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College of Basic and Applied Sciences

Department of Biochemistry, Cell and Molecular Biology

Mapping the kinetics and diversity of adaptive immune responses in children over the course of an acute *Plasmodium falciparum* infection



Molecular Cell Biology of Infectious Diseases (PhD)

In partial fulfilment of the requirements for the award of the Doctor of Philosophy degree in Molecular Cell Biology of Infectious Diseases

By
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WEST AFRICAN CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS

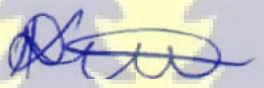
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DECLARATION

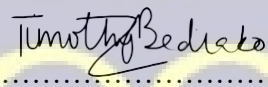
I, Nancy Kemuma Nyakoe, performed the experimental work presented in this thesis at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Accra, Ghana; The Francis Crick Institute, London, England; and the London School for Hygiene and Tropical Medicine, Bloomsbury, England, under the supervision of Dr Yaw Bediako and Prof. Gordon Awandare (West African Centre for Cell Biology of Infectious Pathogens and the Department of Biochemistry, Cell, and Molecular Biology, University of Ghana) and Prof. Jean Langhorne (The Francis Crick Institute, London, England).

All references are duly cited.



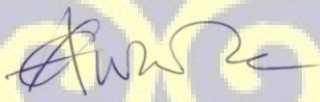
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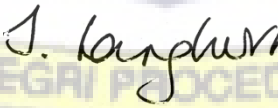
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ABSTRACT

Naturally acquired immunity to malaria is *Plasmodium species* strain- and variant- specific. It is non-sterile and short-lived therefore requiring repeated exposure. These protective immune responses are directed against pre-erythrocytic parasites, blood-stage merozoites, or *Plasmodium* antigens on the surface of infected red blood cells and can be humoral or cellular. The importance of the various immune response components during malaria episodes in exposed populations remains unknown, as conclusive interpretation of data from previous studies has been limited by sampling design, disease definition, patient selection and approaches in natural infections. Long-term malaria protection in humans is likely to be determined by the cumulative effect of multiple low-level immune responses to various antigens, with each person having a unique "protective signature" based on their genetic background.

Differences in transmission intensity observed in malaria-endemic areas significantly influence the acquisition of natural immunity to malaria. Antibody and cytokine responses, which contribute to malaria protective immunity, are exposure-dependent; hence, are significantly influenced by transmission intensity. Cytokine levels change rapidly upon exposure to malaria and often dictate the extent of clinical disease, whereas antibodies increase gradually and are long-lasting, and act to control and eliminate parasites. A variety of molecular mechanisms are involved in regulating these immune responses; however, it is still unclear what combination of immune features is essential for protection. Characterization of these host immune responses is important in understanding the bidirectional host-parasite interactions which will aid in effective vaccine

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development and eventual malaria control and elimination. This study employed a systems immunological approach to profile changes in the cellular, humoral, and molecular components of the immune system during acute *P. falciparum* infection and after treatment to identify immune signatures associated with varying malaria transmission intensity.

Methods: Samples were collected from children of ages 5-14 years in two regions in Ghana with distinct malaria transmission intensities: Accra (low transmission) and Kintampo (high transmission). Whole blood samples were collected and a portion separated to obtain plasma from the participants when they present at the hospital with confirmed *P. falciparum* infection (Day 0), and convalescent phases (Day 7 and Day 21). Levels of 25 plasma cytokines were determined using a multiplex Luminex human magnetic 25-plex bead array kit. A custom-made protein microarray was used to detect IgG responses to 190 antigenic targets for blood-stage, pre-erythrocytic, and gametocyte stage parasite proteins. Antibodies and cytokine levels from the 2 sites were compared during infection and convalescent phases. Additionally, whole-blood transcriptomes were profiled by RNA-sequencing and differential gene expression compared during infection and convalescent in the study participants. Correlation and machine-learning classifier approaches were used to model immune responses to identify predictive responses correlating with distinct transmission intensities. Gene ontology modules associated with changes in immune responses in the two transmission areas were identified using gene set enrichment analysis.

Results: Acute *P. falciparum* infection was associated with pro-inflammatory cytokines IL-6, IFN-γ, IFN-α, MIG, MCP-1, and IP-10, as well as an immunomodulatory profile mediated by IL-10, IL-2R and IL-1RA. In children from low transmission areas, they were significantly higher

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than in children in the high transmission areas, whereas, IFN-α, IL-6, IL-1RA, and MCP-1 were significantly associated with parasitemia. In addition, analysis of the correlation network revealed a distinctive signature between individuals from the low and high transmission areas. Of interest, is a subset of children from the high transmission area with detectable parasitemia at day 21 (D21) after treatment, with a unique cytokine signature dominated by IL-10 and IL-1RA. This was associated with asymptomatic parasitemia.

Of the 190 *Plasmodium* antigens tested, 118 had antibody reactivity in more than 80% of the children in our study cohorts. Though overall breadth and magnitude of antibody responses was similar, the composition of these responses was different between individuals from high and low transmission areas. Hierarchical clustering revealed IgG response clusters to antigens associated with different malaria endemicity, with 48/118 antigens including EBA140, ETRAMP5, GLURP, MSP1 and MSP6 associated to low transmission area and 30/118 antigens associated to high transmission area, including Rh5.1, EBA181, VAR2CSA, ACS5, HSP40, MSP2, MSP3, MSP4, and MSP7. Machine-learning and feature selection approaches further predicted 17 antibody signatures (MSP4, MSP2 3D7, MSP1-19 2A, Etramp 5 Ag1 His var 3, MSP1-19 2B, Etramp 5 Ag1 His var 2, MSP2 [15-46b], MSP2 [5-36A], ACS5 Ag 3, PfMSP1_19, MSP3 FVO, Var2CSA, SE36/SERA 5 (T), HSP40 Ag 3, GLURP R2, Rh4.2_2030, and MSP2 CH150/9) that distinguish individuals from these two regions. Multivariable linear regression models predicted age and transmission intensity as factors that highly affect antibody reactivity to most antigens in our panel.

Gene expression levels differ with transmission intensity More genes were differentially expressed during infection (D0) compared to convalesces (D7 and D21). Comparison of the DEGs between the two sites show that more genes were highly expressed in low transmission area compared to

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the high transmission area at D0. In the high transmission area, the DEGs enriched in gene ontology (GO) modules related to B cell and surface signature, T cell development and activation, platelet activation-actin binding, enriched cell cycle, and regulation of transcription and transcription factor modules, were up-regulated. In the low transmission area, there was DEGs in modules associated with cell cycle and transcription, immune activation- generic cluster, E2F1 targets, plasma cell surface signature, immunoglobulins, enriched monocytes, enriched NK cells, platelet activation, enriched neutrophils, enriched activated dendritic cells, NK cell surface signature, chemokine and inflammatory molecules in myeloid cells, Golgi membrane and TBA modules, were up-regulated. Cellular deconvolution revealed an increased proportion of neutrophils cell type in both high and low transmission areas, with decreased CD4 naïve, CD8, B-cells and T-follicular cells, during infection compared to convalescence in both high and low transmission. Our data identified transcription patterns that are distinguish by malaria transmission intensity, where most genes highly expressed in the high transmission area are those involved in adaptive immune response. While genes highly expressed in the low transmission area are those involve in the innate immune response. These data provide insight into molecular and cellular immune response kinetics in natural infection.

Conclusion: The findings show that cytokine responses during active malaria infection varies significantly between individuals with differing levels of prior exposure, with individuals from low transmission settings having higher levels of pro-inflammatory cytokines. However, these differences are transient and do not persist during recovery. Whereas antibodies levels remained relatively stable across the timepoints for most antigens in our panel. We show that models trained to capture distinct antibody response patterns predicted 17 antibody responses that are key in

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distinguishing between individuals from different intensities. Given the important role that exposure plays in the acquisition of immunity these could be useful antigens as possible targets of protective immunity and provides clues for potential vaccine candidates that can be prioritized in evaluation. Transcriptomic analysis of whole blood over the course of infection revealed molecular signatures that can provide insights into the protective immune responses against *P. falciparum* infection. We identified genes whose expression patterns can help differentiate changes during acute P. falciparum infection and convalesces in varying transmission intensity. Taken together, this study predicts humoral and cellular signatures associated with the acquisition of naturally acquired immunity that can fairly distinguish individuals from two distinct transmission areas based on their immune profile.



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DEDICATION

To God, Thank You for establishing my steps through all seasons.

To my husband, James; Children: Latisha, Merissa, and Jayden. I will love you to the end.

In memory of Dad and Mum, the dream that you nurtured in me, has come to reality.

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ABBREVIATIONS

ACD - Acid Citrate Dextrose

ACT - Artemisinin-based combination therapy

AMA - Apical membrane protein

APC- Antigen Presenting Cells

AUC- Area Under the Curve

BSF-2- B cell stimulation factor 2

BLIMP-1 - B-lymphocyte-induced maturation protein 1

BTM- Blood Transcriptional modules

CCL – Chemokine Ligand

CD- Cluster of Differentiation

CHMI- Controlled Human Malaria Infection

cDNA- complementary Deoxyribonucleic acid

CHAK- CC-Chemokine-activated killer

CSF- Colony-Stimulating Factors

CSP- Circumsporozoite Protein

CTLA-4 - cytotoxic T-lymphocyte-associated protein 4

DCs - Dendritic cells

DNA- Deoxyribonucleic acid

dscDNA- Double-stranded DNA

dAMP- Deoxyadenosine monophosphate

dTMP- deoxythymidine monophosphate

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dUTP- 2′-Deoxyuridine, 5′-Triphosphate

pDCs - plasmacytoid dendritic cells

EBA- Erythrocyte binding Antigen

EC- Endothelial cells

EDTA - Ethylenediamine tetra acetic acid

EPA - ExoProtein

EPCR- Endothelial protein C receptor

ETRAMP- Early transcribed membrane protein

FasL – Fas Ligand

FBC - Full Blood Count

GATA-3 - GATA Binding Protein 3

GC – Germinal centre

GLURP - Glutamate rich protein

GM-CSF- granulocyte-macrophage colony-stimulating factor

G6PD - Glucose 6 phosphate dehydrogenase

GPI - Glycosylphosphatidylinositol

HCT – Haematocrit

HGB- Haemoglobin

HLA-1- Human Leucocyte Antigen 1

ICAM-1- Intercellular Adhesion Molecule 1

ICOS- Inducible T-cell COStimulator

IFN-γ-Interferon-gamma

IL- Interleukin

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JAKs - Janus kinases

KLF13 - Kruppel like factor 13

LAG-3- Lymphocyte Activation Gene-3

MAPK- Microtubule associated protein kinase

MCP-1 – Monocyte chemoattractant protein-1

MCH- Mean Corpuscular Haemoglobin

MCHC- Mean Corpuscular Haemoglobin Concentration

MCV- Mean Corpuscular Volume

MFI- Mean Flourescent Intensity

MIG - Monokine induced by gamma interferon

MIP-1- Macrophage inflammatory protein-1

MPV- Mean Platelet Volume

mRNA- messenger Ribonucleic acid

MSP - Merozoite Surface Protein

MTI- Malaria Transmission Intensity

MHC- Major Histocompatibility Complex

NADPH- Nicotinamide adenine dinucleotide phosphate reduced

NET - Neutrophil extracellular trap

NK - Natural Killer cells

NKT- Natural Killer T cells

OOB- Out-of-Bag

PBMCs- Peripheral Blood Mononuclear Cells

PCA- Principal Component Analysis

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PCR – Polymerase Chain Reaction

pfEMP-1- *Plasmodium falciparum* Erythrocyte Membrane Protein-1

PD-1 - Programmed cell death protein **1**

PGE_2 - Prostaglandin E

PDGF - Platelet-Derived Growth Factor

RANTES- Regulated upon activation, Normal T cell expressed and secreted

RBCs - Red Blood Cells

RDT – Rapid Diagnostic Test

RIN- RNA Integrity number

RNA- Ribonucleic acid

ROS - Reactive Oxygen Species

RPKM- Reads per Kilobase of transcript per 1 million mapped reads

STAT- Signal transducer and activator of transcription

SURFIN- Surface associated interspaced protein

SVM- Support Vector Machines

TBV - Transmission blocking vaccines

TGF-β - Transforming growth factor beta

TIM-3 - T-cell immunoglobulin mucin-3

TNF-α- Tumor Necrosis Factor

TPM- Transcript per million

TRAIL - TNF-related apoptosis-inducing ligand

VCAM-1- Vascular Cell Adhesion Molecule-1

WBC- White Blood Count

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THESIS OUTLINE

Chapter one is a broad overview of the project, describing the continued impact of malaria on human health, current gaps in our understanding of natural immunity to malaria, and a description of the major objectives of this project.

Chapter two is a comprehensive review of the etiology and global distribution of the malaria burden and transmission, with a summary of immune responses during malaria, highlighting the different components of the immune system involved. The systems immunology approach and how it applies to determining the mechanisms involved in protective immunity to malaria is described. The chapter ends with a summary of how defining the dynamics of immune responses can provide a roadmap to the development of an efficacious vaccine.

Chapter three describes the cytokine signatures in children from distinct transmission areas during acute *P. falciparum* infection and convalescence, using a Luminex assay. The results demonstrate that cytokine levels are significantly changed during infection and convalescence, and identify cytokine signatures that distinguish children from the different transmission areas.

Chapter four describes antibody profiling results obtained from protein microarray to identify signatures associated with different transmission areas, using machine-learning modeling algorithms to identify antibody responses that can predict or distinguish individuals with different

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exposure levels. We established antibody signatures that predict individuals from different transmission areas.

Chapter five presents transcriptomics analysis results with RNA- sequencing, showing molecular changes that occur during acute infection and after treatment, and how the transcriptome profile differs in individuals from the two transmission areas in the study cohort.

Chapter six briefly discusses and summarises key findings from the study and conclusions.

Chapter seven outlines the study recommendations and limitations.



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1 CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Despite the considerable public health efforts directed to malaria control worldwide, malaria remains an important parasitic disease globally with almost one quarter of the world's population at risk of *Plasmodium falciparum*. The use of vector control strategies and improved artemisinin-based combination therapies have helped the annual reduction of cases; but the World Health Organisation (WHO) still estimated 228 million cases and 405,000 deaths in 2019 (WHO, 2020). Approximately, 90% of these cases were in Africa, the majority being children less than 5 years. Success in malaria control efforts is challenged by rapid resistance to anti-malarial drugs (Lu *et al.*, 2017), increasing widespread insecticide-resistant mosquito vectors (Ranson & Lissenden, 2016), and the lack of an effective vaccine for long-term protection (Crompton *et al.*, 2010b). The currently approved, RTS,S AS01 vaccine for children from endemic areas only confers partial, short-term protection, even with boosting. (RTS, 2011; Olotu *et al.*, 2013; Adepoju, 2019). Therefore, there is a need for a highly effective and durable vaccine, which will augment anti-malarial drugs and vector control strategies, to eliminate the disease and possibly eradicate the vector. The fact that humans acquire partial immunity to malaria through experimental and natural *P. falciparum* infection, raises hope for the development of a highly effective vaccine.

Naturally acquired immunity (NAI) to *Plasmodium* reflects a complex interaction that balances between anti-parasite effector responses involved in parasite clearance and regulatory immune responses that maintain parasite tolerance without eliciting pathogenic effects (Pinkevych *et al.*, 2012). Understanding these competing responses in natural infection is crucial in contributing to

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the development of a practical vaccine. In *Plasmodium* infection, mechanisms of controlling blood-stage parasite growth are mainly through antibody production, aided by the innate and cellular induced inflammatory responses (Cohen *et al.*, 1961; Langhorne *et al.*, 2008; Dinko & Pradel, 2016; Gomes *et al.*, 2016). While the mechanisms that regulate the immune responses resulting from infection tolerance are unclear, studies have suggested that repeated exposure seen in high malaria-endemic areas is necessary for the establishment of tolerance (Galatas *et al.*, 2016).

Although the role of antibodies in controlling parasite density, symptoms and disease severity has been established (Cohen *et al.*, 1961; Sabchareon *et al.*, 1991), there is a paucity of data on the precise parasite antigens involved and the role of the innate and cellular immune responses that mediate NAI (Doolan *et al.*, 2009). Defining the precise correlates of protection in humans is complicated because most individuals rarely develop sterile immunity to *P. falciparum* infection (Langhorne *et al.*, 2008). Additionally, studies have shown conflicting results, with both evidence for (Dent *et al.*, 2008; Dobbs & Dent, 2016) and against (Yman *et al.*, 2019)natural acquisition of sterile protection. Most studies on cellular immune responses in humans are mainly single time point cross-sectional surveys in previously infected but otherwise healthy individuals. These studies focus on a limited number of immunological parameters; hence, not ideal for determining correlates of protection.

Immuno-epidemiological studies also tend to define immunity as the absence of disease despite exposure. However, it is challenging to measure the definitive level of exposure in individuals resulting in some non-exposed individuals being misclassified as immune. There is currently no consensus on the cellular, molecular or immune mechanisms responsible for naturally acquired or

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experimentally induced immunity to malaria. Identification of these immune cells, mediators, and pathways, linked to protection, could give insights to host-directed immune treatments as well as effective vaccine targeting and efficacy monitoring.

The proper functioning of the host-pathogen immune response depends on the complex, dynamic interaction between these different immune system components at the cellular and molecular levels. Thus, due to the considerable integration and co-regulation, the network cannot be represented by individual elements in isolation. The newly developed field of systems immunology is a promising tool for defining the immune responses to *P. falciparum* because it allows the study of the immune system as a dynamic network of cellular, molecular, and systemic responses (Villani *et al.*, 2018), utilizing high throughput technologies and computational analysis to develop a multi-dimensional, holistic view of the immune system (Braun, 2014). This has increased the need to use high throughput techniques like protein microarray, multiplexed bead-based assay, and next-generation sequencing that allow simultaneous measurement of multiple antibodies, cytokine, and gene transcript levels. However, interpreting the results is often limited by the traditional statistical methods employed to predict individual-level protection from population-wide associations. This is especially true where numerous immune signature combinations and interactions are being tested. Also, these tests are mostly univariate and do not consider the possibility of the interplay between different mediators.

Machine learning-based predictive modeling frameworks offer a better understanding of the contribution of individual components towards the overall function of a system (Cutler *et al.*, 2007; Finney *et al.*, 2014). Computational modeling can provide valuable information on the importance of different immunological components, how they vary during infection, and their interaction with

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each other. This can provide a comprehensive understanding of the immune response during infection and identify potential correlates of malaria immunity (Germain *et al.*, 2011).

The varying epidemiology of malaria in different endemic areas emphasizes the significance of natural exposure in immune development. Areas of unstable transmission are characterized by low levels of immunity, while, endemic areas with high levels of transmission are marked by higher levels of clinical immunity. Further supporting the role of exposure in the acquisition of immunity is the observation that infection prevalence appears to shift into increasingly older age groups as transmission intensity declines immunity (Okiro *et al.*, 2009). Ghana is characterized by distinct regions of malaria transmission intensity, suggesting equally distinct levels of naturally acquired immunity. Since controlled malaria human infection cannot be performed in children, investigating acute infections in children with different levels of immunity presents a good opportunity to understand the factors that determine immunity to malaria in children.

A longitudinal study, such as that employed in this thesis, can broadly characterize the complex framework of naturally acquired immunity during acute *P. falciparum* infection and convalescence in children with different levels of immunity. The study described here aimed to characterise altered immune responses by simultaneously measuring molecular, cellular, and humoral immune responses in children with different levels of immunity.

1.2 PROBLEM STATEMENT

Malaria immunity develops slowly and is non-sterile, quickly waning in the absence of constant exposure to the parasite. This indicates the importance of continuous exposure to *Plasmodium* antigens for the development and persistence of memory and effector cells. However, there is no

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consensus on what components of malaria immunity are lost when there is no persistent exposure. The lack of a clear understanding of the mechanisms by which naturally acquired malaria immunity is achieved and maintained is still a major obstacle to developing an effective malaria vaccine. Hence the need for well-designed cohort studies that will enable mapping of changes in immune signatures during *Plasmodium* infection, which can be correlated to protective immunity. This study broadly characterizes the complex framework of naturally acquired immunity during infection and after treatment by assessing the kinetics and diversity of cellular, humoral and molecular responses to malaria infection in children at different levels of exposure to the malaria parasite and by extension, different levels of immunity.

1.3 RESEARCH QUESTIONS

i. How does transmission intensity influence *P. falciparum* specific cellular, antibody, and molecular signatures during an acute infection?

ii. Which immune signatures distinguish between children from high endemic and low endemic areas, and can these signatures be associated with protective immunity?

1.4 AIMS AND OBJECTIVES

1.4.1 Study aims

To investigate how the difference in malaria transmission intensity affects humoral, cellular, and molecular immune signatures in children during acute *P. falciparum* infection and convalescence.

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1.4.2 Specific Objectives

1.4.2.1 Objective 1

To evaluate plasma cytokine responses during acute *P. falciparum* infection and convalescence in Ghanaian children exposed to different transmission intensities.

Hypothesis: *Cytokine responses of children during P. falciparum infection would be quantitatively and qualitatively different in areas with high and low transmission intensities*.

1.4.2.2 Objective 2

To identify and compare the breadth of antibody responses between children living in areas with varying transmission intensities during acute *P. falciparum* infection.

Hypothesis: *Children living in high transmission areas will have a broad antibody repertoire compared to children of the same age in lower transmission areas.*

1.4.2.3 Objective 3

To investigate gene expression changes over the course of *P. falciparum* infection between children living in areas with varying transmission intensities.

Hypothesis: *Varying gene expression patterns in children living in areas with different transmission intensities would vary.*

1.4.2.4 Objective 4

To generate a model and validate the humoral, cellular, and transcriptomic immune signatures in children from different transmission intensities during *P. falciparum* infection.

Hypothesis: *Immune signatures that correlate with different levels of prior P. falciparum infection exposure can be modelled to predict protective immune correlates.*

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2 CHAPTER TWO: LITERATURE REVIEW

2.1 ETIOLOGY OF MALARIA DISEASE

Malaria, one of the world's main causes of morbidity and mortality, is estimated to kill 409,000 people each year and infect 228 million cases annually (WHO, 2020). Human malaria is causes by five *Plasmodium* species, with *P. falciparum* accounting for the bulk of severe cases and deaths, mainly in Africa (White, 2008). *P. vivax* is the second to *P. falciparum,* in malaria morbidity and mortality, with the highest-burden in Asia. The true burden of *P. vivax* infections is unknown, with estimates of between 14.3 and 22 million cases per year (Battle *et al.*, 2019). Other causes of human malaria include *P. malariae* and *P. ovale*, which exist as two genetically divergent subspecies (*P. ovale curtisi* and *P. ovale wallikeri*) (Sutherland *et al.*, 2010; Miller *et al.*, 2015), and account for a minor proportion of the global malaria burden. *P. knowlesi* is a zoonotic infection that is transmitted from macaques to humans by infected mosquitoes in parts of Southeast Asia, with rare direct human-to-human transmission (Singh & Daneshvar, 2013).

Malaria transmission occurs in 5 tropical and subtropical regions in the world, with the heaviest transmission being in Africa. In malaria-endemic countries, transmission is regional and intensity and seasonality vary throughout the year. Transmission depends on climatic factors such as temperature, humidity, and rainfall. The warmer regions have more intense year-round transmission while the cooler regions have less intense and more seasonal transmission (Figure 2.1).

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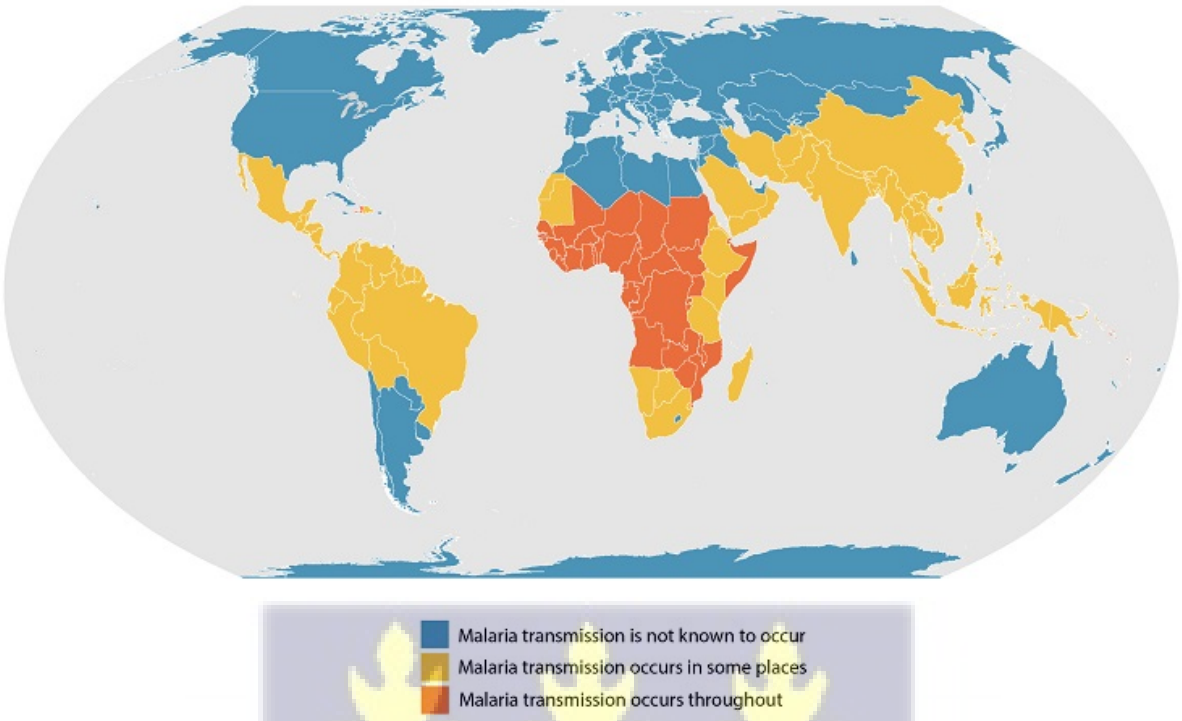


Figure 2. 1: Global distribution of malaria.

Map showing an approximation of the parts of the world where malaria transmission occurs. (Centers for Disease Control and Prevention, 2020, April 9).

Plasmodium is transmitted by bites of infected female *Anopheles* mosquitoes, and the *Anopheles gambiae* complex is responsible for transmission in Africa (Nchinda, 1998). Sporozoites in the mosquito salivary glands are inoculated into the host skin and quickly migrate to the liver, where they invade the hepatocytes multiplying in 10-14 days. Thereafter, they are released as merozoites, which invade red blood cells, where they develop into rings, trophozoite and multinucleated schizont stages over 48 hours. The schizonts then rupture to release merozoites that invade new red blood cells (RBC) (Greenwood *et al.*, 2008), leading to a 10–20-fold increase of *P. falciparum* in the bloodstream every 2 days. This erythrocytic stage is associated with clinical manifestations

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associated with the disease and pathology. The rupture of the RBCs results in the release of various waste products, which activate the immune system causing symptoms such as chills, fatigue, pain, and fever. Malaria pathology is primarily due to the sequestration phenomenon that is associated with late-stage iRBCs (Smith *et al.*, 2013; Hviid & Jensen, 2015). This occurs when parasites developing and maturing within the host RBCs cause alteration to the iRBC membrane to express parasite proteins that adhere to host cells. Such changes make the iRBCs highly susceptible to recognition by host immune cells and specifically splenic filtration. However, *P. falciparum* alters the host cells in ways that make them cytoadhere to vascular endothelium and sequestering in capillaries of less dangerous host organs, to escape splenic clearance (Suwanarusk *et al.*, 2004; Sosale *et al.*, 2015). Rosetting is another cytoadherence phenomenon, in which infected RBC adheres to non-infected RBC via interactions with the A antigens, forming flower-like structures known as "rosettes" (Rowe *et al.*, 1997; Lee *et al.*, 2019). This leads to immune modulation and evasion mechanisms that block the phagocytosis pathway (Champion *et al.*, 2008). Sequestration of iRBCs has important side effects in clinical malaria disease as it leads to severe malarial anaemia, which is as a result of the destruction of non-infected RBCs (Alister *et al.*, 2012). Rosettes can also block blood flow into the capillary bed when they are unable to move into capillaries, causing brain tissues affected to suffer hypoxia and irreversible damage (Idro *et al.*, 2010). Cytoadhesion of iRBCs to endothelial cells, on the other hand directly activates the endothelial cells, which may contribute to endothelial injuries and vascular leakage (Gillrie & Ho, 2017). This has been implicated in the pathogenesis of cerebral malaria, through the interaction of key parasite ligand PfEMP1 expressed on iRBCs with endothelial protein C receptor (EPCR) (Mosnier & Lavstsen, 2016; Kessler *et al.*, 2017; Mkumbaye *et al.*, 2017).

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A percentage of erythrocytic-stage parasites can develop into sexual-stage parasites or gametocytes, which when ingested by a mosquito during a blood meal from the human, escape from the erythrocytes differentiating into gametes in the mosquito gut. The gametes undergo fertilization to form zygotes, which are transformed into ookinetes. The ookinetes traverse the midgut to the basal lamina, where it transforms into an oocyst, producing and releasing sporozoites into the mosquito hemocele, and migrating to the salivary glands. During a blood meal, the infectious sporozoites are released into the human host (Hoffman & Miller, 1996; Cirimotich *et al.*, 2010)

During the early stage of *P. falciparum* infection, most cases are relatively mild with symptoms such as headache, fever, dizziness, chills, malaise, nausea, abdominal discomfort, muscle, and joint ache (Autino *et al.*, 2012). However, more severe complications such as anemia, hypoglycemia, splenomegaly, multi-organ dysfunction, respiratory distress syndrome, and unconsciousness in cases of cerebral malaria, can arise as the disease progresses. (Miller *et al.*, 2002; Cowman *et al.*, 2016). These multi-organ pathological conditions arise from parasite sequestration in the lungs, brain, liver, dermal tissues, intestine, and placenta through host receptors such as CD36, intracellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), and endothelial protein receptor (EPCR) (Smith *et al.*, 2013; Deroost *et al.*, 2016; Wahlgren *et al.*, 2017; Lee & Coban, 2018c). The most severe disease in malaria holoendemic areas occurs in younger children as partial protective immunity is typically acquired between 5-10 years of age (Pemberton-Ross *et al.*, 2015).

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Despite substantial effort and progress in reducing the global malaria burden, by interventions, including the use of insecticide-treated bed nets, rapid diagnosis and effective treatment (using artemisinin combination therapy (ACT), increased resistance to insecticides (Ranson *et al.*, 2016), and of the current ACTs, have challenged these efforts (Lu *et al.*, 2017). This highlights the need for novel control interventions and the development of an effective vaccine to provide a long-term solution to malaria (Crompton *et al.*, 2010b). The currently approved vaccine, RTS, S has only limited efficacy of 29%-36% varying in different age groups even with boosting, and wanes with time (RTS, 2011; Olotu *et al.*, 2013; Adepoju, 2019). Therefore, the development of a highly efficacious vaccine remains a priority in complete malaria eradication.

2.2 MALARIA TRANSMISSION

Malaria transmission is highest in sub-Saharan Africa with up to 1000 infectious bites per year, with *P. falciparum* as the dominant species, and infections are characterized by the circulation of multiple, genetically diverse parasite clones within the population and individuals. The parasites display tremendous antigen variation, frequent superinfection, and highly variable infection lengths (Crompton *et al.*, 2014). Malaria is a holoendemic and symptomatic disease confined to young children in high transmission areas, whereas older children and adults, typically present with asymptomatic parasitemia (Gething *et al.*, 2011). Low transmission areas have an unstable and symptomatic disease that occurs in all age groups. The parasite's transmission dynamics are influenced by the *Anopheles* mosquito vector dynamics, climate (humidity, rainfall, temperature), and geography, all of which have a significant impact on immune responses.

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Immunity to severe disease, symptomatic disease, and the parasite is acquired through prior exposure and infection. This is partly affected by variation in malaria transmission intensity. Immune responses at various ages may be influenced by intensity and seasonality of malaria transmission, with the development of effective NAI occurring primarily in areas with heavy and continuous transmission. The lower transmission areas tend to have monoclonal infections and a higher risk of clinical disease in children. In contrast, the high transmission areas have multiclonal infections that are more chronic, but with reduced risk of clinical malaria (Färnert *et al.*, 2009; Gray *et al.*, 2013). Findings from other studies support the conclusion that increasing transmission intensity is associated with increasing parasite densities in children who present to the hospital with symptomatic malaria (Mobegi *et al.*, 2014; Duffy *et al.*, 2015; Mensah-Brown *et al.*, 2017). Repeated infection can become chronic in asymptomatic individuals who develop "Clinical tolerance", also defined as anti-disease immunity. This immunity prevents the development of clinical symptoms but does not clear or protect from parasite infection. Tolerance by the host is a less understood phenomenon in malaria, it is suggested to be multi-factorial which includes adaptive immune responses and controlled immune-regulatory processes that suppress the excessive immune response in the host (Galatas *et al.*, 2016). Malaria immunological data indicate that tolerance declines in the absence of continuous exposure to the parasite, implying that tolerance levels are transmission-dependent (Portugal *et al.*, 2014b).

The development of tolerance to *Plasmodium* has affected the ability to detect infection and the reintroduction of parasites to previously cleared areas, hence contributing to onward malaria transmission. As a result of the relatively high percentage of clinically silent and low parasite density infections, especially in high endemic settings, measuring disease prevalence and relating

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it to transmission intensity remains difficult. (Recker *et al.*, 2018). With increased malaria control and elimination strategies, many malaria-endemic areas are experiencing declines in transmission (O'Meara *et al.*, 2008; Snow *et al.*, 2015). While this is a positive outcome, it is well established that differences in transmission intensity can influence the acquisition of natural immunity (Eckhoff, 2012). It is therefore likely that declines in transmission will have concomitant impacts on the acquisition of immunity. Therefore, a proper understanding of the impact of changing transmission on immunity is required to provide insight into the development and maintenance of naturally acquired immunity (NAI), which is critical for the development of an effective vaccine.

2.3 LIFE CYCLE OF *P. FALCIPARUM* AND IMMUNITY

P. falciparum is a complex parasite with a large genome of approximately 5400 genes (Gardner *et al.*, 2002) and is characterized by multiple life cycle stages (Florens *et al.*, 2002). These different stages are antigenically distinct and stimulate different parts of the immune system. Specific immune responses involving both humoral and cell-mediated mediators are induced to limit the infection at the different stages of the parasite's life cycle; pre-erythrocytic, erythrocytic and intra-erythrocytic.

2.3.1 Pre- erythrocytic immune responses

Following an infected mosquito bite, sporozoite-specific antibodies are the initial immune responses that inhibit sporozoites from invading the hepatocytes in the liver (Figure 2.2 (a). Sporozoites that do not successfully migrate to the liver die and parasite remnants are taken up by the antigen-presenting cells (APCs), which migrate to the draining lymph nodes and activate the

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adaptive immune response Figure 2.2 (b). At the liver stage, naive CD8+ and CD4+ T cells respond against intrahepatic parasite antigens and are presented through MCH-I and II recognition. Parasite antigen recognition facilitates effector functions of CD8+ cytotoxic T lymphocytes to destroy infected hepatocytes, activate CD4+ helper T cells differentiate into central and effector memory cells, resulting in the production of functional mediators like cytokines Figure 2.2 (c&d). Antibodies against free sporozoites and circumsporozoite protein (CSP) also prevent hepatocyte invasion and activate complement, phagocytosis, and lysis by cytotoxic Natural Killer (NK), NK T cells, and gamma delta ($\gamma\delta$)T cells. These antibodies additionally recognize parasite antigens at the surface of infected hepatocytes and initiate killing by Kupffer cells and NK cells through an antibody-dependent cell-mediated mechanism (Ocaña-Morgner *et al.*, 2003; Casares & Richie, 2009; Dinko *et al.*, 2016) Figure 2.2 (e).

2.3.2 Erythrocytic immune responses

Merozoites released during the erythrocytic cycle in the blood induce antibodies that prevent the merozoites from invading red blood cells (RBCs). Antibodies opsinize parasite antigens on the surface of infected erythrocytes, which activate complement and /or recruit innate immune cells such as macrophages, which eliminate parasite via complement-mediated lysis, particularly in the spleen (Gomes *et al.*, 2016). Antigens from iRBCs can also be presented to CD4+ T cells, to aid in T cell-dependent antibody responses Figure 2.2 (f&g).

2.3.3 Intra-erythrocytic

Finally, antibodies to gametocytes act to hinder uptake and eventual development of parasites in the mosquito vector (Douradinha & Doolan, 2011). Antibodies transferred from the host during

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mosquito bite mediate complement killing of gametocytes, thus preventing gamete fusion in the mosquito. (Dennison *et al.*, 2015). Figure 2.2 (h)

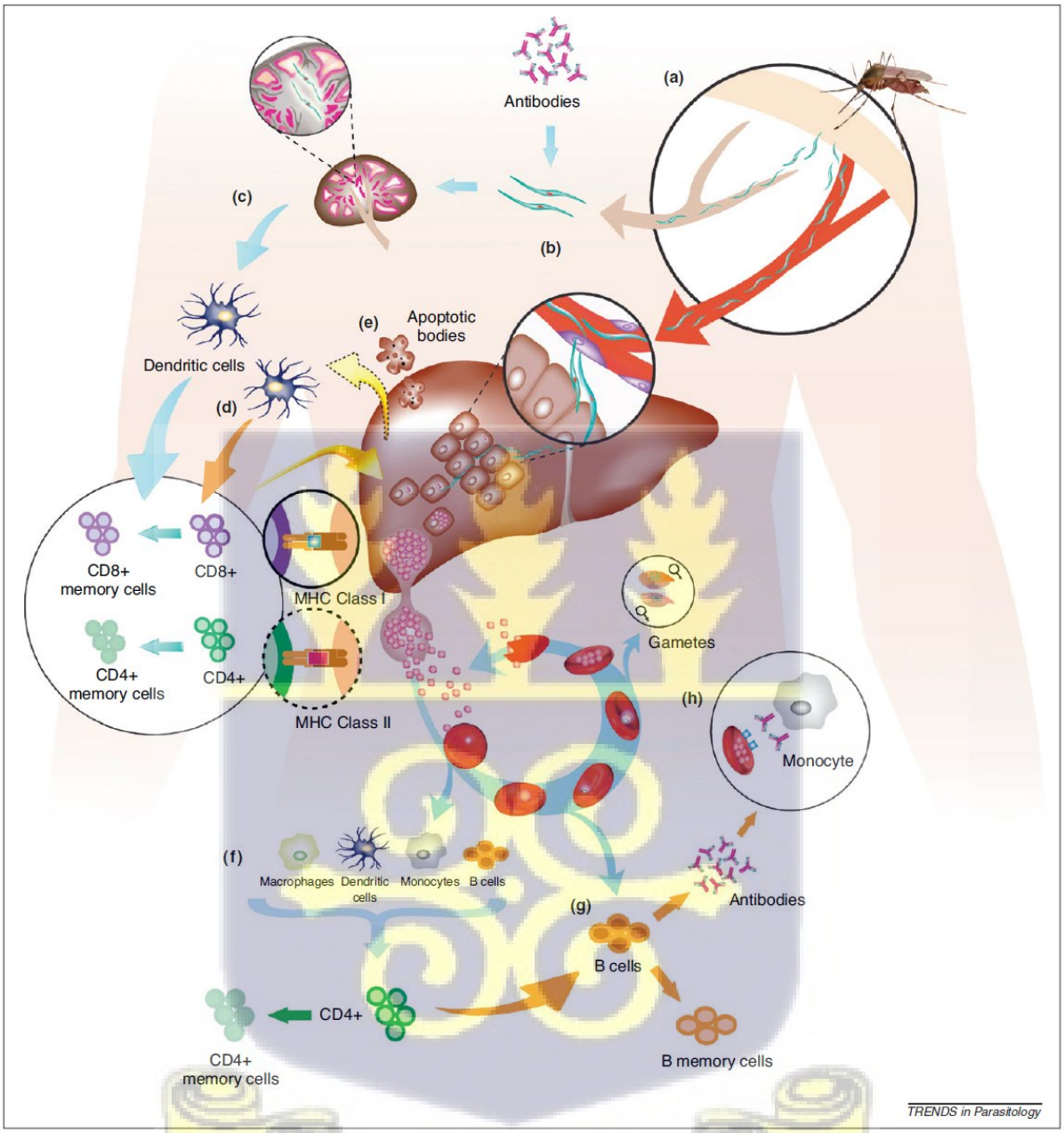


Figure 2. 2: The life cycle of *Plasmodium falciparum* and stage-specific immune responses.

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Describes the stages of the *P. falciparum* life cycle and the immune responses by the host at each stage (a-e) is the Pre-erythrocytic immune responses, (f-g) Erythrocytic immune responses and (h) Intr-erythrocytic

Source: Douradinha, B., & Doolan, D. L. (2011). Harnessing immune responses against Plasmodium for rational vaccine design. *Trends in parasitology, 27*(6), 274-283.

Immune response in humans begins with an infected mosquito bite releasing sporozoites through the skin; these induce specific antibody responses, which are intended to inhibit liver invasion. Sporozoites that evade this response get to the liver. Parasite debris is presented to T naïve cells by antigen-presenting cells (APC) like dendritic cells, activating immune cells such as CD8+ T cells, which detects and kills infected hepatocytes through the production of effector mediators like cytokines. Parasites that escape this attack leave the liver cell and rapidly infect the RBCs. At this stage different parasite antigens are presented to CD4+ T cells to mediate T cell-dependent antibody response, while some antibodies are T cell independent. As the parasite undergoes repeated replication cycle, they go through the system interacting with immune cells and triggering antibodies, cytokines, and reactive free radical production (Douradinha *et al.*, 2011).

Despite a relatively good understanding of the immune response components to *Plasmodium*, it is unclear why the complex-multistage life cycle of malaria parasites typically induces suboptimal immune responses that develop slowly over time. To understand malarial immunity as a whole, these responses need to be studied as a series of spatial, temporal, and antigenically distinct responses (Langhorne *et al.*, 2008; Riley & Stewart, 2013). Furthermore, developing and maintaining immunity is complicated by the intensity and duration of previous infections, as well as the presence of multiple evasion mechanisms employed by malaria parasites (Hisaeda *et al.*,

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2005; Yazdanbakhsh & Sacks, 2010; Crompton *et al.*, 2014). Individuals in high-endemic areas are exposed to multiple infectious bites and cumulative infections, resulting in the development of clinical immunity. However, this immunity is thought to rapidly wane in the absence of further parasite exposure (White *et al.*, 2014b). The relative contribution of immunity against various stages of the parasite life cycle to infection control and disease limitation is not well understood.

2.4 IMMUNITY TO MALARIA

Individuals in malaria-endemic areas gradually acquire immunity with age and exposure to the parasite (Baird, 1998; Doolan *et al.*, 2009; Pemberton-Ross *et al.*, 2015). Though sterile protection is probably never achieved, experimental vaccination has managed to induce sterile though short-lived protection (Hoffman *et al.*, 2002; Doll & Harty, 2014). However, immunity to severe disease is acquired more quickly, mainly after a single or few episodes (Gupta *et al.*, 1999b). While resistance to severe morbidity and death can be achieved in older children and adults, they remain susceptible to infection (Laishram *et al.*, 2012; Kamau *et al.*, 2020). The immune response to malaria is complicated, multi-faceted, parasite stage-specific, and involves both the innate and adaptive immune responses, despite the fact that the mechanisms remain unknown. The next paragraphs will give a detailed review of the current understanding and the studies that have tried to delineate these interactions.

2.4.1 Natural and (innate) immunity

Genetic polymorphisms which affect haemoglobin structure such as sickle cell disease, haemoglobin C, haemoglobin E, or ovalocytosis carriers confer natural protection against *P.*

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falciparum or *P. vivax*. Similarly, deficiency of certain enzymes like glucose 6 phosphate dehydrogenase (G6PD) confers protection against *P. falciparum* infection or severe malaria. Likewise, Duffy negativity in red cells was considered protective against *P. vivax* infection in the African population (Carvalho & Carvalho, 2011; Zimmerman *et al.*, 2013), but recent evidence has shown that *P. vivax* parasite has developed different mechanisms of infection in Duffy negative individuals (Popovici *et al.*, 2020). However, the contribution of these abnormalities in the acquisition of protective immunity is still undetermined.

During acute malaria infection, a rapid and non-specific immune response is stimulated limiting disease progression (Stevenson & Riley, 2004). *Plasmodium* parasite antigens activate innate immune cells, including neutrophils, mast cells, dendritic cells, monocytes/macrophages, natural killer cells, γδ T cells and platelets (Hensmann & Kwiatkowski, 2001). Antigen presentation triggers the adaptive immune response, phagocytosis and production of pro-inflammatory cytokines, all of which contribute to parasite clearance and the induction of adaptive CD4+ and CD8+ T cell responses (Langhorne *et al.*, 2008; Liehl *et al.*, 2014; Kho *et al.*, 2018).

