UNDERSTANDING THE DIFFERENCES IN PLASMODIUM FALCIPARUM GENETIC DIVERSITY AND HOST IMMUNE PROFILES IN ASYMPTOMATIC AND SYMPTOMATIC MALARIA INFECTIONS

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2024

DECLARATION

I, **Kelvin Muteru Kimenyi**, declare that this thesis is my original work and has not been presented for a degree or any other award in any University. To the best of my knowledge and brief, this thesis contains no material previously published or written by another person except where due reference is made.

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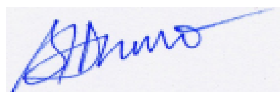
Signature:  Date: 12-09-2024

DEDICATION

This work is dedicated to my wife Anne, son Rick and daughter Janelle who despite not understanding the work, have supported, and inspired me immensely. Not forgetting my parents, who passed on the love of reading and respect for education.

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LIST OF ABBREVIATIONS

COVID-19	Coronavirus Disease 2019
WHO	World Health Organization
RBCs	Red Blood Cells
iRBCs	Infected Red Blood Cells
MDA	Mass Drug Administration
IRS	Insecticide Residual Spray
ITNs	Insecticides Treated Nets
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribose Nucleic Acid
RDT	Rapid Detecting Test
COI	Complexity of Infection
MSP2	Merozoite Surface Protein 2
VSA	Variant Surface Antigens
PBMCs	Peripheral Blood Mononuclear Cells
DEGs	Differentially Expressed Genes
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
FDR	False Discovery Rate
µl	Microliter
RLE	Relative Log Expression
TMT	Tandem Mass Tag
DEPs	Differentially Expressed Proteins
TMM	Trimmed Mean of M-values
PCA	Principal Component Analysis

ABSTRACT

Malaria remains a serious infectious disease in the tropics whose pathogenesis is attributed to a complex interaction between parasites with high genetic diversity and robust host immune responses. In endemic regions, frequent exposure to malaria often leads to acquisition of anti-disease immunity and asymptomatic infections that serve as silent natural reservoirs of infectious parasites thereby sustaining malaria transmission. Although modulation of host immunity has been implicated in the maintenance of chronic symptomless infections, it is still unclear how the host and malaria parasites interactions are involved. To understand this phenomenon, blood samples from 425 children (<15 years) enrolled in a cohort study in Kilifi County, between 2007 and 2019, were studied. Annual cross-sectional surveys conducted in the cohort provided samples from healthy/uninfected and asymptomatic children while weekly follow-ups identified children with symptomatic malaria. Parasite genetic diversity, using merozoite surface protein 2 as a genetic marker, and gene expression profiles as well as host immune responses were then studied by comparing samples from uninfected and/or asymptomatic children with those from subsequent symptomatic malaria infections. Asymptomatic infections had significantly lower parasite density, 800 parasites/µl compared to febrile malaria 28,800 parasites/µl ($p < 0.0001$) that mainly comprised of new parasite clones. They were also more polyclonal (>2 parasite clones per infection) and had a significantly higher complexity of infection (COI) of 2.3 compared to symptomatic malaria infections, 2.0 ($p = 0.016$). Interestingly, children harboring asymptomatic infections had a parasite transcriptional signature featuring a bias towards the trophozoite stage (~12 hours-post invasion), while febrile infections featured increased ring stage parasites (~ 9 hours-post invasion). The host response during febrile/symptomatic children featured increased upregulation of genes associated with inflammatory responses compared to asymptomatic children. Similarly, uninfected children showed upregulation of genes involved in inflammatory responses compared to asymptomatic children. Furthermore, the host-responses during symptomatic malaria infections that followed an asymptomatic infection featured a significant upregulation of genes related to inflammatory responses (TNFRSFIB, TLR4, CCR7, IL1B, IFNGR1), whereas the symptomatic host responses from previously uninfected children were characterized by increased upregulation of genes related to humoral/antibody related immune responses (IGHM, IKC, IGHG4, IGHG2, C5). The results suggest that asymptomatic children in Kilifi harbor highly diverse and polyclonal parasites whereas their subsequent symptomatic

malaria infections consist of new parasite subpopulations unfamiliar to the immune system. Additionally, the priming effect of preceding asymptomatic infection could lead to a reduction in acquisition of antibody responses seen during the subsequent symptomatic malaria infections. This could explain the observed reduction in antibody responses to malaria parasite antigens after vaccination in malaria endemic regions. Therefore, malaria control campaigns should aim to eliminate all parasites, including those in asymptomatic individuals.

Chapter 1: INTRODUCTION

1.1 Background of the study

1.1.1 Introduction and malaria epidemiology

Malaria is a serious public-health problem with devastating socio-economic repercussions, particularly in poor resource settings. In 2022, World Health Organization (WHO) reported about 259 million malaria cases and 618,000 deaths globally caused by malaria (WHO, 2023). Approximately half of the global population is at risk of contracting malaria, with tropical and subtropical areas showing the highest prevalence (WHO, 2016). Over the last 115 years, sub-Saharan Africa has experienced a 40% (1900 – 1929) and 24% (2010 – 2015) reduction in the prevalence of *Plasmodium falciparum*, the main causative agent of malaria (Snow *et al.*, 2017). However, this reduction has been interrupted by periods of rapidly increasing and decreasing transmission (Snow *et al.*, 2017). A case in point is the recent COVID-19 pandemic that added an extra burden on health systems hindering the provision of malaria control and prevention measures that led to the rise in malaria cases and deaths (WHO, 2021b). In Kenya, substantial progress in lowering the prevalence of malaria has been achieved over the last 25 years with 61% of the population living in areas below 1% prevalence (Macharia *et al.*, 2018). The Coastal and Lake Victoria endemic regions have the highest community prevalence of around 4.5% and 18.9%, respectively (*Kenya National Bureau of Statistics. Kenya Malaria Indicator Survey 2020.*, 2020). Malaria infection is caused by protozoan parasites of the genus *Plasmodium* that infect humans. They include *P. falciparum*, *P. vivax*, *P. malariae, P. ovale*, and *P. knowlesi.* Out of the five species, *P. falciparum* is the most deadly worldwide and the most prevalent in sub-Saharan Africa (Snow *et al.*, 2005). Additionally, *P. vivax* and *P. knowlesi* have also been shown to cause severe disease (Daneshvar *et al.*, 2009; Naing *et al.*, 2014). The parasites are transmitted by *Anopheles* mosquitoes. Africa experiences the biggest malaria burden mainly due to the presence of *An. Gambiae* complex that comprises of *A. gambiae sensu stricto*, *An. Arabiensis, An. Melas and An. Merus* (Sinka *et al.*, 2011). Three additional vectors are also present in Africa including *An. moucheti*, An. *funestus* and *An. nili* (Sinka et al 2010). Latest reports have indicated that *An. stephensi*, previously considered as an Asian malaria vector, is rapidly invading and spreading in Eastern Africa (Mnzava, Monroe and Okumu, 2022). A combination of environmental factors, such as vegetation cover, rainfall, temperature, and human factors, such as agriculture,

urbanization, deforestation, influences malaria vectors distribution and complicate the malaria transmission cycle.

Malaria disease spectrum varies in severity from severe, mild, to the little known asymptomatic or symptomless infections. Malaria infections are mainly characterized by a repeated cycle of fever and chills. Additional common symptoms may include arthralgia, vomiting, haemoglobinuria, convulsions and anemia. The symptoms arise approximately 6 to 14 days, after schizont rupture and destruction of red blood cells (RBCs), and peak about every 48 hours upon each successive release of merozoites from RBCs (Trampuz *et al.*, 2003). Sometimes, these symptoms remain unobserved and the infection is labeled asymptomatic particularly in individuals lacking a recent history of antimalarial treatment (Lindblade *et al.*, 2013). In other cases, severe symptoms are observed and are majorly caused by *P. falciparum*. They include cerebral malaria that manifests as coma, respiratory distress characterized by pulmonary oedema, severe anemia characterized by normocytic anemia with hemoglobin level <5g/dl, hypoglycemia, hyperparasitemia, hyperbilirubinemia and renal failure (WHO 2000). Such diverse manifestations have been associated with factors such as host immune response, parasite virulence, host susceptibility and environmental factors (Andrade and Barral-Netto, 2011; Medzhitov, Schneider and Soares, 2012; Galatas, Bassat and Mayor, 2016). In malaria endemic regions, most fevers are presumed to be caused by malaria (D'Acremont *et al.*, 2009). However, studies have shown that majority of these fevers could be due to infection with bacterial, viruses or fungi (D'Acremont, Lengeler and Genton, 2010; Pondei, Kunle-Olowu and Peterside, 2013). Thus, malaria diagnostic capacity should be strengthened and complemented by sufficient clinical diagnosis to accurately diagnosis malaria especially in cases of overlapping clinical manifestations with other illnesses.

1.1.2 Life cycle of *P. falciparum*

The life cycle of *Plasmodium spp.* is complex and utilizes two hosts i.e. humans and female *Anopheles* mosquitoes (**Figure 1**). While taking a blood meal, an infected female *Anopheles* mosquito releases sporozoites from the saliva into the injection site. Within 30-60 minutes, the sporozoites travels through the host blood circulation system into the liver and finally infects liver cells or hepatocytes. After about 7 to 10 days, the sporozoites matures into schizonts without causing any symptoms. The schizonts then rupture and releases daughter parasites (merozoites) into the bloodstream, where they invade RBCs and initiate the erythrocytic stage of infection (Miller, 1969). Notably in *P. vivax* and *P. ovale*, a quiescent stage called hypnozoites can persist

in the liver without causing clinical symptoms but cause relapsed disease many months after initial infection upon reactivation and release into the bloodstream (Wells, Burrows and Baird, 2010). Within RBCs, merozoites evolve from ring forms to trophozoites and later to multinucleated schizonts, containing up to 32 merozoites, that rapture to release merozoites into the bloodstream to perpetuate the cycle of invading other RBCs. The infecting population of parasites tend to undergo this cycle, from invasion into RBC to release of merozoites, synchronously within 48 hours. This rhythmic process is thought to be maintained by a parasite-intrinsic oscillator and not by host circadian processes (Smith *et al.*, 2020). This cyclical rupture of infected RBCs causes releases merozoites and other parasite byproducts like hemozoin and glycosylphosphatidylinositol. These products activate cells of the innate immune system leading to production of inflammatory cytokines which trigger the thermoregulation centers in the brain to raise body temperature eventually leading to fever (Oakley *et al.*, 2011; Punsawad, 2013). Usually, symptoms appear four to eight days after the initial blood invasion. Some merozoites may differentiate into sexual forms that include male and female gametocytes (Smith *et al.*, 2000). This process is usually epigenetically regulated by Apetala 2-G (AP2-G) transcription factor, which belongs to a group of ApiAP2 transcription factors and is a key determinant of sexual commitment, that acts as a transcriptional activator of early gametocyte associated genes (Josling *et al.*, 2020). Finally, the gametocytes that consists of female (macrogametocytes) and male (microgametocytes) are ingested by a female *Anopheles* mosquito during a blood meal and travels to the mosquito midgut where the male gametocytes penetrate the female gametocytes to form zygotes. The zygotes later on transforms into ookinetes that penetrates across the midgut-wall to become oocysts (Angrisano *et al.*, 2012). The oocysts develop and, rupture to release sporozoites that migrates to the salivary glands. Injection of these sporozoites into the next human host occurs during the next blood meal and marks the start of another cycle (Trampuz *et al.*, 2003).

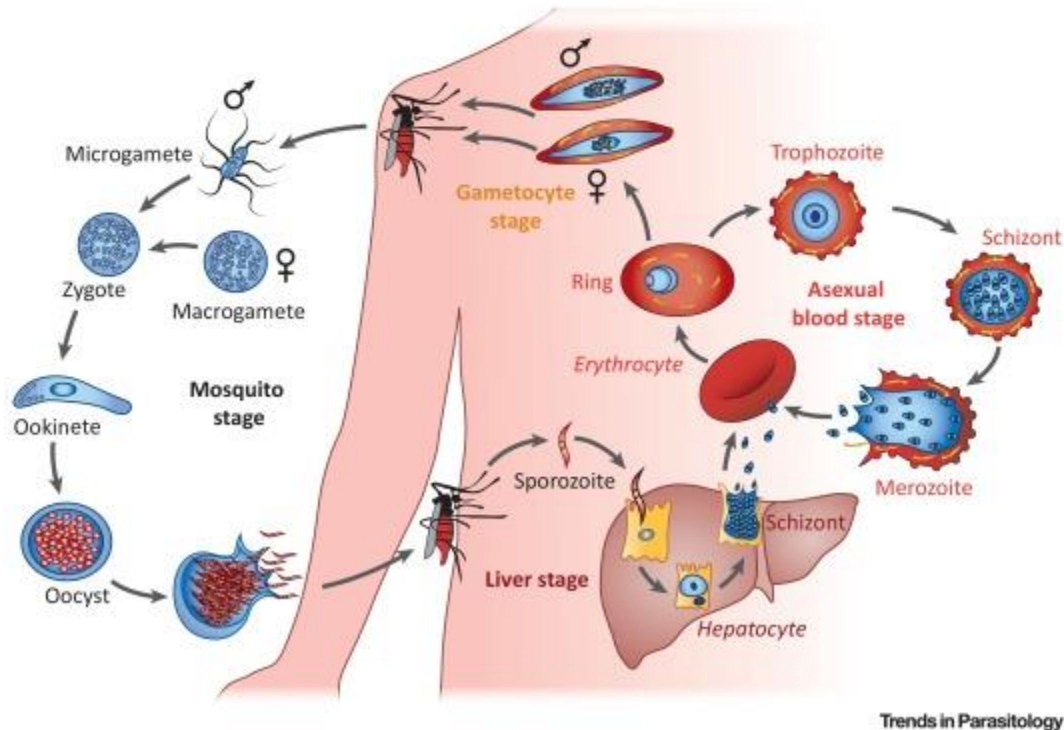

Figure 1: ***P. falciparum*** **life cycle.**

Malaria infection commences when a person is bitten by an infected female Anopheles mosquito that injects sporozoites. These sporozoites quickly migrate to the liver then mature and bursts to release merozoites into the bloodstream. They invade RBCs and multiply asexually leading to bursting that causes fever and other symptoms. Some merozoites develop to become female and male gametes that are ingested by mosquitoes during the next blood meal where they fuse to form oocytes that later divide to become sporozoites. The sporozoites migrate to the mosquito's salivary glands to commence another cycle. Adopted from (Maier *et al.*, 2019).

1.1.3 Prevention and control of malaria

Various interventions exist that aid in preventing malaria transmission. Their purpose is to either prevent the infection by avoiding bites by mosquitoes or prevent disease using antimalarial drugs. The main interventions recommended by WHO Global Malaria Program (WHO/GMP) for effective malaria control include the use of insecticide treated nets (ITNs), indoor-residual spraying (IRS), chemoprevention and larval control strategies that include mass-drug administration (MDA), seasonal-malaria chemoprevention (SMC) and intermittent preventive treatment in pregnancy (IPTp) (WHO, 2016). The use of ITNs involves sleeping under bed nets treated with insecticides as a protective barrier to keep away mosquitoes. This has been very successful in controlling malaria especially in poor resource African settings (Afoakwah, Nunoo and Andoh, 2015; Kanyuka *et al.*, 2016; Dhiman, 2019; Oduma *et al.*, 2021). It has recently been reported that ITNs introduced in 17 malaria endemic African countries between 2019 and 2022 averted 13 million malaria cases and 25,000 deaths (The Global Fund, 2024). The use of IRS involves spraying walls and other surfaces in a house using a residual insecticide hoping that mosquitoes will rest on the sprayed surface after feeding. Studies have shown that IRS has turned out to be successful in reducing malaria incidence by 62% in Ethiopia (Hamusse, Balcha and Belachew, 2012). In Uganda, application of IRS led to a 47% and 71% reduction in malaria incidence among children under 5 years and individuals over 5 years, respectively (Ronald *et al.*, 2023). Larva control involves eliminating mosquitoes before they reach the stage where they can transmit malaria either by reducing larval breeding sites, biological control or use of chemicals. The use of MDA involves the treatment of a population in a specific region with antimalarial drugs, except individuals whom the drugs are contraindicated, without first confirming the presence of the infection and irrespective of the presence of symptoms (Zuber and Takala-Harrison, 2018). This method only results in a short-time reduction in malaria infection and may provide a substantial selective pressure on parasite populations, thus increasing the risk of parasites developing resistance (Zuber and Takala-Harrison, 2018). In areas that experience seasonal malaria transmission, SMC is administered. They are a form of MDA that involves dispensing a curative dose of anti-malarial drugs to children especially those at high risk of severe malaria (WHO 2012).

WHO recommends that malaria patients should be treated within the first 24 hours after the appearance of the symptoms and confirmation of the parasite by microscopy or rapid diagnostic

test (RDT) (WHO 2012). In 2006, the Kenyan government implemented the use of artemether-lumefantrine (AL) as the first line treatment for uncomplicated malaria and dihydroartemisinin-piperaquine as the second line treatment. Artesunate is recommended for severe malaria and sulfadoxine-pyramethrine for IPTp (Musuva *et al.*, 2017), as AL is usually contraindicated in early pregnancy (Mosha *et al.*, 2014).

Despite malaria disease being documented since 2700 BC, development of vaccines became more evident in 2015 (Duffy et al 2020; Millet et al 1995). RTS,S vaccine, which is a pre-erythrocytic candidate, was the first malaria vaccine to pass regulatory assessment after being favorably reviewed by EMA (European Medicines Agency) (Duffy and Patrick Gorres, 2020). Currently, it is recommended by WHO for use among children in regions with moderate to high malaria transmission intensity especially in sub-Saharan Africa (WHO, 2021a). RTS,S aims to train the immune system to eliminate sporozoites before they infect the liver cells. There is evidence that this vaccine significantly reduces severe malaria cases by 30% (Laurens, 2020; Arora, Anbalagan and Pannu, 2021). However, the efficacy of RTS,S vaccine is still undesirable and warrants significant improvements in order to achieve malaria eradication (Mahmoudi and Keshavarz, 2017). The second malaria vaccine to be recommended by WHO was the R21/Matrix-M vaccine which has been shown to reduce malaria symptomatic cases by 75% especially in areas that experience seasonal malaria transmission. Both vaccines work by stimulating the production of antibodies that block sporozoites from infecting liver cells as well as activating T cells that eliminate parasites post the liver stage. Currently, there is no evidence showing which amongst the two vaccines outperforms the other and WHO has recommended that the choice of the vaccine to use in a country be based on vaccine availability (WHO recommends R21/matrix-m vaccine). However, the R21 vaccine is cheaper and easier to make thus it is suggested to have a higher public health impact (Naddaf 2023). Another vaccine that is still undergoing clinical trials is the radiation-attenuated whole sporozoite (PfSPZ) vaccine that has been shown to be safe and conferred over 75% protection to heterologous *P. falciparum* parasites in a controlled human malaria infection (CHMI) study (Datoo *et al.*, 2021; Mordmüller *et al.*, 2022).

Malaria control and eventual elimination efforts are currently under threat by the emergence of drug resistant parasites and insecticide resistance by mosquitoes, presence of highly genetically diverse parasites, prevalence of malaria infections with multiple genetically distinct parasite strains and the perennial presence of asymptomatic *P. falciparum* infections (Alonso *et al.*, 2011; Phillips

et al., 2017; Abukari, Okonu, Samuel B. Nyarko, *et al.*, 2019). The presence of parasites with the ability to resist clearance by artemisinin-based combination therapies was first reported in South East Asia (Dondorp *et al.*, 2009). Since then, surveillance of markers of artemisinin-resistance in Africa have increased as the continent bears the greatest burden of the disease. Independent emergence of artemisinin resistant parasites has recently been shown in Rwanda (Uwimana *et al.*, 2020), Uganda (Balikagala *et al.*, 2021), Eritrea (Mihreteab *et al.*, 2023), Ethiopia (Fola *et al.*, 2023) and Tanzania (Juliano *et al.*, 2023) among clinical samples, further threatening to reverse the gains made in controlling malaria.. Similarly, low frequency unvalidated mutations in artemisinin resistance marker have previously been reported among asymptomatic carriers in temporal survey in Coastal Kenya (Wamae *et al.*, 2019) as well as a recent survey of asymptomatic infections amongst school-aged children in Western Kenya (Osoti *et al.*, 2022). The presence of these mutations necessitates the development of novel approaches to boost malaria control efforts. These approaches should aim to target all parasites i.e. in symptomatic as well as in asymptomatic carriers who remain undetected as they are unlikely to seek treatment (Bousema *et al.*, 2014). Interventions that target only the symptomatic or clinical cases may inadvertently select for ‘asymptomatic parasites’, thereby complicating malaria elimination.

1.2 Problem Statement

Though significant progress has been made in eliminating malaria, it remains a life-threatening disease particularly around the tropics. At the moment, more than 85 territories and countries globally are threatened by the risk of malaria transmission (WHO, 2023). Moreover, over 125 million foreign travelers visit these territories annually further increasing the risk of transmission and complicated malaria elimination (DeVos et al 2023). While the current information regarding malaria disease is based on clinical/symptomatic malaria infections, the biology of symptomless or asymptomatic infections is still unstudied. In addition, there is lack of consensus on the definition of these infections that complicates the comparison of results across studies. Also, there is currently very limited data on the parasite transcriptome and host transcriptome and proteome from asymptomatic carriers.

This thesis was designed to address the current gaps in our understanding of asymptomatic *P. falciparum* infections. First, the study reviewed available literature and came up with propositions on the most suitable approaches to understand these infections. It also studied the temporal genetic diversity of asymptomatic infections based on the merozoite surface protein (*msp*) 2 gene as a genetic marker which has previously been shown to be the most polymorphic and informative in genotyping *P. falciparum* parasite subpopulations compared to other *msp* genes. In addition, the study investigated the differences in *P. falciparum* parasite gene expression profile in asymptomatic infections compared to that of parasites from ensuing symptomatic malaria infections. Finally, the human host gene and protein profiles from individuals harboring asymptomatic infections were compared to those of ensuing symptomatic malaria infections as well as samples from uninfected individuals to provide insights into host responses involved in maintaining symptomless *P. falciparum* infections.

1.3 Study justification

Repeated exposure to genetically diverse parasite sub-populations often results in the acquisition of anti-disease immunity due to the balance between pro and anti-inflammatory immune responses that manifest as asymptomatic infections. Acquisition of anti-disease immunity not only provides great promise that our immunity can be trained to resist malaria infection, but also accelerates vaccine development.

Asymptomatic infections present a big challenge to malaria elimination campaigns as they serve as a silent reservoir of infectious malaria parasites that sustain malaria transmission. Additionally, current malaria interventions only target symptomatic or clinical cases, thus inadvertently selecting for 'asymptomatic parasites' and derailing malaria elimination efforts. Recent evidence suggests that malaria parasites' best survival strategy to overcome numerous selective forces encountered in the host lies in their ability maintain asymptomatic infections. Hence, the presence of these infections threatens to derail malaria control and elimination efforts.

Most malaria studies have focused on clinical malaria leading to the presence of limited data on the parasite genotypes as well as parasite and host transcriptome and proteome from asymptomatic infections. The lack of a standard definition and inclusion criteria of asymptomatic infections has also complicated comparison of datasets across studies.

This work determined whether the decline in malaria transmission intensity, due to intensification of control measures, impacted parasite genetic diversity. It also evaluated whether detectable asymptomatic infections had an impact on the stages of the parasite in circulation, whether the host immune response differed when compared to that of uninfected children and whether asymptomatic infection affects the host response to a febrile superinfection.

1.4 Objectives

1.4.1 General study objective

To study the differences in *P. falciparum* genetic diversity and gene expression profiles as well as host immune profiles between paired asymptomatic and corresponding symptomatic/febrile malaria infections.

1.4.2 Specific objectives

1. To investigate the temporal genetic diversity of *P. falciparum* parasite sub-populations in paired asymptomatic and symptomatic malaria infections using the merozoite surface protein 2 (msp2) genetic marker.
2. To determine differences in *P. falciparum* gene expression profiles in paired asymptomatic and symptomatic malaria infections.
3. To determine differences in host gene and protein expression profiles among asymptomatic, symptomatic and uninfected individuals.

1.5 Null hypotheses

1. There exists no difference in *Plasmodium falciparum* parasite genetic diversity between asymptomatic and symptomatic malaria infections.
2. *P. falciparum* gene expression profiles between asymptomatic and symptomatic malaria infections are similar.
3. Host immune profiles among uninfected individuals, asymptomatic *P. falciparum* carriers and symptomatic malaria patients do not differ.

CHAPTER 2: LITERATURE REVIEW

2.1 Asymptomatic *P. falciparum* infections

These are infections without the classical malaria symptoms mostly fever, but whose parasitaemia can be detected by microscopy, RDTs or molecular methods (Bousema *et al.*, 2014). Currently, there is no standard way of defining these infections, thus complicating the comparative analysis of results across studies (**Table 1**). The basic definition of these infections entails the presence of parasitemia and lack of symptoms, mainly fever (usually axillary temperature less than 37.5℃), seems to be applied across many studies (Lindblade *et al.*, 2013). This definition is ambiguous, hence most studies have modified it by incorporating strict inclusion criteria that includes: 1) the use of longitudinal follow-ups after diagnosis to reduce the chances of "false" asymptomatic parasitemia that are defined during *P. falciparum* incubation towards a clinical outcome; 2) quantification of parasitemia instead of simply reporting as presence or absence and; 3) the incorporation of clinical history data to exclude those who experienced symptoms in the recent past and sought treatment. However, the duration of clinical history is unspecific ranging from 2 weeks to 1 month in some studies **(Table 1).** My first publication from this thesis reviewed the inclusion criteria in asymptomatic *P. falciparum* infections studies and suggest a more feasible approach of defining these infections. Based on our proposition, the inclusion criteria should include longitudinal follow-ups for up to a 6-week period. Four weeks prior to the asymptomatic case and 2 weeks following the case with no history or evidence of the individual having taken antimalarials and no evidence of fever 48 h before and after the case (Kimenyi, Wamae and Ochola-Oyier, 2019).

Normally, most of all *P. falciparum* infections worldwide are usually asymptomatic and they may translate to about five times more widespread than clinical/symptomatic malaria (Lindblade *et al.*, 2013; Galatas, Bassat and Mayor, 2016). One of the earliest reports on asymptomatic infections indicated that their prevalence was over 75% among malaria infections in endemic regions of The Gambia (Greenwood, 1987). Since then, studies have reported varying prevalence of asymptomatic malaria infections across Africa ranging from 40% in Central African Republic (Korzeniewski, Bylicka-Szczepanowska and Lass, 2021), 42% in Western Kenya (Salgado *et al.*, 2021) and as high as 95% in Eastern Uganda (Rek *et al.*, 2022). However, the criteria for inclusion of asymptomatic individuals and the method used to diagnosis the presence of parasites affect the

estimated prevalence of asymptomatic malaria (Laishram *et al.*, 2012). For example, children in Uganda were reported to have a 17% and 47% prevalence of asymptomatic parasitaemia based on microscopy and PCR (Polymerase Chain Reaction), respectively (Nsobya *et al.*, 2004).

Another report in Nigeria showed the prevalence of asymptomatic parasitemia among schooling adolescents to be 9%, 47% and 80% by microscopy, RDT and PCR, respectively (Abdulraheem *et al.*, 2022). A wider deployment of more sensitive diagnostic methods like PCR is needed to provide knowledge on the prevalence of asymptomatic infections so as to aid elimination efforts (Bousema *et al.*, 2014; Ochwedo *et al.*, 2021). Nevertheless, PCR based methods are not cost effective. Asymptomatic malaria infections present a big challenge to malaria elimination efforts, particularly in regions that experience high malaria transmission. Individuals harboring these symptomless infections are unlikely to seek treatment thereby acting as a silent natural reservoir for infectious malaria parasites (Galatas, Bassat and Mayor, 2016). Studies have shown that these infections contain more gametocytes, compared to symptomatic infections, which are infectious to mosquitoes thus sustaining malaria transmission (Andolina *et al.*, 2021; Barry *et al.*, 2021; Sumner *et al.*, 2021; Rek *et al.*, 2022). Asymptomatic carriers migrating to malaria-free areas can develop symptoms when their immunity is compromised leading to imported malaria. Similarly, they can cause transfusion induced or organ-transplantation malaria in these regions (Verra *et al.*, 2018). Finally, asymptomatic infections present a problem for malaria diagnosis as their low parasite density can only be detected by sensitive methods like PCR because they are often missed by less sensitive standard diagnosis methods like microscopy and RDT (Ochwedo *et al.*, 2021).

Table 1: Examples of inclusion criteria used for defining asymptomatic infections in selected transcriptomic studies

Country, year	**Criteria for defining asymptomatic *P. falciparum* infections**	**Study subjects, (sample size)**	**Follow up protocol, duration**	**References**
Cameroon, 2009	Positive thick blood smear and afebrile with no history of fever and antimalarial treatment in the previous 1 and 2 weeks respectively at the time of mass screening	Children <12 years, (18)	No follow-up	(Almelli *et al.*, 2014)
Mali, 2011	PCR detection of *P. falciparum* parasites and lack of fever with no history of antimalarial in the last 30 days and helminths.	Individuals >13, (5)	Bi-weekly surveillance of *P. falciparum* and malaria episode, respectively	(Tran *et al.*, 2016)
Gabon, 2005	Blood smear and lack of clinical symptoms.	Children 0.5 – 6 years (ND)	Follow up for 5 consecutive days	(Boldt *et al.*, 2019)
Uganda, 2007 - 2008	Blood smear and no fever.	Children 4-5 years, (78)	Follow up for upto7 days	(Jagannathan, Kim, *et al.*, 2014)
Mali, 2006	Not stated	5-13yrs (34)	Not defined	(Portugal *et al.*, 2014)
Ghana, 2020	Blood smear and lack of malaria symptoms	Children <15 years (60)	No follow up	(Frimpong *et al.*, 2020)
Mali, 2011-2019	Thick blood smear and no fever or immunosuppressive or antimalarial drugs in the last 30 days	3 months – 45 years (12)	Not defined	(Andrade et al. 2020)
Indonesia, 2022	Blood smear and no clinical symptoms	5 – 45 years (40)	No follow up	(Studniberg *et al.*, 2022)

Yellow highlights indicate inconsistent or lack of inclusion criteria.

