EFFECTS OF GENETIC VARIATIONS AND ALTERED GENE EXPRESSION PATTERNS ON SEVERE MALARIAL ANAEMIA PATHOGENESIS IN CHILDREN FROM SIAYA COUNTY

BY

ONYANGO CLINTON ODHIAMBO

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MEDICAL GENETICS

SCHOOL OF PUBLIC HEALTH AND COMMUNITY DEVELOPMENT

MASENO UNIVERSITY

©2025

DECLARATION

Declaration by the student:

This thesis is my original work and has not been submitted for a degree award at any other University.

Onyango Clinton Odhiambo
PHD/PH/00068/019

Sign: ……. Date: 30/07/2025.

Declaration by the supervisors:

This thesis has been submitted for examination with our approval as university supervisors.

1. Prof. Collins Ouma, PhD
School of Public Health and Community Development
Maseno University

Sign: ……. Date: …….

Dr. Samuel Anyona, PhD
School of Medicine
Maseno University

Sign: ……..……. Date: 30/07/2025.

Prof. Douglas Perkins, PhD
School of Medicine, University of New Mexico

Sign: ……. Date: 30/07/2025.

ACKNOWLEDGEMENT

I express my deepest gratitude to my mentors, Prof. Douglas J. Perkins, Prof. Collins Ouma and Dr. Samuel Anyona, for their invaluable support, guidance and mentorship throughout my research journey and writing of this thesis. Special thanks go to Dr. Qiuying Cheng for his technical support during my research. I also acknowledge the dedicated support of the Siaya County Referral Hospital team and the University of New Mexico-Maseno Global Health Program Laboratories Team. I am highly indebted to all children who participated in the study and their parents/guardians. A heartfelt thanks to my family for their unwavering love, encouragement, and support throughout this journey.

Lastly, I would like to acknowledge the financial support from the National Institutes of Health grants: R01AI130473 (DJP, SBA), R01AI51305 (DJP), and D43TW05884 (DJP, CO), which made this research possible.

DEDICATION

This research is dedicated to children in holoendemic *P. falciparum* transmission areas, who are especially vulnerable to the scourge of severe malarial anaemia.

ABSTRACT

Malaria remains a significant health burden in sub-Saharan Africa. In holoendemic *Plasmodium Falciparum* transmission regions, such as western Kenya, children aged <5 years are vulnerable due to naïve malarial immunity. Among these children, severe malarial anaemia (SMA, haemoglobin (Hb)<5.0 g/dL) is the most prevalent and life-threatening manifestation of severe malaria. Despite its high burden, the molecular mechanism driving SMA pathogenesis, particularly the host genetic and transcriptomic factors as well as parasite gene expression, remains poorly defined. This study determined the effect of human genetic variation, as well as the host and *P. falciparum* gene expression patterns on SMA pathogenesis in children (<5 years) from Siaya County. The specific objectives included: to determine the relationship between genetic polymorphisms in natural cytotoxicity-triggering receptor (*NCR3*) (rs2736191:C>G and rs11575837:C>T) and longitudinal malaria outcomes; to determine the influence of NK cell networks, and other immune signalling pathways on the pathogenesis of paediatric SMA, and to profile the transcriptome of blood-stage *P. falciparum* in children with SMA. The selected *NCR3* SNPs reduced NCR3 transcription, thereby impairing NK cell recognition of iRBCs and cytokine signaling. In contrast, host and parasite transcriptomics provide insights into their expression profiles in SMA. Genomic DNA from dried bloodspots collected at enrollment from 1,515 children was genotyped for the *NCR3* variants using TaqMan® assays, while total RNA from 66 children was used for RNA sequencing via Illumina® sequencing. Poisson regression revealed that the rs11575837 TT genotype increased the risk of malaria episodes [Incidence Rate Ratio (IRR)=1.540, *P*=0.009], while the CC haplotype was protective against SMA (IRR=0.823, *P*=0.009). Host transcriptomics of RNA from whole-blood collected at enrollment from 66 children revealed 6,862 differentially expressed genes (DEGs) in SMA vs. non-SMA, with increased NK cell expression (*P*=0.002) and dysregulated Inflammation_NK cell cytotoxicity network (*P*adj=3.628E-04) in SMA. Furthermore, the HSP60-HSP70-TLR2/4 signalling pathway (*P*adj=5.483E-09) was among the most dysregulated immune pathways, with altered glutamine metabolism in SMA. *P. falciparum* transcriptomics from 60 children revealed 566 DEGs in SMA vs. non-SMA. Weighted gene co-expression network analysis (WGCNA) and protein-protein interaction (PPI) of the DEGs identified parasite pathways potentially contributing to SMA. Additionally, the hub nodes in the WGCNA and PPI networks, which increased the risk of SMA, were identified. Overall, *NCR3* variation influences malaria and SMA risk, while SMA is characterised by dysregulated NK-cell function, impaired HSP60-HSP70-TLR2/4 signalling, altered glutamine metabolism, and distinct parasite transcriptional profiles. These findings advance the understanding of SMA pathogenesis and identify hub nodes as potential therapeutic targets.

TABLE OF CONTENTS

DECLARATION ii
ACKNOWLEDGEMENT iii
DEDICATION iv
ABSTRACT v
TABLE OF CONTENTS vi
LIST OF ABBREVIATIONS AND ACRONYMS x
DEFINITION OF TERMS xi
LIST OF TABLES xii
LIST OF FIGURES xiii
CHAPTER ONE: INTRODUCTION 1
1.1 Background Information 1
1.1.1 Genetic Variations in Immune Response Genes in SMA 1
1.1.2 NK Cell Networks 3
1.1.3 P. falciparum Transcriptome in Malaria 4
1.2 Statement of the Problem 6
1.3 Objectives of the Study 7
1.3.1 General Objective 7
1.3.2 Specific Objectives 7
1.3.3 Hypotheses 7
1.3.3.1 Overall Research Hypothesis 7
1.3.3.2 Null Hypotheses 7
1.4 Significance of the Study 7
1.4.1 Specific Objective One 7
1.4.2 Specific Objective Two 8
1.4.3 Specific Objective Three 9
CHAPTER TWO: LITERATURE REVIEW 11
2.1 P. falciparum Malaria Pathogenesis 11
2.2 NK Cells in Malarial Infection 12
2.2.1 The Role of Natural Cytotoxicity-Triggering Receptor 3 in P. falciparum Malaria 13
2.2.2 NK Cell Networks 14
2.2.3 Dysregulated NK Cell Function in Malaria 16
2.3 Genome-wide Expression Analysis in Malaria 17
2.3.1 Host Gene Expression Analysis 17
2.3.2 Parasite Transcriptome Analysis 19

CHAPTER THREE: METHODOLOGY 21
3.1 Study Area 21
3.2 Study Design 22
3.2.1 NCR3 Variant Analysis 22
3.2.1.1 Study Design and Participants 22
3.2.1.2 Screening and Enrollment 23
3.2.1.3 Inclusion and Exclusion Criteria 24
3.2.1.4 Longitudinal Follow-ups 24
3.2.1.5 Sample Size Calculation 24
3.2.1.6 Collection of Blood Samples 25
3.2.1.7 Laboratory Measurements 25
3.2.1.8 Selection of NCR3 SNPs 26
3.2.1.9 Genotyping of NCR3 rs2736191: C>G and rs11575837:C>T Variants 27
3.2.1.10 Data Analysis 27
3.2.2 Transcriptomic Analysis 28
3.2.2.1 Study Design and Participants 28
3.2.2.2 Screening and Enrolment 29
3.2.2.3 Inclusion and Exclusion Criteria 30
3.2.2.4 Sample Size Calculation 30
3.2.2.5 Sample Collection and Laboratory Procedures 31
3.2.2.6 RNA Isolation 32
3.2.2.7 RNA Sequencing 32
3.2.2.7.1 Library Construction 32
3.2.2.7.2 Clustering, Sequencing and Quality Control 32
3.2.2.7.3 Mapping to Reference Genomes and Quantification 33
3.2.2.7.3.1 Mapping to the Human Reference Genome 33
3.2.2.7.3.2 Mapping to the pfKE01 Reference Genome 33
3.2.2.8 Data Analysis 33
3.2.2.8.1 Host Transcriptomics 33
3.2.2.8.2 Parasite Transcriptomics 34
3.3 Ethical Considerations 36
3.3.1 Inclusion of Children as a Vulnerable Group 36
3.3.2 Ethical Handling of Samples and Clinical Follow-up 36
3.3.3 Consent Process and Counselling Protocols 36
3.3.4 Protections against Risk 37
3.3.5 Potential Benefits to the Participants and the Community 37

3.3.6 Compensation for Research-Related Injury 37
3.3.7 Economic Burden to Subjects 37
CHAPTER FOUR: RESULTS 38
4.1 Objective One 38
4.1.1 Demographic, Clinical and Laboratory Characteristics of Study Participants 38
4.1.2 Characteristics of the NCR3 Variants in the Study Population 39
4.1.3 Longitudinal Risk of Malaria and SMA Episodes for the NCR3 Genotypes/Haplotypes ... 41
4.2 Objective Two 43
4.2.1 Admission Demographic, Clinical and Laboratory Characteristics of Study Participants .. 43
4.2.2 Distribution of Differential Gene Expression in SMA Relative to Non-SMA 45
4.2.3 Leukocytic Immune Cell Profiling 45
4.2.4 Process Network Enrichment Analysis in SMA 47
4.2.5 Immune Response Canonical Signalling Pathways in Children with SMA 48
4.2.6 Unsupervised Hierarchical Clustering Analysis of Gene Expression Profiles in the HSP60-HSP70-TLR Signaling Pathway 52
4.2.7 Differential Expression of Glutamine Transporters and Glutamine Synthetase in SMA 56
4.3 Objective Three 58
4.3.1 Demographic, Clinical and Laboratory Characteristics of Study Participants at Enrollment 58
4.3.2 Distribution of Differentially Expressed P. falciparum Genes in SMA Relative to Non-SMA 60
4.3.3 Principal Component Analysis (PCA) of DEGs 61
4.3.4 P. falciparum Gene Expression Deconvolution Analysis 61
4.3.5 Weighted gene co-expression network analysis (WGCNA) of P. falciparum DEGs 63
4.3.5.1 Co-expression Analysis of P. falciparum DEGs 63
4.3.5.2 WGCNA Module Analysis 66
4.3.6 Protein-Protein Interaction (PPI) Network Analysis 71
4.3.6.1 PPI Network of P. falciparum DEGs 71
4.3.6.2 Functional Enrichment Analysis of PPI Network Clusters 74
4.3.6.3 Relationship between transcript levels of hub genes of blood-stage P. falciparum and malaria severity 77
CHAPTER FIVE: DISCUSSION 79
5.1 Relationship between Genetic Polymorphisms in NCR3 and Longitudinal Susceptibility to Childhood SMA 79
5.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA 82
5.3 Transcriptome Profile of Blood-Stage P. falciparum in Children with SMA 87

5.4 Limitations of the Study 96
CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS 97
6.1 Summary of Findings 97
6.1.1 Relationship between Genetic Polymorphisms in NCR3 and Longitudinal Susceptibility to Childhood Malaria, SMA and All-cause mortality 97
6.1.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA 97
6.1.3 Transcriptome Profile of Blood-stage P. falciparum in Children with SMA 98
6.2 Conclusions 98
6.2.1 Relationship between Genetic Polymorphisms in NCR3 and Longitudinal Susceptibility to Childhood malaria, SMA and All-cause Mortality 98
6.2.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA 99
6.2.3 Transcriptome Profile of Blood-stage P. falciparum in Children with SMA 99
6.3 Recommendations from the Current Study 100
6.3.1 Relationship between Genetic polymorphisms in NCR3 and Longitudinal Susceptibility to Childhood Malaria, SMA and All-cause mortality 100
6.3.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA 100
6.3.3 Transcriptome Profile of Blood-stage P. falciparum in Children with SMA 100
6.4 Recommendations for Future Studies 100
6.4.1 Relationship between Genetic Polymorphisms in NCR3 and Longitudinal Susceptibility to Childhood Malaria, SMA and All-cause Mortality 100
6.4.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA 101
6.4.3 Transcriptome Profile of Blood-stage P. falciparum in Children with SMA 101
REFERENCES 102
APPENDICES 123

LIST OF ABBREVIATIONS AND ACRONYMS

µL	Microlitre
DBL-1α	Duffy Binding Like-1 alpha
DEGs	Differentially Expressed Genes
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
G6PD	Glucose-6-Phosphate Dehydrogenase
Hb	Haemoglobin
HBB	Haemoglobin Beta
HbS	Haemoglobin Sickle
HIV	Human Immunodeficiency Virus
iRBCs	Infected Red Blood Cells
MAF	Minor Allele Frequency
MIF	Macrophage Inhibitory Factor
Mos.	Months
NCR3	Natural Cytotoxicity-triggering Receptor 3
NK cell	Natural Killer cell
PCR	Polymerase Chain Reaction
Pf.	*Plasmodium Falciparum*
***Pf*EMP-1**	*P. falciparum* erythrocyte membrane protein-1
PPI	Protein-protein interaction
RNA	Ribonucleic acid
SMA	Severe Malarial Anaemia
SNPs	Single Nucleotide Polymorphisms
WHO	World Health Organization
WCGNA	Weighted Co-expression Gene Analysis

DEFINITION OF TERMS

Genotype – a set of genes responsible for malaria outcome, i.e., malaria episode or SMA. For instance, wild-type genotype CC for the SNP rs2736191:C>G.