Activated γδ T cells have been proposed to be the initial source of IFN-γ (Teirlinck *et al.*, 2011), that stimulate the other innate cells and release granulysin, which destroy iRBC and merozoites preventing RBC invasion (McCall *et al.*, 2010b). Additionally, γδ T cells are not MHC- restricted and can therefore respond to antigens and migrate to the lymph nodes and act as APCs (Jensen & Chien, 2009; Howard *et al.*, 2018). Recent studies show that γδ T cells are the main source of cytokines and chemokines in severe malaria (Stanisic *et al.*, 2014; Pamplona & Silva-Santos, 2021). Neutrophils being the most abundant white blood cells are also the earliest innate cells to

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be activated after infection through complement-dependent phagocytosis. They function by forming neutrophil extracellular traps (NETs), and producing reactive oxygen species (ROS). The reduction of circulating neutrophils during erythrocytic-stage infection is thought to be due to neutrophil clearance and migration to sites of parasite sequestration (van Wolfswinkel *et al.*, 2017; Knackstedt *et al.*, 2019). Monocytes on the other hand, express complement receptors as well as a variety of pathogen recognition receptors (PRR), which allow for rapid activation by *Plasmodium* DNA, RNA, GPI anchoring, and hemozoin, triggering an immune response via phagocytosis and cytokine release (Gazzinelli *et al.*, 2014; Italiani & Boraschi, 2014).

NK cells are similarly among the initial innate cells associated with malaria protection, they respond by secretion of chemokines, cytokines, and cytotoxins that lyses iRBCs. To produce IFN-γ, NK cells respond to iRBCs via contact-dependent and cytokine-mediated signals from monocytes and myeloid/conventional dendritic cells (cDC) (Newman *et al.*, 2006). NK cells have been shown in studies to be capable of clearing *P. falciparum* and to correlate with parasitaemia and high levels of IFN in endemic areas (Chen *et al.*, 2014). Dendritic cells, despite constituting a small proportion of peripheral blood lymphocytes, play an important role in early response to *Plasmodium* infection by recognizing pathogen-associated molecular patterns (PAMPs) and presenting parasite antigens to T cells through MHC-I or II. Recognition of presented antigen /MHC complexes by naïve CD4+ and CD8+ T cells initiate the adaptive immune response through co-stimulated proliferation and differentiation into effector cells (Orsini *et al.*, 2012).

Non-malaria-specific immunoglobulin levels have also been found to be high during infection; however, the significance of this polyclonal B-cell activation for innate immunity is unknown (Stevenson *et al.*, 2004; Pérez-Mazliah *et al.*, 2020).

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2.4.2 Acquired (adaptive) immunity

Immunity can be acquired in two ways: active or passive. Active immunity is the development of a host's immune response upon encounter with the parasite, whereas passive immunity is the result of the transfer of protective substances pre- or post-natally from mother to child, or by the injection of such substances to infected people (Albrecht & Arck, 2020).

Acquired malaria immunity develops after constant exposure to the parasite, and its protective effectiveness differs depending on host features, place of residence, and the number of infections. It is thought to be an overlap between anti-disease immunity, which protects against clinical disease, anti-parasite immunity, which protects against high parasitemia, and sterilizing immunity (which protects against new infections by maintaining a low-grade, asymptomatic parasitemia; also known as premunition (Doolan *et al.*, 2009). It involves both immune cellular effector T cells and humoral (B cells) responses. Early infections are thought to cause the production of moderately low-affinity antibodies by short-lived plasma cells, while recurrent infections increase the number of antigen-specific effector T cells and memory B cells. Though antibody responses play a vital protective role, the cellular mechanisms underlying the slow and imperfect acquisition of immunity are still undefined (Ryg-Cornejo *et al.*, 2016).

Humoral immunity

P. falciparum infection induces potent polyclonal and specific immunoglobulin responses, both IgM and IgG. These antibodies are crucial for parasite clearance and disease resolution, and are mostly directed against the blood-stage of the parasite's life cycle (Teo *et al.*, 2016; Healer *et al.*,

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2018). Specialized CD4+ T cell, T follicular helper (Tfh) cells, exert important helper function for the selection and maturation of B cells responsible for antibody production (Hale & Ahmed, 2015). Antibody breadth and potency increase with exposure to *P. falciparum* infection, which are crucial in naturally acquired immunity. The rapid expansion of B cells generates more antibody-producing plasma cells and potentially immunoregulatory B cells (Wipasa *et al.*, 2010; Ayieko *et al.*, 2013; Braddom *et al.*, 2020). A small proportion of antibodies are species and stage-specific and recognize a variety of parasite antigens.

Antibodies protect against malaria by inhibiting merozoite invasion (Beeson *et al.*, 2016a), enhancing antibody-dependent cellular killing mediated by cytophilic antibodies (Bouharoun-Tayoun *et al.*, 1995; Boyle *et al.*, 2015; Kurtovic *et al.*, 2020), and opsonizing of iRBCs with antibodies, thereby preventing sequestration in small vessels and enhancing iRBC clearance (Osier *et al.*, 2014a). Moreover, opsonized erythrocyte interaction with effector cells stimulates the release of inflammatory tissue cytokines like TNF, which are toxic to the parasites. Antibodies to glycosylphosphatidylinositol (GPI) can also neutralize parasite toxins preventing the induction of excessive inflammation (Riley *et al.*, 2006; Mbengue *et al.*, 2016). Lastly, antibodies to gametocytes have also been shown to prevent the spread of infection between the host and vector (Bousema *et al.*, 2010; Healer *et al.*, 2018). The comparative significance of each of these mechanisms remains elusive.

Antibodies to *P. falciparum* antigens wane in the absence of re-exposure and re-infection (Ondigo *et al.*, 2014). This has been associated with the expansion of atypical memory B cells, which are hyporesponsive after *P. falciparum* infection; hence, impacting antibody production (Portugal *et*

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al., 2015; Ly & Hansen, 2019). The strength and durability of antibody responses against different malaria antigens are variable, and the mechanisms underlying this variability remain unclear (Akpogheneta *et al.*, 2008; King *et al.*, 2015; Julien & Wardemann, 2019). The variability is also extended to antibody concentration across populations according to exposure level. Studies have suggested that breadth of antibody reactivity to some *P. falciparum* antigens correlate with protection and are good predictors of disease outcome (Osier *et al.*, 2008; White *et al.*, 2014a; Helb *et al.*, 2015). However, the identification of humoral markers for protection has been challenging (Gray *et al.*, 2007; Valletta & Recker, 2017; Obiero *et al.*, 2019), due to the diverse kinetics of antibody responses that provide a varied set of potential immunological signatures of acquired immunity depending on the epidemiologic setting.

The eventual goal of vaccine-induced immunity to malaria is the generation of protective and long-lasting antibodies. However, considering that *P. falciparum* expresses approximately 5300 proteins (Gardner *et al.*, 2002), identifying the best antigens eliciting protective antibodies has been limited by the lack of clearly defined correlates of protection. Several prospective cohort studies have focused on proteins expressed by *P. falciparum* merozoite such as merozoite surface protein (MSP), apical membrane protein (AMA), glutamate rich protein (GLURP), and erythrocyte binding antigen (EBA), which are highly conserved (Fowkes *et al.*, 2010; Beeson *et al.*, 2016b). While other studies have investigated the highly immunogenic proteins expressed on the surface of infected red blood cells (iRBC) like erythrocyte membrane protein (PfEMP-1), Early transcribed membrane protein (ETRAMP), and Surface-associated interspaced protein (SURFIN). Majority of these studies have shown that immune responses to certain *P. falciparum* antigens are associated with higher protection against clinical malaria, with age and exposure being dependent

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factors (Gray *et al.*, 2007; Dodoo *et al.*, 2008; Nebie *et al.*, 2008; Osier *et al.*, 2008; Crompton *et al.*, 2010a; Fowkes *et al.*, 2010; Richards *et al.*, 2010; Greenhouse *et al.*, 2011; Richards *et al.*, 2013; Osier *et al.*, 2014b; Irani *et al.*, 2015). However, differences in sample size and lack of clear criteria for evaluating previous exposure have resulted in contradictory results (Fowkes *et al.*, 2010; Richards *et al.*, 2010). Furthermore, the antigen specificities of protective antibodies and CD4+ helper cells that result in protective antibody production remain unknown (Tran *et al.*, 2012).

Blood-stage merozoites invasion of erythrocytes is responsible for clinical symptoms of malaria. Therefore, an effective blood-stage malaria vaccine would make a significant contribution to lowering disease burden in endemic areas. However, the antigenic targets of malaria-protective immunity remain unknown. Several merozoite antigens have been linked to erythrocyte invasion, with some of them being identified as targets for human invasion-inhibition antibodies and antibody-dependent in vitro cellular inhibition. MSPs are involved in merozoite attachment to RBCs (Beeson *et al.*, 2016b) and MSP-1 and AMA-1 have been linked to merozoite apical reorientation prior to invasion. The EBA(175,181 and140), and Rh are present in the apical organelles and are also required for invasion (Tham *et al.*, 2012). There are several other surface proteins including MSP-2, MSP-3, MSP-4, and GLURP with no known functions (Cowman & Crabb, 2006). The majority of these antigens have genetic polymorphisms, and some of them can be classified into major allelic types. Several of these antigens are being studied or developed as potential candidates for inclusion in a blood-stage malaria vaccine (Richards & Beeson, 2009).

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Antibody responses to various polymorphic merozoite proteins have been associated to a lower risk of malaria, which helps to explain why repeated exposure is required to develop natural protective immunity. MSP-1 is a protein fragmented into 83kDa, 38 kDa, 30 kDa, and C-terminal 42 kDa (MSP-1_{42}) fragments (Koussis *et al.*, 2009). During the invasion, MSP-1_{42} is further processed into MSP-1_{19} and MSP-1_{33} fragments. MSP-1 block 2 has three allelic types, K1-like, RO33-like, and MAD20-like. Antibodies to MSP1 block 2 have been associated with a lower risk of clinical disease (Dobaño *et al.*, 2008; Fowkes *et al.*, 2010; Blank *et al.*, 2020). MSP-1_{42} and MSP-1_{19} are potential vaccine candidates and have been demonstrated to be protective in animal models (Richards *et al.*, 2009; Punnath *et al.*, 2020). The msp-2 locus of *P. falciparum* has two major allelic types, 3D7 and FC27 that are highly polymorphic. Studies have shown an association of anti-MSP-2 antibodies with reduced risk of symptomatic malaria (Taylor *et al.*, 1998; Metzger *et al.*, 2003; Scopel *et al.*, 2007; Osier *et al.*, 2010). The C-terminal region of MSP-3 (MSP-3-Ct) is highly conserved, whereas the rest of the protein is defined by two major allelic types, 3D7 and K1. Studies have indicated a reduction in symptomatic *P. falciparum* in individuals with antibodies against the MSP-3 protein with similar findings also being shown for AMA-1. (Polley *et al.*, 2007; Dodoo *et al.*, 2008; Nebie *et al.*, 2008; Amoah *et al.*, 2018).

The GLURP proteins have also been extensively studied, including GLURP R2 from the carboxy-terminal repeat region and GLURP R0 from the amino-terminal. Although GLURP R2 is the most immunodominant portion of the protein, GLURP R0 is included in the GMZ2 vaccine (Borre *et al.*, 1991; Mamo *et al.*, 2013). The GLURP R2 contains two B cell epitopes and has been shown *in vitro* to elicit antibodies capable of inhibiting malaria parasite growth in collaboration with

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monocytes (Theisen *et al.*, 1998). Antibodies to GLURP R2 are associated with reduced risk of symptomatic malaria infection (Dodoo *et al.*, 2000; Lusingu *et al.*, 2005; Adu *et al.*, 2016).

Antibodies to sexual-stage proteins can also be beneficial in blocking transmission. Pf48/45 and Pf230 gametocyte and gamete proteins have been identified as targets of transmission-blocking monoclonal antibodies that inhibit gamete fertilization in the mosquito gut (Bousema *et al.*, 2007; Bousema *et al.*, 2010; Stone *et al.*, 2018).

Cellular immunity

Malaria-induced cell-mediated immune responses can protect against both pre-erythrocytic and erythrocytic parasite stages. Immune protective functions are mediated by cells such as CD4+, CD8+, and γδ T cells through cytotoxicity, the production of effector molecules and CD4+ T cell-dependent antibody production which aids in the control of the liver and blood-stage parasite (Teirlinck *et al.*, 2011; Kurup *et al.*, 2019). Exo-erythrocytic stage parasites elicit CD8+ T cells which remove infected hepatocytes, after recognition parasite antigens are presented the MHC class I molecule, and CD4+ T cell-dependent antibody production, which prevents sporozoite invasion of the hepatocytes (Bertolino & Bowen, 2015; Heide *et al.*, 2019; Kurup *et al.*, 2019). Activated CD8+ T cells stimulate various mechanisms including cytolytic and cytokine pathways that produce cellular mediators associated with effector functions, such as IFNγ, TNF, and perforin that contribute to protective immunity. Whereas, others like granzymes and FAS-FASL mediated pathways are non-essential for the effector functions (Butler *et al.*, 2010; Halle *et al.*, 2017). The effective response of CD8+ T cells in malaria is dependent on the parasite species and natural host

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defence mechanisms (Schmidt *et al.*, 2011; Bucşan & Williamson, 2020). The pre-erythrocytic stage is short-lived, occurring for 6-7 days, which is a short time for optimal CD4+ and CD8+ responses. To be protective, CD4+ and CD8+ T cells specific to pre-erythrocyte antigens are maintained at relatively high frequency effector cells until the next mosquito bite, or they have to be able to rapidly differentiate into effector-killer cells (Schwenk & Richie, 2011).

During the blood-stage *Plasmodium* infection, iRBCs are indirectly targeted by CD4+ T helper (Th) and γδ T cells which stimulate antibody production or anti-parasitic activity of phagocytes (Moormann *et al.*, 2019). Due to the various protein signatures of the parasite at each stage, the precise CD4+ T cell responses evoked differ between pre-erythrocytic and erythrocytic stages (Nlinwe *et al.*, 2018). The current model assumes that activated CD4+ T cells of the Th1/Th2 lineages have distinct cytokine production patterns and functional capacity. The Th1 (IFN-γ) producing cells are important mediators of intracellular pathogen immunity, while the Th2 (IL-4) producing cells mediate immunity towards extracellular pathogen and are important in development of CD8+ T cell responses in the liver-stage (Mosmann & Coffman, 1989; Carvalho *et al.*, 2002). Significant regulators of inflammation during malaria are specialized CD4 regulatory T cell subsets (Tregs), either expressing FoxP3 when produced from the thymus (tTregs) or inducible T regs (pTregs) that develop regulatory function in the peripheral blood due to exposure to inflammatory stimulation (Haribhai *et al.*, 2011; Frimpong *et al.*, 2018). Studies have shown expansion of Tregs in blood-stage malaria with decreased parasite load (Hansen & Schofield, 2010). Individuals with fewer previous malaria episodes have more inflammation and greater TNF production from CD4 cells compared to those with frequent episodes, producing more IL-10 mostly by the Treg cells (Crompton *et al.*, 2014).

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Another subset of CD4+T cells (Tr1) expresses high levels of B-lymphocyte-induced maturation protein 1 (BLIMP-1) and T-bet but lacks FOXP3 and co-produces IFNγ and IL-10. The Tr1 cells have been shown to restrict protective immunity and parasite control by inhibiting humoral immune responses and perhaps lowering APCs to sustain T cell activity (Zander *et al.*, 2016). Furthermore, Tr1 cells have been associated with both increased parasite levels and lower risk of severe malaria (Boyle *et al.*, 2017). Suggesting that production of IL-10 by Tr1 cells can impair parasite control and also downregulate pro-inflammatory responses (Couper *et al.*, 2008). Therefore, both FOXP3+ Tregs and FOXP3- Tr1 cells are important regulators of anti-disease immunity (Jagannathan *et al.*, 2014a). T cells also express immune checkpoint molecules like CTLA-4, LAG-3, PD-1and TIM-3, which aid in various regulatory mechanisms (Zander *et al.*, 2015). CTLA-4 expression on Tregs has been shown to inhibit communications between Tfh cells and B cells in the germinal centre (GC) reactions, resulting in compromised humoral and cell-mediated immune responses to blood-stage malaria (Kurup *et al.*, 2017). Frequent exposure to *P. falciparum* also alters function of dendritic cells, monocytes (Stanisic *et al.*, 2014) (Turner *et al.*, 2021) natural killer cells, γδ T cells, αβ T cells, B cells and myeloid cells. Repeated exposure can also possibly result in malaria-induced T cell and B cell hypo-responsiveness, a state that has been linked to tolerance or clinical immunity to malaria.

P. falciparum malaria also induces the differentiation of circulating CD4+ T cells to CD4+ (Th1) cells and T follicular helper (Tfh) cells(Ryg-Cornejo *et al.*, 2016). The CD4+ Tfh cells are characterized by the expression of the transcription factor BCL-6, the chemokine receptor CXCR5

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and the inhibitory receptor PD-1 (Vinuesa & Cyster, 2011). The Tfh binds to parasite-specific B cells and triggers the germinal centre (GC) reaction, in which they express inducible T cell co-stimulator (ICOS) and secrete soluble mediators (IL-4 and IL-21) (Wikenheiser *et al.*, 2016). Memory B cells (MBCs), and long-lived antibody-secreting plasma cells are all aided by these mediators, which enhance antibody isotype switching, affinity maturation and somatic hypermutation in GC B cells (Victora & Nussenzweig, 2012). Tfh cells differentiate during *Plasmodium* infection and play a role in protective antibody responses that help in malaria resolution (Obeng-Adjei *et al.*, 2015; Ryg-Cornejo *et al.*, 2016).

Similarly, naïve CD4+ T cells can adopt another function programmed by RORγt transcription factor in the presence of IL-6, IL-26, and TGF-β to a T helper 17 (Th17) cell subsets, which are responsible for the production of IL-17. Th17 has been connected to the recruitment of neutrophils, which has been linked to severe malaria (Feintuch *et al.*, 2016). Th17 can also secrete IL-21, hence may contribute to supporting GC reactions and CD8+ T cell responses (Wei *et al.*, 2007). CD4+ T cells producing IL-27, which have been related to both pro-and anti-inflammatory immunological responses that play a role in reducing immunopathology in blood-stage malaria, are another CD4+ T cell subsets activated during malaria (Findlay *et al.*, 2014). During acute *Plasmodium* infection, IL-27 suppresses IL-2 production by other CD4 T cells (Kimura *et al.*, 2016).

Despite advances in research on cell-mediated immune response, there are vital questions that remain unclear such as; the distinct CD4+ Th cells and Tfh cell populations contributing to protection against repeated exposure to *Plasmodium* parasites. What role inflammation and antigen

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persistence play in malaria-induced tolerance and regulatory responses that control parasite replication observed in most individuals in endemic areas (Portugal *et al.*, 2014b). The role of T cells in immunological memory is still not well studied in humans, and most available data are based on murine models. Addressing these gaps will advance our understanding of cellular immune responses in malaria and aid in the development of effective and practical therapeutic interventions that can control and eliminate the disease. Therefore, studies on *P. falciparum* natural infection are necessary to identify correlates of immunity that can be measured in the population to show protection.

2.4.3 Role of Cytokines in malaria immunity

Cytokines are essential mediators of cellular responses that cause symptoms and pathological alterations in malaria patients (Lyke *et al.*, 2004; Awandare *et al.*, 2006; Clark *et al.*, 2008). *P. falciparum* infection triggers a variety of signaling cascades that result in the production of pro-inflammatory and anti-inflammatory cytokines. Cytokines are secreted by immune cells, they regulate immune responses, maturation, and activation of specific cells. The superfamily cytokines include interleukins (IL), chemokines, colony-stimulating factors (CSF), interferons (IFN), and the transforming growth factors (TGF) and tumour necrosis factor (TNF) families (Turner *et al.*, 2014). The chemokines are a family of chemoattractant cytokines, selectively signaling proteins secreted by cells and mediate chemotactic signals to recruit immune cells to migrate to defined infection or injury sites. The production of pro-inflammatory cytokines such as IFN-γ and TNF-α is necessary for protection; however, excessive production can result in severe disease (Borish & Steinke, 2003).

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Several cytokines have been implicated in protecting against *P. falciparum* infection. Regulation of these pro-and anti-inflammatory responses are important in influencing malaria infection outcome, leading to protection or immunopathology (Rovira-Vallbona *et al.*, 2012). A balanced and timed production of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β is required in regulation of the inflammatory response and disease outcome (Jagannathan *et al.*, 2014a).

Previous research in our group has shown that cytokine expression during acute *P. falciparum* infection differs between individuals with different levels of prior exposure to malaria, reflecting changes in anti-disease immunity and tolerance associated with varying transmission intensity (Ademolue *et al.*, 2017). In addition to exposure, age differences have also been implicated in modulating cytokine responses, with older children having lower anti- and pro-inflammatory responses as compared to younger children (Farrington *et al.*, 2017). Specific cytokine responses like IFN-γ, IFN-α, IL-12, IL-1β, IL-2, TNF-α, MIG, MCP-1, and IP-10, are associated with protection against clinical malaria (Luty *et al.*, 1999; McCall *et al.*, 2010a; Ademolue *et al.*, 2017). In contrast with others like IL-6 that correlate with increased risk of disease, while IL-5 and RANTES are crucial in severe malaria pathogenesis (Rovira-Vallbona *et al.*, 2012). These are early inflammation aimed at controlling parasite growth; however, if not tightly controlled, they can lead to immunopathology. High levels of anti-inflammatory cytokines like IL-10 and TGF-β are thus immunoregulatory responses that dampen pathogenic inflammation while enhancing anti-parasite effector mechanisms (Crompton *et al.*, 2014; Portugal *et al.*, 2014a). Dysregulated production of cytokines can also impede other immune response pathways. Studies in mouse models have shown impaired production of IFN-γ and TNF by Th1 cells hindering the

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development of Tfh cells, suppressing germinal centre reaction, and hence, impacting on the development of immunity by suppressing the production of protective antiparasitic antibodies (Qin *et al.*, 2018).

Alterations of cytokines in malaria are broad and controversial; few studies have simultaneously evaluated various cytokines during infection and convalescent phases of infection. Therefore, characterizing changes in cytokine levels in areas of varying transmission can give more insight into their role in the development of immunity.

2.4.4 Molecular mechanisms involved in immune responses to malaria

Molecular signatures of immunity are important in modelling initial host-parasite interactions and signaling pathways that lead to production of cytokines, chemokines, antibodies, cell-surface molecules and metabolites. These are the mediators involved in cellular proliferation or exhaustion and effector function that eventually shape the quantity and quality of immune response (Parkin & Cohen, 2001). *P. falciparum* natural infections evoke potent immune responses that effectively eliminate the parasite and often induce long-lasting protection against reinfection (Doolan *et al.*, 2009). For malaria, understanding the mechanisms underlying protection against natural infection can provide valid information for vaccine design. The analysis of gene expression has revealed molecular processes underlying the acquisition of malaria immunity, implying that baseline immune activation results from recent or prior cumulative malaria exposure (Hodgson *et al.*, 2019). Therefore, a closer look at the molecular signatures evoked by natural infection can be highly informative. There is a lack of a complete understanding of the molecular mechanisms

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underlying host-parasite interactions, which has slowed the development of an effective malaria vaccine (Dunachie *et al.*, 2015). Transcriptomics allows for the assessment of the several genes in parallel and hence is a powerful tool for assessing changes in the immune system in response to stimuli. Studies to date have used transcriptomic approach to understand the pathogenesis of severe disease (Krupka *et al.*, 2012), mechanism of NAI (Akdis *et al.*, 2016; Bediako *et al.*, 2016; Fowkes *et al.*, 2016; Satchwell, 2016; Talaat *et al.*, 2016; Tran *et al.*, 2016), and identify markers that help in disease diagnosis (Griffiths *et al.*, 2005). In some of these studies, children who had previously experienced a high numbers of malaria episodes had high levels of immune activation, with upregulation of genes involved in IFN and BCR signaling (Bediako *et al.*, 2019). Furthermore, recent *P. falciparum* malaria episodes have been linked to increased activation of innate and adaptive pathways in unstimulated peripheral immune cells during convalescence, with cell composition normalized to baseline (Crompton *et al.*, 2014; Portugal *et al.*, 2014a).

However, interpretation of these results has been challenging given the variations in study design, patient selection, disease definition, and method used.

2.5 SYSTEMS IMMUNOLOGY IN MALARIA

Systems immunology is an application of systems biology approaches that seek to integrate the cellular and molecular components of the immune system using computational methods (Tran & Crompton, 2020) and is ideal for identifying molecular and cellular networks of the human immune response to pathogens. The complexity of malaria immunology makes it an ideal discipline to apply the integrated systems immunology approach for defining the kinetics of the various components of the immune response during infection. Malaria immune responses involve

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complex host-parasite interactions; the innate immune responses providing immediate defense and helping direct adaptive immune cells (B and T cells) (Palm & Medzhitov, 2009). This interaction stimulates innate and adaptive immune cells to convert genes into non-coding and mRNA. The mRNA is then translated into intracellular proteins (such as signaling molecules and antigen-processing machinery), membrane-bound proteins (such as costimulatory and adhesion molecules) and secreted proteins (such as cytokines, chemokines and antibodies), which all contribute to the quality of the immune response to the parasite (Chaplin, 2010).

These complex interactions pose a challenge to defining the host immune response to malaria. Previously, studies aimed at understanding the mechanisms of acquired immunity used conventional approaches that could measure one or a few immunological parameters associated with the severity of malaria. Though these approaches have been able to give understanding to the isolated components of the host immune response to *P. falciparum*, they have not clearly defined the molecular and cellular interactions of the immune system (Moormann *et al.*, 2019). Over the years, studies seeking to comprehend changes in various malaria immune system components have given varying results. This is mainly because most studies have focused on one biological aspect in isolation, on a small group of individuals from different regions and analysing only a few parameters (Bejon *et al.*, 2009; Deroost *et al.*, 2016; Bansal & Kumar, 2018; Chaudhury *et al.*, 2020).

Recent advances in technology now allow for the high-throughput quantification of immune system molecular and cellular components. These large and complex datasets require detailed and integrated data analysis approaches to incorporate the different components of the immune system

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simultaneously (Misra *et al.*, 2019). Recently methods have involved using unbiased computational approaches like unsupervised clustering and pathway analysis to discern the crucial relationships between different immune system components (Li *et al.*, 2014). Despite the challenge of using systems immunology in natural infections, more studies have increasingly recognised the contributions of multiple components and multi-omics platforms in providing new insights into immune responses to *Plasmodium* infections. These studies have applied integration of proteomics, metabolomics, and transcriptomics to enhance understanding of immune responses to *P. falciparum* infection. More focus has been made in understanding immune profiles that distinguish different malaria disease outcomes, ages, level of exposure, and multiple genetic backgrounds. Furthermore, revealed important cell types and immune mediators; applied in predicting the immunogenicity or efficacy of vaccines in humans; defining mechanisms of vaccine stimulated protective immunity; identifying parasite specific targets that can be used as vaccine candidates and possibly predicting immune-related correlates of protective immunity in natural infection (McCall *et al.*, 2010a; Portugal *et al.*, 2014b; Quin *et al.*, 2017; Loiseau *et al.*, 2020).

Previous studies have exploited the integrated approach of systems immunology to discriminate host immune profiles based on disease outcome, such as differences in immune responses in uncomplicated vs severe or symptomatic vs asymptomatic malaria (Tran *et al.*, 2016; Cabantous *et al.*, 2017) . The initial study combined PBMC transcriptomics analysis with the analysis of flow cytometry analysis, *ex-vivo* functionality testing associated with ingenuity pathway and qRT-PCR to examine how the first febrile malaria episode affects the host immune response after the exposure parasites. The analysis was done at baseline, before the malaria season and 7 days after treatment and showed that mainly pro-inflammatory mediators prior to the first episode, but after

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the first episode anti-inflammatory cytokines were more dominant. The polyfunctional IL-10 producing CD4+ CD25+ FoxP3- T cells were also identified as cell signature, which was related with symptomatic protection in individuals exposed to *P. falciparum* (Portugal *et al.*, 2014b). A recent study compared responses in naïve, less exposed and highly exposed children in two distinct endemic areas, combining whole blood transcriptomics, cellular phenotyping, and cytokine analysis to extensively describe the effect of repeated episodes of clinical malaria on the immune system. In children, high repeated exposure was associated with neutrophils, B cells and CD8+ T cells activation, interferon-inducible gene upregulation and IL-10 anti-inflammatory cytokine compared with less exposed and naïve children (Bediako *et al.*, 2019).

System immunology has also been used to evaluate immune mechanisms in CHMI in malaria naïve individuals by assessing 84 miRNAs associated with T-cell and B-cell responses, transcriptomics analysis, immunophenotyping and antibody response. When compared to low miRNA responders, high miRNA responders had more activated B and T cells and a better *P. falciparum* specific antibody response after infection. Furthermore, the identification of miRNA expression could discriminate the ability of an individual to control parasite growth and develop antibodies and T cell responses that affect immune responsiveness and non- response variability (Burel *et al.*, 2017). Recently, another study performed interactive analysis of host metabolome and whole-blood transcriptome, to compare CHMI in naïve and malaria exposed to *P. vivax* individuals, pre- and post-infection. The effect of BTM clusters and metabolite clusters on IFN signature and innate cell activation; chemokines and T cells as well as platelet and complement gene modules were studied (Gardinassi *et al.*, 2018).

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Despite the significant progress that has been made in understanding immune mechanisms to *Plasmosium spp* infection using system immunology, comprehensive studies in malaria are still limited due to challenges of study design, sample collection, sufficient sample size, and financial cost associated with such studies. Therefore, the need for more studies applying systems immunology to further advance deciphering of the host immunity to malaria.

2.6 VACCINE DEVELOPMENT

The possibility of developing a successful malaria vaccine is supported by the fact that naturally exposed people in endemic areas develop considerable protection against clinical disease (Doolan *et al.*, 2009). Furthermore, studies of controlled human infections show sterile protection for several months in 90% of volunteers infected by irradiated sporozoites via infected mosquitoes (Clyde *et al.*, 1975; Sharma & Pathak, 2008). Malaria vaccine development requires a clear understanding of immune responses. This has been challenging because of the complex nature of the parasite with a multi-stage life cycle and significant antigenic diversity, leading to less optimal immune responses, and lack of precise immune correlates of protection; hence a major hurdle in vaccine development.

Most vaccine development strategies are the sub-unit approach that have typically targeted one or a few antigens from the distinct life cycle and whole-parasite vaccine approach (Riley *et al.*, 2013). Various vaccine candidates, focusing on pre-erythrocytic, asexual erythrocytic, sexual, and mosquito stages, have been tried; to prevent infection, reduce infection rate and reduce or prevent transmission (McConkey *et al.*, 2003; Regules *et al.*, 2014; Hoffman *et al.*, 2015; Nikolaeva *et al.*,

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2015). Currently, the emphasis is on malaria vaccines that interrupt transmission (Doumbo *et al.*, 2018). There is also a renewed focus on whole organism vaccines that have a potential of overcoming the limitations of the sub-unit approach.

2.6.1 Types of malaria vaccines

2.6.1.1 Whole organism vaccines

It involves immunisation with killed and adjuvanted *Plasmodium* parasites. The whole parasite vaccine presents a broad array of antigens to the immune system, mostly the conserved proteins between parasites. This overcomes the issues of antigenic polymorphism and immune non-responsiveness that impact sub-unit vaccines. New approaches have been pursued to develop improved killed, live attenuated and genetically modified parasites for pre-erythrocytic or blood stage malaria vaccines (Stanisic & Good, 2015).

2.6.1.2 Pre-erythrocytic vaccines

These vaccines are aimed at targeting the asexual stages of the parasite by preventing infection at the liver stage. Their mechanisms involve preventing sporozoites from invading and developing in the hepatocytes, the release of merozoites from the liver and the resulting clinical outcome. Pre-erythrocytic vaccines that are currently in preclinical or clinical trials are either whole sporozoite vaccines or subunit vaccines. *P. falciparum* Sporozoites (PfSPZ), genetically attenuated parasites (GAP), and chemoprophylaxis (CVac) are examples of whole sporozoite vaccines that are either live attenuated or live sporozoites given under chemoprophylactic treatment. Sanaria Inc produces PfSPZ, an irradiated sporozoite vaccine. The vaccine induces CD4+, CD8+, gamma delta (γδ) T

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cells, as well as antibody responses. Trials in some countries have confirmed efficacy against naturally transmitted parasites, with additional research planned to optimize the dose, incorporate additional vaccine strains, and compare efficacy (Ishizuka *et al.*, 2016; Epstein *et al.*, 2017).

GAP, on the other hand, is a genetically modified parasite vaccine, developed through deletion of genes SAP1, P52, and P36 expressed at the liver stage, and aimed to arrest parasites in the hepatocytes. In mice, it induces protection against sporozoite challenge and provides stage-transcending protection against blood-stage challenge in mice. The vaccine has gone through phase 1 trial and is safe in humans and confer sterile immunity in mice (Kublin *et al.*, 2017). The CVac is a chemically attenuated chemoprophylaxis vaccine that induces both polyfunctional T cells and antibodies. The vaccine has gone through phase 1 trials and confers long-lasting protection against CHMI (Mordmüller *et al.*, 2017).

RTS,S, the most advanced malaria vaccine, is a sub-unit vaccine, made up of the repeat region of circumsporozoite protein (CSP) fused with Hepatitis B virus surface antigen and adjuvanted with AS01 adjuvant. The vaccine has gone through phase 1-IV clinical trials and the only malaria vaccine close to licensure. Although studies have shown a limited efficacy (30-50%), pilot implementation of the vaccine was initiated in some African countries. The vaccine is designed to prevent sporozoite invasion primarily through the action of antibodies against the CSP protein, which is a major component of the sporozoite (Rts, 2014). Clinical trials have shown that protection correlates with CD4+ and CD8+ T cells responses in non-exposed individuals immunised with RTS,S, whereas in endemic areas, protection against clinical disease in immunized infants was correlated with anti-CSP antibodies (Moris *et al.*, 2018).

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Other pre-erythrocytic vaccines that have been tested and showed promising results at preclinical and early phase clinical trials include; cell-traversal protein for ookinetes and sporozoites (CelTOS), exported protein-1 (EXP-1), liver-stage antigen-1 (LSA-1), LSA-3, sporozoite threonine-asparagine-rich protein (STARP), and thrombospondin-related anonymous protein (TRAP). The limitations with these vaccines has been low antibody and T cell responses hence lower protective efficacy even at the preclinical stages. (Tucker *et al.*, 2016). To overcome these limitations, another approach taken has been to combine antigens that would elicit broad immune responses. However, most of the multiantigen pre-erythrocytic vaccines including multiepitope ME-TRAP vaccine, EP1300 multivalent DNA vaccine, L3SEPTL polyprotein vaccine, Multistage DNA vaccine operation five antigens (MuStDO5) and NYVAC-Pf7 attenuated vaccinia virus have not been successful at producing potent immune response (Hodgson *et al.*, 2015).

2.6.1.3 Blood stage (erythrocytic) vaccines

The blood stage vaccine strategies have been designed to protect against disease symptoms by producing antibodies that inactivate merozoites and/or target malarial antigens expressed on the RBC surface, thus inducing antibody-dependent cellular cytotoxicity and complement lysis; they also are intended to elicit T-cell responses that will inhibit the development of the parasite in RBCs. Blood stage vaccine strategies aim to protect from disease symptoms by producing antibodies that inactivate merozoites and/or target malarial antigens expressed on the RBC surface, thus inducing antibody-dependent cellular cytotoxicity and complement lysis; they also are intended to elicit T-cell responses that will inhibit the development of the parasite in RBCs. By reducing the exponential multiplication of merozoites, this type of vaccine primarily serves as a disease-

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reduction vaccine in endemic countries. Blood stage vaccine strategies aim to protect from disease symptoms by producing antibodies which inactivate the RBC surface merozoite invasion and/or target malarial antigens expressed to induce antibody-dependent cellular cytotoxicity and complement lysis; also, they are meant to elicit T-cell responses inhibiting parasite development in RBCs. By reducing the exponential multiplication of merozoites, this type of vaccine primarily serves as a disease-reduction vaccine in endemic countries. Blood stage vaccine development has been slowed by several obstacles including antigenic variation of parasite proteins, redundancy in both sialic acid-dependent and sialic acid-independent merozoite invasion pathways and challenges expressing conformationally correct proteins (Satchwell, 2016). Several blood stage antigens like MSP-1, MSP-2, MSP-3, and glutamate rich protein (GLURP) have been evaluated with limited success. The current vaccines at different stages of clinical trials include; chemically attenuated parasites, AMA-RON2, *P. falciparum* RH5 (*Pf*RH5) and *Pf*EMP-1_VARCSA. The chemically attenuated whole blood parasite vaccines are developed by modifying the parasites with a DNA- binding drug, after which they are used for vaccination. These vaccines induce CD4+ T cell immunity (Raja *et al.*, 2017).

The PfRH5 vaccines are intended to block merozoite invasion and prevent blood-stage infection. Preclinical studies have shown that the vaccine induces broadly neutralizing antibodies and confers protection against virulent heterologous *P. falciparum* challenge (Douglas *et al.*, 2011). The first-generation vaccine is undergoing clinical trials and more studies are being performed to improve the vaccine by identifying critical epitopes and incorporating PfRH5 immunogens into virus- like particles (Wright *et al.*, 2014).

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The AMA1-RON2L vaccine is a peptide complex of AMA1 and RON2 proteins with enhanced immunogenicity, that induces high strain-transcending blocking activity (Srinivasan *et al.*, 2014). The *Pf*EMP-1_VARCSA is a vaccine candidate aimed at preventing pregnancy associated malaria (Gbédandé *et al.*, 2017). Other blood-stage vaccines in early clinical trials include MSP3-LSP, P27A, BK-SE36, AMA-1 DiCo, PfPEBS, EBA-175 RII NG, GMZ2, and ChAd 63RH5/MVA RH5 vaccine (Salamanca *et al.*, 2019a). Most of these vaccines have been shown to be safe and able to induce low to moderate antibody response. Unfortunately, the induced immunogenicity has not been sufficient to induce sufficient protection against malaria (Arama & Troye-Blomberg, 2014). To date no blood stage vaccines have induced significant protection at phase I/II trials. Hence the need for further studies to identify and characterize new *P. falciparum* protein that can be used for functional multi-epitope and multistage approach (Salamanca *et al.*, 2019b).

2.6.1.4 Transmission blocking vaccines (TBV)

The vaccines aim at preventing transmission from mosquitoes to the humans in the sexual stage of the parasite. Pfs25, Pfs230 and Pfs47 are the current vaccines being evaluated at various stages in clinical trials. The Pf25 and Pf230 proteins are conjugate to an immunogenic carrier protein ExoProtein (EPA) to make the Pf25-EPA vaccines and administered with AS01 adjuvant. Clinical trials have shown that Pf25 vaccines can induce functional antibodies to block mosquito parasite transmission (Talaat *et al.*, 2016; Doumbo *et al.*, 2018). Development of TBV has been challenged by the vaccines not been able to achieve sufficient adaptive immune responses that maintain high antibody levels in addition to not having a broad coverage to accomplish herd immunity (Duffy & Gorres, 2020). Despite these limitations TBV are promising to provide a long-lasting solution for malaria eradication.

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Despite the fact that specific antigens predominate at each stage, Proteome analysis of the *P. falciparum* reveals that expression of key malarial antigens is not stage-specific (Florens *et al.*, 2002). A different approach should therefore be considered that combines antigens which cover more than one stage of the parasite life cycle (Wang *et al.*, 2005; Porter *et al.*, 2011). Several methods have been explored in developing a multi-antigen and multi-stage malaria vaccine targeting all the 4 stages of *P. falciparum* life cycle for production of variable antigen-specific protective antibodies, CD4+ and CD8+ T cells (McConkey *et al.*, 2003; Ewer *et al.*, 2013). The most difficult aspect of developing a multistage vaccine is determining the ideal antigen combination.

To date, RTS, S is the most advanced vaccine, has limitations regarding its protection mechanism and efficacy wanes rapidly (White *et al.*, 2015). Other vaccines in trials though promising they have not been able to mount sufficient immune responses to protect against human malaria. These inconsistencies in estimated protection are due to varied reasons like the rate at which natural immunity develops being dependent on the intensity and stability of *P. falciparum* exposure, with immunity to clinical disease developing faster in high transmission areas (Gupta *et al.*, 1999a; Marsh & Kinyanjui, 2006). Therefore, better understanding of the dynamics and longevity of the immune response will greatly accelerate vaccine development.

INTEGRI PROCEDAMUS

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3 CHAPTER THREE: SPECIFIC OBJECTIVE ONE

3.1 PLASMA CYTOKINE AND CHEMOKINE SIGNATURES DIFFERENTIATE CHILDREN WITH DIFFERENT LEVELS OF PRIOR MALARIA EXPOSURE DURING ACUTE INFECTION BUT NOT CONVALESCENCE

Hypothesis: *Cytokine responses during P. falciparum infection would be quantitatively and qualitatively different between children living in areas with high and low P. falciparum transmission intensities.*

3.1.1 Abstract

Background: In naturally exposed individuals, cytokines are crucial in anti-malaria immunity or tolerance. These cellular mediators change rapidly upon exposure to *P. falciparum* infection resulting in symptoms and pathological alterations that determine the disease severity. Variations in exposure caused by geographical differences in transmission intensity influence the rate of acquisition of natural immunity. As a result, it is critical to clearly define cellular immune components that contribute to susceptibility or tolerance that are affected by changes in malaria transmission intensity. The purpose of this study was to investigate cytokine and chemokine responses during the acute and convalescent phases of *P. falciparum* infection in children exposed to different levels of malaria transmission.

Methods: The plasma levels of 25 cytokines and chemokines were compared in children aged 5-14 years who presented to hospital with malaria. The children were recruited from two regions in

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Ghana, Accra (low transmission) and Kintampo (high transmission), and were studied during infection (D0) and convalescent period (D7 and D21). Interleukins (IL), interferons (IFN), Tumor necrosis factor (TNF), IFN-γ-inducing protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), and Monokine induced by (IFN-γ) (MIG) concentrations were measured using Luminex®-based magnetic bead array system. Cytokine levels were compared during infection and convalescent phases and between the two areas, and further correlated with parasite level and interaction with each other in the two areas.

Results: During acute *P. falciparum* infection Pro-inflammatory responses driven by IL-6, IFN-γ, IFN-α cytokines and MIG, MCP-1, and IP-10 chemokines, as well as anti-inflammatory responses mediated by IL-10, IL-2R and IL-1RA were induced. These cytokines and chemokines were significantly higher in children from the low transmission areas compared to those from high transmission areas during the acute infection phase compared to the convalescence phase. Only IL-6, IFN-α, IL-1RA, and MCP-1 significantly correlated with parasitemia levels. Analysis of correlation network revealed a signature that distinguished individuals from the low transmission area. Of interest, a cytokine signature dominated by IL-10 and IL-1RA associated with asymptomatic parasitemia was identified in a subset of children from the high transmission area who had detectable parasitemia at D21 after diagnosis and treatment.

Conclusion: The findings of this study show different regulation ofcytokine responses during active malaria infection (D0) between individuals with varying levels of prior exposure. Individuals from low transmission settings had elevated levels of pro-inflammatory cytokines, suggesting low immune tolerance compared to individuals from high transmission area. However, these differences are temporary and do not persist after treatment (D7 and D21), and they may

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indicate that individuals with higher levels of *P. falciparum* exposure develop a certain level of tolerance to the parasites when compared to individuals in low transmission area.

3.1.2 Introduction

Pro- and anti-inflammatory cytokines play an important role in malaria presentation and outcome, by mediating cell to cell communication, recruiting immune cells to the site of infection and driving the required effector mechanisms (Griffith *et al.*, 2014; Clark & Vissel, 2017). The regulation of these inflammatory responses is a major mechanism for anti-disease immunity or tolerance in naturally exposed individuals (Ademolue *et al.*, 2017). Thus, cytokines are expected to change rapidly on exposure to malaria parasites and may reflect changes in disease tolerance associated with varying transmission. Prior studies of *P. falciparum* infections have suggested an early pro and anti-inflammatory cytokine response associated with protective immunity, including tumour necrosis factor (TNF), interferon gamma (IFN-γ), and interleukin-10 (IL-10) (Lyke *et al.*, 2009). Cytokines like IL-6 have been shown to correlate with risk of clinical malaria, while others like IL-5 and RANTES are important in the pathogenesis of severe malaria (Robinson *et al.*, 2009; Lourembam *et al.*, 2013). This implies that a crucial regulation of inflammatory responses exists during infection to avoid severe disease. Changes in cytokines and chemokine levels during *P. falciparum* infection are broad and controversial; only a few studies have simultaneously evaluated various cytokines during infection and convalescent phases of infection(Rodrigues-da-Silva *et al.*, 2014). Characterizing changes in cytokine levels in areas of varying transmission may give more insight on mediators that are affected by change in transmission and their role in immunity development.