Asymptomatic infections may either clear naturally, remain asymptomatic or become febrile. Age has been associated with longer duration of these infections as repeated exposure over time may lead to the development of a more robust anti-disease immunity compared to less exposed individuals. (Buchwald *et al.*, 2019). Also, transmission intensity has been associated with altering the risk of becoming febrile as asymptomatic individuals residing in moderate to high transmission intensity settings are associated with reduced risk of febrile malaria compared to uninfected individuals (Wamae *et al.*, 2018; Buchwald *et al.*, 2019). While the reverse is true for low transmission settings (Wamae *et al.*, 2018).

Asymptomatic infections can later become symptomatic and exhibit symptoms characteristic of clinical malaria (Bousema *et al.*, 2014). This can be caused by reinfection with new clones containing surface antigens, especially the merozoite surface antigens which are most abundant proteins on the surface of RBCs invading merozoites, different from those previously encountered, hence triggering an increase in parasite density and the development of symptoms (Magesa *et al.*, 2002; Buchwald *et al.*, 2019; Wamae *et al.*, 2022). In the presence of partial immunity, it is expected that clearing of asymptomatic infections would result in an increased risk of symptomatic malaria in the event of *P. falciparum* reinfection. Studies have tested this hypothesis and reported inconsistent results with some studies showing that treatment of asymptomatic parasitaemia is associated with increased risk of febrile malaria and others reported no effect (Owusu-Agyei *et al.*, 2002; Portugal *et al.*, 2017). This presents a gap in our understanding of how the immune system maintains malaria infection in the symptomatic or asymptomatic state.

The ability to be asymptomatic has always been viewed as beneficial to the host as anti-disease immunity may reduce the risk of developing severe disease (Doolan, Dobaño and Baird, 2009). However, studies are now demonstrating that these infections cause significant health implications as they were linked to the presence of recurrent cases of clinical malaria, splenomegaly, anaemia, neonatal and maternal deaths (Chen *et al.*, 2016). A recent study has showed that asymptomatic infections were associated with an immunosuppressive transcriptional signature characterized mainly by the down regulation of T-cell function and an inability to induce efficient anti-parasite immunity (Studniberg *et al.*, 2022). This evidence may also suggest why malaria vaccines fail to induce effective immune responses particularly antibodies, in malaria endemic regions. Previously, malaria exposed adults have been shown to be less immunogenic to attenuated sporozoite vaccine formulations compared to infants and malaria naïve adults (Sissoko et al 2017:

Ishizuka et al 2016).

Asymptomatic infections present the best opportunity for the malaria parasite to survive and overcome numerous selective forces in the host. This can be evidenced by the ability of parasite to survive during vector-free dry seasons, that can last up to 6 months without being cleared by the host or causing disease, only to restart transmission during the ensuring rainy season (Portugal *et al.*, 2017). Recently, a study in Mali showed that the low parasite levels experienced in the dry season result from increased splenic clearance, due to reduced cytoadherence of infected RBCs. In the following wet season, the parasite may senses the presence of the vector and increases its cytoadherence to host tissues thereby reducing splenic clearance and increasing parasitemia that manifests as malaria symptoms (Andrade *et al.*, 2020).

2.2 Naturally acquired immunity to malaria

Immunity to malaria refers to the resistance to infection by the malaria parasite via processes involved in either destroying the parasites or hindering their multiplication. Thus, immunity may either be innate or acquired. Innate immunity is an inherent feature of the host to resist the introduction of the parasite without regard to previous infection or exposure to the parasite. Acquired immunity involves the enhancement of the host defense mechanism due to previous exposure either actively, via previous infection with the parasite or passively through transfer of antibodies. Acquired immunity against the malaria parasite can be described either as anti-disease immunity as it prevents manifestation of malaria symptoms or anti-parasite immunity as it limits parasite growth (Doolan, Dobaño and Baird, 2009; Ademolue and Awandare, 2018). Once someone is infested with malaria parasite, the immune system is tasked with eliminating the parasites, otherwise known as anti-parasite immunity. Also, the immune system is also tasked with averting the occurrence of clinical symptoms, otherwise known as anti-disease immunity. In people with repeated exposure to malaria parasite, acquired immunity is biased towards anti-disease instead of anti-parasite immunity thereby maintaining low-level asymptomatic parasitemia (Ademolue and Awandare, 2018). This acquired immunity provides great promise that our immunity can be trained to resist malaria infection. However, acquired immunity rarely progresses to sterile immunity and often results in asymptomatic infections (Tran *et al.*, 2013). Acquiring natural sterile immunity may be impossible due to the high diversity of pre-erythrocytic and erythrocytic surface antigens (Takala and Plowe, 2009) and the immunosuppressive nature of

chronic malaria infections that is characterized by dysregulation of the immune system (Greenwood *et al.*, 1972; Kijogi *et al.*, 2018). A study in Mali looking for evidence of sterile immunity among individuals living a malaria endemic region concluded that such evidence is non-existent despite the acquisition of clinical immunity as these individuals had chronic infections (Tran *et al.*, 2013). Sterile protection can nevertheless be induced by inoculation with whole sporozoites (Roestenberg *et al.*, 2009).

The immune system is involved in determining the outcome of malaria infection as exhibited by the fact that parasitemia tolerated in high transmission regions is higher than that causing fever in low transmission regions (Roucher *et al.*, 2012; Tran *et al.*, 2016). Individuals residing in malaria endemic regions often harbor persistent asymptomatic infections and are clinically immune due to cross-protection obtained from exposure to multiple genetically distinct *P. falciparum* infections (Bull and Marsh, 2002). Age, a proxy for repeated parasite exposure, has been directly associated with the likelihood of harboring asymptomatic infections (Ladeia-Andrade *et al.*, 2009; Gonzales *et al.*, 2020). Exposure related modulation of the immune system has been shown to be characterized by the increased production of immunoregulatory cytokine interleukin 10 (IL-10) and activation of neutrophils, $CD8^{+}$ T cells and B cells (Bediako *et al.*, 2019). Lack of continuous exposure to malaria may lead to the loss of acquired immunity resulting in elevated pro-inflammatory responses and a high risk of illness (Bediako *et al.*, 2016a). In addition, decreased *P. falciparum* exposure may also result in a reduction in memory B cells and antibodies, explaining the observed delay in acquisition of immunity to malaria (Ayieko *et al.*, 2013). Thus, although a reduction of malaria transmission is a major achievement by the malaria control campaigns, it may seriously impact the development of acquired immunity. Overall, the mechanisms behind acquisition of anti-disease immunity are still not well understood and additional studies are necessary to understand how it develops with implications for application in vaccine development (Ademolue and Awandare, 2018).

Modulation of immune responses has previously been linked to various clinical malaria outcomes (Medzhitov, Schneider and Soares, 2012; Kumar, Ng and Engwerda, 2019). The immune responses are mediated by cytokines which are signaling proteins that regulate inflammation and are thus involved in protective immunity. These cytokines include tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and interleukin 12 (IL-12) (Angulo and Fresno, 2002; Malaguarnera and Musumeci, 2002). Over stimulation of the immune system leading to

overproduction of these cytokines and activation of immune cells is detrimental to the host as they are likely to cause severe malaria symptoms (Lyke *et al.*, 2004; Butler, Harris and Blader, 2013). Luckily, anti-inflammatory cytokines like IL-10, interleukin 27 (IL-27) and tissue growth factor beta (TGF-β) have been shown to dampen pro-inflammatory cytokines thereby minimizing disease severity (Malaguarnera and Musumeci, 2002; Findlay *et al.*, 2010). The presence of anti-inflammatory cytokines, especially IL-10, suppresses parasite clearance, hindering the development of anti-parasite immunity and clinical malaria (Kumar, Ng and Engwerda, 2019), while promoting the development of asymptomatic infections (Portugal *et al.*, 2014). Previously, elevated levels of IL-10 has also been linked to asymptomatic infections in pregnant women (Wilson *et al.*, 2010).

The production of anti-inflammatory cytokines has been shown to increase with repeated exposure to malaria, resulting in asymptomatic infection (Portugal *et al.*, 2014). Conversely, a lack of continuous exposure in historically exposed individuals can lead to the loss of anti-disease immunity (Deloron and Chougnet, 1992; Bediako *et al.*, 2016b). This was exhibited by an increased production of pro-inflammatory cytokines and the proliferation of CD4+ T cells (Bediako *et al.*, 2016b). A study in Ugandan children revealed that the production of cytokines by CD4+T cells is influenced by prior exposure to malaria infections. CD4+ T-cells in more exposed children were shown to produce higher levels of IL-10, while those in less exposed children produced higher levels of TNFα, hence promoting inflammation. The lack of TNFα production was associated with asymptomatic infections (Jagannathan, Eccles-James, *et al.*, 2014). A transcriptomic study in Mali described the activation of pro-inflammatory cytokine (IFN-γ, TNF and IL-1β) production as being influenced by prior exposure to malaria with asymptomatic infections having the least activation of these cytokines (Tran *et al.*, 2016). Another study in Uganda, revealed that the frequent exposure to malaria infection causes decreased levels of pro-inflammatory cytokine (IFN-γ, TNF) producing $V\delta2^+$ γδ T cells and increased expression of immunoregulatory genes potentially dampening symptom development upon subsequent infections (Jagannathan, Kim, *et al.*, 2014). In addition to exposure, age differences have also been suggested to modulate the immune system, with older children having lower anti-inflammatory and pro-inflammatory responses as compared to younger children (Farrington *et al.*, 2017). Lower levels of regulatory T cells (T regs) has also been observed in asymptomatic compared to symptomatic individuals (Boyle *et al.*, 2015; Frimpong *et al.*, 2018). Additionally, high levels of

T regs have been associated with increased parasitaemia, TGF-β production and the development of clinical symptoms (Walther *et al.*, 2005; Minigo *et al.*, 2009; Boyle *et al.*, 2015). Lower T reg levels, on the other hand, may result in a decreased risk of developing symptoms that translates to anti-disease immunity (Frimpong *et al.*, 2018).

Unlike in asymptomatic infections, the Fulani ethnic group who have reduced susceptibility to *P. falciparum* infection compared to sympatric tribes, have a higher ratio of pro-inflammatory to anti-inflammatory cytokines (Boström *et al.*, 2012). The higher levels of pro-inflammatory cytokines have been implicated in causing the reduced symptomatic cases and parasite densities. In a transcriptomic study of their monocytes, an increased up-regulation of gene pathways involved in the production of pro-inflammatory cytokines in uninfected Fulani was observed, potentially priming the immune system to respond more effectively to *P. falciparum* infections (Sanou *et al.*, 2017). Thus, it appears that a balance between inflammatory and regulatory cytokines is important in achieving anti-disease immunity.

Antibodies also play a significant role in malaria protection. Seminal studies in monkeys and humans reported a reduction in fever and parasitaemia following the passive transfer of serum or immunoglobulin G (IgG) antibodies from immune to non-immune subjects with acute malaria (Cohen, McGregor and Carrington, 1961; Mshana *et al.*, 1991). However, antibody responses to malaria seem to be short lived as exposure to malaria may not lead to the production of sufficient antigen-specific memory B cells (Dorfman *et al.*, 2005). In contrast, Swedish residents who had previously travelled to malaria endemic regions and received malaria treatment after developing malaria symptoms were shown to maintain long-lasting memory B cells for 16 years without subsequent exposure (Ndungu *et al.*, 2013). Higher titres of antigen specific IgG have previously been observed in asymptomatic malaria carriers compared to individuals presenting with other malaria outcomes (Braga *et al.*, 2002; Kinyanjui *et al.*, 2004; Moormann *et al.*, 2013). Furthermore, high antigen specific antibody responses were associated with high levels of IFN-γ and IL-10 among asymptomatic Gabonese children, suggesting that antibody responses may exert protective immune mechanisms (Guiyedi *et al.*, 2015). Further studies of immune cells and cytokines (**Table 2**) are necessary to understand the mechanisms underlying immunomodulation and how this can be applied to confer malaria protection. It is evident that there is a complex interplay of various components of the immune system and one way of potentially interrogating this complexity is through an 'omics' approach.

Table 2: A list of selected cytokines and immune cells showing their levels as reported in studies of Africa comparing malaria clinical outcome.

Biomarker	Levels	Study site	Reference
IL-10	High	Uganda	(Jagannathan, Kim, *et al.*, 2014)
IL-10	High	Ghana	(Wilson *et al.*, 2010).
IL-10	High	Mali	(Portugal 2014)
IL-10	High	Gabon	(Guiyedi *et al.*, 2015)
IFN-γ	Low	Uganda	(Jagannathan, Kim, *et al.*, 2014)
IFN-γ	High	Gabon	(Guiyedi *et al.*, 2015)
TNFα	Low	Uganda	Jagannathan, Eccles-James, et al., 2014
TNFα	Low	Uganda	(Jagannathan, Kim, *et al.*, 2014)
Tregs	Low	Uganda	(Boyle et al., 2015)
Tregs	Low	Ghana	(Frimpong, Kusi, Tornyigah, Ofori, & Ndifon, 2018)
$V\delta2^{+}$ γδ T cells	Low	Uganda	(Jagannathan, Kim, *et al.*, 2014)
Natural Killer cells	Low	Kenya	(Kijogi 2018)

To add to this already complex immune process, co-endemicity of *P. falciparum* and helminths is very common in the tropics resulting in increased chances of co-infection (Mwangi, Bethony and Brooker, 2006). Interactions between the two parasites alters immune responses, thus influencing susceptibility to clinical malaria (Druilhe, Tall and Sokhna, 2005; Vaumourin *et al.*, 2015). There are conflicting reports on the outcome of these interactions as some studies have reported enhanced severity (Wilson *et al.*, 2007; Midzi *et al.*, 2010), others reduced severity (Cot *et al.*, 2014; Hürlimann *et al.*, 2019) yet others have revealed no association between helminth co-infection and malaria outcome (Ojurongbe *et al.*, 2011; Lo *et al.*, 2018). These observations may be attributed to differences in the co-infecting helminth species (Shapiro *et al.*, 2005), the host's level of immunity to *P. falciparum* (Mboera *et al.*, 2011) and differences in study design (Mwangi, Bethony and Brooker, 2006). Various helminths elicit different immune responses that have an impact on the immunopathology of malaria due to the imbalance between pro-inflammatory and anti-inflammatory cytokines (Maizels *et al.*, 2004). Although modulation of these cytokines

responses is suggested to be a plausible mechanism through which the immune system is altered, additional well designed asymptomatic *P. falciparum* studies with sufficient sample size are required to elucidate their role in acquisition of anti-disease immunity and how other aspects of the immune system are involved.

2.3 Antigenic variation

The ability of *P. falciparum* to escape host defense mechanisms is a key aspect of malaria pathogenesis. To achieve this, the parasites have adapted to reside inside the RBC to evade the immune response. In addition, they can cause binding of infected RBCs (iRBCs) to endothelial cells otherwise known as cytoadherence or binding of two or more uninfected RBCs around an iRBC, a process known as rosetting (Alexandra Rowe *et al.*, 1997; Smith *et al.*, 2001). If these occurs in small microvasculature of vital organs, it may cause organ failure that manifest as severe malaria. On the contrary, the parasite has also adapted to evade the immune radar by sustaining asymptomatic infections characterized by increased circulation and reduced parasitemia to maximize mosquito transmission (Andrade *et al.*, 2020). The occurrence of these phenomena has been attributed to the differential expression of multicopy gene families encoding highly polymorphic parasite antigens, called variant surface antigens (VSAs), on the surface of iRBCs. The VSAs include *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), repetitive interspersed-families of polypeptides (RIFINs) and sub-telomeric variable open reading frame (STEVORs). PfEMP1 has been studied the most and is encoded by a multi-gene family referred to as var genes that consists of about 60 genes. The parasite expresses these genes on the RBC surface one at a time to avoid simultaneous recognition by host immunity responses, thus maintaining the infection (Borst *et al.*, 1995). Normally, the virulent variants are expressed first followed by the less virulent ones and the switch in the expression patterns is suggested to be controlled by host antibodies to the PfEMP1 variants (Warimwe *et al.*, 2009). Var genes can be categorized into three major groups based on 5’ untranslated sequences i.e. A, B and C (Smith, 2014). They include long 5’ exons that encode two types of extracellular domains i.e., the Duffy Binding Like (DBL) and the Cysteine Rich Interdomain Regions (CIDR). These two domains can further be divided into sub-classes based on sequence similarity (Su *et al.*, 1995). Group A var genes have been associated with parasites causing severe disease and group B with mild disease (Rottmann *et al.*, 2006). Once the initial repertoire of the var genes is exhausted as the host acquires immunity to malaria, like in asymptomatic infections, group C var genes which are more

homogenous, low-abundant and causes minimal cytoadherence in vital organs, may prevail (Warimwe *et al.*, 2013). Var genes have mainly been well characterized in severe and mild malaria outcomes with little information about their expression in asymptomatic infections.

2.4 Molecular approaches to studying malaria

2.4.1 Genetics

P. falciparum infections in malaria endemic areas, primarily in sub-Saharan Africa, are characterised by the co-infection of multiple genetically distinct *P. falciparum* parasites sub-populations or clones in acute and persistent infections (Babiker *et al.*, 1994, 2000; Magesa *et al.*, 2002; Bereczky *et al.*, 2007; Agyeman-Budu *et al.*, 2013). These parasites normally undergo recombination in the mosquito midgut during zygote formation to produce genetically diverse parasites (Babiker *et al.*, 1994). A single *P. falciparum* infection can thus contain distinct merozoites from different clones due to multiple mosquito bites each inoculating a unique clone or a single mosquito harbouring multiple clones (Nkhoma *et al.*, 2020). The total number of genetically unique parasite sub-populations in an individual is referred to as the complexity of infection (COI).

These clones can be distinguished using genetically diverse RBC invasion-associated proteins such as the MSPs, glutamate-rich protein (GLURP) and the apical membrane antigen (AMA) protein (Wright and Rayner, 2014). They are used as genetic markers for malaria genotyping in epidemiological studies because of they are highly polymorphic antigens. Their accessibility to the immune system has made them potential candidates for vaccine development although immune responses may fail to recognizing all the genotypes due to their extensive genetic diversity (Takala and Plowe, 2009). Consequently, acquisition of natural sterile immunity to malaria and vaccine efficacy, especially for antigen-based vaccines, is impacted as it is not possible for an individual to encounter or a vaccine to contain all the genotypes.

Several MSP proteins, that are thought to be involved in RBC invasion and immune evasion, have been described in *P. falciparum* (Beeson *et al.*, 2016). Among them, MSP2 has been shown to be among the most polymorphic and informative in genotyping *P. falciparum* parasite populations as it reveals a high level of genetic heterogeneity (Felger *et al.*, 1994). It is divided into 5 blocks and is flanked by unique variable domains and conserved N- and C- terminal domains **(Figure 2)** (Takala *et al.*, 2006; Ferreira and Hartl, 2007). The polymorphic central block 3 contains repeats

that vary in length, number and sequence and are categorized into two allelic families i.e. FC27 and IC/3D7 (**Figure 2**) (Boyle *et al.*, 2014). MSP2 genotyping has been widely used to study genetic diversity of *P. falciparum* infections (Bereczky et al. 2007; Sondén et al. 2015; Ferreira and Hartl 2007; Amodu et al. 2008; Mwingira et al. 2011).

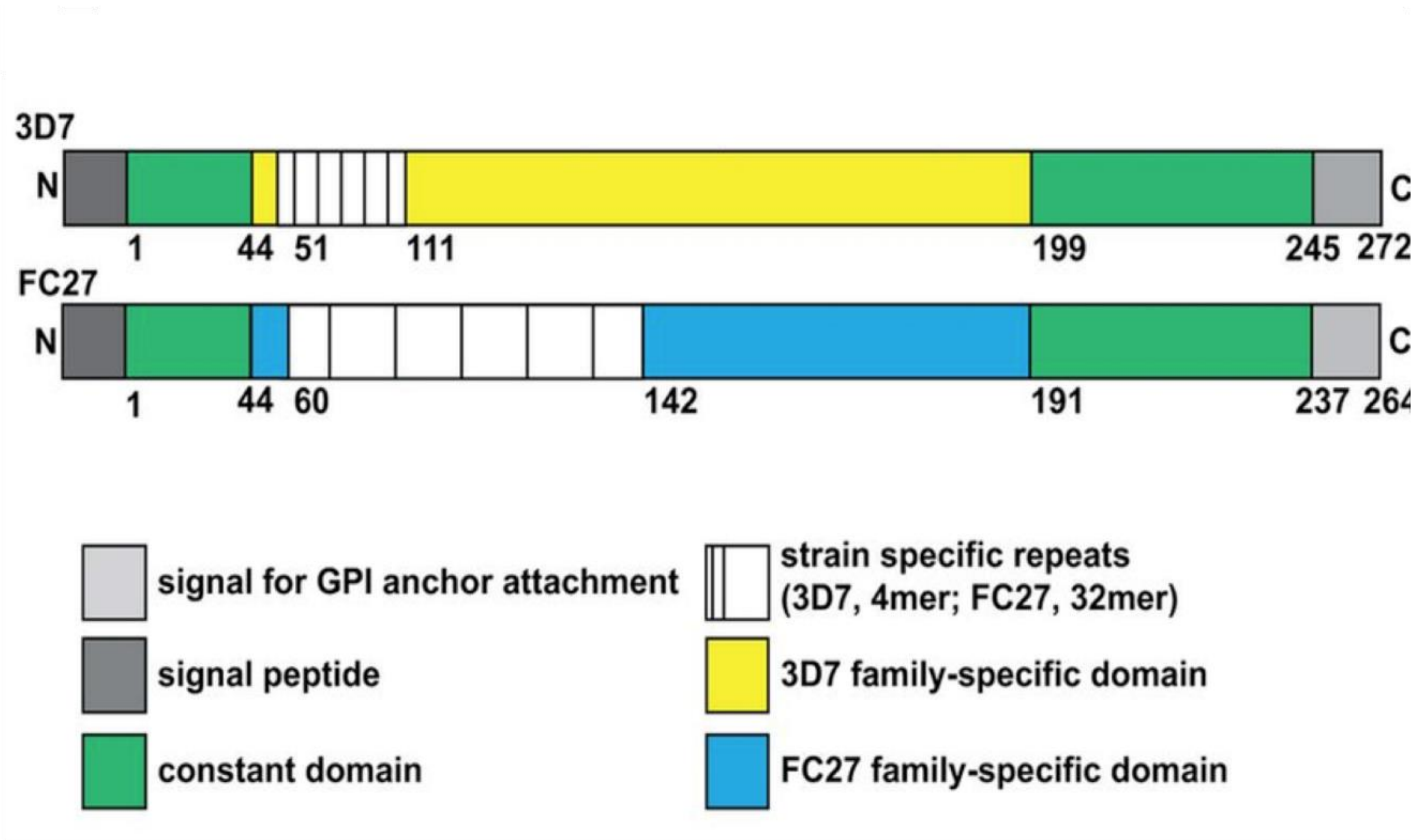

Figure 2: The structure of FC27 and IC/3D7 allelic families found on block 3 of the msp2 gene.

MSP2 alleles are either FC27 or IC/3D7 as per the central variable sections of the gene. These FC27 or IC/3D7 alleles have N-terminally and C-terminally conserved sections. Adopted from (Boyle *et al.*, 2014)**.**

This is especially important in defining treatment outcomes, in clinical drug trials as it can distinguish recrudescent (recurring) from new parasite infections (Snounou and Beck, 1998). This process can also quantify distinct parasite populations in an infection thereby revealing dominant clones that can be associated with clinical episodes of malaria (Mwingira et al. 2011; Kun et al. 2002). Comparative studies of the *P. falciparum msp2* genotypes in various malaria presentations has revealed interesting findings. A study in Thailand suggested that a genetic variation in codon 8 of FC27 and codon 17 of block 4 in 3D7 are parasite virulence markers that could be associated with various clinical outcomes (Chaorattanakawee *et al.*, 2018). Some studies have associated the FC27 genotype with asymptomatic infections and while others have associated it with febrile infections (Amodu *et al.*, 2008; Ibara-Okabande *et al.*, 2012; W. Kidima and Nkwengulila, 2015). Other studies have shown that an increase in COI among asymptomatic infections is correlated with higher risk of febrile malaria in younger children, a lower risk in older children (Felger *et al.*, 1999), while in other studies age had no influence (Ofosu-Okyere *et al.*, 2001).

One of the major limitations of *msp2* genotyping is the presence of repeats that makes sequencing difficult, hence the use of gel and capillary electrophoresis techniques to estimate the size of msp2 fragments. Gel electrophoresis has been used to distinguish PCR products and infer fragment sizes by eye. However, this has very low accuracy since it is not possible to distinguish fragments with small differences in size e.g., 3 base pairs differences. This led to development of a more accurate method called capillary fragment electrophoresis that is performed using automated DNA sequencing machines (Liljander *et al.*, 2009). This method incorporates the use of fluorescently labelled oligonucleotide primers to amplify DNA that is then separated by electrophoresis in capillaries. Laser excitation causes the fragments to fluoresce, and the signal is picked by a detector which is then interpreted as fragment size. Fragments of known sizes are also incorporated into the reaction to help in estimating the base-pair sizes of unknown fragments. This method is more sensitive and detects a higher number of fragments compared to the gel electrophoresis method. Nevertheless, this technique requires rigorous optimization, specialised software to interpret results from the sequencer and an ability to distinguish true peaks from stutter peaks (Liljander *et al.*, 2009). In addition, the presence of identical fragment sizes may not always represent identical sequence lengths and sequencing is recommended for confirmation. Fortunately, a *msp2* sequencing protocol that incorporates the use of Pacific Biosystem’s circular consensus

sequencing has recently been described and promises to improve the utility *msp2* genotyping for describing parasite genetic diversity (Plaza *et al.*, 2022).

Technological advancements have also led to the development of amplicon deep sequencing that utilizes the next generation sequencing platforms (Apinjoh *et al.*, 2019). This has led to the sequencing of several genotyping markers producing high throughput data that gives a better resolution of the genetic diversity of *P. falciparum* parasite populations (Lerch *et al.*, 2019). Some of the studies employing this technique to study COI of *P. falciparum* in asymptomatic and symptomatic infections have corroborated the *msp2* genotyping results. This include *ama1* (Wamae *et al.*, 2022), *cpmp* and *csp* (Sarah-Matio *et al.*, 2022).

2.4.2 Transcriptomics

Transcriptomics is the study of the complete set of RNA transcripts encoded by the genome at a particular time. Various studies have examined the gene expression profile of both the parasite and the host during asymptomatic *P. falciparum* infections and revealed interesting findings that have improved our understanding of these neglected malaria infections. The first study compared parasite transcriptomic patterns between 18 cerebral and 18 asymptomatic malaria infections in Cameroonian children using microarrays. The study reported major differences in genes coding for exported proteins, transcriptional factor proteins, proteins involved in protein transport, variant surface antigen (VSA) proteins such as *P. falciparum* erythrocyte membrane proteins (*PfEMPs*) and repetitive interspersed family (RIFINs) (Almelli *et al.*, 2014). A recent study in Mali studied how parasites survive during the dry season when there is little or no malaria transmission and revealed that the parasites become less virulent by reducing their cytoadhering capacity. This leads to increased circulation time in the blood and therefore, increased chances of clearance by the spleen. The net effect is reduced parasite density undetectable by the immune system (Andrade *et al.*, 2020). Similarly, parasites from low-transmission settings have been shown to invest more in transmission to new hosts and less on replication as compared to those from high-transmission settings (Rono *et al.*, 2017). Studies of asymptomatic parasites are hindered by the reduced parasitemia and lack of longitudinal studies to identify and study true asymptomatic infections.

