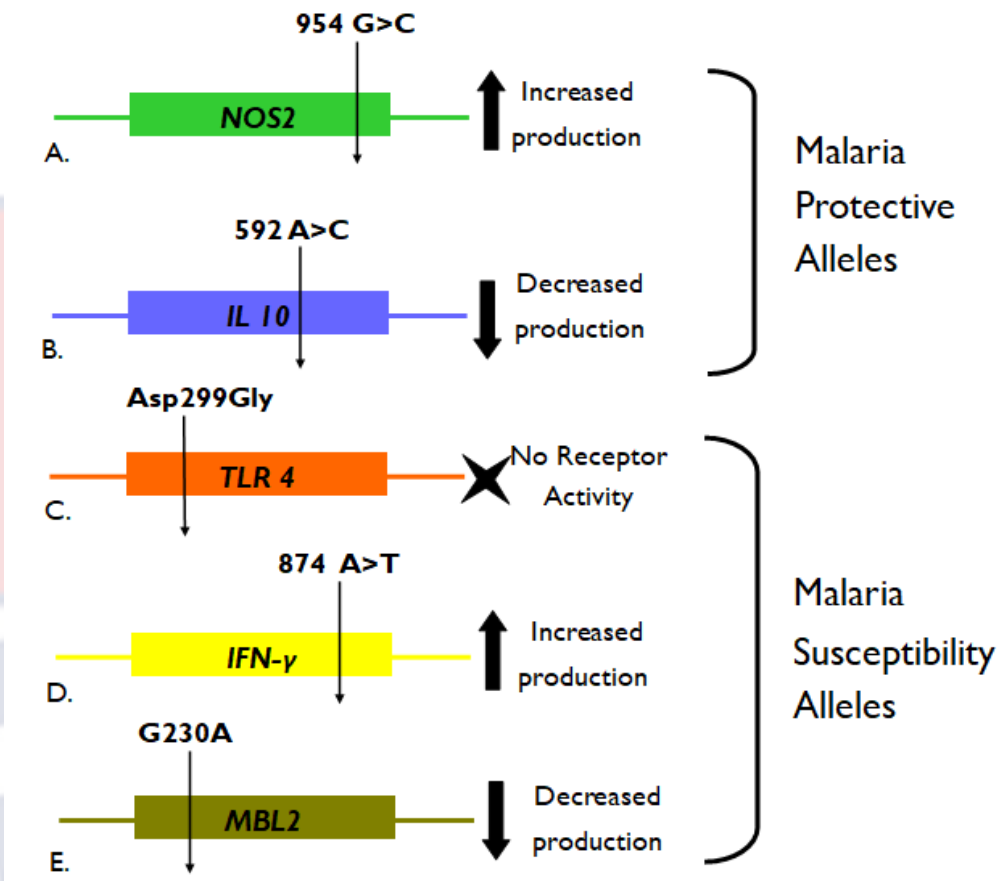



Figure 3 SNPs of Interest and their effects.

(De Mendonça et al., 2012)

***TLR 4*-Asp299Gly (-896 A>G)**

Toll-Like Receptors (TLRs) are Pattern Recognition Receptors, named for their similarity to a membrane protein of *Drosophila spp.*, the Toll. TLRs are proteins on the surface of innate immune cells. TLRs 1, 2, 4, 5, 6 and 10 are located extracellularly, while TLRs 3, 7, 8 and 9 are located intracellularly, in the endoplasmic reticulum and cytoplasmic vesicles. TLRs are characterised by Leucine-Rich Repeats (LRRs) within their extracellular domain that interacts with

pathogen ligands, and a Toll/ Interleukin 1 Receptor domain that interacts with Myeloid Differentiation primary response gene 88, an adaptive protein that causes the expression of pro-inflammatory cytokines, such as TNF-α. TLRs recognize conserved regions on pathogens. For instance, TLR 4 is a receptor for lipopolysaccharide, usually found on Gram-negative bacteria, while TLR 2 is reactive to lipopeptides, peptidoglycans and lipoteichoic acids, usually found on Gram-positive bacteria.

TLR 4 (CD284) is expressed on myeloid cells, such as RBC, granulocytes and macrophages, as opposed to lymphoid cells, such as T and B cells and Natural Killer (NK) cells. It has a molecular weight of 95 kDa. In a malaria infection, TLR 2 and 4 recognize Glycosylphosphatidylinositol (GPI) of *P. falciparum*, while TLR 9 reacts to its hemozoin (Krishnegowda et al., 2005).

*TLR 4* gene is located on chromosome 9q33.1. Its transcription factor is Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB). *TLR 4* has three exons. Exon one codes for a signal protein and the first few amino acids of the extracellular domain. Exon two codes for a part of the extracellular domain, while exon three codes for the remainder of the extracellular domain with LRRs, the transmembrane domain and the cytoplasmic domain, with two Inverted Tandem Repeats (Vaure & Liu, 2014). *TLR 4*-Asp299Gly, also written as *TLR 4*-D299G (rs4986790), occurs in exon three at codon 299 and impacts the stability of the ligand-binding extracellular domain of the receptor (He & Jiang, 2022). As such, the -Asp299Gly polymorphism of *TLR 4*, reduces receptor affinity and increases susceptibility to severe malaria and bacterial infections (Genc et al., 2004; Lorenz,

Mira, Frees, & Schwartz, 2002). *TLR 4*-Asp299Gly is co-segregated with *TLR 4*-Thr399Ile (-1196 C>T) to form the Asp299Gly/ Thr399Ile haplotype (Ohto, Yamakawa, Akashi-Takamura, Miyake, & Shimizu, 2012). This haplotype has been associated with a TLR 4-mediated IL 10 production (Van der Graaf et al., 2006). This is of interest as it expresses the link and functional relatedness of the products of two SNPs of Interest in the present study. An increased level of IL 10 reduces pro-inflammatory action against *Plasmodium spp*., thereby further increasing susceptibility to malaria symptoms.

*TLR 4*-Asp299Gly increased the risk of low birth weight, maternal anaemia, hyperparasitaemia, severe malaria and severe malarial anaemia in primiparous women and children (Mockenhaupt, Cramer, et al., 2006; Mockenhaupt, Hamann, et al., 2006). However, one study reported no effect on *P. falciparum* and *P. vivax* monoinfections (Sirisabhabhorn, Chaijaroenkul, & Na-Bangchang, 2021). The power of this study could not be called to question and may exemplify a weakness of the candidate gene approach study design (Kwiatkowski, 2000; Sinha et al., 2008). In a peculiar fashion, da Silva Santos et al. (2012) reported a reduced risk of mild malaria with *TLR 4*-Asp299Gly variants. This does not necessarily negate the aforementioned increased susceptibility associated with *TLR 4*-Asp299Gly, but may very well illustrate a difference in mechanisms of severe, mild and asymptomatic malarias, as explained in the discussion section. On the subject of asymptomatic malaria, according to reviewed literature my knowledge, the relationship between *TLR 4*-Asp299Gly and asymptomatic malaria has not been explored by any studies.

***NOS2*-954 G>C**

Nitric Oxide (NO) is a reactive oxygen species of the innate immune system that mediates resistance in many infectious diseases. It is a signalling molecule with a short half-life. It can traverse cell membranes because it is soluble in lipids. Cytokines stimulate monocytes (and macrophages), respiratory epithelial cells, hepatocytes, muscle cells, retinocytes, chondrocytes and colorectal adenocarcinoma cells to produce NO. Within these cells, NO production in humans is executed by three isoforms of the Nitric Oxide Synthase (NOS) enzyme: neuronal NOS (nNOS/ NOS1), inducible NOS (iNOS/ NOS2) and endothelial NOS (eNOS/ NOS3). NOS1 functions in the nervous system, NOS2 in the immune and cardiovascular systems, while NOS3 controls the vasodilation of endothelial tissue. NOS2 converts L-arginine to NO in the following reaction:

$$2\ \text{L-arginine} + 3\ \text{NADPH} + 3\ H^{+} + 4\ O_2 \rightleftharpoons 2\ \text{citrulline} + 2\ \text{NO} + 4\ H_2O + 3\ NADP^{+}$$

*NOS2* or *NOS2A* gene encodes the NOS2 enzyme. *NOS2* is located on chromosome 17q11.2-12 and consists 37 kilobase pairs and 26 exons. The transcription site occurs at exon two and the stop codon at exon 26. *iNOS* transcription is mediated by NF-κB and Interferon Regulatory Factor 1. NOS2 is composed of an oxygenase region encoded by exons one through to 13 and a reductase region encoded by exons 14 to 26. Inducible NOS is "induced" or transcribed, usually in the liver, in response to pathogens and pro-inflammatory cytokines, such as IL 1, TNF-α and IFN-γ (Green et al., 1994; Levesque et al., 2010).

Polymorphisms that occur in *NOS2* include CCTTT microsatellite repeats [$(TAAA)_n$ and $(CCTTT)_n$] and a number of SNPs: -954 G>C (rs1800482), -1173 C>T and -1659 A>T. The point mutation at position 954, upstream of the transcription site, has been associated with an increase in enzymatic activity and NO production (Kun et al., 2001). Specifically, *NOS2*-954 mutation presented with a seven times higher baseline enzymatic activity.

*NOS2*-954 G>C has been associated with protection against severe malaria and high parasitaemia (Cramer et al., 2004; Jürgen FJ Kun & Mordmuller, 1998), possibly through parasite clearance (Gramaglia et al., 2006; Serirom et al., 2003; Weinberg, Lopansri, Mwaikambo, & Granger, 2008). *NOS2*-954 G>C exists in linkage disequilibrium with $CCTTT_{(8)}$, and protection against hyperparasitaemia has been reported for the haplotype. The protection associated with *NOS2*-954 G>C heterozygosity is comparable to that of sickle cell trait, as mutants are 2.5 times less likely to develop severe malaria (Dzodzomenyo et al., 2018; Jürgen F Kun et al., 2001). On a tangent, both *HbAS* and *NOS2A*-954 G>C lose their ability to clear infections when parasites are drug-resistant (Amenga-Etego, 2012; Diakite et al., 2011). This is unfortunate as drug-resistant parasite strains are more likely to cause asymptomatic infections. Children are more likely to benefit from the malaria protection of NO and *NOS2A*-954 G>C than adults (Cramer et al., 2005; Parikh, Dorsey, & Rosenthal, 2004), although this age-dependent effect was contested by Lwanira, Kironde, Kaddumukasa, and Swedberg (2017).

On the asymptomatic front, Mombo et al. (2003) reported no association between *NOS2*-954 G>C and asymptomatic malaria. Although an age-dependent effect has not been investigated, the absence of young children in this study sticks out. Additionally, in a cohort of strictly-defined asymptomatic people, *NOS2* G954C did not occur, either indicating its unimportance to the phenotype or complexity of its geo-epidemiological distribution (Boutlis et al., 2003).

Despite the lack of association between *NOS2*-954 G>C and asymptomatic malaria, high levels of NO are produced by mononuclear cells in asymptomatic children (Anstey et al., 1996). According to Anstey et al. (1996) and Perkins et al. (1999), baselevel expression and activity of NOS2 of Peripheral Blood Mononuclear Cells of malaria asymptomatic people in the tropics were higher than that of asymptomatic people in temperate regions (Clair et al., 1996; Weinberg et al., 1995). This higher activity in tropic occupants may be because of chronic NOS2-stimulation by malaria and/ or other infections of poverty and not a result of the genetic background. In other words, a history of consistent immune-exposure is the likely cause of high NO levels reported in asymptomatic *Plasmodium* carriers in tropic regions.

***IL 10-592 A>C***

Interleukin 10 (IL 10) is also called the human Cytokine Synthesis Inhibitory Factor. It is a member of α-helical cytokine class II. The IL 10 protein exists as a 37 kilodaltons (kDa) homodimer and consists 160 amino acids with a molecular weight of 18.5 kDa. IL 10 is produced by monocytes/ macrophages, T and B cells and eosinophils. IL 10 production from $CD4^+$ T cells decreases with

increasing age, independent of *Plasmodium* density (Boyle et al., 2017). It is an immunoregulatory cytokine that regulates pro- and anti-inflammatory responses to ensure homeostasis (Kumar et al., 2020). IL 10 reduces the production of MHC class II and co-stimulatory molecules in Antigen Presenting Cells. IL 10 levels is also a switch factor for Immunoglobulin G1 (IgG1) and IgG3 (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). Excitedly, IL 10 can reduce the pro-inflammatory expression and activities of NOS2 and IFN-γ, the products of two other SNPs of Interest in the present study.

Being immunoregulatory in function, IL 10 is involved in both malaria protection and pathology, as various plasma levels have differing effects. Higher IL 10 titres have been associated with severe malaria in human and animal models, possibly as a result of a diminished pro-inflammatory ability of the host to clear parasites (Gosi, Khusmith, Looareesuwan, & Walsh, 1999; Hugosson, Montgomery, Premji, Troye-Blomberg, & Björkman, 2004; Kobayashi, Ishida, Matsui, & Tsuji, 2000; Lyke et al., 2004; Suguitan Jr et al., 2003; Wenisch, Parschalk, Narzt, Looareesuwan, & Graninger, 1995). A low TNF-α/ IL 10 ratio has also been associated with an increased risk of cerebral malaria and severe malarial anaemia (Kossodo et al., 1997; Kurtzhals et al., 1998; May, Lell, Luty, Meyer, & Kremsner, 2000; Mordmüller et al., 1997; Othoro et al., 1999). This is consistent with the finding of Frimpong et al. (2020), where parasitaemia was a positive predictor of IL 10 levels; as a low IL 10 level was associated with low parasitaemia and increased protection.

Genetics accounts for approximately 50-70% of the variation in IL 10 production in the human body; gender, Body Mass Index and smoking account for the remainder of variation (Reuss et al., 2002; Westendorp et al., 1997). *IL 10* gene is located on chromosome 1q21-q32. *IL 10* has five exons and four introns. Transcription is mediated by the transcription factor Signal Transducers and Activators of Transcription 3 and NF-κB. *IL 10* has over 27 polymorphisms in its over five kilobase pairs promoter region. Common variants in the *IL 10* promoter region include -1082 G>A (rs1800896), -819 C>T (rs1800871) and -592 (or -571) C>A (or A>C) (rs1800872). The latter is of interest to the present study.