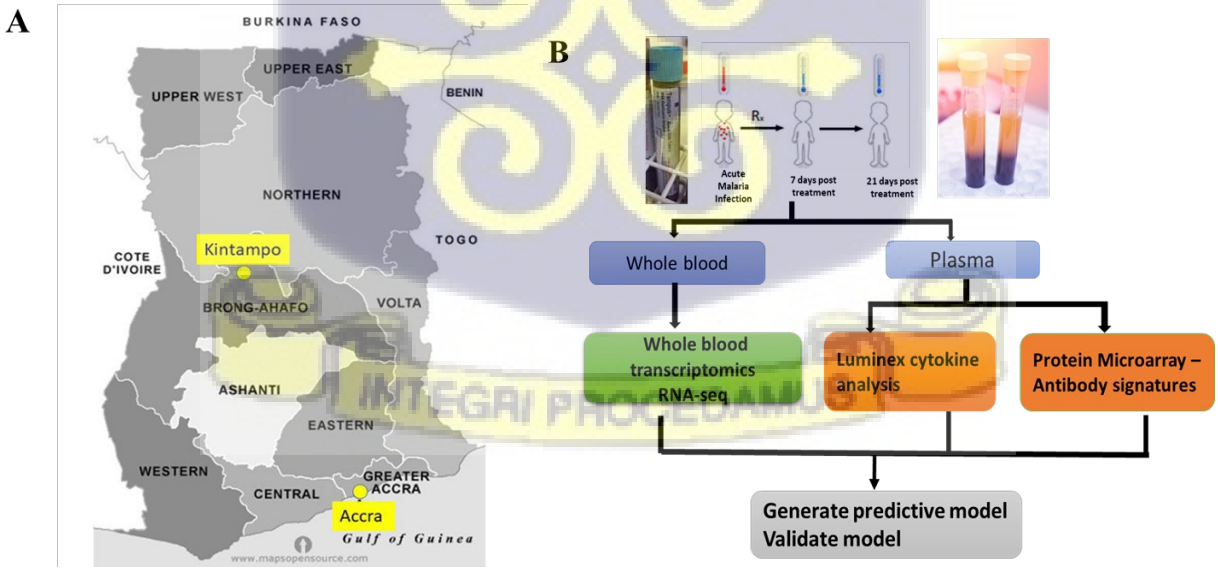
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This study assessed changes in cytokine and chemokine responses in children exposed to different intensities of malaria transmission during acute and recovery phases of *P. falciparum* infection.

3.1.3 Methods

3.1.3.1 Study design and site

This was a longitudinal study of children aged 5-14 years with acute *P. falciparum* from two regions with distinct malaria transmission intensity in Ghana (Kintampo Health Research Centre, Kintampo, with high transmission intensity, and Ledzokuku-Krowor Municipal Assembly Hospital (LEKMAH) in Teshie, Accra (Greater Accra Region), with low transmission). Figure 3.1A is a map showing the location of the sites in Ghana, West Africa. The sites were chosen based on the intensity of local malaria transmission as measured by the entomological inoculation in Kintampo (>250 infective bites/person) and Accra (<50 infective bites/person) per year (Klinkenberg *et al.*, 2008; Dery *et al.*, 2010).



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Figure 3. 1: Overview of study sites, design and analysis.

A) Map of Ghana with highlighted study areas. Accra (low transmission area) and Kintampo (High transmission area). B) Workflow of the study design and analysis.

3.1.3.2 Ethical considerations

Participation in the study was voluntary, and informed consent was obtained from parents or guardians before enrolling participants in the study. Ethical considerations were reviewed and accepted by the Ghana health service (GHS-ERC:002/08/17), Noguchi ethical review committee (008/17-18), and Kintampo Health Research Centre review committees (IEC- 2017-28 and SSC-khrc/adm/2017-11).

3.1.3.3 Participant recruitment

Children between 5-14 years presenting at the hospitals with acute *P. falciparum* infection, and confirmed by a positive rapid diagnostic test (RDT) and Giemsa-stained thin blood smear were recruited for the study, after obtaining informed consent. One hundred and twenty-one children were recruited from both sites: 60 participants from Kintampo District Hospital (KDH) and 61 from Ledzokuku-Krowor Municipal Assembly Hospital (LEKMAH). Blood samples were taken at recruitment: day 0 (D0) and at two follow up timepoints: day 7 (D07) and day 21 (D21). Additionally, clinical, haematological and parasitological data were collected at each sampling time point. In all cases, treatment was initiated on D0, and all participants had no parasites and malaria symptoms by Day 7, this was confirmed by negative RDT and Giemsa-stained blood smear. Acute malaria was defined as infection with *P. falciparum* parasite accompanied by fever and any two other malaria symptoms.

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3.1.3.4 Clinical and hematological indices testing

Hematological parameters such as haemoglobin levels, erythrocyte indices [red blood cell (RBC) counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and red cell distribution width (RDW)], leucocyte indices [total white blood cell (WBC) count, lymphocytes count (LYM), monocytes count (MON), neutrophil count (NEU), and platelet indices [platelet count (PLT), mean platelet volume (MPV), and platelet distribution width (PDW)] were measured using Systemex and Abacus analyzers. Hemoglobin typing for sickle cell traits was performed using high-performance electrophoresis and sickling tests. Parasitemia was determined using thick and thin blood films for malaria microscopy and real-time PCR for both genus and *P. falciparum* as previously described (Kamau *et al.*, 2011). A brief focused history was conducted with parents/guardians to obtain clinical and epidemiologic information, including age, the community of residence and duration, history of illness, the number of malaria cases the child had in a year and socioeconomic status.

3.1.3.5 Geospatial data collection

Additional consent was sought from parents/ caregivers to collect geospatial data on each participant. This included Geographic information system (GIS), spatial video and spatial video geo-narratives (SVG), used to assess the malaria endemicity and environmental malaria risk factors. This information was collected using GPS embedded cameras to obtain environmental coordinates and visuals in each identified location. The SVG method included collecting both videos and interview narratives simultaneously as the participants made spatial reference to specific items in the environment. This data was further used to generate maps with defined

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boundaries for the two study areas. Reported malaria cases in our study were mapped to the specific locations on the map.

3.1.3.6 Luminex Cytokine detection

A total of 25 soluble plasma mediators including; interleukins (IL)-1β, IL-1 receptor antagonist (IL1-ra), IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, interferon (IFN)-γ, (IFN)-α and tumor necrosis factor (TNF-α) as well as chemokines IL-8/ CXCL 8, eotaxin 1/CCL11, IFN-γ-inducing protein 10 (IP-10/CXCL10), monocyte chemotactic protein-1 (MCP-1/CCL2), Monokine induced by (IFN)- γ (MIG), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory proteins 1a (MIP-1α/CCL3) and MIP-1β/CCL4 were measured using a Human Cytokine Magnetic 25-Plex Panel kit (Novex, Life Technologies, Carlsbad, CA, USA). The assay was performed according to the manufacturer's instructions. Briefly, 25 µL of antibody-coated magnetic beads were added to each well and washed. Incubation buffer (50 µL) with 100 µL of diluted samples, standards and blanks were added to respective wells and plates incubated overnight under agitation on an orbital plate shaker at 2ºC to 8ºC. Plates were then washed, and 100 µL of biotinylated detection antibody mixture added to each well and incubated for 1 hour at room temperature. Finally, 100 µL of diluted streptavidin- Phycoerythrin solution was added to each well and after 30 minutes, the beads were washed and re-suspended in 150 µL wash solution and analyzed using MAGPIX system (Luminex Corporation, Austin, TX, USA) and xPONENT software. A minimum of 100 beads per region was analyzed. A curve fit was applied to each standard curve (% PL logistic weighted) and sample concentrations

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interpolated from the standard curve. Cytokines below detectable range (where we had more than 80% of values in participants below the lower detection limit) were excluded from further analysis (IL-5, IL-7, IL-17, and GM-CSF). There were no cytokine measurements above the upper detection limit of the assay used.

3.1.3.7 Statistical analysis

The Kruskal-Wallis non-parametric test was used to examine the time point differences. If significant differences were found, the Mann-Whitney non-parametric test was used to compare the two sites, and p-values were adjusted for multiple testing by the Benjamini–Hochberg method. When less than 0.05 after adjustment, all p-values were considered statistically significant. Spearman's correlation was used to identify potential correlation between cytokine levels and parasite density. All statistical tests were performed using Prism v.6 (GraphPad Software Inc, San Diego, CA, USA) and R version 3.0 (4.0.2). All p-values less than 0.05 were deemed statistically significant.

3.1.3.8 Cytokine network analysis

Correlation networks were generated by analysing the relationship between cytokine levels that were significant. To investigate multivariable relationship, a weighted partial correlation network was built as a force-directed graph for cytokines that were significantly different between the two areas or across different times. Using igraph package in R software, the Fruchterman-Reingold modelling algorithm was used to graph force-directed networks, with each node representing a cytokine and each edge the correlation between the connecting nodes. Edges attracted node pairs with spring-like forces that reflected partial correlations, whereas nodes behaved like atomic particles and naturally repulsed each other. The Spearman rank correlation coefficient was used to

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test for correlation and p-value of 0.05 was used as a cut-off. Strongly correlated nodes are close to one another. Only edges with rho >0.5 were displayed in the network for ease of interpretation, and all the edges with rho < 0.5 were zeroed.

3.1.3.9 Cytokine modular analysis

To identify cytokine modules and assess their clinical phenotype association, we used a modular-based approach called CytoMod (Cohen *et al.*, 2019). Using a data driven clustering approach, and based on their pairwise correlation using an unsupervised hierarchical clustering algorithm, this approach classified cytokines into putative functional modules. Modules were formed over absolute and adjusted cytokine levels. To identify co-signalling cytokines, modules were also compared across datasets. The pearson correlation coefficient across all subjects for each area was used to determine the similarity of each cytokines pair. We generated cytokine pairwise similarity matrices for each of the transmission areas. Cytokine concentrations were adjusted by regressing all subject levels against the mean cytokine concentration.

3.1.4 Results

3.1.4.1 GIS mapping of malaria cases in the study sites

GIS mapping was used to show the distribution of reported malaria cases in the study areas (Figure 3.2). Since the areas did not have defined boundaries, artificial boundaries were created to help in defining cases. We compared malaria cases reported between 2017 – 2019 from our study areas (Figure 3.2). A comparison of malaria cases between the two study areas indicated a higher number of reported cases in the high transmission area than in the low transmission area. However, it is important to note that the high transmission site is a rural area with a limited number of health care

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facilities; while the low transmission area is a city with more health care facilities, limiting the number of individuals who report to a particular facility. Generally, however, from the proportions of malaria reported cases in the two areas and reports of malaria incidence in Ghana, we were reasonably confident that there is a higher incidence of malaria in the high transmission area compared to the low transmission area.

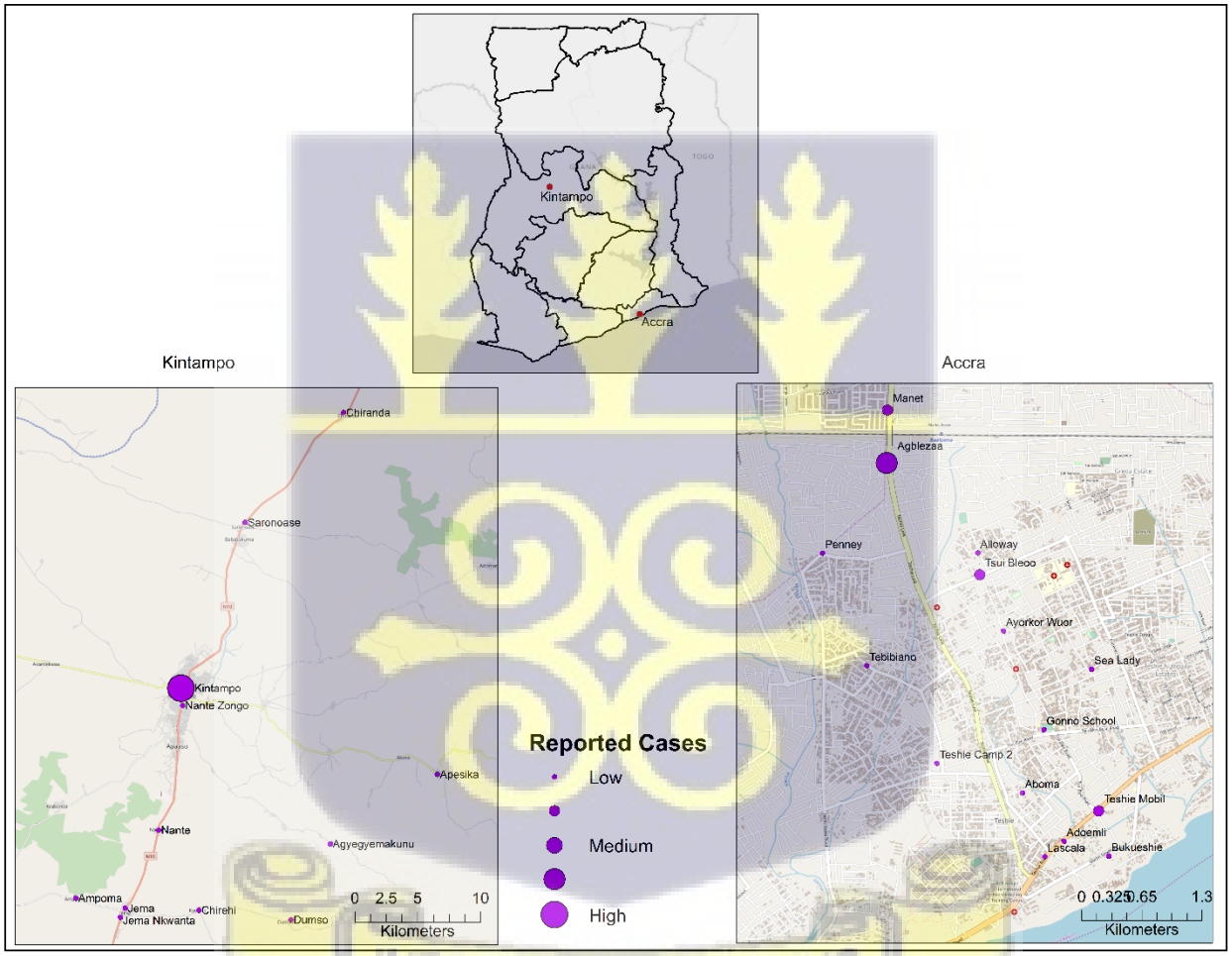


Figure 3. 2: Mapped malaria cases in the study areas.

Represents a map of our study areas, Kintampo-High transmission, and Accra-Low transmission. The purple dots represent locations where the patients indicated to be their homes and the size of dots indicates the malaria reported cases in that location.

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3.1.4.2 Patients' demographics and haematological data

1.1.1.1 Patient Demographics

A total of 121 patients were recruited for the study, including 61 participants from Ledzokuku-Krowor Municipal Assembly Hospital (LEKMAH) in Teshie, Accra, and 60 from Kintampo District Hospital (KDH). Whole blood samples were collected in Ethylenediamine tetra acetic acid (EDTA) tubes on days 0, 7, and 21. The EDTA tube samples were used to perform Full Blood Count (FBC) analysis, thick and thin blood films for malaria microscopy, sickling test analysis, and malaria real time Polymerase chain reaction (RT-PCR). The second whole blood sample collected in the Acid Citrate Dextrose (ACD) tube was processed to separate the plasma for cytokine and antibody profiling, and the buffy coat for PBMC isolation. General characteristics of patients recruited are summarised in Table 3.1. The male to female ratio was comparable in the 2 study sites. We aimed to recruit children of similar age from each site; however, this was not possible since the majority of children with malaria that reported to the high transmission area with malaria were significantly younger than those who reported to the low transmission area. The mean age of study participants recruited was 7.0 (5-10) years and 10.0 (5-15) years, from high and low transmission areas respectively. We suspect that this reflects the acquisition of immunity to malaria, which likely occurs much earlier in high transmission areas than in low transmission areas (Mensah-Brown *et al.*, 2017). For the sickle cell trait, 95 % and 99 % of children were negative for the trait in high and low transmission areas respectively. Children from the high transmission (82 %) had normal haemoglobin (Hb) type (AA) compared to 90 % from the low transmission area. We also observed other Hb variants (CC, AC, and AS) in a small proportion of the participants (12 %) in the high transmission area and 10 % in the low transmission area). Children

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from the high transmission areas (53 %) had more malaria episodes in a year (2- 4 times) than those in the low transmission areas where 54 % of children had only one episode per year. Parasite density by microscopy was not significantly different in the two transmission sites (p=0.2724).

Table 3. 1: General characteristics of the study population

Characteristic	**Kintampo**	**Accra**	**P value**
Sample analysed	**60**	**61**	NA
Mean Age (Range)	7.00 (5-10) yrs	9.5(5-15) yrs	< 0.0001
Gender (M:F)	63/37	57/43	
Mean Temperature (Range)	37.35 (35.4-39.60)	38.3 (34.7-41.0)	< 0.0001
Episodes on malaria in a year	2 to 4 times	1	
Parasite Density (P/ul)-Range	105,935p/ul (315-804,600)	42,608p/ul (111-336,000)	0.2724
Hb Type (%)			
AA	82%	90%	
AS	4%	2%	
AC	12%	5%	
CC	0	2%	
Mean Hb levels (Range) g/dl	9.9(4.6-12.4)	11.4 (6.7-13.6)	< 0.0001
Parasitemia at D21	5	0	
Coinfections	24	17	

The screening was done using Malaria rapid diagnostic test (RDT) kit and microscopy. Parasite density determined by microscopy and PCR were categorised into Low (1-10,000parasites/µL),

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Medium (>10,000 – 100,000) and High (>100,000). The results showed that 77% of the children from low transmission areas had parasitemia of >10,000 parasites/µL compared to 68% for high transmission areas. However, more participants from the high transmission area had parasitemia of >50,000 parasites/µL than those from the low transmission area (Adjusted p-value = 0.044). (Figure 3.3).

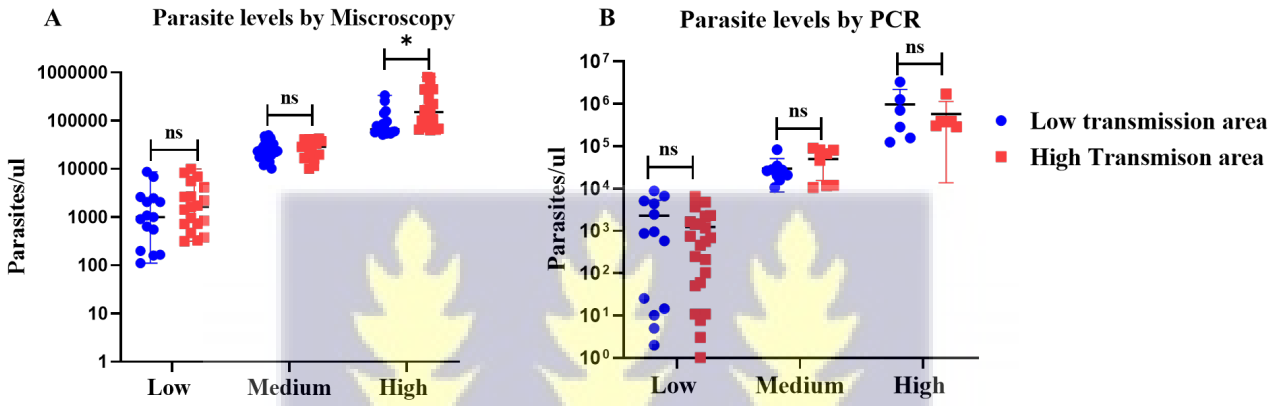


Figure 3. 3: Parasite density distribution in the study population based on A. microscopy and B. Real-time Polymerase chain reaction (PCR).

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Hematological indices

Table 3. 2: Hematological values in the study population

Hematological Indices	High endemic area				Low endemic area				Ref Range	High Vs Low		
	Medians (IQR)			p value Kruskal-Wallis test	Medians (IQR)			p value Kruskal-Wallis test		Mann-Whitney U test		
	Day 0 n=51	Day 7 n=34	Day 21 n=38		Day 0 n=61	Day 7 n=49	Day 21 n=52			Day 0 Adj p value	Day 7 Adj p value	Day 21 Adj p value
White Blood Cell (WBC) Indices												
White Blood count (WBC) (10^9/L)	6.9(5.5-9.2)	7.4(5.8-8.3)	7.2(5.6-9.1)	0.945	7.3(5.9-9.3)	6.3(5.1-9.2)	5.4(4.5-6.7)	<0.001	4.0 – 10.0	0.873	0.873	0.105
Monocytes (MON) (10^3/µL)	0.9(0.5-1.2)	0.65(0.5-0.8)	0.6(0.5-0.8)	0.012	0.6(0.4-0.9)	0.7(0.5-1.1)	0.5(0.4-0.7)	0.039	0 – 0.7	0.036	0.708	0.708
Neutrophils (NEU) (10^3/µL)	3.9(2.2-6.3)	2.7(1.8-3.3)	2.7(1.8-3.3)	0.004	5.5(4.3-6.6)	2.8(2.0-3.7)	2.0(1.5-2.5)	<0.001	1.5 – 7.0	0.345	0.223	0.223
Lymphocytes (LYM) (10^3/µL)	2.0(1.5-3.1)	3.6(2.8-4.5)	3.6(2.6-4.3)	<0.001	1.1(0.7-1.6)	2.7(2.2-3.8)	2.7(2.3-3.3)	<0.001	1.0 – 3.7	0.028	0.032	0.041
Red Blood cell Indices (RBC) Indices												
RBCs (10^6/ ul)	3.8(3.5-4.4)	4.0(3.3-4.4)	4.2(3.8-4.5)	0.281	4.5(4.1-4.8)	4.1(3.7-4.3)	4.4(4.1-4.7)	<0.001	2.5 – 5.5	<0.001	0.080	0.037
Hemoglobin (HGB) (g/dl)	10.5(8.7-11.2)	10.1(8.9-11.05)	11.2(10.2-11.6)	0.005	11.6(10.6-12.5)	10.7(9.4-11.3)	11.4(10.7-11.8)	<0.001	13.5 – 18.0	<0.001	0.213	0.271
Hematocrit (HCT) (%	32.0(13.5-37.5)	31.4(20.3-39.5)	33.9(25.1-39.6)	0.001	35.4(20.3-41.6)	32.1(21.4-38.4)	33.9(27.4-40.8)	0.001	36-53	<0.001	0.397	0.421
MCV(fL)	79.0(60.0-89.0)	78.0(60.0-94.0)	81.0(62.0-95.0)	0.310	79.7(64.4-93.4)	80.4(64.2-96.3)	81.9(63.9-95.6)	0.790	77-100	0.398	0.557	0.543
MCH(pg)	26.7(18.5-33.6)	25.8(18.8-29.5)	26.6(19.0-31.4)	0.710	25.9(16.8-30.3)	25.9(19.5-29.3)	26.2(19.8-29.6)	0.960	25-35	0.212	0.677	0.161
MCHC(%)	33.7(26.1-40.1)	33.0(30.7-35.4)	33.1(30.9-35.4)	0.030	32.5(25.6-35.3)	32.4(29.1-34.3)	32.0(29.1-34.3)	0.790	32-36	<0.001	0.003	<0.001
Platelet Indices												
Platelet (10^9/L)	141(93-207)	357.5(316.5-446)	264.5(199.3-316.5)	<0.001	82(113-163.5)	393(338.5-462.6	280.5(246.5-374	<0.001	150.0 – 450.0	0.045	0.384	0.082
MPV(fL)	8.3(6.8-11.2)	7.9(5.5-9.9)	8.3(6.4-10.7)	0.004	7.9(3.8-24.6)	6.8(3.4-11.2)	7.3(3.7-12.5)	0.400	6.5-10.0	0.748	0.081	0.174

* Kruskal-Wallis test was used to compare medians for the different time points (a), and the Mann whitey test used to compare medians between the two sites (Accra and Kintampo). Bonferroni-Dunn's test was used to adjust for multiple comparisons.

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3.1.4.3 Changes in hematological indices in children from different transmission intensities during infection and convalescence

Changes in White blood count (WBC), Red blood count (RBC), and Platelet indices were compared in children from both the high and low transmission areas (Table 3.2). Some of the indices found to be significantly changing included monocytes, neutrophils, lymphocytes, RBCs, haematocrit (HCT), and platelets. Though monocytes levels were within normal ranges in the low transmission area (median count = 6.0 $x10^2/\mu L$) during infection and (median count of 7.0 $x10^2/\mu L$ at day 7 and 5.0 $x10^2/\mu L$ at day 21) convalescence, the comparison over the different time points was significantly different (P=0.039). The high transmission area had significantly increased levels of monocytes during infection compared to convalescence (P= 0.012 for comparison across the different time points). Levels between the two areas also varied significantly (adjusted P = 0.036) as shown in Table 3.2 and Figure 3.4A. Neutrophils were significantly increased during infection compared to convalescence (high transmission area comparison across time points, P = 0.004, median count D0=3.9 $x10^3/\mu L$, D7=2.7 $x10^3/\mu L$ and D21=2.7 $x10^3/\mu L$ and the low transmission area P = <0.001, median count D0= 5.5 $x10^3/\mu L$, D7=2.8 $x10^3/\mu L$ and D21=2.0 $x10^3/\mu L$). The levels were also significantly different between the two transmission areas during infection (D0) (P=0.038, Figure 3.4B). However, these levels were still within the normal acceptable ranges.

Lymphocytes, on the other hand, were significantly decreased during infection in individuals from both transmission areas in comparison to after treatment (High transmission area p-value < 0.0001, median count D0=2.0 $x10^3/\mu L$, D7=3.6 $x10^3/\mu L$, D21=3.6 $x10^3/\mu L$ and low transmission area p-value <0.0001, median count D0=1.1 $x10^3/\mu L$, D7=2.7 $x10^3/\mu L$ and D21=2.7 $x10^3/\mu L$, Table 3.2 and figure 3.4C). Interestingly lymphocyte levels were consistently significantly higher in the high

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transmission area during infection and after treatment (D0(P<0.028), D7(P=0.032) and D21(P=0.041)). HGB and HCT levels were significantly higher during infection than after treatment. However, the levels for both were below normal ranges. The HGB median levels for the high transmission area was D0=10.5g/dL, D7=10.1g/dL and D21=11.2g/dL (P=0.0054), and D0=11.6g/dL, D7=10.7g/dL and D21=11.4g/dL(P=0.0001) for the low transmission area (Table 3.2). The levels were significantly different in children from the two areas (P = 0.001). The HCT median levels for the high transmission area D0=32.0%, D7=31.4% and D21=33.9%, P = 0.001 and D0=35.4%, D7=32.1% and D21=33.9% (P=0.0007) for the low transmission area (Table 3.2). During infection, HCT was significantly higher in the low than the high transmission (P<0.0001).

Platelet levels were decreased below normal levels in children from the two areas (P<0.0001), Table 3.2.) and did not seem to be affected by transmission intensity. The platelets were also significantly lower than the normal ranges in both the high and low transmission areas (P<0.0001), with median levels of 141 x10^9/L and 83 x 10^9/L, respectively (Table 3.2). There were, however, no significant differences between the two transmission areas during infection and after.

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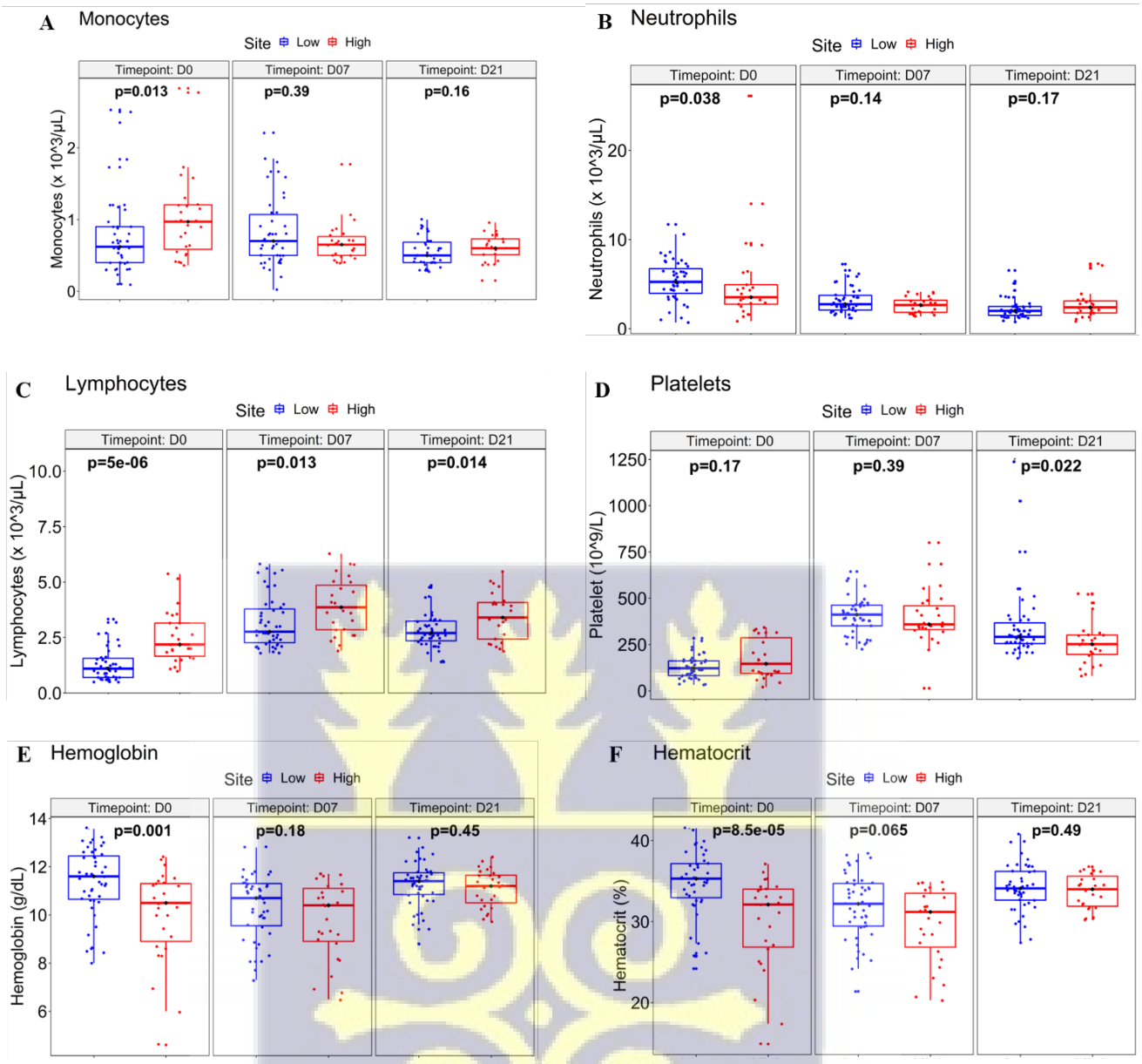


Figure 3. 4: Changes in haematological indices.

Plots are stratified by time point and grouped by site. Plots show median and interquartile range; (A) Monocytes, (B) Neutrophils, (C) Lymphocytes, (D) Platelets, (E) Haemoglobin, and (F) Haematocrit

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Table 3. 3:Descriptive statistics for cytokine levels in plasma samples.

Cytokine/ Chemokine		Low endemic area				High endemic area				Low Vs High		
		Medians			Kruskal-Wallis test (a)	Medians			Kruskal-Wallis test (a)	Mann-Whitney test (b)		
		Day 0	Day 7	Day 21	P value	Day 0	Day 7	Day 21	P value	P value Day 0	P value Day 7	P value Day 21
Pro-inflammatory Cytokines	IL-1b	27.95	23.44	23.49	0.666	22.81	19.39	22.45	0.874	0.854	0.992	0.962
	IL-2	20.91	14.38	14.25	0.192	13.64	9.726	14.32	0.695	0.07	0.045	0.664
	IL-5	10.12	10.12	10.12	0.585	10.01	9.567	10.12	0.776	0.064	0.156	0.487
	IL-6	118.1	13.3	11.73	<0.001	74.61	14.62	16.83	**<.001**	**0.031**	0.786	0.132
	IL-7	18.97	18.97	18.97	0.083	18.97	18.97	18.97	0.359	0.083	1	0.122
	IL-12	477.8	550.2	532.6	0.976	482	498.7	540.7	0.496	0.922	0.797	0.469
	IL-15	154.3	104.8	94.5	**0.022**	115.9	69.31	104.8	0.143	0.111	0.261	0.97
	IFN-g	82.68	45.94	46.61	**0.022**	39.7	37.66	42.07	0.999	**0.009**	0.588	0.634
	IFN-α	152.8	82.58	80.26	**<.001**	108.7	79.05	92.87	**0.028**	0.021	0.89	0.654
	TNF-α	19.21	15.42	14.78	0.265	17.29	23.42	20.72	0.951	0.887	0.149	0.117
Pro-Inflammatory chemokines	MIP-1α	93.93	75.38	68.72	0.237	112.7	101.2	124.9	0.586	**0.033**	0.189	**0.013**
	MIP-1β	306.5	255.6	208	0.068	404.8	250	303.7	0.314	0.137	0.435	**0.022**
	MCP-1	230.2	176	164.9	**0.001**	200.3	160.7	184.3	0.114	0.387	0.717	0.197
	MIG	740.3	356.5	302.3	**<.001**	460.7	268.8	270	**<.001**	**0.03**	0.755	0.415
	IL-8	51.01	44.45	42.04	0.288	59.92	43.22	48.47	0.447	0.963	0.633	0.284
	GM-CSF	4.094	3.944	3.845	0.235	3.914	3.674	3.914	0.439	0.605	0.557	0.79
	IP-10	91.01	20.37	16.03	**<.001**	60.62	15.02	15.7	**<.001**	**0.005**	0.142	0.667
	RANTES	3746	5518	5325	**0.001**	4796	6100	5356	0.319	0.167	0.532	0.777
	Eotaxin	23.22	29.72	29.61	0.234	18.44	22.55	28.32	0.228	0.755	0.463	0.963
Ant-inflammatory cytokines	IL-10	454.7	36.77	26.39	**<.001**	258.1	26.16	27.39	**<.001**	0.082	0.8	0.74
	IL-4	66.5	62.83	62.83	0.488	62.83	62.83	62.93	0.626	0.508	0.192	0.963
	IL-13	25.77	22.89	23.31	0.769	24.96	21.64	22.48	0.628	0.804	0.941	0.698
	IL-17A	29.89	29.89	29.89	0.871	29.89	29.89	29.89	0.647	0.515	0.35	0.029
Cytokine Receptors	IL-1RA	1909	532.2	503.5	**<.001**	1173	498.3	522.7	**<.001**	0.06	0.985	0.405
	IL-2R	960.6	688.7	630.1	**0.005**	1081	627.6	713	**0.019**	0.715	0.84	0.23

*Kruskal-Wallis test was used to compare median levels for the different time points. p-values were adjusted for multiple testing using the Benjamini–Hochberg approach (a), and Mann whitey U test (b) used to compare medians between the sites (Low and High) transmission areas.

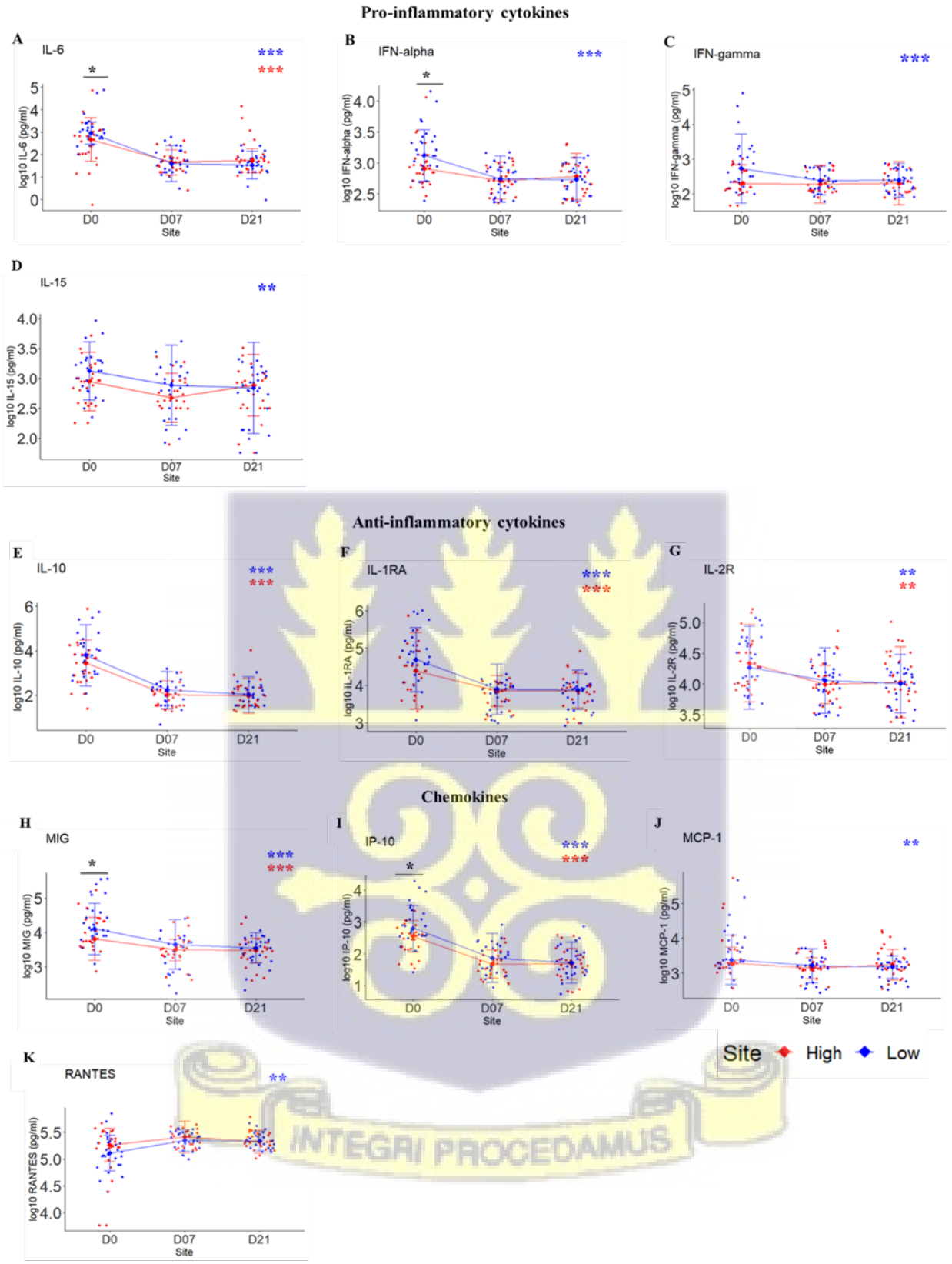
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3.1.4.4 Magnitude of plasma cytokine and chemokine response to acute malaria infection differs between individuals living in high and low transmission areas.

As expected, we observed significant changes in plasma cytokine levels throughout the infection period in both groups (Table 3.3). The majority of cytokines were at their highest at Day 0 (coinciding with detectable parasites) and declined over the next two time points after treatment, except for RANTES/CCL5 which had a contrary pattern (P=0.001). Notedly, children from the low transmission area demonstrated a more dynamic cytokine response compared to those from the high transmission area. Out of the 25 cytokines analyzed, 11 (IL-6, IL-15, IFN-γ, IFN-α, MCP-1, MIG, IP-10, RANTES, IL-10, IL-1RA and IL-2R) were found to change significantly over the time course among individuals from the low transmission group (P<0.001, P= 0.022, P= 0.022, P<0.001, P=0.001, P<0.001, P<0.001, P=0.001, P<0.001, P<0.001 and P=0.005) respectively. However, only 7 (IL-6, IFN-α, IL-10, IL-1RA, IL2R, MIG and IP-10) of these cytokines changed over the same time course among the high transmission group (P<0.001, P=0.028, P<0.001, P<0.001, P=0.019, P<0.001 and P=0.005) respectively. The magnitude of pro-inflammatory cytokines: IL-6 (P=0.031), IFN-γ (P=0.009) and IFN-α (P=0.021) (Figure3.5A-C), immunomodulatory cytokine: IL-1RA (P=0.06) (Figure 3.5F) and the chemokines: MIG (P=0.03) and IP-10/CXCL10 (P=0.005) were higher in LT areas (Figure 3.5 H-I). Overall, our results reveal that plasma cytokine levels change significantly due to *Plasmodium* infection. However, individuals living in a high transmission area demonstrate a degree of hypo-responsiveness, mounting a less robust cytokine response than those in a low transmission area.

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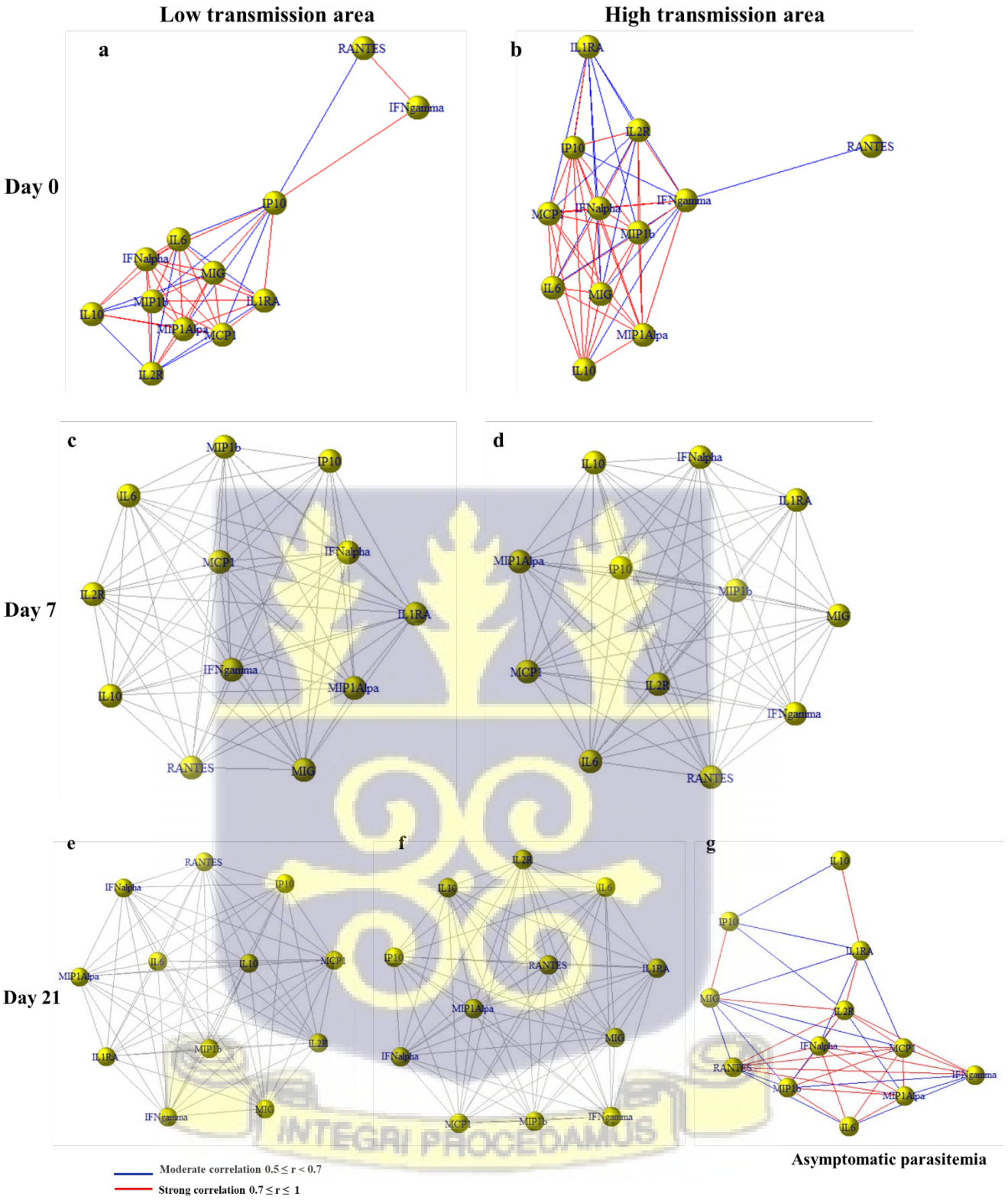
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Figure 3.5: Pro and anti-inflammatory cytokines distinguishing between high and low transmission areas.

Comparative analysis of cytokines, [(A) IL-6, (B) INF-γ, (C) IFN-α, (D) IL-15, (E) IL-10, (F) IL-1RA, (G) IL-2R, (H) MIG, (I) IP-10, (J) MCP-1, (K) RANTES, during acute infection (D0) and convalescence (D7&21) in two different endemic sites. Kruskal–Wallis test was used to compare the levels between the timepoints, and p-values were adjusted for multiple testing using the Benjamini–Hochberg approach.

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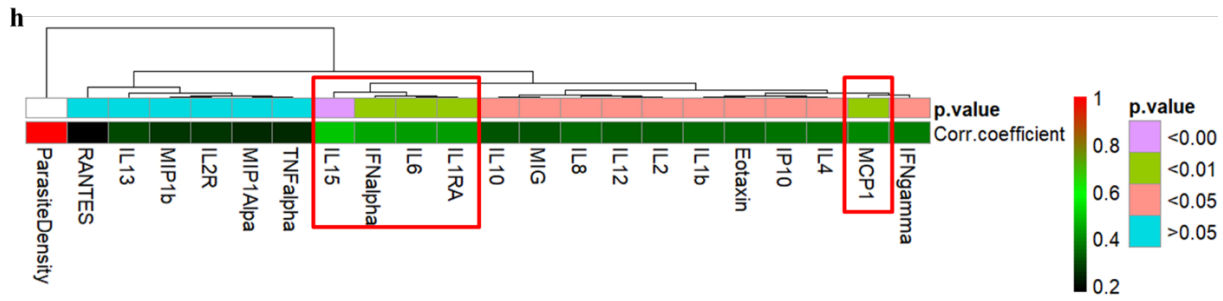


Figure 3.6: Cytokine signature that distinguish people from low and high transmission areas identified by correlation network analysis.