Haplotype – a group of alleles that is inherited together from a single parent, i.e., GT haplotype from rs2736191:C>G and rs11575837:C>T genotypes.

Holoendemic – an area where essentially all individuals in a population are infected, and malaria symptoms do not appear equally across age groups. In such cases, the young are most likely to express pathogenic responses, while older hosts will carry the disease asymptomatically due to adaptive immunity.

Polymorphisms – variations at single positions in the DNA sequence among individuals.

Transcriptomics – the study of all RNA transcripts that are produced by the genome of an organism.

Module – a cluster of highly interconnected genes based on their similar expression patterns across a set of samples.

Cluster – a group of proteins that interact more frequently with each other than with proteins outside the group.

Seed node – a gene or protein in a network with the highest weight in a network (highly connected).

Hub node – a highly connected node within a network, having significantly more connections (edges) than the average node.

Dysregulated – an abnormal or uncontrilled immune response.

Impaired – immune function that is weakened, deficient or not functioning adequately

LIST OF TABLES

Table 4.1: Admission demographic, clinical and laboratory characteristics of study participants 39
Table 4.2: Distribution of rs2736191:C>G and rs11575837:C>T genotypes and haplotypes..... 41
Table 4.3: Susceptibility to malaria and SMA over 36 mos. of follow-up 42
Table 4.4: Survival analysis for all-cause mortality..... 43
Table 4.5: Admission demographic, clinical and laboratory characteristics of study participants 44
Table 4.6: Differentially expressed transcripts in the HSP60-HSP70-TLR2/4 signalling pathway in children with SMA 51
Table 4.7: Top process networks of DEGs in clusters 1 and 2 of the heatmap 55
Table 4.8: Differential expression of transcripts of glutamine transporters and glutamine-metabolizing enzymes in children with SMA 58
Table 4.9: Admission demographic, clinical and laboratory characteristics of study participants 59
Table 4.10: 10 Most down-regulated and up-regulated P. falciparum genes in SMA vs. non-SMA 61
Table 4.11: List of PPI network clusters identified in the PPI analysis of P. falciparum DEGs .. 73
Table 4.12: Differential expression of genes encoding the hub nodes of the PPI network clusters 73
Table 4.13: Relationship between transcript levels of P. falciparum hub genes and malaria severity 78

LIST OF FIGURES

Figure 3.1 Location of Siaya County and the Siaya County Referral Hospital in Western Kenya. 22
Figure 3.2 Study design: Objective one. 23
Figure 3.3 Sample size calculation for objective one. 25
Figure 3.4 Selection of the NCR3 SNPs. 27
Figure 3.5 Study design: Objectives two and three. 29
Figure 3.6 Sample size calculation for objective two. 31
Figure 4.1 NCR3 variants investigated. 40
Figure 4.2 Differential gene expression analysis and leukocytic immune profiling in children with severe malarial anaemia. 46
Figure 4.3 Inflammation- NK cell cytotoxicity process network. 48
Figure 4.4 Top emergent immune response canonical pathway maps in children with severe malarial anaemia. 49
Figure 4.5 Unsupervised hierarchical clustering of RNA-seq data showing differentially expressed genes in the HSP60-HSP70-TLR2/4 pathway. 54
Figure 4.6 Model showing significantly dysregulated glutamine transporters and glutamine synthetase. 57
Figure 4.7 Volcano plot showing the magnitude and significance of differentially expressed *P. falciparum* genes in patients with SMA relative to non-SMA. 60
Figure 4.8 *P. falciparum* gene expression deconvolution analysis and relative proportions of *P. falciparum* developmental stages in non-SMA and SMA. 62
Figure 4.9 Identification of co-expression modules using WGCNA and enrichment analysis of WGCNA networks. 66
Figure 4.10 Brown module. 67
Figure 4.11 Yellow module. 68
Figure 4.12 Blue module. 69
Figure 4.13 Turquoise module. 70
Figure 4.14 Green module. 71
Figure 4.15 Visualization of PPI network of *P. falciparum* DEGs. 72
Figure 4.16 Functional enrichment terms of clusters 1-5 in the *P. falciparum* PPI network. 75
Figure 4.17 Functional enrichment terms of clusters 6-10 in the *P. falciparum* PPI network. 76
Figure 4.18 Functional enrichment terms of clusters 11-17 in the *P. falciparum* PPI network. 77

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Malaria is a major threat to public health globally, accounting for an estimated 249M cases and 608,000 deaths (WHO, 2023). The World Health Organization (WHO) African region bears the greatest burden, accounting for 94% of malaria cases and 95% of malaria deaths globally, with children under five comprising approximately 80% of this mortality (WHO, 2023). Moreover, most of the morbidity and mortality in the WHO Africa region is attributed to *Plasmodium Falciparum* (WHO, 2023). Life-threatening complications of *P. falciparum* malaria include hyper-parasitemia, hypoglycemia, hyper-lactatemia, kidney failure, metabolic acidosis, cerebral malaria (CM), severe malarial anaemia [SMA, haemoglobin (Hb)<5.0g/dL with peripheral parasitemia], and respiratory distress (Marsh *et al.*, 1995). In holoendemic *P. falciparum* transmission areas, such as western Kenya, children are particularly vulnerable to SMA, a primary manifestation of severe malaria (Akech *et al.*, 2019; Anyona *et al.*, 2021; Okoyo *et al.*, 2021). The aetiology of SMA includes haemolysis [destruction of infected and uninfected red blood cells (RBCs)], splenic sequestration of RBCs, dyserythropoiesis, and bone marrow suppression, often complicated by co-infection with other pathogens (Perkins *et al.*, 2011). Despite malaria control efforts in western Kenya, SMA has remained one of the major causes of morbidity and mortality in children less than five years of age (Okoyo *et al.*, 2021; Paton *et al.*, 2021).

1.1.1 Genetic Variations in Immune Response Genes in SMA

The outcome of malaria infection, particularly its severe forms such as SMA, is strongly influenced by host genetic factors that modulate immune responses. For instance, polymorphisms in immune response genes that regulate innate immunity have been associated with an increased susceptibility to SMA in children from western Kenya. These polymorphisms occur in genes involved in complement activation (e.g., complement component 3 (C3), C5, cytokine production (e.g., interleukin-1 beta (IL-1β), IL-7, IL-10, IL-12, IL-13, interferon gamma (IFN-γ),), chemokine signalling (e.g., regulated on activation, normal T cell expressed and secreted (RANTES)) and leukocyte regulation (e.g., cluster of differentiation (CD) 32, colony stimulating factor (CSF) 2, leukocyte-associated immunoglobulin-like receptor (LAIR) 1 (Achieng' *et al.*, 2019b; Anyona *et al.*, 2020; Kisia *et al.*, 2022; Kisia *et al.*, 2019; Munde *et al.*, 2017; Okeyo *et al.*, 2013; Ong'echa *et al.*, 2011; Ouma *et al.*, 2008a; Ouma *et al.*, 2012; Ouma *et al.*, 2008b; Ouma *et al.*, 2006; Raballah *et al.*, 2022a; Raballah *et al.*, 2022b).

A key genomic region implicated in malaria susceptibility is the 6p21.3 locus within the major histocompatibility complex (MHC), which has been linked to mild malaria in African populations (Brisebarre *et al.*, 2014; Flori *et al.*, 2003; Jepson *et al.*, 1997). Centrally located in this MHC region is the natural cytotoxicity-triggering receptor 3 (*NCR3*, also known as *NKp30* or *CD337*). NCR3 encodes a receptor that is constitutively expressed on natural killer (NK) cells and plays a pivotal role in their activation, degranulation, and cytotoxic function. (Moretta *et al.*, 2001; Wang *et al.*, 2012a). Importantly, NCR3 can directly bind to the Duffy binding-like (DBL1α) domain of *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP1) on infected red blood cells (iRBCs), independently of MHC class I molecules, initiating NK cell-mediated cytotoxicity and release mediators: IFN-γ, TNF-γ, MIP-1α/1β, and RANTES, known to influence the pathogenesis of SMA (Artavanis-Tsakonas *et al.*, 2003a; Baratin *et al.*, 2007; Fauriat *et al.*, 2010; Korbel *et al.*, 2004; Perkins *et al.*, 2011).

These findings highlight that a mutation in the *NCR3* could potentially affect the function of the receptor in orchestrating NK-cell-mediated immunity. Specifically, previous studies have reported that the promoter variant rs2736191: C>G is associated with an increased risk of mild malaria in Burkina Faso and Congo. The mutant allele G reduces promoter activity, leading to lower transcription and expression of the receptor NCR3 on NK cells. This reduced NCR3 expression impairs NK cell recognition of *P. falciparum*-infected red blood cells, resulting in weakened NK cell-mediated cytokine signaling (notably IFN-γ and TNF-α), which is critical for parasite clearance (Baaklini *et al.*, 2017; Delahaye *et al.*, 2007).

Another variant, rs11575837:C>T, located in the untranslated exon 1 region, has been linked to reduced transcription of *NCR3* and autoimmune conditions such as primary Sjögren's syndrome (Auton *et al.*, 2015; Rusakiewicz *et al.*, 2013). Both Sjögren's syndrome and SMA display immune dysregulation and inflammatory cytokine signalling. Thus, the reduced NCR3 transcription linked to the rs11575837 T allele, translating to lower NK cell activation in Sjögren's syndrome, could affect the host's ability to control parasitaemia or modulate inflammation in SMA. (Baaklini *et al.*, 2017; Rusakiewicz *et al.*, 2013). This reduced transcription could lead to lower NCR3 expression, which may impair NK cell recognition of iRBCs, weaken NK-mediated cytokine signalling in response to *P. falciparum* infection, thereby contributing to ineffective parasite clearance and increased risk of severe malarial anaemia.

However, the role of *NCR3* variants in the development of severe forms of malaria, such as SMA, particularly in high-transmission settings like western Kenya, remains unexplored. Interestingly, these SNPs are in strong linkage disequilibrium (88%) in the study population, but no studies to date have comprehensively investigated how rs2736191 and rs11575837 influence

the risk of SMA or longitudinal clinical outcomes such as recurrent malaria episodes and all-cause mortality in endemic regions. Given NCR3's essential role in NK cell recognition of iRBCs and its localization within the MHC locus linked to malaria susceptibility, reduced NCR3 expression and impaired NK cell function could contribute to ineffective parasite clearance and the pathogenesis of SMA. The present study, therefore, investigated the relationship between rs2736191 and rs11575837, as well as longitudinal clinical outcomes, including the incidence of malaria, SMA episodes, and all-cause mortality, in a pediatric cohort from western Kenya.