Strangely, the nomenclature of *IL 10*-592 A>C is used interchangeably with *IL 10*-592 C>A in different publications (Bahadori et al., 2014; Sghaier et al., 2022; Shih et al., 2005; Turner et al., 1997; Vázquez-Villamar et al., 2016), and once in the same publication (Sobti et al., 2010). However, considering the nomenclature of the reference sequence used in sanger sequencing analyses in the present study, the A allele was considered the wild-type in the present study. The -1082 G>A, -819 C>T and -592 A>C polymorphisms in the *IL 10* promoter region are in linkage disequilibrium and form the ATA and GCC haplotypes. By virtue of the disequilibrium, an A mutation at position -592 indicates the presence of the ATA haplotypes, associated with low IL 10 plasma levels (Berglundh, Donati, Hahn-Zoric, Hanson, & Padyukov, 2003; Crawley et al., 1999; Eder et al., 2007; Lowe, Galley, Abdel-Fattah, & Webster, 2003; Yılmaz, Yentür, & Saruhan-Direskeneli, 2005).

The *IL 10*-592 mutation have been associated with lower levels of the immunosuppressive IL 10 cytokine, and thus a lower risk of developing clinical malaria, including a lower parasitaemia and higher antibodies levels against *P. falciparum* NANP repeats (Asn-Ala-Asn-Pro amino acids of circumsporozoite of sporozoites) (Dewasurendra et al., 2012; Pereira et al., 2015). Extrapolating from the functionality of IL 10, the symptomatic MPA property of *IL 10*-592 A>C is suspected to extend to the asymptomatic parasitaemia. In support of this hypothesis, high levels of IL 10 have been linked to the incidence of asymptomatic malaria (De Jong et al., 2017; Ibitokou et al., 2014; Wilson et al., 2010).

***MBL2* G230A**

Mannose Binding Lectin (MBL/ MBL2) is a liver-produced, calcium-dependent, acute phase reactant C-type serum lectin that activates the complement system. It is a part of the collectin family. MBL2 is a 400-700 kDa oligomer with three identical 32 kDa peptide subunits. Each subunit is made of a N-terminal cysteine-rich cross-linking region, a collagenous region, an α-helical coil (the "neck" region) and a C-terminal carbohydrate-binding domain. MBL-Associated Serine Proteases (MASPs) and Mannose-binding lectin Associated protein 19 (MAp19) are associated with MBL2 function.

MBL2 is involved in complement activation, complement-independent opsonophagocytosis, inflammation modulation and apoptosis. MBL2 binds to sugars (carbohydrates) moieties, such as N-acetyl-D-glucosamine, N-acetyl-mannosamine, glucose, fucose and mannose on pathogens. After binding, it interacts with MASPs-1, -2, -3 and MAp19 to activate the complement system. The

complement system kills pathogens by phagocytosis and a Membrane Attack Complex. In similar fashion, MBL2 binds to the surface of infected erythrocytes and merozoites in a malaria infection and opsonizes parasites for phagocytosis and controls the release of pro-inflammatory cytokines.

*MBL2* gene is located on chromosome 10q11.1-q21. *MBL1* is a nonfunctional pseudogene in the liver, lungs and testes, while *MBL2* encodes the functional protein in the liver. *MBL2* consists four exons. Exon one of *MBL2* encodes a signal peptide, a cysteine-rich domain and a portion of the glycine-rich collagenous region. Exon two encodes the remainder of the collagenous region, while exon three encodes an α-helical coiled structure ('neck' region). The fourth exon encodes the carbohydrate-recognition domain.

Variations in MBL2 functionality and plasma levels are almost completely determined by genetics. Genetic variants in exon one of *MBL2* include *B*, *C* and *D* alleles found in codons 54, 57 and 52, respectively. They cause the following amino acid and nucleotide changes: D (Arg52Cys), B (Gly54Asp/ G230A) and C (Gly57Glu). All the variants are collectively known as "O" allele, while the wild-type is known as "A" allele. These polymorphisms cause a ~10% reduction in MBL2 function and alter MBL2 plasma levels by modifying the collagenous structure of the oligomer into fast degrading molecules (Madsen, Satz, Hogh, Svejgaard, & Garred, 1998). Resultantly, carriers and mutants for these SNPs, have lower plasma MBL2 levels than the wild-type.

Variants in the promoter region of *MBL2* are also relevant to MBL2 plasma concentrations, being in linkage disequilibrium with the aforementioned variants in the coding regions. Relevant polymorphisms in the promoter region of *MBL2* gene include -550 (H/L), -221 (X/Y) and +4 (P/Q). These form a number of haplotypes with O allele, such as HYPA, LYQA, LYPA, LXPA, HYPD, LYQC and LYPB (Casanova & Abel, 2004). This is a list of such haplotypes in decreasing MBL concentrations: HYPA = LYQA = LYPA > LXPA >> HYPD = LYPB = LYQC = LYPD (A. Boldt et al., 2006). Interestingly, plasma MBL levels can still vary among individuals with similar genotypes (Garred, Larsen, Madsen, & Koch, 2003; Steffensen, Thiel, Varming, Jersild, & Jensenius, 2000).

*MBL2**B may be written as *MBL2* G230A, *MBL2* G54A, $MBL2_{Gly54Asp}$ or *MBL2* 161 G>A (Ceylan, Karkucak, Coban, Karadag, & Yakut, 2017; Fugtagbi et al., 2022; Lewandowska, Jędrychowska-Dańska, Zamerska, Płoszaj, & Witas, 2017; Medetalibeyoglu et al., 2021; Olszowski, Adler, Janiszewska-Olszowska, Safranow, & Kaczmarczyk, 2012). It is a missense genetic variant that is inherited in an autosomal dominant pattern. As expected, considering the importance of the complement system in malaria immunity, *MBL2**B was a risk factor for severe malaria (Jha et al., 2014; Luty, Kun, & Kremsner, 1998). A Gambia study, however, did not support this; it should be noted that only adults were enrolled in this study (Bellamy et al., 1998). The relevance of age has been reviewed in the next paragraph.

MBL2 has been reported to be relevant in infants (six months to two years of age), after maternal antibodies have waned, but before adaptive immunity is fully developed. In lockstep, MBL2 is also important in adults suffering immunodeficiency or immunosuppression (Bouwman, Roep, & Roos, 2006). This may explain the lack of association between MBL2 variants and clinical malaria in studies which excluded children (Bellamy et al., 1998; A. Boldt et al., 2006; Holmberg et al., 2008; Luty et al., 1998; Mombo et al., 2003).

For *MBL2* G230A and asymptomatic parasite carriage, there exists some literature on the relationship. One peculiar study, posited the role of transmission setting in the relationship been *MBL2**B and asymptomatic malaria. In a study by Fugtagbi et al. (2022), carrier and mutant genotypes of the *MBL2* codon 54 variant were associated with asymptomatic parasite carriage in a low transmission area (with a 4-fold increase in risk of asymptomatic malaria parasite carriage) but not in a high transmission setting. Mombo et al. (2003), however, did not find a significant association between *MBL2* G230A and asymptomatic malaria among schoolchildren, despite being conducted in a low transmission study site. Perhaps worth noting, *MLB2*C* heterozygosity, another variant associated with MBL deficiency, increased the odds of asymptomatic malaria infection by 55% in hyperendemic areas (Holmberg et al., 2008).

The *MBL2**LYPA/LYPA haplotype was associated with the absence of asymptomatic infection in Gabonese adults, while *MBL2**YA/YO and *MBL2**YO/YO haplotypes (-221 Y allele in the promoter region and any of the O alleles in exon one) were associated with asymptomatic parasitaemia (A. B. Boldt

et al., 2009). Interestingly, *MBL2**YO/YO was also associated with parasitaemia in clinical malaria episodes (Garred, Nielsen, et al., 2003), supporting the hypothesis that allele functionality extended across different malaria forms.

***IFN-γ*+874 T>A**

Interferon-gamma (IFN-γ) is the only member of the IFN type II family. It is a homodimer with six alpha helices and no beta sheets. IFN type I, IL 12, IL 15 and IL 18 trigger IFN-γ production mainly in NK cells. IFN-γ production is also triggered in Natural Killer T (NKT) and myeloid cells. On the adaptive arm of immunity, $CD4^+$ $T_H1$ and $CD8^+$ cytotoxic T lymphocyte cells, $\alpha\beta$T cells and $\gamma\beta$T cells produce IFN-γ.

IFN-γ is relevant to the activities of the innate and adaptive immunities, particularly, immune responses against infections and tumours. It is popularly known for its role in antiviral immunity. IFN-γ is involved in the signalling of cellular effectors of the immune system, triggering phagocytosis and increasing expression of MHC. IFN-γ also influences the differentiation, growth and activation of NK cells, NKT cells, endothelial cells and fibroblasts. On the adaptive arm of immunity, IFN-γ induces IgG class switching to cytophilic isotopes and promotes the differentiation of T and B cells (Schroder, Hertzog, Ravasi, & Hume, 2004).

IFN-γ can have either protective or immunopathological effects during a malaria infection. In malaria, IFN-γ activates macrophages to attack parasites in the liver and opsonize infected RBCs in the bloodstream (Akdis et al., 2011; Yildiz

Zeyrek, Kurcer, Zeyrek, & Simsek, 2006). $CD4^+$ and $CD8^+$ T cells also produced IFN-γ against liver- and blood-stages parasites of *Plasmodium* infections (Connelly et al., 1997); specifically, this production of IFN-γ has been associated the development of uncomplicated malaria (Walther et al., 2009), reduction in severe malarial anaemia (Ong'echa et al., 2003) and protection against cerebral malaria (Cabantous et al., 2005).

With its pro-inflammatory properties, IFN-γ also possesses immunopathological potential, when unregulated (Cabantous et al., 2009; Torre et al., 2002). Several studies have largely registered a correlation between increasing IFN-γ plasma levels and febrile/ symptomatic malaria (Dodoo et al., 2002; Harpaz et al., 1992; Mordmüller et al., 1997; Mshana, Boulandi, Mshana, Mayombo, & Mendome, 1991; Riley et al., 1991; Torre et al., 2002; Walther et al., 2006). This occurs when IFN-γ triggers the release of pyrogenic cytokines (Munder, Mallo, Eichmann, & Modolell, 1998; Otani et al., 1999), causes inflammation in the brain and lung against sequestrated infected RBCs (Villegas-Mendez et al., 2012), and activates $CD8^+$ T cells to produce perforin and granzymes (Haque et al., 2011). There appears to be a threshold plasma level about which IFN-γ is the "Jekyll and Hyde of Malaria" (King & Lamb, 2015). This is the reason anti-IFN-γ immune-therapy has not been effective in controlling cerebral malaria (Inoue, Niikura, Mineo, & Kobayashi, 2013).

The *IFN-γ* gene is located on chromosome 12q15 in humans. With four exons and three introns, the gene spans approximately four (~4) kilobase pairs. The first intron has a highly polymorphic CA-repeat microsatellite (Pravica, Perrey,

Stevens, Lee, & Hutchinson, 2000). At the 5' end of this repeat and 3' terminal of *IFN-γ* gene is *IFN-γ*+874 T>A (rs2430561), which occurs in the transcription binding site for NF-*κβ* (Koch et al., 2005). The *IFN-γ*+874 A mutation, which interrupts with NF-*κβ* binding, is associated with a lower mRNA and IFN-γ productions (Maniero et al., 2007; Medina et al., 2011). As such, *IFN-γ*+874 A has been linked to malaria (Medina et al., 2011) and Chagas disease susceptibilities (Torres et al., 2010). According to the reviewed literature, there are no reports on the influence of *IFN-γ*+874 T>A on mild and asymptomatic malarias.

**Hardy-Weinberg Equilibrium**

The Hardy-Weinberg non-evolutionary model, often known as the Hardy-Weinberg Equilibrium (HWE), is a fundamental hypothesis in population genetics and evolutionary biology. This equilibrium describes how allele and genotype frequencies remain constant in a population throughout generations, under the following conditions:

1. There is no migration in and out of the population.
2. There is no natural selection.
3. There are no mutations.
4. There is random mating (equal chance of breeding).
5. The population is infinitely large.

Manifestly, only a theoretical population can be in HWE. However, HWE remains a cornerstone in genetics because it can help detect evolutionary factors in existing populations. For instance, in case-control studies, cases do not need to be

in HWE, as deviations indicate disease associations with a particular allele (Lee, 2003). However, deviations of controls or a sample with unselected controls (as with the present study) may indicate the failure of one or more of the conditions of HWE to hold in the population (Salanti, Amountza, Ntzani, & Ioannidis, 2005), assuming quality control of the genetic data had been ensured.

In genetic studies, HWE serves as a null hypothesis model of expected allele frequencies in a population for statistical testing (Telleria & Tibayrenc, 2010). However, before failure of one or more of conditions of HWE are considered, a deviation from the HWE (also known as Hardy-Weinberg Disequilibrium) may indicate problems with genetic data sets (Khoury, Beaty, Beaty, & Cohen, 1993; Khoury, Little, & Burke, 2004; Sham, 1998). Genotyping error is the most relevant problem (Hosking et al., 2004; Wang & Shete, 2012), requiring repetition and/ or double-checking of allelic discrimination. Other problems may include population stratification (Cardon & Palmer, 2003), methodological artefacts (i.e., selection bias) (Gillespie, 2004), non-randomly missing genotype data (Graffelman, Sánchez, Cook, & Moreno, 2013), allele-specific genetic effect bias (Sasieni, 1997; Schaid & Jacobsen, 1999) and relatedness among study participants.

Assuming genotyping error and other quality control parameters have been checked, deviations of a sample (which may include unselected controls) from HWE may indicate the failure of one or more of the conditions of the equilibrium to hold in the population (Salanti et al., 2005). For instance, natural selection of the heterozygote advantage of sickle cell mutation against malaria, and cystic fibrosis against cholera, typhoid and Ashkenazi Jewish diseases, as well as, consanguinity

in Africa caused a deviation of HWE in the Genome Aggregation Database (Piel et al., 2015).

**Summary**

In summary, the chapter has delved into the field of malaria and malaria host genetics, providing the reader with the adequate knowledge to understand the importance of the present study and the relevance of the finding. As a reference section, the reader can appreciate the following chapters.

# CHAPTER THREE

# RESEARCH METHODS

## Introduction

The third chapter of this thesis provides the relevant details about how the study was conducted to answer the research question or aim. Details in the chapter include the study site and design, laboratory examinations in *P. falciparum* detection and allelic discrimination and the analysis of statistical data.