Several studies have investigated the host transcriptomic signatures during asymptomatic infections and revealed insights that have improved our understanding of how the host is able to harbor the parasites without showing symptoms. A study in Gabon compared the host

asymptomatic transcriptomic profiles versus that of healthy controls and uncomplicated malaria infections. Among 4,643 differentially expressed genes, 36 genes were exclusively expressed during asymptomatic infections compared to healthy children and mild malaria. Functional analysis on these genes revealed that they are involved in nucleotide binding and RNA processing suggesting that gene regulation via chromatic remodeling is a potential mode of maintaining asymptomatic infections (Boldt *et al.*, 2019). Chromatin remodeling changes the chromatic architecture, making condensed genomic DNA accessible to transcription proteins leading to transcription and translation of the DNA. This process leads to activation of immune cells such as macrophages and monocytes (Schultze, 2017). It is postulated that during asymptomatic infections, chromatin remodeling decreases the expression of immunoglobulin genes leading to reduced antibody mediated responses (Boldt *et al.*, 2019). On the contrary, chromatin remodeling may have resulted in a stronger transcriptional activity in the monocytes of the Fulani ethnic group, resulting in a pro-inflammatory state and reduced susceptibility to malaria infections when compared to sympatric ethnic groups (Quin *et al.*, 2017). Monocytes from asymptomatic *P. falciparum* carriers have also been shown to increase the expression of genes associated with the P53 pathway which is associated with dampened inflammatory immune responses that hinders acquisition of anti-parasite immunity (Tran *et al.*, 2019). In Mali, pre- and post-infection profiles of adults who were either naïve, asymptomatic, or febrile were compared. Intriguingly, asymptomatic individuals had the least transcriptional changes in gene pathways that are regulated by pro-inflammatory cytokines IFN, IL-1B and TNF (Tran *et al.*, 2016). This was probably caused by the downregulation of genes encoding pro-inflammatory cytokines as a result of modulation of immune responses due to previous exposure (Portugal *et al.*, 2014). This can be confirmed by the fact that increased expression of immunoregulatory genes in Vδ2+ T cells has been associated with chronic exposure to *P. falciparum* (Jagannathan, Eccles-James, *et al.*, 2014). A recent study conducted in a malaria endemic region of Indonesia revealed that asymptomatic infections were characterized by an immunosuppressive transcription signature that featured diminished T-cell function due to the expression of CTLA-4 that outcompetes T-cells in binding the costimulatory molecule B7. The net effect is suppression of immune responses leading to asymptomatic infections (Studniberg *et al.*, 2022). This evidence points to an active immune response during asymptomatic infections that can be further interrogated to provide insights into how anti-disease immunity is induced with implications for vaccine development. Studies on transcriptomics of

asymptomatic infections have suggested that gene regulation mechanisms like transcription factors or chromatin remodeling may play a key role in regulating inflammatory mechanisms that maintain malaria infections in the asymptomatic state (Boldt *et al.*, 2019). However, these studies are faced with numerous limitations including the lack of a standard definition of asymptomatic infections (**Table 1**) that makes it difficult to compare data and results. The ubiquitous challenge faced by transcriptome studies of whole blood associated samples in malaria is the lack of cellular homogeneity of the samples. Often, it is difficult to establish whether gene expression differences revealed among sample groups are due to differential gene regulation or due to differences in the relative proportions of immune cell subsets or parasite development stages. Single cell transcriptomics or flow cytometry may help to overcome this challenge although these methods are expensive and difficult to set up in limited resource settings. Studies of parasites transcriptome face the challenge of having abundant host RNA that mask the parasite RNA. In addition, culturing parasites may also help to synchronize parasite development stages. However, culture conditions and lack of host immune pressure hinders replicating parasite expression patterns observed *in vivo*. To overcome these challenges, the most suitable alternative is to infer the cellular proportions in the transcriptome data computationally using gene expression deconvolution by comparing the normalized transcriptome to that of known cells. Specific challenges faced by transcriptome studies of asymptomatic *P. falciparum* infections include lack of enough power due to a small sample size. Finally, poor study designs that limit the identification of true asymptomatic infections as most of them lack longitudinal samples and sufficient clinical history of the study participants (Kimenyi, Wamae and Ochola-Oyier, 2019). Future studies should focus on addressing these limitations to provide valuable insights into the mechanisms involved in the acquisition of natural acquired immunity to malaria with implications for vaccine development. In addition, understanding of chromatic accessibility of genome regions encoding inflammatory factors should be well studied as it may shed light on how different malaria outcomes are maintained.

2.4.3 Proteomics

Proteomics is the large-scale identification and quantification of the proteome which represents the complete set of proteins expressed in a cell, tissue or an organism. Genes undergo transcription and translation to form proteins. This is a complex process and is often followed by post-

translational modifications. Hence, validation of genomic and transcriptomic results at the proteomic level is important in confirming gene function by revealing gene products in a cell or tissue. Proteomics studies have previously been applied in the identification of diagnostic markers, vaccine candidates and understanding disease pathways (Aslam *et al.*, 2017). Several proteomic studies have examined *P. falciparum* infections and reported interesting findings. These studies have associated selected proteins with the pathology of different clinical manifestations of malaria. Some of the key proteins implicated include RBC membrane proteins that are involved in protein trafficking and variant surface antigens (VSAs) protein export and their interaction with the immune system. These proteins have been highlighted as potential vaccine candidates especially in the case of cerebral malaria (Bertin *et al.*, 2016). A study in Brazil identified parasite proteins expressed on the infected RBC membrane, which caused differential antibody responses in asymptomatic and symptomatic infections and associated them with the absence or presence of malaria symptoms. These proteins include antigenic variation proteins like a truncated var1CSA (variant surface antigen 1 chondroitin sulphate A) probably involved in pathogenesis and cytoadherence, while membrane proteins involved in infected RBCs (iRBC) remodeling like heat shock proteins that were suggested to be involved in protein folding after translocation (Cabral *et al.*, 2017).

Proteomics studies that compare asymptomatic infections versus other malaria presentations promises to increase our understanding of asymptomatic infections by validating results from transcriptomic studies and potentially revealing interesting diagnostic and prognostic markers that can predict disease outcome.

Chapter 3: METHODOLOGY

3.1 Study design

Samples utilized here were collected prospectively between 2007 and 2018 from a cohort that is based in Junju, Kilifi County. This area experiences a moderate to high malaria transmission intensity **(Figure 3)**. Here, about 425 children were enlisted into the study at birth and followed-up every week by active case-surveillance up to when they turned 15 years. Usually, this region experiences two rain seasons yearly that are characterized by increased malaria transmission. They consist of the long rains of May to July as well as the short rains in October to November (O'Meara *et al.*, 2008). Annual cross-sectional surveys were carried out in this cohort just before the long rains from 2007 to 2018 and individuals categorized as being uninfected (healthy), having febrile malaria, having non-malarial fever or being asymptomatic *P. falciparum* carriers. *P. falciparum* malaria was diagnosed using RDT, confirmed with microscopy and treated. After these surveys, weekly active surveillance wase conducted and symptomatic malaria infections identified and sampled **(Figure 4)**.

Figure 3: Study area.

A map of Kenya (inset) and a map of Kilifi County showing the area covered by the Junju cohort. Adopted from (Olotu et al., 2010).

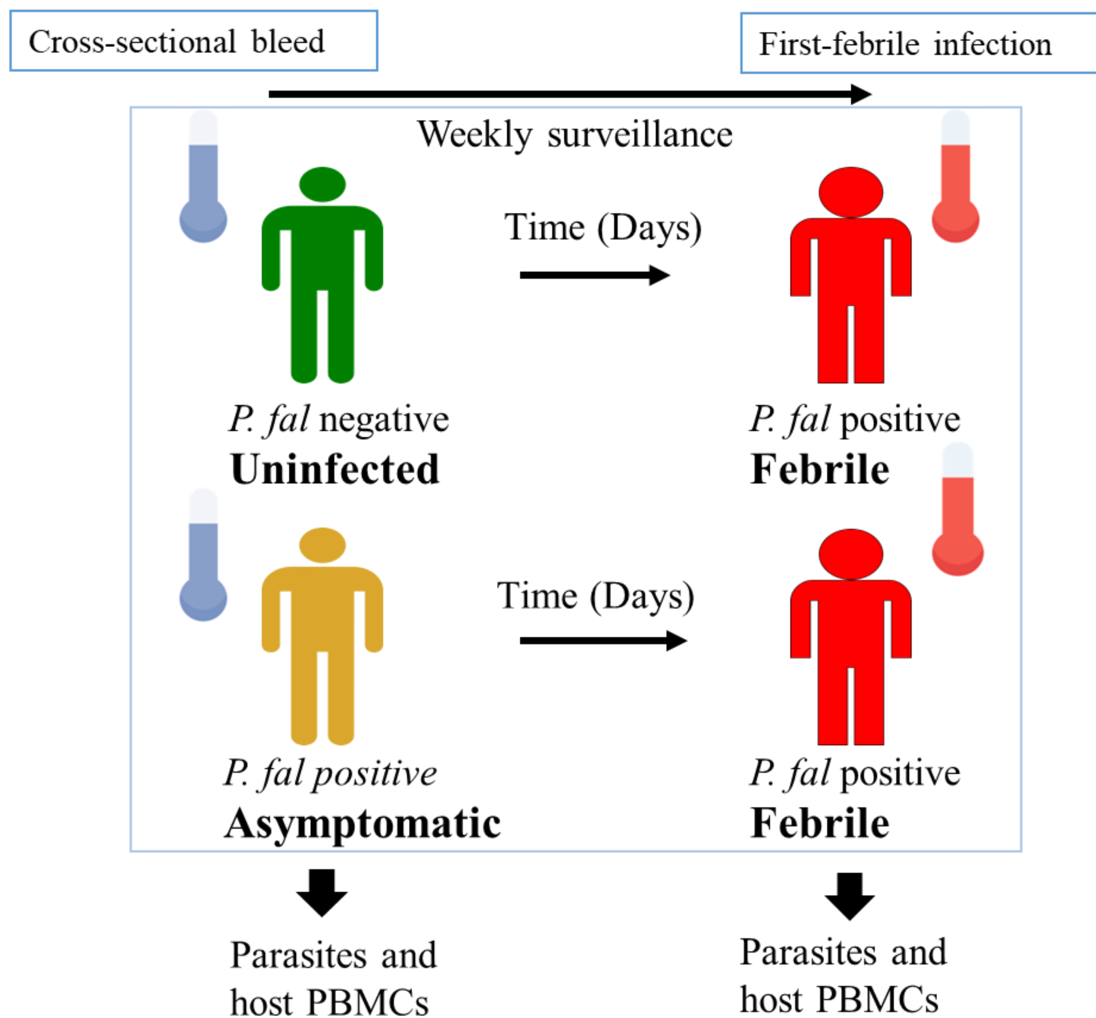

Figure 4: Study design

DNA was extracted from whole blood. Parasite and host Peripheral Blood Mononuclear Cells (PBMCs) samples were collected retrospectively from individuals during the cross-sectional bleed and at their first febrile malaria infection.

3.2 Study Site

The study was conducted at the Kenya Medical Research Institute (KEMRI) – Wellcome Trust Programme (KWTRP) in Kilifi, Kenya.

3.3 Ethical approval

The ethical approval was obtained from KEMRI's Scientific Ethics and Review Unit (SERU), under protocol number 3149. Parents or guardians of all study participants were required to provide written informed consent before sample collection.

3.4 Inclusion criteria

Uninfected children were defined as being parasite negative by RDT and microscopy. Asymptomatic individuals had to have presence of the parasite as diagnosed by RDT and confirmed by microscopy and had the following additional inclusion criteria: 1) axillary temperature of less than 37.5°C and without fever during the cross-sectional survey, 2) lack of a recent febrile malaria case or evidence of antimalarial drug use about a month prior to the survey, and 3) lack of fever within seven days after the survey (Wamae *et al.*, 2018). Sub-microscopic asymptomatic infections were excluded as they yielded insufficient RNA material for RNAseq during optimization assays. The subsequent first febrile malaria infections from individuals who were initially uninfected their parasitaemia was defined as greater than 2500 parasites/µl by microscopy and axillary temperature greater than 37.5°C as previously defined in this cohort (Mwangi *et al.*, 2005; Bejon *et al.*, 2006). For individuals who were previously asymptomatic, their subsequent first-febrile malaria infections were defined as having any parasitaemia by microscopy and a tympanic temperature greater than 37.5°C. Individuals with any evidence of severe malaria were not included in the study.

About 2801 uninfected, 1710 asymptomatic and 3205 febrile samples collected between 2007 and 2018 were selected from the biobank database for use in this work. A total of 3068 uninfected, 1348 asymptomatic and 3500 febrile samples were collected between 2007 and 2018. Out of these, about 838 samples from children who were asymptomatic during the survey and 147 corresponding febrile malaria DNA samples were available in the biobank for *msp2* genotyping. While 154 paired samples from children who were asymptomatically infected on the survey and

subsequently experienced febrile infections were identified for parasite and PBMC transcriptomic and proteomic analysis but only 21 pairs were available in the biobank. Additionally, 22 paired uninfected and corresponding febrile infections were available for PBMC transcriptomic and proteomic analysis out of 1500 pairs identified in the database. Sample size used in this study was determined pragmatically based on availability of retrospective samples with sufficient clinical data in the KWTRP Biobank.

3.5 Sample collection

Blood samples used in this study had been collected, processed and the various blood components archived in the biobank. Briefly, blood samples were drawn by venipuncture from all the participants during the cross-sectional bleed and the ensuing febrile malaria episode. The samples were collected in sodium citrate-containing cell preparation tubes (Vacutainer CPT Tubes, BD) and transported to the laboratory where they were separated into peripheral blood mononuclear cells (PBMCs) and red blood cell (RBC) pellets and remaining sample was used for DNA extraction. The PBMCs and RBC pellets were harvested and processed before storage in liquid nitrogen (N_2). PBMCs were isolated using the Lymphoprep^{TM} density gradient solution and carefully collected using a wide mouth Pasteur pipette then washed twice with RPMI media before storage in LN2.

3.6 Parasite DNA genotyping

3.6.1. Parasite DNA extraction

DNA extraction from whole blood was performed using the QIAamp® DNA mini-kit (QIAGEN). Briefly, about 20µl of protease enzyme for every 200µl of blood was added to each tube and mixed thoroughly to remove any contaminating proteins. The cells were lysed using 200µl of lysis buffer and incubated for 10 minutes at 56^0C. Then, 200µl of 100% ethanol was added to each tube to promote DNA aggregation, the solution passed through a QIAamp spin column and centrifuged at 9000 revolutions per minute (rpm) for 1 minute. The columns were washed twice using wash buffer. Finally, DNA was eluted using 50µl of elution buffer and stored in 1.5ml Eppendorf tubes. DNA concentration and purity were then assessed using NanoDrop Lite Spectrophotometer (Thermo Scientific) and stored in -80^0C freezer until use.

3.6.2 *MSP2* genotyping

Genotyping of *msp2* gene was conducted using a nested polymerase chain reaction (PCR) assay

comprising of a primary amplification of block 3, followed by a nested reaction that used fluorescently labelled oligonucleotide primers **(Table 3)** to target the FC27 and IC/3D7 allelic families (Liljander *et al.*, 2009).

Table 3: Sequence of primers for msp2 genotyping

Primer	Amplification	Sequence	Modification
***Msp2* F**	Primary	5'- AATACTAAGAGTGTAGGTGCARATGCTCCA-3'	-
***Msp2* R**		5'- CTTTGTTACCATCGGTACATTCTT-3	-
FC27 F	Secondary	5'- AATACTAAGAGTGTAGGTGCARATGCTCCA-3'	7 bp-tail
FC27 R		5'- TTTTAT TTG GTGCAT TGCCAGAACTTG AAC-3'	6-FAM™ (blue)
IC/3D7 F		5'- AGAAGTATGGCAGAAAGTAAKCCTYCTACT3'	7bp-tail
IC/3D7 R		5'- GATTGTAATTCGGGGGATTCAGTTTGTTCG-3'	VIC® (green)

The total reaction volume for primary and nested PCRs was 10µl per reaction. The primary reaction consisted of 1µl of 1 x PCR buffer, 1.25µl of 125µM dNTPs, 0.1U of AmpliTaq® DNA polymerase (5units/µl including 10x Buffer II, $MgCl_2$ 25mM), 250nM each of *msp2* forward (F)/reverse (R) and 1µl DNA template, 2.15µl of DNase/RNase free H_20. The PCR was performed by first denaturing the DNA at 95°C for 5 min followed by 24 cycles comprising primer annealing at 58°C for 2 min and DNA extension at 72°C for 2 min. A final denaturation step at 94°C for 1 min and 1 cycle comprising final primer annealing at 58°C for 2 min and DNA extension at 72°C for 5 min were then carried out.

The nested reaction consisted of 1µl of 10 x PCR buffer, 1µl of 125µM dNTP (each), 1µl primary PCR product, 3µl of 300nM each (F/R) *msp2* FC27/3D7 allele-specific primers and 0.1 unit of AmpliTaq® DNA polymerase, and 0.65µl of DNase/RNase free H_20. The PCR was performed at an initial denaturation at 95°C for 5 min followed by 26 cycles of primer annealing at 58°C for 1 min and DNA extension at 72°C for 1 min. A final denaturation at 94°C for 30 sec was done.

PCR fragments from each nested reaction were diluted 10 times with DNase/RNase free H_20 and mixed with 9µl of deionized (Hi-Di) formamide and 0.5µl size standard GS-LIZ that consisted of

73 single-stranded DNA fragments with varying sizes from 20bp to 1200bp. The solutions were transferred to 96-well Optical reaction plates and sent to ILRI (International Livestock Research Institute) in Nairobi, Kenya, for capillary electrophoresis on the 3730XL DNA sequencer (Applied Biosystems).

3.6.3 Quality control

Good clinical laboratory practices (GCLP) were observed. Laboratory cultured HB3 and 3D7 parasite DNA were used as a positive control for FC27 and IC/3D7 alleles, respectively. Master mix reaction without DNA served as the negative control. Samples were analyzed by gel electrophoresis before sending for capillary electrophoresis. Samples that failed to generate an amplicon were repeated using two times the DNA amount. If non-amplifications persisted after the second PCR reaction, it was deemed unsuccessful.

3.6.4 Data analysis

Data from capillary electrophoresis was analyzed using GeneMapper Software version 4.0 (Applied Biosystems) to determine the number of alleles present in each sample. The output was analyzed using custom R scripts in R statistical environment (R, 2020). A fluorescent cut-off of 300 relative fluorescent units (rfu) was applied to simplify identification of true alleles by removing fluorescent background noise and non-specific low background noise (Falk *et al.*, 2006). Msp2 alleles were considered similar if fragments differed by <1bp. Stutter and artefact peaks were defined as peaks having a height of less than 10% the height of the true peak (Liljander *et al.*, 2009). Otherwise, they were considered as true peaks. COI was defined as the total number of fragments in an individual infection, and the mean COI was calculated as the sum of COI values from individual infections in a year divided by the number of individuals in the same year.

3.7 Parasite RNA work

3.7.1 Thawing of cryopreserved parasites

Vials containing infected RBCs were retrieved from the liquid Nitrogen (N_2). Parasites frozen in glycerolyte (storage media) were thawed using decreasing concentrations of NaCl to remove glycerolyte. Briefly, 0.1X volume of 12% Na Cl was added gently while shaking and incubated for 5 minutes. The process was repeated with 10 volumes of 1.6% NaCl and finally with 10 volumes of 0.9% NaCl or normal saline. 10ml of 0.2% saponin was added and the solution

centrifuged at maximum speed to recover the parasitophorous vacuole containing the parasite. The supernatant, containing RBC contents, was aspirated to avoid contamination with host RNA. The cell pellet containing the parasite was then used for RNA extraction.

3.7.2 Parasite RNA extraction

RNA extraction from the parasite (RBC pellet) was performed using ISOLATE II RNA Mini Kit (Bioline). First, about 350µl of lysis-buffer was included then the solution vortexed vigorously to lyse the cells. About 4µl of Beta-mercaptoethanol was added to denature any RNAses and centrifuged. Then, 250µl of absolute ethanol was added and the solution transferred to RNeasy spin columns and later centrifuged. The spin columns were washed two times with the wash buffer and eluted with 50µl nuclease-free water. Messenger RNA (mRNA) was enriched using NEBNext^{TM} Poly(A) mRNA magnetic-isolation module. The nature of the eluted RNA was accessed using NanoDrop^{TM}. The first-strand complimentary DNA (cDNA) synthesis was performed using superscript III reverse transcriptase (Invitrogen) with the following PCR cycling conditions: 25^{0}C for 10min, 42^{0}C for 60min and a final hold of 4^{0}C. RNase inhibitor was added to prevent RNA digestion while actinomycin D was added to prevent transcription. The primers used included random hexamers and oligo (T) (Qiagen). Dithiothreitol (DTT) was added to stabilize the reverse transcriptase. The final product was cleaned with 1.8 X volume of RNAcleanXP beads then washed twice with 80% freshly prepared ethanol while on a magnetic strand. Finally, elution of the first strand cDNA was done using 20.5µl of Qiagen elution buffer with the beads being left inside the wells for use when cleaning the second-strand cDNA reaction.

Synthesis of the second strand was performed using NEBNext^{TM} mRNA Second Strand Synthesis Module (New England Biolabs) using the following conditions: 16^{0}C for 2.5 hours and a final hold at 4^{0}C. The cDNA was cleaned using 1.8 X volumes of 20% PEG (polyethylene-glycol) and 2.5M NaCl solution. This was important for restoring the binding abilities of the beads. Finally, the beads, still in the magnetic stand, were washed twice using 80% ethanol and cDNA eluted using Qiagen elution buffer.

Next 13.5µl of cDNA was used to prepare libraries using the NEBNext^{TM} Ultra II FS DNA library-preparation kit from New England Biolabs in a three-step process. First, cDNA was enzymatically fragmented to standardize the length using the following cycling conditions: 37^{0}C for 10 minutes and 65^{0}C for 30 minutes. Second, the fragments were ligated/joined to NEXTflex adaptor oligos that contained barcodes by incubating the reaction mix at 20^{0}C for 15 minutes and a final hold of

4^0C. Finally, Uracil Specific Excision Reagent (USER) enzymes from Biolabs used make the libraries stranded using the following cycling conditions: 37^0C for 15 minutes, 95^0C for 10 minutes to digest the second-strand and a final hod at 4^0C. The libraries were finally amplified in 17 cycles, following optimization, using the KAPA library Amp primer mix (KAPA Biosystems) and P7 and P5 Illumina primers used to increase the yield. The they were finally cleaned using 0.8 X volumes of RNAcleanXP beads and eluted with 11µl of Qiagen elution buffer. The Agilent DNA 1000 chips on the Agilent Tape Station 2200 system were used to assess the quality of the libraries, which were then quantified using KAPA Library Quantification Kit – Complete Kit (ABI Prism). The samples were finally pooled into equimolar amounts based on their DNA concentrations and submitted for paired-end sequencing on an Illumina HiSeq 4000 platform at the Wellcome Sanger Institute (WSI), Hinxton, United Kingdom.

3.7.3 Parasite transcriptome analysis

Raw sequence data was received from the sequencing platform in FASTQ file format and checked for sequence quality using FastQC v0.11.9 software. The reads were pseudo-aligned to the reference *P. falciparum* 3D7 latest transcriptome (release 55, downloaded from Plasmodb.org) using a fast aligner known as Kallisto v046.1 (Bray *et al.*, 2016). Lowly expressed genes were filtered using a cut-off of 2 counts per million (CPM) in a least 10 samples. *In silico* deconvolution of the RNAseq was done for each sample to determine the abundance of five parasite life cycle stages i.e. rings, early trophozoites, late trophozoites, schizonts and gametocytes, using available reference data (López-Barragán *et al.*, 2011). This data comprised of five of the six *P. falciparum* life cycle stages sequenced and include: ring stage, early and late trophozoite stage, schizont stage and the gametocyte II and V stages. The ookinete stage was not used for deconvolution as it is normally found in the mosquito, thus samples obtained from the human host are least likely to contain this stage. The count data was first normalized for sequencing depth and gene length using the RPKM (reads per kilobase of exon per million reads) method. A mixture model was then fitted to estimate the proportions of each parasite stage per sample. The RPKM values of the reference data and the test data were used to fit the linear model described below:

$$\sum_{i=1}^{N}(g_{i,sample} - \sum_{s \in S} \pi_s g_{i,s})^2$$

π represents the proportions of stages that will minimize the preceding expression. g is the gene

expression. $\sum_{s \in S} \pi_s = 1 \; and \, \pi_s \geq 0$ (Tonkin-Hill *et al.*, 2018). The model was fitted using the solve.QP function in quadprog package (Berwin and Turlach, 2022). The trimmed mean of M-value (TMM) method was implemented to normalize for library size and RNA composition (Robinson and Oshlack, 2010). In addition, three factors of unwanted-variation were identified using RUVSeq package (Risso *et al.*, 2014) and insilico empirical negative controls that consisted of 3240 least differentially expressed genes as determined prior to this step of the analysis. Quality control to check for outlier samples was determined using the relative log expression (RLE) plots at each of the following stages i.e., after normalization, after accounting for proportions of ring and early trophozoite stages, and finally after removing factors of unwanted variation.

Differential expression analysis (DEA) was conducted using edgeR package (Robinson, McCarthy and Smyth, 2010) and in addition to the normalized counts matrix, proportions of ring and early trophozoite stages and factors of unwanted variation were also included as covariates in the negative binomial model. Genes with P values < 0.05 (Benjamini-Hochberg adjusted) were considered as differentially expressed and retained for further analysis. Functional analysis of differentially expressed genes was done using clusterProfiler v4.2.2 (Wu *et al.*, 2021) to determine over-representation of specific Gene-ontology (GO) terms and Kyoto-Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) *P. falciparum* pathways and accessed via the KEGG API (Application Programming Interface) using KEGGREST package (Tenenbaum *et al*., 2023) and Org.Pf.plasmo.db v.3.1.4 (Carlson, 2021).

3.8 Host PBMCs transcriptome analysis

3.8.1 Peripheral blood mononuclear cells (PBMCs) processing

PBMCs from all the samples used in this study were available in the biobank. They were processed immediately after the samples were obtained from the study participants and stored in N_2 for future use. About 1 ml of PBMCs per sample was previously isolated using the Lymphoprep™ density gradient medium. Briefly, whole blood was centrifuged at 2000 revolutions per minute (rpm) for 10 minutes. This was done to separate plasma from the rest of the blood components. The supernatant and plasma components were carefully extracted and discarded. Then 5ml of Lymphoprep™ medium was added into a new tube. Slowly and carefully, the cell suspension was layered on the Lymphoprep™ medium. The solution was then centrifuged for 20 minutes at 2000 rpm at room temperature. After centrifugation, the PBMCs form a distinct band at the sample/medium interphase. The cells (PBMCs) in the interphase were isolated using a sterile wide

mouth Pasteur pipette without including the lower layer that would contaminate the cells. The cells were then washed twice with R2 media to wash off any contaminating plasma and other blood cells. The isolated PBMCs from each sample were then divided into two components, one component for transcriptomics analysis and the other for proteomics. Thawing of cryopreserved samples was done by placing them on ice and later in a 37^{0}C water bath.

3.8.2 Transcriptional analysis of PBMCs

RNA from PBMCs was extracted and libraries prepared using a similar protocol to the same protocol as the parasite samples above. Paired-end sequencing was performed on a NovaSeq 6000 (Illumina) system at the advanced sequencing facility (ASF), The Francis Crick Institute. Quality of the reads was assessed using FASTQC program and adapters were removed using Cutadapt (Martin, 2011). The remaining reads were aligned to the human reference genome GRCh38 release 95 obtained from Ensembl using STAR aligner (Dobin *et al.*, 2013) and reads overlapping the different gene features counted using the RSEM (RNA-Seq by Expectation-Maximization) tool (Li and Dewey, 2011). Only samples with greater than 10 million aligned reads were considered. Hemoglobin genes described in (Harrington *et al.*, 2020) were removed from the analysis together with genes having 5 counts per million or higher in less than 13 (number of samples in the least popular group) samples, then the filtered counts were normalized using TMM normalization. To determine differential expressed genes among the uninfected, asymptomatic and febrile samples groups, gene analysis was performed by fitting a negative binomial generalized log-linear model in the edgeR package (Robinson, McCarthy and Smyth, 2010). Using the following model matrix formular: ~ 0 + treatment + pair + batch, where treatment was the sample group i.e., uninfected, asymptomatic and febrile, pair indicated the pairing of samples per individual while batch was the RNA processing batch. Differentially expressed genes were partitioned into four clusters, as determined using the elbow method, with the least intra-cluster variation using K-means clustering and plotted using a heatmap using ComplexHeatmap package v2.14.0 (Gu, Eils and Schlesner, 2016). Additional pairwise analyses were conducted to identify differences between asymptomatic and uninfected sample groups. Genes in each cluster were used as input during functional overrepresentation analysis to identify enriched pathways using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets in clusterProfiler v4.4.4 package (Yu *et al.*, 2012). Pathways below a Benjamin-Hochberg adjusted p-value cut of 0.05 were retained.

3.8.3 In silico deconvolution of PBMCS

The cellular composition and abundances of PBMCs cell types was assessed by performing in-silico deconvolution of bulk TPM normalized counts in CIBERSORT (https://cibersortx.stanford.edu/about.php) using the LM22 signature gene set that served as the reference dataset (Newman *et al.*, 2015). The LM22 dataset contained 547 genes that can distinguish 22 mature human hematopoietic cell populations isolated from peripheral blood. These cells include memory and naïve B cells, NK (Natural Killer) cells, plasma cells, seven T cells types and myeloid subsets (Chen *et al.*, 2018).

3.9 Proteomics analysis of host PBMCs

3.9.1 Proteomics sample processing and Liquid chromatography – Mass spectrometry (LC-MS/MS)

The PBMC component for proteomics was resuspending in 5µl of 6M UREA (Thermo scientific). The protein samples were then adjusted with 50mM Triethylamonium bicarbonate (TEAB, Sigma-Aldrich) to 100 ul and the protein concentration ascertained using BCA (Bicinchoninic acid) protein assay (Thermo scientific). Proteins reduction was done using 40mM Dithiothreitol (Sigma-Aldrich) while incubated at 70°C for 1 hour. The protein solution was then alkylated away from light for 1hour using 80mM iodoacetamide from Sigma-Aldrich. Excess iodoacetamide was later quenched with 80mM Dithiothreitol (Sigma-Aldrich). The proteins were then precipitated for 1 hour at -20°C with 400µl of pre- chilled (-20°C) acetone. Then, samples were centrifuged for 10min at 15,000 x g at room temperature. The supernatant was aspirated and discarded. The protein pellet was later air-dried at room temperature for 5 minutes and resuspended in 80µL of 100mM TEAB. Protein digestion was done using trypsin from Sigma-Aldrich which was mixed with the samples using a trypsin-protein sample ratio of 1:20 and incubated for 16 hours at 37°C in a shaker. Peptide samples were labelled using the Tandem Mass Tag (TMT) 10-plex kit from Thermo-scientific. The labelled peptides were later mixed to get separate pools that were then desalted with P10 C18 pipette Zip Tips (Millipore). The peptides that were eluted were dried using a Speedvac concentrator (Thermo Scientific) and resuspended in 15µl loading solvent composed of 98% H_2O, 2% acetonitrile and 0.05% formic acid. Finally, the protein concentration per sample was determined using Qubit Protein Assay Kit (Thermo Fisher Scientific). A standardized protein

concentration of 5 µg was finally injected into the LC-MS/MS equipment available at the KWTRP for analysis

3.9.2 Mass spectrometry (MS) protein identification and quantification

Raw MS files were then processed using MaxQuant software version 2.0.3.0 (Cox and Mann, 2008) by searching against Uniprot human proteome (downloaded on 10/06/2021) using the Andromed search system (Cox *et al.*, 2011). Methionine oxidations and N-terminal acetylation were set as variable modifications while cysteine carbamido-methylation and TMT-10plex labelled N-terminus and lysine were set as a fixed modification. The FDR (false discovery rate) cut-off was set as 0.01 for both peptides and proteins with a minimum length of seven amino acids. Enzyme specificity was put as C-terminal to lysine and arginine while trypsin was set as the protease. Only up to two missed cleavages were permitted in the database search. Peptided identification was performed using an accepted fragment mass deviation maximum of 20ppm (parts per million) and an initial precursor mass deviation maximum of 7ppm. Default parameters for Orbitrap-type data were used. The pooled sample channels were used for batch correction. The 10-plex reported ion intensity matrix was retrieved from the protein groups, an output file from MaxQuant, and used for downstream analysis. Proteins matching the reversed part of a decoy database, potential contaminants and proteins only identified by a modification site, were excluded.