1.1.2 NK Cell Networks

NK cells are the main lymphocyte subset of the innate immune system, accounting for 8-20% of circulating lymphocytes in humans. (Cooper *et al.*, 2001). Following activation, NK cells produce various effector molecules, including cytokines (IFN-γ, tissue necrosis factor alpha (TNFα), granulocyte colony-stimulating Factor (G-CSF), macrophage inflammatory proteins (MIP)-1α/β, chemokines (RANTES) and cytotoxic granules (granzymes and perforins) (Abel *et al.*, 2018; Artavanis-Tsakonas *et al.*, 2003a; Baratin *et al.*, 2007; Fauriat *et al.*, 2010). Additionally, NK cells have been shown to shape and amplify adaptive immune responses, linking innate and adaptive immune responses (Hart *et al.*, 2019). In addition to NK cells, other immune cell populations play critical roles in malaria pathogenesis. Monocytes and macrophages contribute to both parasite clearance and immunopathology through phagocytosis and cytokine production (Perkins *et al.*, 2011). In the adaptive arm, $CD4^+$ T cells producing IFN-γ are elevated, while memory-like IL-17^+ $CD4^+$ T cells are diminished, suggesting dysregulated responses. $CD8^+$ T cell expansion and activation also correlate with SMA, potentially mediating parasite clearance and erythrocyte destruction. These findings underscore the complex interplay between innate and adaptive immune cells (Anyona *et al.*, 2023; Raballah *et al.*, 2017; Royo *et al.*, 2019; Safeukui *et al.*, 2015).

NK cell activation is achieved by the up-regulation of complementary activating signals and the down-regulation of inhibitory signaling. (Lanier, 2008). NK cell has a repertoire of activating receptors on their surface (e.g., NCR1-3) that bind ligands on infected cells or tumour cells to initiate signal transduction (Barrow *et al.*, 2019; Dushek *et al.*, 2012). Activating signalling in NK cells can occur through antibody-mediated mechanisms, such as when the fragment crystallizable gamma receptor [FcγRIIIa (CD16)] on NK cells recognizes immunoglobulin G (IgG) bound to infected red blood cells (iRBCs) in *P. falciparum* malaria. (Arora *et al.*, 2018; Sulica *et al.*, 1993). Additionally, NK cell activation can be cytokine-mediated through receptors for cytokines such as IL-2, IL-12, and IL-18 on NK cells in the context of *P. falciparum* malaria. As a result, NK cell activation leads to the release of cytotoxic molecules, such as perforin and granzymes, as well as pro-inflammatory cytokines, including IFN-γ (Artavanis-Tsakonas &

Riley, 2002; Horowitz *et al.*, 2010; Nielsen *et al.*, 2016). Conversely, NK cells also express a plethora of inhibitory receptors that suppress NK cell activation (Kärre, 2008; Vivier *et al.*, 2004).

In severe malarial anemia (SMA), patients present with dysregulation of innate immune signaling (Perkins *et al.*, 2011). Interestingly, the overproduction of pro-inflammatory mediators by NK cells can exacerbate the innate immune response, leading to immunopathology (Wolf *et al.*, 2017). Since NK cell function is regulated by a delicate balance of activating and inhibitory signalling (Lanier, 2008), it remains unclear whether this dynamic equilibrium is disrupted in SMA, contributing to a dysregulated innate immune response. Furthermore, despite the crucial role played by NK cells in innate immunity, the signalling pathways of NK cells in SMA are yet to be elucidated. Transcriptomic analysis revealed altered immune cell expression patterns, including NK cells in acute malaria in Malaysia (Dooley *et al.*, 2023). In Uganda, transcriptional analysis showed up-regulation of NK inhibitory receptors and transcriptional characteristics of exhausted (impaired functionality) NK cells in Ugandan children with repeated malaria episodes (Ty *et al.*, 2023). While transcriptome profiling has been employed to elucidate molecular mechanisms in *P. falciparum* infection, these findings have not been extended to populations with high SMA prevalence.

Transcriptomic studies in western Kenya have identified altered expression of immune-related genes in children with SMA (Achieng' *et al.*, 2019a; Kempaiah *et al.*, 2016). For instance, previous array-based and global transcriptome profiling studies on children with SMA have shown altered expression profiles of immune response genes, such as LAIR1 and heat shock protein (HSP) 70 (Achieng' *et al.*, 2019a; Kempaiah *et al.*, 2016). Moreover, targeted transcriptional analyses revealed altered expression of genes in the ubiquitin-proteasomal proteolysis process (Anyona *et al.*, 2021). However, these analyses are yet to uncover the specific immune signalling networks, particularly NK cell-associated pathways that may drive disease severity. To address these gaps, a high-throughput next-generation RNA sequencing was employed to characterise the entire transcriptome in children with SMA to determine the NK cell networks as well as other immune signalling pathways contributing to the pathogenesis of paediatric SMA.

1.1.3 *P. falciparum* Transcriptome in Malaria

Clinical manifestations of malaria present during the intraerythrocytic developmental cycle when merozoites invade and replicate within RBCs(Cowman *et al.*, 2012; Miller *et al.*, 2002). These blood-stage parasites have been demonstrated to be highly transcriptionally active and with tightly regulated gene expression patterns that govern their survival, replication, and virulence

(Bozdech *et al.*, 2003; Painter *et al.*, 2018). Since *P. falciparum* mRNAs possess a poly(A) tail, next-generation RNA sequencing on whole blood samples captures both human and *P. falciparum* gene transcripts (Erath *et al.*, 2019). This has allowed for transcriptomic profiling of *P. falciparum* gene expression in human infection, providing critical insights into parasite biology during the complex life cycle of *P. falciparum* (Bradwell *et al.*, 2020; Guillochon *et al.*, 2022; Lee *et al.*, 2018b; Tonkin-Hill *et al.*, 2018). For instance, RNAseq carried out on blood from Gambian children showed 236 parasite DEGs between severe and uncomplicated malaria (UM), enriched for gene ontology (GO) terms including RNA processing, haemoglobin catabolic process and protein transport (Lee *et al.*, 2018a). Similarly, another gene expression study identified parasite DEGs in Malian children with uncomplicated malaria (Bradwell *et al.*, 2020).

Enrichment analyses of parasite DEGs across other studies have uncovered vital biological processes such as glycolysis, histone methylation, protein folding pathways and tricarboxylic acid (TCA) cycle in patients with severe malaria (excluding SMA) (Guillochon *et al.*, 2022; Tonkin-Hill *et al.*, 2018). Collectively, these studies demonstrate the utility of transcription profiling in examining gene expression patterns of *P. falciparum* during its asexual blood stages in malaria, uncovering key molecular mechanisms driving parasite development, survival, replication, virulence and host immune evasion. However, no study to date has specifically characterized the *P. falciparum* transcriptome during the erythrocytic stages in children with severe malarial anemia (SMA), a predominant and life-threatening malaria complication in high-transmission areas such as western Kenya. Additionally, while previous work has illuminated parasite biology in other severe malaria phenotypes, the transcriptional profile of the Kenyan *Pf*KE01 strain in the context of SMA remains undefined.

Current treatment for SMA focuses primarily on parasite clearance using intravenous artesunate, a potent schizonticide (Li & Weina, 2010; MoH, 2020). However, SMA is also marked by profound immune dysregulation, partly driven by phagocytosis of *P. falciparum*–derived haemozoin (*Pf*Hz), which contributes to the pathogenesis independent of parasite load (Perkins *et al.*, 2011). This suggests that parasite clearance alone may not be sufficient for effective therapeutic management in SMA patients. As such, the current study employed high-throughput RNAseq to explore the entire *P. falciparum* transcriptome in peripheral blood from children with SMA to uncover key parasite molecular mechanisms that drive parasite development, virulence, and immune evasion within the host, offering insights that may inform future therapeautic strategies for SMA.

1.2 Statement of the Problem

Although severe malarial anaemia (SMA) remains a leading cause of malaria-related morbidity and mortality in children under five, especially in high-transmission regions like western Kenya, the specific immunogenetic mechanisms contributing to its pathogenesis are not fully defined, particularly in the context of longitudinal malaria outcomes. Preliminary data from my master's research suggest that NK cell function may influence malaria pathogenesis, as polymorphisms in the NCR3 gene were associated with SMA in children under 5 years in western Kenya. However, these findings were limited to a cross-sectional analysis. To address this knowledge gap, the present study investigated the longitudinal influence of NK cells on malaria outcomes by examining associations between *NCR3* genetic variants (rs2736191: C>G and rs11575837:C>T, including haplotypes) and longitudinal clinical outcomes: malaria episodes, SMA episodes, and all-cause mortality in Kenyan children.

NK cell function is regulated by a dynamic balance of inhibitory and activating signalling. However, how these signalling pathways contribute to the immunopathogenesis of SMA in malaria naïve children remains poorly defined. Although prior transcriptomics studies have revealed altered NK cell responses, these findings have not been specifically contextualized within pediatric SMA, characterised by innate immune dysregulation. Furthermore, while blood-stage *P. falciparum* infection drives substantial host immune activation, the downstream immune signalling networks, especially those involving NK cell pathways, have not been comprehensively characterized in children with SMA. Therefore, this study utilized high-throughput RNA sequencing to define NK cell networks and other immune signaling pathways involved in SMA pathogenesis, identifying top-ranked gene networks across the entire host transcriptome.

P. falciparum engages in complex interactions with the host immune system, contributing to malaria-related morbidity and mortality. Despite extensive malaria research on *P. falciparum* biology and host-pathogen interactions, the gene expression profiles of blood-stage *P. falciparum* specifically in children with SMA remain largely unexplored. Current treatment for SMA focuses primarily on parasite clearance. Since clinical symptoms arise during the erythrocytic stages of infection, understanding the transcriptional activity of *P. falciparum* during this phase is crucial for elucidating mechanisms of virulence, immune evasion, and disease severity. Current treatment of SMA focuses on parasite clearance; however, SMA pathogenesis also involves immune dysregulation driven by parasite-derived products like haemozoin, which are not addressed by antiparasitic therapies alone. This persistent burden highlights the need to uncover parasite factors that may contribute to SMA pathogenesis. Therefore, this study profiled the transcriptome of blood-stage *P. falciparum* in children with SMA to reveal the parasite's gene

expression patterns and identify potential molecular mechanisms that may serve as targets for improved therapeutic strategies beyond parasite clearance alone.

1.3 Objectives of the Study

1.3.1 General Objective

To determine the effects of human genetic variation as well as altered host and *P. falciparum* gene expression patterns on the pathogenesis of SMA in children (<5 years) from Siaya County.

1.3.2 Specific Objectives

i. To determine the influence of NK cells on malaria outcomes by examining the relationship between genetic polymorphisms in *NCR3* (rs2736191: C>G and rs11575837:C>T) and longitudinal clinical outcomes.
ii. To determine the influence of NK cell networks and other immune signalling pathways on the pathogenesis of paediatric SMA by determining the top-ranked genes/gene pathways from the entire transcriptome.
iii. To profile the transcriptome of blood-stage *P. falciparum* in children with SMA.

1.3.3 Hypotheses

1.3.3.1 Overall Research Hypothesis

Human genetic variation, as well as the host and *P. falciparum* gene expression patterns, do not affect the pathogenesis of severe malarial anaemia in children (<5 years) from Siaya County.

1.3.3.2 Null Hypotheses

i. Genetic polymorphisms in *NCR3* (rs2736191:C>G and rs11575837:C>T) do not affect the longitudinal malaria outcomes.
ii. NK cell networks and other immune signalling pathways do not significantly influence the pathogenesis of paediatric SMA.
iii. The transcriptome of blood-stage *P. falciparum* is not significantly different between children with SMA and those without SMA.

1.4 Significance of the Study

1.4.1 Specific Objective One

Malaria immunity in children develops gradually over repeated infections, making it critical to study host genetic contributions to disease outcomes longitudinally during early childhood. This study provides insights into how human genetic variation, specifically in *NCR3* (rs2736191 and rs11575837), influences the development of SMA and longitudinal malaria outcomes in children from holoendemic regions. By identifying the TT genotype of rs11575837 as a risk factor for

increased malaria episodes and the protective effect of the CC haplotype against SMA, this study contributes to the genomic epidemiology of pediatric malaria.

The findings of the study advance current knowledge by linking NCR3 variants to functional changes in transcription factor binding sites and associating these changes with clinical outcomes. This may explain how altered NK cell activity contributes to SMA pathogenesis. These insights are relevant for the development of polygenic risk scores to estimate an individual's genetic predisposition to SMA, facilitating early identification of high-risk children who may benefit from targeted interventions such as vaccines, prophylaxis, or enhanced clinical monitoring.