(a-g) Network graphs represent cytokine interactions determined by correlation analysis of 12 significantly different mediators for low and high transmission separated by different timepoint. Each node is a cytokine, and each edge represents is a Spearman's correlation between two cytokines. The red edges represent strong correlations, while the blue edges represent moderate correlations. The width of the edge corresponds to the correlation's absolute value: the higher the correlation, the shorter and more saturated is the edge. (h) Heatmap representation of cytokine levels correlation with parasitemia. Correlation was calculated using *spearman's rank test*.

3.1.4.5 Correlation of cytokine levels with parasite density

Considering changes in cytokine levels during infection and differences with transmission intensity, we explored the correlation between parasitemia with cytokine levels that were significantly different. Parasite levels had an effect on some of the cytokines as there was a significant positive correlation between parasitemia and cytokine levels for IL-6, IL-15, IFN-α, MCP-1, and IL-1RA, with Spearman's correlations above 0.60 (0.2 -0.7) and P values of >0.05 to <0.001 (Figure 3.6h). This may indicate that parasite levels affect regulation of these cytokines directly and not the underlying immunity, but could equally be suggestive that certain cytokines may interfere with parasite clearance.

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3.1.4.6 Network analysis identifies cytokine signatures

Since *P. falciparum* infection simultaneously activates the production of several cytokines, we used network correlation analysis to examine multivariable cytokine interaction during infection and after treatment in individuals from the different transmission intensity areas. Individuals from each transmission area displayed a distinct network pattern during acute infection (Day 0), with IFN-γ appearing to play a much more dominant role in individuals from the low transmission area than in individuals from the high transmission area. With most cytokines returning to baseline levels by Day 7 after treatment, the network analysis revealed no difference between groups after treatment (Day 7 and Day 21) (Figure 3.6B). A few individuals from the high transmission area were found to have detectable parasitemia at Day 21 after treatment. Interestingly, network analysis identified a different signature associated with these individuals dominated by the immunoregulatory cytokines IL-10 and IL-1RA. These demonstrate increased activation of pro-inflammatory cytokines during infection and more in low transmission area. It also suggests that asymptomatic parasitemia that occurs after treatment stimulates dominantly immunoregulatory mediators, which help in controlling disease outcome. However, these differences do not persist during convalescence.

3.1.4.7 Modular cytokine analysis reveals Co-signaling cytokines

Using CytoMod, cytokine measurements from each transmission area during acute infection were clustered into modules to capture the underlying correlation structure induced by co-signaling. Each module represents a subset of cytokines that vary across individuals in each location. When these cytokine modules were compared across the two transmission sites, they revealed specific subsets of co-expressed cytokines that cluster together across the two transmission sites. We

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computed cytokine pairwise similarity for each site (Figure 3.7A and B), and unsupervised hierarchical clustering algorithm to group cytokines based on their pairwise similarity. We observed different clusters for the two areas, with the low and high transmission areas having 6 and 5 cluster modules respectively. Overall, the different cytokine clusters identified by the modular analysis were highly correlated in the low compared to the high transmission area. This may be because most individuals in low transmission area had higher cytokines levels compared to the high transmission area. These results suggest that most cytokines co-regulate with each other to mediate effector functions. Module 6, whose constituent were IFN-γ and RANTES in the low transmission area and module 1 (IL-13, IL-1β, GM-CSF and IL-2) in the high transmission area were interesting clusters that uniquely distinguished cytokine that co-varies in the two areas.

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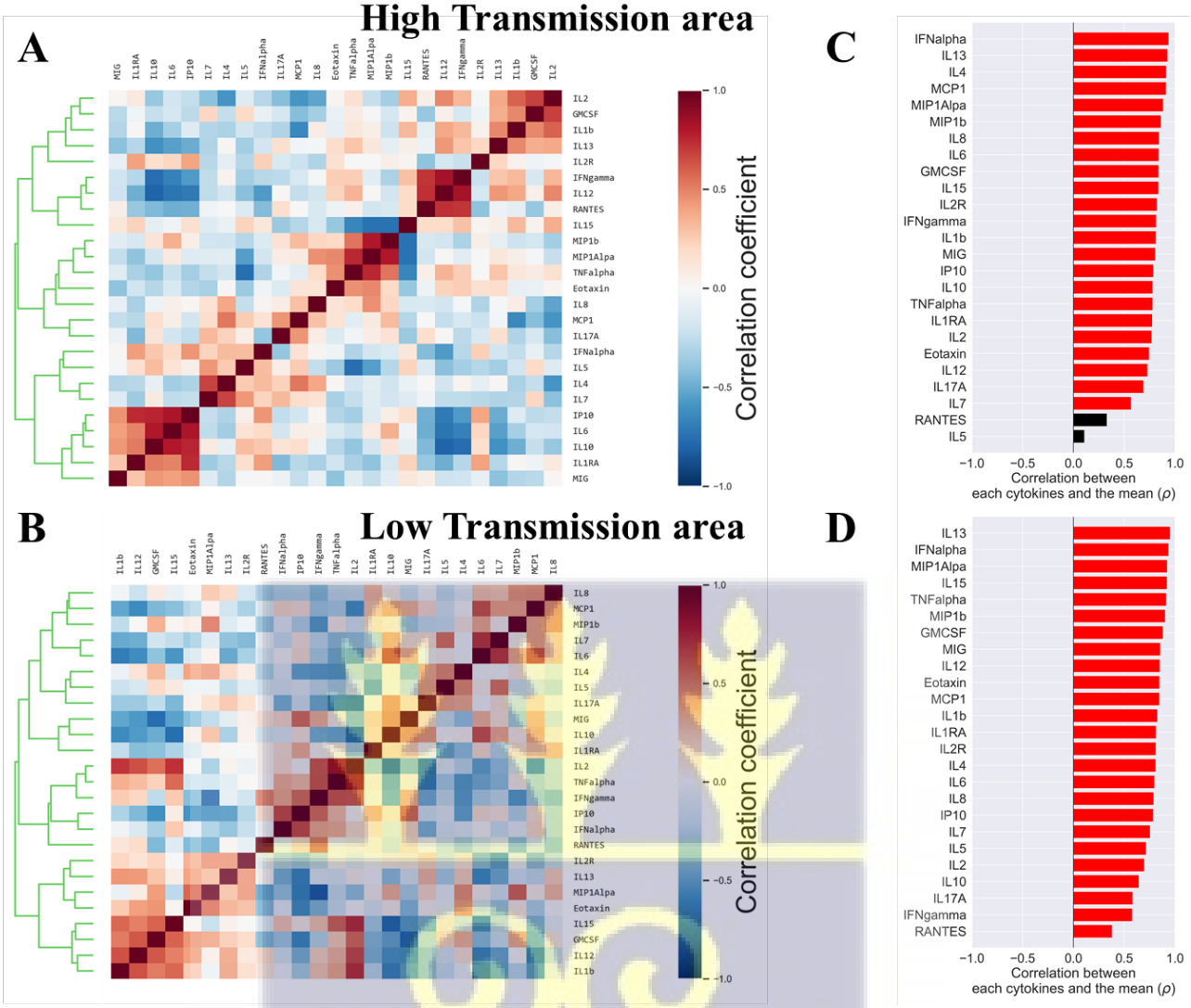


Figure 3. 7: Cytokine concentrations are correlated with each other and with each individual's mean levels.

(A). Pairwise correlation between cytokines following adjustments to the mean cytokine levels for high transmission area and (B). For low transmission area. (C&D). Correlation between cytokine levels and mean cytokine levels for each individual for high and low transmission areas.

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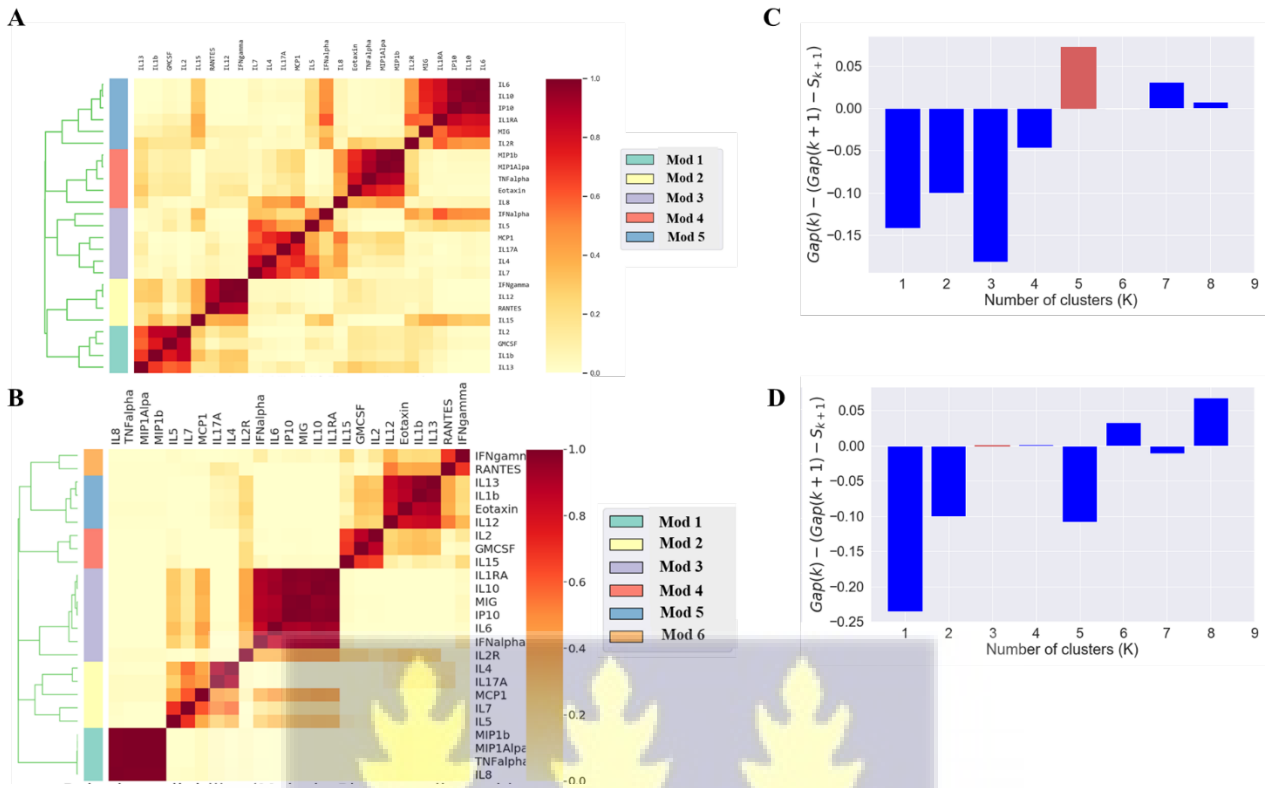


Figure 3. 8: Defining cytokine modules on the adjusted plasma cytokine profiles.

(A) Cytokine module heatmap for the high transmission area – To group cytokines into *K* modules, complete linkage clustering over the Pearson pairwise correlation similarity measure is used, with *K* determined by gap statistic. A clustering reliability score is calculated based on 1, 000 replacement samplings of subjects. Each cytokine pair's represents the percentage of times they clustered together across 1, 000 random samples. The *K* = 5 reliability score is shown here. The final modules are then built by clustering the pairwise reliability scores, and they are represented by the coloured stripes next to the clustering dendrogram. (B) Heatmap of cytokine modules for low transmission area, the reliability score of *K* = 5 is presented here

(C) Automated selection of the optimal number of modules for high transmission area.

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3.1.5 Discussion

Malaria transmission has declined over the last decade, due to intensive interventions in endemic areas. The impact of the change in transmission intensity on malaria immunity is an important aspect to be considered for sustained interventions. Ghana is one of the countries with a high malaria burden and has well demarcated regions with varying transmission intensities. This study assessed the influence of varying malaria transmission intensities on cellular immune profiles through quantification of several cytokines and haematological indices. Data presented shows changes in cytokines and haematological parameters during infection with acute *P. falciparum* and after treatment. Additionally, the data demonstrates that change in transmission affects levels of most of the cellular mediators measured.

Changes in hematological parameters are frequently encountered during acute malaria, hence, playing a major role in malaria pathology. However, these changes vary depending on the level of malaria endemicity, background haemoglobinopathy, demographic factors, and level of malaria immunity. The pathophysiological processes causing the changes in malaria are multiple and unclearly defined. Hematological indices have been generally useful in malaria diagnosis and have been useful in epidemiological studies (Chandra & Chandra, 2013).

We measured hematological parameters in the study participants and compared changes of the different parameters during acute infection (Day 0) and after treatment (Day 7 and 21), respectively, in the different transmission intensity (Low and High) areas. Results show that malaria-infected children (Day 0) tended to have significantly lower platelets, lymphocytes, and haemoglobin levels. At the same time, monocyte and neutrophil counts were significantly higher

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than after treatment, where the parameter resolved to levels within the reference ranges. Suggesting that acute *P. falciparum* infection is associated with thrombocytopenia, lymphocytopenia, anaemia, neutrophilia, and monocytosis which have also been shown in previous studies (Maina *et al.*, 2010). However, the parameters were also significantly different between the low and high transmission areas.

The decrease of the platelet and lymphocyte counts in the study participant is an important association with malaria disease. The platelets decrease (thrombocytopenia) has been previously associated with anaemia and shown to be a good *Plasmodium* infection marker (Lacerda *et al.*, 2011). Low platelet count during infection (Day 0) observed in 72% and 60% of the children in the high and low transmission areas in our study, respectively is thought to occur as a result of excessive removal of platelets by spleen pooling, peripheral destruction, reduced life spans and usage, through disseminated intravascular coagulopathy (Okafor, 2017; Thachil, 2017). Platelets provide initial response and produce platelet factor 4, that destroys parasite vacuole and presents antigens to class 1 MHC (Love *et al.*, 2012). The decreased lymphocyte counts may also be due to redistribution of lymphocytes with sequestration in the spleen and re-location to inflammation sites, rather than the actual exhaustion of the cells (McMorran, 2019) Likewise, malaria-induced apoptosis has been suggested as a cause of decreased lymphocyte count (Toure-Balde *et al.*, 1996). Thrombocytopenia has been associated with high parasitaemia and low haemoglobin levels (Maina *et al.*, 2010), which was also observed in this study.

The increased monocyte level may result from activation during *plasmodium* infection, producing inflammatory cytokines essential for parasite clearance and has been associated with severe

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clinical outcomes in anaemia and cerebral malaria (Royo *et al.*, 2019). This was observed in 52% and 32%, respectively, of high and low transmission areas study participants with malaria.

An increase in neutrophil levels was observed during infection, probably due to increased activation during parasites clearance through phagocytosis, formation of Neutrophil extracellular trap (NET) and production of reactive oxygen species (Aitken *et al.*, 2018). Neutrophils have also been implicated in severe malaria, though the mechanism is not understood. Several studies have hypothesised the involvement of neutrophil products in cerebral malaria (CM) pathology, with elastase damaging the endothelium (Smedly *et al.*, 1986; Yang *et al.*, 1996; Feintuch *et al.*, 2016). Also, with inflammatory mediators such as TNF and ROS increasing expression of the iRBC intercellular adhesion receptor (ICAM-1) on endothelial cells to promote parasite adhesion (Berendt *et al.*, 1989; Meager, 1999; Mittal *et al.*, 2014). Recently, a Gambian study showed that several neutrophil proteins correlate with protection (Georgiadou *et al.*, 2019), hence, high neutrophil levels suggest increased immunity levels.

The results from our study showed decreased RBC count, haemoglobin, and haematocrit levels, which was consistent with previous studies (Akinosoglou *et al.*, 2012; Kotepui *et al.*, 2015). This has been associated with anaemia during *P. falciparum* infection. The causes of anaemia are multifactorial and are not clearly defined, however, it is thought to be due to a combination of hemolysis of parasitised RBCs, removal of parasites and in the process destruction of unparasitized RBC and ineffective erythropoiesis (White, 2018; Nsiah *et al.*, 2020).

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The study also assessed the effect of different malaria transmission intensity (MTI) patterns with the cellular immune profiles, in children during infection and after treatment. Previous research has revealed that the balanced regulation of pro-inflammatory and anti-inflammatory cytokines that determines disease outcome (Lyke *et al.*, 2004; Prakash *et al.*, 2006), and this is also similar for chemokines (Ioannidis *et al.*, 2014). Given the importance of these mediators in immune response and the consequent control of immunopathology, we hypothesized that they could be affected by changes in MTI.

The study also presents a descriptive analysis of plasma cytokine and chemokine levels in children during *P. falciparum* infection (D0) and after treatment (D7 and D21) from areas with varying transmission intensity. We applied multivariate data analysis in combination with predictive modelling approaches to identify distinct cytokine signatures from the two transmission areas, as possible functional protective features. The results indicate differences in some of the cellular mediators in individuals from the different transmission intensity areas, suggesting the involvement of different cytokines in acute *P. falciparum* infection. Except for MIP-1α, MIP-1β and RANTES, all measured cytokines were higher in the low transmission area compared to high transmission area, indicating more immune activation in individuals from low transmission area. This also suggests the possibility of a dampened cytokine response in the high transmission area, which could be associated with host tolerance or up-regulated response in the low transmission area which contributes to clinical malaria. Whereas, antibody responses to most tested antigens are maintained even after infection, the cytokine responses were transient rapidly changing after treatment.

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As indicated in previous studies (Ademolue *et al.*, 2017; Boyle *et al.*, 2017), the study demonstrate that some specific cytokine responses including; pro-inflammatory responses, IL-6, IFN-γ, IFN-α MIG, MCP-1/CCL2, IP-10/CXCL10, RANTES/CCL5, and immunomodulatory profiles, IL-10, IL-2R and IL-1RA, contribute to parasite elimination during (Day 0). In this study transmission intensity was used as a proxy for exposure; therefore, the results imply that less exposure in low transmission areas, leads to reduced host immune tolerance, hence more clinical disease, compared to individuals from high transmission areas. Repeated exposure to *P. falciparum* has been shown to lead to establishment of tolerance (Jagannathan *et al.*, 2014b), which can be associated with the loss or altered function of immune cell types that show less proliferation and cytokine production (Horne-Debets *et al.*, 2013; Illingworth *et al.*, 2013). Our results support this implication showing pro-inflammatory cytokine signatures were significantly different during the acute infection phase compared to convalescence phase in only the low transmission area (IFN-γ, IFN-α, MCP-1 and RANTES). The anti-inflammatory responses (IL-10, IL-2R and IL-1RA) on the other hand are not significantly different in the two transmission areas, proposing different ability in pro-inflammatory and anti-inflammatory response regulation associated with exposure, and possible development of tolerance in more exposed individuals (Boutlis *et al.*, 2006).

The results also revealed cytokines (IFN-α, IL-6, IL-1RA and MCP-1) were affected by parasitaemia during infection, indicating that parasitaemia is associated with elevated cytokines. However, there was no significant difference in the parasitaemia between high and low transmission, therefore the association to cytokine level is dependent on cytokine level and not transmission intensity. Parasite density determines production of these cytokines, while for others

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the underlying type of immune response is important. Different cytokine interaction patterns shown in the results demonstrates that an individual's immune status influences the combination of different interacting cytokines to establish balanced protective immunity. The presence of infection primarily influences overall changes in cytokines. Hence, they temporarily and rapidly go back to normal after the infection is cleared. This implies that cytokines are involved in controlling infection and disease outcome, hence may contribute to development of anti-disease immunity as recently reported in other studies (Ademolue *et al.*, 2017). High parasitaemia in high endemic areas are associated with high parasite antigens; therefore, expected to stimulate more cytokine production. Nevertheless, this was not observed in our study. Thus, suggesting high parasitaemia tolerance in areas of high malaria transmission intensity resulting in unresponsiveness to immune cells stimulation due to continuous exposure to high levels of malaria antigens (Mueller, 2010). Recent studies have shown loss or altered function of several immune cells like γδ T cells, αβ T cells, B cells and myeloid cells, due to continuous exposure to *P. falciparum* infection that may be involved in tolerance to infection, hence less proliferation, activation and cytokine production by these cells.

This study additionally reveals a cytokine signature associated with the different transmission intensities, suggesting more pro-inflammatory cytokine stimulation in less exposed individuals compared to the highly exposed. The results also indicate that individuals from both transmission areas had a similar capacity to regulate anti-inflammatory cytokines. In comparison, the difference in pro-inflammatory cytokines suggests the different ability of individuals to control disease outcome and pathogenesis. Hence, the differences in cytokine responses during active malaria infection between individuals with varying levels of prior malaria exposure, including elevated

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pro-inflammatory cytokines levels, found in individuals from low transmission settings. Interesting, a profile dominated by immunoregulatory cytokines (IL-1RA and IL-10 were seen to be unique to asymptomatic infections observed in individuals from the high transmission area after treatment. This provides some evidence that previous exposure shapes immune responses leading to less inflammatory responses upon re-infection. Our data are in agreement with several studies that analysed cytokine response in children with malaria. These studies report shifts in cytokine production from pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) to anti-inflammatory (IL-10, TGF-β) (Aguilar *et al.*, 2019). These findings suggest that children acquire exposure-dependent *P. falciparum*–specific immunoregulatory responses that dampen pathogenic inflammation in re-exposure while enhancing anti-parasite effector mechanisms (Crompton *et al.*, 2014; Portugal *et al.*, 2014a). This study has demonstrated that the degree of modulation of response is directly associated with the levels of exposure/malaria endemicity, generally showing that pro-inflammatory cytokine responses during malaria infection were increased with reducing transmission intensity.

Though we used local transmission as a proxy for exposure some individuals in low transmission areas may get exposed equally depending on the environmental conditions. This was partially overcome by collecting data on the number of previous episodes individuals had in a year.

3.1.6 Conclusion

Individuals with varying levels of prior exposure had different cytokine and chemokine profiles during active malaria infection. Individuals from low transmission areas had higher levels of pro-inflammatory cytokines. Pro-inflammatory cytokines IL-6, IFN γ, IFN α, MIG, MCP-1 and IP-10,

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as well as the immunomodulatory cytokines IL-10, IL-2R and IL-1RA were associated with acute infection and were elevated in the low transmission area This could be due to lack of tolerance due to low transmission versus a high transmission area with increased exposure to *P. falciparum* and thus increased tolerance. These differences, however, were short-lived and did not persist during convalescence. IFN-α, IL-6, IL-1RA and MCP-1 were significantly associated with parasitaemia levels and a signature dominated by IL-10 and IL-1RA associated with asymptomatic parasitaemia in a subset of children from the high transmission area at day-21. These findings highlight the importance of transmission intensity in regulation of immune response and eventual development of regulated protective immunity to Malaria.

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4 CHAPTER FOUR: SPECIFIC OBJECTIVE TWO

4.1 ANTIBODY SIGNATURES ASSOCIATED WITH DIFFERENT MALARIA TRANSMISSION INTENSITIES IN CHILDREN DURING ACUTE *P. FALCIPARUM* INFECTION AND CONVALESCENCE.

Hypothesis: *Children living in high transmission areas will have a different P. faciparum antibody repertoire compared to children of the same age in lower transmission areas.*

4.1.1 Abstract

Background: Understanding natural acquired immunity is key to improving malaria surveillance, diagnostics and effective vaccine development. Antibodies play a crucial role in malaria immunity and can serve as bio-signatures of protective immunity. The acquisition and maintenance of immunity is significantly influenced by differences in transmission intensity as observed in malaria-endemic areas. This varied exposure to *P. falciparum* causes accumulation of specific antibody responses to various antigens distinct to these differences. However, the distinct antigen repertoire that is responsible for inducing protective antibody response is still unclear. Traditional approaches to understanding the host humoral immune response have failed to provide a comprehensive understanding of the antibody repertoire generated in response to infection. In this study, we used protein microarray-based serological profiling and machine learning methods to identify predictive antibody signatures and classify the associations of antibody features with varying transmission intensity and infection.

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Methods: Antibody responses in children aged 5-14 years were profiled during acute *P. falciparum* infection (Day 0) and convalescence (Day 7 and Day 21) by probing protein microarrays consisting of 190 *P. falciparum* antigens using plasma samples. This was done through a longitudinal hospital-based study in two regions in Ghana with distinct malaria transmission intensities: Accra (low transmission) and Kintampo (high transmission).

Results: Antibody profiles to specific malaria antigens were distinct in individuals from the two regions, reflective of their malaria transmission intensities. Hierarchical clustering revealed distinct IgG response clusters of antigens associated with the different malaria endemicity. Immunoreactivity was identified against 118/190 antigens tested, among these 48 antigens including EBA140, ETRAMP5, GLURP, MSP1 and MSP6 were significantly more reactive in low transmission area and 30 antigens significantly more reactive in high transmission area including Rh5.1, EBA181, VAR2CSA, ACS5, HSP40, MSP2, MSP3, MSP4, and MSP7. Most antibodies' levels persisted during the convalescence period and we demonstrated a wide magnitude and breadth of humoral responses in the two sites. The breadth of antibody responses was more heterogeneous in the low transmission site, but there were no significant differences in antibody breadth and magnitude between the two sites. Multivariable linear regression models predicted age and transmission intensity as factors that positively affect antibody reactivity to most antigens in our panel. Machine learning modelling predicted a further 17 signature antibody signature (MSP4, MSP2 3D7, MSP1-19 2A, Etramp 5 Ag1 His var 3, MSP1-19 2B, Etramp 5 Ag1 His var 2, MSP2 [15-46b], MSP2 [5-36A], ACS5 Ag 3, PfMSP1_19, MSP3 FVO, Var2CSA, SE36/SERA 5 (T), HSP40 Ag 3, GLURP R2, Rh4.2_2030 and MSP2 CH150/9) that can distinguish children from the two different transmission areas.

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Conclusion: Results from this study contribute to the overall understanding of the acquisition and maintenance antibody responses during *P. falciparum* infection. The study also identified antibody repertoires that distinguish individuals with different levels of pre-existing immunity. The findings also showed the kinetics, breadth and magnitude of antibody response during acute *P.falciparum* and after treatment. The 17 identified antigens could serve as possible key targets of protective immunity, that would be useful for identifying potential vaccine candidates.

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4.1.2 Introduction

Antibodies are important component of naturally acquired malaria immunity, and they are especially important during the blood stage of the infection, where they target both free merozoites and infected red blood cells (iRBCs) (Osier *et al.*, 2014b). Adoptive transfer of antibodies from immune adults to infected children reduced parasitaemia, demonstrating the importance of antibodies in protecting against blood stage malaria (Cohen *et al.*, 1961; Sabchareon *et al.*, 1991).As a result, understanding of the acquisition and maintenance of antibody responses during malaria is critical for improving prospects of developing an effective vaccine (White *et al.*, 2014a). This necessitates a thorough characterisation of antibody responses during and after natural infection in individuals with varying levels of exposure.

Immunity to malaria in humans is shown to develop after repeated exposure (Hviid, 2005; Crompton *et al.*, 2014; Bansal *et al.*, 2018) and several studies have shown an association between specific antibodies and protection against malaria in natural infection (Osier *et al.*, 2008; Daou *et al.*, 2015; Gonzales *et al.*, 2020). Although protective antibodies are acquired with time, the responses are short lived and interpretation is complicated by exposure heterogeneity; hence, it still remains unclear which parasite antigens induce protective antibodies (Fowkes *et al.*, 2010; Richards *et al.*, 2010). Furthermore, natural exposure occurs with diverse and unknown genotypes, complicating the implications of protective immunity (Bowman *et al.*, 2016; Oyedeji *et al.*, 2020). Due to this genetic diversity in *Plasmodium*, there is a need for malaria vaccines that can protect against these different variants for a sustained public health impact. The antibody-mediated responses are essential in controlling individuals' parasite levels through prevention of invasion,

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increased clearance of antibody-bound erythrocytes by phagocytosis and antibody-dependent cellular cytotoxicity mechanisms (Bouharoun-Tayoun *et al.*, 1995; Langhorne *et al.*, 2008; Osier *et al.*, 2014a; Gonzales *et al.*, 2020).

Antibodies to several parasite antigens are generated after infection and boosted upon subsequent exposure (White *et al.*, 2014a). Understanding which antibody specific responses generated in natural infection correlate with immunity is key for developing next generation vaccines. However, this has been a challenge due to the complex life cycle of *P. falciparum* that presents several antigens, making it arduous for traditional approaches, such as the use of immunofluorescent antibody (IFA) test and enzyme-linked immunosorbent assay (ELISA), to provide rapid interrogation of the antibody response to infection. Furthermore, previous findings have provided contradictory and inconsistent results of most studies that have used a small number of antigens to identify targets of protective immunity The reliable quantification of previous exposure has also challenged the accurate identification of protective antibodies (Beeson *et al.*, 2016b) as it is difficult to differentiate markers of exposure and protective immunity (Bejon *et al.*, 2009).

Development of high throughput techniques like protein microarray have allowed the study of multiple antibody responses to a large panel of antigens in parallel and has enabled investigation of the breadth and dynamics of the whole anti-*Plasmodium* antibody response to infection. There have been extensive research studies that have used protein microarray for multiple serological profiling in malaria. This includes studies that have shown longevity of antibody responses before and after malaria season, which showed increase of malaria antibodies during transmission that

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were short-lived and declined after the season (Crompton *et al.*, 2010a; Valletta *et al.*, 2017). Several studies have used protein array for simultaneous detection of antibody profiles associated with protection from malaria (Osier *et al.*, 2014a; Obiero *et al.*, 2019; Gonzales *et al.*, 2020). Antibody profiling has also been useful in determining ongoing transmission and evaluating control strategies by identifying antigenic markers that correlate with recent *P. falciparum* infection (Helb *et al.*, 2015; King *et al.*, 2015; Kobayashi *et al.*, 2019).

The challenges of discriminating protective immune responses with high-throughput protein microarray are the high dimensionality of data produced and the limitation of standard statistical approaches in providing details of the strength of the associations and sensitivity to small changes in immune responses to multiple targets (Robotti *et al.*, 2014). This has restricted the success of identifying protective antibody signatures.

This study aimed to descriptively analyse antibody responses of children against *Plasmodium* antigens in two epidemiologically diverse malaria endemic areas in Ghana using protein microarray. This was done by assessing the relationship between exposure and acquisition of antibodies and further identifying immunogenic antigens based on antibody responses at these two sites, and predicting antibodies associated with the varying transmission intensities.

Samples were collected in a longitudinal hospital-based study to characterise antibody responses to *Plasmodium* antigens at two varying transmission intensity areas in Ghana: Accra which is a low transmission area and Kintampo in northern Ghana, which is a high transmission area. Antibodies to 180 *P. falciparum* and 10 non- f*alciparum* antigens were measured in plasma samples using a protein microarray platform with expressed proteins printed onto a support

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surface. Individuals from the two sites showed reactivity to 118 antigens despite the transmission setting. Predictive modelling approach based on machine learning provides the opportunity for systematic analysis that considers all possible antibody response combinations. Further hierarchical clustering of antibody responses in the two transmission areas revealed 54 reactive antigens in the low transmission than 24 reactive antigens in the high transmission area. However, the overall magnitude and breadth of antibody response were not significantly different, but there was more heterogeneity observed in the breadth of antibody profiles in the low transmission site. Machine learning methods predicted 17 antibody responses that can differentiate individuals from distinct transmission areas.

4.1.3 Methods

4.1.3.1 Samples

Plasma samples collected in a longitudinal study conducted in two regions in Ghana were probed using protein microarray for detection of antibody responses for various antigens. Details of the study are described in chapter three.

4.1.3.2 Protein microarray platform

With the exception of *Pf*AMA1, which was expressed as a histidine tagged protein in *Pichia pastoris*, recombinant proteins were generated and expressed in Escherichia coli glutathione S-transferase (GST)-tagged fusion proteins. For GST and His tagged proteins, affinity chromatography (Glutathione Sepharose 4B (GE Healthcare Life Sciences) or HisPur Ni-NTA (Invitrogen) resins were used to purify the proteins. The Bradford protein assay was used to determine protein concentration, and 4-20 percent SDS-PAGE was used to determine protein

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quality and purity. Purified proteins were diluted to a final concentration of 0.001% Tween 20 before being printed onto nitrocellulose-coated slides (AVID, Grace Bio-Labs, Inc., Bend, OR, USA) with an Omni Grid Accent microarray printer (Digilabs, Inc., Marlborough, MA, USA). To allow for background normalisation of purified proteins, buffer (PBS) spots were included alongside proteins of interest as controls (Oulton *et al.*, 2020). The protein microarray platform was developed at the London school of Hygiene and Tropical Medicine, London, UK (Oulton *et al.*, 2021) Protein Microarray Antibody detection

The IgG responses to 190 antigenic targets in *Plasmodium spp* blood-stage, pre-erythrocytic and gametocyte stages, were determined using the above-mentioned custom-made protein microarray platform (Oulton *et al.*, 2021). Each slide was sub-divided into 16 arrays, each containing the whole complement of antigenic targets printed in duplicate. Within each array a total human IgG standard curve (beginning with concentration 200 µg/ml, fivefold series of 6 dilutions) was printed in duplicate. In deep wells, plasma samples were diluted in blocking buffer [Phosphate buffered saline (PBS)/BlockIt buffer (ArrayIt Corporation, United States)] at 1:200 dilution. Multiple well hybridization cassettes were used to hold the printed nitrocellulose slides and blocked with 200 µl of blocking buffer for 1 hour on a rotary shaker (100 rpm) at room temperature (RT). Blocking buffer was removed and wash buffer (200 µl, PBS/Tween 0.05 percent) was immediately added, and the HC shaken for 2 minutes on the rotary shaker. After washing the slides three times, the wash buffer was removed by sharply flicking buffer into a sink. Following the final wash, the buffer was aspirated one column at a time with a multichannel pipette and study participant samples were analysed alongside European malaria-naïve control samples. The naïve samples were collected as part of a different study at the London School of Hygiene and Tropical Medicine

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and were donated to be used to generated background seropositivity cut-off. To avoid inter-slide variability influences during assay processing, where possible samples from same participants for the 3 time points were grouped on the same slides. The slides were washed three times and incubated for 1 hour at room temperature on the rotary platform. Samples were incubated for 1 hour at room temperature on the rotary platform with the IgG-specific goat anti-human secondary antibody (TX9052-32RD goat anti-human IgG; Southern Biotech) at a dilution of 1:1000. After three more washes, slides were centrifuged at 3000 rpm for 5 minutes at room temperature to dry. The slides were immediately stored at 4°C and scanned with the GenePix 4300A scanner at a wavelength of 635 nm (Molecular Devices, United States). Background correction was applied at the median fluorescence intensity (MFI), and an average of the duplicate measurements used (Pearson's correlation coefficient 0.99, $p < 0.001$). The MFI-background values less than or equal to zero were log-transformed and replaced with the average value of blank responses.

4.1.3.3 Statistical Analysis

Microarray spot intensities were quantified using the Genepix pro 6 software. Foreground target intensities were corrected using background correction function for each spot in the R limma package. The background-corrected signal intensities for each antigen were then transformed to give a normal distribution. Further correction was done for antigens with the GST tag, by subtracting GST signal from the background-corrected signals. Samples with a high background or that differed between the duplicated were removed from further analysis (one sample was eliminated from further analysis due to high background), and the data was checked for deviant antigens. Foreground signal intensities were confirmed relative to buffer signals, and cut-offs

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generated using mean ($\overline{X}$) + 3 standard deviation (SD) (spots above the cut-off were excluded). R studio and GraphPad prism8 were used for all statistical analyses. Log-transformed MFI minus background values were used to express antibody responses (IgG) (appendix, Figure A.3.1). The average response of eighty naïve participants ($\overline{X}$+ 2SD) for each antigen was used as the threshold for seropositivity. Positive proteins were defined as having a transformed signal intensity greater than the $\overline{X}$+ 2 SDs of the transformed negative-control spots. Boxplots with medians, and interquartile ranges (IQR), by Mann-Whitney tests, and p-values adjusted for multiple comparisons using the Holm approach were used to compare of IgG levels ($\log_{10}$ MFI) across antigens. Analyses included all subjects or subjects analyzed separately by timepoint, with some cases stratifying by site, age, and age group within each site. The proportion of positive responses out of the total of 190 *Plasmodium* proteins for each participant was used to calculate the breadth of the antibody response. The Mann-Whitney test was used to compare the breadth of responses across groups, with p-values adjusted for multiple testing using the Benjamini–Hochberg method. We fitted multivariable linear regression models (coefficient, 95% confidence interval [CI], p-values) that included the following predictors: Age, parasite density, malaria transmission site, and sickle cell status (using the same antigen/IgG levels as the outcome variable) to evaluate factors affecting the IgG levels to each antigen.

4.1.3.4 Machine learning predictive modelling

To model and identify predictive immune signatures associated with immunity to malaria, we used 7 different machine learning approaches. These included: Decision tree (or CART), support vector machines (SVM), Neural networks, extremely randomized tree, random forest, adaboost and extreme gradient boosting, with cross-validation setting that assessed their ability to predict

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antibody responses from individuals in the low or high transmission intensity areas (Camacho *et al.*, 2018; Eraslan *et al.*, 2019). These classifiers were built using R and trained on default parameters. With a train/test split of 70/30, i.e., 70% of the shuffled data was dedicated to training, and 30% to testing. The R caret package was used for predictive modelling. The training dataset is used to train an algorithm to understand how to apply machine learning technologies to produce complex results, while the testing data set is used to assess how well the algorithm was trained. We used the measured antibody responses as an input variable for our models. The predicted response variables were antibody responses that could identify individuals from the two transmission areas. To validate that the algorithms were learning an inherent pattern in the data to meaningfully classify samples but not randomly guess, a control randomised experiment was set up to evaluate if the achieved classification accuracy was better than expected by chance. In this experiment, the four machine learning algorithms were used to build classifiers trained on the antibody response data but tested on random data. Due to some shortcomings of using accuracy alone as an evaluation metric for a classification model, the recall and precision of each classifier were calculated. To evaluate if classification could be improved by incorporating response to multiple antigens, the Boruta algorithm, a wrapper method built around random forest classification was further used for feature selection to capture all important and interesting responses (Kursa *et al.*, 2010). The Boruta algorithm was fitted to antibody responses for all reactive antigens.

Since each of the combinations of antibody response identified was unique to each classifier, an intersection of all four set of combination was found to establish a base set of antibodies that is common to all classifiers. To further verify that the above combination as sufficient to differentiate

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individuals from the two transmission intensities areas, a new set of four classifiers were trained, using the data of only the antibodies identified in the first classification.

4.1.3.5 Predictive accuracy

We used confusion matrix measures and receiver operating characteristic (ROC) curves to assess and report on the predictive accuracies of the best models. The confusion matrix compares instances of predicted classes (low or high transmission areas) to instances of observed classes. The misclassification-rates are calculated using out-of-bag (OOB) errors (Liaw & Wiener, 2002). These are derived by testing the model's performance against subsets of data that were left out during the fitting process iteratively. The OOB errors are an estimate of the generalization error (how well the model would fare against previously unseen data). The receiver operating characteristic (ROC) analysis was used to determine whether specific antibody responses could be used to differentiate individual from high and low transmission areas. The curves were created by plotting the true-positive rate versus the false-positive rate (i.e., the observed incidence versus the false predicted incidence) at various threshold settings. The area under the curve (AUC) was a measure of predictive accuracy (AUC = 1 equates to zero error, and an AUC = 0.5 equates to random guessing).

4.1.3.6 Multivariate analysis

Principal component analysis (PCA) was used for discriminant analysis, to shown clustering of all the 190 antibody responses and compare with clustering based on the 4 different classifiers and the 18 predicted antibody responses. We looked at the factors that influence IgG levels to the predicted antigens, and we fitted multivariable linear regression models (coefficient, 95% confidence interval [CI], p-values) that include the predictors: age, parasite density, malaria

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transmission site and sickle cell status (using the same antigen/IgG levels as the outcome variable).

4.1.4 Results

4.1.4.1 Characteristics of antigens tested by protein microarray

We used a protein microarray containing 180 *P. falciparum* antigens and 10 *non-falciparum* antigens for blood-stage, pre-erythrocytic and gametocyte stage parasite proteins. Plasma samples from children of the two study sites, 68 and 54 participants from the low and high transmission areas, respectively, and European naïve controls, were analyzed for IgG antibody reactivity. Appendix, Table A.3.1-3.4 shows the characteristics of antigens included in the protein microarray.

Each target background MFI plots for all samples, spots, sub-array and array were generated. A cut-off for each spot was generated from the $\overline{X}$+ 3SD of the buffer spot (Figure 4.1 and Appendix Figures A.3.1-A.3.3), and one sample with the high background was removed from further analysis. Seropositivity cut-off for each antigen was generated using the $\overline{X}$+ 3SD MFIs of the European naïve controls. Comparison of antibody responses between all plasma samples identified 118 of the 190 tested antigens to be reactive with significantly higher intensity in the samples from both endemic regions compared to the unexposed European naïve controls. Sixty-nine of these antigens were reactive in at least 50% of participants from both high and low transmission areas across all time points. The top five reactive being MSP-2, Etramp 4 and 5, MSP 1_19, GLURP R0 and HSP40.

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4.1.4.2 Protein microarray data analysis

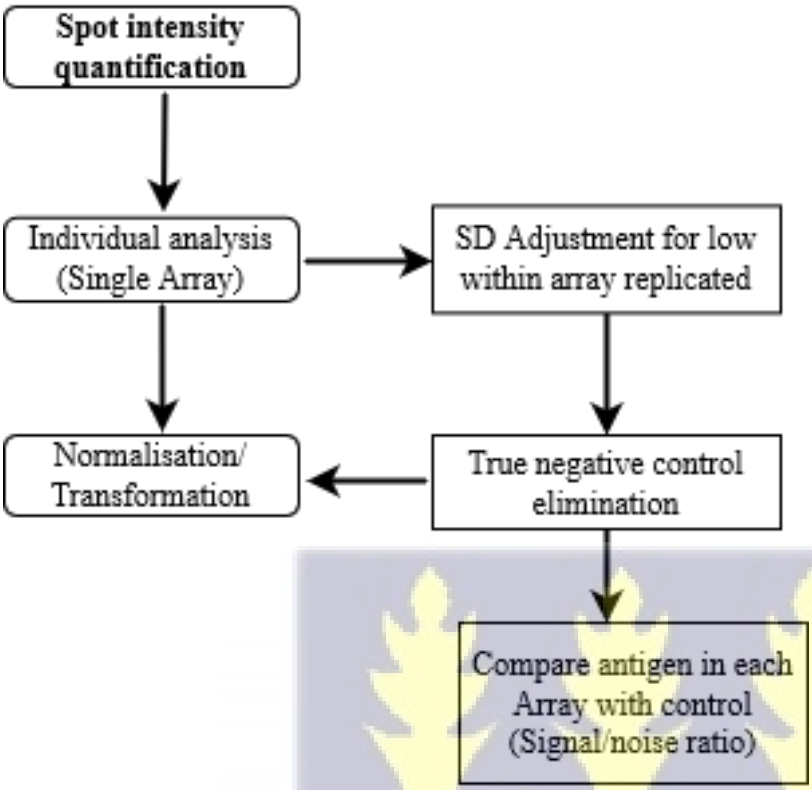


Figure 4. 1: Flow chart for the protein microarray data analysis pipeline

4.1.4.3 Breadth and magnitude of antibody response do not differ between low and high transmission areas.

We investigated the breadth of antibody response against the 190 *Plasmodium ssp* antigens immobilized on the peptide array, the total number of sero-reactive antigens were analyzed in children from the two transmission areas. Antigen recognition varied widely among individual participants, with breadth ranging from less than 5 to more than 130 antigens in both areas. Additionally, we examined the magnitude of the signal intensity for each antigen and detected no significant difference in the total number of antigens recognized between transmission areas

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(breadth) nor the overall magnitude of antibody responses over the time course (Figure 4.2). These suggest that antibody responses to dominant proteins do not vary by transmission intensities, and that there could be suppression of response to other proteins, hence broader number of less reactive antigens. Though the dynamics of the antibody response varied by antigen, the majority of the antibody responses increased in magnitude after acute infection (D0), with a peak in antibody response at D7, and a gradual decrease at D21.

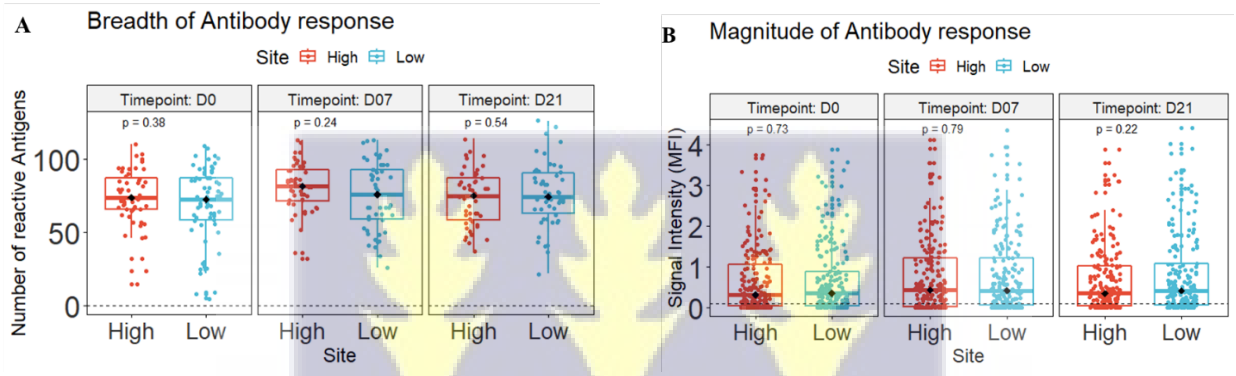


Figure 4. 2: Breadth and Magnitude of antibody responses.