3.9.3 Analysis of proteomics data

Differential protein abundance analysis of the labelled samples intensities generated by MaxQuant were performed using PERSEUS v2.05.0 workstation (MaxPlanck Institute of Biochemistry, Martinsried, Germany) (Tyanova and Cox, 2018). Briefly, reverse and contaminant proteins were removed from the data. The data was normalized using Z score normalization and annotated using Homo sapiens (9606) annotations. Multiple sample tests were performed using Analysis of Variance (ANOVA) followed by post hoc Tukey's HSD (honestly significant difference) test to determine significant proteins. The FDR threshold was set to $q < 0.05$, respectively. The differentially expressed proteins were loaded into STRING database version 11.5 (https://string-db.org/) which is a curated database for functional annotation of genes and proteins that relies on evidence from high throughput genomic, co-expression and proteomic studies, for protein-protein interaction and Gene Ontology (GO) functional analyses.

3.10 Statistical analyses

The student's t-test and ANOVA were used for comparison of mean COI between asymptomatic and febrile malaria infections. In addition, associations between categorical variables i.e., the two treatment groups versus the frequency of the two msp2 alleles among paired infections, were tested using the Fisher's exact test. The analysis of microscopy positive data trends over time was done using the Mann-Kendall trend test function as implemented in the trend package (Pohlert, 2020). Multivariate logistic regression models were fitted to associated asymptomatic and febrile infections with COI after adjusting for age, parasitaemia and microscopy positivity as a categorical variable (high positivity from 2007 to 20012 and low positivity from 2013 to 2018). The expected heterozygosity (*He*) was defined as the probability that two randomly selected clones from this population will contain different *msp2* alleles. *He* was thus used to estimate *msp2* allelic diversity at each time-point based on the formula below:

$$H_e = [n/(n-1)]\ [(1-\sum P_i^2)],$$

where n is the sample size and P*i* is the frequency of *i*th allele in the population (Nei, 1978).

All Statistical analyses were performed in R environment v.4.2.1 (R Core Team, 2020) by using RStudio 2021.09.0 Build 351. All plots were generated using R packages ggplot2 v3.3.2 (Kassambara, 2020a) and ggpubr v.0.4.0 (Kassambara, 2020b), unless otherwise stated. Differences between paired samples were analyzed using Wilcoxon rang sum test where appropriate. Statistical significance was set at a corrected FDR cutoff of 0.05.

Chapter 4: RESULTS

4.1 Distribution of asymptomatic and malaria cases in Kilifi from 2007 – 2018

About 425 children (< 15 years) in the Junju cohort who were under active and passive surveillance were bled during the annual cross-sectional survey that takes place before the long rains to identify *P. falciparum* negative and positive samples. They were then followed up weekly and all malaria cases (temperature $\geq 37.5^0$; parasitemia $\geq$ 2500 parasites/µl of blood) cases diagnosed and samples taken. The distribution of malaria febrile cases from 2007 to 2018, when the samples used for this study were collected, shows that malaria transmission in this region is perennial, as it occurs throughout the year without greater variation in intensity, except for the dip in transmission in 2012 (**Figure 5**). The distribution of *P. falciparum* positive samples identified during the cross-sectional bleed from 2009 – 2017, and identified as asymptomatic samples, is shown in **Figure 6A**. The proportion of *P. falciparum* positive infections among the asymptomatic samples was highest in 2010 at 25.2% and lowest in 2017 at 4.2%. Malaria positivity in this region was also evaluated using malaria positive samples (n = 838) collected during the cross-sectional bleed from 2007 – 2018. A significant decline in malaria positivity rate was observed ($P < 0.0001$, Mann-Kendall test) (**Figure 6B**). We also checked whether there was change in the median age of malaria positive children in the cohort as a measure of change in malaria transmission intensity. The median age of malaria positive children in the cohort during the cross-sectional bleed was shown to be increasing over time (**Figure 6C**).

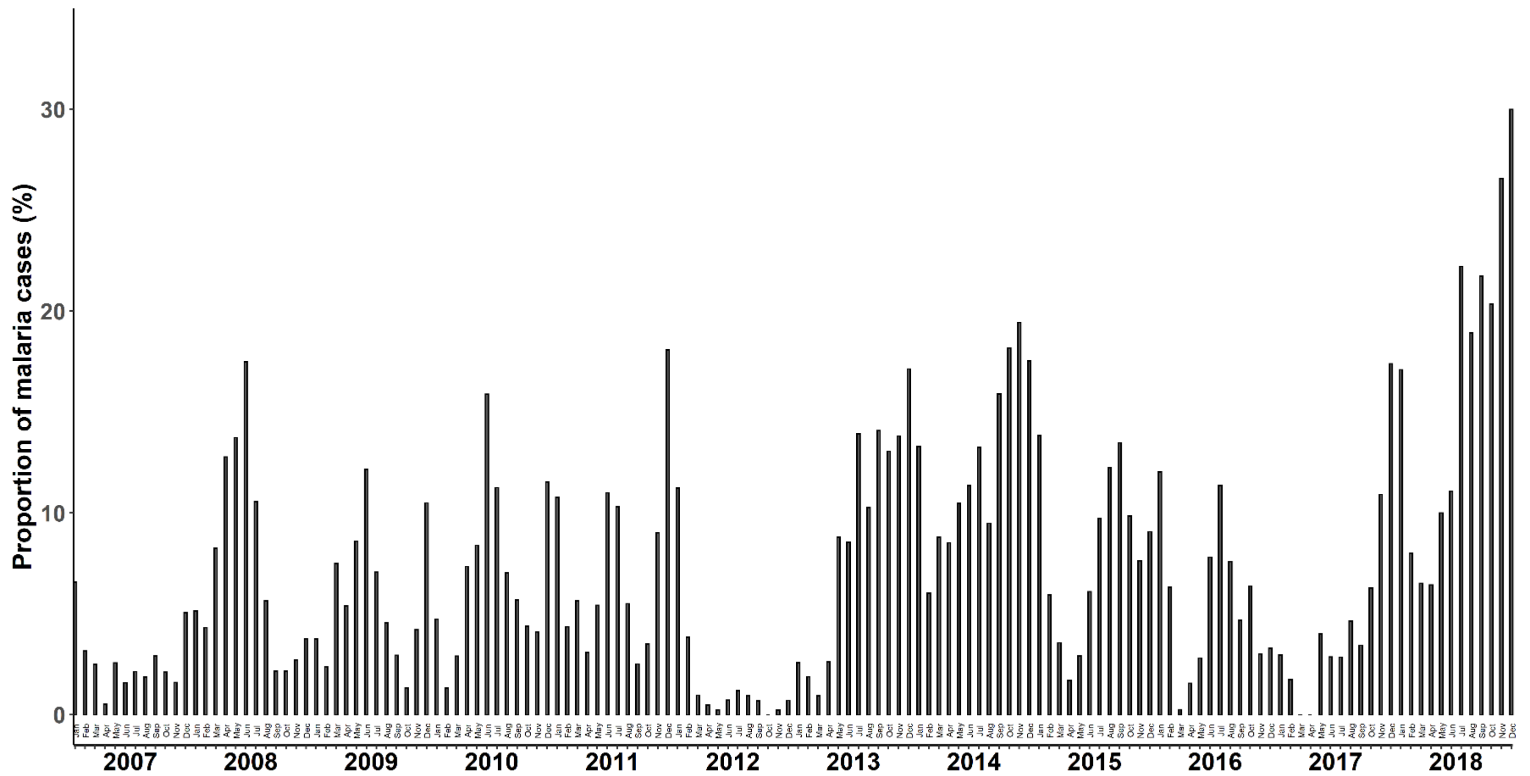

Figure 5: Perennial malaria transmission in Junju among febrile samples

The proportion of symptomatic malaria cases (diagnosed by presence of fever (temperature ≥ 37.5^{0}C) and parasitemia ≥ 2500 parasites/µl) recorded in the cohort during the weekly active surveillance. The proportion of malaria cases is the percentage of malaria cases reported per month. The samples were collected from ~ 425 children aged between 0 and 15 years recruited in the Junju cohort.

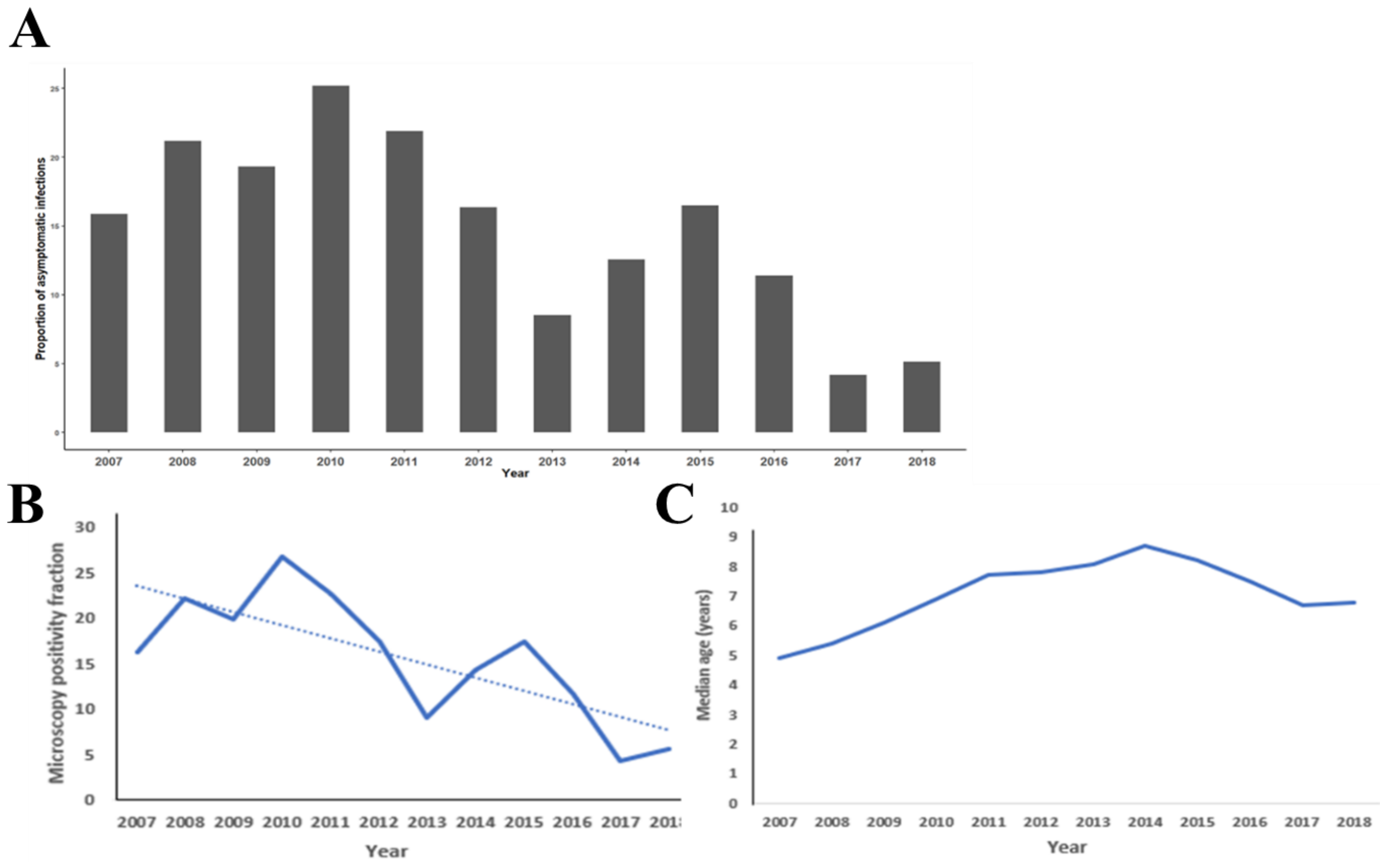

Figure 6: Perennial malaria transmission in Junju among samples from asymptomatic individuals

(A) The frequency of asymptomatic *P. falciparum* infections (diagnosed by presence of parasites without fever) recorded during the annual cross-sectional bleed. The samples were collected from ~425 children aged between 0 and 15 recruited in the Junju cohort. **B)** The temporal trend of asymptomatic malaria positive fraction during the cross-sectional bleed from 2007 to 2018. **C)** The temporal trend in the mean age of malaria positive children during the cross-sectional bleed from 2008 to 2018.

4.2 Genetic diversity of asymptomatic *P. falciparum* infections and febrile malaria infections

4.2.1 Demographics of study participants

Out of the 838 asymptomatic and 147 febrile samples identified, respectively, 411 and 92 asymptomatic and febrile samples, respectively, collected from 217 children (median age = 7.9 years) between 2007 and 2019 were successfully amplified and sized **(Table 4)**. The raw data generated is available as indicated in **Appendix I.** The reason for the lack of amplifications in 2014 and 2019 was due to low parasitemia because of multiple DNA dilutions during previous studies. The age of the children ranged from 5.7 to 10.3 years (IQR) and 50.2% (109) were male while 49.8% (108) were female. The mean parasitemia was significantly lower in asymptomatic infections 14,055 parasites/µl (6,180 – 21,929) compared to febrile infections 116,843 parasites/µl (85,386 – 148,300) (t-test $p < 0.0001$).

Table 4: Characteristics of the cohort and number of samples successfully genotyped from 2007 to 2018.

Year	Microscopy positivity rate* (%)	Median Age in years	Asymptomatic Episode		First Febrile Episode	
			n	Samples available [% genotyped]	*n*	Samples available [% genotyped]
2007	16.2	4.9	85	55 [80.0]	18	0
2008	22.1	5.4	122	110 [59.1]	33	15 [6.7]
2009	19.9	6.1	119	96 [46.9]	34	20 [95.0]
2010	26.8	6.9	237	223 [22.4]	115	39 [64.1]
2011	22.6	7.7	146	62 [77.4]	47	28 [64.3]
2012	17.4	7.8	179	68 [61.8]	16	4 [25.0]
2013	9.0	8.1	105	52 [69.2]	32	7 [100.0]
2014	14.2	8.7	165	41 [0]	73	15 [46.7]
2015	17.4	8.2	98	60 [73.3]	27	5 [100.0]
2016	11.7	7.5	73	36 [58.3]	13	4 [100.0]
2017	4.3	6.7	14	13 [76.9]	4	4 [75.0]
2018	5.5	6.8	27	22 [27.3]	7	6 [33.3]
2019	5.7	7.0	21	14[0]	5	5[0]

NB: In 2007 and 2019, there were no corresponding febrile samples in the biobank while in 2014 and 2019 asymptomatic samples failed PCR amplification. Positivity rate was determined by microscopy, *there was a significant decline (Mann-Kendall trend analysis $p<0.001$). % genotyped is the percentage of PCR amplified amplicons that yielded successful fragments. *n* refers to number of individuals in the cohort each year defined as asymptomatic and first febrile during follow up visits.

4.2.2 *msp2* genetic diversity

A total of 128 [31.2%] IC alleles were observed in the asymptomatic infections compared to 101 [24.6%] FC27 alleles (**Table 5**). The sizes of these genotypes ranged from 180 – 673bp and 315 – 805bp for the FC27 and IC allelic families, respectively. There were at least 5 FC27 alleles (291bp, 327bp, 362bp, 365bp and 411bp) at a relatively high frequency (dominant alleles) that persisted over the 12-year study period out of a total of 45 FC27 alleles in asymptomatic infections **(Figure 7)**. Though there was a lot more genetic variation in the IC allelic family and only three (497bp, 548bp, 555bp) IC fragments out of 78 were persistent over time **(Figure 7).** Compared with asymptomatic infections, the first febrile infections contained fewer alleles (i.e.- 29 FC27 alleles and 38 IC alleles, with allele sizes ranging from 217–545bp for the FC27 and 327–724bp for the IC allelic families **(Figure 8)**. An overlap of 19 FC27 and 32 IC alleles between asymptomatic and febrile infections were detected. A high expected heterozygosity of greater than 0.95 was revealed in both asymptomatic and febrile malaria infections over time (**Table 4**).

Figure 7: Prevalence and occurrence per year of each msp2 allelic family FC27 (left) and IC/3D7

The total number of FC27 and IC/3D7 alleles were 45 and 78, respectively. The size range was 180 – 673 bp for FC27 alleles and 315 – 805 bp for IC/3D7 alleles. m represents the total number of alleles per year while n is the number of successfully genotyped samples per year. k is the number of times a specific allele was seen across the 11 years.

Figure 8: Prevalence and occurrence per year of each msp2 allelic family FC27 (left) and IC/3D7 (right) in febrile malaria infections.

The total number of FC27 and IC/3D7 alleles were 29 and 38, respectively. The size range was 217–545bp for FC27 alleles and 327–724bp for IC/3D7 alleles. Highlighted in grey is the number of successfully samples per year. m represents the total number of alleles per year while n is the number of successfully genotyped samples per year. k is the number of times a specific allele was seen across the 11 years.

4.2.3 Complexity of infections

Asymptomatic infections were characterized by more (281, 68.4%) polyclonal (>2) infections with a median COI of 2.0 (1 - 10) while febrile infections were generally monoclonal with a median COI of 1.0 (1 – 6) **(Figure 9)**. In addition, the proportion of samples with different number of clones i.e. COI, over time is shown in **Table 4**. The mean COI for asymptomatic infections was 2.4 (95% CI, 2.2 – 2.5). The lowest COI was 1.7 in 2008, while the highest COI, 3.0, was observed in 2016 (**Table 4**). While the mean COI for febrile infections was 2.0 (95% CI, 1.7 – 2.3) with the lowest COI of 1.5 being observed in 2016 and the highest, 2.5, observed in 2018 **(Table 4)**. Overall, there was a statistically significant difference in COI between asymptomatic and febrile infections, $p = 0.016$. In addition, multivariate analysis revealed the risk of being febrile reduced by 22.9% (adjusted odds ratio (AOR): 0.771; 95% CI 0.611 – 0.95) for every unit increase in COI, after adjusting for parasitemia, age and microscopy positivity.

Table 5: Distribution of msp2 alleles and Complexity of infections over time

Year	Asymptomatic infections						Febrile infections					
	n	Allelic type			COI (Range)	*He*	n	Allelic type			COI (Range)	*He*
		FC27 n (%)	IC/3D7 n (%)	FC27 + IC/3D7 n (%)				FC27 n (%)	IC/3D7 n (%)	FC27 + IC/3D7 n (%)		
2007	44	4 (4.0)	22 (17.2)	18 (9.9)	2.4 (1-7)	0.986	NA	NA	NA	NA	NA	NA
2008	65	20 (19.8)	33 (25.8)	12 (6.6)	1.7 (1-5)	0.969	1	1 (2.0)	NA	NA	1	NA
2009	45	12 (11.9)	11 (8.6)	22 (12.2)	2.7 (1-6)	0.959	19	13 (26.5)	3 (23.1)	3 (10.0)	2.1 (1-6)	0.968
2010	50	18 (17.8)	13 (10.2)	19 (10.5)	2.0 (1-7)	0.967	25	14 (28.6)	3 (23.1)	8 (26.7)	2.0 (1-6)	0.965
2011	48	13 (12.9)	16 (12.5)	19 (10.5)	2.5 (1-5)	0.975	18	12 (24.5)	2 (15.4)	4 (13.3)	1.6 (1-5)	0.953
2012	42	17 (16.8)	5 (3.9)	20 (11.0)	2.7 (1-8)	0.964	1	1 (2.0)	NA	NA	1	NA
2013	36	9 (8.9)	3 (2.3)	24 (13.3)	2.6 (1-5)	0.962	7	4 (4.2)	NA	3 (10.0)	2.4 (1-4)	0.963
2014	NA	NA	NA	NA	NA	NA	7	2 (4.1)	1 (7.7)	4 (13.3)	2.4 (1-4)	0.971
2015	43	3 (3.0)	13 (10.2)	27 (14.9)	2.2 (1-5)	0.959	5	1 (2.0)	1 (7.7)	3 (10.0)	2.2 (1-4)	NA
2016	21	2 (2.0)	6 (4.7)	13 (7.2)	3.0 (1-10)	0.975	4	1 (2.0)	2 (15.4)	1 (3.3)	1.5 (1-2)	NA
2017	10	1 (1.0)	5 (3.9)	4 (2.2)	2.7 (1-5)	0.966	3	NA	1 (7.7)	2 (6.7)	1.7 (1-2)	NA
2018	6	2 (2.0)	1 (0.8)	3 (1.7)	2.3 (1-5)	0.934	2	NA	NA	2 (6.7)	2.5 (2-3)	NA
2019	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Total (%)	**410**	**101 (24.6)**	**128 (31.2)**	**181 (44.2)**	**2.3 (1-10)**	**0.975**	**92**	**49 (53.3)**	**13 (14.1)**	**30 (32.6)**	**2.0 (1-6)**	**0.964**

n corresponds to the number of successfully genotyped samples per year while % is the frequency per year. NA indicates non available data. The lack of amplifications in 2014 and 2019 was due to low parasitemia because of multiple DNA dilutions from previous studies. *He* refers to the expected heterozygosity. *He* was only calculated for 2009 to 2011 and 2013 to 2014 among the febrile where n was sufficient.

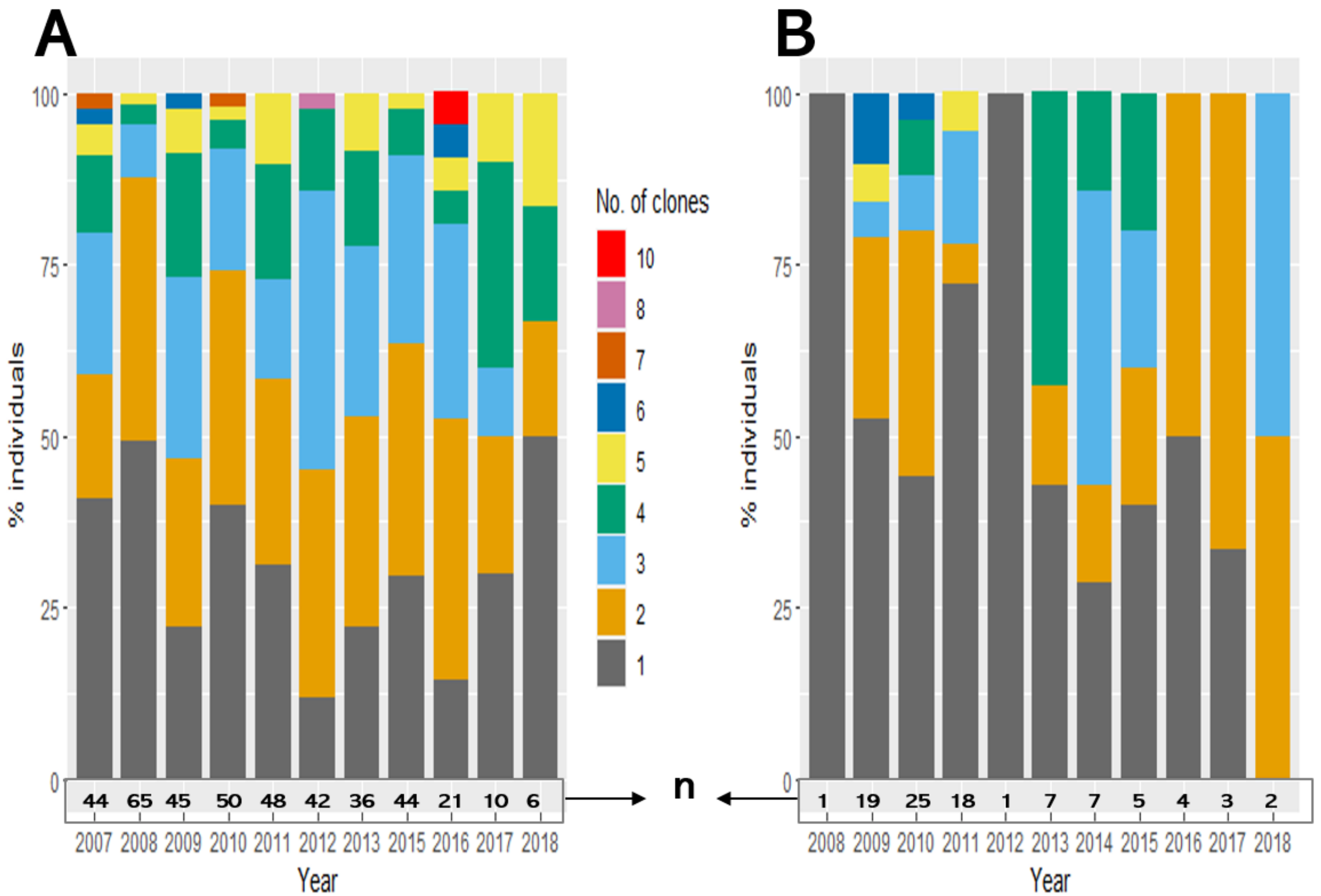

Figure 9: Proportion of samples with diverse number of *P. falciparum* clones per year, based on block 3 of msp2.

(A) Asymptomatic and **(B)** Febrile infections. n shows the total number of successfully genotyped samples annually. The different colors represent the various number of clones: dark grey (1), orange (2), light blue (3), green (4), yellow (5), blue (6), brown (7), pint (8) and red (10).

4.2.4 *msp2* genetic diversity in paired asymptomatic and febrile samples

Out of the 425 children recruited in the study, 26 had paired samples i.e., samples during asymptomatic and corresponding follow-up febrile infection, that were successfully genotyped. Interestingly, only 2 individuals maintained a persistent allele during their asymptomatic and febrile infections. The persistent alleles were FC27 (327bp and 411bp) alleles (**Table 5**) which were also the most dominant in the population (**Figures 8 and 9**). Eight FC27 and 3 IC genotypes were common among the paired asymptomatic and febrile samples out of a total of 18 FC27 and 35 IC alleles, respectively (**Table 5**). All except 5 individuals had an IC allele in their asymptomatic infection, while 14 (53%) of the individuals had no IC genotypes in their febrile infections. However, 24 (92.3%) of the febrile infections harbored FC27 alleles. In contrast, about 7 (26.9%) of the asymptomatic infections did not have an FC27 allele. Subsequently, a modest association between the outcome of *P. falciparum* infection and the *msp2* allelic family groups, either asymptomatic FC27 alleles (6), IC alleles (7) and mixed alleles (13) or symptomatic FC27 alleles (14), IC alleles (2) and mixed alleles (10) (Fisher's Exact Test, $P = 0.057$). However, there was a significant difference (Fisher's Exact Test, $p = 0.041$) when only the FC27 and IC allelic families were considered as majority of FC27 and IC alleles were observed in febrile and asymptomatic infections, respectively. Like the observation in the population, mean parasitemia was significantly higher ($P < 0.01$) while COI was significantly lower ($P = 0.011$) during febrile infections compared to asymptomatic infections among the paired samples (**Figure 10**).

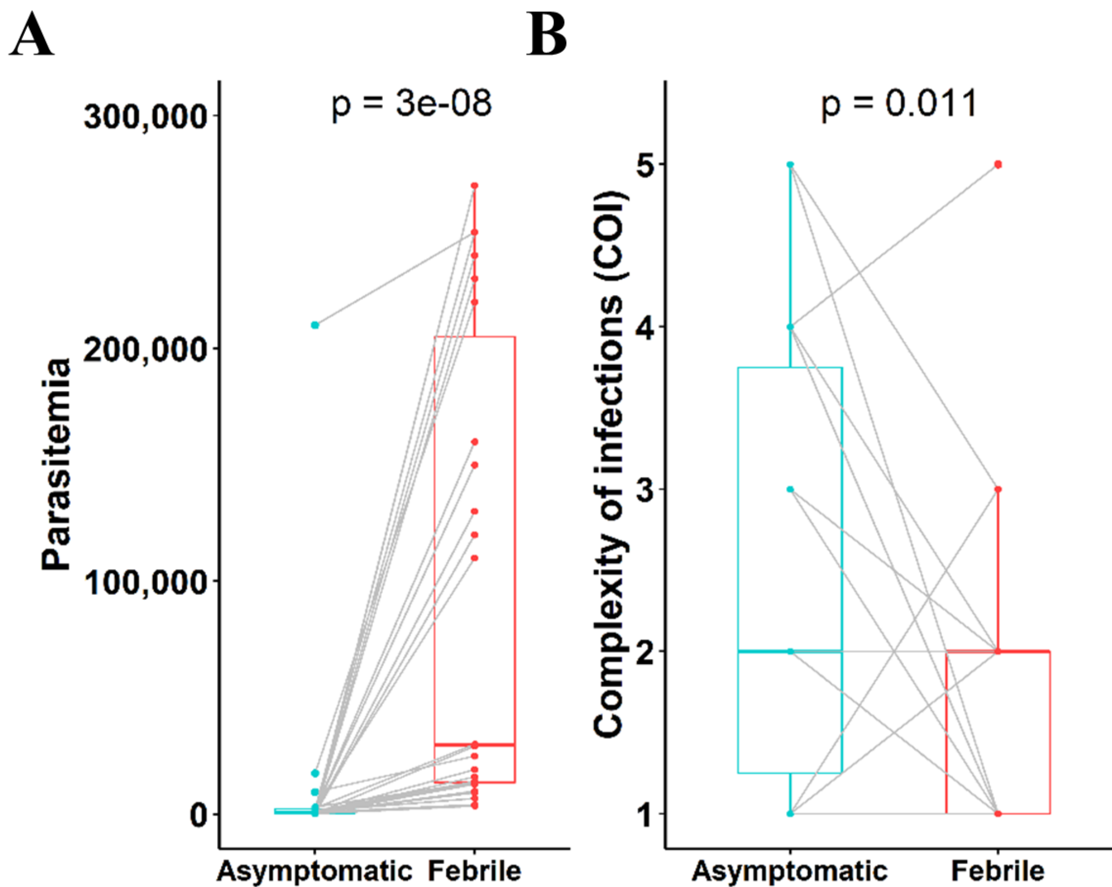

Figure 10: Comparative analysis of parasitemia and COI among the paired samples

A) A comparison of parasitemia between asymptomatic and febrile infections among the 26 paired samples**.** There was a significant increase in parasitemia during the febrile infection ($P < 0.001$). **B**) A comparison of COI between asymptomatic and febrile infections among the 26 paired samples**.** There was a significant reduction in COI during the febrile infection ($P = 0.011$). Each dot represents a sample while the grey lines connect paired samples.