The study directly benefits malaria researchers, geneticists, immunologists, and public health stakeholders involved in malaria control and personalized medicine initiatives in sub-Saharan Africa. Additionally, the results lay the groundwork for future mechanistic studies exploring how specific transcription factor binding site changes affect *NCR3* expression and function, and how these molecular alterations influence NK-cell-mediated immune responses during *P. falciparum* infection.

1.4.2 Specific Objective Two

Despite the clinical significance of SMA, the molecular mechanisms driving SMA pathogenesis remain poorly understood. This study provided a comprehensive transcriptome-wide analysis of whole blood samples from children with SMA, offering insights into the immunological and metabolic dysregulation underlying the condition. The study identified 6,862 DEGs genes in children with SMA compared to non-SMA controls, with key findings highlighting the dysregulation of immune signaling networks such as the Inflammation_NK cell cytotoxicity process and the HSP60-HSP70-TLR2/4 pathway. These results suggest that SMA is associated with altered natural killer (NK) cell function, impaired pattern recognition receptor signaling, and activation of compensatory immune pathways. Furthermore, the data revealed significant changes in genes involved in glutamine transport and metabolism, pointing to the role of metabolic adaptation in SMA.

These findings significantly advance the current understanding of SMA pathogenesis by connecting immune and metabolic dysregulation to disease severity. The identification of specific genes and pathways that are consistently up- or down-regulated in SMA provides a valuable basis for developing novel therapeutic interventions. For example, small molecule compounds targeting dysregulated immune pathways such as TLRs, NF-κB, and IRAK1 may help restore immune balance. In addition, the observed upregulation of glutamine transporters

and glutamine synthetase suggests that glutamine supplementation could be explored as an adjunct therapeutic strategy to support immune function and meet metabolic demands during severe infection.

The implications of this study extend to multiple beneficiaries. Researchers in molecular immunology and infectious diseases can use these findings to guide mechanistic studies of immune dysfunction in malaria. Pediatric clinicians and global health practitioners may benefit from improved understanding of SMA pathogenesis to better stratify risk and tailor treatment approaches. Moreover, the study provides pharmaceutical scientists and translational researchers with potential targets for therapeutic development. Future research should investigate the cytotoxic activity of NK cells in children with SMA to validate their functional relevance. It is also critical to measure protein expression levels of key genes to determine the correlation between transcriptomic changes and functional protein abundance. Additional RNA-seq studies in independent cohorts and plasma proteomics validation will strengthen the evidence base and generalizability of these findings. Finally, the study supports the need for in vitro evaluation of therapeutic compounds targeting the identified dysregulated pathways, as well as clinical trials assessing the impact of glutamine supplementation on immune function and clinical outcomes in children with SMA.

1.4.3 Specific Objective Three

This study provides important insights by profiling the transcriptome of blood-stage *P. falciparum* in pediatric patients with SMA. Through this analysis, the study uncovered 566 differentially expressed parasite genes: 499 of which were upregulated and 67 downregulated in children with SMA compared to non-SMA. These genes were found to encode mitochondrial and ribosomal proteins, DNA methyltransferases, proteasome subunits, and trafficking proteins, all of which play key roles in the parasite's rapid growth and replication within red blood cells. The study also showed accelerated transition of the parasite from the ring stage to the trophozoite stage in children with SMA, as well as the increased proportions of schizonts. These changes suggest that faster developmental progression may be linked to more frequent rupture and invasion of red blood cells, contributing to the extensive hemolysis that characterizes SMA. In addition, the study observed a higher male-to-female gametocyte ratio, which may reflect an adaptive strategy by the parasite to maximize reproductive success under the stress of a hostile host environment.

This research contributes to existing knowledge by identifying parasite genes and pathways that are active during blood-stage infection in children with severe disease. These include processes such as cell division, protein synthesis and degradation, RNA splicing, metabolic regulation, and

molecular transport, which were significantly enriched in the SMA group. Importantly, the study identified hub genes such as glutamine–tRNA ligase, ribosomal proteins, proteasome subunits, and trafficking complex proteins, which may serve as biomarkers for disease severity or as promising therapeutic targets. The findings have practical applications in the development of novel anti-malarial therapies. By identifying genes and pathways essential for parasite survival and virulence during the blood stage, the study highlights opportunities for targeted intervention. Therapies that disrupt mitochondrial function, protein synthesis, or the ubiquitin-proteasome system could impair the parasite's ability to grow and replicate, thereby reducing disease severity. Moreover, a combination of drugs that target multiple key pathways simultaneously may improve treatment outcomes and reduce the likelihood of drug resistance.

A wide range of stakeholders stand to benefit from this work. Researchers and scientists gain a clearer understanding of parasite biology and the pathogenesis of SMA. Clinicians may use these insights to inform diagnosis, prognosis, and case management. Public health agencies and policy-makers can apply these findings to refine strategies for malaria control and elimination. Ultimately, the primary beneficiaries are the children living in malaria-endemic regions, whose health outcomes could be significantly improved through the development of better diagnostic tools and treatments based on this research. In light of these contributions, the study proposes several directions for future research. First, the functional characterization of the top upregulated genes and hub genes, especially those with unknown functions, is needed to elucidate their roles in parasite biology and pathogenesis. Second, host-parasite co-expression studies are recommended to uncover interactions between the human immune system and the parasite during infection. Third, longitudinal transcriptomic analyses could provide valuable insight into how parasite gene expression changes throughout infection and in response to treatment. Finally, integrating transcriptomic data with other 'omics' datasets, such as proteomics and metabolomics, would provide a more comprehensive understanding of the regulatory networks that enable the parasite to adapt and thrive within the host.

CHAPTER TWO
LITERATURE REVIEW

2.1 *P. falciparum* Malaria Pathogenesis

Human malaria is an infectious disease caused by unicellular parasites of the *Plasmodium* genus (Miller *et al.*, 1994). Transmission occurs through the bite of an infected female *Anopheles* mosquito, inoculating the sporozoites into the host's bloodstream. The sporozoites migrate to the liver, where they invade hepatocytes, mature and undergo asexual reproduction, resulting in the production of merozoites (Miller *et al.*, 1994). Upon parasite-induced hepatocyte rupture, these merozoites are released into circulation, facilitating their invasion of RBCs (Langlois *et al.*, 2018). Merozoites recognize and attach to surface receptors on RBCs, reorient themselves and form a tight junction with the RBC membrane, facilitating the formation of a parasitophorous vacuole around the merozoite, separating it from the host cell cytoplasm (Cowman *et al.*, 2012). The merozoites grow and replicate (48-72 hours), rupture the RBCs, and are released into the bloodstream (asexual cycle). However, a fraction of merozoites differentiate into gametocytes which are taken up by a mosquito during a blood meal (sexual cycle) (Cowman *et al.*, 2012; Miller *et al.*, 2002). Clinical manifestations of malaria present during the asexual erythrocytic stage when merozoites invade and develop in RBCs (Cowman *et al.*, 2012; Miller *et al.*, 2002).

P. falciparum is the most virulent of the 5 *Plasmodium* species: *P. falciparum, P. vivax, P. malariae, P. knowlesi, and P. ovale*, accounting for the highest mortality (Perkins *et al.*, 2011; WHO, 2023). Clinical manifestations of falciparum malaria range from typical presentations such as fever, headaches, chills, vomiting, sweating and muscle pain to severe life-threatening complications including hypoglycemia, hyper-parasitaemia, hyperlactataemia, metabolic acidosis, kidney failure, respiratory distress, cerebral malaria and SMA (Cheaveau *et al.*, 2019; WHO, 2020). The aetiology of SMA is multi-faceted, and includes the destruction of infected and uninfected RBCs, dyserythropoiesis, bone marrow suppression, and the sequestration of RBCs in the spleen (Perkins *et al.*, 2011). SMA remains one of the major causes of morbidity and mortality in children under five years old in holoendemic *P. falciparum* transmission areas, such as western Kenya (Okoyo *et al.*, 2021; Paton *et al.*, 2021). These children are especially vulnerable due to naïve anti-malarial immunity (Akech *et al.*, 2019; Anyona *et al.*, 2021; Okoyo *et al.*, 2021) and rely on their innate immunity. NK cells are key mediators of innate immunity against malaria and facilitate the development of acquired immunity (Riley & Stewart, 2013; Ye *et al.*, 2018).

2.2 NK Cells in Malarial Infection

NK cells are the main lymphocyte population in innate immunity, accounting for 8-20% of circulating lymphocytes in humans (Cooper *et al.*, 2001). They are characterised by the expression of the cluster of differentiation (CD) 56 surface markers and the absence of the CD3 marker ($CD56^{+}$/ $CD3^{-}$). NK cells are further classified based on surface expression of CD56 and CD16 (FCγRIII) markers: $CD56^{dim}CD16^{+}$ subset (largely cytotoxic) and $CD56^{bright}CD16^{-}$ (mainly produce cytokines)(Björkström *et al.*, 2016; Cooper *et al.*, 2001). NK cells develop and mature in both the bone marrow and secondary lymphoid tissues. Naïve NK cells interact with target cells expressing MHC class I molecules via their receptors to learn what is "self" (NK cell licensing) (Tu *et al.*, 2016). However, they require no priming or prior activation to be cytotoxic (Scoville *et al.*, 2017). Mature functionally competent NK cells are released in the blood, spleen, liver and uterus and are attracted by chemokines to infected and/or inflamed tissues(Scoville *et al.*, 2017; Tu *et al.*, 2016; Yokoyama *et al.*, 2004).

Hepatic NK cells, consisting of both transient conventional NK cell subsets and resident NK cells, represent almost half the lymphocyte population within the liver (Mikulak *et al.*, 2019). These NK cells mediate pre-erythrocytic immunity against malaria by producing IFN-γ (Burrack *et al.*, 2019). Similarly, TNF-α production inhibits *P. falciparum* liver-stage development (Depinay *et al.*, 2011; Wang *et al.*, 2012b). NK cells in circulation directly recognise *P. falciparum* iRBCs and are one of the earliest sources of IFN-γ during infection (Artavanis-Tsakonas *et al.*, 2003a; Artavanis-Tsakonas & Riley, 2002; Korbel *et al.*, 2004). Decreased IFN-γ production has been associated with increased malarial anaemia severity and high-density parasitaemia in paediatric patients (Oyegue-Liabagui *et al.*, 2017). Timely IFN-γ production controls *P. falciparum* infection in both hepatic and erythrocytic stages and prevents the development of severe malaria, while aberrant expression can lead to pathology (King & Lamb, 2015).

NK cells also secrete chemokines such as migration inhibitory factor and C-C motif Ligand 5 (CCL5, RANTES), which are suppressed in children with SMA (Awandare *et al.*, 2006; Fauriat *et al.*, 2010; Were *et al.*, 2006). Additionally, NK cells release cytotoxic granules containing perforin and granzymes, initiating the eryptosis of the iRBCs (Burrack *et al.*, 2019; Voskoboinik *et al.*, 2015). Granzyme B was associated with reduced parasitaemia (Kapelski *et al.*, 2015). The significance of NK cells in adaptive malarial immunity is underscored by their ability to activate adaptive immune cells through cytokine production, recall pro-inflammatory cytokine-mediated and receptor-mediated activation through memory-like NK cells, and orchestrate antibody-dependent cellular cytotoxicity (ADCC) through adaptive NK cells (defined by the loss of

transcription factor PLZF and Fc receptor γ-chain) (Arora *et al.*, 2018; Hart *et al.*, 2019; Odera *et al.*, 2023; Pahl *et al.*, 2018; Ty *et al.*, 2023).

P. falciparum-infected RBCs induce the up-regulation of activation markers, CD25 and CD69, on NK cells (Baratin *et al.*, 2005). NK cells can discriminate between iRBCs and uninfected RBCs and form stable contacts with iRBCs (Artavanis-Tsakonas *et al.*, 2003b; Chen *et al.*, 2014). They recognise iRBCs through a combination of activating and inhibitory receptors, i.e., inhibitory receptors detect the downregulation of MHC-1 molecules on iRBCs, reducing the inhibitory signal of the NK cell, while the activating receptors bind directly to pathogen-derived or stress-induced ligands on RBCs, transducing activating signals triggering NK cell activation (Lanier, 2008). NK cell depletion leads to a significant increase in *P. falciparum* parasitaemia in murine models (Chen *et al.*, 2014). Despite these elaborate effector functions of NK cells in *P. falciparum*, their role in SMA in paediatric residents in a holoendemic malaria transmission area, such as western Kenya, remains undetermined.