Comparison of breadth and magnitude of responses to the microarray protein targets (n = 190). (A). Represents the number of antigens reactive above background in each individual, within the given groups. (B). The magnitude of antibody response to microarray protein targets. Each dot represents the average Signal Intensity (SI) of all individuals within the given groups in response to each protein target. For differences in count and MFI signals between the two study sites, p-values for intensity data and response breadth are based on Mann-Whitney test.

4.1.4.4 Composition of antibody response differs between Low and high transmission areas

Although the number of antigen specificities recognized by individual participants did not differ significantly between sites, there was a possibility that specific antigens are more highly associated with one transmission area than the other. Using hierarchical clustering, we compared the relative

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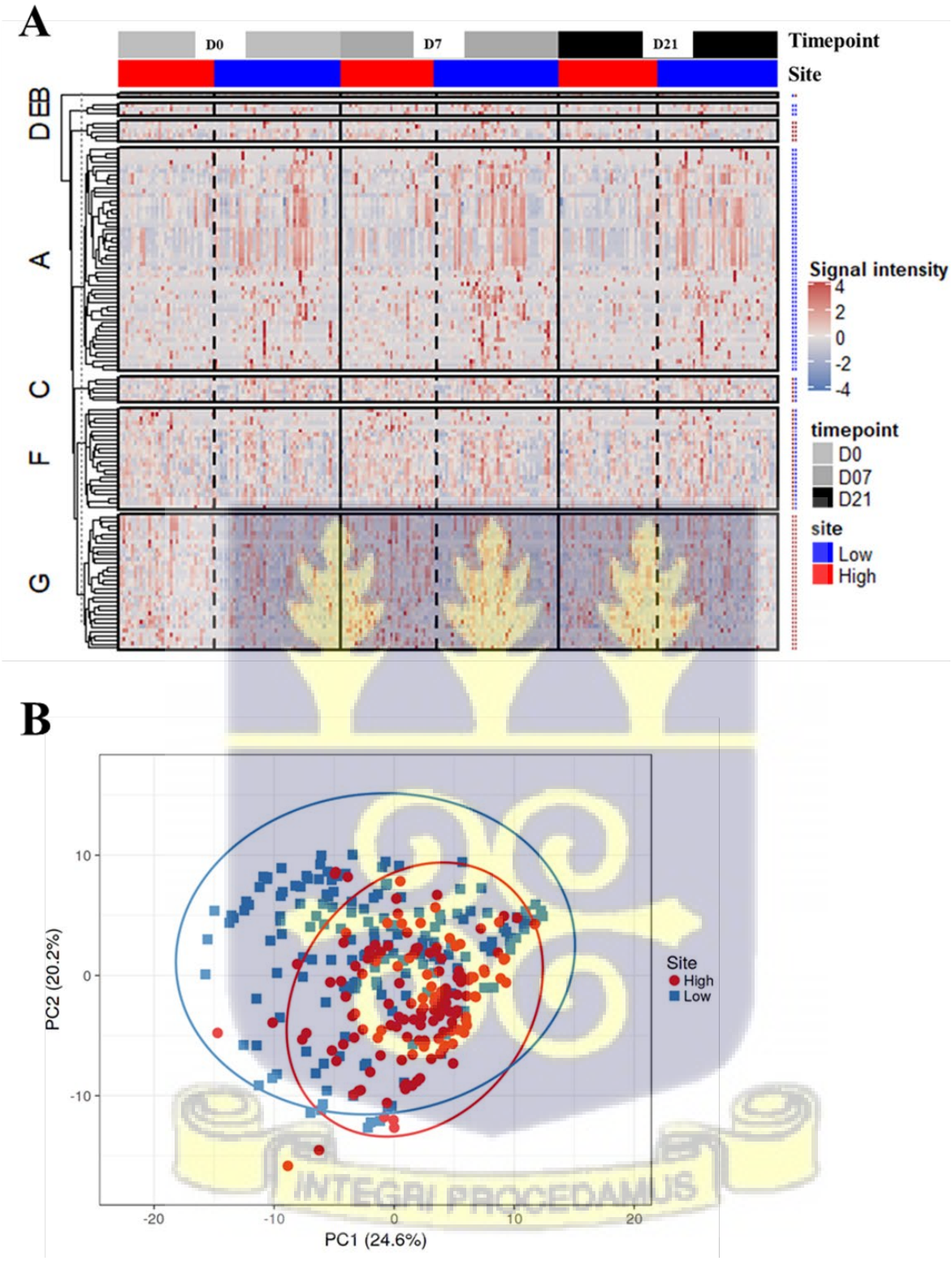
intensities of the 118 antigens recognized in at least 80% of participants. Our results demonstrate antibody profiles to specific proteins that are clearly clustered by transmission intensity of the two groups. Although some antigens appear to be reactive in both groups (40), certain antigens had high reactivity to either the low or high transmission groups. The low transmission group (Accra) was highly reactive to 54 distinct antigens compared to the high transmission (Kintampo) which showed preferential reactivity to 24 antigens (Figure 4.3). Despite the distinct clustering by transmission intensity, antibody responses showed a varied reactivity across the participants with some displaying broader recognition and stronger signal than others.

The clustering reveals 7 clusters (Figure 4.3, A-G and appendix table A3.2, A3.3) based on the reactivity of each antigen in the two locations and across the 3 time points. Cluster A are antigen specificities associated with high magnitude response in the low transmission area throughout the 3 time points, including EBA140, ETRAMP5, GLURP, MSP1 and MSP6, while cluster G are those associated with the high transmission area, including Rh5.1, EBA181, VAR2CSA, ACS5, HSP40, MSP2, MSP3, MSP4, and MSP7. This suggests that responses to specific antigens may provide information on effect of exposure.

The hierarchical clustering data is supported by unsupervised principal-component analyses (PCA) of the antibody profiles that separated the two sites. Despite the two sites having similar signal intensities for most reactive antigens, the low transmission site has broader and overlapping responses than the high transmission site which has a more distinct cluster. These results possibly reflect the influence of transmission intensity to antibody responses.

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Figure 4. 3: Immunoreactive *P. falciparum* antigens clusters identified by hierarchical clustering distinct to the different transmission sites.

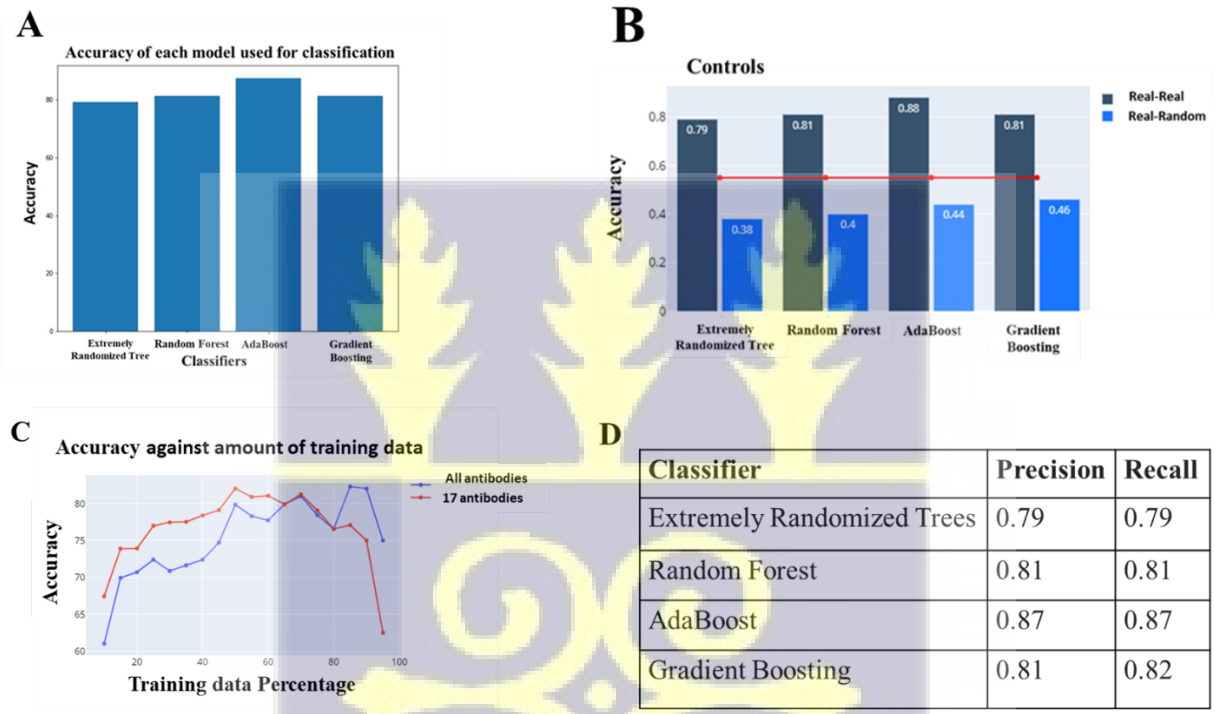
A). Heatmap of normalized median fluorescent intensity (MFI) of the antibody responses to 118 most reactive *P. falciparum* antigens in the microarray chip. Columns represent individual samples antibody responses grouped by time points and sites transmission intensity. Rows represent the different antigens clusters identified by antibody response that are significantly different for the two sites over the different time points. For the colour code for signal intensity, light blue (-4) represents no reactivity, while red (4) represents high reactivity. Cluster A; Reactivity is high in the low transmission site than the high transmission site for D0, D7 and D21, Cluster G. Antibody reactivity is higher in high transmission site for all time points. Clusters B-F had varied reactivity on different time points for the different sites. B). The PCA using antibody responses against all the antigens tested in the study.

4.1.4.5 Machine learning identifies antibody signature that distinguishes between high and low transmission areas

Protective antibodies are often as a result of a combination of responses to several antigens instead of one antigen. Given our findings that transmission intensity appears to affect the composition of the *Plasmodium ssp*-specific antibody repertoire, we employed machine learning tools to evaluate the specific antigens that most accurately discriminate between groups and capable of predicting an individual's epidemiological group. We initially built 7 different machine learning-based classifiers. The classifiers differ slightly in their accuracy each scoring between 74% - 83%. The 7 classifiers and their respective accuracies are classification and regression tree (CART) or Decision tree algorithm (81%), support vector machine (74%), Neural Network (81%), Extremely Randomized Trees classifier (79%), Random Forest (83%), AdaBoost (83%), and Xtreme Gradient Boosting (82%) (Figure 4.4A). Since using accuracy alone as an evaluation metric for a classification model can be misleading, the recall and precision of each classifier was calculated

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(Table 4.4D). Four of the classifiers were further validated in a control experiment using random data. The accuracy significantly dropped when the classifiers were applied to random data: Extremely Randomized Trees classifier (38%), Random Forest (40%), AdaBoost (44%), and Gradient Boosting (46%) (Figure 4.4B). This provides a certain degree of confidence that the 4 algorithms selected were learning meaningful patterns from the antibody responses and making highly accurate predictions in distinguishing responses from the two transmission areas.



Classifier	Precision	Recall
Extremely Randomized Trees	0.79	0.79
Random Forest	0.81	0.81
AdaBoost	0.87	0.87
Gradient Boosting	0.81	0.82

Figure 4. 4: Accuracy performance of the four models used for prediction.

A). Percentage accuracy of the 4 classifiers used; (Extremely randomized trees, random forests, Adaboost and gradient boosting models. B). The average accuracy of the 4 classifiers used when the different percentage of training and testing data set is used. C). Validation of the different models with random data. D. Precision and recall of the 4 classifiers.

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4.1.4.6 Predictive antibody signatures that distinguish high and low transmission areas

The classifiers identified antibody signatures of varying numbers from the 190 antibodies, including Extremely Randomized Trees classifier (77), Random Forest classifier (70); AdaBoost classifier (56), and Gradient Boosting classifier (34). To identify a core signature, we determined antigen specificities that were conserved between the 4 classifiers selected. This common set includes 17 antigen specificities (Figure 4.6C&D): MSP4, MSP2 3D7, MSP1-19 2A, Etramp 5 Ag1 His var 3, MSP1-19 2B, Etramp 5 Ag1 His var 2, MSP2 [15-46b], MSP2 [5-36A], ACS5 Ag 3, PfMSP1_19, MSP3 FVO, Var2CSA, SE36/SERA 5 (T), HSP40 Ag 3, GLURP R2, Rh4.2_2030 and MSP2 CH150/9 capable of discriminating high versus low transmission areas. The 4 classifiers predicted children from the varying malaria transmission areas using the core antibody signature against 17 antigens with accuracy ranging between 80%-83%, sensitivity range of 80% - 85%, specificity of 79% - 86% and AUC of 0.89 - 0.92 (Figure 4.5).

To separate individual antibody responses with respect to the transmission site, principal component analysis (plotted in 2-dimensional) was performed for antibody responses predicted by the 4 classifiers, the 17 antibodies that were common with 4 classifiers and compared to using antibody responses to all the 190 antigens (Figure 4.6).

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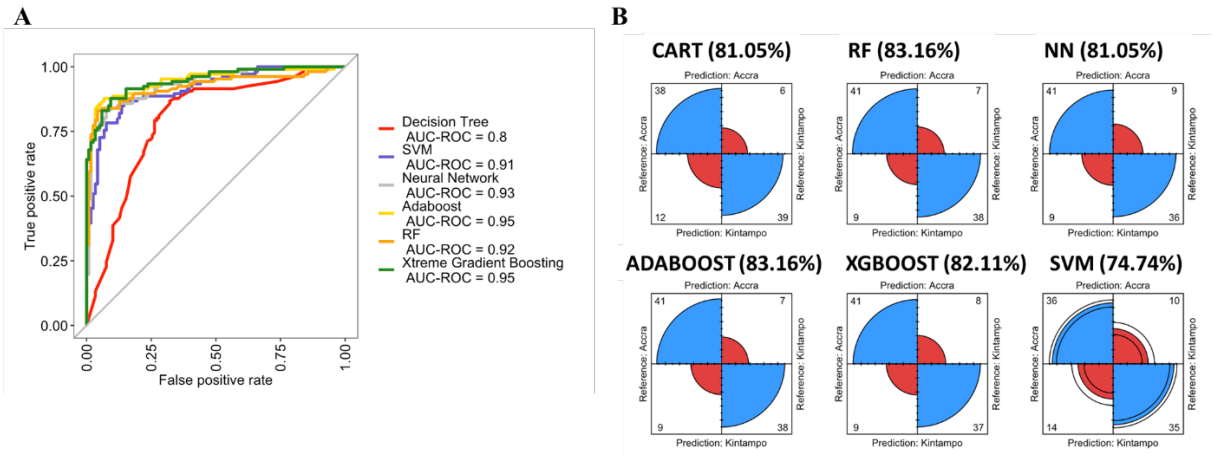


Figure 4.5: Predictive performance of the 6 classifiers.

A). ROC area under curve plots derived from the six models' decision tree (red line), SVM (purple line), Neural network (grey line), Adaboost (yellow line), Randomforest (orange line) and Extreme gradient boosting (green line) to predict performance for all the antibodies tested. Area under the curve (AUC) is given in their respective legend, whilst the grey line represents an AUC = 0.5 B. Confusion matrices derived from a model of all the antibodies tested in low and high transmission areas.

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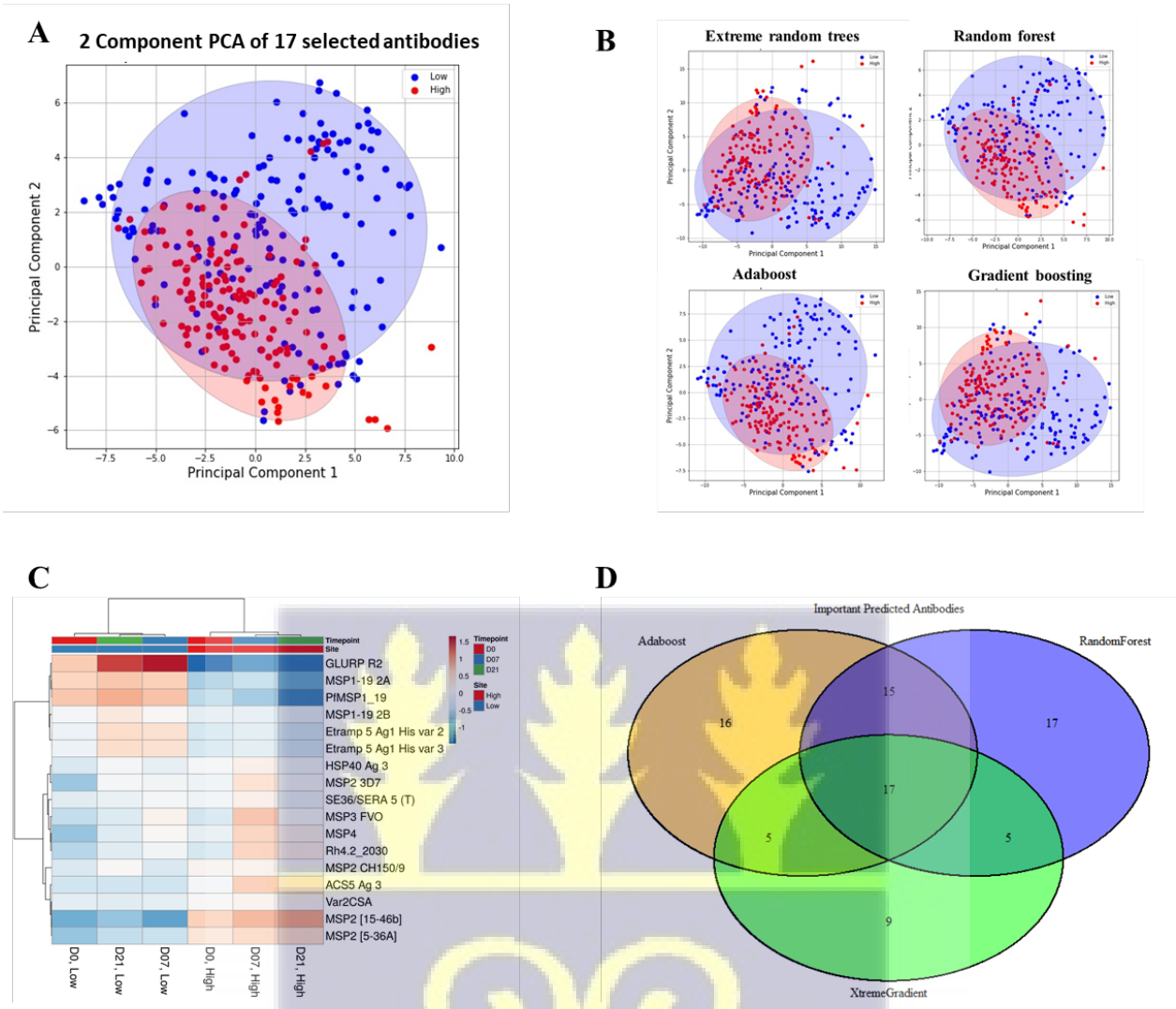


Figure 4.6: **Principal component analysis (PCA) of antibody responses showe a separation between individuals from the high and low transmission areas.**

A). PCA using antibody responses against 17 antigens tested in the study. B). PCA for the antibody responses predicted by the 4 predictive models used is separately plotted Extremely randomized trees, Random forests, Adaboost and Gradient boosting). C). Heatmap representation of median signal intensities (MFI) of antibody response to the 17 selected antigens comparison by transmission intensity. D). Venn diagram showing the intersection of the 3 best predictive models and antibodies intersecting within the models.

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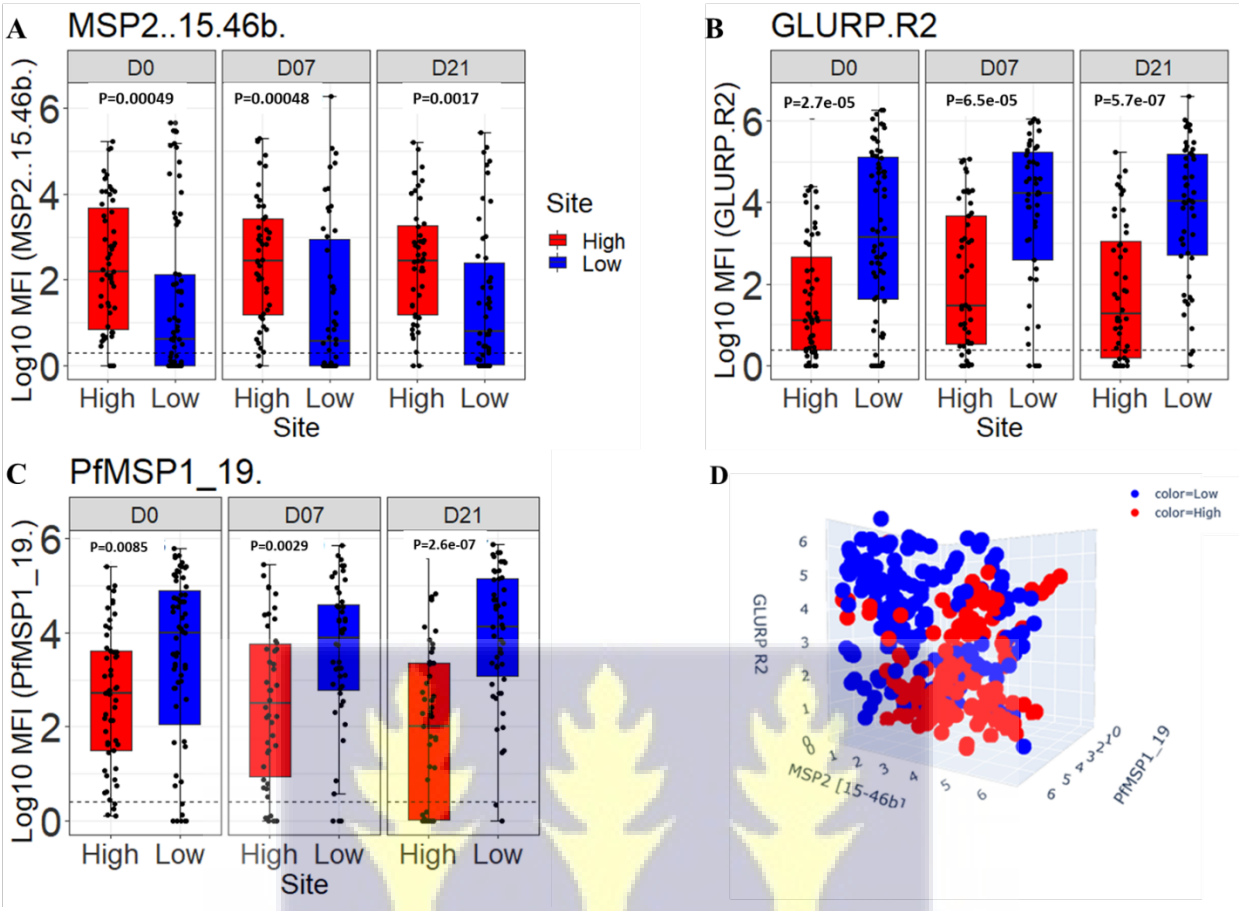


Figure 4.7:Signal intensity comparison of the top 3 selected antibody signatures discriminating individuals from the high and low transmission intensities.

A, B and C- Boxplots representing magnitude of antibody response to the top 3 selected antigen signatures (median and interquartile range (IQR) of signal intensities (MFI) over time in individuals from high (red) and low (blue) transmission intensity areas (P values, Mann-Whitney test). The antigens are MSP2.15.46, GLURP.R2 and PfMSP1_19, respectively. (D). 3-D PCA plots for the top 3 antibody signatures using MFIs further selected from the 17.

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4.1.4.7 Antibody responses selected as important features to distinguish the low and high transmission areas

We used a feature selection Boruta algorithm to identify important antigens in differentiating antibody responses from the two sites. We identified 53 antigens that contributed significantly to classification of whether individuals were from the high or low transmission area based on the antibody response to these antigens (Figure 4.8A). The greatest relative importance for classification of individuals in the high transmission area was contributed by Pep18, CIDR, VarCSA, MSP2 variants, ACS5, *Pf*AMA, HSP40, Rh4.2_2030, MSP3, MSP4 and MSP6. While antibody responses to MSP1 variants, RALP-1, Etramp 5 and GLURP were considered important for classification in the low transmission area (Figure 4.8B). Similar to the result based on the 4 classifiers, the 17-antibody signature was also among the 53 identified by the Boruta algorithm.

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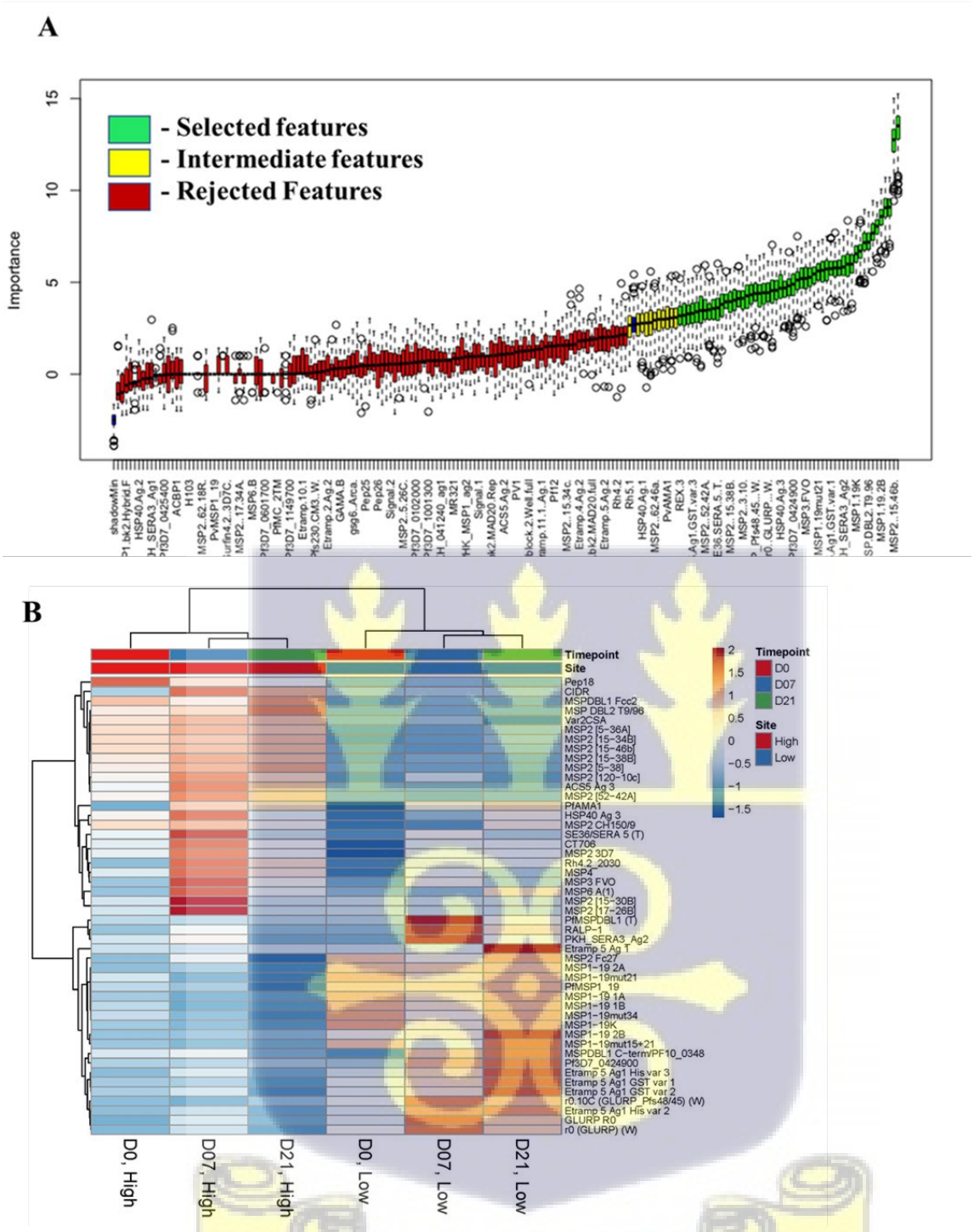


Figure 4. 8: Feature selection of antibody responses that distinguish the low and high transmission areas.

A). Variable importance plot for classification analysis using the Boruta feature selection algorithm. Antibody responses are ordered from left to right, with importance defined as the Z-scores of the mean decreasing accuracy.

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Green boxes represent the 53 selected antibody responses that contribute significantly to classification, red boxes represent antibody responses not contributing significantly and yellow boxes represent intermediate response. B). Heatmap clustering the antibody responses predicted by Boruta model.

4.1.4.8 Multivariate linear regression analysis for selected antigens

To evaluate factors affecting the IgG levels, specific antigens predicted by the models were fitted with multivariable linear regression models (coefficient, 95% confidence interval [CI], p-values), including the following predictors: Age, Parasite density, malaria transmission site and Sickle cell status (using the same antigen/IgG levels as the outcome variable), (See Table 5). The results indicate that age and transmission intensity are categorical factors that are likely to affect antibody reactivity.

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Table 4. 1: Multiple Linear regression analysis for selected proteins

Multiple regression analysis

	MSP.DBL2.T9.96			**MSP1.19.1A**			**MSP1.19mut34**			**MSP1.19mut34**			**MSP1.19mut15.21**		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	-0.04	0.72 – 0.63	0.904	3.01	2.18 – 3.83	**<0.001**	2.57	1.83 – 3.31	**<0.001**	2.57	1.83 – 3.31	**<0.001**	1.55	0.93 – 2.17	**<0.001**
Age	0.14	0.07 – 0.21	**<0.001**	-0.04	0.12 – 0.04	0.348	-0.04	0.11 – 0.03	0.285	-0.04	0.11 – 0.03	0.285	0.00	0.06 – 0.06	0.952
Site: Kintampo	1.15	0.78 – 1.53	**<0.001**	-1.38	-1.84 – 0.92	**<0.001**	-1.22	-1.62 – -0.81	**<0.001**	-1.22	-1.62 – -0.81	**<0.001**	1.01	-1.36 – -0.67	**<0.001**
ParasiteDensity	-0.00	0.00 – 0.00	0.684	0.00	0.00 – 0.00	0.414	0.00	0.00 – 0.00	0.471	0.00	0.00 – 0.00	0.471	0.00	0.00 – 0.00	0.379
SicklingStatus: Positive	-0.88	1.80 – 0.03	0.058	-0.40	1.52 – 0.72	0.486	-0.63	1.64 – 0.37	0.215	-0.63	1.64 – 0.37	0.215	0.64	1.47 – 0.20	0.134
Observations	310			310			310			310			310		
R^2 / R^2 adjusted	0.126 / 0.115			0.113 / 0.101			0.112 / 0.100			0.112 / 0.100			0.128 / 0.116		

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Multiple regression analysis

	GLURP.R0			GLURP.R2			r0.10C..GLURP_Pfs48.45..W.			Rh4.2_2030			Rh5.1		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.55	0.02 – 1.08	**0.041**	1.99	1.21 – 2.77	**<0.001**	0.31	-0.22 – 0.84	0.253	0.14	-0.48 – 0.77	0.654	1.52	1.14 – 1.90	**<0.001**
Age	0.12	0.07 – 0.17	**<0.001**	0.17	0.09 – 0.25	**<0.001**	0.12	0.06 – 0.17	**<0.001**	0.12	0.06 – 0.19	**<0.001**	0.02	-0.02 – 0.06	0.365
Site: Kintampo	-0.30	-0.59 – -0.01	**0.043**	-1.28	-1.71 – -0.85	**<0.001**	-0.24	-0.53 – 0.05	0.111	0.77	0.42 – 1.11	**<0.001**	0.20	-0.01 – 0.41	0.059
ParasiteDensity	-0.00	-0.00 – 0.00	0.573	-0.00	-0.00 – 0.00	0.556	-0.00	-0.00 – 0.00	0.563	-0.00	-0.00 – 0.00	0.140	-0.00	-0.00 – 0.00	0.410
SicklingStatus: Positive	-0.68	-1.39 – 0.03	0.062	-1.75	-2.80 – -0.70	**0.001**	-0.60	-1.32 – 0.11	0.099	-1.16	-2.00 – -0.31	**0.007**	-0.57	-1.08 – -0.05	**0.030**
Observations	310			310			310			310			310		
R^2 / R^2 adjusted	0.125 / 0.113			0.253 / 0.244			0.107 / 0.095			0.097 / 0.085			0.027 / 0.015		

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Multiple regression analysis

	Etramp.5.Ag1.GST.var.1			Etramp.5.Ag.1			Etramp.11.1..Ag.1			EBA175RII_F2			EBA181.RIII.V		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.29	0.71 – 1.87	**<0.001**	0.42	-0.17 – 1.01	0.159	0.34	0.07 – 0.60	**0.013**	0.91	0.38 – 1.43	**0.001**	-0.31	-0.76 – 0.15	0.185
Age	0.09	0.03 – 0.14	**0.004**	0.09	0.03 – 0.15	**0.004**	-0.00	-0.03 – 0.03	0.921	0.09	0.04 – 0.14	**0.001**	0.08	0.03 – 0.12	**0.001**
Site: Kintampo	-0.52	-0.84 – -0.20	**0.001**	-0.52	-0.84 – -0.19	**0.002**	0.05	-0.10 – 0.20	0.497	0.18	-0.11 – 0.46	0.224	0.46	0.21 – 0.71	**<0.001**
ParasiteDensity	-0.00	-0.00 – 0.00	0.466	-0.00	-0.00 – 0.00	0.346	0.00	-0.00 – 0.00	0.611	-0.00	-0.00 – 0.00	0.809	-0.00	-0.00 – 0.00	0.329
SicklingStatus: Positive	-0.57	-1.36 – 0.21	0.150	-0.33	-1.13 – 0.47	0.417	-0.12	-0.48 – 0.24	0.509	-0.20	-0.90 – 0.51	0.586	0.10	-0.51 – 0.72	0.744
Observations	310			310			310			310			310		
R^2 / R^2 adjusted	0.105 / 0.093			0.097 / 0.086			0.005 / -0.009			0.037 / 0.024			0.055 / 0.042		

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4.1.5 Discussion

Naturally acquired immunity requires antibody response for effective protection from disease. Antibody responses can, therefore, be used to provide important signatures that can be measured as correlates of immunity. During *Plasmodium* infection, several antigens are presented by the parasite, mounting antibody responses. Despite years of research in this area, it is still unclear which antigens play a vital role in the induction of protective antibody responses. Previous studies have suggested that protection could result from the accumulation of responses against a set or specific combination of antigens (Osier *et al.*, 2008; Osier *et al.*, 2014b). However, the number of possible interactions is still not clear. Development of technologies and analytical platforms for complex data have allowed large-scale analyses of immune responses, that have provided new insights into immunity and possible correlates of protection.

This study sort to determine the antibody repertoire to various malaria antigens using protein microarray covering *P. falciparum* and non-falciparum proteins. We measured antibody responses to 190 blood-stage, pre-erythrocytic- and gametocyte-stage *Plasmodium* antigens and using predictive modelling approaches, we identify a predictive antibody signature that can be associated with different exposure or transmission intensities. We used a modelling framework based on machine learning and feature selection to predict a combination of antibodies that can distinguish varying exposure (in this case, using individuals from different malaria transmission areas). The results show a broad antibody reactivity to various antigens and identified 118 antigens reactive in more than 80% of the individuals. This reflects the complex nature of individual antibody profile that works as a combination of high antibody reactivity to a few numbers of proteins, or equally as low reactivity to many proteins. This also shows that not all parasite antigens are important in

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eliciting a functional antibody response or that they are different immunogenically; hence, unable to trigger an immune response. Studies have also suggested that antibody responses to dominant antigens may supress responses to other less-dominant antigens (Proietti *et al.*, 2020). The breadth of the responses was more heterogeneous in the low than high transmission area but differences were not significant. Breadth of antibody response has been associated with reduced risk of malaria and anti-malaria protection due to the sum protective immunity across the different antigens (Osier *et al.*, 2008; Daou *et al.*, 2015). However, our data show that breadth and antibody response magnitude is not directly associated with transmission intensity, indicating an equal underlying immune profile of wide antigen recognition in both groups of children.

Antibody reactivity varied by transmission areas, with some antibody levels higher in either of the 2 areas or levels remaining the same in both sites. Most of the antibody reactivity that was uniquely high in the low transmission site were to MSP1, MSPBDL and GLURP antigens (Appendix A.3.1 shows details of antigens), while those in the high transmission site were to mostly MSP2 antigens. This demonstrates that there are exposure dependent differences in antibody responses and has been shown by other studies (Adu *et al.*, 2016; Cherif *et al.*, 2017).

The machine learning approach identified 17 antibody signatures distinct to the two transmission areas, that could likely predict the individual's immune status from the two transmission intensities. Most of the identified antigens have been proposed to induce protection and individually tested as vaccine candidates, but have shown poor efficacy. Therefore, suggesting that functional protective antibody response stems from multiple antigens, with high, moderate and low reactivity, due to the possible synergistic effect. Previous studies have suggested that

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combination of antibody responses to specific antigens are associated to immunity (Osier *et al.*, 2014b; Daou *et al.*, 2015; Schussek *et al.*, 2017). While other studies have argued that a more effective combination would be from antigens targeting multiple stages (Proietti *et al.*, 2020).

It is interesting to note that we observed that some antigens are reactive only in one transmission site and not in the other, indicating that immune status selects antibody response, or that dominant antigen suppresses others and that some antigens are differently immunogenic or more polymorphic. It could also mean that antigen prioritization is different in individuals from the two transmission areas; hence showing variability in antigen presentation and antibody production in different individuals (Yman *et al.*, 2019). These observations suggest that vaccine design based on one or a few antigens may not be effective in providing long term and high-level protection. Therefore, future vaccines designs should be considered based on the need to target a combination of many antigens to induce the required synergistic effect.

The vital challenges in correctly identifying antibody correlates of protection are finding biosignatures of previous and cumulative exposure. These signatures are important in assessing the difference between true protection from clinical symptoms and low exposure risk. Previous studies have used predictive framework to estimate recent exposure by identifying key antigens predictive since the last infection (Finney *et al.*, 2014), while others suggested antigens of continuous exposure (Valletta *et al.*, 2017). Our study took a different approach by assessing antibody responses in individuals with different exposure using differences in malaria transmission intensities as proxy for exposure variation, hence predicting key antigens that vary with exposure. We also used a longitudinal approach so as to monitor changes over time after a clinical episode.

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Machine learning models are currently widely applied to develop effective, sensitive and specific predictive models. However, the excess features in the training dataset for these models can increase the risk of overfitting and prediction variability. We therefore used various classifiers to increase the accuracy of the predicted signatures. We also incorporated feature selection by Boruta to provide a more robust model.

Several studies have associated naturally acquired antibodies to dominant Merozoite proteins, AMA-1, MSP-1_{42}, EBA-175, for protective immunity (Conway *et al.*, 2000; Dodoo *et al.*, 2008; Osier *et al.*, 2008; Osier *et al.*, 2010; Richards *et al.*, 2010; Dobaño *et al.*, 2012). However, antibody responses to these antigens were not predicted in our model as signatures that could distinguish responses between the two transmission areas. There was high reactivity to these antigens in individuals from both transmission areas. The antigens EBA140, ETRAMP5, GLURP, MSP1 and MSP6, elicited high antibody responses in the low transmission area, while higher antibody response was observed for Rh5.1, EBA181, VAR2CSA, ACS5, HSP40, MSP2, MSP3, MSP4, and MSP7 in the high transmission area. This is a similar trend to other studies, suggesting that the acquisition of immunity is dependent on malaria transmission intensity (Cherif *et al.*, 2017).

The continuous significant recognition of VAR2CSA in individuals from the high transmission areas is worth exploring, being one of the antibody responses selected by our model as distinguishing between the two transmission areas. The VAR2CSA is a family of variant antigens *P. falciparum* usually associated with parasites sequestering in the placenta (Babakhanyan *et al.*, 2014; Fried & Duffy, 2017), however, several studies have shown its presence in malaria exposed

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children (Beeson *et al.*, 2007; Fodjo *et al.*, 2016). This supports previous studies that suggest that children acquire antibodies to VAR2CSA and shows the importance on highly polymorphic antigens in boosting malaria immunity. Therefore, VAR2CSA should be considered in a multicomponent vaccine to allow the immune system to experience the variability presented by exposure to different parasite strains (Oleinikov *et al.*, 2012).

We further explored multiple linear regression analysis to check which participants characteristics were good antibody reactivity predictors. The results show that age and transmission intensity (high and low) were good predictors of antibody reactivity to distinct set of *P. falciparum* antigens. The rates of antibodies acquisition are antigen-specific, and different antigens produce varied antibody kinetics. Another study on the protection against clinical malaria in Ghana reported that the level of IgM and IgG, anti-MSP1-19, anti-MSP3, anti- MSP2, anti-GLURP and anti-AMA1, increased with age (Dodoo *et al.*, 2008). We identified several antigens recognized with the highest intensity in plasma from children in both transmission sites such as Rh4.2_2030, RH5.1, ACS5.Ag2, MSP5, uncharacterized antigens (Pf3D7_0102000 and Pf3D7_1419400_F3) and gametocyte stage antigens (Pfs48.45 and Pfs230_CM3), which may serve as exposure indicators. However, a recent study has identified GAMA, pfSEA1, PF3D7_1136200, MSP8 and PTEX150 as important antigens to identify recent exposure (Yman *et al.*, 2020).

The results suggest that antibodies to specific antigens that varied with transmission intensity and age could be targets of protective immunity, and could therefore be considered in multistage vaccine development to boost the naturally acquired immunity.

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In summary, we show that using multivariate and predictive modelling approaches allow a systemic interpretation of the complex antibody interactions. We see that despite having several immunoreactive antigens, only a few antibody responses are dependent on transmission intensity and can be used to predict the immune status of individuals. In our study, we specifically show that the detection of 17 antigens is adequate to distinguish individuals from different transmission areas, which could be tested as possible protection correlates. We propose further validation of these signatures in a different cohort of individuals from the same regions and other areas with similar transmission intensities.

4.1.6 Conclusion

The results of the study highlight the importance of a multiparameter approach to address malaria immunity complexity and contribute to the understanding of naturally acquired immune response to malaria. Comparison between different transmission area/exposure allowed us to identify antibody signatures that distinguish individuals with different immune status. Antibody responses to EBA140, ETRAMP5, GLURP, MSP1 and MSP6 were associated with low transmission while antigens associated with high transmission included Rh5.1, EBA181 RIII-V, VAR2CSA, ACS5, HSP40, MSP2, MSP3, MSP4, and MSP7. Feature selection and machine learning approaches identified a core signature of 17 antigens. The antigens identified could be useful as possible targets of protective immunity and provide clues for potential vaccine candidates that can be prioritized for evaluation.

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5 CHAPTER FIVE: SPECIFIC OBJECTIVE THREE

5.1 LONGITUDINAL BLOOD TRANSCRIPTOME PROFILING DURING ACUTE *P. FALCIPARUM* INFECTION IN CHILDREN FROM VARYING TRANSMISSION INTENSITY

Hypothesis: *Varying gene expression patterns in children living in areas with different transmission intensities.*

5.1.1 Abstract

Background: The mechanism of acquired immunity from febrile malaria remains unclear despite the increase in malaria research. Identifying these molecular interactions during *P. falciparum* infection is essential for understanding acquisition of immunity. Transcriptomics profiling can therefore provide an integrative description of immune responses used to dissect the mechanism of naturally acquired immunity. Though previous studies have contributed a lot to understanding immune responses to malaria, conclusive interpretation of these data has been limited by sampling design, disease definition, patient selection and approaches in natural infections. Longitudinal approach was used to assess the gene expression changes of naturally acquired immunity over the course of *P. falciparum* infection using whole-blood transcriptomes in children from areas with distinct malaria transmission intensity.

Method: We profiled whole-blood transcriptome by RNA-sequencing in children with acute *P. falciparum* infection. Peripheral blood sample were collected at time of presentation to the hospitals before treatment (D0) and after (D7 and D21) from two sites with different transmission

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intensity in Ghana. Differential gene expression was compared between the two sites and across the different time points.

Results: Gene expression levels differs with transmission intensity. More genes were differentially expressed during infection (D0) compared to convalesces (D7 and D21). Comparison of the DEGs between the two sites show that more genes were highly expressed in low transmission area compared to the high transmission area at D0. In the high transmission area, the DEGs enriched in gene ontology (GO) modules related to B cell and surface signature, T cell development and activation, platelet activation-actin binding, enriched cell cycle, and regulation of transcription and transcription factor modules, were up-regulated. Whereas, the low transmission area, had DEGs in modules associated with cell cycle and transcription, immune activation- generic cluster, E2F1 targets, plasma cell surface signature, immunoglobulins and enriched of multiple innate immune cells. Cellular deconvolution revealed an increased proportion of neutrophil cell type in both the high and low transmission areas, with decreased CD4 naïve, CD8, B cells and T follicular cells, during infection compared to convalescence samples. Our analysis revealed several distinct patterns of gene expression over time that play a role in TLR and inflammatory signaling, TBA, cell cycle and transcription and enriched activated dendritic cells. Gene sets that were significant in all the different comparisons done included those involved in adaptive and innate immune system, cytokine signaling, programmed cell death, activation of APCs and DNA damage checkpoint.