Table 6: Msp2 gene diversity in paired asymptomatic and febrile infections

Sample	Year	Days to	Identity	Asymptomatic							Febrile				
ID		febrile		FC27 Genotypes				IC/3D7 Genotypes			FC27 Genotypes		IC/3D7 Genotypes		
Pair_01	2008	250	Novel	397	411			542	558		291				
Pari_02	2008	300	Novel					443	562		362				
Pair_03	2009	38	Novel	400	545			509	627		327				
Pair_04	2009	43	Novel	545							411				
Pair_05	2009	71	Novel		362			469			336	365	516		
Pair_06	2009	233	Novel	327				454			400				
Pair_07	2010	15	Novel	327				558			365				
Pair_08	2010	216	Novel					490	599		236		555		
Pari_09	2010	24	Novel	217	291	327		490			411		565		
Pair_10	2010	69	Novel					624			327				
Pair_11	2010	106	Novel	362				505			236	327			
Pair_12	2010	23	Novel	299							327				
Pair_13	2011	40	Novel	336				454	497	525	452	462	463	606	724
Pair_14	2011	35	Novel					463			365				
Pair_15	2011	25	Novel	400	545			463	497	582				539	
Pair_16	2011	36	Novel					463	582		236			512	
Pair_17	2012	289	Novel	362				548	669		299				
Pair_18	2015	99	Novel					542			484				
Pair_19	2016	121	Novel	556				612	653		327		555		
Pair_20	2016	221	Novel					555	656				500		
Pair_21	2017	563	Novel	217	314	411		532			291		606	570	
Pair_22	2017	175	Novel	217	314	411					327		473		
Pair_23	2018	114	Novel	365							327		520		
Pair_24	2018	172	Novel	217	314	327	411	497			236		469		
Pair_25	2011	51	Persistent	327				616			327			528	
Pair_26	2009	30	Persistent	391	411						291	411			

The persistent clones are highlighted in grey.

4.3 Analysis of parasite transcriptome

4.3.1 Characteristic of study participants

Parasites were successfully isolated from 16 paired individuals, consisting of 12 females and 4 males, during the asymptomatic and their febrile follow-up malaria infections (**Table 7**). The participants had a mean age of 7.5 years during the asymptomatic *P. falciparum* infections and were followed for an average of 138.3 days before developing malaria symptoms. The median parasitaemia was significantly lower during asymptomatic infections compared to febrile infections ($P = 0.02$) (**Table 6**).

Table 7: Characteristics of the study participants

Variable	Asymptomatic infection	Febrile malaria	P value
Number of participants	16 individuals		
Median Age in years (Range)	8.5 (0.8 – 13.1)	78.7 (1.4 - 13.6)	
Parasites/ul (95% confidence interval)	28,017.5 (-8,023.9 – 64,058.9)	165,630.0 (-3,425.6 – 334,685.6)	0.0204*
Gender F/M (n)	3.0 (12/4)		
Time to febrile	114.6.25 (22 – 235 days		
Total reads (95% confidence interval)	14,310,879 (5,034,540 – 23,587,218)	17,743,126 (3,187,348 – 32,298,904)	0.7072*
Mapped reads (95% confidence interval)	6,863,892.5 (801,052.5 – 12,926,732.5)	11,820,355 (1,599,510 – 22,041,200)	0.1556*

*** -** Wilcoxon rank sum exact test

4.3.2 All gene expression analysis

On average, about 15.4 million pair-end reads were generated per sample (range = 0.6 – 111 million reads)) (**Table 6**). The raw data generated are available as indicated in **Appendix II.** The median parasite age, as expressed in hours post-infection (hpi) and determined using the maximum likelihood estimation method (Lemieux *et al.*, 2009), was statistically higher (p = 0.0004) in asymptomatic infections (median =11.7 hpi, IQR[10.48 - 11.9]) compared to febrile infections (median = 8.7 hpi, IQR(7.6 - 9.55) (**Figure 11)**. A mixture model was used to estimate the proportion of ring, early trophozoite, late trophozoite, schizont and gametocyte parasite stages present in each sample using reference data obtained from Lopez et al. (López-Barragán *et al.*, 2011) (**Figure 12**). The model identified statistically significant higher proportion of ring stage parasites in the febrile samples as compared to asymptomatic infections (p= 0.012). On the contrary, the model estimated that asymptomatic infections had a higher representation of more developed parasites i.e., parasites in the early trophozoite, schizont and gametocyte parasite stages, compared to febrile samples. However, this difference was statistically significant only in the early trophozoite stage (P = 0.0025) (**Figure 13**).

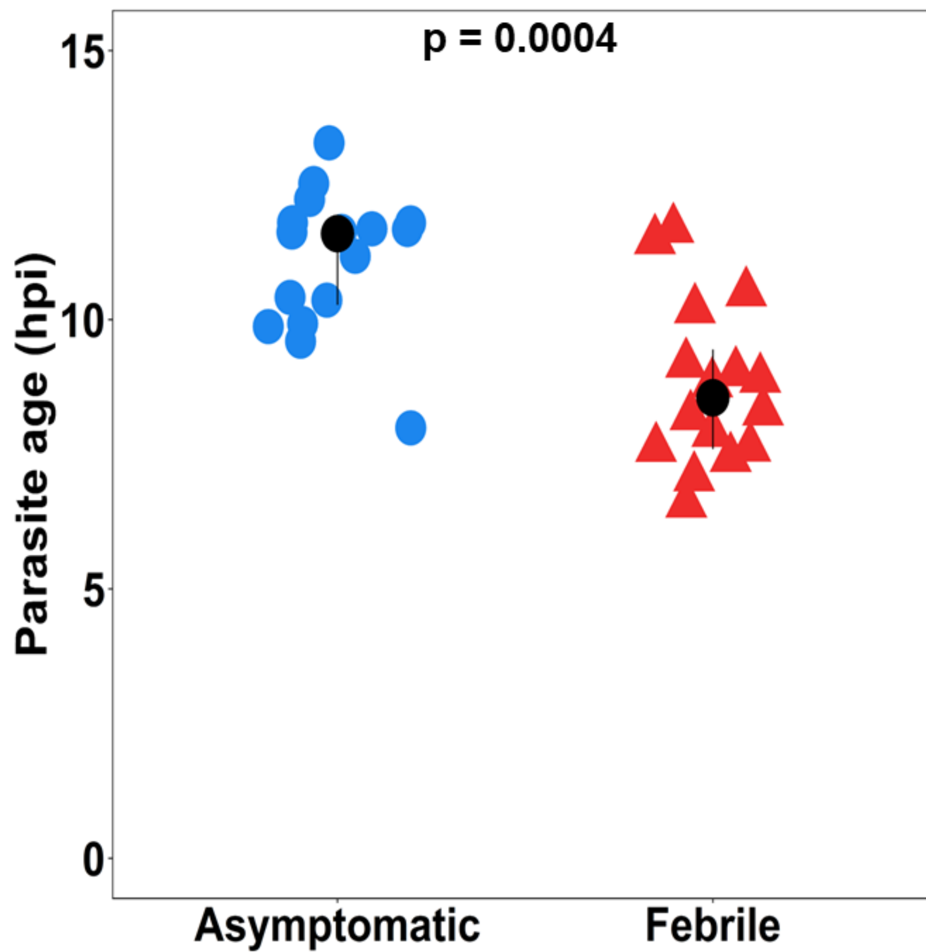

Figure 11: Asymptomatic infections are characterized by older parasites compared to ensuing febrile malaria.

Dot plot showing the maximum likelihood estimation (MLE) of the hours post-invasion (hpi) determined using reference dataset from (Bozdech *et al.*, 2003). The samples are colored by clinical phenotype, asymptomatic infections (blue) and febrile malaria (red). The black dot shows the median hpi.

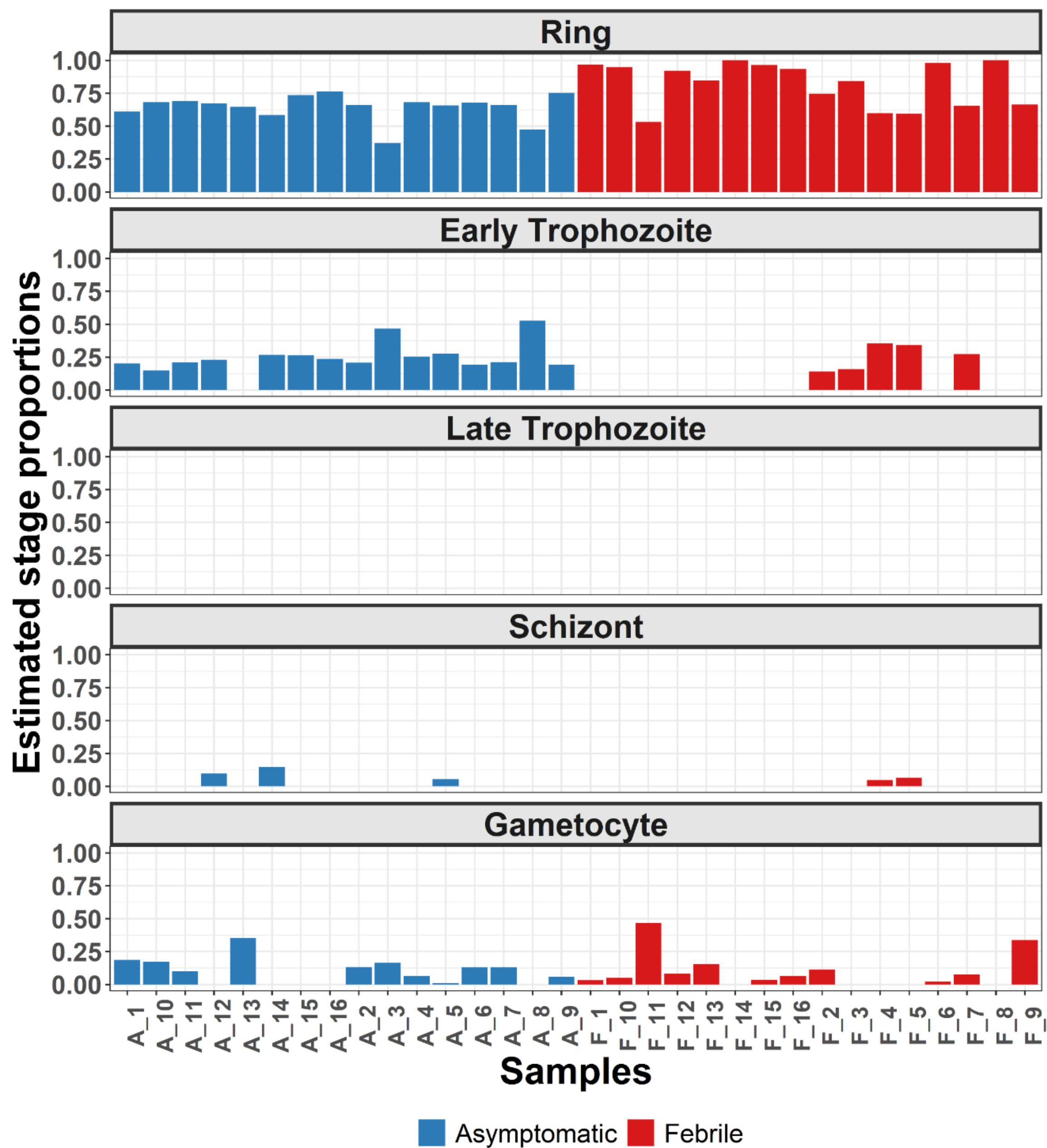

Figure 12: Estimated *P. falciparum* parasite stage proportions for each sample in asymptomatic and ensuing febrile malaria infections.

Bar plots illustrating the relative estimated proportions of the different parasite development stages in asymptomatic and febrile parasite samples estimated using a mixture model and reference dataset from López-Barragán et al. (2011). The stage proportions must add to 1 for each sample. The samples are colored by clinical phenotype, blue for asymptomatic infections and red for febrile malaria.

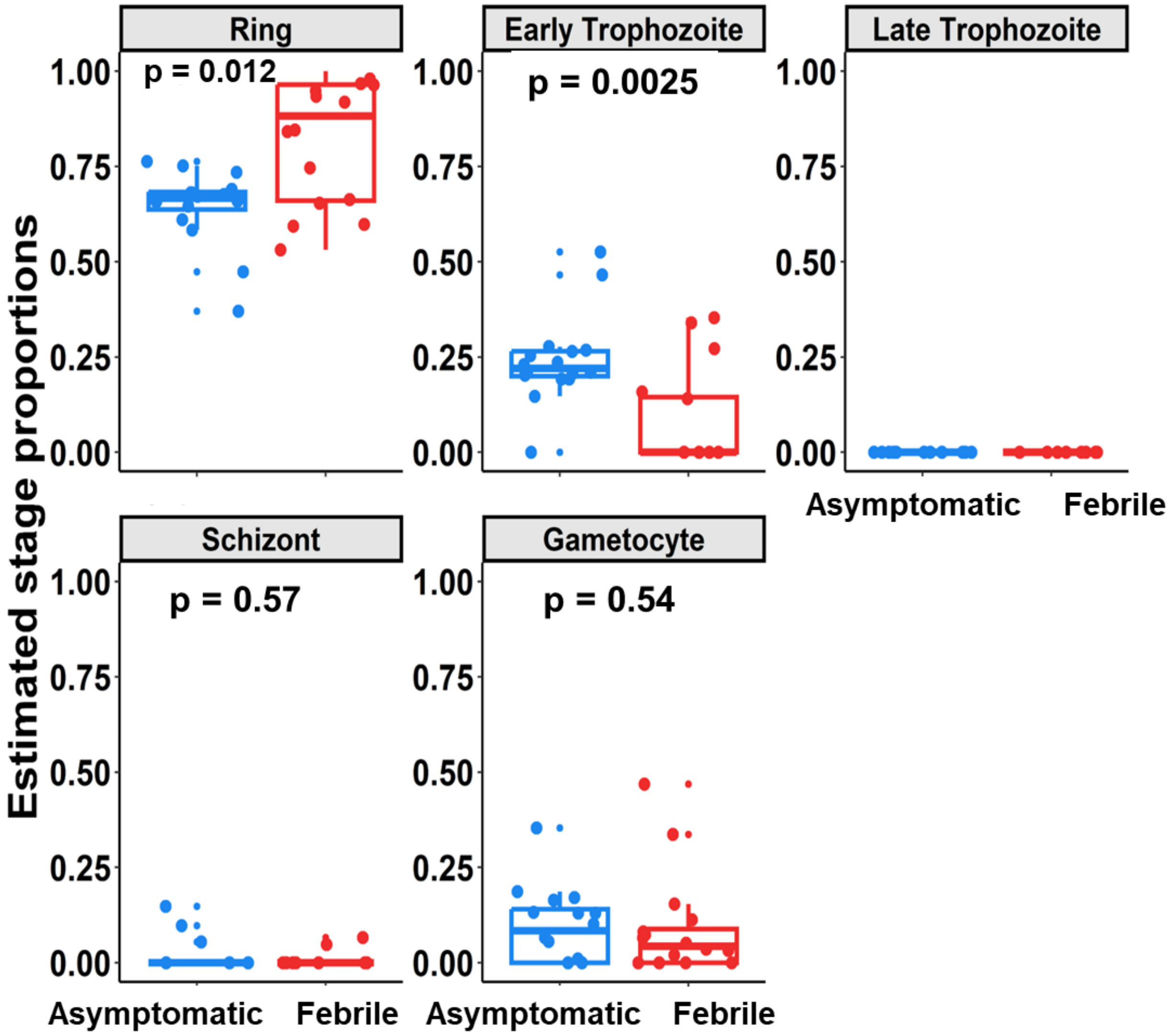

Figure 13: Comparison of the estimated *P. falciparum* parasite proportions between asymptomatic and febrile malaria infections per each life cycle stage.

Boxplots showing the estimated proportions of parasite blood stage development in asymptomatic infections and febrile malaria as estimated using a mixture model and reference dataset from López-Barragán et al. (2011). For each parasite stage, Wilcoxon test (corrected for multiple testing using Benjamini & Hochberg) was performed to evaluate the difference in the proportions of parasites between asymptomatic and febrile malaria. The samples are colored by clinical phenotype, blue for asymptomatic infections and red for febrile malaria.

To test the hypothesis that more developed parasites are associated with lower parasite density, the proportion of rings and non-rings was correlated to parasite density across all the samples. The rings were positively correlated with parasite density (r = 0.53: $P = 0.0017$) (**Figure 14**) while the non-rings were negatively correlated (r = - 0.45; $P = 0.01$) (**Figure 15**).

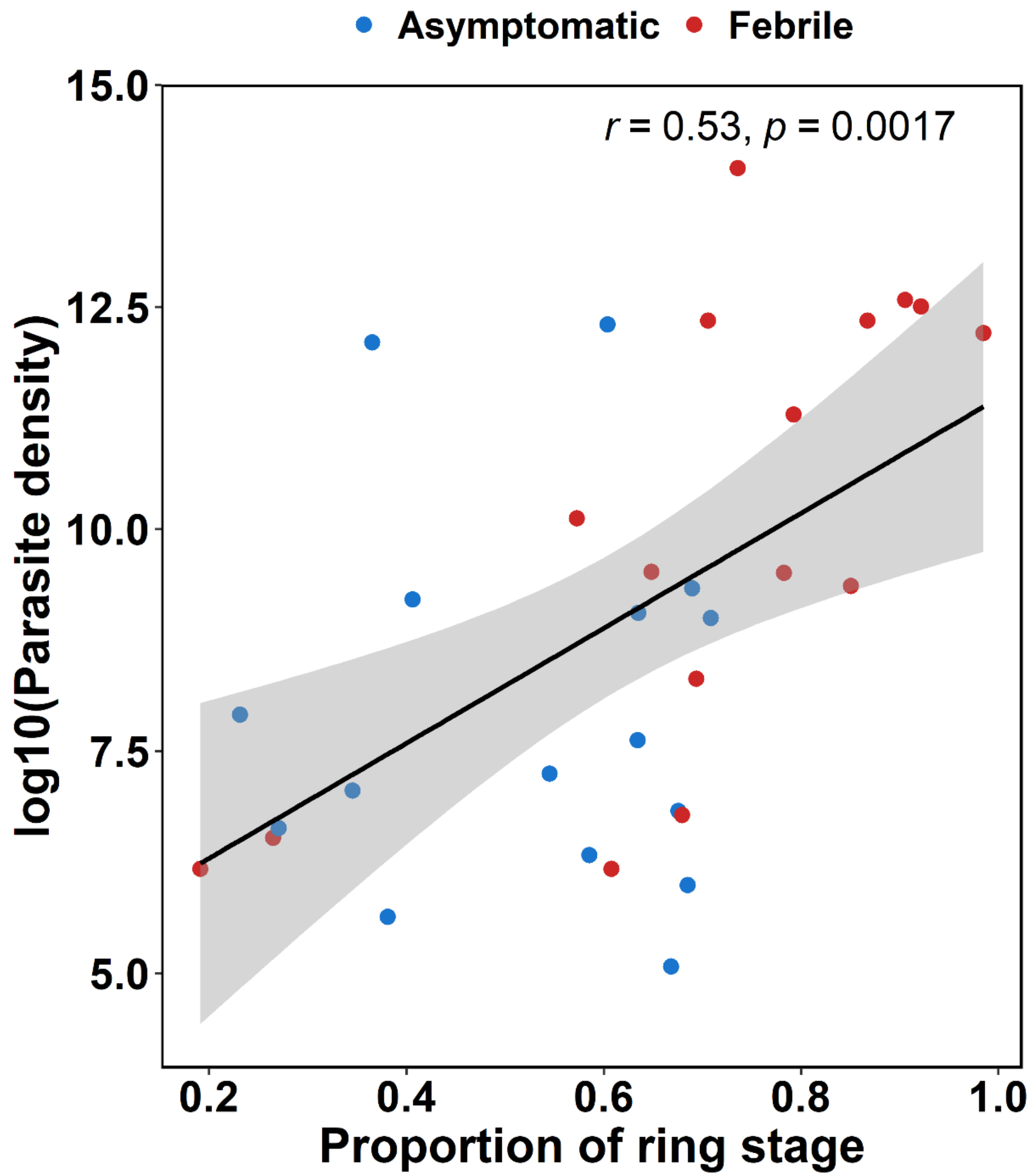

Figure 14: Association between ring stage proportions and parasite density across all samples.

Scatter plot showing the correlation between the parasite density (log10) and the estimated proportions of ring stages across all the samples.

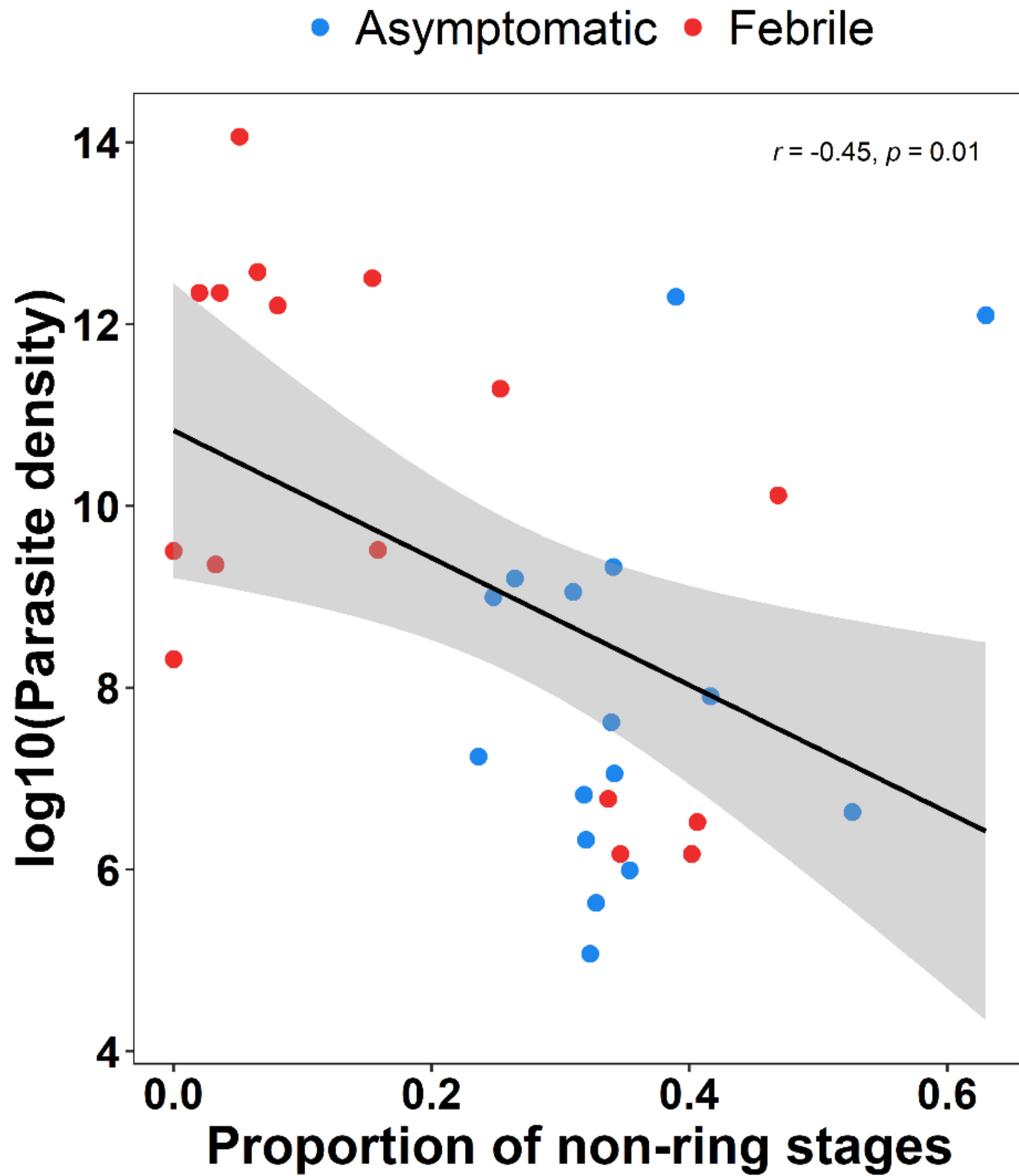

Figure 15: Association between non-ring stages and parasite density across all samples.

Scatter plot showing the correlation between the parasite density (log10) and the estimated proportions of non-ring stages across all the samples. The samples are colored by the clinical phenotype, blue for asymptomatic infections and red for febrile malaria.

Prior to differential expression analysis, we investigated how the parasite life-cycle stages affect the separation of the two outcomes by the first component as illustrated by principal component analysis (PCA). PCA of all the genes, after accounting for different library sizes using TMM normalization, showed that parasite life-cycle stages may influence the separation of the two outcomes (**Figure 16A**). A Relative Log Expression (RLE) plot showed outlier samples with high variability in their gene expression mainly due to life-cycle stages hence the need to account for these stages when conducting differential expression (**Figure 16B**). Adjusting for the life-cycle stages improved the separation of the two outcomes and reduced outlier samples (**Figure 17A**). There were still several samples with RLE above or below the horizontal line hence warranting further analysis to account for the unwanted variation (**Figure 17B**).

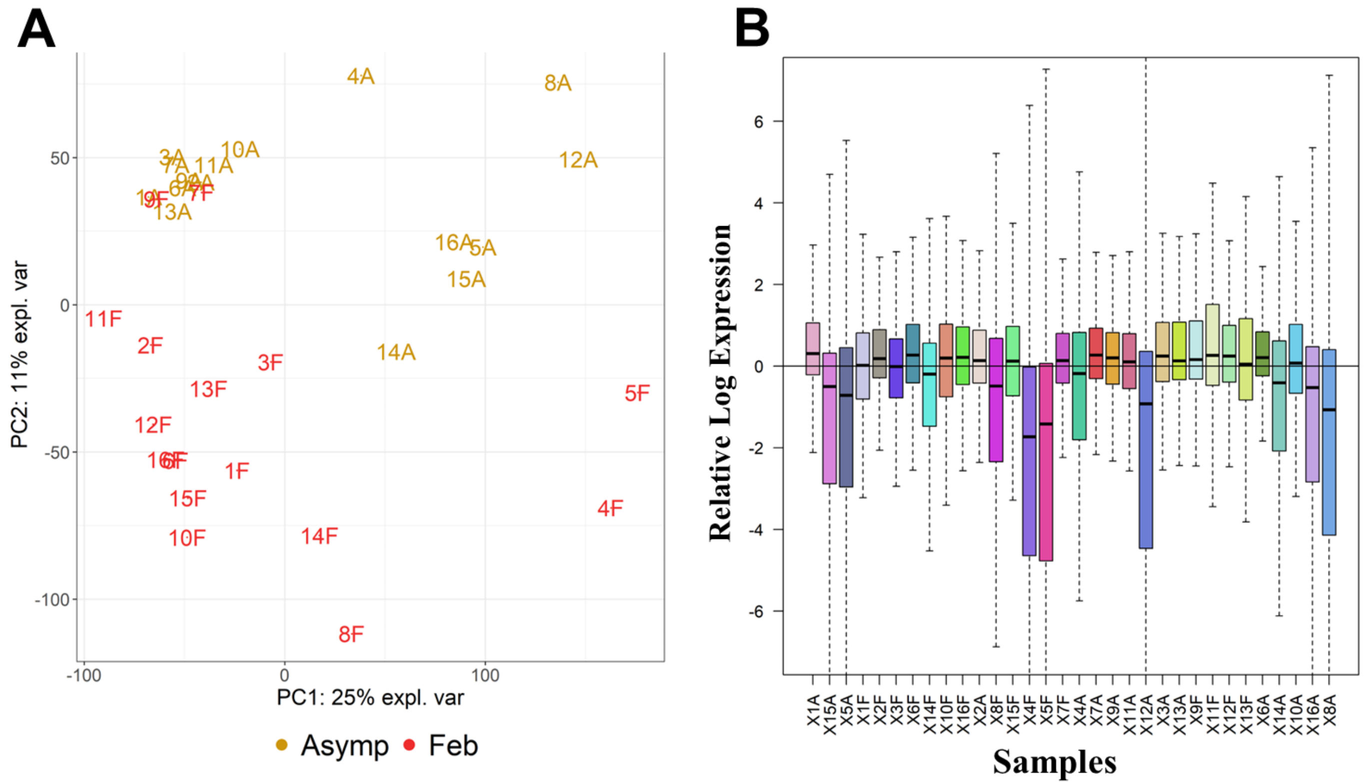

Figure 16: Genome-wide analysis of *P. falciparum* transcriptome data after normalizing for sequence depth.