## Study Site

The study was conducted in Simiw, a peri-urban community in the Komenda-Edina-Eguafo-Abrem Municipal Assembly of Central Region, Ghana. The community is situated in the coastal savannah of southern Ghana, approximately 11 km from Elmina, the district capital, and two kilometres (2 km) from the Ankaful Maximum Prison. Specifically, Simiw is located on longitude 1°15' W and latitude 5°06' N, with an elevation of approximately 36 m above sea level.

Malaria transmission in Simiw is perennial, peaking during the rainy season, from May to July. The dry season in Simiw begins in November and ends in April. Simiw has a high prevalence of asymptomatic *P. falciparum* infections; 50% during the dry season (Acquah et al., 2020; Acquah et al., 2021). The most prevalent *Anopheles gambiae* complex species identified in the locality is *Anopheles coluzzii*.

**Study Design**

This Candidate Gene Approach study was conducted using archived DNA samples from a parent study, "Gametocytogenesis". Gametocytogenesis is a longitudinal study aimed at understanding asymptomatic malaria parasite carriage and identifying the triggers of malaria gametocytogenesis. Gametocytogenesis enrolled 249 school-going children permanently living in the study site and attending Simiw Basic School. This population was selected for its high prevalence of asymptomatic *P. falciparum* infections (Obboh, Okonu, & Amoah, 2020). From October, 2020 to August, 2021, Dried Blood Spots (DBS) had been collected at 12 time points (hereby known as visits) from malaria asymptomatic school-going children (Table 1). There were three (3) sampling time points in the post-peak (October, 2020 to November, 2020) and pre-peak (February, 2021 to March, 2021) transmission seasons (dry season), as well as, six (6) sampling time points in the peak/ wet season (May, 2021 to August, 2021) all spaced out by two weeks. The use of the term "peak" is in reference to the peak of clinical malaria cases which coincides with the wet season. The enrolled children were asymptomatic within the observational window and were not treated with antimalaria treatment at any time. The use of samples from Gametocytogenesis allowed the inclusion of unselected controls.

Between sampling time points, the clinical states of the children were checked and recorded in order to obtain a fuller picture of the asymptomatic malaria phenotype within the observational window. Some of the children did develop clinical symptoms over the course of Gametocytogenesis (either on the sampling

day or before). These children were tested for *P. falciparum* infection by RDT and given antimalaria drugs when positive.

Although Simiw experiences a year-round transmission of malaria parasites, there is a recognised peak transmission period. Although, conventionally and meteorologically, two malaria seasons are recognised in most malaria study sites (dry and wet seasons), in line with its objectives, Gametocytogenesis recognised three seasons based on the peak transmission season (Table 1). These were also recognised in the present study.

For the present study, genotyping was performed on DNA, obtained by the Tween-Chelex Extraction method from DBS, of all participants in Gametocytogenesis. However, data on *P. falciparum* infections, as detected by Multiplex Photon-induced Electron Transfer (PET)-PCR, was only available for 96 participants, who were present for all 12 sample collection times; tests of associations were focused on these.

Table 1 Dates of the 12 Sampling Visits in the Gametogenesis Longitudinal Study

| Visit | Date | Season |
|---|---|---|
| 1 | 8th October, 2020. | |
| 2 | 22nd October, 2020. | Post-peak Season (Dry Season) |
| 3 | 5th November, 2020. | |
| 4 | 19th February, 2021. | |
| 5 | 5th March, 2021. | Pre-peak Season (Dry Season) |
| 6 | 19th March, 2021. | |
| 7 | 28th May, 2021. | |
| 8 | 11th June 2021. | |
| 9 | 25th June 2021. | |
| 10 | 9th July, 2021. | Peak/ Wet Season |
| 11 | 23rd July, 2021. | |
| 12 | 6th August, 2021. | |

**Inclusion and Exclusion Criteria**

Children were recruited into the Gametocytogenesis study (classified as asymptomatic), if they had an axillary body temperature of ≤ 37.5 °C with no observable and/ or self-reported malaria symptoms, such as malaise, headaches or vomiting.

**Ethical Considerations**

The Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, approved Gametocytogenesis (NMIMR-IRB CPN 005/19-20). Gametocytogenesis was also approved by the KEEA Municipal Director of Education. Informed consent had been obtained from a parent or legal representative of every participant. For the present secondary study, participant information remained coded and confidential at all times.

**Selecting Candidate Alleles**

Secondary to a parent study, a modest-few genetic variants were available to this study. In order to test the study's hypothesis, an economic number of five polymorphisms were selected; two MPA alleles and three MSA alleles (NCBI, 2022) (Table 2). SNPs were selected if they had been reviewed to have a purported biological role in symptomatic malaria populations (De Mendonça et al., 2012) and a Minor Allele Frequency (MAF) greater than or equal to four percent (≥ 4%) in African populations, according to NCBI.

Table 2 RS Numbers and MAFs of SNPs of Interest in African Populations

| | SNP | RS number | MAF in African populations |
|---|---|---|---|
| **Symptomatic MPA SNPs** | *NOS2*-954 G>C | rs1800482 | 0.06 |
| | *IL 10-592 A>C* | rs1800872 | 0.41* |
| **Symptomatic MSA SNPs** | *MBL2* (G230A) | rs1800450 | 0.04 |
| | *TLR 4*-Asp299Gly (A>G) | rs4986790 | 0.07 |
| | *IFN-γ*+874 T>A | rs2430561 | 0.20 |

*MAF occurred in the normal allele.

**Determining *P. falciparum* Infections**

Gametocytogenesis had run PET-PCR on samples from the 96 participants who were involved in all 12 sample collections. The unprocessed data was made available to the present study. Cycle threshold (Ct) values were converted into positive/ negative infection statuses and parasite densities. A heat map was designed using this information. The heat map also reflected symptomatic episodes for participants over the observational period.

**Allelic Discrimination**

**Genotyping**

Samples were amplified using OneTaq® DNA Polymerase (NEB, USA), genotyped by Restriction Fragment Length Polymorphism (RFLP) and Allele-Specific PCR (AS-PCR), and confirmed by Sanger Sequencing. Details are outlined in Appendices A, B and C. RFLP was repeated for samples of randomly selected initially-genotyped mutants and carriers to crosscheck.

**Preparing Samples for Sanger Sequencing**

Q5® High-Fidelity DNA Polymerase (>280 times the fidelity of Taq polymerase) (NEB, USA) was employed in High-Fidelity PCR (HF-PCR) to amplify an economic number of 32 samples at three genetic loci (*NOS2*, *IL 10* and *MBL2*) for sanger sequencing, using their respective primers (Appendix A). Twenty-five microliters (25 µl) of successfully amplified samples, as determined by gel electrophoresis, were shipped off to Macrogen-Europe, Netherlands, for Sanger sequencing. Details are outlined in Appendix D.

**Gel Electrophoreses**

Products of PCR, restriction enzyme digestion, AS-PCR and HF-PCR were electrophoresed in agarose gels stained with Ethidium bromide. Briefly, two percent agarose gels were cast using 1X Tris-Acetate-Ethylenediaminetetraacetic (TAE) buffer stained with 0.5 mg/ml of Ethidium Bromide. Gels were moulded in casting trays set up with combs. Products of PCR (20µl), restriction enzyme digestion (20 µl), AS-PCR (15 µl) and HF-PCR (5 µl) were mixed with 6X Orange G loading dye (5:1), and loaded into the wells of the moulded gels. Gels were run

in a tank of 1X TAE at 120 volts and 200 amperes for 45 to 60 minutes with four microliters (4 µl) of a 100-base pair (bp) ladder (50 µg/ml) (NEB, USA). The gels were subsequently viewed in a Gel Documentation System (BIO-PRINT CX4, Vilber) employing ultraviolet light. Gel images were captured and scored by two dependent persons.

**Sanger Sequence Analyses**

Chromatograms of sanger sequences were trimmed off of bad base calls using Chromas (Technelysium, Australia) version 2.6.6. Consensus sequences were built using Bioedit Sequence Alignment Editor© (Hall, 1999). Consensus sequences were aligned to their respective reference/ ancestral sequences obtained from the National Center for Biotechnology Information (NCBI) (Maryland, USA) Genbank® (accessed November, 2022) in the online versions of Multiple Alignment using Fast Fourier Transform version 7.505 (accessed in October, 2022) (Katoh & Standley, 2013) and The Benchling R&D Cloud (San Francisco, USA) (accessed January, 2023). The reference sequences provided by the RefSeqGene project of NCBI under the Locus Reference Genomic Project based on the current Genome Reference Consortium Human Build 38 patch release 14 (GRCh38.p14) assembly (O'Leary et al., 2016).

**Statistical Analyses**

Data entry was carried out in Microsoft Excel, 2019 (Washington, USA). Microsoft Excel, 2019 was used to generate descriptive statistics. Tests of associations were carried out in the online tool, SNPStat (Barcelona, Spain) (accessed in January, 2023) (Sinnwell & Schaid, 2005; Solé, Guinó, Valls, Iniesta,

& Moreno, 2006; Warnes, Gorjanc, Leisch, & Man, 2005) at an alpha value of 0.05. SNPStat was customised to provide descriptive statistics for allele and genotype frequencies, exact tests for HWE and single-SNP associations with parasite densities, adjusted by age and season (Gadalla et al., 2016; Mayer et al., 2009; Odongo-Aginya, Ssegwanyi, Kategere, & Vuzi, 2005; Ouédraogo et al., 2008). In testing a particular response, the homozygous genotype (wild type or mutant) with the higher frequency was used as a reference. Thus, the mutant genotypes for *IL 10*-592 A>C and *IFN-γ*+874 T>A were used as reference genotypes. Thirteen (13) children who became symptomatic were excluded from the analysis.

SNPStat performed Single-SNP analyses across various genetic models (Dominant, Recessive, Codominant, Overdominant, Log-additive/ Multiplicative) and generated the respective Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) values. AIC and BIC are Likelihood Ratio Tests used to determine goodness-of-fit of genetic models. BIC was considered in the present study because AIC is suitable when $n/k > 40$, where n is the sample size and k is the number of test parameters. The genetic model with the lowest BIC was considered the model with the best fit; this prevents genetic model misspecification and its resultant loss of statistical power. The aphorism popularised by George Box, "All models are wrong but some are useful", was acknowledged in the process.

**Summary**

In summary, this methods chapter provided details of the study in Simiw, Ghana in the years 2020 and 2021. A thorough description of laboratory examination and statistical analyses show allow a convenient repetition of th study.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## Introduction

Arguably the "star for show", this fourth chapter presents the results of the study and its discussion in a clear and easy-to-understand way. In the Results section, the reader will learn the demographics of the study participants, prevalence of *P. falciparum* infections in the Simiw for the period, Minor Allele Frequencies of the SNPs of Interest and the results of the tests of associations according to the study objectives in the first chapter. Ensuing the Results are the discussion and insights one might hope to glean from such results in light of existing knowledge. Some of the results do break new ground as avantgardes.

## An Overview of the Study

The present study is a Genetic Association Study aimed at identifying associations, if any, between hypothesis-driven candidate SNPs and the characteristics of parasite carriage in asymptomatic *P. falciparum*-infected children in a malaria endemic community in southern Ghana. The study design employed a Candidate Gene Approach, which uses the current understanding about the mechanism of malaria disease to select candidate genetic variants to test for possible associations with a disease condition. This was preferred over a Genome-Wide Association Study (GWAS), which is capital- and time-intensity, in the context of a resource-constrained Africa, and subject to a number of ethical and social conundrums (Chokshi & Kwiatkowski, 2005; Damena, Denis, Golassa, & Chimusa, 2019). As a compromise, a preceding Candidate Gene Association study

is recommended for a more robust and detailed GWAS. As expressed by Loucoubar et al. (2011) "… prior genetic analysis of carefully defined phenotypes, both spatially and temporally delimited, must surely not only be a prerequisite to more detailed GWA studies, but also may be informative for the potential importance of pathogen genetics and the occurrence of host-pathogen interactions."

## Results

### Study Demographics

This study involved two groupings of the study participants from the Gametocytogenesis study. Genotyping was performed on all participants of Gametocytogenesis (Table 4), while tests for association with parasite density were conducted on approximately a third of participants, who participated in all 12 sample collections (Table 5). Parasite data, obtained by PET-PCR was available for the latter group. The parent study employed, virtually, an equal proportion of male and female children with a median age of eight years.

Table 3 Demographics of all 249 Participants Enrolled in Gametocytogenesis

| **Sex** | **Frequency (%)** | | | | |
|---|---|---|---|---|---|
| **Male** | 127 (51) | | | | |
| **Female** | 122 (49) | | | | |
| **Total** | 249 | | | | |
| | **Median** | **IQ1** | **IQ3** | **Min.** | **Max.** |
| **Age (years)** | 8 | 5 | 10 | 4 | 13 |

Table 4 Demographics of 96 Participants of Gametocytogenesis Who Participated in all 12 Sample Collections

| **Sex** | **Frequency (%)** | | | | |
|---|---|---|---|---|---|
| **Male** | 49 (51) | | | | |
| **Female** | 47 (49) | | | | |
| **Total** | 96 | | | | |
| | **Median** | **IQ1** | **IQ3** | **Min.** | **Max.** |
| **Age (years)** | 8.5 | 5 | 10 | 4 | 13 |

**Asymptomatic *P. falciparum* Infections in Simiw**

Figure 4 is a heat map of the 96 participants offering an easy overview of infection statutes and occurrence of symptomatic malaria episodes associated with or without RDT positivity. As shown, most children were asymptomatic and positive for *P. falciparum* by PET-PCR with scattered symptomatic episodes. The heat map also shows symptomatic episodes before the day of sampling. According to figure 4, 13 children experienced at least one symptomatic malaria episode and were RDT positive. Four children experienced at least one symptomatic episode and were RDT negative.

Figure 5 displays the number of infected children and their median parasite densities at every visit of the study period. The peak of the prevalence of asymptomatic infections over the study period did not coincide with the peak transmission season but preceded it. The median number of all parasite densities for all visits was 40.5 p/µl (Figure 5). Interestingly, none of the parasite densities fell below the microscopic limit of detection of 30 p/µl.