2.2.1 The Role of Natural Cytotoxicity-Triggering Receptor 3 in *P. falciparum* Malaria

Natural cytotoxicity-triggering receptor 3 (NCR3, also NKp30) is one of the main activating receptors of NK cells. It is encoded by the NCR3 gene (*NCR3*) and is expressed constitutively on the surface of NK cells (Moretta *et al.*, 2001). During *P. falciparum* infection, NCR3 directly recognizes iRBCs by binding to the DBL1-α domain of the parasite-derived surface protein, *Pf*EMP-1, triggering the activation of NK cells(Artavanis-Tsakonas *et al.*, 2003a; Korbel *et al.*, 2004; Mavoungou *et al.*, 2007; Wang *et al.*, 2012a). Additionally, *NCR3* is highly upregulated during controlled human malaria and *NCR3* expression was associated with NK cell activation (Walk & Sauerwein, 2019). Activated NK cells produce cytokines and chemokines known to influence the pathogenesis of SMA: IFN-γ, TNF-α, G-CSF, MIP-1α/β, and RANTES (Baratin *et al.*, 2007; Fauriat *et al.*, 2010; Perkins *et al.*, 2011). In addition, activated NK cells release granzymes and perforins for the cytotoxicity of iRBCs (Artavanis-Tsakonas *et al.*, 2003a; Korbel *et al.*, 2005).

The selective pressure of malaria on the human genome is illustrated by a high frequency of the sickle haemoglobin (HbS) allele of haemoglobin beta gene (*HBB*), for which heterozygotes are protected against severe malaria in areas of high malaria prevalence (Flint *et al.*, 1998; Kwiatkowski, 2005; Tishkoff & Williams, 2002). Previous studies in western Kenya illustrate that genetic variants, especially in innate immune response genes, influence susceptibility to malaria and the subsequent development of SMA(Achieng' *et al.*, 2019b; Awandare *et al.*, 2009; Kisia *et al.*, 2019; Munde *et al.*, 2017; Okeyo *et al.*, 2013; Ong'echa *et al.*, 2011; Ouma *et al.*, 2008b). Furthermore, studies in different ethnic groups from Africa have linked the 6p21.3 locus

of the MHC region and susceptibility to mild malaria (Brisebarre *et al.*, 2014; Flori *et al.*, 2003; Jepson *et al.*, 1997). Centrally located in the MHC region is *NCR3* (Brisebarre *et al.*, 2014; Flori *et al.*, 2003).

A single nucleotide polymorphism (SNP) located in the promoter region of *NCR3*, rs2736191:C>G, has been associated with increased mild malaria episodes in carriers of the mutant allele in the Congo and Burkina Faso (Baaklini *et al.*, 2017; Delahaye *et al.*, 2007). Furthermore, carriage of the mutant allele was associated with decreased expression of *NCR3* and reduced affinity of the *NCR3* promoter to transcription factors, suggesting impaired NK cell function (Baaklini *et al.*, 2017). It is important to note that these studies defined the SNP as a G to C transition, in contrast to conventional NCBI nomenclature used in our study i.e., C to G. Research conducted on the rs2736191 polymorphism in a Senegalese population revealed no relationship between the rs2736191 SNP and cerebral malaria.

Both rs2736191 and another *NCR3* variant rs11575837:C>T (located in the non-coding region of exon 1) are inversely associated with risk of primary Sjögren's syndrome (pSS), and the minor T allele of rs11575837 is associated with reduced *NCR3* transcription (Rusakiewicz *et al.*, 2013). Currently, no literature links rs2736191 or rs11575837 to SMA in immune-naïve children in holoendemic *P. falciparum* malaria transmission areas. Therefore, to extend previous studies on the potential role of NK cells in the pathogenesis of malaria, the current study examined the relationship between *NCR3* variants (rs2736191:C>G and rs11575837:C>T) and longitudinal clinical outcomes (i.e., malaria, SMA episodes and mortality) in a cohort of children residing in western Kenya.

2.2.2 NK Cell Networks

Elevated expression of the activating receptor NCR3, observed in controlled human malaria infections, results in the up-regulation of CD69 (NK cell activation marker), as well as increased production of IFN-γ and granzyme B (Walk & Sauerwein, 2019). Apart from NCR3, NK cells express a plethora of other activating receptors, i.e., NCR1 (NKp46), NCR2 (NKp44), NKG2D, CD226 (DNAM-1), and CD244 (2B4) (Pegram *et al.*, 2011). These receptors recognize pathogen-associated ligands on target cells and transduce activating signals into the cytosol via adapter molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs). These ITAM-bearing adaptor molecules include Fc Receptor γ-Chain (FCRγ), Cluster of Differentiation 3 zeta-chain (CD3ζ), and DNAX activation protein of 12kDa (DAP12) (Arnon *et al.*, 2006; Barrow *et al.*, 2019; Kaifu *et al.*, 2011). Following ligand binding, ITAM-containing adapter molecules are phosphorylated by membrane-anchored enzymes, SH2 domain-containing kinases

Syk or ZAP70, initiating downstream signalling cascades leading to NK activation, cytotoxicity and cytokine production (Dushek *et al.*, 2012).

Activating signals can also be either antibody- or cytokine-mediated (Stevenson & Riley, 2004). ADCC has been reported, whereby fragment crystallisable gamma receptor [FcγRIIIa (CD16)] on NK cells recognises immunoglobulin G (IgG) bound to iRBCs via *Pf*EMP-1 or repetitive interspersed family (RIFINS), triggering the release of perforins and granzymes, as well as production of IFN-γ (Arora *et al.*, 2018; Sulica *et al.*, 1993). In addition, ADCC of *P. falciparum* iRBCs by NK cells requires a stable adhesion involving an interaction of integrin αMβ2 and receptor CD2 on NK cells, with ICAM-4 and CD58 on iRBCs, respectively (Sekar *et al.*, 2023). NK cells also express cytokine receptors on their surface (Meazza *et al.*, 2011). Signalling through receptors for IL-2 and IL-15 mediates NK cell proliferation, differentiation, and cytotoxicity (Becknell & Caligiuri, 2005). Moreover, IL-18 signalling synergises with IL-2, IL-15, and IL-21 signalling at low cytokine concentrations, enhancing NK cell effector functions (Nielsen *et al.*, 2016). In *P. falciparum* malaria, NK cells respond to the inflammatory cytokines IL-12 and IL-18 by producing IFN-γ (Artavanis-Tsakonas & Riley, 2002). Furthermore, a cellular crosstalk between IL-2-secreting $CD4^+$ T and NK cells has also been demonstrated, activating NK cells to produce IFN-γ against iRBCs (Horowitz *et al.*, 2010).

Conversely, NK cells express a range of inhibitory receptors, such as killer-cell immunoglobulin-like receptors (KIRs), which detect the downregulation of self-markers. KIRs specifically recognize MHC class I molecules, including HLA-A, -B, and -C, and suppress NK cell activation to prevent aberrant immune responses (Kärre, 2008; Tukwasibwe *et al.*, 2020). Unlike activating receptors, inhibitory receptors have intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail for signal transduction (Burshtyn *et al.*, 1996). These receptors function to regulate NK cell activation by dephosphorylating tyrosine residues in ITAMs associated with activating signalling (Gunturi *et al.*, 2004; Lanier, 1997; Tukwasibwe *et al.*, 2020).

Korbel *et al* demonstrated that NK cell response to microbial ligands was dependent on the strength of the activating signal and the regulatory effect of inhibitory receptor KIR (Korbel *et al.*, 2009). This finding suggests that NK cell activation against pathogens could be influenced by both activating signalling and the presence/absence of inhibitory signals. During *P. falciparum* infection, NK cells' inhibitory receptors detect downregulation of MHC-1 molecules on iRBCs, dampening the inhibitory signal of the NK cell. Meanwhile, the activating receptors directly bind to pathogen-derived iRBCs, transducing activating signals that trigger NK cell effector functions (Artavanis-Tsakonas *et al.*, 2003a; Chen *et al.*, 2014; Lanier, 2008).

Therefore, despite the transduction of NK cell-activating signals through contact- or cytokine-mediated signalling, these signals must still overcome the NK cell inhibitory signals for NK cell activation to occur. Notably, NK cells express co-stimulatory receptors that potentiate NK cell activating signalling, to enable them to surpass a critical threshold of activation necessary to overcome the inhibitory signals (Moretta *et al.*, 2002).

SMA displays a dysregulated innate immune response, characterised by pathway perturbations resulting in an imbalance of inflammatory mediators of innate immunity (Perkins *et al.*, 2011). Since NK cell function is regulated by a delicate balance of activating and inhibitory signalling (Lanier, 2008), it is unclear whether this dynamic equilibrium is disrupted in SMA, potentially contributing to the dysregulated innate immune response. However, it has been reported that the overproduction of pro-inflammatory mediators by NK cells can exacerbate the innate immune response, leading to immunopathology (Wolf *et al.*, 2017). Furthermore, despite the crucial role played by NK cells in innate immunity, the signalling pathways of NK cells in SMA are yet to be elucidated. As such, this study aimed to determine the influence of NK cell networks and other immune signalling pathways on the pathogenesis of paediatric SMA by identifying the top-ranked genes/gene pathways for the entire transcriptome.

2.2.3 Dysregulated NK Cell Function in Malaria

Altered NK cell function has been linked to immunopathology in diverse conditions(Forconi *et al.*, 2018; Ty *et al.*, 2023), indicating that NK cell dysregulation can contribute to disease pathogenesis, and deciphering the immune signalling pathways may uncover molecular mechanisms underlying disease development. During the disease process of severe malaria (CM), NK cells are recruited to the brain of malaria-infected mice, where they induce CM by stimulating the migration of T lymphocytes to inflammation sites within the brain, underscoring the role of NK cell response in severe malaria pathogenesis (Hansen *et al.*, 2007). A longitudinal study conducted on malaria-exposed children residing in a malaria-endemic area of Mali showed a significantly reduced NK cell degranulation response to *P. falciparum* iRBCs compared to malaria-naïve individuals (Kouriba *et al.*, 2023). In another study in Tanzania, NK cells from *P. falciparum* seronegative individuals exhibited significantly reduced innate IFN-γ response to iRBCs compared to those from seropositive individuals due to the absence of malaria- specific antibodies for ADCC and lack of priming from accessory and T cells (Obiero *et al.*, 2015). Taken together, these observations suggest that repeated exposure to malaria may result in lowered NK cell response, potentially as a mechanism to prevent an exacerbated inflammatory response.

The proportion of the total NK cell population ($CD56^{+}/CD3^{-}$) was reported to be higher in patients with severe malaria than in those with acute malaria in a malaria-endemic region of Columbia. Specifically, $CD56^{bright}$ (mainly mediators of cytokine response) and $CD56^{dim}$ (mostly mediators of cytotoxicity) were elevated, with the $CD56^{dim}$ subset being 4-fold higher than the $CD56^{bright}$ subset. Similarly, NK cell production of cytokines IFN-γ and TNF-α was significantly higher in severe malaria patients. These findings suggest that, in severe malaria, increased NK cell cytotoxicity may help suppress parasitaemia, but prolonged production of pro-inflammatory cytokines could contribute to disease pathology (Agudelo *et al.*, 2012). These findings agree with previous studies that SMA children have lower parasitaemia relative to non-SMA but an elevated production of IFN-γ and TNF-α (Perkins *et al.*, 2011).