A) PCA plot of read counts after normalizing for sequencing depth and RNA composition using TMM (Trimmed Mean of M values) method. Samples are colored by phenotype i.e., yellow for asymptomatic and red for febrile malaria. **B)** A Relative Log Expression (RLE) plot showing the distribution of read counts across the samples after normalization. The RLE per sample (log counts for each sample divided by the reference counts determined by the median counts across all samples) are plotted.

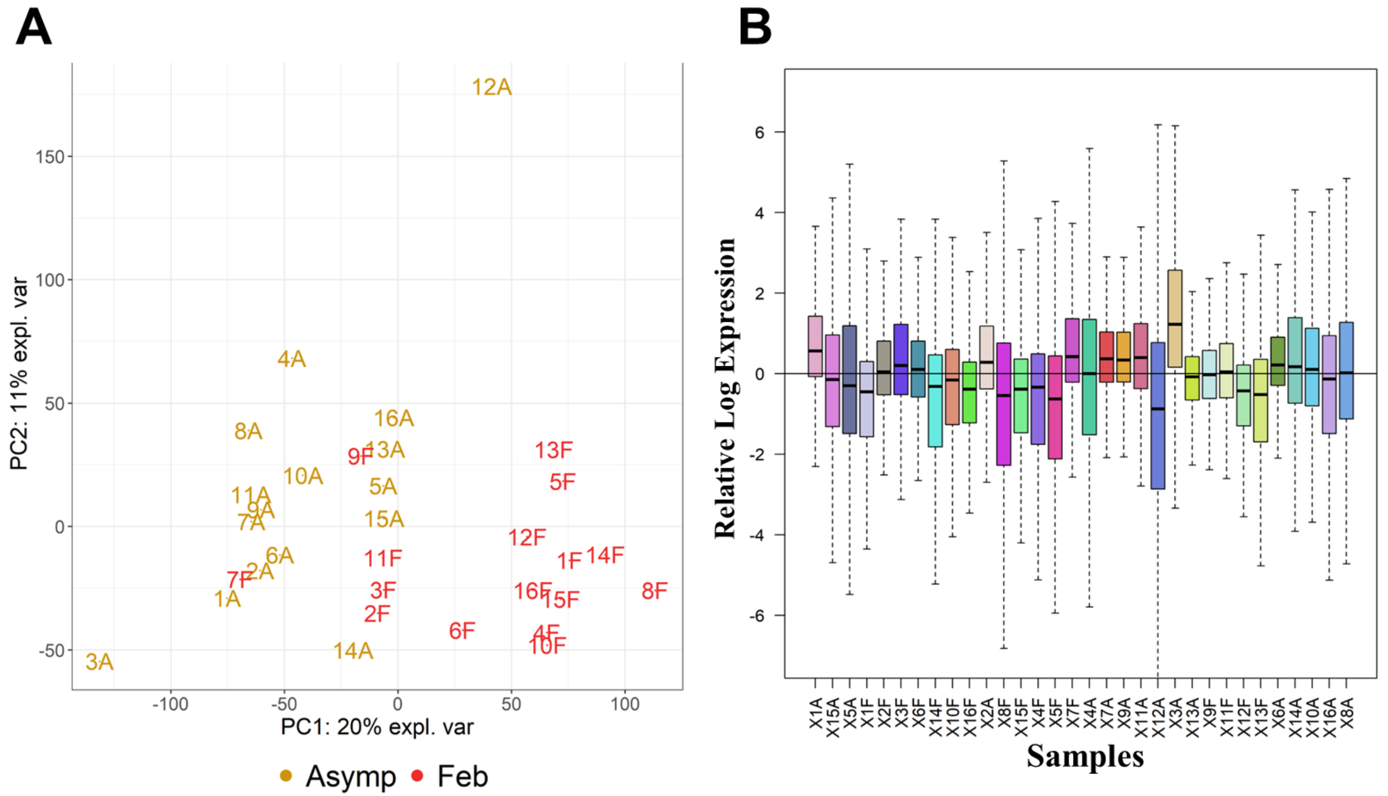

Figure 17: Genome-wide analysis of *P. falciparum* transcriptome data after normalizing and adjusting for parasite life cycle state proportions.

A) A PCA plot of read counts after normalization and removing life-cycle stage effects. The samples are colored by phenotype i.e., yellow for asymptomatic and red for febrile malaria. **B)** A Relative Log Expression (RLE) plot showing the distribution of read counts after normalization and accounting for staging effects.

To handle the outlier samples without losing statistical power we estimated factors of unwanted variation. Taking care of the library size, parasite life cycle stage and unwanted-variations handled outlier samples and improved the distinction between febrile malaria and asymptomatic infections (**Figure 18A, B,**).

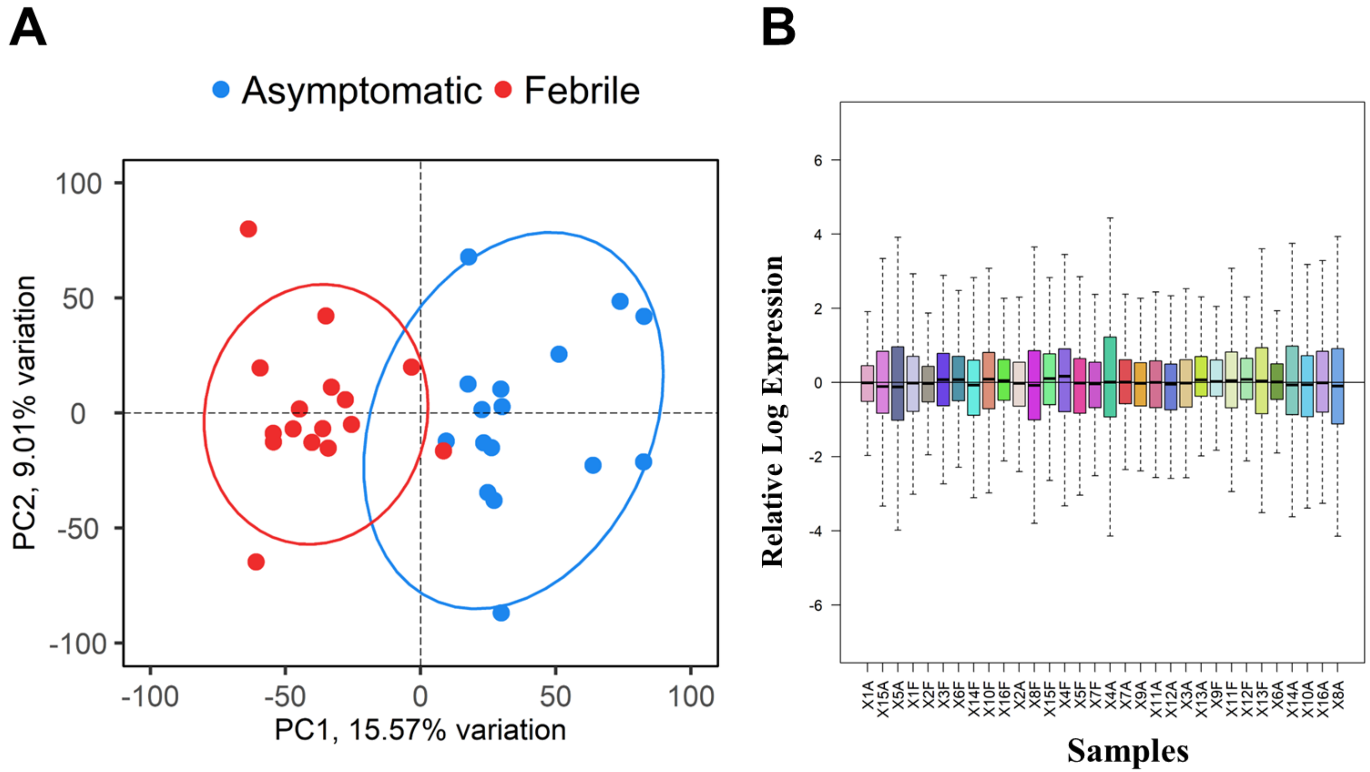

Figure 18: Genome-wide analysis of *P. falciparum* transcriptome data after normalizing for library size, life cycle staging effects and 3 unwanted factors of variation.

A) PCA plot of read counts normalized for library size, life cycle staging effects and 3 unwanted factors of variation estimated by RUV4 package. Samples are colored by clinical phenotype i.e., blue for asymptomatic and red for febrile malaria. Ellipses represent 95% confidence intervals. **B)** A Relative Log Expression (RLE) plot showing the distribution of relative log counts per sample after normalizing for library size, life cycle staging effects and 3 unwanted factors of variation estimated by RUV4 package. The RLE per sample was determined by dividing the log counts for each sample by the reference counts i.e. the median counts across all samples.

4.3.5 Differential gene expression analysis

Differential expression analysis resulted in 1,351 differentially expressed genes (DEGs) determined at a false discovery threshold of 5% (**Supplementary information 2**). About 744 genes were upregulated, and 607 genes were downregulated during the febrile infections as compared to asymptomatic infections (**Figure 19)**. Hierarchical clustering of the samples revealed segregation of the expression profiles based on the condition while the DEGs were grouped into two clusters using k-means clustering (**Figure 20**). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs revealed a significant (FDR < 5%) enrichment of pathways related to fatty acid biosynthesis, ribosomal biogenesis and spliceosome during febrile malaria infections. While genes associated with glycerophospholipid metabolism and phagosome pathways were upregulated in asymptomatic infections (**Figure 21**).

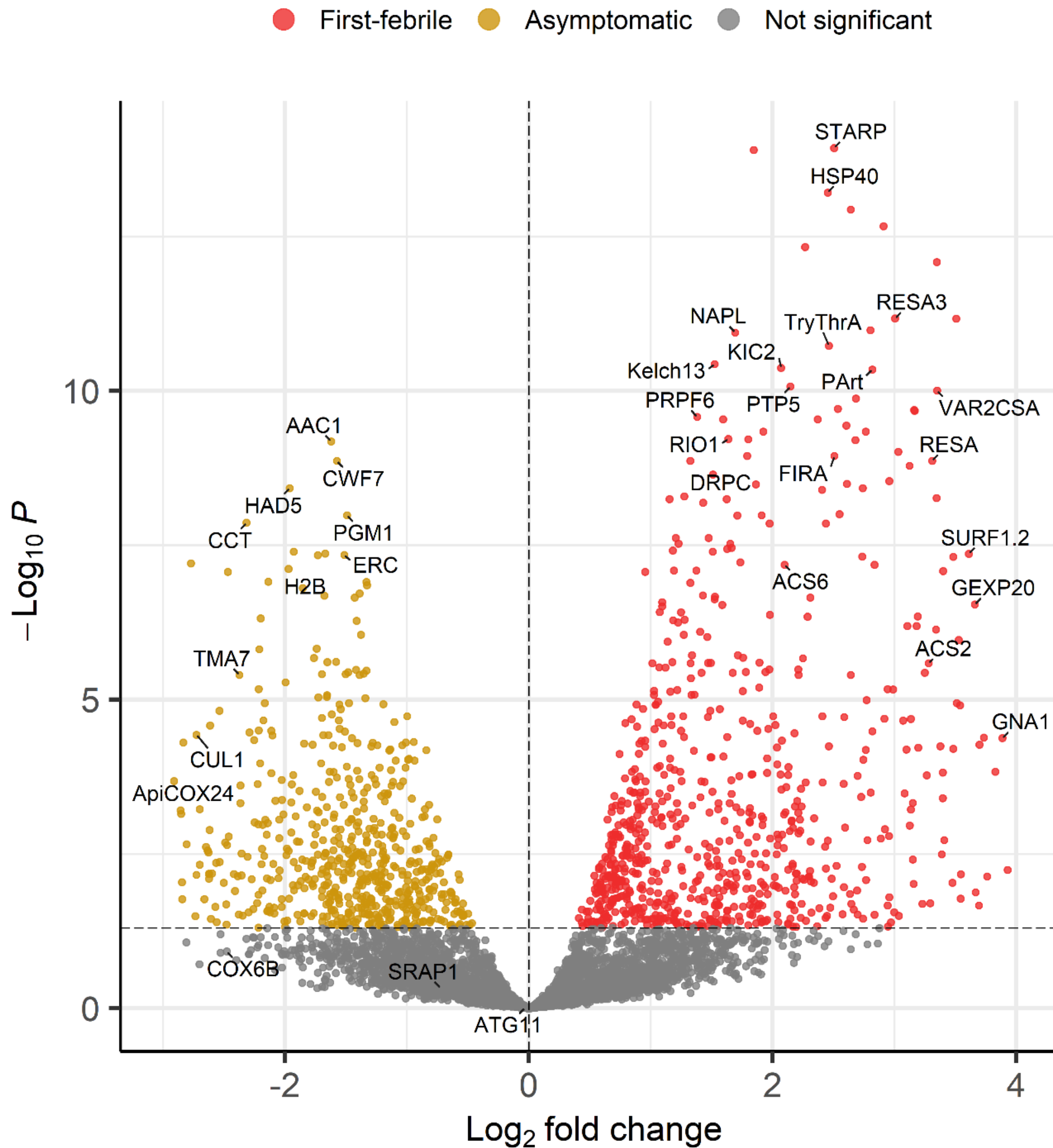

Figure 19: Volcano plot showing differentially expressed genes

Volcano plot showing 1,351 significantly differentially expressed genes (DEGs) at a false discovery rate (FDR) threshold of 5%. Red dots represent 744 genes upregulated in first-febrile while yellow dots represent 607 genes upregulated in asymptomatic infections. Grey dots represent non-significant (FDR > 5%) genes.

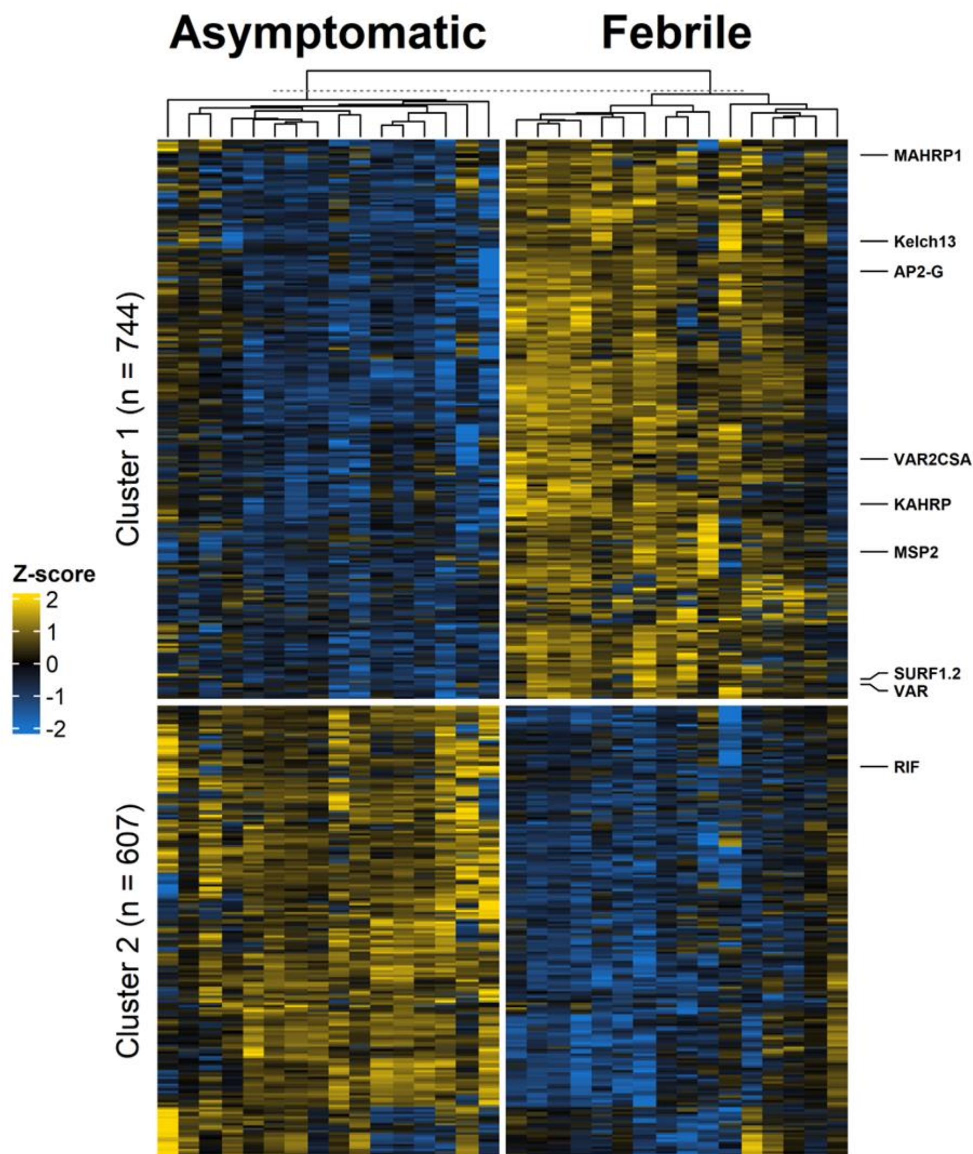

Figure 20: Heatmap visualization of the differentially expressed genes in the parasite transcriptome.

The 1351 differentially expressed genes (DEGs) are shown as rows while the asymptomatic and febrile malaria samples are the columns. K-means clustering was used to cluster the genes into two clusters as indicated on the y axis whereby 744 genes were upregulated in febrile infections and were clustered in Cluster 1. The remailing 607 genes were upregulated in asymptomatic infections and were clustered in Cluster 2.

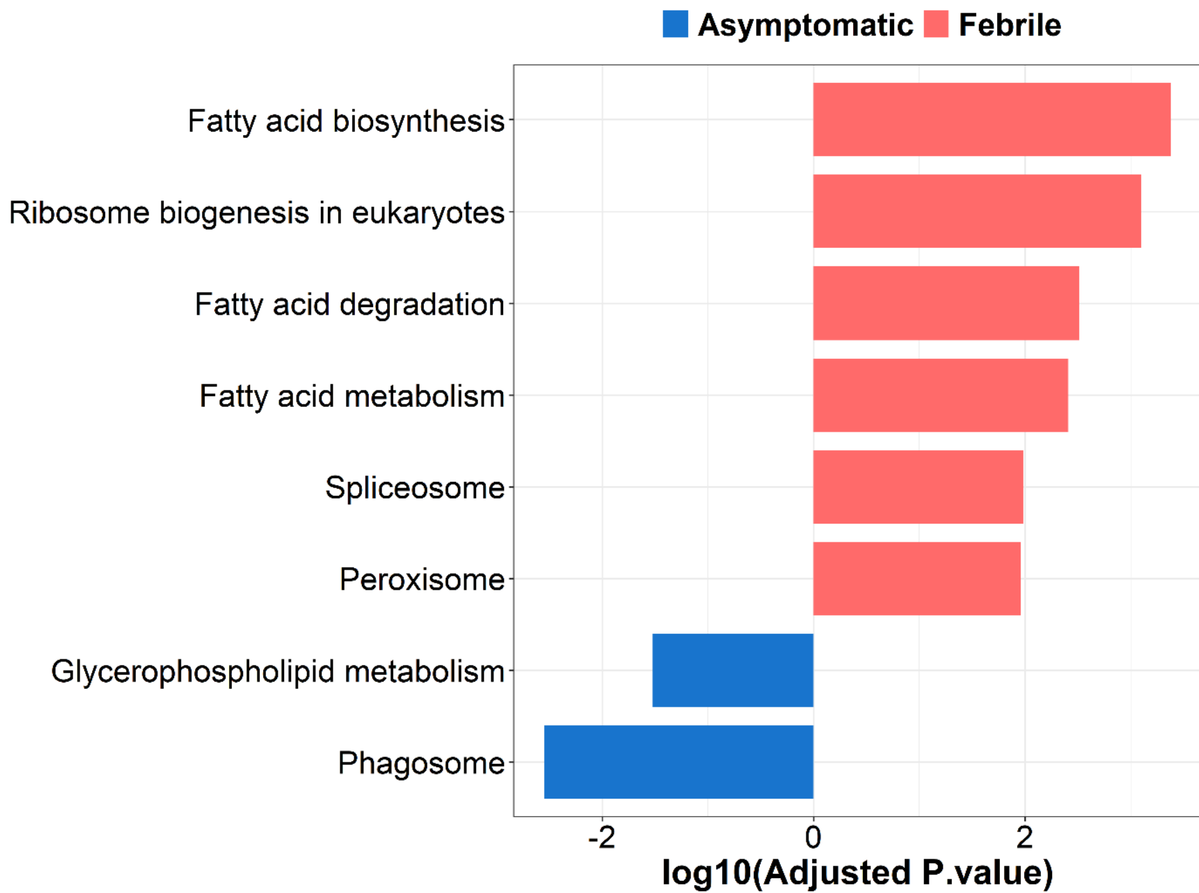

Figure 21: Functional analysis of parasite transcriptome.

Barplot visualization of the top enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Pathways enriched in asymptomatic infections are in blue while those enriched in febrile malaria infections are in red. Log10 (adjusted *P*. value) represents log10 of the Benjamini-Hochberg adjusted *p*-values.

4.4 Transcriptomic analysis of host PBMCs

4.4.1 Characteristics of study participants

A total of 49 PBMC samples from uninfected (n = 14), asymptomatic (n = 13) and febrile (n = 22) individuals were successfully sequenced. Of these, 20 were paired samples, i.e., 8 uninfected (U) to febrile (UF) and 12 AF paired samples. The other nine samples were unpaired and comprised of 6 uninfected, 1 asymptomatic and 2 febrile samples. The mean age for uninfected individuals (8.15 years) was not significantly different from asymptomatic individuals (7.36 years) ($P = 0.56$). There was no significant difference in parasitemia observed between the febrile infections ensuing from uninfected or asymptomatic infections ($P = 0.504$). On average, about 60 million reads mapped to each sample with a range of 11.5 – 374.4 million paired end reads. The raw data generated are available as indicated in **Appendix III.**

4.4.2 Differential expression analysis

Differential expression analysis revealed 4311 differentially expressed genes (DEGs) among the U, A and F treatment groups. Principal component analysis (PCA) using DEGs separated the samples into febrile and non-febrile groups on PC1, which is associated with 21.64% of the variation, while PC2 (7.52%) partially separated U from A and the febrile infections into those who were initially asymptomatic (FA) and uninfected (FU), suggesting a transcriptional distinction in the febrile infections (**Figure 22**). Gene clustering using DEGs identified four gene clusters 1 to 4 (c1-c4). Overall, c1 (1506 genes) was upregulated in non-febrile children (A and U groups), while c4 (1325 genes) was upregulated in the febrile children (FU and FA). There was a marked upregulation of c2 (552 genes) in the FA group and a contrasting pattern of expression in c3 with an upregulation of 928 genes in FU samples (**Figure 23**).

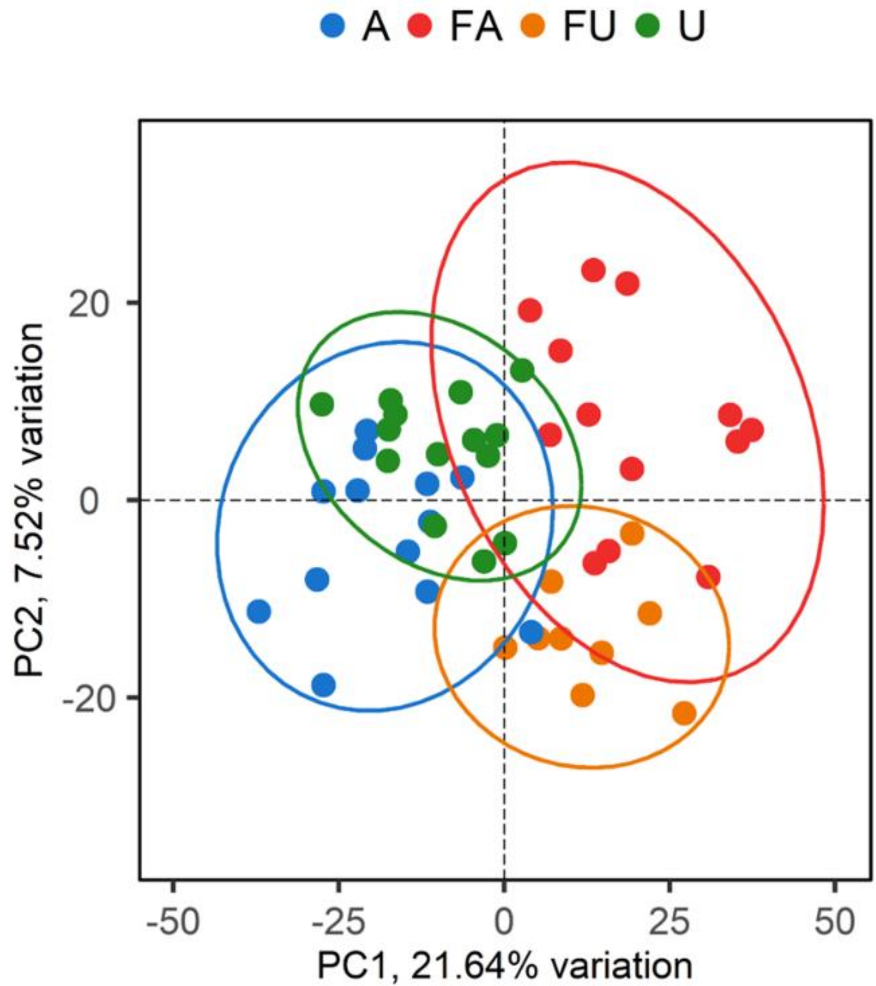

Figure 22: Principal component analysis of differentially expressed genes across the four clinical phenotypes.

Principal-component analysis of the 4,311 differentially expressed genes (DEGs) among the four clinical phenotypes. Ellipses represent 95% confidence intervals. Percentages along x and y axes show the degree of variance explained by each principal component. The samples are coloured by the clinical phenotype i.e., A = Asymptomatic, U = Uninfected, FU = Febrile malaria ensuing from uninfected individuals and FA = febrile malaria ensuing from asymptomatic individuals.

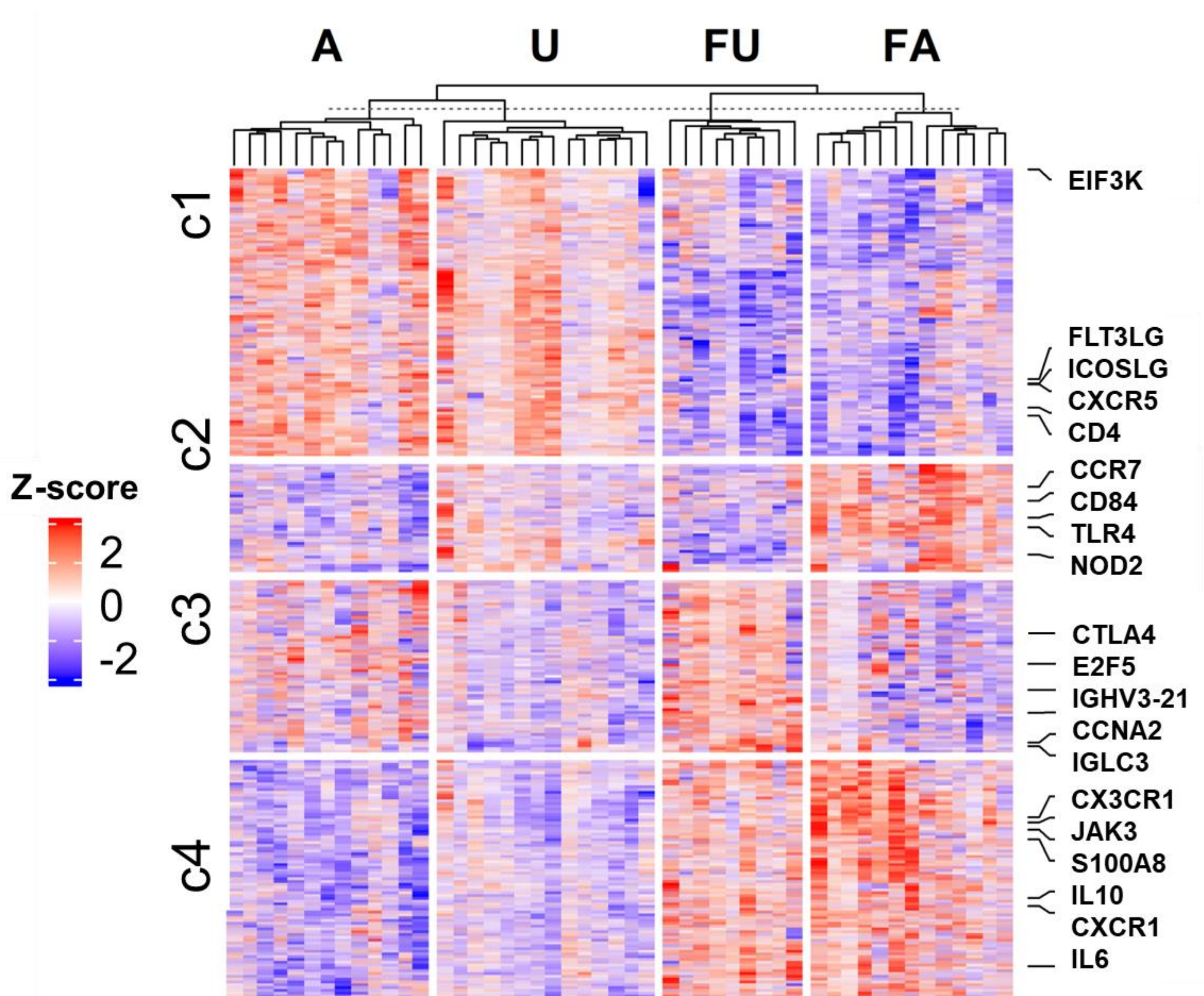

Figure 23: Host Peripheral Blood Mononuclear Cells (PBMCs) differential gene expression analysis.