The median number of infected children per visit was 65 (Figure 6). All the children were infected, at least twice in the year (Figure 6). A significant proportion of the children were constantly infected in all visits of the year; more than twice the proportions of those infected a lesser number of times in the study period (Figure 6).

| Case | V1 | V2 | V3 | V4 | V5 | V6 | V7 | V8 | V9 | V10 | V11 | V12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 3 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 |
| 4 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 |
| 5 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| 6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| 8 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| 9 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 11 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 12 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 13 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 14 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 15 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 16 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 17 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| 18 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| 19 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |
| 20 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 21 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| 22 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 23 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 24 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| 27 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| 29 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 30 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| 31 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| 32 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |

| Case | V1 | V2 | V3 | V4 | V5 | V6 | V7 | V8 | V9 | V10 | V11 | V12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 34 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 35 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |
| 36 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 37 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 38 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 39 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 40 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 41 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| 42 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| 43 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 44 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 45 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| 46 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 47 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 48 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 49 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 50 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 51 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 52 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 53 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 54 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 55 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 56 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| 57 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 58 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| 59 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 60 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| 61 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 |
| 62 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| 63 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 64 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |

| Case | V1 | V2 | V3 | V4 | V5 | V6 | V7 | V8 | V9 | V10 | V11 | V12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 65 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 66 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 67 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 68 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 69 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| 70 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 71 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 72 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 73 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 74 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 75 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 76 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 77 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| 78 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| 79 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 |
| 80 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 |
| 81 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 |
| 82 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 83 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 84 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 85 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| 86 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 87 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| 88 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| 89 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 90 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 91 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 92 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |
| 93 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 |
| 94 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| 95 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| 96 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

Key

a) V1, V2 etc – Visit one, visit two etc.
b) 0 - PET-PCR-negative on sampling day
c) 1 - PET-PCR positive on sampling day
d) Green - Asymptomatic.
e) Red - Symptomatic and RDT-positive on sampling day.
f) Pink - Symptomatic and RDT-positive a week or more before sampling day.
g) Ash - Symptomatic and RDT-negative on sampling day.
h) Yellow - Symptomatic and RDT-negative a week or more before sampling day.

Figure 4 Heat map of participants showing their infection statuses, asymptomatic malaria phenotypes and symptomatic malaria episodes in all visits (n=96).

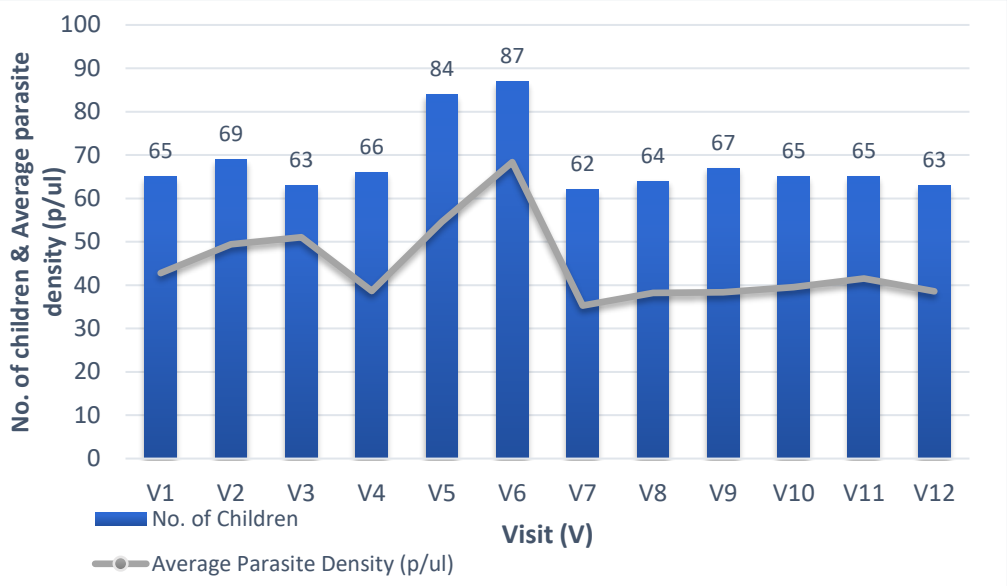



Figure 5 Number of asymptomatic *P. falciparum*-infected children and the median parasite density at every visit (n=96).

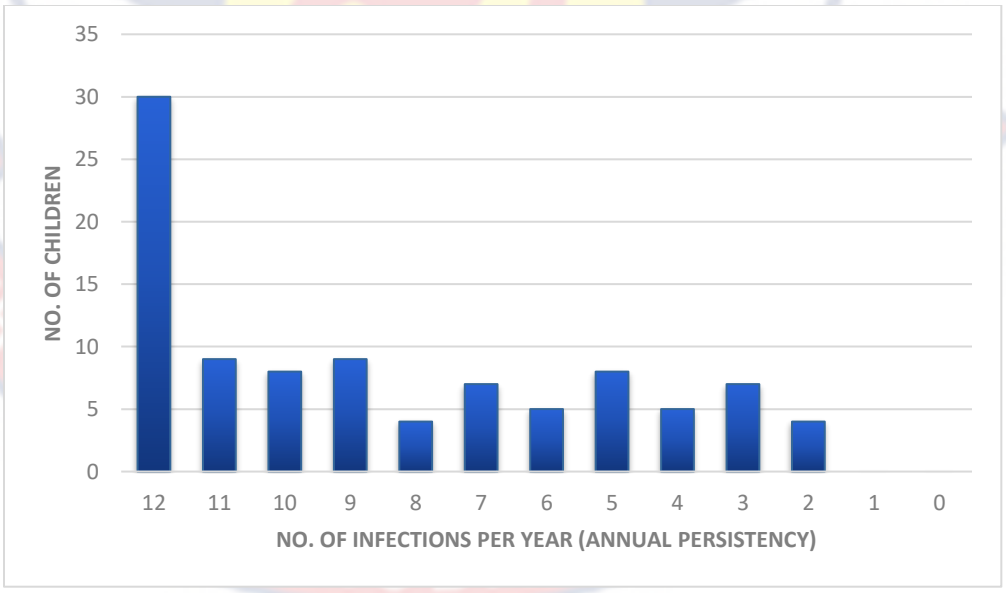



Figure 6 Number of children with various number of asymptomatic malaria infections in the year (n=96).

**MAFs of SNPs of Interest**

The MAFs of the SNPs of Interest in Simiw and African populations (NCBI, 2022; Sherry et al., 2001) and tests for HWE are presented in Table 6. *NOS2*-954 G>C deviated from HWE. *MBL2* G230A presented as a monomorphic SNP in the sample.

Table 5 MAFs of SNPs of Interest in Simiw and Africa and Tests for HWE (All 249 participants)

| SNP | Number | MAF | MAF in African Populations (NCBI) | HWE Test |
|---|---|---|---|---|
| ***TLR 4*-Asp299Gly** | 207 | 0.07 | 0.07 | 0.26 |
| ***NOS2*-954 G>C** | 229 | 0.21 | 0.06 | 0.00061 |
| ***IL 10*-592 A>C** | 210 | 0.38* | 0.41* | 0.66 |
| ***MBL2* G230A** | 226 | - | 0.04 | - |
| ***IFN-γ*+874 T>A** | 190 | 0.32* | 0.20 | 0.40 |

*MAF occurred in the normal allele.

Table 6 MAFs of SNPs of Interest in Simiw and Africa (The 96 participants)

| SNP | Number | MAF | MAF in African Populations (NCBI) |
|---|---|---|---|
| ***TLR 4*-Asp299Gly** | 96 | 0.07 | 0.07 |
| ***NOS2*-954 G>C** | 96 | 0.21 | 0.06 |
| ***IL 10*-592 A>C** | 90 | 0.37* | 0.41* |
| ***MBL2* G230A** | 93 | - | 0.04 |
| ***IFN-γ*+874 T>A** | 86 | 0.33* | 0.20 |

*MAF occurred in the normal allele.

**Confirming Genotyping**

Thirty-two (32) samples were amplified by HF-PCR at three genetic loci (*NOS2*, *IL 10* and *MBL2*) for sanger sequencing. Success rate of RFLP, as determined by sequencing, was >99%.

**Associations of SNPs of Interest with Parasite Density**

Table 7 shows the results of single-SNP analyses conducted in SNPStat. In summary, there was a statistically significant result for the dominant, codominant and overdominant models of IFN-γ+874 T>A at an alpha value of 0.05. Although this significant association occurred in the wildtype, it should be transposed to the mutant and carrier genotypes, since the mutant genotype (AA) was set as the

reference genotype. As such, the mutant allele (AA) of IFN-γ+874 T>A was associated with a higher parasite density than the wildtype and mutant genotypes. Measures of associations could not be computed for *MBL2* G230A, because the data was monomorphic.

Table 7 Association of SNPs of Interest with Parasite Density (Adjusted by Age and Season)

| SNP | Inheritance Model | Genotype | n | Transposed Count | Response Mean (SE) | Difference (95% CI) | p-value | AIC/ BIC |
|---|---|---|---|---|---|---|---|---|
| *TLR 4-* Asp299Gly | Codominant | A/A | 67 | 804 | 46.67 (1.37) | 0.00 | 0.3 | 9580.2/ 9614.2 |
| | | A/G | 11 | 132 | 48.55 (3.33) | 1.45 (-5.49 - 8.39) | | |
| | | G/G | 1 | 12 | 65.16 (5.21) | 16.57 (-4.93 - 38.07) | | |
| | Dominant | A/A | 67 | 804 | 46.67 (1.37) | 0.00 | 0.43 | 9580/ 9609.1 |
| | | A/G-G/G | 12 | 144 | 49.93 (3.11) | 2.70 (-3.99 - 9.40) | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | A/A-A/G | 78 | 936 | 46.94 (1.26) | 0.00 | | |
| | Recessive | G/G | 1 | 12 | 65.16 (5.21) | 16.36 (-5.11 - 37.83) | 0.14 | 9578.4/ 9607.5 |
| | Overdominant | A/A-G/G | 68 | 816 | 46.94 (1.35) | 0.00 | 0.73 | 9580.5/ 9609.6 |
| | | A/G | 11 | 132 | 48.55 (3.33) | 1.20 (-5.74 - 8.14) | | |
| | Log-additive | --- | --- | --- | --- | 3.40 (-2.56 - 9.35) | 0.26 | 9579.4/ 9608.5 |
| ***NOS2*-954 G>C** | | G/G | 47 | 564 | 45.86 (1.65) | 0.00 | 0.24 | 9579.8/ 9613.8 |
| | | G/C | 31 | 372 | 372 | 372 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Codominant | C/C | 1 | 12 | 43.26 (5.14) | 4.37 (-17.32 - 26.06) | | | |
| | G/G | 47 | 564 | 45.86 (1.65) | 0.00 | | | |
| Dominant | G/C-C/C | 32 | 384 | 49.09 (1.91) | 4.20 (-0.69 - 9.10) | 0.093 | 9577.8/ 9606.9 | |
| | G/G-G/C | 78 | 936 | 47.22 (1.27) | 0.00 | | | |
| Recessive | C/C | 1 | 12 | 43.26 (5.14) | 2.60 (-19.01 - 24.20) | 0.81 | 9580.6/ 9609.7 | |
| | G/G-C/C | 48 | 576 | 45.8 (1.62) | 0.00 | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Overdominant | G/C | 31 | 372 | 49.28 (1.97) | 4.10 (-0.82 - 9.02) | 0.1 | 9577.9/ 9607.1 |
| | Log-additive | --- | --- | --- | --- | 3.90 (-0.74 - 8.55) | 0.1 | 9577.9/ 9607 |
| ***IL 10*-592 A>C** | Codominant | C/C | 28 | 336 | 49.73 (2.12) | 0.00 | | |
| | | C/A | 37 | 444 | 46.14 (1.87) | -2.80 (-8.20 - 2.59) | 0.14 | 9233/ 9266.7 |
| | | A/A | 11 | 132 | 41.68 (3.26) | -7.67 (-15.33 - 0.01) | | |
| | | C/C | 28 | 336 | 49.73 (2.12) | 0.00 | 0.13 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Dominant | C/A-A/A | 48 | 576 | 45.11 (1.62) | -3.92 | (-9.05 - 1.20) | | 9232.7/ 9261.6 |
| Recessive | C/C-C/A | 65 | 780 | 47.68 (1.4) | 0.00 | | | |
| | A/A | 11 | 132 | 41.68 (3.26) | -6.07 | (-13.09 - 0.94) | 0.09 | 9232.1/ 9261 |
| Overdominant | C/C-A/A | 39 | 468 | 47.46 (1.78) | 0.00 | | | |
| | C/A | 37 | 444 | 46.14 (1.87) | -0.64 | (-5.59 - 4.32) | 0.8 | 9234.9/ 9263.8 |
| Log-additive | --- | --- | --- | --- | -3.59 | (-7.22 - 0.04) | 0.053 | 9231.2/ 9260.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***MBL2*** **G230A** | | | | Monomorphic SNP | | | |
| ***IFN-γ*+874** **T>A** | A/A | 34 | 408 | 50.17 (1.87) | 0.00 | | |
| | A/T | 27 | 324 | 42.5 (2.08) | -7.39 (-12.84 - -1.94) | 0.022 | 8473.6 / 8506.8 |
| Codominant | T/T | 9 | 108 | 52.41 (3.75) | -0.14 (-8.14 - 7.86) | | |
| | A/A | 34 | 408 | 50.17 (1.87) | 0.00 | | |
| Dominant | A/T-T/T | 36 | 432 | 44.98 (1.83) | -5.60 (-10.66 - -0.54) | 0.03 | 8474.6 / 8503 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Recessive | A/A-A/T | 61 | 732 | 46.78 (1.4) | 0.00 | | 8478.7/ |
| | T/T | 9 | 108 | 52.41 (3.75) | 3.09 | (-4.57 - 10.76) | 0.43 | 8507.1 |
| Overdominant | A/A-T/T | 43 | 516 | 50.64 (1.68) | 0.00 | | | |
| | A/T | 27 | 324 | 42.5 (2.08) | -7.36 | (-12.56 - - 2.16) | 0.0056 | 8471.6 / 8500 |
| Log-additive | --- | --- | --- | --- | -2.20 | (-5.85 - 1.45) | 0.24 | 8478/ 8506.4 |

NB: Data was transposed from wide to long and presented with a transposed count for analysis.