Conclusion: These data provide insight into the kinetics of molecular immune responses in natural infection. We identified genes whose expression patterns can help differentiate changes during acute *P. falciparum* infection and after treatment in varying transmission intensity on a functional

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molecular level. The low transmission areas had high number of DEG compared to high transmission area, suggesting increased immune activation in the low transmission area. We showed significantly DEGs related to cytokine genes that correlated well with the increased levels of cytokines measured (IL10, CXCL9, CXCL10, CXCL17, CXCL11 and CCRL2).

These results suggest that variation in transmission intensity contributes to the variable host immune response that affect individual's ability to fight *P. falciparum* infection.

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5.1.2 Introduction

Malaria remains a significant public health concern with approximately 200 million new clinical cases annually and 400,000 deaths, annually (WHO, 2020). *Plasmodium*, the parasite responsible for malaria, has multiple developmental stages both in the human host and mosquito vector (Phillips *et al.*, 2017). Clinical manifestations of different forms of disease severity are as a result of various host pathogen interactions. *P. falciparum* infections induce potent immune responses, aimed at eliminating the pathogen and coordinated interaction is required to induce long-lasting protection against reinfection. However, there is limited understanding of these complex interactions, and the development of naturally acquired immunity (NAI). Hence, the need for a clear understanding of immune responses to malaria as a series of responses to spatial, temporal and antigenically distinct life cycle stages.

Over the years, a lot of studies have contributed to the understanding of the immune responses in malaria (Deroost *et al.*, 2016), the data has however not been sufficient to clearly define the mechanisms of acquired immunity to malaria. Therefore, an integrative look at the gene expression in immune cells induced during the course of natural infection can be important for identifying and understanding immune responses at a systems level. Transcriptome analysis studies have helped understand the molecular basis of immune responses in malaria disease (Lee *et al.*, 2018a). This includes studies that have described transcriptome analysis of peripheral blood mononuclear cells (PBMC), or of whole blood showing the DEGs profile of genes related to innate and adaptive immune responses in controlled human malaria infection (CHMI) and natural *Plasmodium* infection.

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Early gene expression analysis in controlled human malaria infections (CHMI), presented an opportunity to monitor early immune responses after exposure to parasites. One study found upregulation of cell surface and intracellular pattern recognition receptors, antigen-processing and presentation for major histocompatibility complex class I (MHC-I) and MHC-II, proinflammatory cytokines, phagocytic and scavenger receptors, glycolysis pathway genes and NADPH oxidase components (Ockenhouse *et al.*, 2006). Another study showed enrichment of genes related to platelet activation, CORO1A-DEG6 network as well as translation and transcription (Rothen *et al.*, 2018). Together indicating a harmonized activation of different components of the innate immune response. Transcriptome analysis later in infection, when individuals first developed fever, showed similar results, with additional downregulation of T and B cell genes noted (Tran *et al.*, 2016). These findings however cannot be translated directly to what happens in natural infection because of the difference in dynamics of infection, where infection and disease outcome is controlled. Additionally, CHMIs have only been done in adults whose immune response is more robust than children who are the vulnerable group in malaria endemic areas.

Transcriptomics analysis have also been useful in identifying the complex interaction of immune system after RTS,S vaccination, to better predict vaccine-induced protective immune responses(Moncunill *et al.*, 2021; Moncunill *et al.*, 2022). Comparison of PBMC transcriptome in volunteers before and after vaccination with adjuvanted RTS,S vaccine and showed significant gene expression changes in plasmablast-associated transcriptional signatures, cell cycle genes, and type I interferon genes after vaccination, however no differences were observed between the protected and non-protected(Tran *et al.*, 2020). GSEA in the same study revealed upregulation of immunoproteasome degradation pathway, cell cycle and apoptosis function in the protected,

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suggesting differential antigen presentation and processing (Vahey *et al.*, 2010; Kazmin *et al.*, 2017). A more-focused transcriptomic analysis on the same vaccinated groups using modelling approach to predict protection status, identified 110 genes, 42 related to immune functions, including those within IFN-γ signalling pathway in the models to be sufficient in accurately predicting protection (van den Berg *et al.*, 2017). Whole-blood transcriptomics profiling assessing *ex vivo* immune response in Dutch volunteers vaccinated with CSP vaccine showed up regulation of T cell and NK cells modules in protected individuals 28 days after first vaccination (Tran *et al.*, 2019a). The studies on CHMI challenge and vaccine trials have advantages of precise timing of immunological changes, individuals can be properly followed and biological samples collected in ideal conditions. Therefore, can provide detailed assessment of the complex immune response in the host.

Despite the several challenges associated with interpreting transcriptome analysis data in *Plasmodium* natural infection (Tran *et al.*, 2012). Studies have used whole-blood transcriptomics to decipher mechanisms of immune response during *Plasmodium* natural infection. Studies comparing whole-blood transcriptomes in children with uncomplicated *P. falciparum* malaria and negative controls, showed that acute malaria induced upregulation of innate immune response; IFN-inducible genes, neutrophil-related modules and markers of Fcγ- receptor mediated phagocytosis and downregulated adaptive signatures including T cells, B cells and cytotoxic T cell pathways (Idaghdour *et al.*, 2012). Similarly, studies that examined differences in gene expression in severe malaria and uncomplicated malaria showed similar upregulation of neutrophil related genes (Lee *et al.*, 2018b). Recent studies have also examined whole-blood transcriptomes to investigate the impact of cumulative malaria exposure on the immune system in age-matched

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children who had low or high previous malaria episodes and malaria naïve. Differential gene expression showed significant DEGs between high and low exposure groups related to innate immunity, T cell differentiation, and regulation of adaptive immune system, but it could not differentiate between low and malaria naïve groups (Bediako *et al.*, 2019). A prospective cohort study aimed to understand cellular mechanisms in naturally acquired protection showed that immune individuals also had enriched molecular signature associated to B cell and interferon activation (Tran *et al.*, 2019b).

While most studies agree (Idaghdour *et al.*, 2012; Bediako *et al.*, 2019; Tran *et al.*, 2019b) on some gene expression profiles, others have presented contrasting data, especially when comparing whole blood gene expression in symptomatic exposed versus previously naïve individuals, showing less upregulation of interferon responses but greater upregulation of genes responsible for B-cell receptor signalling (Tran *et al.*, 2016) These conflicting findings may represent genetic or clinical episode differences between the different groups, differences in parasite loads (Idaghdour *et al.*, 2012), or changes in leukocyte subpopulations proportions in infection. The effect may be dependent on whether RNA is extracted from PBMCs or whole blood. Differences in results between studies may also be due to different patient cohorts with different symptoms under different transmission conditions, variation in study design such as cross-sectional studies, and using different transcriptomics platforms. Additionally, few studies have longitudinally profiled gene expression changes during acute *P. falciparum* infection and after treatment in areas of varying transmission intensity. Thus, although many DEGs can be associated with a certain outcome it is still difficult to select a transcriptional profile that can best predict protection or immunity to malaria in all settings.

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We hypothesised that children living in high malaria transmission areas will have different transcriptome compared to children living in low transmission areas. Transcriptome differences of children living in high transmission ("immune" / "highly exposed") compared to those in low transmission ("non-immune" / "less exposed") over the course of acute *P. falciparum* infection may predict markers of protective immunity. Therefore, this study used a longitudinal approach to characterise gene expression signatures during acute *P. falciparum* infection in children with different exposure levels. The study identified genes upregulated in the low transmission area during acute infection that were involved in organ and tissue specific immune response, type 2 immune response, interleukin and chemokine production, interferon-gamma production, neutrophil mediated immunity and humoral response. Other genes also upregulated in this area were responsible for negative regulation of cell activation, anti-inflammatory response and regulation of innate immune response. Whereas in the high transmission area upregulated genes were involve in chemotaxis, cell surface signalling, lymphocyte migration, MAPK cascade and innate immune response. Pathway analysis revealed p53 signaling, JAK-STAT signaling, cytokine-cytokine receptor interaction and Th17 differentiation as the major pathways enriched from DEGs in both the high and low transmission area. The study identified cell signatures such as neutrophils, CD4 and CD8 T cells as the most important cells that express the gene signatures during the interaction of the human host with *Plasmodium* parasites.

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5.1.3 Methods

5.1.3.1 Study design and site

The study was a longitudinal study of children aged 5-14 years with acute *P. falciparum* infection from two distinct malaria transmission intensity regions in Ghana. Kintampo Health Research Centre, Kintampo (high transmission intensity) and Ledzokuku-Krowor Municipal Assembly Hospital (LEKMAH) in Teshie, Accra (Greater Accra Region) (low transmission intensity). Malaria transmission intensity in the selected recruitment sites have been determined by the entomological inoculation rates and are high in Kintampo (>250 infective bites/person per year), and low in Accra (<50 infective bites/person per year) (Klinkenberg *et al.*, 2008; Owusu-Agyei *et al.*, 2009).

5.1.3.2 Ethical considerations

Participation in the study was voluntary, and each participant's parent or guardian signed an informed consent form before recruitment to the study. All ethical considerations were reviewed and accepted by the Ghana health service (GHS-ERC:002/08/17), Noguchi ethical review (008/17-18) committee, and Kintampo health research Centre review committees (IEC- 2017-28 and SSC-khrc/adm/2017-11).

5.1.3.3 Participant recruitment

Forty-three children age 5-14 years, who presented with acute *P. falciparum* infection confirmed by a positive RDT and Giemsa-stained blood smear at the hospitals, with 2 timepoint sample collection at D7 and D21 post-treatment, were included in RNA-seq analysis. A total of 127

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samples were analysed from the high transmission site (D0 =20, D7 = 14 and D21 = 18) and the low transmission site (D0 = 23, D7 = 25, D21 = 24).

5.1.3.4 Blood collection

Peripheral whole blood was collected into Tempus™ Blood RNA Tubes (ThermoFisher Scientific) from participants during infection (D0) and follow-up visits on days 7 and 21 after treatment. The sample was stored at -80°C until RNA extraction.

5.1.3.5 RNA extraction

RNA was extracted using Tempus™ Spin RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Whole blood in Tempus tubes was transferred to a 50 mL tube, diluted with sterile PBS and vigorously vortexed. The blood lysate was then centrifuged at 4°C at 3000 × *g* (rcf) for 30 minutes. The supernatant was carefully poured out and the RNA pellet resuspended with RNA purification resuspension solution. Resuspended RNA was then transferred into a purification filter and eluted into a microcentrifuge. All steps were done on ice to preserve RNA quality and integrity. The RNA quality and integrity were checked using the Nanodrop, Qubit and Agilent Bioanalyzer Samples with concentrations above 70 ng/µl and RNA integrity number (RIN) greater than 7 (Representative results provided as Appendix) were used for the next step involving depleting globin mRNA from the sample.

5.1.3.6 Globin clear

The GLOBINclear™ Kit (Ambion, Inc.) was used for globin mRNA removal following manufacture's protocol. To capture the mRNA, the kit uses a bead capture separation method that takes advantage of the strength of biotin/streptavidin binding, the specificity of nucleic acid hybridization, and the convenience of magnetic separations. Total RNA samples were incubated

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for 15 minutes with a biotinylated capture oligo mix specific for human globin mRNA to allow the biotinylated oligonucleotides to hybridize with the globin mRNA. After that, streptavidin magnetic beads were added and incubated for 30 minutes. The streptavidin was used to bind the biotinylated oligonucleotides, allowing the globin mRNA to be captured on the magnetic beads. The streptavidin magnetic beads were then pulled to the side of the tube with a magnet, and the RNA was transferred to a new tube, depleted of the globin mRNA. The treated RNA was then purified using a rapid magnetic bead-based purification method that involved adding an RNA binding bead suspension to the samples and then using magnetic capture to wash and elute the globin cleared RNA. The globin depleted RNA was quality checked (representative results are provided as appendix) and used for cDNA library preparation.

5.1.3.7 Library preparation and RNA sequencing

Globin cleared mRNA was used to prepare RNA sequencing libraries with the KAPA mRNA Hyper Prep Kit (kapabiosystems) as per the manufacture's protocol. This included capturing mRNA with magnetic oligo-dT beads and fragmenting it with heat and magnesium. The first-strand of cDNA was then synthesized using random priming, and the second strand was synthesized using A-tailing. This resulted in double-stranded cDNA: RNA hybrid to double-stranded cDNA (dscDNA). The second cDNA strand was then incorporated with dUTP, and the 3' ends of the resulting dscDNA were capped with dAMP. An adapter ligation was the next step, in which dsDNA adapters with 3' dTMP overhangs were ligated to library insert fragments. The library amplification step was then carried out, in which library fragments with appropriate adapter sequences at both ends were amplified using high-fidelity, low-bias PCR. The dUTP-

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marked strand was not amplified, allowing for strand-specific sequencing. Prepared library qualities were checked by confirming library fragment size distribution using the Agilent Bioanalyzer 2100 system (Representative results provided as appendix). The libraries were sequenced to an average depth of 30 million 1 x 100 base pair single-end reads per sample on an Illumina, v4 chemistry Hiseq 2500. The sequencing was done at The Francis Crick institute's advanced sequencing facility.

5.1.3.8 Data processing and statistical analysis

Read adaptor removal and quality trimming was carried out with Trimmomatic (version 0.36). Raw data reads were aligned to the human genome, using Ensembl GRCh38 - release 89 as reference using STAR (v.2.5.2a) and annotated with RSEM package (v.1.2.31). DESeq2 (v 1.28.0) within the R programming environment (R version R-4.0.2) was used to qualify reads within coding sequences and identify differentially expressed (DE) genes. Pairwise comparisons between conditions were performed in DESeq2 using a negative binomial generalized log-linear model through the glmLRT fit function. Significantly differentially expressed genes (DEG) had a false discovery rate FDR–adjusted *P*-value of <0.01 from DESeq2 and an FDR-adjusted *P*-value of <0.05 from the intensity-difference test. The Benjamini-Hochberg correction [false discovery rate (FDR)] was used to adjusted the *P*-values for multiple comparisons using.

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5.1.4 Functional annotation

5.1.4.1 Gene expression estimation and differentially expressed genes analysis

The DESeq2 was used to assess statistically significant DEGs between the high and low transmission areas for each time point using a negative binomial model. This pairwise comparison tests the null hypothesis that the two groups have equal expression distribution (i.e., the gene is not differentially expressed) for each gene and rejects this hypothesis if the two groups have significantly different expression distributions (i.e., the gene is differentially expressed). ANOVA was used to estimate the variance of genes across the 3 time points for the two groups. The ANOVA was used to look for DEGs between any set of groups, with the null hypothesis that the mean gene expression is the same across all groups. *P*-values were corrected for multiple testing using Benjamini-Hochberg FDR and adjusted *P*-value < 0.05 and a minimum 1.0-log2 fold change used to select the DEGs. An independent data set was generated using multiple comparisons. The results were therefore generated using multiple comparison, in which each patient had no more than one measurement. Independent samples were used to differentiate between location at the other time points (D7 vs D0, D7 Vs D21 and D21 Vs D0). To assess the variability of transcriptional signatures induced during infection and after in subjects from the two distinct transmission areas, we analysed the expression data with a total average of 22,500 genes in the 127 individuals, using unsupervised principal component analysis (PCA).

The data analysis was supported by the bioinformatics and biostatistics facility (Miriam Llorian Sopena and Gavin Kelly) at The Francis Crick institute.

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5.1.4.2 Gene set enrichment analysis

To determine significant associations between blood transcriptional patterns of each timepoint comparison at both sites, Gene Set Enrichment Analysis (GSEA) was used. This is a computational method that determines if predefined genes are significantly enriched by a concordant with each group (Subramanian *et al.*, 2005). The GSEA software was used to perform pathway enrichment analysis of the DEGs; this is a threshold free method that analyzes all genes based on their differential expression score, with no gene filtering. By checking from the top to bottom of the DEGs list, the software calculates an enrichment score (ES) and increases the ES if a gene is included in the pathway or reduces the score if that is not the case. The running sum values are weighted so that the top and bottom DEGs are enriched, while the moderately expressed ones are not enlarged. The maximum value of the running total, normalized in relation to the pathway size, is determined by the ES to give a normalized enrichment score (NES), which reflects the enrichment of the pathways. Positive and negative NES values represents enrichment at the top and bottom of the list respectively. A permutation-based *P* is then calculated and corrected for multiple testing to yield a permutation-based false-discovery rate (FDR) Q value ranging from 0 as highly significant to 1 as not significant. The resulting pathways were selected using the FDR Q value threshold $Q < 0.05$ and ranked using the NES. We used the phenotype permutation method to determine significance (*P* value) of the ES for comparisons of our groups. The software has published gene sets and pathways database that can be selected or provide custom database. We used modules identified by hallmark (Liberzon *et al.*, 2015) and Blood transcription module (BTM) (Li *et al.*, 2014), which were loaded to the software as gene sets.

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5.1.4.3 Blood transcription module analysis (BTM)

We used GSEA which was previously developed using broad institute (http://software.broadinstitute.org/gsea/index.jsp), based on Blood Transcriptional Modules (BTM) framework that includes 30,000 human blood transcriptomes derived from over 500 publicly available studies in public databases. The GSEA parameters contained weighted enrichment statistic and Signal2Noise metric, with 1,000 permutations (Ampomah *et al.*, 2014). The DEGs were all matched to a BTM gene list to generate a combined dataset with fold-change expression, gene symbol, and BTM gene set at each time-point.

5.1.4.4 Blood cell deconvolution for estimation of cell-type proportion

To estimate the relative abundance of each cell-type from whole blood expression profiles, we employed the CIBERSORT online software. That uses reference gene expression signatures called Leukocyte signature matrix (LM22) modules previously used to estimate the relative proportion of cell types from microarray data using a machine learning support vector regression (SVR) method. Cibersortx, which extends this framework to infer cell-type-specific gene expression profiles without physical cell isolation (https://cibersort.stanford.edu/), was used to deconvolve 22 functionally defined human hematopoietic subsets on the DEGs. (Newman *et al.*, 2019). This LM22 is a signature matrix file consisting of 547 genes established from microarray studies that accurately identifying 22 mature human hematopoietic cell phenotypes isolated from peripheral blood or *in vitro* culture conditions (Table S1), including seven T cell types, naïve and memory B cells, plasma cells, NK cells, and myeloid subsets (Chen *et al.*, 2018).

The raw expression counts were converted to transcripts per million (TPM) for input into CIBERSORT, and the TPM for our 127 samples and the LM22 gene signature matrix as input,

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was run online with 100 permutations and quantile normalization disabled as recommended for RNA-seq data. The output matrix is made up of deconvolution results with relative fractions of cell types normalized to one across all cell subsets for each sample.

5.1.5 Statistical analysis

For paired data from the same individual at different time points, all statistical tests for cell-type frequencies assumed nonparametric data; two-group comparisons were made using either two-tailed Mann-Whitney tests or Wilcoxon tests. All multiple testing was subjected to the FDR (or Benjamini-Hochberg) adjustment. The PCA was used to visualised variation of expressed genes. The distance between sample was calculated and represented by the correlation between samples using Spearman's rank correlation coefficient, which describes the direction and strength of the relationship between variables. The PCA plot and the Spearman's rank correlation heat map was generated using normalized reads per kilobases of transcript per 1 million mapped reads (RPKM) counts.

5.1.6 Results

5.1.6.1 Study participant characteristics

The total number of samples analysed was 127. The was a significant difference (*P =0.012)* in the median age between the high transmission (6 years (5-10) and low transmission areas (8 years (5-15). Each group had comparable gender proportions with thirteen (56%) males in the low transmission area and thirteen (61%) males in the high transmission area. To minimize uninformative variation, the analysis eliminated gender-specific genes, calculated normalization

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factors in order to scale the raw library sizes, and included the subject (which accounts for almost all batch effects) as a co-variate as per methods.

Table 5. 1: General Characteristics of the study population

Characteristic	High Transmission	Low transmission	P-value
Study Participants	**n = 21**	**n = 23**	NA
Median Age (Range), years	6.00 (5-10)	8(5-15)	0.0124
Gender (M:F)	62:38	57:43	
Episodes of malaria in a year	2 to 4	1	
Median Parasite Density (Range), per µl	16,354(380-779,100)	23,102(549-143,372)	0.3649
Median Hb levels (Range) g/dl	10.5(4.6-12.4)	11.6 (6.7-13.6)	< 0.0001
Parasitemia at D21	**2**	0	

5.1.6.2 Library QC

Libraries QC show that most libraries were sequenced on average 30 million reads with a duplication rate around 60%. There is one library that had > 100 mill reads (Figure 5.1A) which was excluded from analysis. Some libraries seem to map less to the human genome (< 80%) than others and they seem to associate with D0, at the peak of the infection, where there is high parasite concentration (Figure 5.1C).

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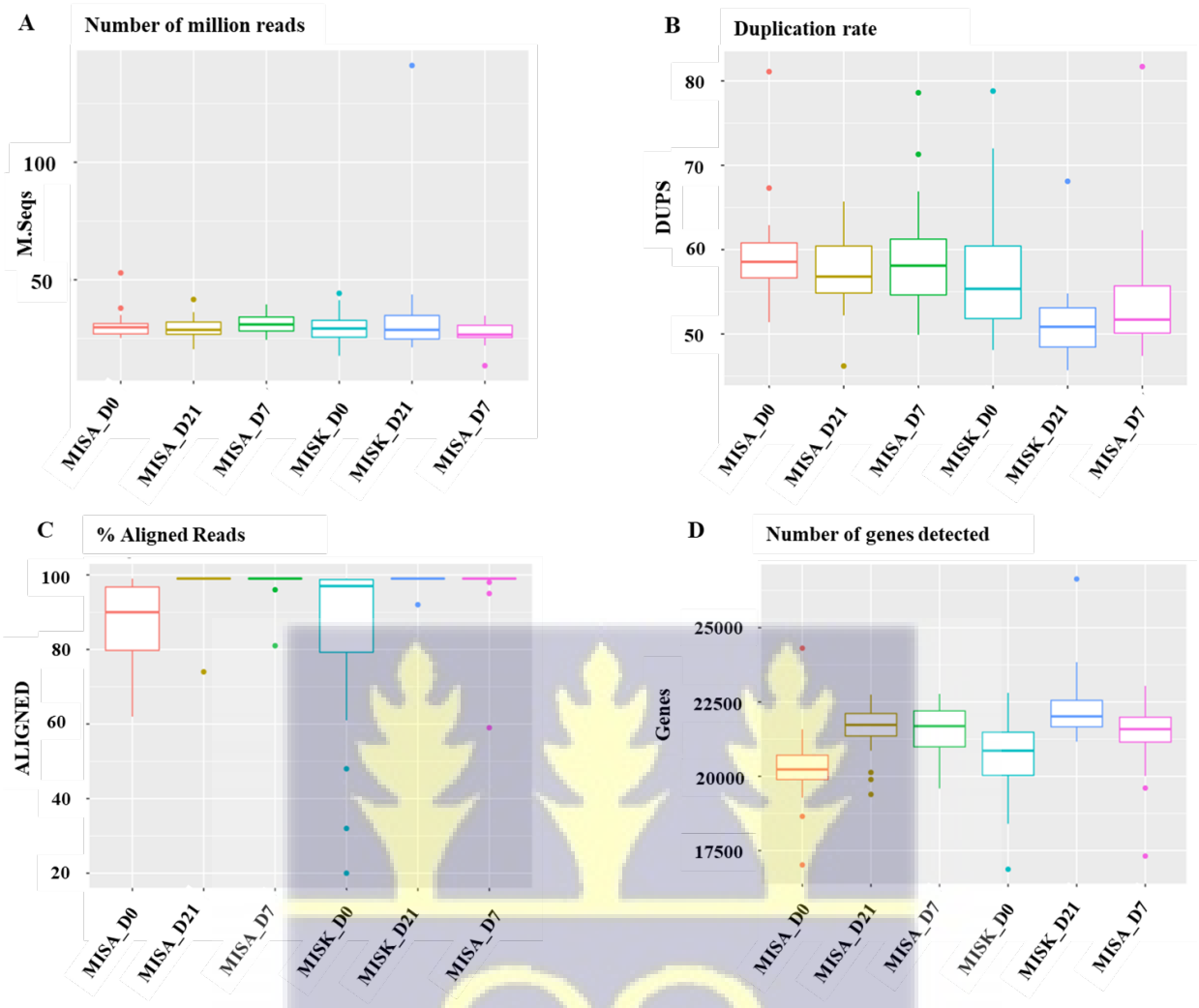


Figure 5. 1: Represents Library QC for sequenced libraries.

5.1.6.3 Sample variability in gene expression (PCA Plots)

We first performed principal component analysis (PCA) to assess the variability of gene expression patterns corresponded with sample group assignments. The PCA revealed a broad cluster by timepoint and site, although the clusters are not clearly separate. Variation along PC1 was best

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explained by time point (Figure 5.2A) and accounted for 29% of the variance, while variation along PC2 additionally explained 20% of the differences between the two transmission areas. There is variability between individuals, with the exception of samples from the low transmission area (MISA), which separate quite closely together at D0 (Figure 5.2A), while for samples from the high transmission area (MISK), there is more variability with a few samples clustering with MISA D. Most of the MISK D0 samples cluster with the D7 and D21 of both MISA and MISK. Gene expression patterns at day 7 and 21 from the two transmission areas are not clearly separated between the two-time points. This suggests similar gene expression profile between the two time points. This variation in gene expression patterns during infection (D0) in the two groups could be possible interindividual variations or differences in levels of disease severity and parasite levels. Overall, these results indicate substantial changes to blood transcriptional signatures during acute infection in the low transmission area compared to the high transmission area.

5.1.6.4 Sample correlation analysis based on genome-wide expression (Heatmap)

Sample to sample distances was used to determine inter sample variations and were represented with spearman's rank correlation (Figure 5.2B). A Heat map was generated using normalized reads per kilobases of transcript per 1 million mapped reads (RPKM) counts.

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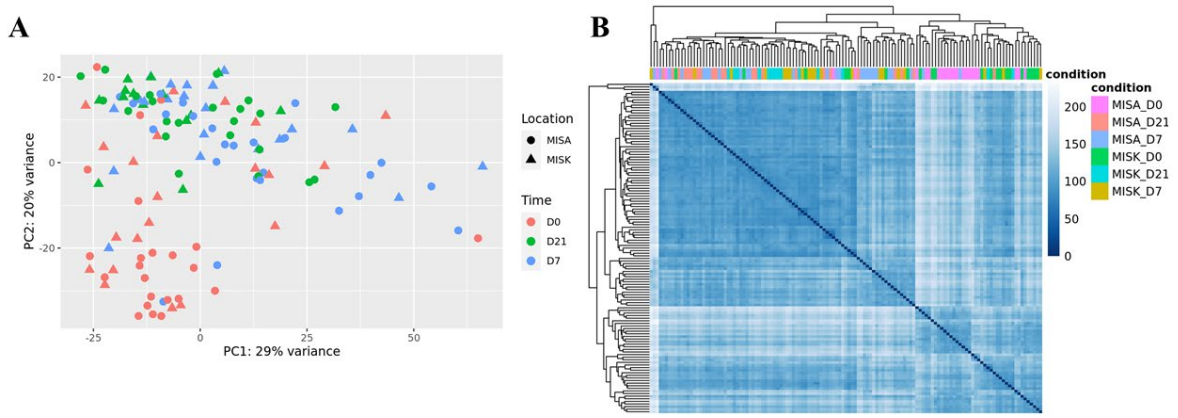


Figure 5. 2: Assessing sample variability:

(A) principal component analysis (PCA) displaying all 127 samples along PC1 and PC2 which describes 29% and 20% of the variability in the expression data set, respectively. The standardized PC analysis was applied to normalized (reads per kilobases of transcript per 1 million mapped reads). (B) Spearman's rank correlation plot visualizing sample- to-sample distance.

5.1.6.5 Differential gene expression during *P. falciparum* infection and after treatment in the distinct transmission areas

To determine the differences in gene transcription induced during *P. falciparum* infection and after treatment between individuals from two different transmission areas, we employed pairwise comparison analysis of samples from our study cohort at days 0, 7 and 21. Gene expression profiles were compared between the two areas for different time points, significantly DEGs were considered as genes with adj $P < 0.05$ and then $-1 < \log2FC < 1$. Our results show that *P. falciparum* infection-induced a multitude of transcriptional changes in the low and high transmission area, 532 and 423 genes were found among the highly DEGs in low and high transmission areas respectively.

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(Figure 5.3, A and Appendix Table S2). Genes upregulated in the low transmission area included genes involved in regulation of cell cycle phase transition like (HSP90AA1, CDT1, CDCA5, CDC25A, CDC20, CDC6, CDC45, CDC25C BUB1 and UBE2C). Other genes upregulated are those involved in regulating signalling pathway for immune response like (HSPD1, HSP90AA1, HSP90AB1, HSPA1A, TIFA, CTLA4, CLEC6A, TREML4, NFKBIA and FCGR1A), regulating innate immune response (SLAMF8, SOCS3, SOCS1, HMGB2, S100A9, S100A8, CLEC4E, IL18RAP and CEACAM1) and adaptive immune response (BATF, CCR2, HLX, IL27, CD274, IL31RA, IL18R1 and SAMSN1). Whereas, genes significantly upregulated in the high transmission area are those involved in angiogenesis (NR4A1, SPRED1, PLXDC1, NOTCH3, MMRN2, NRARP, HEY1 and THBS4), in the MAPK pathway (RET, SPRED1, ALOX15, TEK, NDRG4, HTR2B, FBLN1 and AXL) and regulation of developmental growth like (SYT17, SYT1, OLFM1, NRCAM, NOG, SOX15 and SEMA4G). Genes involved in negative regulation of the immune system like (CLEC12B, ALOX15 and SFTPD), leukocyte differentiation (LRRC17, NRARP and AXL) and the leukocyte activation cardiotrophin like cytokine factor 1 (CLF1) are among the immune related genes that were up regulated in the high transmission area. These results indicate a robust immune response in the low transmission area compared to the high transmission area considering more immune genes were highly expressed in the low transmission area. Genes significantly expressed at convalescence (D7) were 252 and 108 for the high and low transmission area, respectively after applying the threshold cut-off (Figure 5.3, supplementary data, Table A. 5.2). In the low transmission area these included genes involved in neutrophil mediated immunity (DEFA1, DEFA4, ELANE, SERPINB10, PRTN3, TARM1, OLFM4, HP, CD177 and MS4A3), humoral immune response and leukocyte migration (CXCL17, AZU1, SDC1, CEACAM8, OLR1, MMP1, MMP9, CXCL10 and CXCL9). Whereas those in the high transmission area were

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TBC1D3C, AC008758.1, AC233968.1, MEG3 and NAALAD2. At Day 21 (D21), 253 and 183 genes were DE in the low and high transmission areas, respectively (Figure 5.3, Day 21 supplementary data Table S4). Genes involved in platelet activation (MYL9, HBB, VWF, ITGA2B, PF4V1, TREML1, GP1BB, GP9, and DGKK) were unique in the low transmission area, while genes involved detoxification (MT1E, HBQ1, HBZ, HBA2, HBA1, HBM, HBD, and HBB), cofactor catabolic process (HBQ1, HBZ, HBA2, HBA1, HBM, HBD, HBB, BLVRB, and CYP4F2) and drug catabolism process (HBQ1, SMOX, HBZ, HBA2, HBA1, HBM, HBD, HBB, GCAT, OXCT2, and MAOB were similar in the both areas. Generally, more genes were differentially expressed in the low transmission area in all the 3 time points compared to the high transmission area, with Day 0 having the highest number of DEGs in both locations compared to Day 7 and Day 21.

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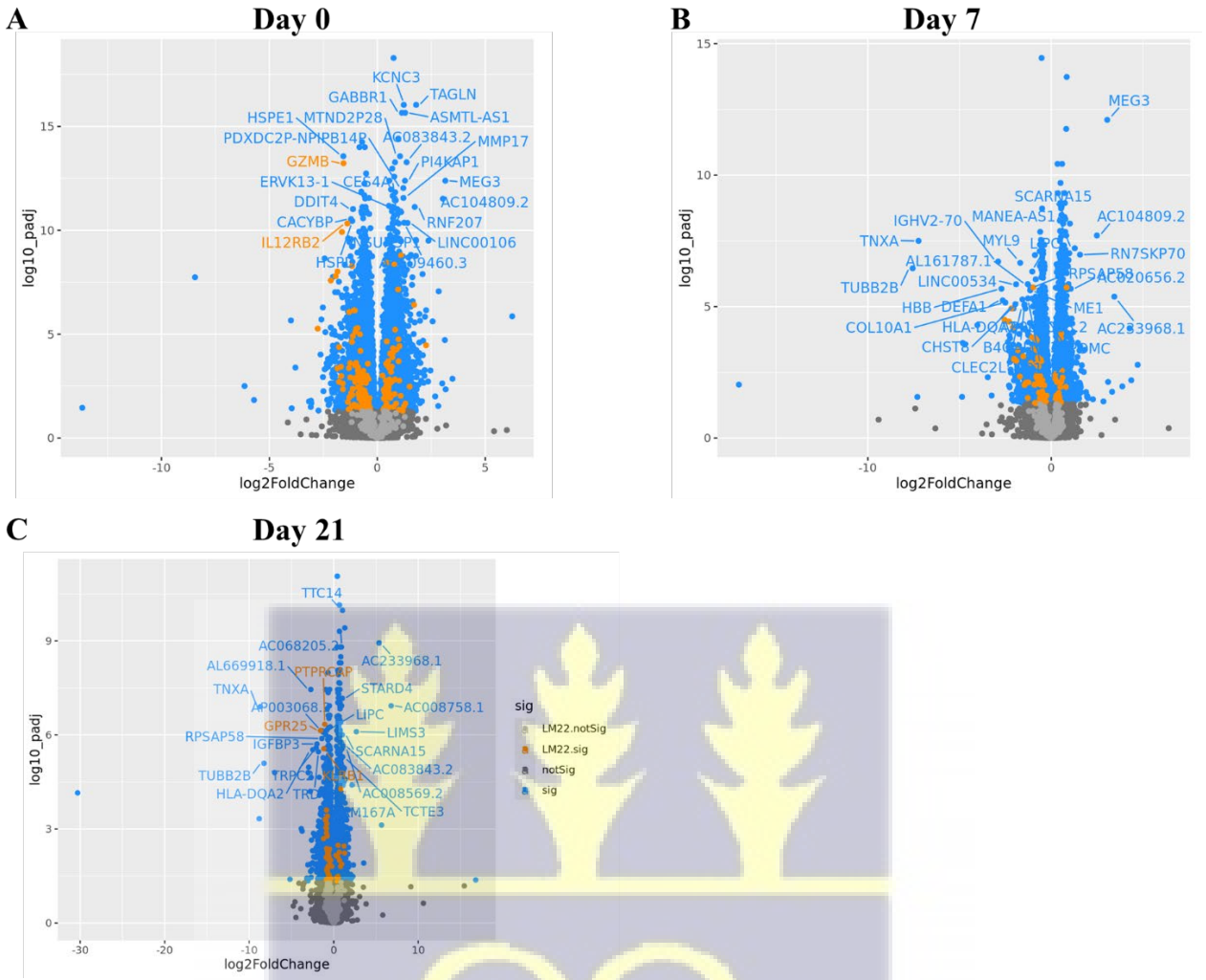


Figure 5. 3: Differential gene expression between the two study areas for the different study timepoints (D0, D7 and D21) displayed by volcano plots.

Differentially expressed genes with positive log fold change values represent the high transmission area, while those with negative log fold change values are for the low transmission area. Each dot represents one gene and is displayed according to the log fold change in expression (**x**-axis) and the statistical significance of the association (**y**-axis, in $-\log_{10}$ of the p-value). Blue dots indicate

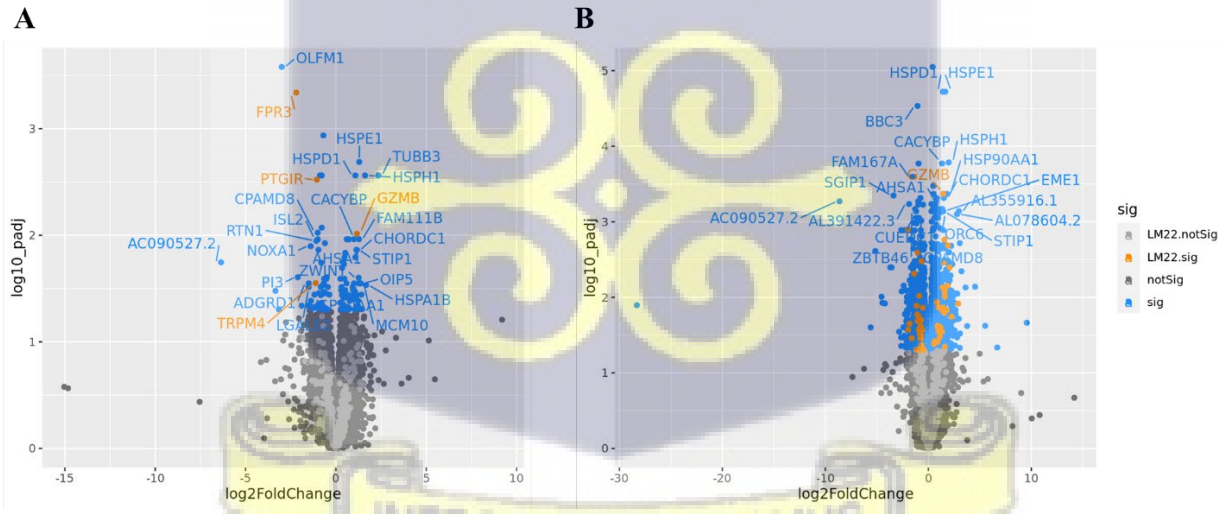
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genes deemed to be significantly differentially expressed (adj p-value < 0.05), orange dots indicate significantly expressed LM22 genes and grey dots indicate non-differentially expressed genes.

Extended pairwise comparative analysis was done to identify genes with differential expression at D0, D7 and D21 in the two transmission areas. Setting D7 and D 21 as the comparators we had 3 different at D7 (D7 Vs D0), D21 (D21 Vs D0) and (D7 Vs D21) after treatment, that revealed a substantial number of DEGs in blood. Overall comparison for the two sites, D21 Vs D0 had 622 genes that were upregulated at D0 compare to D21, these include genes involved in malaria immune response (IL10, IFNG, GYPC, HGF, SDC2, HBB, HBA2 and HBA1), antigen processing and presentation (HSP90AA1, HSP90AB1, IFNG, HSPA5, TAP1, HLA-DQA2, HSPA1B and HSPA1A) and p53 signaling pathway (CCNB2, RRM2, CCNE2, CHEK1, CDK1, FAS and BBC3). Other immune related genes that were differentially upregulated during infection are genes involved in E2F targets, IFN-α and IFN- γ response, TNF-α signaling via NF-kB and IL-6/JAK/STAT3 signaling. while the D7 Vs D0 comparison had 45 DEGs above the fold change threshold cut-off. There were no DEGs between D7 Vs D21 comparison in the two transmission areas. Comparing gene expression between the low and high transmission areas revealed that 2881 genes were significantly up-regulated at D0 compared to D7 and 3777 for the D0 versus D21 comparison in the low transmission area. This included; METTL7B, FAM20A, MTHFD2, SLC1A3, SLCO4A1, G0S2, SAMSN1, BCL2A1, POMP, VIPR1 and ANKRD22. A similar comparison showed 799 genes significantly up-regulated in high transmission area at D0 versus D7 and 665 genes at D21 versus D0, namely; SMPDL3A, C3AR1, MARCO, ITGA7, MS4A4A, RNF144B, TMEM176A, and METTL7B.

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Some of the genes with high fold change and high expression in the low transmission compared to the high transmission area that were up-regulated at D0 than D7 and 21 included; Granzyme B (GZMB), Cysteine And Histidine Rich Domain Containing 1) (CHORDC1), CUB Domain Containing Protein 1 (CDCP1), FKBP Prolyl Isomerase 4 (FKBP4), Heat Shock Protein 90 Alpha Family Class A Member 1 (HSP90AA1), Family With Sequence Similarity 111 Member B) (FAM111B), Heat Shock Protein Family E (Hsp10) Member 1 (HSPE1), Minichromosome Maintenance 10 Replication Initiation Factor (MCM10), Opa Interacting Protein 5 (OIP5), Regulator Of Calcineurin 1 (RCAN), and Tubulin Beta 3 Class III (TUBB3). Genes with high expression and high fold change in the high transmission area that were up-regulated at D0 compared to D7 and 21 are; Tryptase Beta 2 (TPSB2), N-formyl peptide receptor 3 (FPR3), Olfactomedin 1 (OLFM1), Peptidase Inhibitor 3 (PI3), Adhesion G Protein-Coupled Receptor D1 (ADGRD1), NADPH Oxidase Activator 1 (NOXA1), Synaptotagmin 17 (SYT17), and Reticulon 1 (RTN1).



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Figure 5. 4:Volcano plots showing DEGs with different patterns across the three time points between the two transmission areas.

(A). Represents D7 versus D0 comparison, (B). Represents D21 versus D0 comparison. Each dot represents one gene and is displayed according to the log fold change in expression (x-axis) and the statistical significance of the association (y-axis, in $-\log_{10}$ of the p-value). Blue dots indicate genes deemed to be significantly differentially expressed (adj p-value < 0.05), orange dots indicated significantly expressed LM22 genes and grey dots indicate non-differentially expressed genes.

5.1.6.6 Gene expression changes between early days of infection compared to after treatment

We further investigated the number of DEGs at the different time points in children from the two areas separately to understand the kinetics of induced transcription changes during *P. falciparum* infection and after treatment. Independent samples were used to differentiate between location at the other time points (D7 vs D0, D7 vs D21 and D21 vs D0). The DEGs (log fold change threshold -1 to 1 and adj p-value < 0.05) was determined at each time point relative to baseline to assess the transcriptomic changes induced by natural infection. The results that gene expression patterns are not only varied between the two sites but also at the different timepoints for each site separately. The low transmission area had 3,777 DEGs above the cut-off threshold and 371 genes of the LM 22 group for the D21 vs D0 comparison. The D7 vs D0 comparison had 2881 DEGs above the fold change threshold and 349 genes of the LM 22 group, while the D21 vs D7 had 79 DEGs. The high

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transmission area had 799 genes above the fold change threshold and 248 significantly DEGs of the LM22 group for the D7 vs D0 comparison. For the D21 vs D0 comparison, 4279 genes were differentially expressed with 665 genes above the fold change cut-off and 169 genes in the LM22 group. No genes were differentially expressed in the high transmission area in the D21versus D7 comparison (Appendix-Figure A 5.2). Overall, various genes with different expression patterns were observed between the locations over the different time points. These expression dynamics are visualised as heatmap (Figure 5.5)

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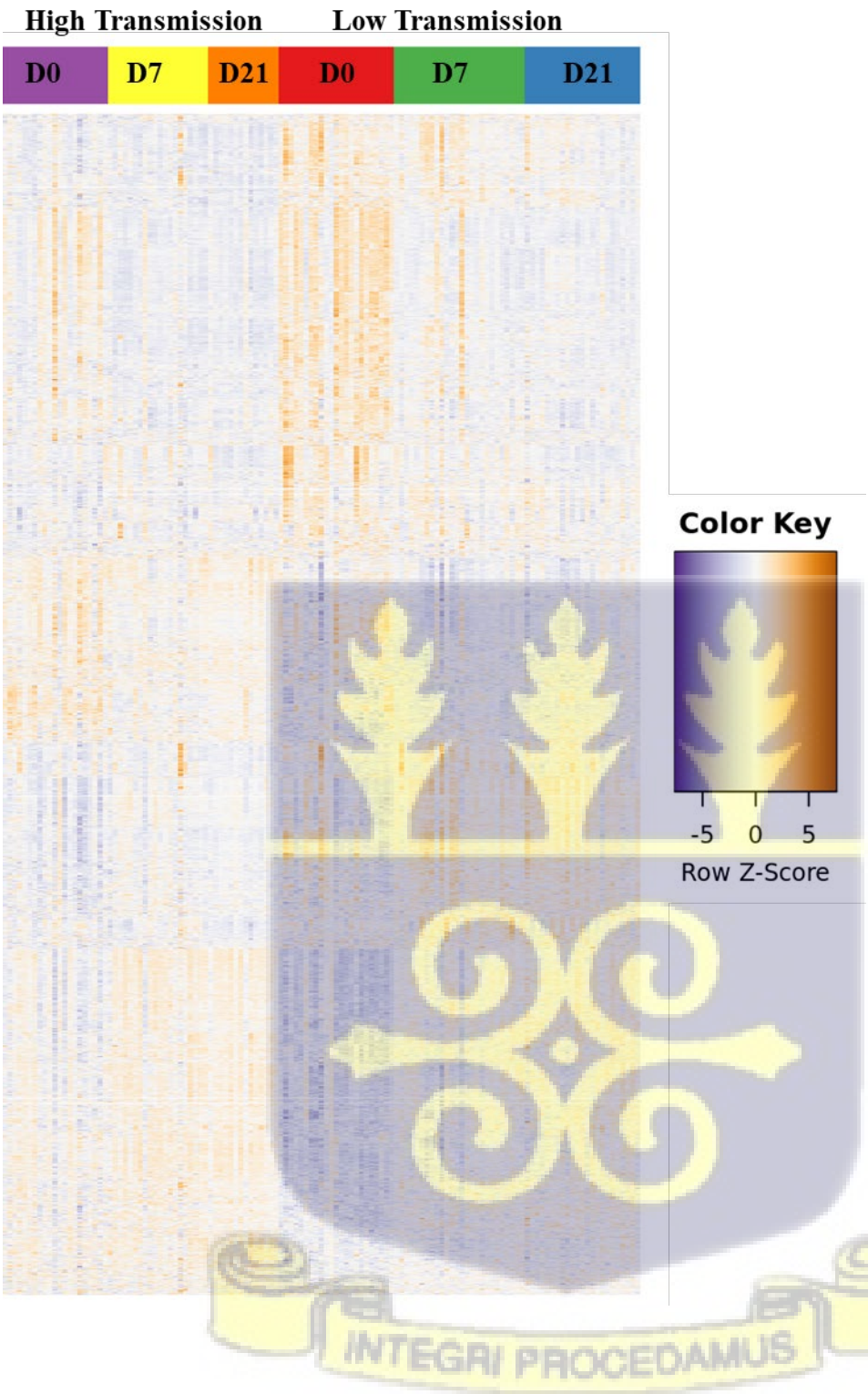


Figure 5.5: Gene expression trends clustering visualised as Heatmap.