Heatmap of the 4,311 DEGs (rows) and samples (columns). The samples have been split into four groups each corresponding to each clinical phenotype i.e., A for Asymptomatic, U for Uninfected, FA for febrile infections ensuing from U and FA for febrile infections ensuing from A. The genes were split into 4 clusters using K-means clustering and labelled c1 – c4.

Gene ontology analyses was used to study the genes enriched in each of the four clusters defined in **Figure 23**. GO terms for cytoplasmic translation, ribosomal activity, T cell activation and cell adhesion (**Figure 24A)** predominated in c1, including genes associated with protein translation factors (EIF3D, EIF3E, EIF3G and EIF3K) and ribosomal proteins (RPL18A, RPL19, RPS12, RPS16, RPL35, RPL8, RPS11, RPS27A) and genes associated with T cell activation and adhesion (CD3D, CD3E, CD3G, CD4, CD96, CD247, CD5, ZAP70, LCK, CXCR4, CXCR5, CD74 and TBX21, GATA3), Figures 6B and 6C. These data suggest that protein synthesis and T cell activation were down regulated in the febrile cases.

By contrast, c4 genes, were enriched for GO terms involving immune effector processes, adaptive and innate immunity, and cytokine pathways (TLR2, STAT3, IL6, IL6R, ANXA3, S100A11, CXCR1, C3AR1, LRG1, S100A8, S100A12, S100A9, JAK3) and regulation of immunity (CD274, CD276, IL1RN, IL10, CR1, CD55, IDO1, IL2RA, BST2) (**Figure 24D).** This data shows, as expected, that markers of immune activation, particularly neutrophil are upregulated in febrile infections (FA and FU).

Cluster 2 and 3 drives the separation of the febrile infections based on whether they were initially from asymptomatic or uninfected individuals. Cluster 2 was enriched for GO terms involving immune effector processes, leucocyte migration, host defense and TNF production (**Figure 24B**). The greatest differences in expression were in the FA group particularly in genes associated with innate immune effector processes (TLR4, CD14, CD68 TGFB1, EOMES, CCR7, CCL3R1, CD84, TNFRSF1B, IL1B, IFNGR1). There were also some differences between the A and U groups, a pairwise analysis of the two groups revealed an upregulation of several genes related to inflammatory responses (CCR7, CCL2, NOD2, SIRPA. TNFSF14, NFKB1) in the U group. The innate and inflammatory responses were strongly upregulated in FA compared to the FU group.

The GO terms enriched in c3 were nuclear division and chromosomal segregation, humoral responses, genes for pathways associated with cell cycle regulation (E2F5, CCNA2, CDC6, CDC14B, CCNB1, CDKN2C), and immunoglobulin, phagocytosis and complement (IGHM, IKC, IGHG4, IGHG2, C5) (**Figure 24C**). There was a clear distinction in gene expression of c3 genes between the FA and FU samples, where many genes were upregulated in the FU samples (**Figure 24B**), indicating a relatively stronger humoral response. Pairwise analysis of A and U groups revealed an upregulation of humoral responses (C1QB, IGHG4, IGKC, IGLC3, IGHV5-51, IGHV3-15, IGHV3-21) in the A group.

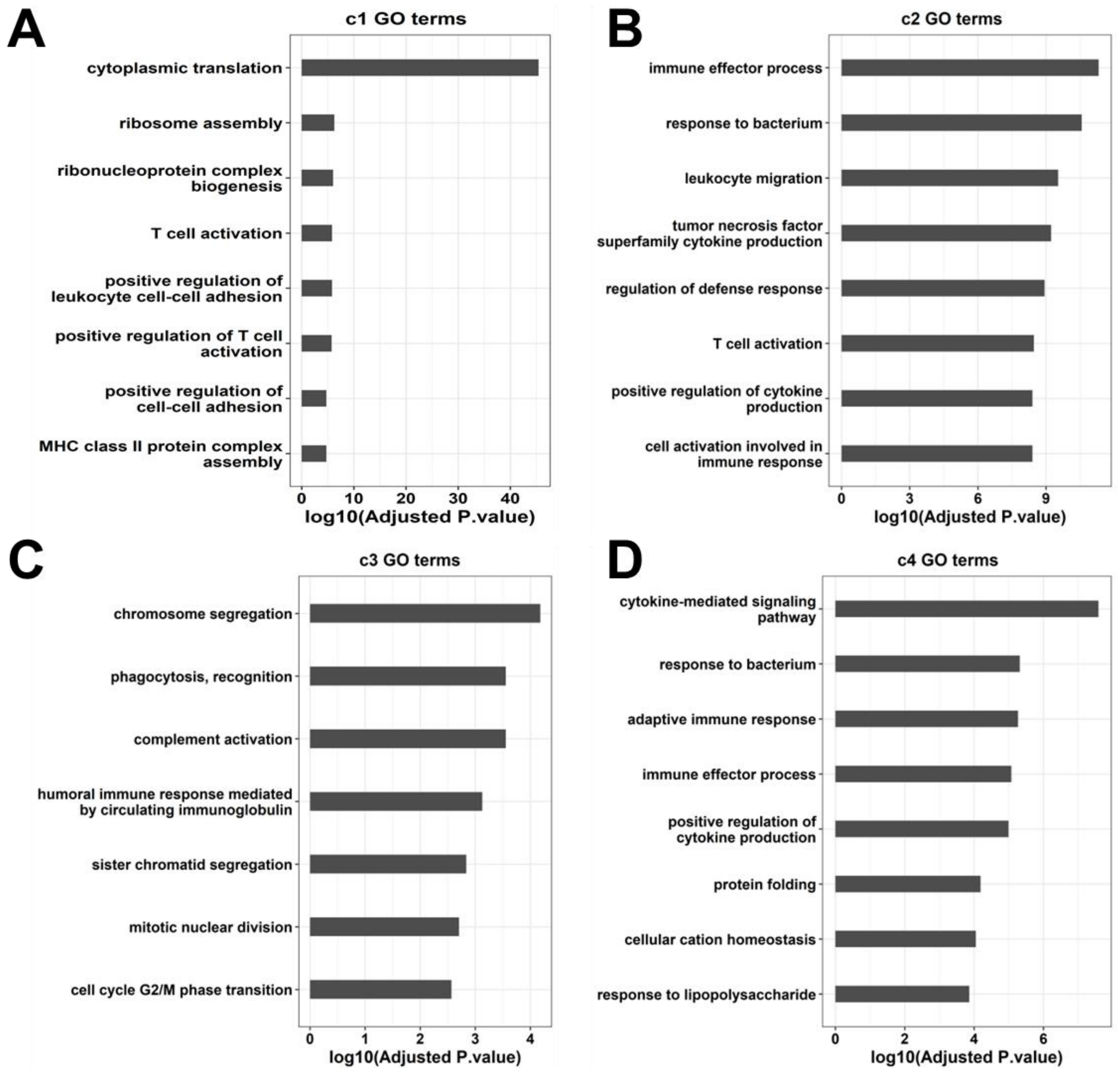

Figure 24: Functional analysis host Peripheral Blood Mononuclear Cells' (PBMCs) differentially expressed genes.

A, B, C and D) Barplots showing enriched Gene Ontology (GO) terms in clusters c1, c2, c3 and c4, respectively, from the heatmap in **Figure 23**.

The 10 most highly differentially expressed genes of selected ontology terms; humoral responses, cytokines, TNF and T-cell activation, in each group is shown in **Figure 25**, with a comparison of the normalization expression of a selection of relevant genes (**Figure 26**). Both the A and U samples show upregulated T-cell activation compared to the respective febrile samples. The FU samples had the strongest expression of immunoglobulin-related genes and humoral response whereas the FA samples had the greatest gene expression for cytokines and TNF production.

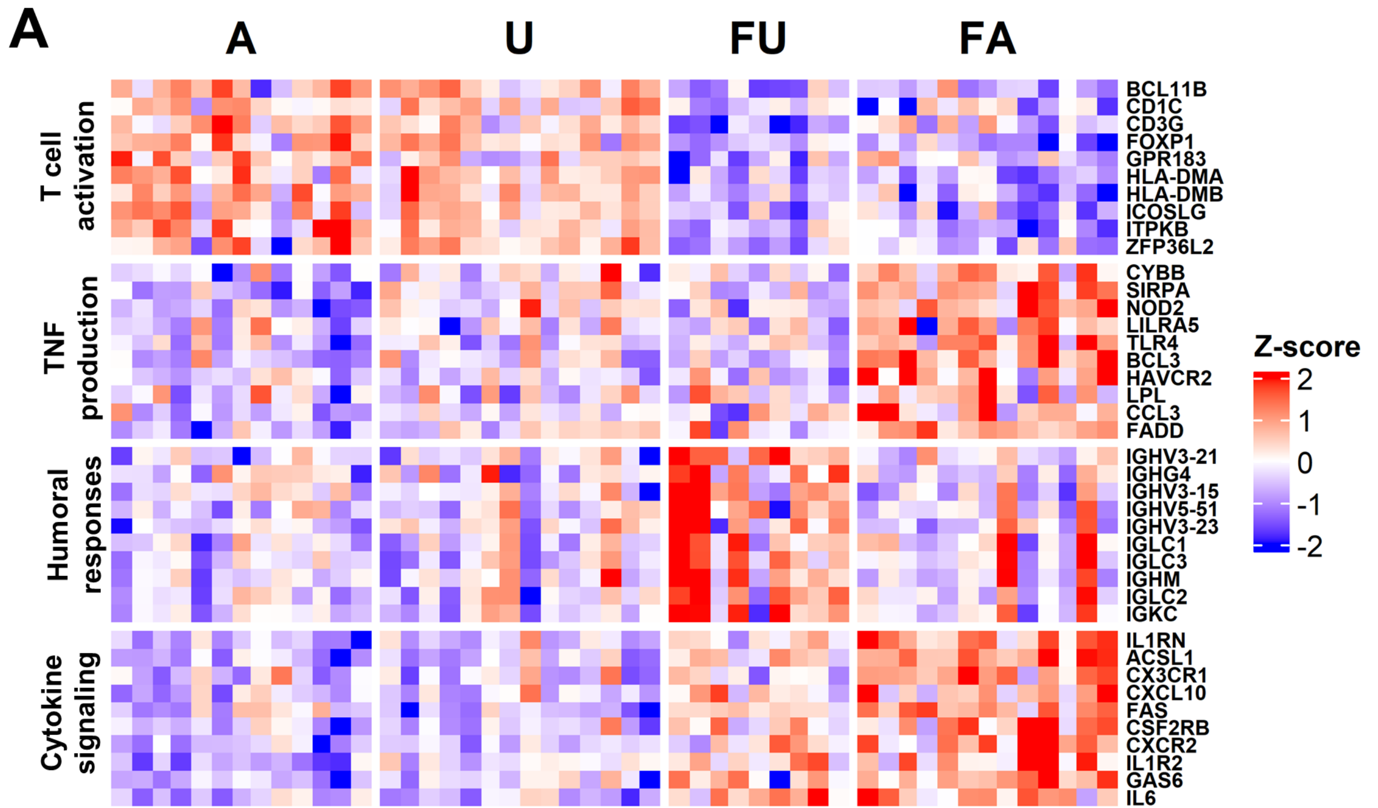

Figure 25: Analysis of selected Gene Ontology (GO) term

Heatmap showing the top 10 differentially expressed genes (DEGs) (based on FDR) enriched in humoral responses, cytokine signaling, TNF production and T cell activation GO terms. The genes (rows) are grouped by the clinical phenotype: A = Asymptomatic, U = Uninfected, FU = Febrile malaria ensuing from Uninfected, FA = Febrile malaria ensuing from Asymptomatic.

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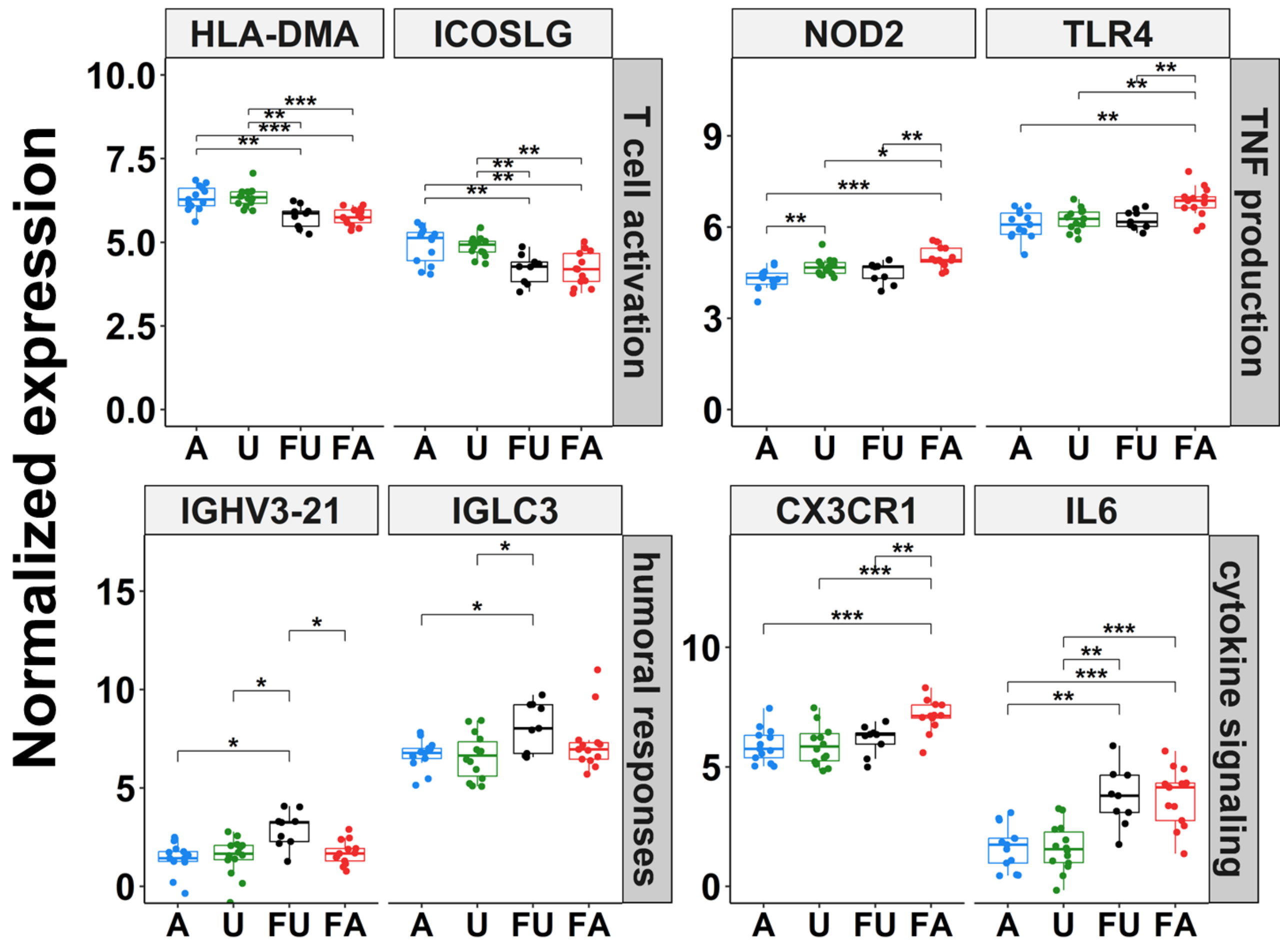

Figure 26: Analysis of expression levels of selected genes per Gene Ontology (GO) term

Boxplots showing the normalized expression levels of selected DEGs per GO term displayed in **Figure 24**. The boxplots are labelled based on the clinical phenotype: A = Asymptomatic, U = Uninfected, FU = Febrile malaria ensuing from U and FA = febrile malaria ensuing from A. Pairwise statistical analyses indicated in the plots are Wilcoxon tests corrected for multiple testing (Benjamini & Hochberg, *=< 0.05, **=<0.01 and ***=<0.001).

Deconvolution analysis revealed major differences in estimated proportions of PBMC cell among the four conditions (**Figure 27**). A lower proportion of naïve B cells was observed in AF while a higher proportion of plasma cells was observed in UF. The proportion of $CD8^{+}$ T cells was higher in U and A while that of naïve and activated memory $CD4^{+}$ T cells was higher in AF and UF. A higher proportion of activated mast cells was observed in AF (**Figure 28**).

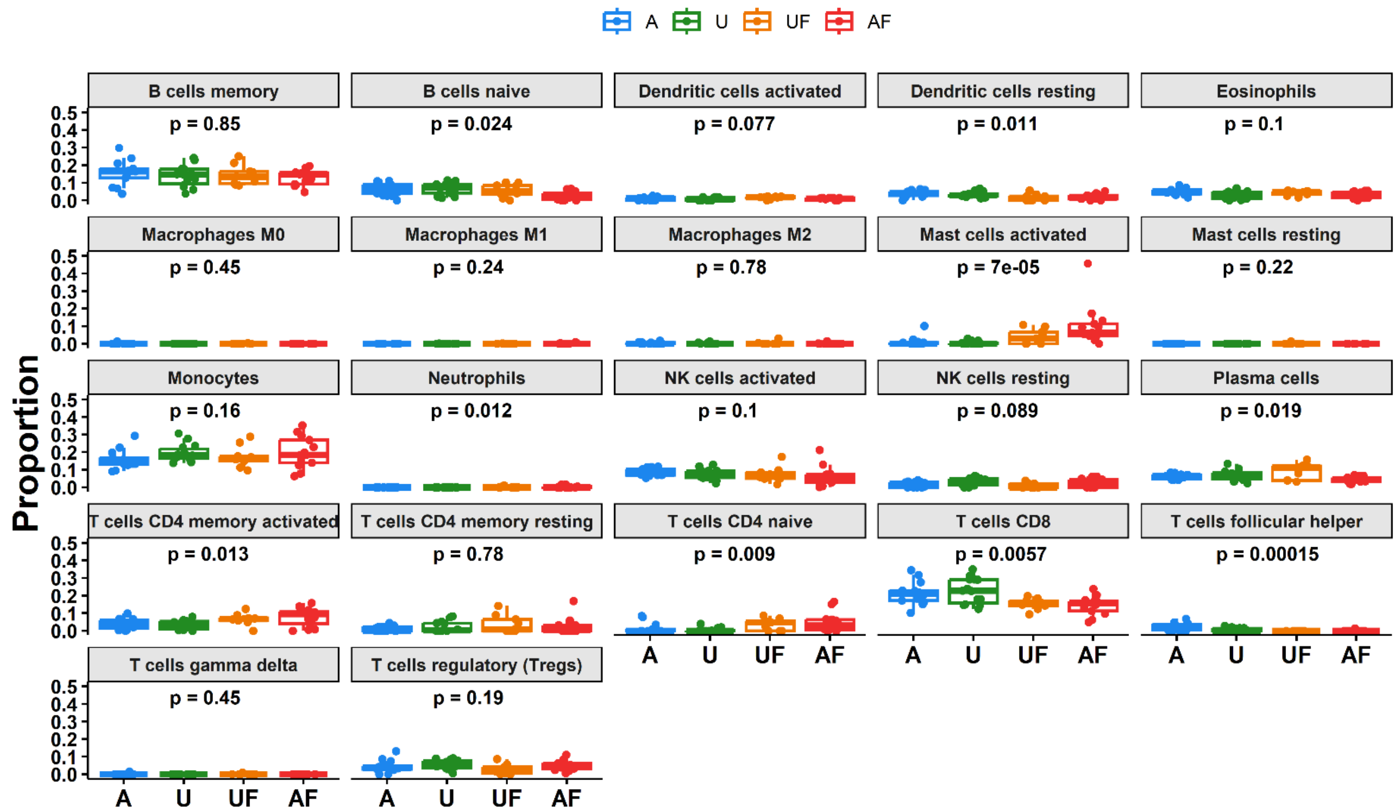

Figure 7: Deconvolution analysis of host Peripheral Blood Mononuclear Cells' (PBMCs)

PBMC subpopulations proportions were estimated for each sample using the deconvolution analysis in CIBERSORT and a gene signature matrix. Samples are colored based on the clinical phenotype: A = Asymptomatic, U = Uninfected, FU = Febrile malaria ensuing from uninfected individuals and FA = febrile malaria ensuing from asymptomatic individuals. A Kruskal-Wallis test was used to determine significant differences in the proportion of cell types across the four sample groups.

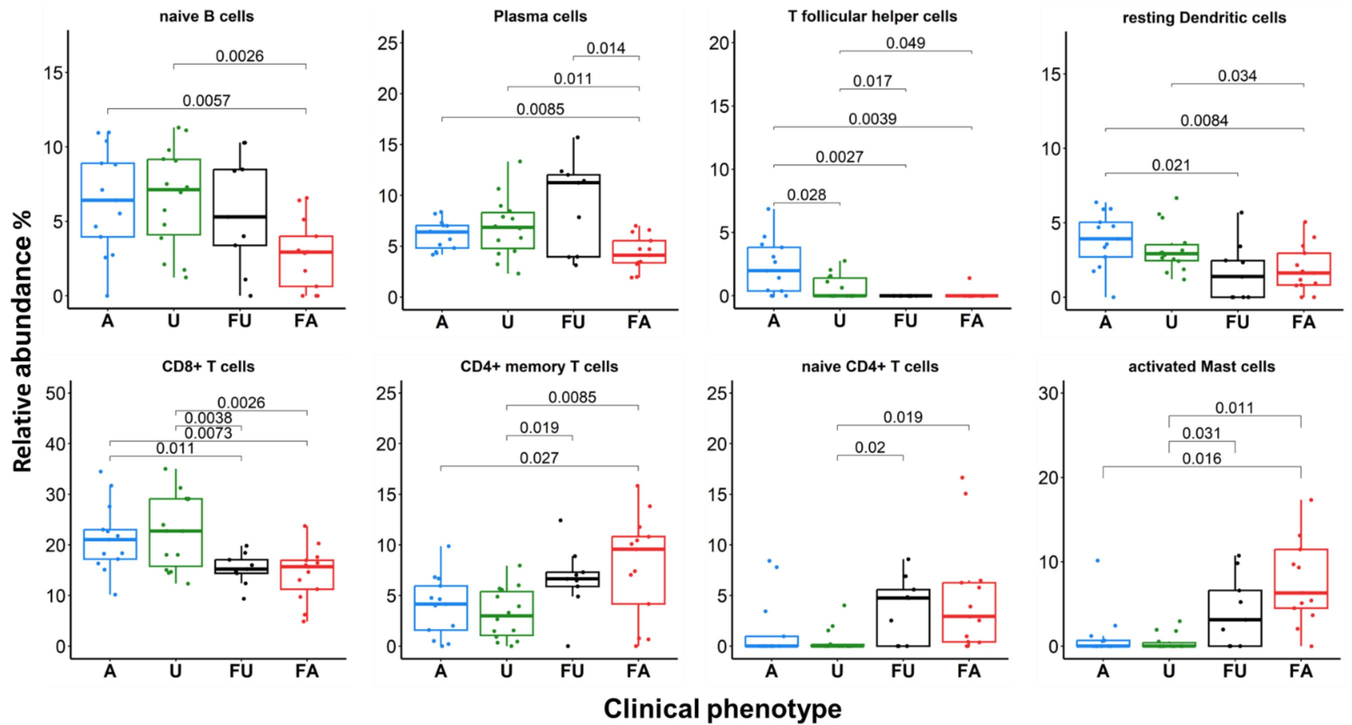

Figure 27: Deconvolution analysis of specific Peripheral Blood Mononuclear Cells' (PBMCs) cell types.

PBMC subpopulations proportions were estimated for each sample using the deconvolution analysis in CIBERSORT and a gene signature matrix. Samples are coloured based on the clinical phenotype: A = Asymptomatic, U = Uninfected, FU = Febrile malaria ensuing from uninfected individuals and FA = febrile malaria ensuing from asymptomatic individuals. All pairwise statistical tests indicated in the plots are Wilcoxon tests corrected for multiple testing (Benjamini & Hochberg).

4.5 Proteomics analysis of host PBMCs

4.5.1 Effect of UREA concentration on total protein concentration

The effect of UREA on the protein concentrations was studied determining the effect of varying UREA concentrations on protein concentration. The protein concentration was determined using Bicinchoninic acid (BCA) protein assay. Varying UREA concentration were shown not to have no effect on the protein concentration (**Figure 28**).

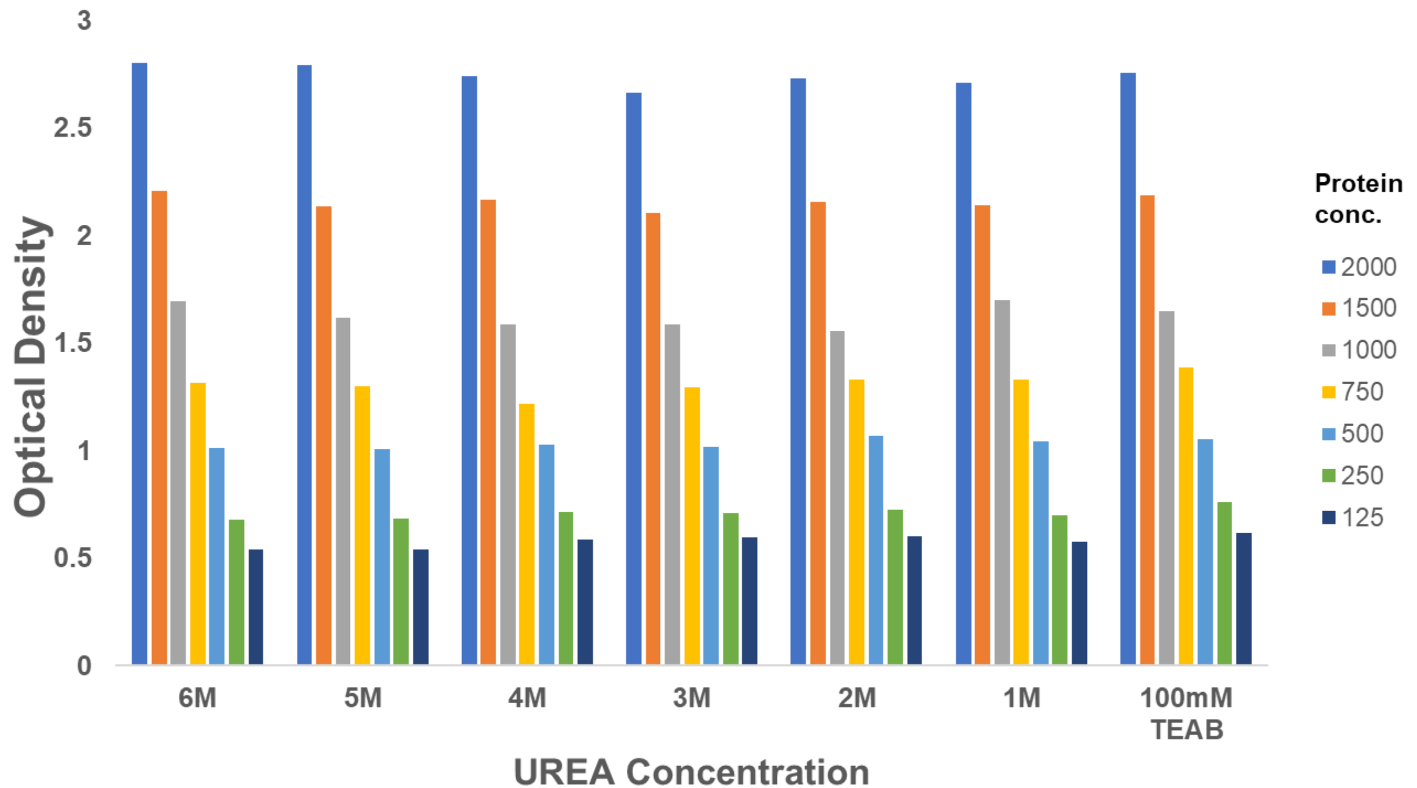

Figure 28: Effect of UREA concentration on protein concentration

The protein concentration as determined using BCA (Bicinchoninic acid assay). Triethyammonium bicarbonate (TEAB) was the diluent used to prepare the different concentrations. Protein conc. – protein concentration.

4.5.2 Analysis of the PBMC proteome

To identify proteomic signatures in uninfected, asymptomatic and their corresponding febrile infections, isobaric labelling-based quantitative proteomics of 16 asymptomatic to febrile pairs (A - FA) and 22 uninfected to febrile (U - FU) pairs was performed. The raw data generated are available as indicated in **Appendix IV**. A total of 328 proteins identified (q-value < 0.1) after filtering out reverse and contaminant proteins. Differential protein expression analyses of the four conditions identified 35 proteins **(Table 8)** that clustered into two groups i.e., febrile (FA and FU) and Non-febrile (A and U) (**Figure 29A**). Protein-protein interactions (PPI) analysis in STRING database revealed significant enrichment of proteins in either febrile or non-febrile groups (PPI enrichment P value <0.001) (**Figure 29B**). Consequently, 16 proteins were upregulated in the non-febrile samples and were enriched for protein-DNA complex and nucleosome (HIST1H1E, HIST1H2BC, H2AFJ, HIST1H4A) cellular components (**Table 9**). The remainder were upregulated in febrile infections and were enriched for chemokine production (MPO, HP, S100A8, MMP9, ANXA1, DEFA1) and neutrophil aggregation and chemotaxis (S100A8, S100A9, S100A12) and chronic inflammatory responses (CAMP, S100A8) biological processes (**Table 9**). Though, a low number of proteins were detected, it indicates chromatin changes in asymptomatic and uninfected individuals, and innate immune responses in febrile infections.

Table 8: Differentially expressed proteins identified PBMCs from uninfected and asymptomatic samples and ensuing febrile malaria.