## Disquisition

### About the Study Design

The longitudinal nature of Gametocytogenesis, with samplings at regular intervals, allowed for an adequate evaluation of the parasitological statuses of participants; this is recommended because asymptomatic parasitaemia has been reported to fluctuate periodically (Jafari-Guemouri, Boudin, Fievet, Ndiaye, & Deloron, 2006; Snounou, Rooth, & Bjorkman, 1997). This longitudinal study also allowed for a greater accuracy in determining the asymptomatic malaria disease phenotype according to its definition. The involvement of young children in the present secondary study allowed the investigation of the associations of genetic variations in the innate immune system with parasite carriage without the cloud of a fully developed adaptive immunity (Migot-Nabias et al., 2006).

### Asymptomatic *P. falciparum* Prevalence and Parasite Density in Simiw

School-going children in Simiw had a higher asymptomatic parasite prevalence (~65% to 90%), as detected by a highly sensitive PET-PCR, than other studies in Ghana (~30% to 45%), which varied with season and age groups (Figure 5) (Amoah et al., 2019; Ayanful-Torgby, Quashie, Boampong, Williamson, & Amoah, 2018). This finding is in agreement with other studies in Sub-Saharan African, which reported higher malaria infection burdens in school-going children than younger children and adults (Mwandagalirwa et al., 2017; Nankabirwa et al., 2015; Walldorf et al., 2015; Zhou et al., 2016).

More than half of the children were asymptomatically infected in all visits, similar to reports by Obboh et al. (2020), Acquah et al. (2020) and Acquah et al.

(2021) in Simiw, with a peak of asymptomatic infections just before the peak transmission season (Figure 5). These studies reported an asymptomatic malaria parasite prevalence of ~50 % in October, 2017 and from November, 2019 to January, 2019, both in the dry season. Gametocytogenesis is the only study that has investigated parasite prevalence in the dry and wet seasons in Simiw. According to the findings of Gametocytogenesis, the ~50% prevalence of asymptomatic *P. falciparum* infections in the dry season may continue throughout the wet season, notably peaking in the pre-peak season (visits four to six) (Figure 5). The peaking of asymptomatic infections in the pre-peak season is strikingly different from the peaking of symptomatic infections in the peak season in malaria endemic areas. It is difficult to discuss this finding, as reported changes in rainfall patterns in the study site in recent years (data not available) may have had a significant influence. However, one possible reason for the rise in parasite densities in the pre-peak season is a rise in sexual parasite densities in preparation for the transmission season (Oduma et al., 2020, 2021). This phenomenon, investigated by Gametocytogenesis, theories that *P. falciparum* gametocytogenesis is triggered by a direct and/ or indirect detection of vector salivary proteins in preparation for vector arrival in the wet season. Lastly, it should be noted that parasite data for the 96 participants can be confidently extrapolated to all participants for the parent study (~250) because the two groups share virtually equal gender and MAF proportions (Tables 4 to 7).

The median parasite density of asymptomatic children at all visits was 40.5 p/µl. This is well below the pyrogenic threshold of *Plasmodium*, estimated at 2,000

p/µl (Greenwood & Armstrong, 1991; McMorrow, Aidoo, & Kachur, 2011), explaining the afebrility of infections. Interestingly, none of the median parasitic densities per visits were submicroscopic (Figure 5). This is unusual, as most asymptomatic infections are submicroscopic (Bousema et al., 2014). However, this may be explained by the age limits of the study population, which, unlike an adult population in malaria endemic areas, is able to maintain lower parasite densities in the asymptomatic state.

Based on a 1:0.05 ratio of asexual parasites to gametocytes (Taylor & Read, 1997), the median number of gametocytes in the children over the year was estimated to be two (2) gametocytes/µl (submicroscopic), sufficient for a perennial transmission (Schneider; et al., 2007), as observed in the present study and reported for Simiw. With a third of the children constantly infected throughout the entire year (Figure 6), it can be assumed that most of their infections were polyclonal, which have been reported to last for more than five months (Nassir et al., 2005).

**SNPs of Interest in Simiw**

Before I dive into the choicest of the study, I will briefly discuss the genotype data (Table 6). The findings, when compared to data from NCBI and the research hypothesis, were quite interesting. MAFs should, however, be considered in this light: NCBI pools data from different studies in different research fields of different populations with diverse ethnicities and demographics. Additionally, malaria studies pooled by NCBI may not discriminate between malaria-symptomatic and -asymptomatic populations.

***TLR 4*-Asp299Gly**

MAF of *TLR 4*-Asp299Gly in Simiw (0.07) conformed exactly with data published in NCBI (Table 6). Even though *TLR 4*-Asp299Gly increased susceptibility to severe malaria (Mockenhaupt, Hamann, et al., 2006; Sirisabhabhorn et al., 2021), it was surprising to find it maintained its proportion in a malaria asymptomatic population. This may imply that the study hypothesis is false.

***NOS2*-954 G>C**

The MAF of *NOS2*-954 G>C (0.21) was significantly higher than data published in NCBI (0.06) (Table 6), as well as, the MAF in a similar location among malaria symptomatic children (9%) (Dzodzomenyo et al., 2018). Other studies in malaria endemic populations have reported much lower MAFs ranging from three percent (3%) to 15% among populations of all malaria disease states (Kun et al., 2001; Levesque et al., 1999). MAFs of one to four percent (1-4%) have been reported in low malaria endemic areas (Dzodzomenyo et al., 2018). A pattern does not immediately emerge, however the Hardy-Weinberg Disequilibrium of *NOS2*-954 G>C may have the solution (Table 6).

*NOS2*-954 G>C did not fit HWE in this study sample. Usually, this can indicate problems with genetic data sets (Khoury et al., 1993; Khoury et al., 2004; Sham, 1998). However, this disequilibrium cannot easily be attributed to genotyping error, the typical cause of deviation (Hosking et al., 2004; Wang & Shete, 2012), because RFLP results were confirmed by sequencing. One might then point to the relatedness of participants, as a possible cause for the deviation; but

that would not explain equilibrium found in the other SNPs of Interest (Table 6). Aside from other quality control arguments that can still be mounted, such as mutations in the binding sites of primers for sequencing, it remains possible that one or more of the conditions for HWE were not met. Obviously, deviations from a hypothetical, non-evolutionary model (HWE) can be practically relevant. Natural selection of an advantageous variant and consanguinity in Africa immediately stand out (Abramovs, Brass, & Tassabehji, 2020; Garnier-Géré & Chikhi, 2013).

***IL 10*-592 A>C**

*IL 10*-592 A>C registered a similar MAF (0.38) compared to data published by NCBI (0.41) (Table 6). In lockstep with data from NCBI, the MAF occurred in the wild-type. This is unexpected in an asymptomatic population, as the wild-type is associated with symptomatic malaria protection, relative to the variant. Along with *TLR 4*-Asp299Gly, the study hypothesis did not agree with the reported MAF.

*IL 10*-592 A>C influences the production of IL 10, unique in this study for its functional relationship with TLR 4 and NO productions (Van der Graaf et al., 2006). As such, a possible SNP combination analysis was considered, however, it was beyond the scope of this study.

***MBL2* G230A**

As published by NCBI, a 0.04 MAF for *MBL2* G230A was expected for African populations. However, there was no incidence of the mutant allele of *MBL2* G230A in this study, despite a respectable statistical power (Table 6). A possible explanation for its absence is the asymptomatic boundary of the study population.

Of course, the absence of this polymorphism should be taken with caution, as a more powerful study may unearth remnants.

The oddity of this finding is not without company. Boutlis et al. (2003) reported an unexpected absence of the MPA genetic variations for *NOS2* G-954-C and C-1173-T in the Papua New Guinea, a place of intense malaria transmission. Boutlis et al. (2003) rationalised that the functionality of the alleles in malaria infection may not be universal, and the selection of the variants may be complex. Notable is the employment of asymptomatic participants in the study (similar to the present study). On the other side of the spectrum is the high frequency of *MBL2**C, a MSA allele, in Sub-Saharan Africa, which cannot be explained by Haldane's malaria hypothesis (Hanley, 2001).

Finally, an interesting study by Fugtagbi et al. (2022) reported, peculiarly, that transmission intensity influenced the susceptibility of hosts with *MBL2* G230A to asymptomatic parasite carriage. Following this lead, a null association was expected in this study conducted in a high transmission area. Surprisingly, the genetic factor was absent.

*IFN-γ*+874 T>A

*IFN-γ*+874 T>A, a MSA allele, registered a significantly higher proportion of the mutant allele (A) in the study sample (0.68) than data provided by NCBI for African populations (0.20) (Table 6). This difference was so wide, the MAF in the present study occurred in the wild type. The difference in frequencies is not in

tandem with *TLR 4*-Asp299Gly and *IL 10*-592 A>C, which passed the test for HWE and agreed with NCBI.

The high frequency of *IFN-γ*+874 A may have been influenced by factors, such as population sub-sampling and founder effect. This unusualness can also be seen in some MPA alleles in other locations. For instance, hemoglobinopathies occurred at high frequencies in Polynesia, where malaria is absent, and absent in South-America, where malaria is endemic (López, Saravia, Gomez, Hoebeke, & Patarroyo, 2010). There is also the random rise and fall in the allele frequencies of other hemoglobinopathies, such as $\alpha^{+}$-thalassemia (López et al., 2010).

The high frequency of *IFN-γ*+874 A allele may also be explained by its influence on hosts' behaviour and reproduction. IFN-γ is a serotonergic modulator, and therefore, a modifier of human behaviour. It has been associated with proactive defensive behaviours that reduce the risk of infections among healthy people. Demonstrative of this phenomenon is the work of MacMurray, Comings, and Napolioni (2014). Similar to the present study, MacMurray et al. (2014) reported a high-than-expected frequency of *IFN-γ* +874 A in the study sites of southern California and Brazil (Medina et al., 2011), which were endemic for malaria and Chagas disease. In this immune-behaviour study, the *IFN-γ*+874 T>A mutation increased susceptibility to infectious diseases, such as malaria, Chagas disease and tuberculosis. *IFN-γ*+874 A also increased "harm avoidance (such as asthenia) and decreased extroversion and exploratory excitability". Described as a "genetic trade-off or antagonistic pleiotropy" (Elena & Sanjuán, 2003), the heightened genetic risk for infectious diseases by *IFN-γ*+874 A, was compensated for by "disease-risk

avoidant behavioural traits". Interestingly, harm avoidance has a heritability of 50-60% (Ando et al., 2002; Stallings, Hewitt, Cloninger, Heath, & Eaves, 1996). Finally, *IFN-γ*+874 A was associated with an increased reproductive success in women; they were protected against recurrent pregnancy losses (Daher et al., 2003; Prigoshin, Tambutti, Larriba, Gogorza, & Testa, 2004).

A previous study found an association between a malaria-relevant SNP and asymptomatic parasitaemia, reporting transmission intensity as a possible confounder (Fugtagbi et al., 2022). With an adjustment for age and season in the present study, a significant difference in parasite densities was found for the wildtype of IFN-γ+874 T>A within the dominant, co-dominant, and overdominant inheritance models. The overdominant model was considered to be more likely because it had a lower BIC than the codominant model. The mutant genotype in the overdominant model was associated with a higher parasite density of 7.36 p/µl over the wildtype and mutant genotypes (Table 7). This is consistent with the antiparasite role of the T and A alleles of the SNP in symptomatic malaria (Table 2).

**_IFN-γ_+874 T>A was Associated with Parasite Density in Asymptomatic Infections**

According to the single-SNP analyses (Table 7), there was a higher register of mean parasite density for *IFN-γ*+874 T>A by 7.36 p/µl. Previously, IFN-γ+874 T>A has been associated with parasite susceptibility in symptomatic malaria, although the study was limited to *P. vivax* (Medina et al., 2011). According to reviewed literature, the present study is the first report of the influence of *IFN-γ*+874 in asymptomatic *P. falciparum* infections. Consistent with intuition, *IFN-*

*γ*+874 T>A was associated with a lower parasite density than the wildtype. Unfortunately, this intuitive expectation was not met by the other SNPs of Interest, casting a shadow of complexity over the study hypothesis. Of course, no less is expected in genetic studies and much more is welcomed for malaria research.

The remaining SNPs of Interest (*TLR* 4 Asp299Gly, *NOS2*-954 G>C, *IL 10*-592 A>C, *MBL2* G230A and *IFN-γ*+874 T>A) were not associated with asymptomatic *P. falciparum* density (Table 7). This finding can be presented alongside others that line up in support. The antiparasitic protection of *HbAS*, *HbCC*, *HbAC* and blood group O in symptomatic malaria does not extend to the asymptomatic parasitaemia (Migot-Nabias et al., 2000; Taylor et al., 2012; Vafa et al., 2008), although the protection of G6PD does (Amoah et al., 2016; Mombo et al., 2003; Ouattara et al., 2014). *TLR 4*-Asp299Gly has been associated with severe malaria, but not mild malaria (da Silva Santos et al., 2012; Mockenhaupt, Cramer, et al., 2006; Mockenhaupt, Hamann, et al., 2006). Additionally, Gerbich blood group negativity (deletion of exon 3) does not influence the prevalence and density of asymptomatic parasitaemia (Patel, King, Mgone, Kazura, & Zimmerman, 2004; Patel et al., 2001), although it protects against severe malaria (Maier et al., 2003). With that said, it remains to be known the association of these SNPs of Interest with asymptomatic submicroscopic parasite density, better examined among an adult population, which has a lower pyrogenic threshold and parasite density (Smith et al., 2004).

Another possible explanation for the lack of association in this study is that, although MPA alleles might be associated with antiparasitic protection in asymptomatic groups, the effect size is not comparable to antiparasitic protection in symptomatic groups. The effect may have been below the detection limit of this study. A pattern may be identified in literature. As reviewed in this thesis, there is an eight percent (8%) lesser contribution of host genetics to the variation in mild malaria than severe malaria (Kariuki & Williams, 2020). Additionally, *HbS* was associated with relatively lesser protection against mild malaria than severe malaria. Possibly, the influence of host genetics on asymptomatic malaria may be even lesser, registering much lower additive effect sizes not detectable by this moderately (traditionally adequate) powered study.