Malian children displayed upregulation of the programmed death-1 (PD-1) inhibitory receptor on NK cells and increased IL-6 production during acute malaria. Similarly, NK cells of malaria-naïve adults upregulated PD-1 following *P. falciparum* stimulation *in vitro* (Moebius *et al.*, 2020). In acute infection, this PD-1 upregulation likely acts as a regulatory constraint to prevent excessive NK cell activation, leading to reduced natural cytotoxicity against stressed or infected self-cells (Moebius *et al.*, 2020). In the context of chronic immune activation from repeated malaria exposure, PD- 1 expression on NK cells remains associated with diminished natural cytotoxicity; however, these cells appear to shift toward enhanced ADCC (Moebius *et al.*, 2020). Collectively, despite evidence indicating altered NK cell function in malaria, it remains unclear whether dysregulation of the finely tuned regulation and complex integration of NK cell signalling pathways occurs in immune-naïve children presenting with SMA. Furthermore, transcriptomic analysis has been suggested as a method to comprehensively characterise complex biological networks and pathways involved in malaria, such as those involving NK cells (Lee *et al.*, 2018a).

2.3 Genome-wide Expression Analysis in Malaria

2.3.1 Host Gene Expression Analysis

Dysregulation in immune response pathways has been studied using transcriptomic analysis in various conditions to gain insights into mechanisms underlying disease pathogenesis and clinical manifestations(Gallego *et al.*, 2024; Zhong *et al.*, 2024). For instance, network analysis of transcriptome data revealed key cellular networks involved in the progression of systemic lupus erythematosus (Wang *et al.*, 2024). Single-cell RNA-seq analysis revealed altered immune cell expression profiles, including NK cells, in acute *P. falciparum* malaria, and activation of type I IFN signalling in NK cells in response to malaria (Dooley *et al.*, 2023). Transcriptional analysis of blood samples from a longitudinal cohort of malaria-exposed Ugandan children showed

transcriptional features of exhausted adaptive-like NK cells, which were observed to increase in children repeatedly exposed to malaria. These cells expressed higher levels of inhibitory receptors (KIRs, LILRB1, and LAG-3), as well as enhanced ADCC capacity, and were associated with reduced parasite densities and malarial symptoms longitudinally (Ty *et al.*, 2023).

Upon conducting transcriptomic analysis on peripheral blood mononuclear cells of 13 pediatric malaria patients (7 with CM and 8 with UM) using a microarray platform, Cabantous *et al.* identified several top significant pathways, including the malaria pathway (hsa05144), cytokine-cytokine receptor interaction (hsa04060), TNF signalling pathways (hsa04668), chemokine signalling pathways (hsa04062), as well as the NOD-like receptor signalling pathway (has04621) which highlights the key role of the inflammasome in CM development (Cabantous *et al.*, 2020). Another transcriptome study of microarray data from CM patients showed aberrant signalling, neutrophil degranulation, and gene repression as the over-represented pathways in CM (Karikari *et al.*, 2021). Moreover, gene expression profiling of microarray data from CM patients in Senegal identified pathways involved in immune cell activation, including NK cells. Specifically, pathways such as TLR, FcεRI, FcγR, and RIG-I-like receptor signalling, as well as the natural killer cell-mediated cytotoxicity pathway, were found to be enriched (Thiam *et al.*, 2019).

Other previous transcriptomic studies conducted in cases of severe malaria defined as overlapping clinical manifestations i.e., respiratory distress, anaemia (Hb<5 g/dL), hypoglycaemia, and lactataemia (WHO, 2000), revealed overrepresentation of complement and TLR pathways with C1q, TLR-2, TLR-4, TLR-8, and CR1 genes being differentially expressed between children with severe and UM in Mali (Sobota *et al.*, 2016). Another study reported diminished NK cell immune response in severe malaria patients relative to those with UM. Functional network analysis showed that the top 10 pathways were primarily related to immune responses and pathogen recognition, with IFN signalling ranking as the top pathway (Ye *et al.*, 2018). Toll-like receptors and the RIG-I-like receptor pathways emerged as the primary pattern-recognition receptor pathways implicated in NK cell interactions with iRBCs (Ye *et al.*, 2018). While transcriptome profiling has proven crucial in elucidating molecular mechanisms in *P. falciparum* malaria infections, the cellular networks and pathways explicitly associated with SMA as the primary manifestation of severe malaria remain unexplored. Therefore, high-throughput next-generation RNA sequencing of the entire expressed transcriptome in whole blood was employed to investigate the NK cell networks and other cellular pathways in immune-naïve children presenting with SMA in a holoendemic malaria transmission region.

2.3.2 Parasite Transcriptome Analysis

Previous studies have utilised transcriptomic analysis to investigate gene expression patterns during the complex life cycle of the *P. falciparum* parasite. Notably, assessing *P. falciparum* gene expression *in vivo* is crucial to capturing variations that might not be observed under the standardised conditions utilised to culture the parasite *in vitro* (Lee *et al.*, 2018a). Gene expression analysis of whole blood samples from Malian children with UM enabled the identification of DEGs for *P. falciparum* phospholipase A2, Alba 2, and glyceraldehyde-3-phosphate dehydrogenase, with no significant enrichment of parasite-related pathways revealed (Bradwell *et al.*, 2020). However, in a group of naïve (first-time infected) adult patients, the base excision repair pathway, which facilitates the maintenance of genome integrity by repairing small base lesions in DNA, was the most enriched (Wichers *et al.*, 2021).

Analysis of transcriptomes of *P. falciparum* isolates from patients with severe malaria (not including SMA) showed that up-regulated genes were enriched in glycolysis, histone methylation, folate metabolism and protein folding pathways. In contrast, down-regulated genes were enriched in pathways associated with the tricarboxylic acid (TCA) cycle, nucleoside diphosphate (pyrimidine) metabolism and regulation of guanosine triphosphatase (GTPase) (Tonkin-Hill *et al.*, 2018). RNA-seq data analysis also found glycophorin binding protein (PF3D7_1016300) as the most highly expressed, while AP2 domain transcription factor AP2-G (PF3D7_1222600) was the most down-regulated in children with CM in Gambia(Lee *et al.*, 2018b). Additionally, GO enrichment analysis identified terms including protein translation, RNA splicing and processing, protein deneddylation and regulation of nucleobase-containing compounds (Lee *et al.*, 2018b).

Another study conducted on whole blood samples from children with CM in Benin identified dysregulated pathways related to DNA metabolic processes, protein metabolic processes, organelle organisation and entry into the host, as well as KEGG pathways involved in vitamin B6, pyruvate metabolism and glycolysis/gluconeogenesis (Guillochon *et al.*, 2022). Further analysis involving protein-protein interaction (PPI) network construction of DEGs uncovered sub-networks related to peptide biosynthetic process, host entry, biological adhesion and protein phosphorylation (Guillochon *et al.*, 2022). These studies highlight the utility of transcription profiling in examining gene expression patterns of *P. falciparum* asexual blood stages, uncovering key molecular mechanisms driving disease progression to severe malaria. Surprisingly, the profiling of the parasite transcriptome in SMA, one of the most prevalent manifestations of severe malaria in high *P. falciparum* transmission areas, remains to be determined.

Profiling the transcriptome of blood-stage *P. falciparum* has been of growing interest since clinical manifestations of malaria primarily arise from the *P. falciparum's* asexual stages in blood (Bradwell *et al.*, 2020; Cowman *et al.*, 2012; Guillochon *et al.*, 2022; Lee *et al.*, 2018a; Miller *et al.*, 2002; Painter *et al.*, 2018; Tonkin-Hill *et al.*, 2018). These stages have been demonstrated to be highly transcriptionally active and tightly regulated (Bozdech *et al.*, 2003; Painter *et al.*, 2007). Therefore, understanding the gene expression dynamics of the parasite during this stage of infection can provide insights into the molecular basis of parasite factors, i.e., parasite virulence and host immune evasion in malaria. Moreover, *P. falciparum* pre-erythrocytic antigens have been selected as vaccine candidates based on the analysis of transcriptome databases (Aguiar *et al.*, 2015). In addition, PPI networks have been utilised to predict potential drug targets for both pathogen- and host-directed malaria drug discoveries (Agamah *et al.*, 2021). Taken together, to decipher the blood stage parasite's molecular mechanisms driving severe malaria and identify potential immune-related biomarkers for disease prognosis and therapeautic targets, the current study profiled the transcriptome of *P. falciparum* asexual blood stages in children with SMA.

CHAPTER THREE
METHODOLOGY

3.1 Study Area

The study was conducted at Siaya County Referral Hospital (SCRH) in western Kenya (**Figure 3.1**), a holoendemic *P. falciparum* malaria transmission region (Hollowell *et al.*, 2023; Okoyo *et al.*, 2021; Ong'echa *et al.*, 2006). Malaria is the primary cause for admission in western Kenya, accounting for 63.4% of admissions and 61 deaths for every 1,000 malaria admissions among children under 5 years old(Kapesa *et al.*, 2018). The common malaria vectors in this region are *Anopheles gambiae s.s., A. arabiensis,* and *A. funestus* (Minakawa *et al.*, 2012). The area is mainly inhabited by the Luo ethnic group (94.8%) (Wiesmann, 2016), providing a homogeneous population for a genetic-based study. Since malaria is ubiquitous, all ages are at risk of exposure, but the paediatric population is especially susceptible to malaria as they are yet to develop a robust immunity to malaria(Hollowell *et al.*, 2023; Stich *et al.*, 2006). Severe malaria is predominant in paediatrics and majorly manifests as SMA and/or HDP (Akech *et al.*, 2019; Bloland *et al.*, 1999; Obonyo *et al.*, 2007). Moreover, SMA is one of the major causes of paediatric mortality and morbidity in the study region (Perkins *et al.*, 2011). Siaya County has an equatorial climate, 1,140 to 1,430 m above sea level, with an annual rainfall between 800-2,000 mm and average annual temperatures ranging from 15 ℃ to 30 ℃ (Ong'echa *et al.*, 2006). The population is 993,183 and children (0-14 years) are 403,845 (38.1%) (DNMP. & ICF., 2021)

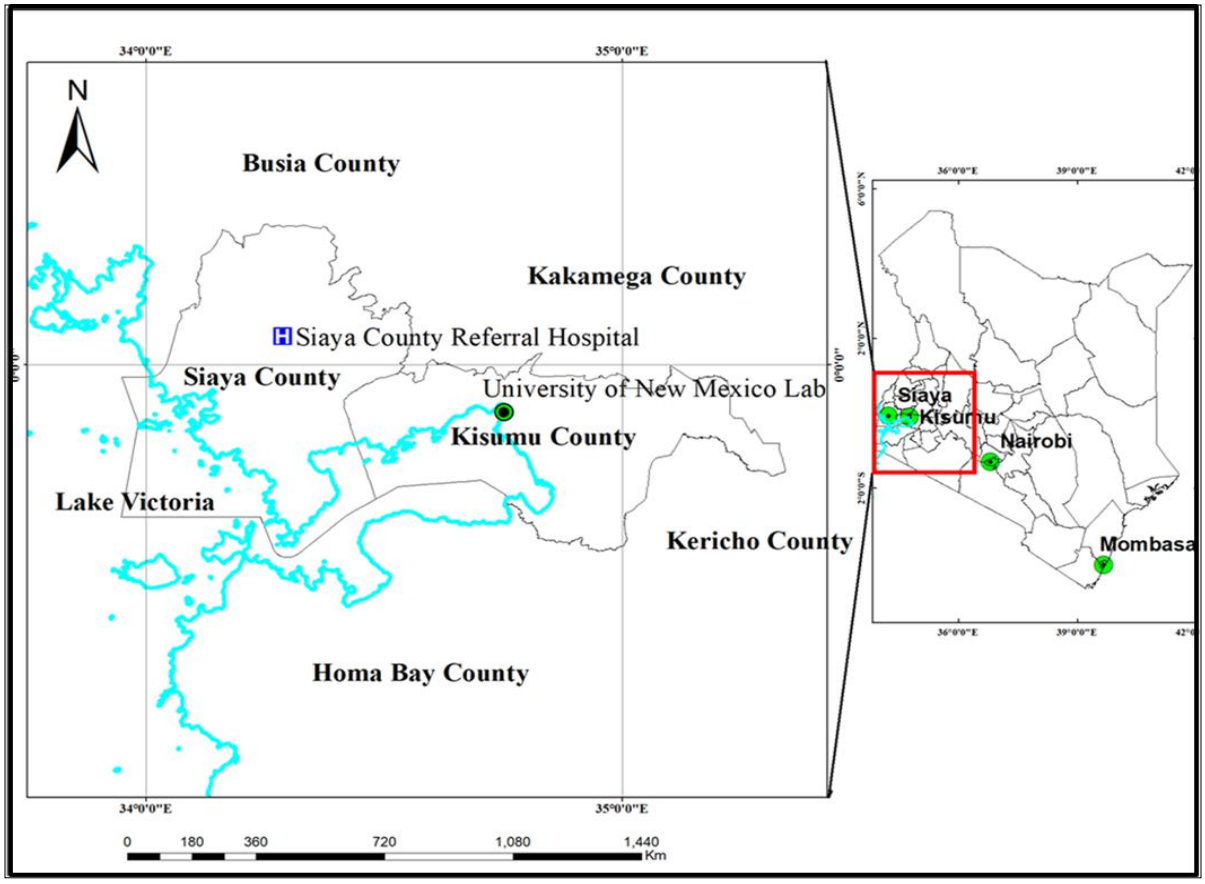


Figure 3.1: Location of Siaya County and the Siaya County Referral Hospital in Western Kenya

3.2 Study Design

3.2.1 *NCR3* Variant Analysis

3.2.1.1 Study Design and Participants

A longitudinal study design was employed to investigate the relationship between NCR3 variants and susceptibility to malaria outcomes (**Figure 3.2**). A pediatric cohort (n=1,640) of both sexes presenting at SCRH who either reported for their first documented hospital visit for febrile episodes or routine childhood vaccinations was enrolled in the study from March 2004 to December 2012 and followed for 36 months. (>19,000 visits) (**Figure 3.2**). Parasitaemic (cases) and aparasitaemic (controls) children were enrolled at baseline (day 0) and followed prospectively over three years to capture longitudinal clinical outcomes, including malaria episodes, severe malarial anemia (SMA), and mortality.