Protein names	Protein ids	Gene names	Unique peptides (%)	Coverage (%)	FDR
Myosin-9	P35579	MYH9	98.8	42	0
Transketolase	B3KSI4	HEL107	92.9	29.6	0
Heterogeneous nuclear ribonucleoproteins A2	A0A024RA28	HNRPA2B1	100	23.9	0
Histone H2A	A3KPC7	HIST1H2AH	33.3	35.9	0
Haptoglobin	Q6NSB4	HP	100	18.5	0
Nucleophosmin	Q9NX34	NPM1	100	29.6	0
Cathelicidin antimicrobial peptide	J3KNB4	CAMP	100	17.9	0
Annexin A1	P04083	ANXA1	91.7	39.6	0
Histone H1.4	Q4VB24	HIST1H1E	14.3	54.3	0
Lymphocyte-specific protein 1	A8K2L4	LSP1	100	16.5	0
Lysozyme	B2R4C5	LYZ	100	56.1	0
Histone H4	B2R4R0	HIST1H4H	100	64.1	0
Histone H2B	B2R4S9	HIST1H2BI	8.3	72.2	0
Nucleolin	Q9BQ02	NCL	100	12.7	0
Lactotransferrin	B3VMW0	LTF	93.5	47	0
Actin_like	B4DUI8	-	16.7	29.2	0
Matrix metalloproteinase-9	B7Z507	MMP9	100	7.4	0
Protein-tyrosine kinase 2-beta	C9JHV9	PTK2B	100	4.2	0.007481
14-3-3 protein zeta/delta	D0PNI1	YWHAZ	77.8	32.7	0
C-X-C motif chemokine	D3JV41	PPBP	100	42.9	0
Anion exchange protein	G4V2I9	SLC4A1	100	9.5	0
Myosin light polypeptide 6	F8W1R7	MYL6	100	55.2	0
DnaJ homolog subfamily C member 13	O75165	DNAJC13	100	1.1	0.009828
Fibrinogen beta chain	V9HVY1	HEL-S-78p	84.8	62.1	0
Platelet factor 4	P02776	PF4	100	36.6	0
Protein S100-A8	P05109	S100A8	100	60.2	0
Myeloperoxidase	P05164	MPO	83.3	44.2	0
Protein S100-A9	P06702	S100A9	92.9	82.5	0
L-lactate dehydrogenase B chain	P07195	LDHB	85.7	22.8	0
Cofilin-1	V9HWI5	HEL-S-15	100	67.5	0
Neutrophil defensin 3	Q6EZE9	DEFA3	100	26.6	0
Actin, cytoplasmic 1	Q1KLZ0	PS1TP5BP1	2.6	79.7	0
Protein S100-A12	P80511	S100A12	100	43.5	0
Neuroblast differentiation-associated protein AHNAK	Q09666	AHNAK	100	6.4	0
Resistin	Q9HD89	RETN	100	26.9	0

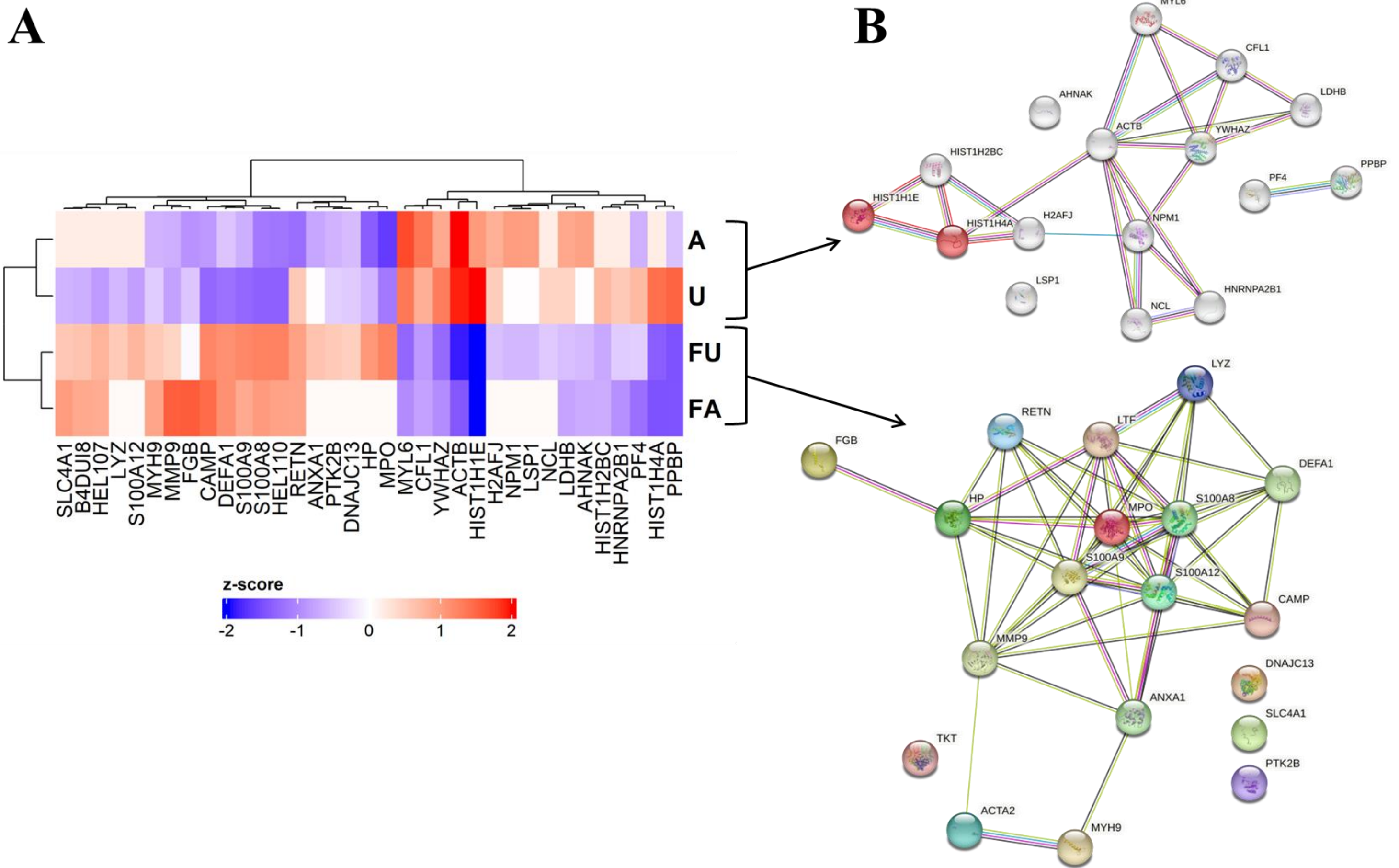

Figure 29: Differential expression of proteins among the paired asymptomatic to febrile and uninfected to febrile samples.

A) Heat map of the 35 significantly differentially expressed protein in the clinical phenotypes identified using isobaric labeling-based on quantitative proteomics. The columns represent the proteins and the rows the clinical phenotypes, A (Asymptomatic), U (Uninfected) and F (Febrile from Asymptomatic [FA] and Uninfected [FU]). **B)** Protein-protein interaction network analysis using STRING database. Proteins are shown as nodes, and the color of each link is based on the type of evidence available for the interaction between two proteins (e.g., purple- experimental, light blue – homology, blue – co-occcurence, black – coexpression, purple – experimental, green – text mining).

Table 9: Gene ontology (GO) analysis of differentially expressed proteins.

	GO term	Strength	FDR
Non-febrile (A & U)	protein-DNA complex	1.5	0.0001
	Nucleosome	1.66	0.00035
	Focal Adhesion	1.18	0.0024
	Actin cytoskeleton	1.11	0.0044
	Host intracellular part	1.26	0.0493
Febrile (FU & FA)	Sequestration of zinc ion	2.71	0.0026
	Chemokine production	2.31	0.0096
	Neutrophil aggregation	3.01	0.011
	Chronic inflammatory responses	2.23	0.0119
	Leukocyte migration involved in inflammatory response	2.06	0.0192

Strength is a measure of the enrichment effect while FDR is the adjusted *P*-value after correcting for multiple testing using the Benjamini-Hochberg.

Chapter 5: DISCUSSION

The longitudinal Junju cohort provided a unique opportunity to identify "true" asymptomatic *P. falciparum* infections and follow them up until they developed symptomatic malaria. The presence of follow-up samples helped to control for inter-individual differences, enhancing our ability to detect intra-individual differences i.e., infection driven differences between the different conditions. In addition, the cohort provided data for evaluating malaria positivity in the Junju area which has previously been classified as a region of moderate to high malaria transmission (Mwangi *et al.*, 2005). Analysis of febrile and asymptomatic *P. falciparum* cases between 2007 and 2019 showed that malaria transmission in this region is perennial, as it occurs throughout the year without great difference in intensity (World Health Organization, 2021). This contrasts with other areas of varying malaria transmission in Africa which experiences seasonal malaria transmission characterized by few or no malaria cases during dry season and increased malaria episodes during rainy season (Portugal *et al.*, 2017). Analysis of malaria positivity in this cohort using 810 microscopy positive samples processed during the cross-sectional bleed for the 12-year period between 2007 and 2018 showed that there was a notable decrease in malaria positivity (16.2% – 5.5.%; $P < 0.001$). This reduction was most likely attributed to the intensification of malaria control measures as reported in other parts of the country (Okech *et al.*, 2008; Dulacha *et al.*, 2022). The dip in malaria transmission in 2012 could not be ascertained although there may have been improper data collection or other unknown reasons.

Despite the observed reduction in malaria transmission in Junju for over 12 years, genotyping *P. falciparum* parasites showed that malaria is still characterized by genetically diverse infections with high complexity. The decline has not been significant enough to change the parasite genetic landscape as demonstrated by stability of *msp2* alleles. In the larger study area, Kilifi County, a major decline in malaria hospital-admissions was shown between 2002 upto 2009. There was also an increase in the mean age of hospital admissions due to malaria potentially caused by the slow acquisition of acquired immunity to malaria due to reduction in transmission intensity (Mogeni *et al.*, 2016). A more recent study looked at the admission of severe malaria cases in Kilifi County Hospital between 1989 and 2016 and showed an overall reduction in the number of cases (Njuguna *et al.*, 2019). There is a possibility that a genetically diverse malaria parasite population was preserved by the sustained transmission in the county notwithstanding the local decline in Junju area. This hypothesis agrees with findings of serological surveys, at a household level, that

revealed evidence of diverse parasite populations in homesteads at low malaria risk while the surrounding area was at high malaria transmission and vice versa (Bejon *et al.*, 2011).

Contrastingly, a dramatic reduction in malaria transmission as observed in Grande Comore Island, Union of Comoros, from 108,260 cases in 2006 to 1072 in 2015, was followed by a commensurable decline in MOI based on *msp2* genotyping from 2.75 to 1.35 in healthcare facility samples obtained from 2006/2007 and 2013-2016 (Huang *et al.*, 2018). Furthermore, there was a significant reduction in *msp2* alleles between the two time-points (Huang *et al.*, 2018). Altogether the *msp2* genetic profile corresponded to the decline in malaria transmission. Likewise, enhancement of malaria control efforts in Senegal between 2006 and 2011 caused a reduction in genetic diversity of parasite populations (Daniels *et al.*, 2013). However, reduction in malaria transmission in the Kingdom of Eswatini did not result into low parasite genetic diversity mainly due to malaria importation from neighbouring countries, particularly Mozambique and South Africa (Roh *et al.*, 2019). These contrasting reports on the impact of reducing malaria transmission intensity on the parasite genetic diversity implies that inference of malaria transmission intensity from parasite genetic data ought to consider the external factors, like malaria importation, that influence parasite population genetics.

The evident preference for the FC27 alleles was a key feature of febrile malaria infections in the asymptomatic-febrile paired analysis. This observation has previously been made in Congo and Tanzania whereby FC27 alleles were associated with severity of disease and were more predominant in children who had more than two febrile malaria episodes (Ibara-Okabande *et al.*, 2012; W Kidima and Nkwengulila, 2015). Similarly, in a case control study done in Papua New-Guinea, FC27 genotypes were twice as likely to be found in symptomatic than asymptomatic malaria individuals (Engelbrecht *et al.*, 1995). Thus, FC27 allelic family is potentially an important set of genetic variation to interrogate further to determine their impact on immunity.On the contrary, IC/3D7 family has been associated with asymptomatic *P. falciparum* carriers and is thought to protect against clinical malaria (W Kidima and Nkwengulila, 2015; Botwe *et al.*, 2017; Abukari, Okonu, Samuel B Nyarko, *et al.*, 2019). However, contradicting findings have been reported showing that parasites harboring FC27-like alleles are more prevalent among asymptomatic carriers (Amodu *et al.*, 2008; Gnagne *et al.*, 2019). There is no clear consensus on whether the two *msp2* allelic families are likely to be found in asymptomatic or symptomatic infections. Larger studies in regions with different transmission intensities are needed to gain more

insights into the effect of each allelic family on clinical outcome.

The high COI and large proportion of polyclonal asymptomatic infections is a result of the frequent and repeated exposure to genetically distinct malaria parasites in endemic areas, as described in previous studies (Touray *et al.*, 2020). This leads to the development of partial immunity that results in a reduction in clinical symptoms and carriage of low-level parasitemia (Smith *et al.*, 1999; Bereczky *et al.*, 2007). The paired samples showed a major change in *msp2* alleles between asymptomatic infections and febrile malaria infections, which is expected owing to the ongoing malaria transmission in Junju. In this study, the febrile infections were characterized by more monoclonal infections, an overall lower COI and new alleles unobserved in the prior asymptomatic infection. The new alleles likely escape immune responses, rapidly increasing parasitemia thereby causing massive tissue damage that manifests as symptoms. Similar findings have been reported in other studies, implicating the lack of protective immune responses against the new alleles (Contamin *et al.*, 1996; Roper *et al.*, 1998; Kun *et al.*, 2002; Nsobya *et al.*, 2004; Wamae *et al.*, 2022).

The high *msp2* genetic diversity maintained across the study period was expected as Kilifi is a region of moderate to high malaria transmission. The 291bp, 327bp and 411bp FC27 and 555bp IC/3D7 fragment sizes were common in both asymptomatic and febrile infections. Strikingly, some of these genotypes have been reported in other countries such as Mali (Sondén *et al.*, 2015) as the most common genotypes, suggesting that they can be selected as candidates for malaria vaccine development. However, identical fragment lengths may not always represent identical sequence lengths and sequencing is the only way to confirm. Sequencing *msp2* gene has been a challenge due to the presence of insertions, deletions and tandem repeats that complicates sequencing using short-read sequencing and mapping to the reference during assembly (Ferreira and Hartl, 2007). The recently developed *msp2* sequencing protocol that incorporates the use Pacific Biosystem's long-read circular consensus sequencing (CCS) promises to provide more insights into sequence variation in *msp2* isolates from diverse clinical malaria outcomes (Plaza *et al.*, 2022).

Parasite factors that contribute to the observed heterogeneity in malaria clinical manifestations are still not well understood. Febrile infections have been shown to harbor new circulating parasite clones, unfamiliar to the host immune system, that are unique from the preceding asymptomatic infection (Kun *et al.*, 2002; Buchwald *et al.*, 2019; Wamae *et al.*, 2022). This is not unusual in

moderate to high transmission settings, where infection rates are high and on average it was observed there were more than four months (138 days) between asymptomatic and the ensuing febrile infection. In our study, febrile infections featured a significant increase in parasitemia, a larger proportion of ring-stage parasites and a smaller proportion of non-ring parasites compared to asymptomatic infections. An excess of ring stage parasites was also observed among febrile infections compared to parasites from asymptomatic infections in Mali (Andrade *et al.*, 2020). A similar profile was observed among parasites from severe malaria and cerebral malaria cases compared to those from non-severe malaria which exhibited a higher proportions of trophozoites (Tonkin-Hill *et al.*, 2018; Guillochon *et al.*, 2022). The reason for this occurrence has been attributed to differential cytoadhering capacity that potentially dictates the severity of malaria infection outcomes (Thomson-Luque *et al.*, 2021). Increased cytoadherence by shorter circulating parasites may result in higher parasitemia and increased malaria severity compared to longer circulating parasites (Andrade *et al.*, 2020). It is not entirely understood how parasites change their cytoadherence capacity although host antibodies against variant surface antigens (VSAs) have been shown to select against parasites with high binding ability (Warimwe *et al.*, 2009) hence inadvertently selecting for parasites with low binding ability in semi-immune asymptomatic carriers. Studies of differential expression of VSAs between different malaria presentations using transcriptome data have not been informative mainly due to their highly polymorphic nature. Future studies should aim to use longitudinal samples to characterize this antigens by amplifying their conserved regions (Andisi and Abdi, 2022).

Body temperature has also been shown to affect cytoadherence of infected RBCs (Singhaboot *et al.*, 2019) and since asymptomatic infections are not accompanied by hyperthermia as seen in febrile malaria, this could result in reduced cytoadherence. Another important contributing factor to the reduced cytoadherence in asymptomatic infections could be the host response. How the parasite senses its environment and effects these changes is still not well understood. Transcriptomic analysis of PBMCs from these asymptomatic children revealed that some inflammatory responses such as cytokine signaling and tumor necrosis factor (TNF) production, which are known to play a role in upregulation of adhesion molecules such as the intercellular adhesion molecule-1 (ICAM1) (Hubbard and Rothlein, 2000) and other cell surface ligands recognized by VSA proteins (Tuikue Ndam *et al.*, 2017), are lower than in febrile cases.

In addition to the differences in the proportions of parasite stages, unique pathways were revealed

in asymptomatic infections as compared to febrile infections. The metabolic pathways glycerolphospholipid metabolism and fatty acid biosynthesis were upregulated and downregulated in asymptomatic infections, respectively. A similar expression pattern of these pathways in asymptomatic and febrile malaria infections was also reported during seasonal malaria transmission in Mali (Andrade *et al.*, 2020). This alludes to suppression of central-carbon metabolism pathways during asymptomatic infection and aligns with previous findings which revealed that *P. falciparum* parasites adapt to amino acid starvation by suppressing the central-carbon metabolism intermediates (Babbitt *et al.*, 2012). The spliceosome pathway that was upregulated among febrile infections was also previously associated with higher parasitemia in severe malaria (Milner *et al.*, 2012; Lee *et al.*, 2018; Tonkin-Hill *et al.*, 2018), while the phagosome pathway revealed in asymptomatic infections was also upregulated among asymptomatic infections (Andrade *et al.*, 2020). This overlap in pathways points to a common transcriptional signature by parasites during asymptomatic infections that enables persistence at low parasitemia to overcome selection pressure from host immunity. However, this necessitates further research.

Altogether, this evidence suggests that parasites can sense the host environment and respond to present selection forces to increase their chances of survival. While the changes in mosquito transmission were cited as the main selection force in areas of seasonal transmission (Andrade *et al.*, 2020). In an area of perennial malaria transmission like Kilifi, though a reduction in mosquito biting cannot be ruled out, immune pressure that may select for less virulent var genes, seems to be responsible for the reduction in cytoadhering capacity of the parasites during chronic asymptomatic infections. However, upon infection with new parasite clones unfamiliar to the immune system, iRBCs bind to the endothelial cells and escape clearance by the spleen leading to increased parasitemia and malaria severity. How the parasite senses its environment and effects these changes is still not well understood although epigenetic regulation of variant surface antigen expression has been implicated.

To investigate the changes in immune responses in asymptomatic and uninfected individuals and their subsequent symptomatic malaria infections, we studied their blood transcriptomic and proteomic profiles. Transcriptomic analysis of asymptomatic versus uninfected samples collected during the cross-sectional surveys were broadly similar. However, asymptomatic samples showed the downregulation of pathways related inflammatory responses. They also displayed greater

expression of some genes associated with the humoral response, perhaps indicating that persistent infection may stimulate greater antibody responses. It is also possible that the immune response has been modified to tolerate the continued parasite presence. Comparison of asymptomatic and healthy individuals in Papua, Indonesia also revealed evidence of anti-inflammatory responses during asymptomatic infections (Studniberg *et al.*, 2022). Acquired immunity in asymptomatic individuals has been associated with increased production of anti-inflammatory cytokines (Jagannathan, Eccles-James, *et al.*, 2014; Jagannathan, Kim, *et al.*, 2014). In addition, gene pathways involved in the production of pro-inflammatory cytokines were least activated in asymptomatic individuals compared to febrile malaria (Tran *et al.*, 2016). Probably due to increased expression of CTLA-4, an immune regulator, by memory $CD4^+$ T cells as previously shown in mouse models (Studniberg *et al.*, 2022). Alternatively, it could be due to chromatin remodeling leading to down-regulation of gene expression in immune cells (Schultze, 2017) ultimately sustaining asymptomatic infections (Boldt *et al.*, 2019). On the contrary, chromatin remodeling among monocytes from the Fulani ethnic group was previously associated with an inflammatory gene signature that enhances their ability to resist infection and clear the parasites (Sanou *et al.*, 2017).

Follow-up febrile infections among both asymptomatic and uninfected were characterized by upregulation of pathways related pro-inflammatory responses to fight the new infection. These results agree with previous studies that compared pre-infection and early febrile malaria infection and revealed marked activation of pro-inflammatory responses pathways (Ockenhouse *et al.*, 2006; Tran *et al.*, 2019; Studniberg *et al.*, 2022). We also compared the febrile infections ensuing from asymptomatic and uninfected individuals. Interestingly, greater enrichment of genes involved in innate immune responses was observed in febrile malaria ensuing from uninfected individuals. Superinfection during asymptomatic infections, due to new *P. falciparum* parasite sub-populations (clones) that are unfamiliar to the host immune system as confirmed by genotyping of parasites in our cohort (Kimenyi *et al.*, 2022; Wamae *et al.*, 2022), may expose the host to parasite components unfamiliar to the immune system thus stimulating more of a "primary" inflammatory-type response that causes symptomatic malaria. In contrast, febrile infections in previously uninfected individuals were characterized by a greater upregulation of humoral immune responses. The increase in transcripts associated with humoral immunity could be due to the higher proportions of plasma cells seen in the deconvolution analysis rather than increased transcription on a per-cell

basis. Single cell gene transcriptional analysis may be required clarify this. Previously, strong inflammatory responses have been shown to impair the ability of T follicular helper (Tfh) cells to activate B cells to produce antibodies in the germinal centers via increased expression of the T helper 1 defining transcription factor T-bet in the T_{FH} cells and germinal center B cells (Obeng-Adjei *et al.*, 2015; Ryg-Cornejo *et al.*, 2016; Soon, Nalubega and Boyle, 2021). Thus, enrichment of humoral responses in febrile infections ensuing from uninfected individuals may have been facilitated by a reduced inflammatory environment compared to febrile infections ensuing from asymptomatic infections. All in all, it is still unclear why febrile infections ensuing from uninfected individuals showed an increased upregulation of gene expression characteristic of B cells and humoral responses. However, plasma antibody responses were not measured, which might reflect more accurately the humoral responses. As suggested by the transcriptome data, it is possible that immunoglobulin M (IgM) antibodies producing B-cells characteristic of a more primary response, are elevated in the uninfected group, suggesting a less well-developed state of acquired immunity. Follow-up studies, incorporating parasite levels, cellular studies and antibody responses, to determine whether the differences in host responses have a detrimental or positive effect on the ability to control a superinfection and pathology would be required.

Proteomics analysis of the uninfected and asymptomatic samples and their ensuing febrile samples revealed only 328 proteins out of which 35 were differentially expressed and only allowed the separation of febrile and non-febrile samples. It was not clear what caused the reduced number of proteins although the use of UREA for PBMCs sample processing could be implicated. Although UREA helps in protein solubilization and denaturation when lysing cells, it can also cause carbamylation, binding of cyanates to free amino groups, of peptides. This reaction negatively affects protein digestion and reduces protein detection (Luebker, Wojtkiewicz and Koepsell, 2015). Since proteins are the end-products of gene expression, we could not correlate transcript levels with their corresponding protein levels. Studies have reported that this correlation is usually poor mainly due to post-translational modifications (Pascal *et al.*, 2008; Edfors *et al.*, 2016). However, a study on ovarian cancer shown that differentially expressed transcripts correlated significantly better with their corresponding proteins products compared to non-differentially expressed transcripts (Koussounadis *et al.*, 2015). Future studies planning to use UREA for cell lysis should consider lowing the temperature during protein extraction to minimize carbamylation and ultimately improve protein detection (Luebker and Koepsell, 2016; Betancourt *et al.*, 2018).

The study had several key limitations. First, since participants were only sampled once during the cross-sectional bleed and next when they became febrile/symptomatic, it was impossible to detect asymptomatic parasitemia between the two timepoints. Therefore, the febrile malaria episodes sampled may not necessarily be the first febrile episode after the cross-sectional bleed surveys as assumed in the study. Second, sub-patent malaria infections that could only be detected by PCR may have been missed among the uninfected/healthy individuals as the study relied on RDT and microscopy. Finally, single point sampling was done making it difficult to determine the course and nature of humoral responses and parasitemia.

Chapter 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Asymptomatic *P. falciparum* infections in Junju, Kilifi County, polyclonal and highly diverse thus warranting the attention of malaria control interventions like the attention given to symptomatic malaria cases.

Asymptomatic carriers are associated with the downregulation of inflammatory responses. In contrast, their ensuing febrile infection were observed to elicit increased inflammatory responses compared to febrile infections ensuing from uninfected individuals. The clear segregation of febrile outcomes based on whether the preceding state was uninfected or asymptomatic provides further evidence that the presence of parasitemia in asymptomatic individuals modifies the host, offering an immunological advantage to the children. However, impaired antibody responses among asymptomatic individuals upon superinfection align with the reduced malaria vaccine efficacy observed among malaria exposed individuals' residing in endemic regions.

Overall, the parasites' ability to maintain asymptomatic infections is touted as one of its best assets for overcoming the numerous selective forces encountered in the host. Therefore, to achieve malaria elimination, malaria control campaigns should aim to eliminate all parasites, including those in asymptomatic individuals because they not only harbor a pool of parasites with extensive genetic diversity that sustain malaria transmission, but may also impair host immune responses leading to reduced efficacy of malaria vaccine.

6.2 Recommendations

The study design and definition of asymptomatic infections addressed the challenge of a lack of a standard definition, which is a major limitation in most studies that makes it difficult to compare results across studies. The presence of follow-up samples of up to 6 weeks, 4 weeks prior to the diagnosis and 2 weeks after, with no history of antimalarials or fever 48hrs before and after the diagnosis was suggested as the most feasible definition of asymptomatic infections. Future studies of asymptomatic *P. falciparum* infections should incorporate this definition to enable standardization of inclusion criteria and comparison of results across studies.

The presence of stutter peaks in the capillary electrophoresis data also presented technical challenges in the definition of true peaks. Future studies should consider using more sensitive methods like targeted amplicon deep sequencing (TADS) or long-read circular consensus sequencing (CCS) to define COI. A single cell approach could be applied to study specific parasite

clones and track their progression in the host in the presence of potential competition from other clones.

Parasite transcriptome segregated asymptomatic and febrile infection mainly due to life-cycle stage differences. Asymptomatic infections were characterized by longer circulating and more developed parasites compared to febrile infection possibly due to reduced cytoadhering ability conferred by VSAs. Unfortunately, differential expression of VSAs between the two conditions using transcriptome data was not within the scope of this study. Future studies should aim to use longitudinal samples to characterize these antigens by amplifying their conserved regions as described in (Andisi and Abdi, 2022) or characterizing var gene expression in *P. falciparum* transcriptome data using computational pipelines (Tonkin-Hill *et al.*, 2018; Wichers *et al.*, 2021; Andradi-Brown *et al.*, 2023). This promises to provide more insights into how the parasites adapt to the host environment which is still not well understood.

Based on the results of this work, immune modulation in asymptomatic infections was shown to underlie anti-disease immunity. We also confirm using host transcriptomic and proteomic data that febrile infections are characterized by the activation of the immune system to initiate a cascade of immunological events that mainly features activation of pro-inflammatory cytokines. Total PBMCs were used to describe the transcriptomic signatures between the two groups, thus limiting the ability to identify interesting signatures associated with specific cell types. Future studies should aim to use single cell transcriptomics to study important signatures associated with each cell type.

A clear segregation of febrile outcomes based on whether the preceding state was uninfected or asymptomatic, provides further evidence that the presence of parasitemia in asymptomatic individuals modifies the host. The priming effect of prior asymptomatic *P. falciparum* infections may explain the blunted acquisition of antibody/humoral responses to malaria antigens following natural exposure or vaccination in malaria endemic regions. Follow-up studies that will monitor parasitemia, severity of the febrile infections, cell and antibody responses to determine whether the differences in host response have a detrimental or positive effect on the ability to control a superinfection and pathology are recommended.

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APPENDICES

Appendix I: Datasets and R scripts for msp2 genotyping

The datasets and R codes used for this work are publicly available in the Harvard Dataverse repository: https://doi.org/10.7910/DVN/3UE1NB

Appendix II: Differential expressed genes (DEGs) between asymptomatic and symptomatic/febrile malaria using parasite RNAseq data.

The processed counts data used to determine the DEGs were deposited in the National Center of Biotechnology Information's Gene Expression Ominibus (GEO) database under the GEO accession number **GSE240643**.

Appendix III: Differential expressed genes (DEGs) among the uninfected, asymptomatic and symptomatic/febrile malaria samples using host RNAseq data.

The processed counts data used to determine the differentially expressed genes were deposited in the National Center of Biotechnology Information's Gene Expression Ominibus (GEO) database under the GEO accession number **GSE241467**.

Appendix IV: Differential protein analysis (DEP) among the uninfected, asymptomatic and symptomatic/febrile malaria samples

The raw mass spectrometry files used for the DEP analysis are available in the online Dryad repository:
https://datadryad.org/stash/share/8jLfceeQfJ8B1FTcGlYzXYu-RQ4c7yddZw2PA4BugE