Overall, the findings of this study confirm a shared relevance of host genetic variants in all forms of malaria disease phenotypes and indicate a complex relationship therein. What remains to be found is a better hypothesis that would inform a robust selection of candidate alleles to advance the field of malaria host genetics. A good starting point could be G6PD and chromosomal loci 5q31, 12q21-q23 and 20p11q11. Chromosomal loci 5q31 and 12q21-q23 has been associated with parasite carriage (Rihet, Traoré, et al., 1998; Sakuntabhai et al., 2008) and maximum parasite density (Sakuntabhai et al., 2008) respectively, in the asymptomatic state of malaria infections. Interestingly, chromosomal region 5q31-33 has also been shown to influence symptomatic malaria parasite density (Flori et al., 2003; Garcia et al., 1998; Rihet, Traoré, et al., 1998). With a heritability of more than 40%, the locus has been duly named *PfIL* for *P. falciparum* Infection Level.

Finally, chromosomal region 20p11q11 was also associated with asymptomatic *P. falciparum* parasitaemia in a GWAS, the fourth in Africa (Milet et al., 2010).

**Summary**

In summary, this chapter presented and discussed the finds of the present study. In a systematic fashion, the results of the various SNPs of Interest were discussed. *IFN-γ*+874 T>A was associated with a lower asymptomatic parasite density while *MBL2* G230A was monomorphic in the population. As such, a relationship between asymptomatic malaria parasite density and host genetic appears evident warranting further study in the same direction.

# CHAPTER FIVE

# SUMMARY, LIMITATIONS, RECOMMENDATIONS AND CONCLUSION

## Introduction

This chapter provides a panoramic portrayal of the present study and summarises the salient points from previous chapters. This chapter comprises a summary section with key highlights, a recommendations section, which might prove useful for investigators, policy makers and other interest parties and a limitations section, which airs the weaknesses of the present study, all of which culminate in a captivating conclusion to sign off.

## Summary of the Study

Malaria can present with one of three phenotypes/ outcomes: severe, mild and asymptomatic malarias. The asymptomatic malaria phenotype is not properly understood and its parasitic characteristics have not been fittingly studied. The natural history of malaria is determined by host genetics and immunity, parasite genetics and immunity and environmental factors. Host genetics contribute approximately a third of the variation in the malaria phenotypic outcome (Kariuki & Williams, 2020). *HbS*, a popular variant, contributes only two percent to this variation, indicating the presence of several unknown additive genetic factors. The present study sought to determine the association between certain candidate biallelic genetic markers and *P. falciparum* carriage density among asymptomatic school-going children in a malaria hotspot in southern Ghana. The hypothesis tested was that clinical MPA alleles protect against parasite density in the nonclinical state

of malaria infections. This was a secondary study of the Gametocytogenesis Study, which followed malaria asymptomatic children longitudinally. Allelic discrimination was carried out on DNA samples using RFLP and Sanger sequencing. *P. falciparum* infection statuses and parasite densities, as obtained by PET-PCR, were available from the parent study. Tests of associations were carried out in SNPStat. At the end of the study, *IFN-γ*+874 T>A was associated with a lower parasite density in the asymptomatic state in the young children. The lack of positive results among the other SNPs of Interest may indicate the function of much more complex immunogenetic mechanisms against asymptomatic parasite density than hypothesised in the present study.

**Limitations of the Study**

Recognised limitations of this study include the possible relatedness of participants and a lack of statistical power to detect a smaller effect size (Cohen's $f^2 = 0.02$). The family-wise error rate was not corrected; although its necessity has been contested by Perneger (1998) and Nakagawa (2004). There was also the obvious limitation of almost any other study in this field; a null-consideration for sequestrated parasites, solvable by skin biopsies and post-mortem examinations. Least but not last, helminth co-infections, nutrition and stress among participants were not considered. The genetic heterogeneity of parasites also adds a layer of complexity that was not considered in this study (Eldh et al., 2020; Nsobya et al., 2004). Another possible limitation was drug resistance in parasites (Amenga-Etego, 2012; Diakite et al., 2011).

**Recommendations from the Study**

Absence of evidence is not evidence of absence. Much like any other study, this study should be repeated without the outlined limitations in this and many other study sites, using these and many other genetic variants. A mechanistic theory behind the action of G6PD in asymptomatic parasitaemia may inform a better selection of candidate genetic variants. Research focus could also be directed towards chromosomal loci 5q31, 12q21-q23 and 20p11q11, and the variants therein. These have been identified to influence parasitaemia in the asymptomatic infection state (Hernandez-Valladares, Rihet, & Iraqi, 2004; Milet et al., 2010; Rihet, Abel, et al., 1998; Sakuntabhai et al., 2008). With consanguinity being a major genetic factor in Africa, a fixation index, $F_{IS}$, otherwise known as Wright's Inbreeding Coefficient, may be calculated to determine the measure of consanguinity (Balding, Bishop, & Cannings, 2008; Excoffier, 2001). Hardy Weinberg Equilibrium could subsequently be calculated (not only a simple test of deviation), correcting for $F_{IS}$; this process is more complicated than expressed. If any positive results are found, an epistatic direction would be interesting to pursue.

**Conclusion**

Malaria is a debilitating illness in some of the poorest regions in the World. The control and elimination of this complex and obdurate disease requires a comprehensive understanding of all dimensions of the disease, including human host genetics and its most elusive disease state, an asymptomatic malaria infection. The present study is one of few to investigate and report the influence of human genetic variants on the asymptomatic malaria parasite density. For future

deliberations of malaria host genetics, the present study is earmarked to be bookmarked in the annals of scientific literature.

# APPENDICES

## Appendix A: Primer Sequences, Expected Band Sizes and Restriction Enzymes of SNPs of Interest

| SNP | Primer Sequences (5'-3') | PCR (bp) | PCR-RFLP (bp) | Restriction Enzyme |
|---|---|---|---|---|
| ***NOS2*-954 G>C** | F: CATATGTATGGGAATACTGTA TTTCAG<br>R: TCTGAACTAGTCACTTGAGG | 573 | GG: 437+136<br>GC:573+437 +136<br>CC: 573 | *BsaI* |
| ***IL 10-592 A>C*** | F: GGTGAGCACTACCTGACTAGC<br>R: CCTAGGTCACAGTGACGTGG | 412 | CC: 412<br>AC:412+236 +174<br>AA: 236+176 | *RsaI* |
| ***MBL2* (G230A)** | F: GCACCCAGATTGTAGGACAGA G | 340 | GG: 245+84 | *BanI* |

| | | | | |
|---|---|---|---|---|
| | R: CAGGCAGTTTCCTCTGGAAGG | | GA:340+245 +84 AA: 340 | |
| ***TLR 4*-Asp299 Gly (A>G)** | F: GATTAGCATACTTAGACTACT ACCTCCATG R: GATCAACTTCTGAAAAAGCAT TCCCAC | 263 | AA: 263 AG:222+263 GG: 222 | *NcoI* |
| ***IFN*-γ+874 T>A** | F: TTCTTACAACACAAAATCAAA TCT (T allele) F:TTCTTACAACACAAAATCAA ATCA (A allele) R: TCAACAAAGCTGATACTCCA | T: 262 A: 262 | - - | - - |

NB: *TLR 4*-Asp299Gly, *NOS2*-954 G>C, *IL 10*-592 A>C and *MBL2* G230A were genotyped by RFLP. *IFN*-γ+874 T>A was genotyped by AS-PCR.

**Appendix B: PCR and AS-PCR Reaction Conditions**

| | *NOS2*-954 G>C | *IL 10-592 A>C* | *MBL2* (G230A) | *TLR 4*-Asp299Gly (A>G) | *IFN-γ*+874 T>A |
|---|---|---|---|---|---|
| | | | **FINAL CONCENTRATIONS** | | |
| **Distilled water** | - | - | - | - | - |
| **Buffer** | 1X* | 1X** | 1X** | 1X** | 1X** |
| **$MgCl^{2-}$ (mM)** | 0.5 | 0.2 | 0.2 | 0.7 | 0.2 |
| **dNTP mix (mM)** | 0.17 | 0.2 | 0.2 | 0.2 | 0.2 |
| **Forward Primer (µM)** | 0.5 | 0.5 | 0.07 | 0.2 | 0.5 |
| **Reverse Primer (µM)** | 0.5 | 0.5 | 0.07 | 0.2 | 0.5 |
| **OneTaq® DNA Polymerase (U/µl)** | 0.01875 | 0.00667 | 0.025 | 0.025 | 0.00667 |
| **Template (ng/µl)** | ~10 | ~10 | ~10 | ~10 | ~10 |

| | | | | | |
|---|---|---|---|---|---|
| **Reaction volume (µl)** | 20 | 20 | 20 | 20 | 15 |
| | **CONDITIONS** | | | | |
| **Initial denaturation** | 94°C – 2 mins | 94°C – 2 mins | 94°C – 2 mins | 94°C – 2 mins | 94°C – 2 mins |
| **Cyclic denaturation** | 94°C – 45 secs | 94°C – 30 secs | 94°C – 30 secs | 94°C – 30 secs | 94°C – 30 secs |
| **Cyclic annealing** | 62°C – 45 secs | 62°C – 45 secs | 61°C – 30 secs | 60°C – 30 secs | 56°C – 40 secs |
| **Cyclic elongation** | 68°C – 45 secs | 68°C – 55 secs | 72°C – 60 secs | 68°C – 30 secs | 68°C – 40 secs |
| **No. of cycles** | 35 | 35 | 35 | 35 | 35 |
| **Final elongation** | 68°C – 5 mins | 68°C– 5 mins | 72°C – 7 mins | 68°C – 5 mins | 68°C– 5mins |
| **Hold** | 4°C | 4°C | 4°C | 4°C | 4°C |

NB: *5x PCR buffer (NEB, USA); **5x GC buffer (NEB, USA); dNTP - Deoxynucleotide Triphosphate.

**Appendix C: Conditions for Restriction Digest Reactions**

| | ***NOS2* -954 G>C** | ***IL 10* -592 *A>C*** | ***MBL2* (G230A)** | ***TLR* 4-Asp299Gly (A>G)** |
|---|---|---|---|---|
| | **FINAL CONCENTRATIONS/ VOLUME** | | | |
| **Distilled water** | - | - | - | - |
| **rcutsmart buffer** | 1X | 1X | 1X | 1X |
| **Restriction Enzyme (U/µl)** | 0.1 | 0.1 | 0.1 | 0.1 |
| **Template/ PCR product (µl)** | 10 | 10 | 10 | 10 |
| **Reaction volume (µl)** | 20 | 20 | 20 | 20 |
| | **CONDITIONS** | | | |
| **Enzyme Activation** | 37ºC for 1 hour | 37ºC for 1 hour | 37ºC for 1 hour | 37ºC for 1 hour |
| **Hold** | 4ºC | 4ºC | 4ºC | 4ºC |

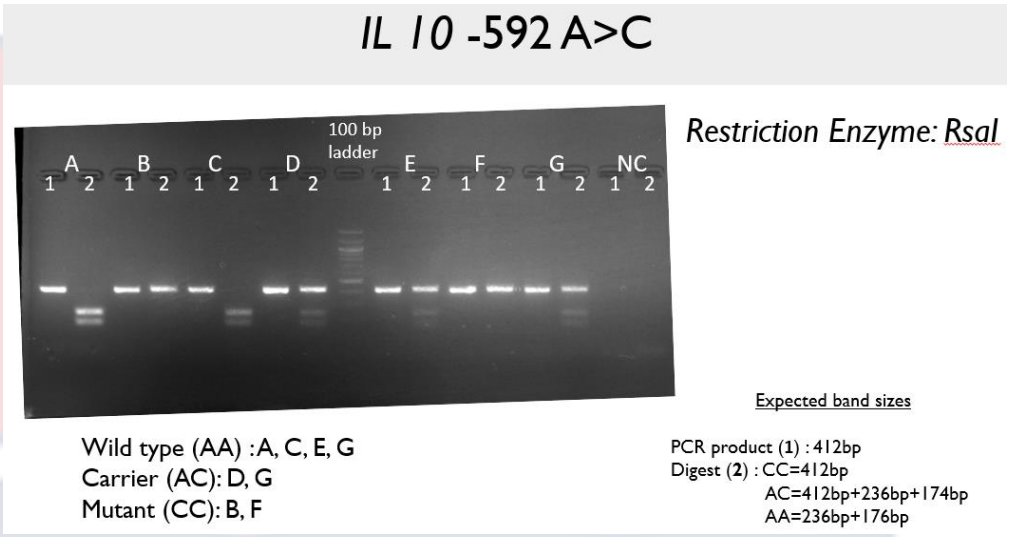
NB: the volume of template/ PCR product used for digest is indicated instead of the concentration

**Appendix D: HF-PCR Reaction Conditions**

| | *NOS2*-954 G>C | *IL 10-592 A>C* | *MBL2* (G230A) |
|---|---|---|---|
| | **FINAL CONCENTRATIONS** | | |
| **Distilled water** | - | - | - |
| **Q5 Reaction buffer** | 1X | 1X | 1X |
| **dNTP mix (mM)** | 0.2 | 0.2 | 0.2 |
| **Forward Primer (µM)** | 0.5 | 0.5 | 0.5 |
| **Reverse Primer (µM)** | 0.5 | 0.5 | 0.5 |
| **Q5® High-Fidelity DNA Polymerase (U/µl)** | 0.01 | 0.01 | 0.01 |
| **Template (ng/µl)** | ~2 | ~2 | ~2 |
| **Reaction volume (µl)** | 30 | 30 | 30 |
| | **CONDITIONS** | | |