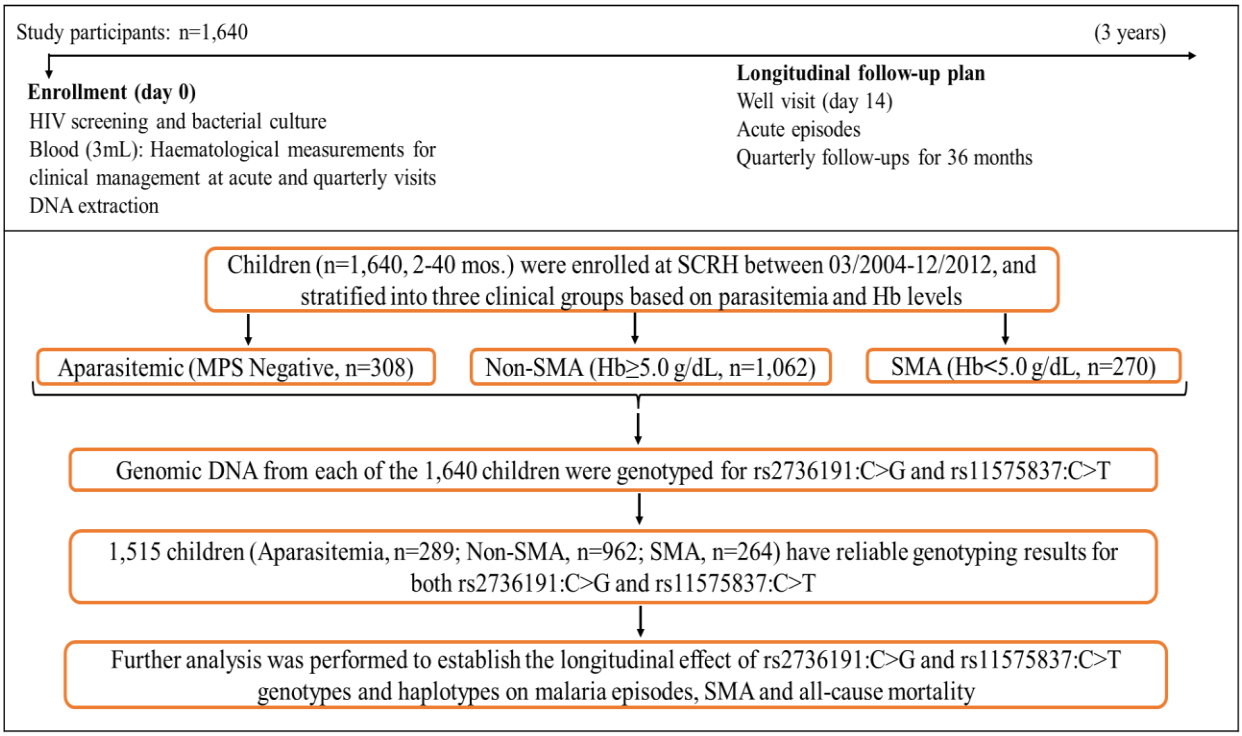


Figure 3.2: Study design: Objective one.

3.2.1.2 Screening and Enrollment

The recruitment process involved screening and enrollment (**Figure 3.2**). Cases were defined as children with severe malarial anaemia (positive smear for asexual *P. falciparum* parasitaemia and haemoglobin <5.0 g/dL) based on the global WHO definition of SMA (WHO, 2014), whereas controls were children of similar age and gender with non-severe malarial anaemia (positive blood smear for asexual *P. falciparum* parasitaemia and haemoglobin ≥5.0 g/dL). The following features determined the presence of parasitaemia: a positive blood smear showing asexual *P. falciparum* malaria and oral/axillary temperature >37.5 °C or two of the following: nausea/vomiting, diarrhoea, headache, myalgia, poor feeding, etc. Asexual malaria parasites were counted against 300 leukocytes on thick blood smears, and parasite densities were estimated by assuming a standard leukocyte count of 8,000 white blood cells per microliter of blood, when actual WBC counts are not available.

A comprehensive explanation of the study was given to the parent/legally authorised representative of a child before signing an informed consent form. The informed consent in the language of choice (i.e., English, Kiswahili, or Dholuo) was obtained from the parents or legally authorised representatives of all children participating in the study. Moreover, questionnaires were used to collect demographic information during enrollment.

Venous blood samples were collected before any treatment or supportive care as part of routine clinical evaluation for hematological measurements, HIV testing, and bacterial culture. These procedures were conducted per standard patient care practices. Based on the HIV-1 status, malaria parasitemia, and hemoglobin levels, children meeting all inclusion criteria were subsequently enrolled in the study. All participants received prompt antimalarial treatment and supportive therapy according to the Kenya Ministry of Health (MoH) guidelines.

3.2.1.3 Inclusion and Exclusion Criteria

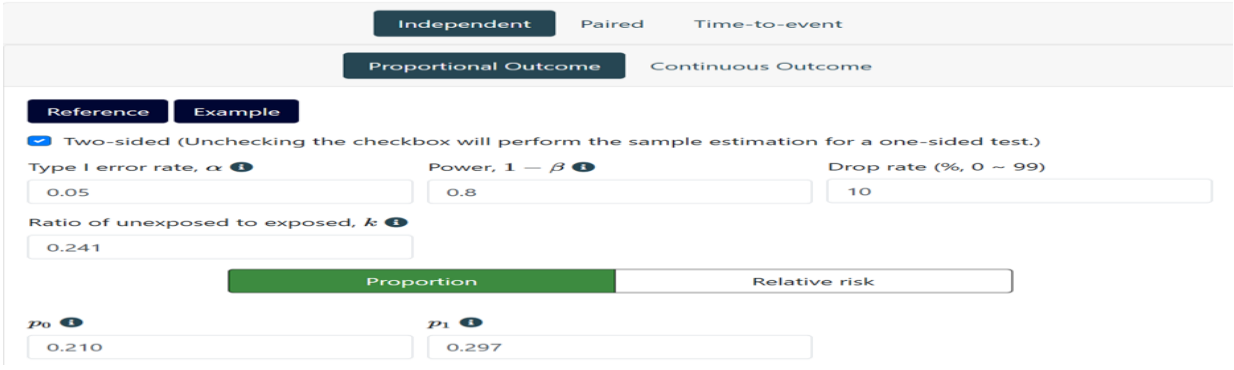
Inclusion criteria included: malaria parasitaemia (any density, SMA and non-SMA) and aparasitaemic controls; age ≤ 5 years; parent/legally authorised representative willing to sign the consent form; parent/legally authorised representative able to keep schedule and study appointments; parent/legally authorised representative living ≤ 15 km to the hospital, and ability to provide two contacts who are familiar with the child's whereabouts during the study period according to previously established protocols (Ong'echa *et al.*, 2006). Exclusion criteria included: children with cerebral malaria; history of any HIV-1 related symptoms such as oral thrush; clinical evidence of acute respiratory infection; prior hospitalisation; intent to relocate during the study period; and unwillingness of the parent or guardian to enrol the child in the study.

3.2.1.4 Longitudinal Follow-ups

Enrolled children were stratified into 3 clinical groups based on parasitaemia and Hb levels: aparasitaemic [Malaria parasites (MPS) negative], non-SMA (Hb≥5.0 g/dL), and SMA (Hb<5.0 g/dL). Following enrollment, parents/guardians were requested to bring their child to the hospital quarterly (every three mos.) throughout the longitudinal follow-up period (36 months) and during any acute febrile episode (**Figure 3.2**). A complete physical exam and clinical laboratory tests were performed at each quarterly and acute visit. All-cause mortality data were collected throughout the follow-up period. The geographic information coordinates of each child's address were recorded upon enrollment using a GIS/GPS surveillance system. For the participants who did not report for a scheduled follow-up visit, the health status of the child, which included a verbal autopsy in cases of mortality, was determined by a visit to the child's residence.

3.2.1.5 Sample Size Calculation

The sample size was calculated using a web server: https://riskcalc.org/samplesize/. A two-sided type I probability of 5%, with a power of 0.8, an attrition rate of 10%, and a 0.241 ratio of unexposed to exposed (Anyona *et al.*, 2020) will require a minimum sample size of 1,505 (**Figure 3.3**).



Sample size	
2-side significance level	0.05
Power (1-beta)	0.8
Ratio of sample size, unexposed/exposed	0.241
Probability of event in the unexposed group	0.21
Probability of event in the exposed group	0.297
Drop rate (%)	10

Result

	Fleiss	Fleiss with correction for continuity
Sample Size - Exposed	1147	1212
Sample Size - Unexposed	277	293
Total sample size	1424	1505

Figure 3.3: Sample size calculation for objective one.

3.2.1.6 Collection of Blood Samples

For children who met the inclusion criteria, blood samples (<100 µL) were obtained from heel or finger pricks and used to prepare thin and thick blood smears. The blood smears were then stained using Giemsa reagent for microscopic diagnosis of *P. falciparum* parasitaemia and determining parasite density. In addition, peripheral blood (<3.0 mL) was collected before anti-malarial treatment into EDTA BD Vacutainer® tubes (Becton, Dickinson and Co., Franklin Lakes, New Jersey, USA) for haematological measurements, HIV testing, bacterial culture, HbAS status, $\alpha^{3.7}$-thalassemia deletion, and G6PD deficiency. About 100 µL of the blood sample was blotted onto FTA Classic® cards (Whatman Inc., Clifton, New Jersey, USA), air dried, and stored with desiccants at room temperature for use in DNA extraction.