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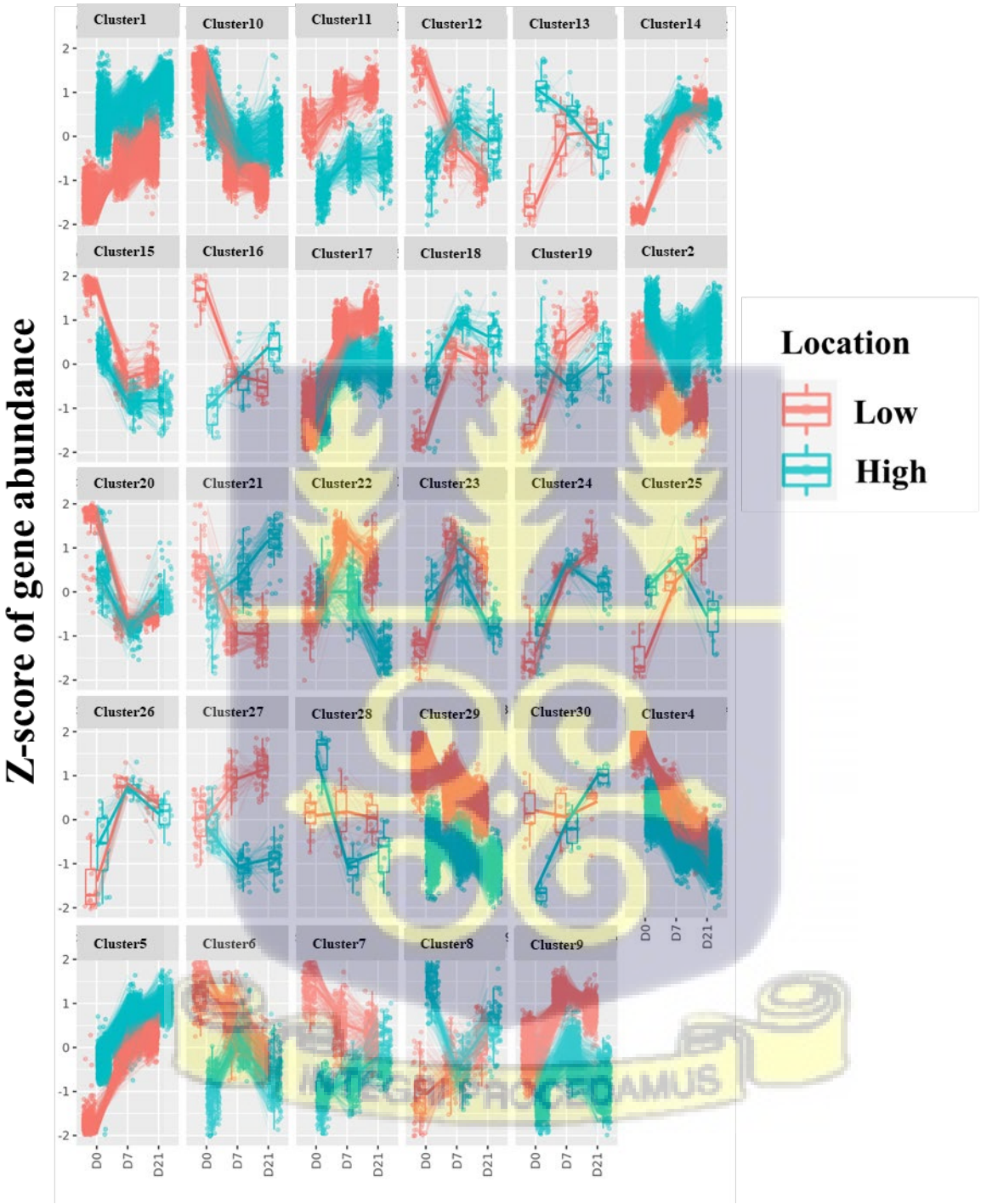
Represents gene expression trends for the low transmission and high transmission areas during infection (D0) and after treatment (D7 and D21). The rows represent DEGs for the two-transmission area, and the columns represent individuals clustered by transmission area and time points. The colour key represents gene expression levels normalised z-scores. The column colour bars indicate the grouping of individuals by time point for each transmission area as described in the scale. Orange colour intensity represents increased expression, while the blue intensity indicates decreased expression. The z-score gives the log-normalized gene expression value, i.e the number of standard-deviations that the value is away from the mean.

5.1.6.7 Gene clustering patterns in the low and high transmission areas

A total of 30 clusters were clearly distinguished in the two groups based on expression patterns at D0, D7 and D21 (Figure 5.6). Cluster 1, 2, 5, 8 and18 reveal DEGs which are upregulated in the high transmission area compare to the low transmission at the time of infection (D0). However, the expression patterns for each cluster were varying at convalesces (D7 and D21). Gene expression in cluster 1 and 5 increased at D7 and 21 compared to D0, clusters 2 and 8 showed a decreased expression at D7 and 21, while cluster 18 increased at D7 and reduced at D21. Clusters 3, 4, 6, 7, 9, 11 and 15 comprised of highly expressed genes in the low transmission area, but with varied kinetics across D7 and D21. Cluster 10, 17 and 26 have similar expression levels at D0, but cluster 17 and 26 genes had increased expression at D7 and 21, while cluster 10 genes showed decreased expression at D7 and 21. Some of the interesting clusters 22 and 27 where gene expression levels were similar during infection (D0), but decreased at D7 and 21 in the high transmission area; however, the gene expression levels increased for the two time-points on these

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days in the low transmission area. Clusters 13, 16, 25 and 30 had different expression patterns at D0, with similar expression at D7 and a different expression pattern at D21. The list of genes in each cluster is presented in Table A 5.4.



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Figure 5. 6: Gene cluster analysis: Represents 30 clusters with genes that have similar patterns.

Gene's clustering patterns in the low and high transmission areas

5.1.6.8 Gene set enrichment analysis (GSEA) using Blood Transcriptional modules (BTM)

Aiming to translate gene expression patterns to specific immunological molecular functions that are modulated during *P. falciparum* infection and resolution, we applied GSEA with BTM gene sets for enrichment (Li *et al.*, 2014). Functional analysis of 21,128 DEGs, ranked in terms of differential expression (no fold change cut-off) was done using the GSEA (Broad institute) tool to determine the most significant functional terms. GSEA takes into account subtle fold expression changes as well as concurrently increased and decreased DEGs in a given gene module. As a result, using all genes allowed us to identify gene dynamics even when there were no DEGs, such as D7VsD21 comparison between the high and low transmission areas.

GSEA identified 15 out of 105 BTMs that are significantly enriched (FDR < 25%, p-value < 0.05) to be upregulated in the high transmission area during infection (D0). These are linked to modules associated with Enriched B cell and surface signature (M47.0, S2, M47.1, M69, S8, M47.2, S9), T cell development and activation (M62.0), Platelet Activation-Actin binding (M196), TBA (M201, M70.0, M80.0), enriched cell cycle (M167) and regulation of transcription and transcription factor modules (M213). In contrast, children from low transmission area have 69 out of 105 BTMs upregulated during infection (D0). These modules include mitotic cell cycle in stimulated CD4 T cells (M4.5, M4.6) DNA and Mismatch repair [M76, M22.0]), immune

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activation- generic cluster (M37.0), E2F1 targets (M10.0, M10.1), Plasma cell surface signature, immunoglobulins (S3, M156.1)), enriched monocytes (M11.0, M118.0, S4, M81) enriched NK cells (M7.2, M61.0), Platelet activation (M32.0), enriched neutrophils (M37.1), enriched activated dendritic cells (M165, M64, M168, S5), NK cell surface signature (S1), chemokine and inflammatory molecules in myeloid cells (M86.0).

Functional analysis for BTM comparison between the high and low transmission areas at D7 used 20,375 genes, which identified 10 out 104 modules up regulated in high compared to the low transmission area with 68 of 104 gene sets were upregulated. Most of the enriched gene sets in the high transmission area (D7) were related to TBA (M137, M72.0, M72.1, M174), enriched B cells (M69, M47.0, M47.1), and naïve B cell surface signature (S8, S2). Whereas, in the low transmission area, majority of the enriched gene sets were associated with immunological function, including enriched T cells (M7.0), NK cells, monocytes, activated dendritic cells, regulation of antigen presentation and immune response, T cell activation and surface signature, chemokine cluster, platelet activation, leukocyte migration and MHC-TLR7-TLR8 cluster. Comparison at D21 was done using 19,785 DEGs, modules upregulated in the high transmission area are 20 while the low the low transmission area has 43. Modules enriched in the high transmission area relate to TBA, TLR and inflammatory signalling, Immune activation cluster, enriched monocytes, neutrophils, and activated dendritic cells. While modules in the low transmission area associate with cell cycle, T cell activation, enriched NK cells, platelet activation, plasma cells, B cells and immunoglobins

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Modules that were significantly enriched in all the 3 comparisons are presented as a dot plot (Figure 5.7). Generally, the high transmission area has more differentially expressed gene sets in all the 3 comparisons in contrast to the low transmission area. Modules uniquely enriched at D0 in the high transmission area are all related to enriched B cells and B cell surface signature. The D7 has only TBA related modules, and D21 has TLR and inflammatory signalling, TBA and intracellular transport. The low transmission area has genes sets that are enriched during infection and convalescence like T cell surface signature, T cell activation, respiratory electron transport, platelet activation, Plasma surface signature, MHC-TLR7-TLR8 cluster, enriched T cells and NK cells. Of interest are genes modules enriched only at D0 like PLK1 signalling events, mitotic cell division, mitotic cell cycle in stimulated CD4 T cells, mitochondrial cluster, mismatch repair, leukocyte differentiation, KLF12 targets network, enriched neutrophils, E2F1 targets, cell division in stimulated CD4 T cells, cell division-E2F transcription network and cell cycle. Some gene sets are only in D0 and D7 but not D21 like immune activation-generic cluster, enriched monocytes, enriched dendritic cells and cell cycle and transcription. Finally, other BTMs were only enriched at D7 and D21 like cell movement, adhesion and platelet activation, enriched antigen presentation and enriched in myeloid cells and monocytes.

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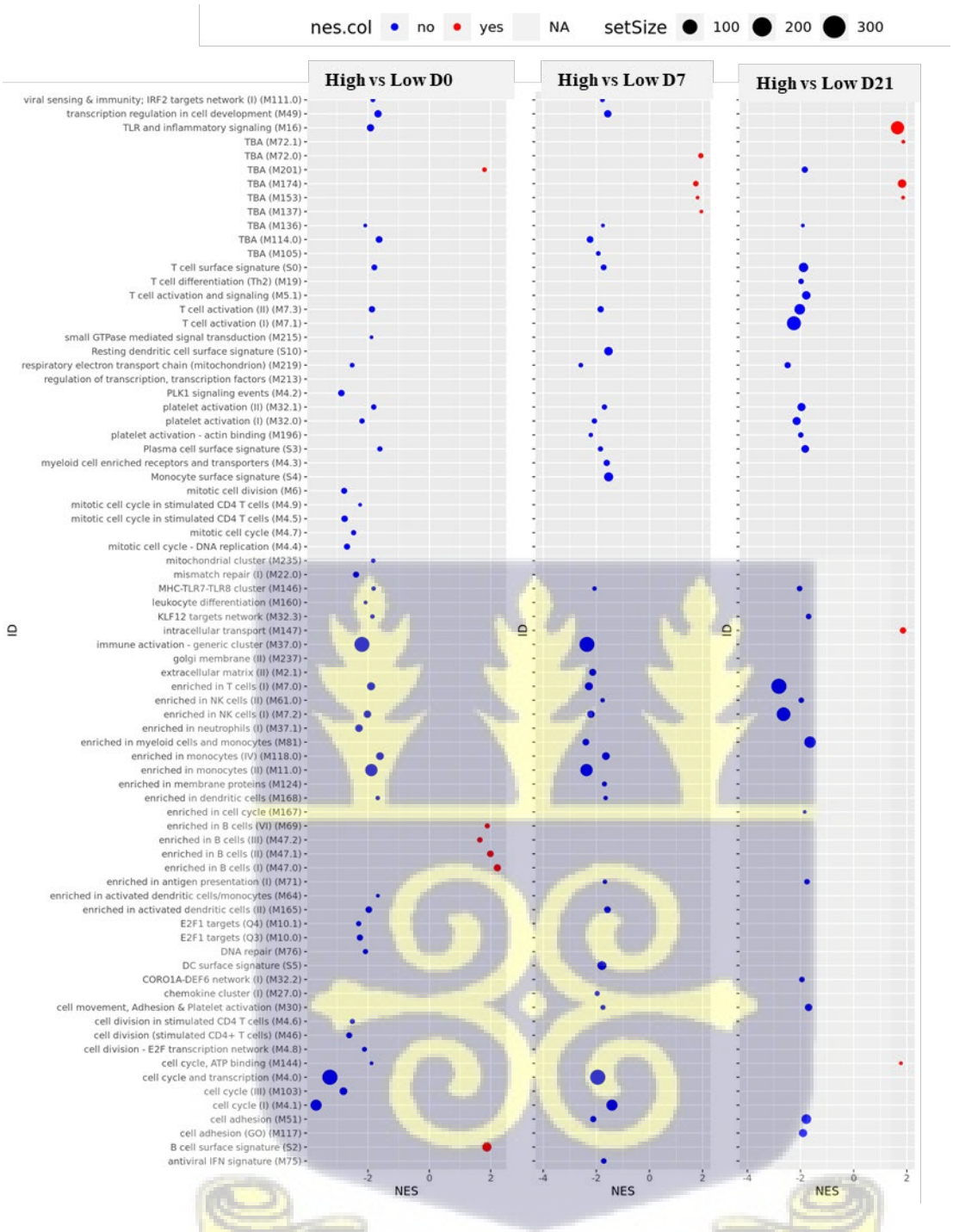


Figure 5.7:Blood transcriptional modular analysis showing differential induction of functional and cell subset modules in individuals during acute infection and after treatment from the two transmission areas.

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Dot plots representation of BTM, produced using gene sets that were significant in at least one comparison. The normalised enrichment score (NES) was used for comparison between the two sites (high Vs low transmission areas) during infection (D0) and after treatment (D7and D21). The x-axis represents NES and the y-axis are enriched gene sets in at least one of the comparisons. The dot sizes depict the number of genes in each gene set present in the of DEGs for each comparison. The red dots show positive NES, which are significantly enriched gene sets in the high transmission area and the blue dots show negative NES values which are significantly enriched gene sets in the low transmission area. Gene sets that were not significant in one comparison were not represented in the dot plot.

5.1.6.9 Functional enrichment for DEGs across time

Multiple comparisons were made across the three time-points for differentially enriched genes between the two transmission areas (High_Low_D7VsD0, D21VsD0 and D21VsD7). Across time in both transmission areas, 18,122 DEGs were analysed for BTM GSEA. For the D7 versus D0 comparison, 59 of 101 gene sets had a positive enrichment score. Forty-seven of these were significant at FDR < 25%, with 37 significantly enriched gene sets at nominal p-value < 0.05. Forty-two gene sets had a negative enrichment score, of which 28 were significantly enriched at FDR < 25%, with 20 significantly enriched gene sets at nominal p-value < 0.05. The D21 Vs D0 comparison had 61 out of 101 gene sets with a positive enrichment score. Fifty-one of these were significantly enriched at FDR < 25%, with 42 significantly enriched gene sets at nominal p-value < 0.05. Forty gene sets had a negative enrichment score, with 23 gene sets significantly enriched at FDR < 25%, and 19 significantly enriched gene set at nominal p-value < 0.05. The most

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significantly enriched modules in both study areas were enriched monocytes, immune activation-generic cluster and regulation of antigen presentation and immune response. Several modules were significantly enriched in genes in individuals from both areas. However, more immune modules appeared in the low transmission area with a high ranking of innate and adaptive immunity modules (Figure 5.8). This is probably due to increased immune activation in the low transmission area because the children are less exposed to infection.

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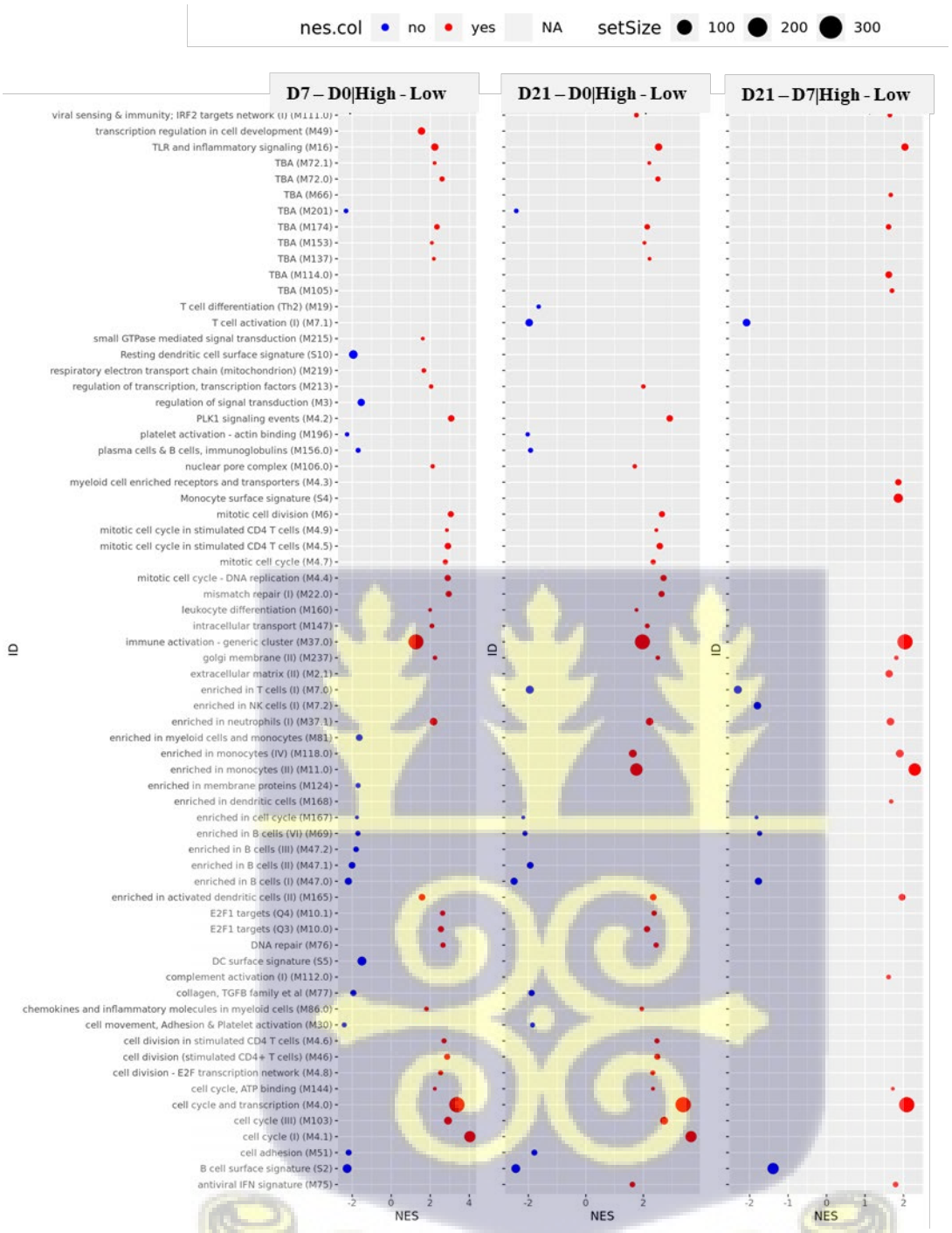


Figure 5. 8: Dot plot representing gene sets enriched in at least one comparison, using the NES value for representation.

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The x-axis is NES and the y-axis are gene sets enriched in at least one of the comparisons. Blue represents negative NES values and red for positive NES values. The size of dots corresponds with the number of genes from the gene set.

5.1.6.10 Gene expression deconvolution shows differences in the relative proportion of WBC

Computational deconvolution of gene expression data with CIBERSORT, using a leukocyte gene signature matrix of 22 blood cell-type signatures (Newman *et al.*, 2019) to determine relative proportions of white blood cell subsets. The percentage of white blood cell subsets extrapolated from the RNA-seq data corresponded to what is predictable in the human whole-blood (Palmer *et al.*, 2006). Neutrophils are one of the cell types that were significantly expanded during infection in both locations compared to convalescence. Comparison by Wilcoxon rank-sum test was: in the low transmission area, D0 Vs D21 (p< 0,001) and D0 Vs D7 (p< 0.001), and in the high transmission area, D0 Vs D21 (p-value = 0.026) and D0 Vs D7 (p-value = 0.03). While the proportion of T cells CD4 Naïve and CD8, were significantly decreased during infection (D0) compared to convalescence. Comparison of CD4 Naïve cells in low transmission area D0 Vs D21, $p < 0.001$ and D0 Vs D7, $p < 0.001$, while in the high transmission area D0 Vs D21, p-value = 0.024 and D0 Vs D7, p-value = 0.018. Similarly, the proportion of naïve B cell in both locations was decreased during infection (D0) compared to convalescence D7 and 21. In the low transmission area, D0 Vs D21, $p < 0.001$, and D0 Vs D7, $p < 0.001$ while in the high transmission area, D0 Vs D21, p-value = 0.002 and D0Vs D7, p-value = 0.07. Naïve B cells were also significantly different between high and low transmission areas during infection (p-value = 0.03). For the T follicular helper cells, they were also decreased during infection for both locations, D0

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Vs D21, $p < 0.001$ and D0 Vs D7, $p < 0.001$ for low transmission area in comparison to D0 Vs D21, p-value = 0.021 and D0 Vs D7, p-value = 0.001 in the high transmission area.

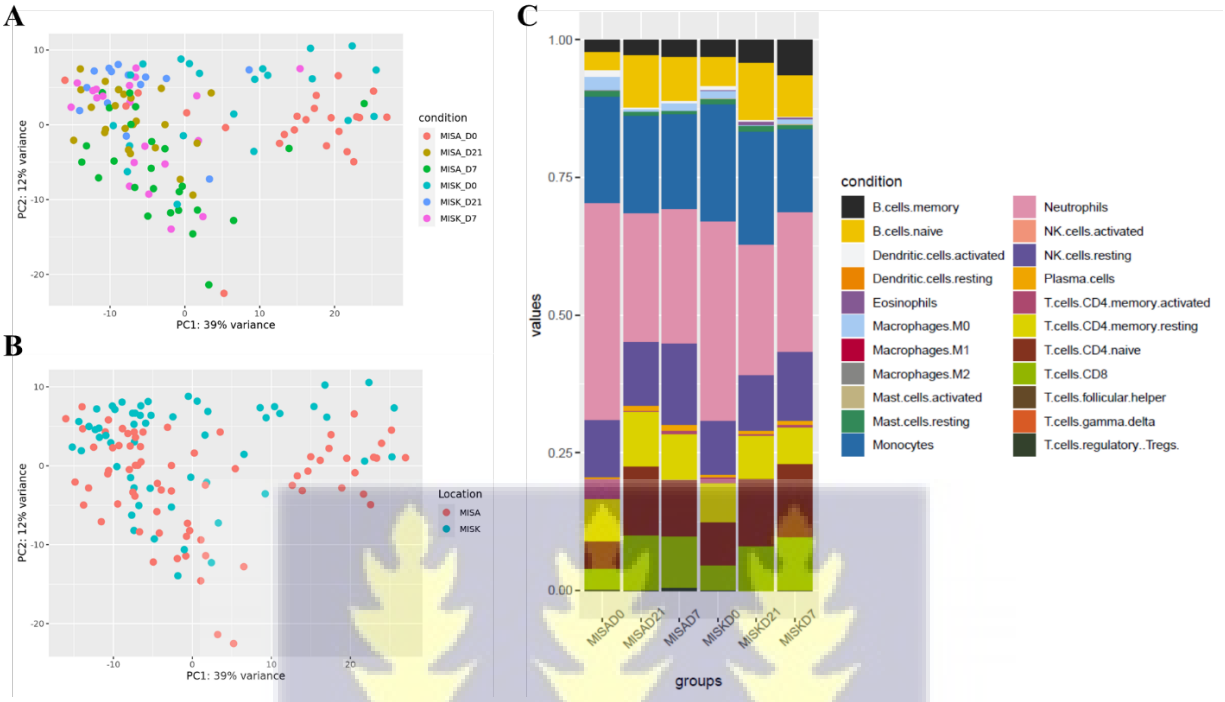


Figure 5. 9: Gene expression deconvolution results.

A&B). PCA plots clustering by timepoint (A) and location (B) of genes from LM22. (C) Relative percentage of white blood cell subsets deconvoluted from DEGs. Inferred composition of 22 immune cell subsets in children during acute infection (D0) and after treatment (D7 and D21) from two study areas. The results were generated using CIBERSORT and the built-in LM22 immune cell gene signature, and the CIBERSORT webserver automatically generated the stacked bar plot figure.

These results suggest involvement of neutrophils, CD4+ and CD+8 T cells in immune response during acute infection, where CD4+ and CD8+ are recruited to sites of infection hence lower proportions circulating in the peripheral blood and more neutrophil expansion.

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5.1.7 Discussion

The processes driving advancement in disease during infection with *P.falciparum* parasites remain unclear. This study employed the RNA-seq approach to investigate changes in whole-blood transcriptome during acute infection and after treatment in Ghanian children from two study areas with different transmission intensities, associating these molecular signatures to immune responses. We discovered gene expression profiles that highlight on pathways and gene ontology modules linked to varying transmission intensities during acute *P.falciparum* and after treatment. Notably, transcriptional signatures identified in this study distinguish between children from low transmission areas and high, during disease and after disease resolution. Transcriptional changes of several genes that increased or decreased relative expression levels between D0 (day reported to hospital), 7 and 21 were identified. In contrast with comparable studies that investigated transcriptional responses in controlled human infection or following vaccination (Tran *et al.*, 2016; Kazmin *et al.*, 2017), this study examined natural infection with *P. falciparum* parasites.

The results show strong transcriptional changes during acute infection and after treatment, using pathways, modular and deconvolution analysis. The results were interpreted as quantitative and qualitative differences in molecular responses between children from areas with distinct transmission intensities. Significant differences in DEG between individuals from the two areas indicate changes in induced gene expression during infection and after treatment, suggesting that the amount of prior exposure plays a crucial role in the induced molecular signatures. We speculate that changes in gene expression levels are a combined result of infection and treatment in response to parasite clearance and induced cellular immune responses. These findings indicate that most transcriptional changes occurred during infection (D0). Genes identified include those related to

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innate immune signalling [(FPR3) N-formyl peptide receptor 3 and (TPSB2) (Tryptase Beta 2)], immune regulation [(CST7) cystatin 7, (PTGIR) prostaglandin I2 receptor and (FKBP4) FKBP Prolyl Isomerase 4], pro-inflammatory stimuli [(IL1RL1) Interleukin 1 Receptor-Like 1, (IFNG) Interferon-gamma, (CXCL10) C-X-C Motif Chemokine Ligand 10, (CXCL9) C-X-C Motif Chemokine Ligand 9 and (CXCL11) C-X-C Motif Chemokine Ligand 11], anti-inflammatory related [(CXCL17) C-X-C Motif Chemokine Ligand 17]. Although these results provide a quantitative measure of transcriptional activity, we cannot infer that any specific differentially expressed genes are associated with immunity. However, we provide an assessment of the transcriptional activity in children from areas with different transmission intensities during infection and convalescences based on BTM enrichment and cell deconvolution analysis.

We detected substantial changes in the BTM in the low than the high transmission area, with more significant changes during infection compared to convalescence. In the low transmission area multiple modules related to innate immune activation were detected including; transcription modules, TLR and inflammatory signalling, platelet activation, NK cells, monocytes, neutrophils and cell cycle modules. The genes in these modules could be important in the development of innate immune response. In contrast, the high transmission area had mostly modules related to adaptive immune response like enriched B cells and TBA modules in each time point comparison. This suggests an upregulation of mostly innate immune responses in the low transmission area and upregulation of adaptive immune responses in the high transmission area. Although several significantly represented immune and inflammatory gene sets were enriched in the low transmission area, such as TLR and inflammatory signalling, immune activation cluster, chemokines and inflammatory molecules pathways, there was a similar adaptive immunity gene

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sets ranking (B and T cell signalling) in both the high and low transmission areas. Notably, RTS, S vaccination and homologous challenge have all been shown to have an effect on cell cycle, NK, and monocyte related modules (Kazmin *et al.*, 2017). The discovery of differentially regulated genes involved in platelet regulation in response to subsequent infections is remarkable. This is due to platelets' involvement in parasite killing and clumping of *P. falciparum*-infected erythrocytes, leading to thrombocytopenia, one malaria complication (Deroost *et al.*, 2016).

We had some striking results, identifying 30 clusters that showed different clustering of differentially expressed genes with similar patterns. We show that substantial transcriptional changes during infection translated into remarkable differences in the immunological response between the high and low transmission areas.

We used the RNA-sequencing data to determine the relative proportions of WBC subsets by gene expression deconvolution, in addition to testing for gene expression differences (Newman *et al.*, 2019). These results show that valuable information can be obtained from whole-blood RNA-sequencing that allow for examining changes in WBC subsets over time, as well as the use of differential gene expression analyses to distinguish changes in cell population. We noted differences in Neutrophils, Naïve CD4 T cells, CD8 T cells, Naïve B cells, T follicular helper cells, Memory B cells and activated CD4 memory T cells. Upregulation of neutrophil associated gene expression signature in both the high and low transmission areas can be explained by the high proportion of neutrophils in blood. However, using gene expression deconvolution, it was difficult to accurately differentiate and quantify cell populations with similar or low transcriptional profiles. Notably, in our analysis, we were not able to reliably differentiate Dendritic cells, macrophages

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and NK cells subsets in our analyses, though recent advances in gene expression deconvolution methods such as single cell sequencing could address this issue (Monaco *et al.*, 2019). This is possibly due to the fact that the LM22 dataset used is originally from Microarray analysis but has been tested using RNA-seq datasets and the cells are *in vitro.* Altogether, the data suggest T and B cell activation in both transmission areas during infection compared to after treatment.

The study tried to overcome potential confounders associated with measuring immune response by recruiting age-matched children from the two sites, collecting data on previous malaria episodes, checking for the haemoglobinopathies and excluding individuals with co-infections. However, the study was unable to overcome other variables like spatial factors, parasite biomass, nutrition status, effect of microbiota, timing of blood sampling, parasite genetics, and host genetics which have been shown to affect immune response

5.1.8 Conclusion

Transcriptomics analysis of whole blood during a natural *P. falciparum* infection reveal molecular signatures that can potentially distinguish immune responses in individuals from varying transmission intensity. The study showed significant differences in DEGs in the low and high transmission areas, DEGs in low transmission area were related to innate immune signalling, immune regulation, pro-inflammatory stimuli, anti-inflammatory while in the high transmission areas DEGs were associated with adaptive immune response, including; Enriched B cells, cell surface signalling, lymphocyte migration and MAPK cascade. Deconvolution of significant DEGs for relative proportion of immune cell population showed that neutrophils were expanded during

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infection while the B cell, CD8+ and CD4+ cells were decreased. This study provides a guide to defining transcriptomic changes kinetics during a natural infection.

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6 CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSION

6.1 Discussion

Immunity to *P. falciparum* is slowly acquired over a long period due to the parasite's complex nature. To date, clearly defined immune markers of protection remain elusive, and there is still inadequate knowledge of the protective mechanisms. Previous efforts to understanding the mechanism of acquired malaria immunity have made significant progress in evaluating and identifying various components that contribute to immune responses in malaria. However, data analysis in these studies was limited by use of reductionist approaches, that measure one or a few immunological parameters. These approaches cannot resolve the complexity of molecular and cellular interactions of the innate and adaptive immune responses that together are responsible for malaria immunity. A comprehensive understanding of malaria immunity is necessary, however analyzing complex immune data sets has previously posed challenges limiting the insights of these data. Therefore, in-depth profiling of naturally-induced cytokines, antibodies and transcriptomic responses ("immunoprofiling") combined with machine-learning holds the promise of identifying *Plasmodium*-specific immune response that can guide malaria vaccine development. We broadly profiled cellular, humoral and molecular naturally induced immune signatures and further integrated these signatures to identify immune features combination that distinguish naturally induced responses in children with different exposure levels.

We used a multi-parameter system approach to profile plasma cytokines, antibody and whole blood transcriptome in naturally induced immune signatures in children from areas with distinct malaria transmission and further integrated these signatures to identify the combination of immune

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features that distinguish natural induced responses in children with different exposure. Differences in immune responses in these two groups over the course of infection could provide immune signatures that change with exposure and hence potential protection correlates that can be used for active surveillance.

Given the complexity of the host's immune response during *P. Falciparum,* we sought to measure how the various components change in the course of an infection in individuals from varying malaria transmission intensities. Both cellular and humoral components of the adaptive immune system are key in eliminating parasites from the body. To investigate the cellular immune component, we measured plasma cytokine levels in two groups of children from high and low transmission areas during infection (D0), and after treatment (D7 and D21). Results show that specific cytokines were increased during infection; pro-inflammatory cytokines IL-6, IFN-γ, IFN-α MIG, MCP-1/CCL2, IP-10/CXCL10, RANTES/CCL5 and immunomodulatory profile IL-10, IL-2R and IL-1RA. With the exception of MIP-1α and MIP-1β, all measured pro-inflammatory cytokines were generally higher in the low transmission area compared to high transmission area, suggesting more immune activation in individuals from low transmission area. This also suggests a different ability to regulate pro-inflammatory and anti-inflammatory response according to exposure and possible development of tolerance in individuals who are more exposed (Boutlis *et al.*, 2006). Individuals in the low transmission areas seem to be less exposed hence infection by P. falciparum immediately triggers a more robust immune response to clear parasites compared to those in high transmission areas whose immune response is already primed and they trigger ant-inflammatory response faster to regulate the responses.

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Correlation with parasitemia also revealed cytokines that are dependent on parasite levels (IFN-α, IL-6, IL-1RA and MCP-1), suggesting a specific host-parasite interaction is necessary for production of these cytokines while others are dependent on underlying immunity. Different cytokine interaction patterns shown in our results (Figure 3B), is an indication that the combination of different interacting cytokines is influenced by individual's immune status to establish a balanced protective immunity. Overall changes in cytokines are largely influenced by presence of infection, hence temporary and rapidly go back to normal after infection is cleared. This implies that cytokines have a major role in symptoms of disease and hence contribute to anti-disease immunity and here we propose cytokine signature that contribute in proving this protective immunity.

We also profiled the humoral response by measuring antibody responses to 190 *Plasmodium* proteins. Antibodies play a key in parasite clearance during infection and have been shown to be important biosignatures of protective immunity (Doolan *et al.*, 2009). However, the parasite expresses many proteins and the host mounts antibody response to most of these proteins. Despite the progress that has been made by several studies in identifying antibodies that contribute to protective immunity, it is still not clear which correlates that can be measured as signatures of functional protection. We assessed antibody response in the two groups of children in our study and used predictive modelling approach based on machine learning to identify antibody signatures that can distinguish response in the two groups of children. We used malaria transmission intensity as a proxy for exposure and proposed that the difference in antibody responses in these two areas could suggest signatures of protection.

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Our results showed reactivity to 118 out of the 190, and using hierarchical clustering we identified antigens that were in the two transmission areas. Antibody levels to EBA140, ETRAMP5, GLURP, MSP1 and MSP6 were high in the low transmission area while in the high transmission area there were higher antibody responses to Rh5.1, EBA181, VAR2CSA, ACS5, HSP40, MSP2, MSP3, MSP4, and MSP7. This is a similar trend to that seen in other studies suggesting that the acquisition of immunity is dependent on malaria transmission intensity(Cherif *et al.*, 2017). Several studies have associated naturally acquired antibodies to dominant Merozoite proteins AMA-1, MSP-1_{42}, EBA-175 with protective immunity(Conway *et al.*, 2000; Dodoo *et al.*, 2008; Richards *et al.*, 2010; Dobaño *et al.*, 2012). However, antibody responses to these antigens were not predicted as signatures in our model, that could distinguish responses between the two sites as there was high reactivity to these antigens from individuals from both transmission areas. Multiple linear regression analysis showed that age and transmission area were good predictors of antibody responses. Antibody responses to most antigens appeared to increase with age in both transmission areas such as; EBA175, Rh4.2, Etramp5, MSRP5, PfMSPDBL1, Pf230 and GLURP. This suggests these responses as possible indicators that can be used to measure exposure. Overall, the breath and magnitude of antibody responses was not different in the two transmission areas. We identified 18 antibody signatures that are distinct between the two transmission areas, which could likely predict the immune status of individuals from these two areas with different transmission intensities. These results indicate that functional protective antibody response is possibly due to multiple antigens, having high, moderate and low reactivity, suggesting the possibility of synergy (Osier *et al.*, 2008).

The antigens identified could be possible targets of protective immunity and thus potentially useful vaccine candidates.

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Our last objective investigated changes in whole-blood transcriptomics during the course of infection in these two groups of children to determine molecular signatures related to immune responses. Our results revealed differences in transcriptional signatures between the two areas during infection and after treatment. Most of the gene expression differences were seen at D0, that is when children were infected with parasites. This suggests that presences of parasites modulate gene expression. Genes identified to be differentially expressed were related to innate immune response, immune regulation, pro-inflammatory response and cell cycle. Using GSEA, we identified BTM that were enriched by the DEGs. Enrichment of DEGs from the low transmission area detected transcription modules, TLR and inflammatory signalling, T cells, Platelet activation, immune activation, NK cells, monocytes, neutrophils and cell cycle modules, these are associated with innate response to disease. While the high transmission area had mostly enriched B cells and TBA modules in each time point comparison, suggesting more adaptive immune response. This suggests an upregulation of mostly innate immune responses in the low transmission area compared to upregulation of adaptive immune responses in the high transmission area.

We had some striking results that showed different clustering of differentially expressed genes with similar patterns, 30 clusters were identified. Using the gene cluster analysis, we show strong transcriptional changes during infection translated into outstanding differences in the immunological response between the high and low transmission areas. The relative proportions of WBC subsets were determine by gene expression deconvolution (Newman *et al.*, 2019) . Our results demonstrate that whole-blood RNA-seq can provide important information and facilitate measurement of changes in WBC subsets over time, and using differential gene expression analyses to distinguish changes in cell proportions from a difference in gene regulation in a specific

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cell population. We noted differences in Neutrophils, Naïve CD4 T cells, CD8 T cells, Naïve B cells, T follicular helper cells, Memory B cells and activated CD4 memory T cells. This suggests that molecular signatures can be used to make inferences to immune responses at the cellular level and estimating cell proportions.

6.2 Conclusion

Overall, this study highlights the importance of multiparameter approach to address the complexity of malaria immunity and suggests that multiple pathways lead to development of protective immunity. Comparison between different transmission area/exposure allowed us to identify cytokine, antibody and transcriptomics signatures that distinguish individuals with different immune status. The difference in cytokine signature suggests different regulation of cellular immune response and shows the importance of exposure to the *P. falciparum* in development of tolerance. The antibody signatures identified could be used as possible targets of protective immunity and provide clues for potential vaccine candidates that can be prioritized in evaluation.

Transcriptomics analysis of whole blood during a natural infection reveal molecular signatures that can provide insights into the protective immune responses against *P. falciparum* infection. This study provides a guide to defining the kinetics of transcriptomic changes over the course of a natural infection. The longitudinal approach provide guide to defining kinetics of these changes over the course of an infection. This study provides understanding to immunological mechanisms involved in naturally acquired immunity and shows the importance of transmission in modulating immunity. This is crucial in providing insights to development of effective vaccine, identifying surrogate markers to monitor vaccine efficacy and understanding disease pathogenesis. Predictive model generated in our study can be used to estimate malaria endemicity level. The integration of

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antibody features within machine learning provides an objective approach to discovering and assessing multivariate immune correlates.

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7 RECOMMENDATIONS AND LIMITATIONS

7.1 Recommendations

The overall aim of the study was to analyse the different components of the immune response, including cellular, humoral and molecular responses. However, for the analysis of the cellular component was done for cytokine responses. We would recommend future studies to consider analysing T cell responses and intra-cellular cytokine responses, to enable give a more comprehensive conclusion. We also recommend single sequencing to identify which immune cell subpopulation correlated with DEG. We also recommend large studies that can recruit participants from other regions in Ghana with similar transmission intensities to compare if there will be similar findings, since this study only looked at two regions. Antigens identified to have high magnitude and distinct antibody responses can be prioritised in multicomponent vaccine design.

Further validation of the immune signatures identified in this study can be done in a different cohort of individuals for the same areas and other malaria endemic areas of similar transmission intensities. Since transmission is key in modulating immune responses, it is important for the health service to maintain active surveillance to monitor transmission intensity in malaria endemic areas.

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7.2 Limitations

The study was not able to make clear correlation between the identified immune signatures and protection since we looked at participants who were already infected with malaria. However, we used transmission intensity as a proxy to define immune status of study participants.

Besides looking at age, transmission intensity, hemoglobin variants and coinfection, other factors like nutritional status, genetic and geographical difference of the two areas studied were not factored in participant recruitment. This could have impacted the results of some participants and maybe a chance for bias. Also, PBMC samples collected during the study for T cell work were lost due to cold room malfunctioning, this limited the amount of data we could generate on cellular immune responses

A limited number of samples was analyzed for transcriptomic profiling due to limited resources, it will be great if similar work was done in a large group of participants.