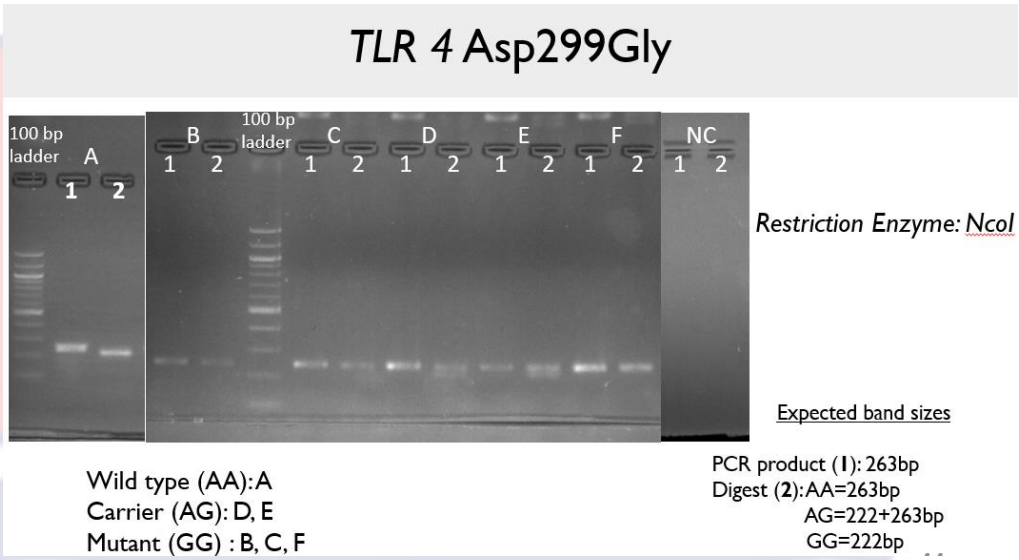
| | | | |
|---|---|---|---|
| **Initial denaturation** | 98°C – 30 secs | 98°C – 30 secs | 98°C – 30 secs |
| **Cyclic denaturation** | 98°C – 10 secs | 98°C – 10 secs | 98°C – 10 secs |
| **Cyclic annealing** | 62°C – 30 secs | 70°C – 30 secs | 68°C – 30 secs |
| **Cyclic elongation** | 72°C – 10 secs | 72°C – 10 secs | 72°C – 10 secs |
| **No. of cycles** | 35 | 35 | 35 |
| **Final elongation** | 72°C – 2 mins | 72°C – 2 mins | 72°C – 2 mins |
| **Hold** | 4°C | 4°C | 4°C |

**Appendix E: Gel Image Showing Electrophoresed Products of *IL 10* PCR and *IL 10*-592 A>C Restrictive Digest**

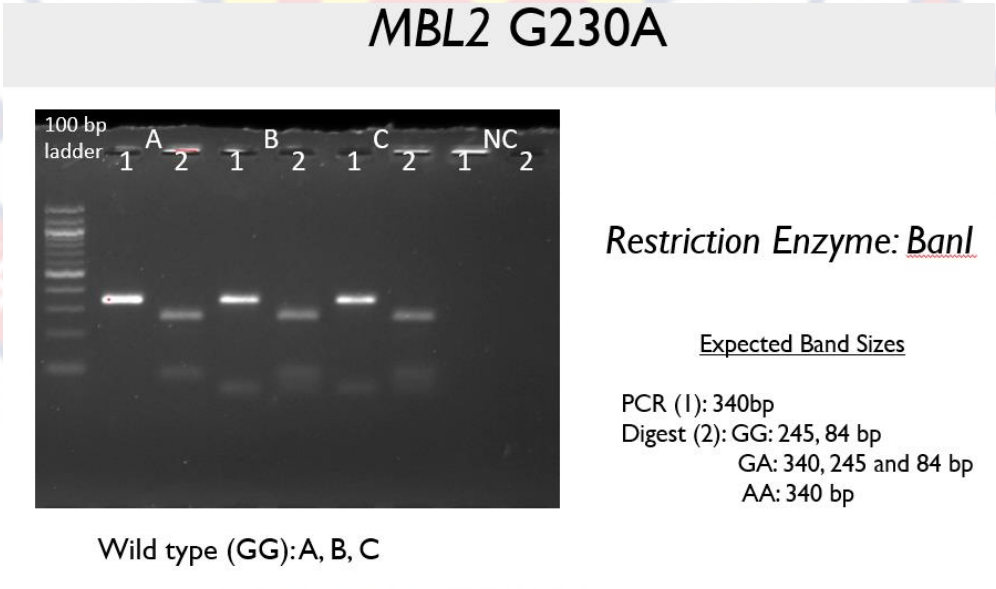



**Appendix F: Gel Image Showing Electrophoresed Products of *NOS2* PCR and *NOS2*-954 G>C Restrictive Digest**

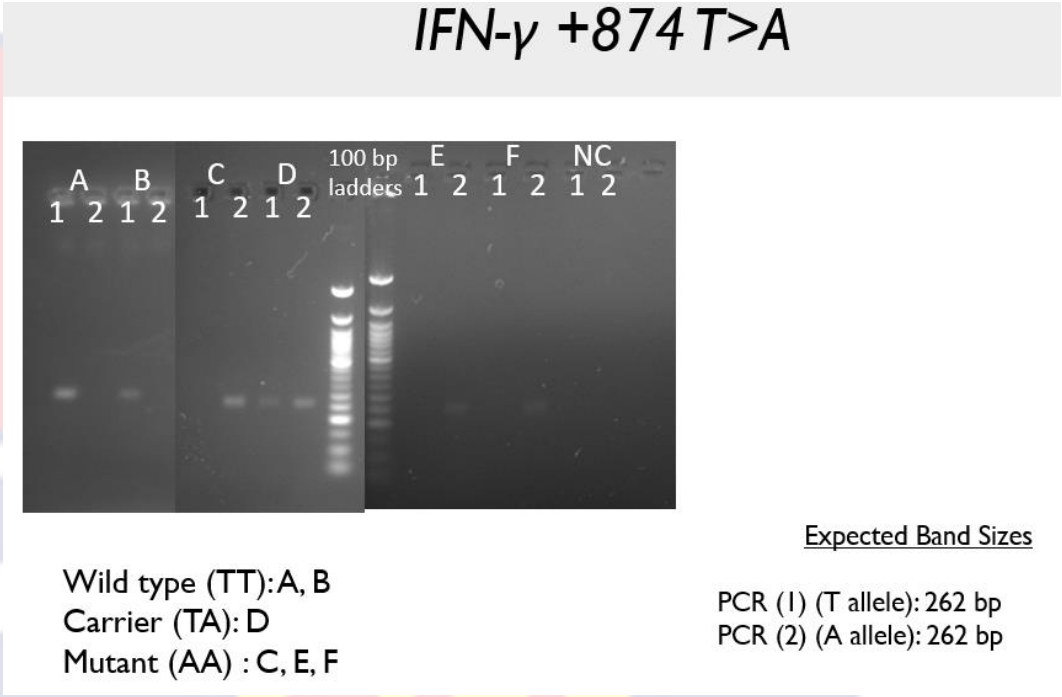
**Appendix G: Gel Image Showing Electrophoresed Products of *TLR 4* PCR and *TLR 4*-Asp299Gly Restrictive Digest**



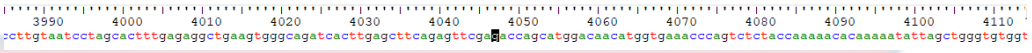

**Appendix H: Gel Image Showing Electrophoresed Products of *MBL2* PCR and *MBL2* G230A Restrictive Digest**



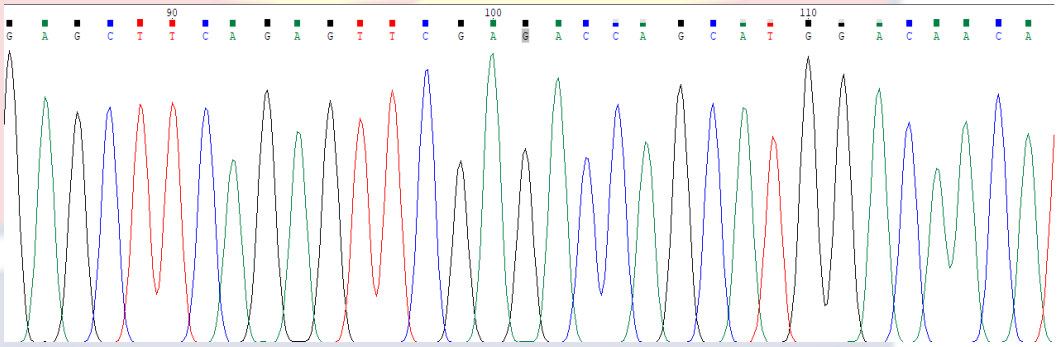
**Appendix I: Gel Image Showing Electrophoresed Products of *IFN-γ*+874 T>A (AS-PCR)**



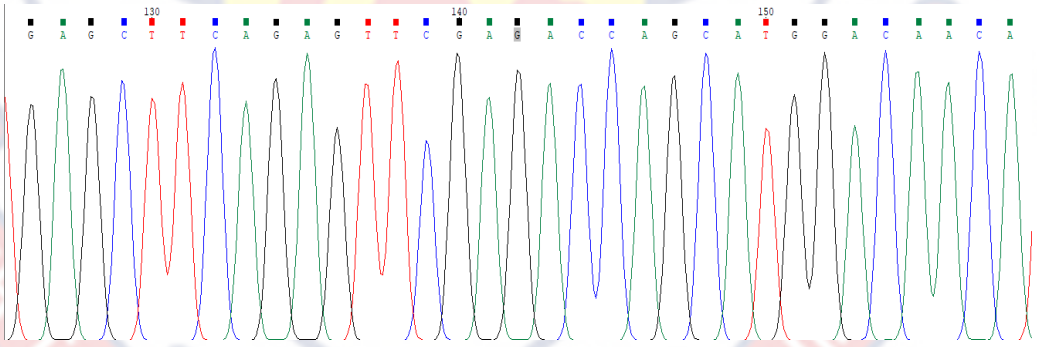
**Appendix J: Chromatographs of NOS2 locus and Reference Sequence (the relevant SNP positions are highlighted).**



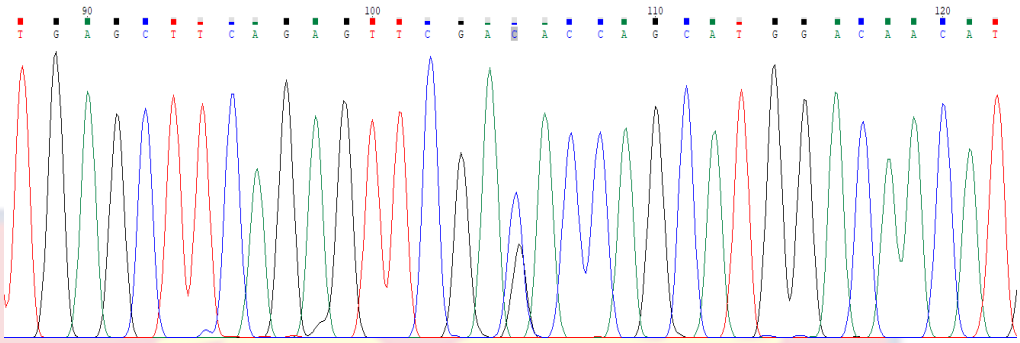

NCBI Reference Sequence: NG_011470.1 (Trimmed)



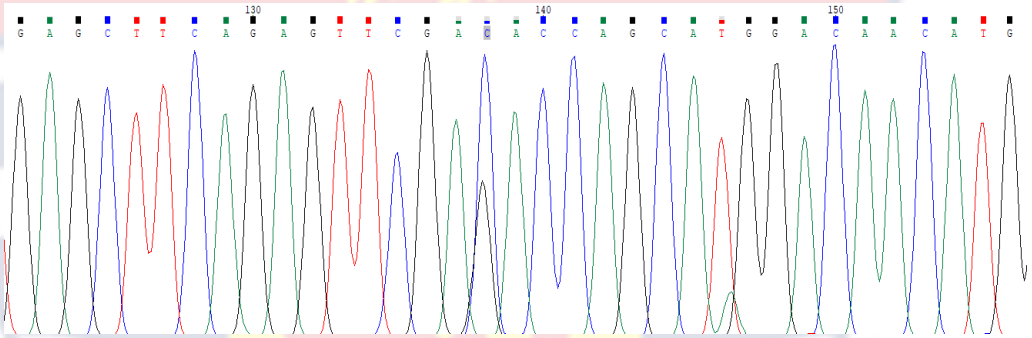

Chromatogram of *NOS2*-954 G>C homozygous wild type sequenced using forward primer (Trimmed Trace Data; 5'-3' direction)



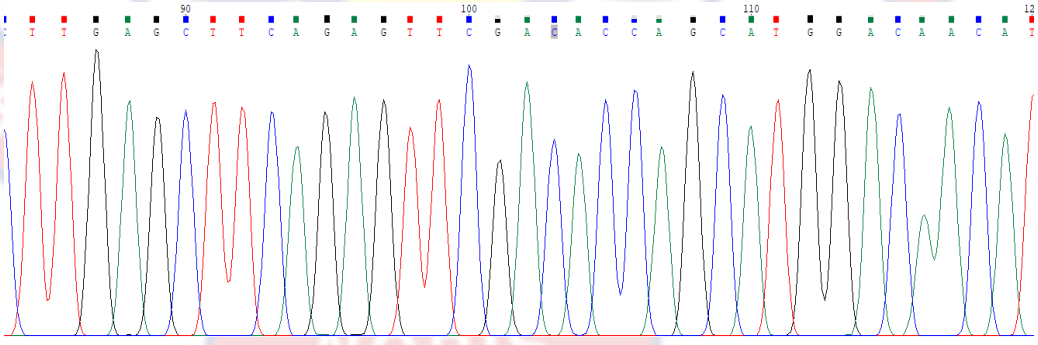

Chromatogram of *NOS2*-954 G>C homozygous wild type sequenced using reverse primer (Trimmed Trace Data; reverse complement; 5'-3' direction)



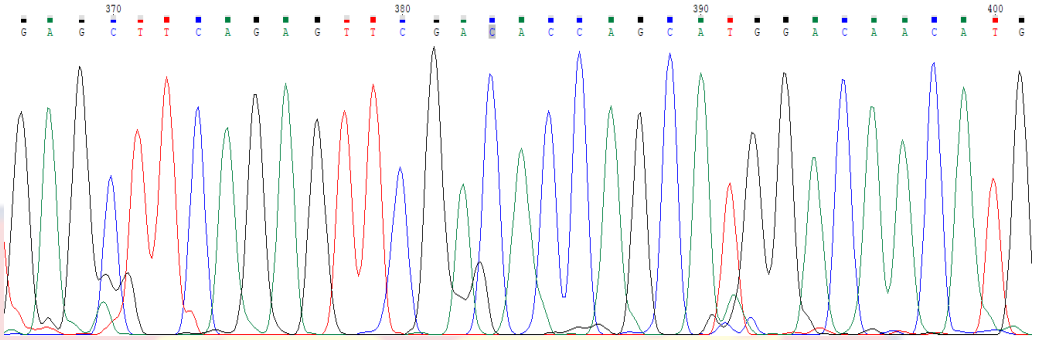
Chromatogram of *NOS2*-954 G>C heterozygous mutation sequenced using forward primer (Trimmed Trace Data; 5'-3' direction)