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Appendix

Table A. 3.1: Characteristics of antigens tested by protein microarray

No. of Antigens	Recombinant Antigen (Acroynm)	Protein family/Description	Location	Expression tag	Allele/Strain	Source/ Reference
1	ACBP1	acyl-CoA binding protein, isoform 1	iRBC	GST	3D7	Tetteh/LSHTM
4	ACS5 Ag 1,2,3&4	Acyl-CoA synthase_Antigen	iRBC	GST	3D7	Tetteh/LSHTM
1	CIDR	Cysteine-Rich Interdomain Region	iRBC			
10	MSP3 (N&C terminal), H101,H103	merozoite surface protein 3	Merozoite surface	?	3D7,K1,FVO	Theisen
27	MSP1 (Block 1&2), Full length, Flanked, Mutants	Merozoite surface protein 1 block 2	Merozoite Surface	GST	MAD20-R033, 3D7, mutan	Tetteh/Conway/LSHTM
29	MSP2 [Variable fragraments], Full length	Merozoite surface protein 2 variable region	Merozoite Surface	GST	Ghana clone, CH150/9, 3D	Tetteh/Riley/Theisen
1	MSP4	Merozoite surface protein 4	Merozoite Surface	GST		Coppel
1	MSP5	Merozoite surface protein 5	Merozoite Surface	GST		Coppel
3	MSP6 A(1), B & N-MSP5	merozoite surface protein 6	Merozoite Surface	GST	3D7	Tetteh/LSHTM/Theisen
3	MSRP 2B, 4B & 5B	MSP7-like protein	Merozoite Surface	GST	?	Holder/NIMR/Crick
1	MSP7	merozoite surface protein 7	Merozoite Surface	GST		Holder/NIMR/Crick
7	MSPDBL1 C&N-term/PF10_0348, Fcc2, (T)	Duffy binding-like merozoite surface protein 1 C-terminal	Merozoite Surface	GST	3D7,Fcc2	Tetteh/LSHTM/Theisen
1	C-pf38	Pf38	Merozoite surface	?		Theisen
1	CSP	circumsporozoite protein Component of RTS,S vaccine	Sporozoite	n/a		commercial
1	CyRPA		Apical tip			
2	DBL1 &2	Duffy Binding-1&2	iRBC			Theisen
4	EBA140 RIII-V, 175 RIII-V&RII_F2, 181	Erythrocyte binding antigen-140, 175, 181	Apical organelles, micro	GST	3D7,Region 2, F2 domain c	Beeson/Chetan
2	EPF1&3v2	exported protein family 1 &3	iRBC, maurer's cleft	GST	3D7	Tetteh/LSHTM
20	Etramp2,4,5,8 10.1,10.2,11.1,11.2,13.1,14.1	Early transcribed membrane protein 2,4,5,9 10.1,11.1,13.1,14.1	iRBC, PVM	GST	3D7	Tetteh/LSHTM
2	GAMA-A &B	GPI-anchored micronemal antigen -A &B	micronemes	?		Holder/NIMR/Crick
1	GEXP18	Gametocyte exported protein 18. Unknown function.	iRBC/Gametocyte	GST	3D7	Tetteh/LSHTM
4	GLURP R0, R2, (W) & Pfs48/45) (W)	glutamate-rich protein	Merozoite Surface	His		Thiesen
2	gsg6 (Arca) & gSG6_protein (LSHTM)	Anopheles gambiae Salivary Antigen gSG6 & gSG7	Exposure			
3	HSP40 Ag 1,2 &3	heat shock protein 40, type II Antigen 1,2 &3	iRBC	GST	3D7	Tetteh/LSHTM
1	Hyp 2	Plasmodium exported protein (hyp2)	iRBC	GST	3D7	Tetteh/LSHTM
1	N-RON4	Rhoptry neck protein - 4	Apical/ Micronemes			Theisen
3	Pep18,24,25,26	CMP	Unknown	n/a		
2	PF10_0350_B & A	M712; probable protein, unknown function: C&N-terminal	Unknown	GST	3D7	Tetteh/LSHTM
2	PF10_0351 C-term & Repeat	probable protein, M566; probable protein, unknown function	Unknown	GST	3D7	Tetteh/LSHTM
1	Pf12	Pf12	Merozoite Surface			Theisen
8	Pf3D7_0102000	Plasmodium exported protein (PHISTa),unknown function	iRBC cytoplasm	GST	3D7	
1	PF3D7_1419400 F3 [1]	conserved Plasmodium membrane protein	iRBC	GST	3D7	Tetteh/LSHTM
1	Pf41	Pf41	Merozoite Surface			Theisen
2	Pf & PvAMA1	apical membrane antigen 1 (pf&Pv)	micronemes/Apical	His	FV0	Blackman/Crick
8	Pfs230 CM1,3,4,7,B (W), C(T),C0(T),C1(T)	Pfs230 CM1,3,4,7,B,ProDI,II,II,Pro,ProD1	Gametocyte surface			Radboud/Jore/de Jong
1	PfMC_2TM	Maurer's cleft transmembrane protein	iRBC cytoplasm	GST	3D7	
5	Pfs48/45 10C (W),6C(T),10N(W),6C(W),full(	Pfs48/45	Gametocyte surface			Radboud/Jore/de Jong
1	PfSEA-1	schizont egress antigen-1	iRBC	GST	3D7	Tetteh/LSHTM
2	PKH_031930_Ag1&2	hypothetical protein, conserved in P.knowlesi	Unknown			
1	PKH_041240_ag1	cysteine protease, putative	Merozoite Surface	GST	strain H	Herman/Tetteh/LSHTM
1	PKH_080030	Plasmodium exported protein		GST	strain H	Herman/Tetteh/LSHTM
3	PKH_SERA3_Ag1,2&5	Serine Repeat Antigen -Ag1&2	Merozoite Surface/Exposure			
1	PKH_SSP2	thrombospondin-related anonymous protein	Sporozoite	GST	strain H	Herman/Tetteh/LSHTM
1	PV1	parasitophorous vacuolar protein 1	iRBC	GST	3D7	Tetteh/LSHTM
1	RALP-1	Rhoptry-associated, leucine zipper-like protein 1	Apical tip			Theisen
1	REX 3	ring-exported protein 3	iRBC	GST	3D7	Tetteh/LSHTM
4	Rh 4.2, 4.2_2030, 4.9, 5.1	reticulocyte binding protein homologue 4,4	Apical tip	?	?	Beeson
1	RIPR		Apical tip			
1	SBP1	skeleton-binding protein 1; essential for translocation of PfEMP1 to iRBS surface via Maurer's cleft.	iRBC	GST		Tetteh/LSHTM
4	Signal 1,2,6&7	Pf3D7_0301700 F1,Pf3D7_0505000 F2,Pf3D7_0826100 Ag3,Pf3D7_0936400 F1	Unknown	GST	3D7	
	Surfin4.2 [3D7B,C],[K1A,B,C],[conserved]	surface-associated interspersed protein 4.2	iRBC	GST	3D7,K1,Conserved	Tetteh/LSHTM
	Var2CSA		iRBC/ Exposure			
	VAR2CSA DBL5		iRBC/ Exposure			

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Table A. 3.2: List of antigens that clustered to Accra (Group A)

Antigens Distinguishing Low Transmission area	Protein family/Description	Location	Allele/Strain	Reference
PF10_0351.Repeat	M566; probable protein, unknown function	Unknown	3D7	Tetteh/LSHTM
Rh4.2	reticulocyte binding protein homologue 5	Apical tip		Beeson
EBA140.RIII.V	Erythrocyte binding antigen-140	Apical organelles, mic	3D7	Beeson
Pfs230C1..T.	Pfs230ProD1	Gametocyte surface		Theisen
Pfs230C0..T.	Pfs230Pro	Gametocyte surface		Theisen
Pfs230C..T.	Pfs230ProDI,II,II	Gametocyte surface		Theisen
Pfs48.45.10C..W.	Pfs48/45 10C	Gametocyte surface		Radboud/Jore/de Jong
Pfs48.45.6C..T.	Pfs48/45 6C	Gametocyte surface		Radboud/Jore/de Jong
Pfs48.45.6C..W.	Pfs48/45-6C	Gametocyte surface		Theisen
RALP.1		iRBCs		Theisen
PfSEA.1	schizont egress antigen-1	iRBCs	3D7	Tetteh/LSHTM
Hyp.2	Plasmodium exported protein (hyp2)	iRBCs	3D7	Tetteh/LSHTM
PKH_SERA3_Ag1		iRBCs		
Pf3D7_0424900	Plasmodium exported protein (PHISTa),	iRBCs	3D7	Tetteh/LSHTM
Etramp.14.1.Ag.1	Early transcribed membrane protein 14.1 antigen (exon) 1	iRBCs	3D7	Tetteh/LSHTM
Etramp.10.2	Early transcribed membrane protein	iRBCs	3D7	Tetteh/LSHTM
PV1	parasitophorous vacuolar protein 1	iRBCs	3D7	Tetteh/LSHTM
Etramp.11.1..Ag.2	Early transcribed membrane protein 11.1 antigen (exon) 2	iRBCs	3D7	Tetteh/LSHTM
Etramp.2.Ag.1	Early transcribed membrane protein 2 antigen (exon) 1	iRBCs	3D7	Tetteh/LSHTM
Etramp.5.Ag.1	Early transcribed membrane protein 5 antigen (exon) 1	iRBCs	3D7	Tetteh/LSHTM
Etramp.5.Ag1.His.var.1	Early transcribed membrane protein 5 antigen (exon) 1	iRBCs	3D7	Tetteh/LSHTM
PfMSPDBL1..T.	PfMSPDBL1 (Leucine)	Merozoite surface		Theisen
MSP1.bk2.MAD20.Rep	Merozoite surface protein 1 block 2	Merozoite surface	MAD20; repeat only	Tetteh/Conway
MSPDBL1.C.term.PF10_	Duffy binding-like merozoite surface protein 1 C-terminal	Merozoite surface	3D7	Tetteh/LSHTM
MSP2..15.42C.	Merozoite surface protein 2 variable region [15-42C]	Merozoite surface	Ghana clone 15-42C	Tetteh/Riley
MSP6.A.1.	merozoite surface protein 6	Merozoite surface	3D7	Tetteh/LSHTM
MSP2..15.38.	Merozoite surface protein 2 variable region [15-38]	Merozoite surface	Ghana clone 15-42C	Tetteh/Riley
MSP2..15.34c.	Merozoite surface protein 2 variable region [15-34c]	Merozoite surface	Ghana clone 15-42C	Tetteh/Riley
MSP2.Dd2	Merozoite surface protein 2 full-length [Dd2]	Merozoite surface	Dd2	
MSP1.bk2.3D7.full.length	Merozoite surface protein 1 block 1	Merozoite surface	3D7; full-length	Tetteh/Conway
Pf12	Pf12	Merozoite surface		Theisen
GLURP.R2	glutamate-rich protein	Merozoite surface		Theisen
GLURP.R0	glutamate-rich protein	Merozoite surface		Theisen
r0..GLURP...W.		Merozoite surface		Theisen/Radboud
r0.10C..GLURP_Pfs48.45...W.		Merozoite surface		Theisen/Radboud
MSP1.19mut15.21	Merozoite surface protein 1-19	Merozoite surface	MSP1-19 mutants	Holder/NIMR/Crick
MSP1.19.2B	Merozoite surface protein 1-19 3D8	Merozoite surface	EGF doimain 2	Holder/NIMR/Crick
MSP1.19.2A	Merozoite surface protein 1-19 3D7	Merozoite surface	EGF doimain 1	Holder/NIMR/Crick
MSP1.19K		Merozoite surface		Theisen
MSP1.19mut34	Merozoite surface protein 1-19	Merozoite surface	MSP1-19 mutants	Holder/NIMR/Crick
MSP1.19mut21	Merozoite surface protein 1-19	Merozoite surface	MSP1-19 mutants	Holder/NIMR/Crick
MSP1.19.1A	Merozoite surface protein 1-19 FVO/Well	Merozoite surface	EGF doimain 1	Holder/NIMR/Crick
MSP1.19.1B	Merozoite surface protein 1-19 FVO/Well	Merozoite surface	EGF doimain 2	Holder/NIMR/Crick
PfMSP1_19	Merozoite surface protein 1-19	Merozoite surface		Holder/NIMR/Crick
MSP2.Fc27	Merozoite surface protein 2 full-length [Fc27]	Merozoite surface	Fc27	Theisen

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Table A. 3.3: List of antigens that clustered to Kintampo (Group G)

Antigens Distinguishing High transmission area	Location	Protein family/Description	Allele/Strain	Reference
Rh5.1	Apical	reticulocyte binding protein homologue 5		Draper/Jenner
Rh4.2_2030	Apical	reticulocyte binding protein homologue 4.2		Beeson
EBA181.RIII.V	Apical	Erythrocyte binding antigen-181		Beeson
Pfs48.4510N..W.	Gametocyte surface	Pfs48/4510N		Radboud/Jore/de Jong
Pfs230.CM1..W.	Gametocyte surface	Pfs230 CM1		Radboud/Jore/de Jong
Pf3D7_0102000	iRBC	Plasmodium exported protein (PHISTa)	3D7	
REX.3	iRBC	ring-exported protein 3	3D7	Tetteh/LSHTM
CIDR	iRBC			
Var2CSA	iRBC			
ACS5.Ag.3	iRBC	Acyl-CoA synthase_Antigen 3	3D7	Tetteh/LSHTM
MSP1.bk2.3D7.Rep	Merozoite surface	Merozoite surface protein 1 block 2	3D7; repeat	Tetteh/Conway
MSP7	Merozoite surface	merozoite surface protein 7		
MSP2..5.38.	Merozoite surface	Merozoite surface protein 2 variable region [5-38]	Ghana clone 5-38	Tetteh/Riley
MSP2..15.46b.	Merozoite surface	Merozoite surface protein 2 variable region [15-46b]	Ghana clone 5-38	Tetteh/Riley
Pep18	Unknown	CMP		
MSP1.block.2.R033	Merozoite surface	Merozoite surface protein 1 block 2	R033; full-length	Tetteh/Conway
MSP2..120.10c.	Merozoite surface	Merozoite surface protein 2 variable region [120-10c	Ghana clone 120-10c	Tetteh/Riley
MSP4	Merozoite surface	Merozoite surface protein 4		Coppel
MSP2.3D7	Merozoite surface	Merozoite surface protein 2 full-length [3D7]	3D7	Theisen
MSP2.CH150.9	Merozoite surface	Merozoite surface protein 2 full-length [CH150/9]	CH150/9	
MSP.DBL2.T9.96	Merozoite surface	Duffy binding-like merozoite surface protein 2	T9/96	Tetteh/LSHTM
MSPDBL1.Fcc2	Merozoite surface	Duffy binding-like merozoite surface protein 1	Fcc2	Tetteh/LSHTM
MSP2..3.10.	Merozoite surface	Merozoite surface protein 2 variable region [3-10]	Ghana clone 3-10	Tetteh/Riley
MSP2..52.42A.	Merozoite surface	Merozoite surface protein 2 variable region [52-42A]	Ghana clone 52-42A	Tetteh/Riley
MSP2..5.60B.	Merozoite surface	Merozoite surface protein 2 variable region [5-60B]	Ghana clone 5-60B	Tetteh/Riley
MSP2..17.26B.	Merozoite surface	Merozoite surface protein 2 variable region [17-26B]	Ghana clone 17-26B	Tetteh/Riley
MSP2..15.30B.	Merozoite surface	Merozoite surface protein 2 variable region [15-60B]	Ghana clone 15-60B	Tetteh/Riley
MSP2..15.38B.	Merozoite surface	Merozoite surface protein 2 variable region [15-38B]	Ghana clone 15-38B	Tetteh/Riley
MSP2..5.36A.	Merozoite surface	Merozoite surface protein 2 variable region [5-36A]	Ghana clone 5-36A	Tetteh/Riley
MSP2..15.34B.	Merozoite surface	Merozoite surface protein 2 variable region [15-34B]	Ghana clone 15-34B	Tetteh/Riley
MSP2..62.46a.	Merozoite surface	Merozoite surface protein 2 variable region [62-46a]	Ghana clone 15-34B	Tetteh/Riley

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Table A. 3.4: List of antigens that clustered to both Kintampo and Accra at various timepoints (Group B-F)

Antigens Distinguishing Transmission areas (Clusters B-F)	Protein family/Description	Location	Allele/Strain	Reference
(Cluster B)				
Pfs230 CM3 (W)	Pfs230 CM3	Gametocyte surface		Radboud/Jore/de Jong
(Cluster C)				
MSP3 FVO	merozoite surface protein 3	Merozoite surface		
MSP3-3D7	merozoite surface protein 3	Merozoite surface	3D7	Conway
MSP5	Merozoite surface protein 5	Merozoite surface		Coppel
PKH_031930_Ag1	hypothetical protein, conserved in P.knowlesi		strain H	Herman/Tetteh/LSHTM
MSP1 bk 2 K1SRv2	Merozoite surface protein 1 block 2	Merozoite surface	synthetic multiepitope string (based on K1-type allele)	Tetteh/LSHTM
MR152	Merozoite surface protein 1 block 2	Merozoite surface	MAD20-R033 parasite native hybrid; clone 152	Tetteh/LSHTM
(Cluster D)				
MR321	Merozoite surface protein 1 block 2	Merozoite surface	MAD20-R033 parasite native hybrid; clone 321	Tetteh/LSHTM
Etramp 11.1 Ag 1	Early transcribed membrane protein 11.1 antigen (exon) 1	infected erythrocyte	3D7	Tetteh/LSHTM
Pfs230 CM7 (W)	Pfs230 CM7	Gametocyte surface		Radboud/Jore/de Jong
Pfs230 CM4 (W)	Pfs230 CM4	Gametocyte surface		Radboud/Jore/de Jong
(Cluster E)				
HSP40 Ag 2	Heat shock protein 40	infected erythrocyte	3D7	Tetteh/LSHTM
PvAMA1	apical membrane antigen 1	micronemes	Pv	Kocken/BPRC
EBA175 RIII-V	Erythrocyte binding antigen-175	Apical tip	3D7	Beeson
(Cluster F)				
ACS5 Ag 2	Acyl-CoA synthase_Antigen 2	infected erythrocyte	3D7	Tetteh/LSHTM
ACS5 Ag 4	Acyl-CoA synthase_Antigen 4	infected erythrocyte	3D7	Tetteh/LSHTM
Etramp 5 Ag 2	Early transcribed membrane protein 5 antigen (exon) 2	infected erythrocyte	3D7	Tetteh/LSHTM
EPF1v2	exported protein family 1	iRBC, maurer's cleft	3D7	Tetteh/LSHTM
MSPDBL1 N-term/PF10_0348	Duffy binding-like merozoite surface protein 1 N-terminal	Merozoite surface	3D7	Tetteh/LSHTM
PF3D7_1419400 F3 [1]	conserved Plasmodium membrane protein		3D7	Tetteh/LSHTM
Etramp 4 Ag 2	Early transcribed membrane protein 4 antigen (exon) 2	infected erythrocyte	3D7	Tetteh/LSHTM
MSP3-K1	merozoite surface protein 3	Merozoite surface	K1	Conway
MSRP 5B	MSP7-like protein	Merozoite surface		Holder/NIMR/Crick
MSP2 [47-34A]	Merozoite surface protein 2 variable region [47-34A]	Merozoite surface	Ghana clone 47-34A	Tetteh/Riley
MSP2 [15-34a]	Merozoite surface protein 2 variable region [15-34a]	Merozoite surface	Ghana clone 15-34a	Tetteh/Riley
GAMA-B				Holder/NIMR/Crick
HSP40 Ag 3	heat shock protein 40, type II	infected erythrocyte	3D7	Tetteh/LSHTM
HSP40 Ag 1	heat shock protein 40, type II	infected erythrocyte	3D7	Tetteh/LSHTM
MSP2 [62-22A]	Merozoite surface protein 2 variable region [62-22A]	Merozoite surface	Ghana clone 62-22A	Tetteh/Riley
PfAMA1	apical membrane antigen 1	micronemes	FV0	Blackman/Crick
EBA175RII_F2	erythrocyte binding antigen-175	Apical tip	3D7	Chetan
Pep24	CMP			
Pep25	CMP			
Pep26	CMP			
CyRPA				

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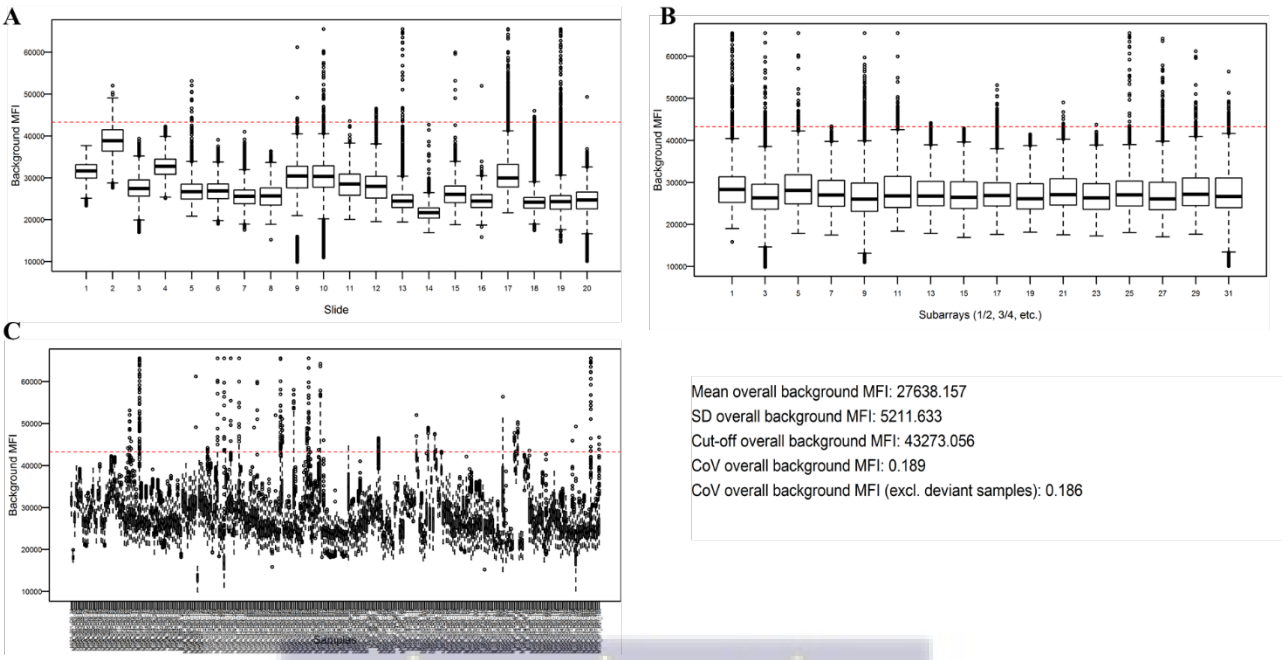


Figure A.3.1: Represents background Median fluorescent intensities by; A) slide, B) subarrays and C) samples.

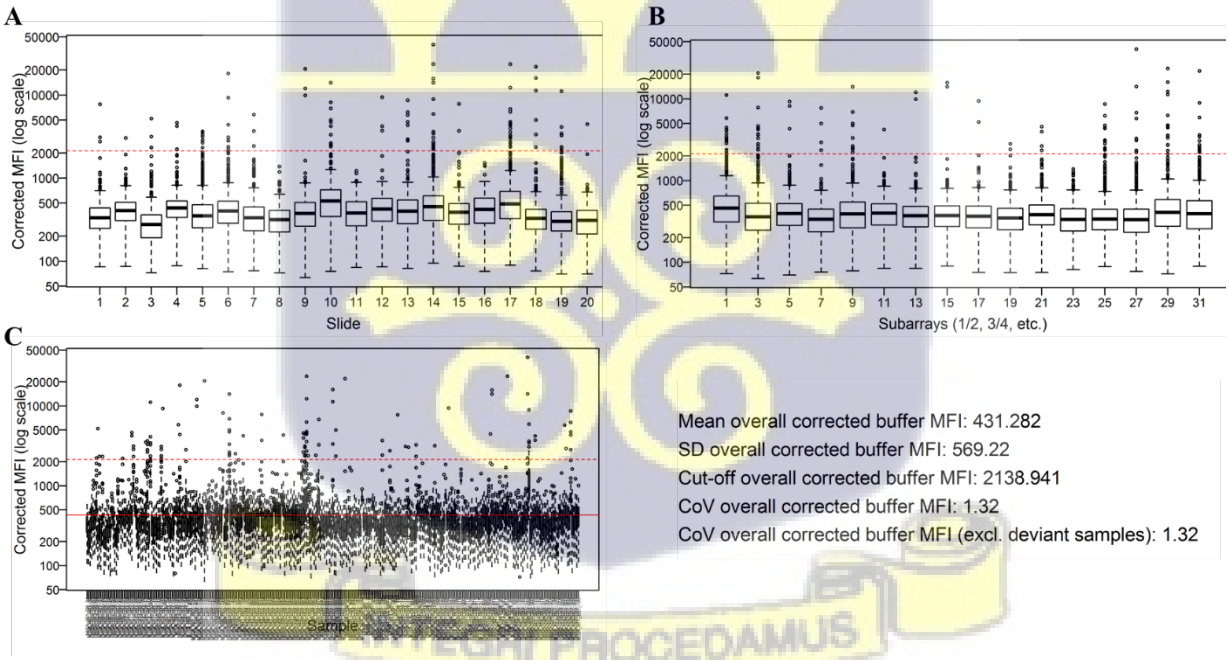


Figure A.3.2: Represents corrected buffer Median fluorescent intensities by; A) slide, B) subarrays and C) samples.

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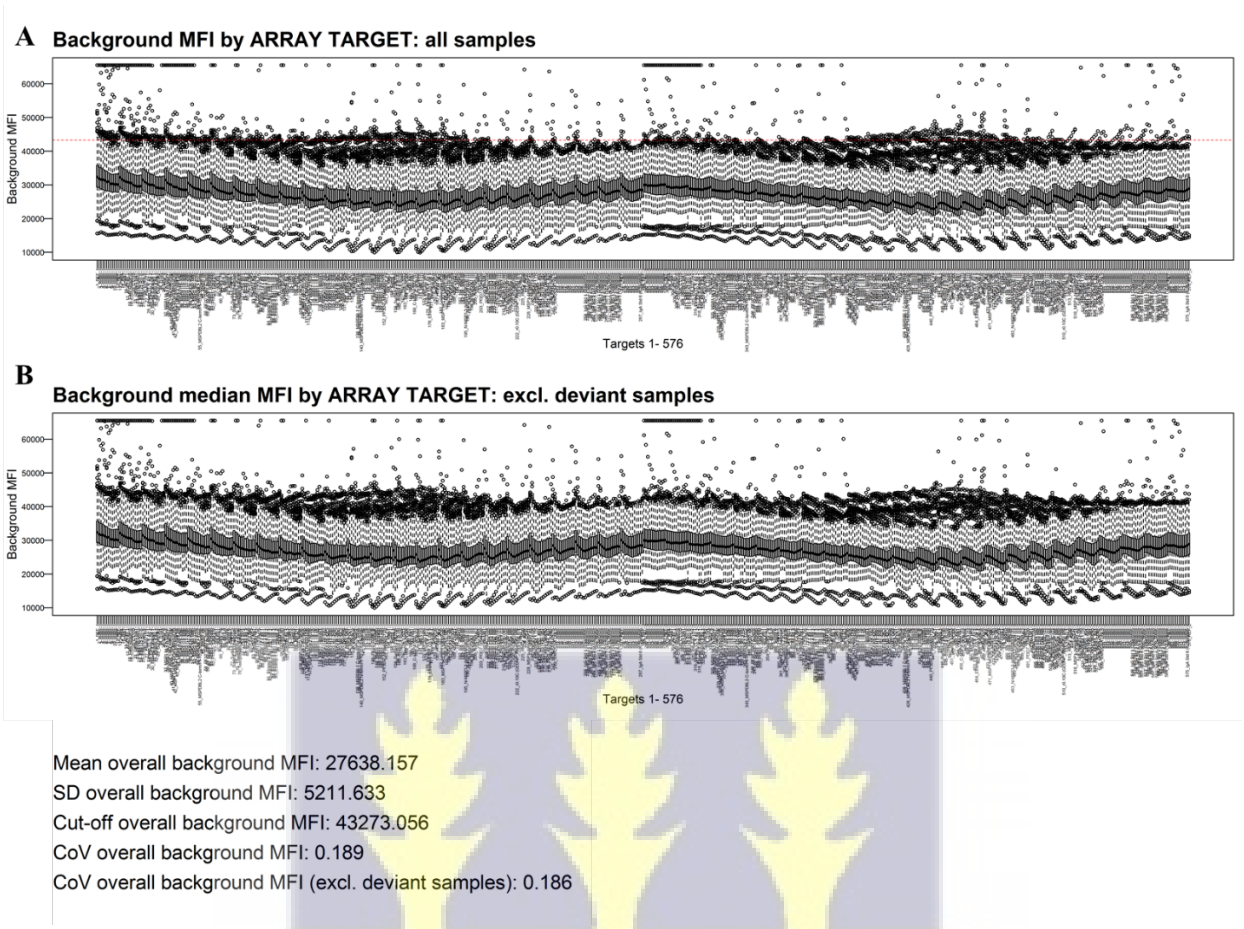


Figure A.3.3: Represents corrected buffer Median fluorescent intensities by array targets; A) all samples and B) with deviant samples excluded.

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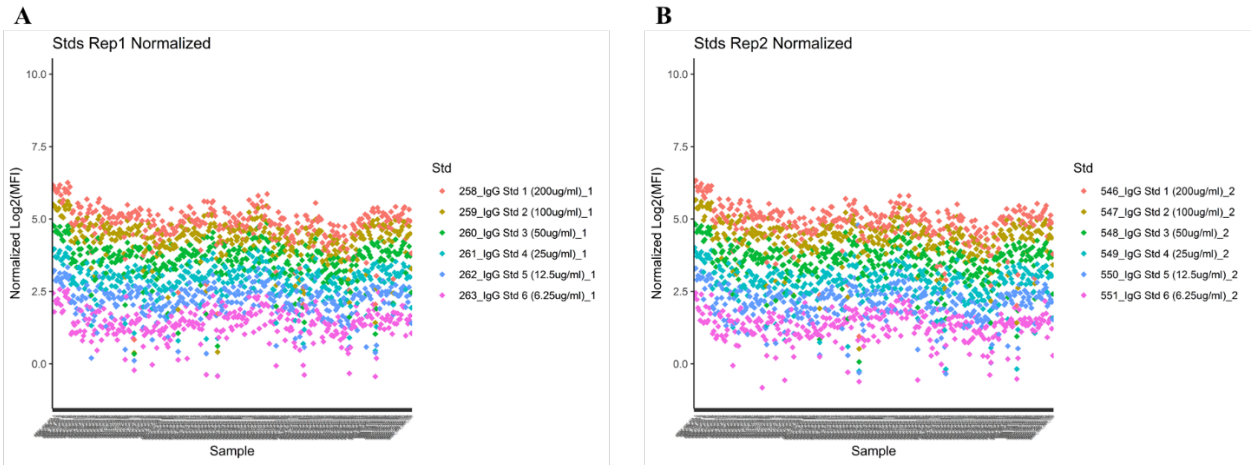


Figure A.3.4: Represents normalized Median fluorescent intensities for standards in all the samples; A) Standard Replicate 1 and B) Standard Replicate 2.

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Table A. 5.1: Leukocyte reference cell types ordered and clustered by function

Leukocyte reference cell types
B cells naive
B cells memory
Plasma cells
T cells CD8
T cells CD4 naive
T cells CD4 memory resting
T cells CD4 memory activated
T cells follicular helper
T cells regulatory
T cells gamma delta
NK cells resting
NK cells activated
Monocytes
Macrophages M0
Macrophages M1
Macrophages M2
Dendritic cells resting
Dendritic cells activated
Mast cells resting
Mast cells activated
Eosinophils
Neutrophils

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Table A. 5.2. Differentially expressed genes High Vs Low comparison at D0

Top 30 Genes Upregulated in Low transmission area at D0				Top 30 Genes Upregulated in high transmission area at D0			
Gene_symbol	Gene Name	log2Fold Change	pvalue	Gene_symbol	Gene Name	log2Fold Change	pvalue
RMRP	RNA component of mitochondrial RNA processing endoribonuclease(RMRP)	-13.6670	0.0097	AC068234.1	Uncharacterised	6.2493	4.45E-08
TUBB2B	tubulin beta 2B class IIb(TUBB2B)	-8.4450	0.0000	AC008758.1	Uncharacterised	3.4825	1.84E-04
AC008695.1	Uncharacterised	-6.1515	0.0005	TBC1D3	TBC1 domain family member 3(TBC1D3)	3.1645	7.54E-04
AC105052.1	Uncharacterised	-5.7100	0.0033	MEG3	maternally expressed 3 (non-protein coding)(MEG3)	3.1502	3.79E-16
CXCL17	C-X-C motif chemokine ligand 17(CXCL17)	-4.0008	0.0000	AC233968.1	Uncharacterised	3.1286	1.05E-06
CD177P1	CD177 molecule pseudogene 1(CD177P1)	-3.9661	0.0104	AC104809.2	Uncharacterised	3.0395	4.55E-15
NR2E1	nuclear receptor subfamily 2 group E member 1(NR2E1)	-3.7957	0.0000	PRR5-ARHGAP8	PRR5-ARHGAP8 readthrough(PRR5-ARHGAP8)	3.0100	3.48E-04
AP002748.4	Uncharacterised	-3.1621	0.0040	NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2(NAALAD2)	2.8417	1.48E-09
TNXA	tenascin XA (pseudogene)(TNX	-3.0830	0.0087	RPL23AP25	ribosomal protein L23a pseudogene 25(RPL23AP25)	2.8209	7.49E-03
AC018512.1	Uncharacterised	-3.0762	0.0036	DDX11L1	DEAD/H-box helicase 11 like 1(DDX11L1)	2.6821	2.01E-03
PRELID2P1	PRELI domain containing 2 pseudogene 1(PRELID2P1)	-3.0573	0.0051	PRTG	protogenin(PRTG)	2.5633	8.82E-08
CXCL11	C-X-C motif chemokine ligand 11(CXCL11)	-2.7605	0.0000	HMGA2	high mobility group AT-hook 2(HMGA2)	2.4597	6.00E-05
ATP6V1G2-DDX	ATP6V1G2-DDX39B readthrough (NMD candidate)(ATP6V1G2-DDX39B)	-2.7278	0.0146	AC100803.3	Uncharacterised	2.3687	1.45E-12
AL139300.1	Uncharacterised	-2.6302	0.0000	C2orf81	chromosome 2 open reading frame 81(C2orf81)	2.3514	2.67E-04
APOL4	apolipoprotein L4(APOL4)	-2.5911	0.0000	GPR20	G protein-coupled receptor 20(GPR20)	2.2589	1.97E-08
SEMA6B	semaphorin 6B(SEMA6B)	-2.5204	0.0000	ALOX15	arachidonate 15-lipoxygenas	2.2569	2.05E-06
GLDN	gliomedin(GLDN)	-2.5164	0.0001	OLFM1	olfactomedin 1(OLFM1)	2.2077	4.74E-08
OLAH	oleoyl-ACP hydrolase(OLAH)	-2.4959	0.0010	SLC28A3	solute carrier family 28 member 3(SLC28A3)	2.1978	5.70E-05
TUBB3	tubulin beta 3 class III(TUBB3)	-2.4406	0.0000	PGAM1P7	phosphoglycerate mutase 1 pseudogene 7(PGAM1P7)	2.1864	1.23E-04
HIST1H1B	histone cluster 1 H1 family member b(HIST1H1B)	-2.4367	0.0001	BX088651.4	Uncharacterised	2.1449	3.18E-04
IGLV3-10	immunoglobulin lambda variable 3-10(IGLV3-10)	-2.4055	0.0000	AL645608.8	Uncharacterised	2.1208	1.14E-06
CD177	CD177 molecule(CD177)	-2.3756	0.0000	TSPEAR-AS1	TSPEAR antisense RNA 1(TSPEAR-AS1)	2.1069	7.88E-06
SPATA20P1	spermatogenesis associated 20 pseudogene 1(SPATA20P1)	-2.3515	0.0000	ADGRE4P	adhesion G protein-coupled receptor E4, pseudogene(ADGRE4P)	2.0931	1.49E-10
RAB4B-EGLN2	RAB4B-EGLN2 readthrough (NMD candidate)(RAB4B-EGLN2)	-2.3211	0.0032	FIBCD1	fibrinogen C domain containing 1(FIBCD1)	2.0669	3.86E-03
ACOD1	aconitate decarboxylase 1(ACO	-2.2834	0.0000	LCN8	lipocalin 8(LCN8)	2.0330	2.39E-04
IGKV2D-30	immunoglobulin kappa variable 2D-30(IGKV2D-30)	-2.2810	0.0000	SNORA48	small nucleolar RNA, H/ACA box 48(SNORA48)	1.9933	2.23E-05
AC244154.1	Uncharacterised	-2.2568	0.0066	CLEC4F	C-type lectin domain family 4 member F(CLEC4F)	1.9845	7.87E-05
CDO1	cysteine dioxygenase type 1(CDO1)	-2.2194	0.0053	GPR173	G protein-coupled receptor 173(GPR173)	1.9783	7.88215E-05
SLC1A3	solute carrier family 1 member 3(SLC1A3)	-2.1557	0.0000	AC099521.3	Uncharacterised	1.9768	8.06268E-08
SOCS1	suppressor of cytokine signaling 1(SOCS1	-2.1540	0.0000	KIAA1549L	KIAA1549 like(KIAA1549L	1.9741	0.005026521

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Table A. 5.3. GSEA for significantly enriched gene sets

Top 20 enriched GO gene sets in Low transmission area			Top 20 enriched GO gene sets in High transmission area		
NAME	NES	NOM p-val	NAME	NES	NOM p-val
CELL CYCLE AND TRANSCRIPTION (M4.0)	-9.879	0	ENRICHED IN B CELLS (I) (M47.0)	3.01812	0.0000
CELL CYCLE (I) (M4.1)	-9.8358	0	B CELL SURFACE SIGNATURE (S2)	2.9179	0.0000
IMMUNE ACTIVATION - GENERIC CLUSTER (M37.0)	-5.286	0	ENRICHED IN B CELLS (II) (M47.1)	2.63281	0.0000
PLK1 SIGNALING EVENTS (M4.2)	-5.1109	0	ENRICHED IN B CELLS (VI) (M69)	2.53329	0.0000
MITOTIC CELL DIVISION (M6)	-4.7814	0	TBA (M201)	2.38062	0.0000
MITOTIC CELL CYCLE - DNA REPLICATION (M4.4)	-4.7699	0	NAIVE B CELL SURFACE SIGNATURE (S8)	2.08918	0.0078
MITOTIC CELL CYCLE IN STIMULATED CD4 T CELLS (M4.5)	-4.5301	0	CELL MOVEMENT, ADHESION & PLATELET ACTIVATION (M30)	1.76971	0.0203
CELL CYCLE (III) (M103)	-4.3706	0	ENRICHED IN CELL CYCLE (M167)	1.68597	0.0323
ENRICHED IN MONOCYTES (II) (M11.0)	-4.1221	0	REGULATION OF TRANSCRIPTION, TRANSCRIPTION FACTORS (M213)	1.6667	0.0292
CELL DIVISION (STIMULATED CD4+ T CELLS) (M46)	-3.9463	0	RESTING DENDRITIC CELL SURFACE SIGNATURE (S10)	1.59014	0.0360
RESPIRATORY ELECTRON TRANSPORT CHAIN (MITOCHONDRION) (M219)	-3.8589	0	TBA (M177.0)	1.57306	0.0600
MISMATCH REPAIR (I) (M22.0)	-3.8149	0	ENRICHED IN B CELLS (III) (M47.2)	1.51179	0.0587
ENRICHED IN NEUTROPHILS (I) (M37.1)	-3.685	0	TBA (M70.0)	1.37115	0.1027
CELL DIVISION IN STIMULATED CD4 T CELLS (M4.6)	-3.6239	0	COLLAGEN, TGFB FAMILY ET AL (M77	1.36392	0.1324
MITOTIC CELL CYCLE (M4.7)	-3.4459	0	PLATELET ACTIVATION - ACTIN BINDING (M196)	1.33832	0.1352
E2F1 TARGETS (Q3) (M10.0)	-3.4182	0	EXTRACELLULAR MATRIX (I) (M2.0)	1.23843	0.2082
CELL DIVISION - E2F TRANSCRIPTION NETWORK (M4.8)	-3.3783	0	LEUKOCYTE MIGRATION (M88.0)	1.2003	0.2324
E2F1 TARGETS (Q4) (M10.1)	-3.211	0	MEMORY B CELL SURFACE SIGNATURE (S9)	1.14081	0.2789
MITOTIC CELL CYCLE IN STIMULATED CD4 T CELLS (M4.9)	-3.1463	0	T & B CELL DEVELOPMENT, ACTIVATION (M62.0)	1.09085	0.3293
TLR AND INFLAMMATORY SIGNALING (M16)	-3.0631	0	TBA (M80)	0.9896	0.4511
PLATELET ACTIVATION (I) (M32.0)	-3.0451	0	TRANSMEMBRANE TRANSPORT (II) (M191)	0.96822	0.4902

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Table A. 5.4. Genes represented in each cluster

Gene Cluster	No. of Genes	LM22 genes
Cluster 1	1407	13
Cluster 2	644	6
Cluster 3	859	14
Cluster 4	1420	51
Cluster 5	929	25
Cluster 6	176	10
Cluster 7	172	3
Cluster 8	95	5
Cluster 9	533	19
Cluster 10	575	9
Cluster 11	185	5
Cluster 12	67	2
Cluster 13	19	0
Cluster 14	186	5
Cluster 15	100	2
Cluster 16	20	0
Cluster 17	551	24
Cluster 18	47	1
Cluster 19	48	1
Cluster 20	93	4
Cluster 21	70	2
Cluster 22	120	0
Cluster 23	53	0
Cluster 24	40	2
Cluster 25	14	0
Cluster 26	17	1
Cluster 27	46	0
Cluster 28	17	1
Cluster 30	10	1

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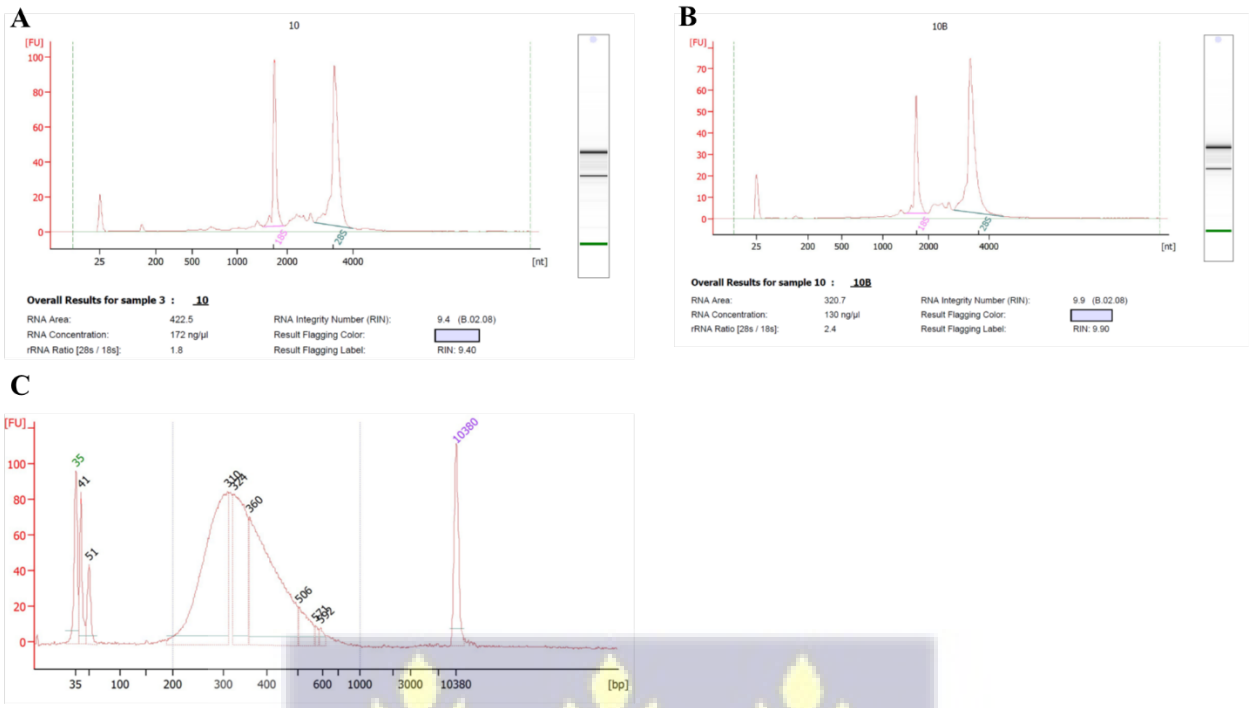


Figure A.5.1: Representative Electropherograms results for RNA quality checks. A. after RNA extraction, B. After Globin clear and C. prepared cDNA libraries.

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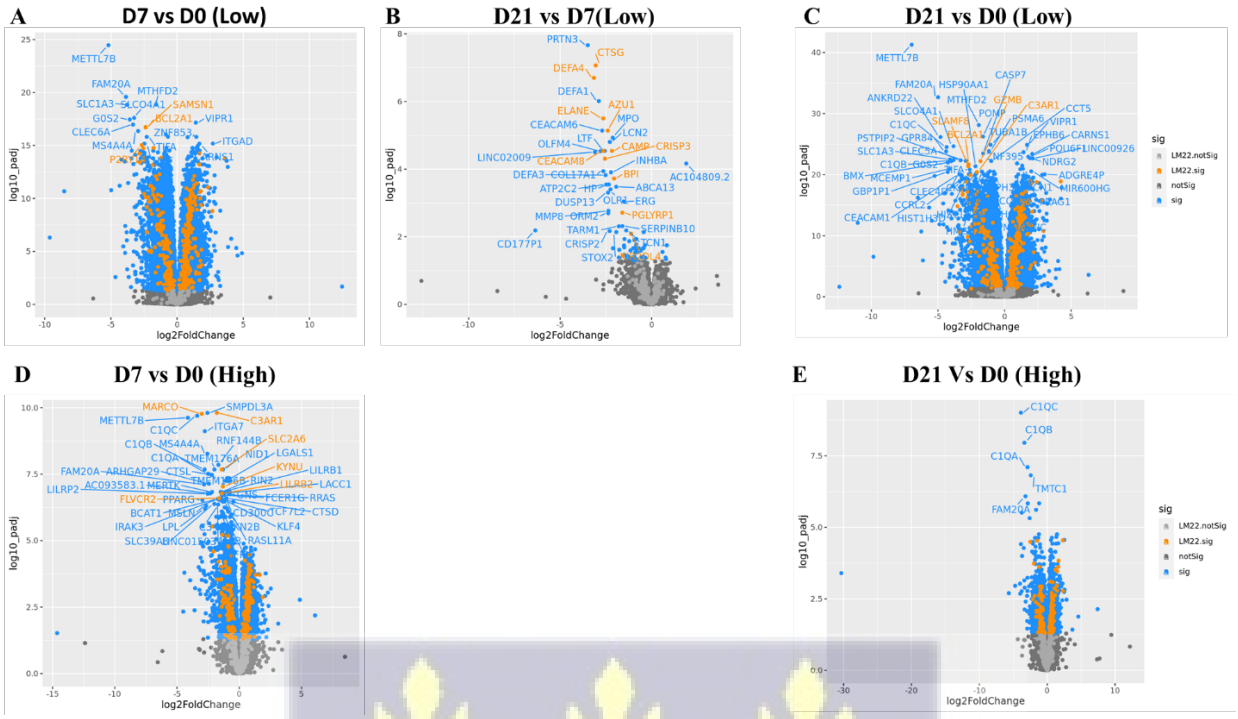


Figure A 5.2: Volcano plots showing DEGs with different comparisons across the three timepoints between the two transmission areas. Each dot represents one gene and is displayed according to the log fold change in expression (x-axis) and the statistical significance of the association (y-axis, in $-\log_{10}$ of the p-value). Blue dots indicate genes deemed to be significantly differentially expressed (adj $p < 0.05$), orange dots indicated significantly expressed LM22 genes and grey dots indicate non-differentially expressed genes. A). Represents D7 versus D0 comparison, B). Represents D21 versus D7 comparison and C) represents D21 versus D0 comparison in the low transmission area. D and E are D7 versus D0 and D21 versus D0 comparison respectively in the high transmission area. There were no DEGs for D21 Vs D7 in the high transmission area.