Chromatogram of *NOS2*-954 G>C heterozygous mutation sequenced using reverse primer (Trimmed Trace Data; reverse complement; 5'-3' direction)

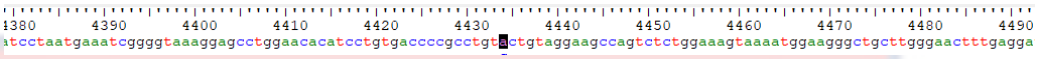


Chromatogram of *NOS2*-954 G>C homozygous mutation sequenced using forward primer (Trimmed Trace Data; 5'-3' direction)

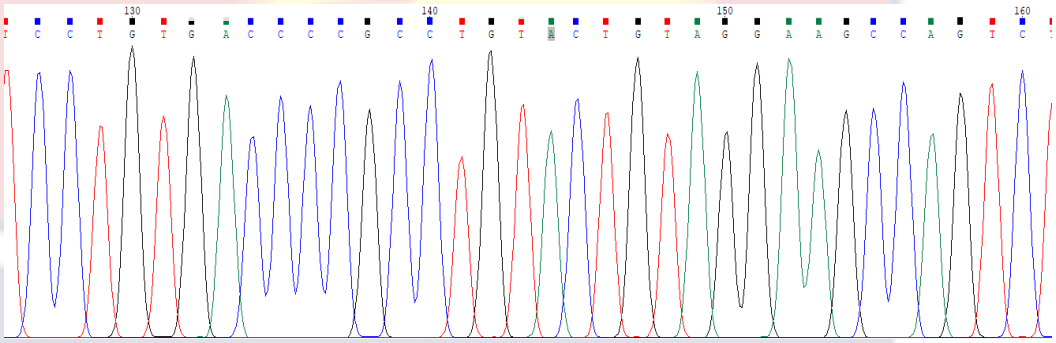



Chromatogram of *NOS2*-954 G>C homozygous mutation sequenced using reverse primer (Trimmed Trace Data; reverse complement; 5'-3' direction)

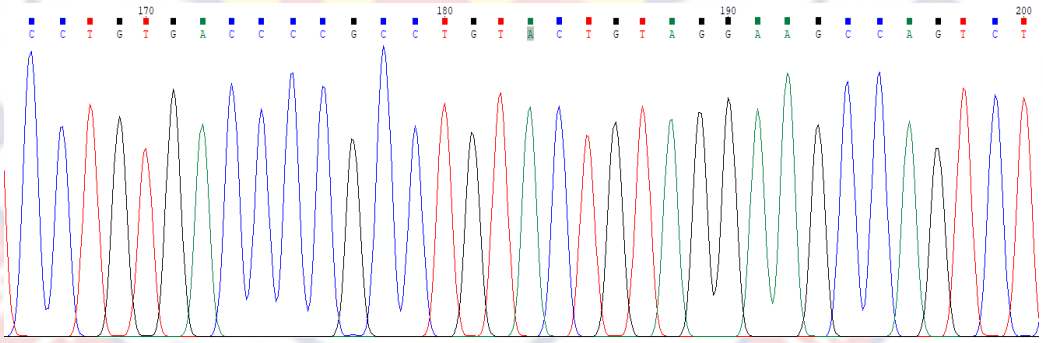
**Appendix K: Chromatographs of IL 10 locus and Reference Sequence (the relevant SNP positions are highlighted).**



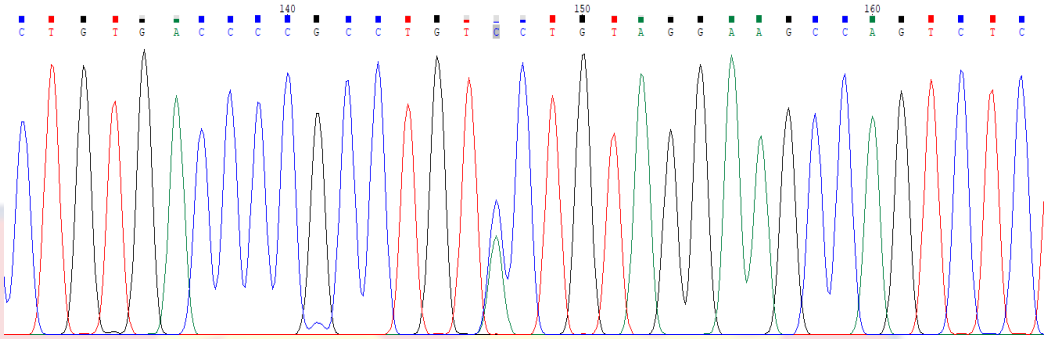

NCBI Reference Sequence: NG_012088.1 (Trimmed)



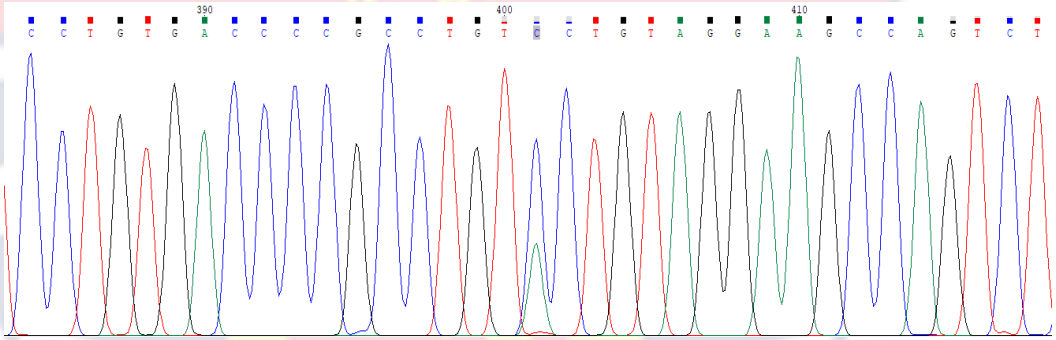

Chromatogram of *IL 10*-592 A>C homozygous wild type sequenced using forward primer (Trimmed Trace Data; 5'-3' direction)



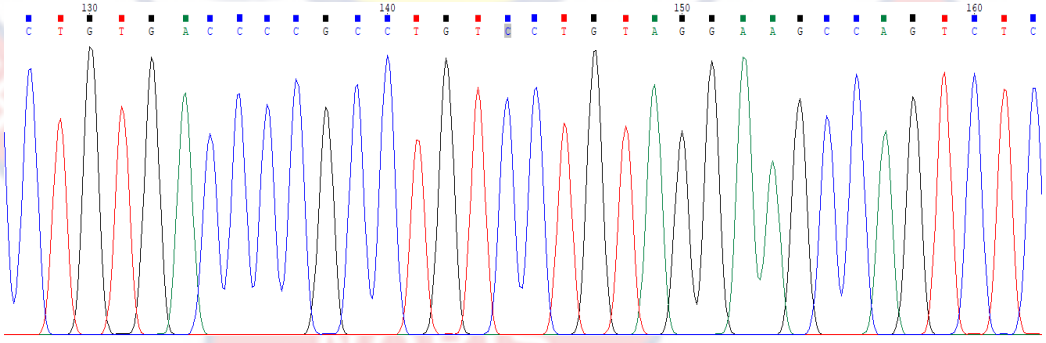

Chromatogram of *IL 10*-592 A>C homozygous wild type sequenced using reverse primer (Trimmed Trace Data; reverse complement; 5'-3' direction)



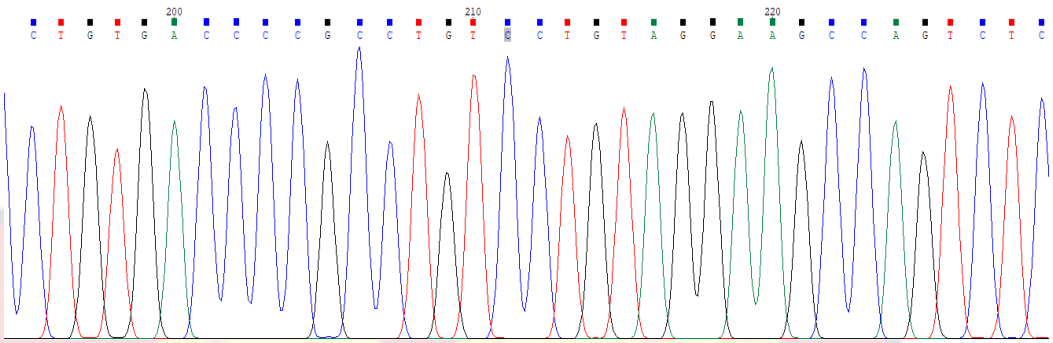
Chromatogram of *IL 10*-592 A>C heterozygous mutation sequenced using forward primer (Trimmed Trace Data; 5'-3' direction)



Chromatogram of *IL 10*-592 A>C heterozygous mutation sequenced using reverse primer (Trimmed Trace Data; reverse complement; 5'-3' direction)

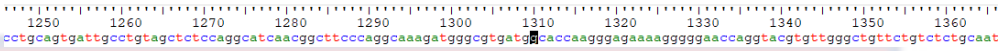


Chromatogram of *IL 10*-592 A>C homozygous mutation sequenced using forward primer (Trimmed Trace Data; 5'-3' direction)



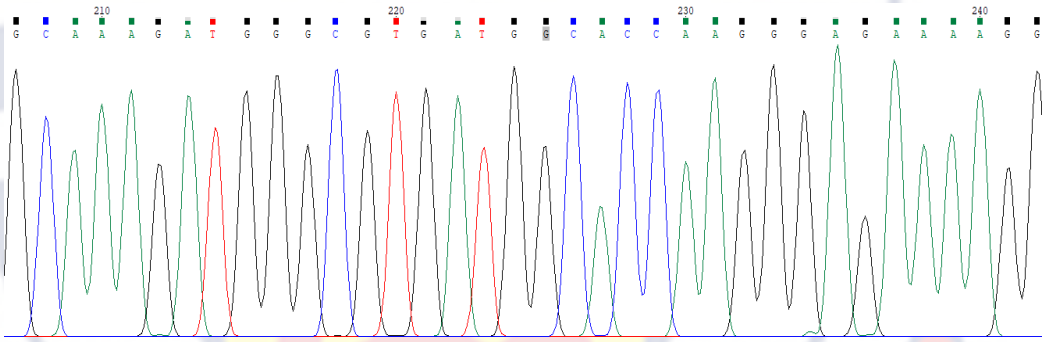
Chromatogram of *IL 10*-592 A>C homozygous mutation sequenced using reverse primer (Trimmed Trace Data; reverse complement; 5'-3' direction)

**Appendix L: Chromatographs of MBL2 locus and Reference Sequence (the relevant SNP positions are highlighted).**

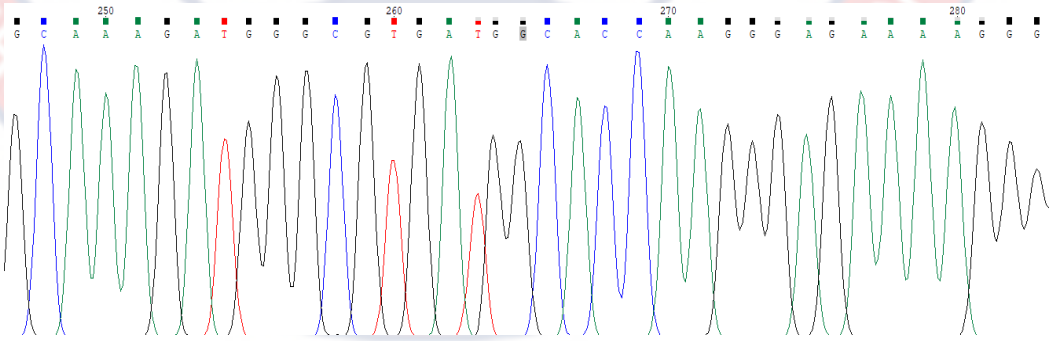



Reference sequence: NCBI Reference Sequence: NC_000010.11 (Trimmed)

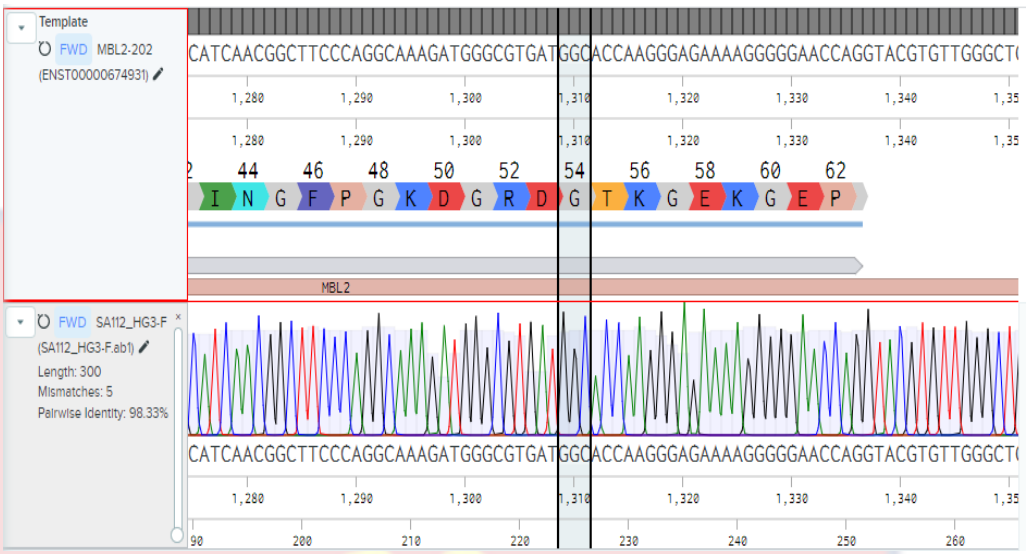
NB: Nucleotides to the immediate right and left of the SNP position of interest are the remaining two members of the codon. This is illustrated in the chromatogram from The Benchling R&D Cloud below.




Chromatogram of *MBL2* G230A homozygous wild type sequenced using forward primer (Trimmed Trace Data; 5'-3' direction)




Chromatogram of *MBL2* G230A homozygous wild type sequenced using reverse primer (Trimmed Trace Data; reverse complement; 5'-3' direction)




Chromatogram of *MBL2* G230A homozygous wild type sequenced using forward primer (Trimmed Trace Data in The Benchling R&D Cloud; 5'-3' direction).

NB: Polymorphic site occurs at the medial position of the highlighted codon.

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