3.2.1.7 Laboratory Measurements

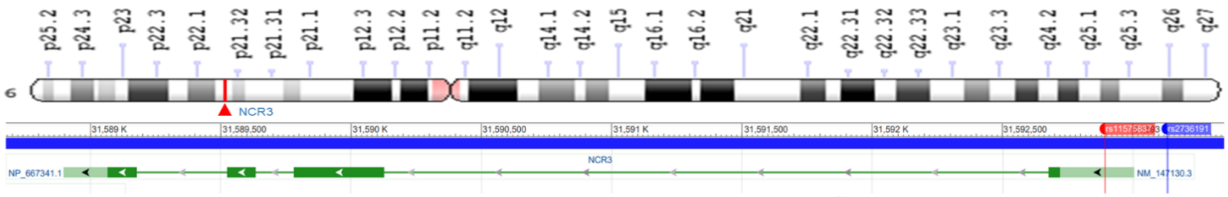
Hb levels and complete blood counts (CBCs) were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter Corporation, Miami, Florida, USA). To account for the common causes of severe anaemia in the study region, we evaluated anaemia-promoting conditions, including HIV-1 exposure/infection, bacteremia, sickle-cell trait (HbAS) status, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and $\alpha^{3.7}$-thalassemia deletion. HIV-1 exposure

was determined through rapid serological testing using Uni-gold™ specific for HIV-1 and Determine™ specific for HIV-1/2. Samples that yielded single or double positive results were subjected to confirmatory nested pro-viral DNA PCR testing as previously described (Otieno *et al.*, 2006). Pre- and post-test HIV counselling was provided to all parents/guardians of all the participants. Bacteraemia was detected by inoculating ~1.0 mL of venous blood into an automated BACTEC 9050 culture system (Becton-Dickinson, Franklin Lakes, New Jersey, USA) to detect bacterial growth. Positive alerts were then examined by Gram staining to classify the bacteria as Gram-positive or Gram-negative. To isolate and identify causative bacteria, cultures were plated on blood agar, chocolate agar, or MacConkey agar, depending on expected growth characteristics. Blood agar supports general bacterial growth and allows detection of hemolysis; chocolate agar is used for fastidious organisms; and MacConkey agar is selective for Gram-negative enterics and differentiates lactose fermenters. These culture methods enable accurate bacterial recovery and characterization, which is critical for confirming bacteraemia and informing clinical relevance (Were *et al.,* 2011). Bacteria grown from the cultures were then identified using API biochemical galleries (bioMérieux, Louvres, France), which apply a series of miniaturized biochemical tests to classify bacteria based on their metabolic characteristics. G6PD deficiency was assessed using a fluorescent spot test, a qualitative assay that measures the activity of the glucose-6-phosphate dehydrogenase enzyme. Blood samples were processed and examined under ultraviolet light, where fluorescence indicates normal enzyme activity (Trinity Biotech Plc., Bray, Ireland). The presence of the sickle cell trait (HbAS) was determined by cellulose acetate electrophoresis, a method that separates hemoglobin variants based on their charge and mobility, as per manufacturer's instructions (Helena Bio-Sciences, Oxford, United Kingdom). $\alpha^{3.7}$-Thalassaemia deletion was determined using a nested PCR method (Dodé *et al.,* 1993). This technique involves two successive rounds of PCR to enhance the specificity and sensitivity of detecting the 3.7-kilobase deletion in the alpha-globin gene cluster. The first round amplifies a broader region containing the target sequence, while the second round uses internal primers to selectively amplify the deletion-specific fragment, allowing precise identification of individuals carrying the $\alpha^{3.7}$-Thalassaemia mutation.

3.2.1.8 Selection of *NCR3* SNPs

The *NCR3* SNPs (rs2736191:C>G and rs11575837:C>T) were selected based on the following criteria: a) proximity to *NCR3* transcription start site (TSS); b) likelihood of corresponding SNP to potentially create or ablate transcription factor binding sites (TFBS), which was predicted using TFBIND software (Tsunoda & Takagi, 1999) and c) strong linkage disequilibrium (88%) between the SNPs determined using Multiallelic Interallelic Disequilibrium Analysis (MIDAS) software version 1.0 (Gaunt *et al.,* 2006) (**Figure 3.4)**.

A



B

Gene variants	Minor Allele Frequency		TFBS	
	dbSNP LWK	Study Population	Major allele	Minor allele
rs2736191:C>G	0.23	0.30	TFAP2A (AP2)	TCF3 ZBTB6
rs11575837:C>T	0.01	0.03	TFAP2A (AP2)	HNF4A

C

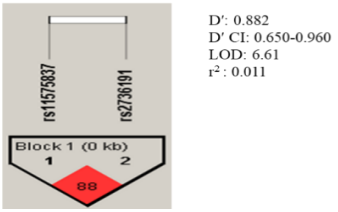


Figure 3.4: Selection of the NCR3 SNPs.
A) Chromosome location of NCR3 and the variants under investigation. B) Selection criteria for rs2736191:C>G and rs11575837:C>T variants. C) Linkage disequilibrium.

3.2.1.9 Genotyping of *NCR3* rs2736191: C>G and rs11575837:C>T Variants

Genomic DNA from each participant was extracted from blood spots on FTA Classic® cards using the Gentra System protocol (Gentra System Inc., Minneapolis, MN, USA). Primers specific to the human housekeeping gene encoding human glucose-3-phosphate dehydrogenase (*GAPDH*) were used in a conventional PCR reaction to confirm the presence of human DNA. The genomic DNA was then amplified using the GenomiPhi® system (GE Healthcare, Midland Park, New Jersey, USA) to obtain sufficient quantities for genotyping. The *NCR3* rs2736191 and rs11575837 polymorphisms were genotyped using the TaqMan® 5' allelic discrimination Assay-By-Design high-throughput method [Assay ID: C_16286876_10 for rs2736191 and C_27834902_10 for rs11575837] (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. PCR was conducted in a total reaction volume of 10 µL with the following amplification cycles: initial denaturation (60 °C for 30 s and 95 °C for 10 min) followed by 40 cycles of (95 °C for 15 s and 60 °C for 1 min) and a final extension (60 °C for 30 s) using allele-specific fluorescence on the StepOnePlus™ Real-Time PCR Systems. StepOne™ Software (Version 2.3) was used for allelic discrimination (Thermo Fisher Scientific).

3.2.1.10 Data Analysis

The SPSS® statistical software package version 23.0 (IBM SPSS Inc., Chicago, Illinois, USA) was used to analyze data at enrollment. Demographic and clinical data across study groups were compared using Pearson's Chi-square ($\chi 2$) test and the Kruskal-Wallis test. Differences in parasitological variables between SMA and non-SMA were computed using the Mann–Whitney U test. *NCR3* (rs2736191 and rs11575837) haplotypes were constructed using HPlus software version 2.5 (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). Moreover,

the proportions of alleles, genotypes and haplotypes of the *NCR3* variants were compared across the study groups using Chi-Square (χ^2), and the Hardy–Weinberg Equilibrium (HWE) was evaluated using a χ^2 goodness of fit test.

The relationship between *NCR3* genotypes/haplotypes and longitudinal malaria outcomes was analysed in R (version 3.6.1) by fitting a Poisson rate regression using a forward-backwards model selected using the Akaike Information Criterion (AIC). The following covariates were subject to model selection: age, sex, co-infections (HIV-1 and bacteremia), G6PD deficiency, sickle-cell trait and $\alpha^{3.7}$-thalassemia since these covariates could influence malarial anaemia (Aidoo *et al.*, 2002; Otieno *et al.*, 2006; Preuss *et al.*, 2012; Were *et al.*, 2011). Poisson regression was used to determine the incidence rate ratio (IRR). The Poisson regression model accounted for the varying lengths of observation by including the logarithm of the follow-up period as an offset. This offset was added to the model to adjust the logarithm of the expected number of events, ensuring that the model correctly estimated incidence rates (Poisson rate regression).

The Poisson rate regression was also performed to determine the IRR for the genotypes/haplotypes under an additive genetic model, rather than treating genotypes/haplotypes as categorical variables. In this model, the genotypes/haplotypes were coded metrically, representing the number of mutant alleles (0, 1, or 2 copies of the mutant allele or haplotype) carried by an individual. This approach allowed for the assumption of a cumulative effect of the mutant allele or haplotype on the malaria outcomes, where each additional copy was expected to incrementally modify the incidence rate. Survival analyses were performed by a non-parametric Cox proportional hazard model to determine the hazard ratio (HR) of all-cause mortality associated with *NCR3* genotypes/haplotypes over 36-month follow-up period. For each model, Holm-Bonferroni correction was applied to control familywise error rates in multiple comparisons. The statistical significance for all analyses was set at $P \leq 0.050$.

3.2.2 Transcriptomic Analysis

3.2.2.1 Study Design and Participants

A cross-sectional study design was utilised for the human host and parasite transcriptome analysis (**Figure 3.5**). The study participants were parasitaemic children (n=66, 1-59 mos.) presenting at SCRH for their first documented hospital visit due to febrile (>37.5 ℃) episodes, who were selected from 577 participants enrolled between 03/2017-09/2020 (**Figure 3.5**).

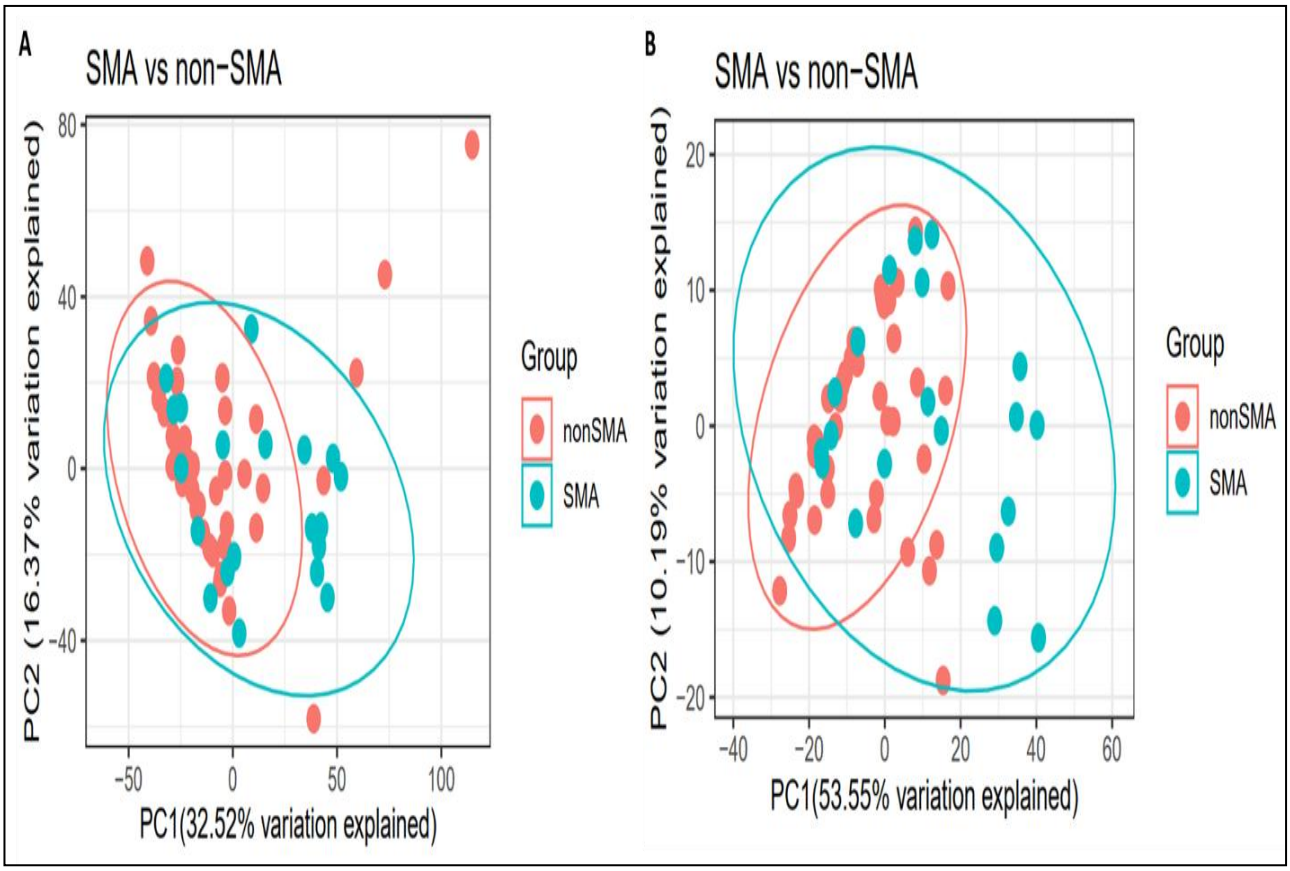


Figure 3.5: Principal component analysis of *P. falciparum* transcriptomics data.
The first two components of the principal component analysis of the TPM of differentially expressed genes in Kenyan children presenting with non-SMA and SMA. **A)** PCA of all 3,155 annotated P. falciparum genes. **B)** PCA of 566 DEGs. Each point represents a sample; red represents the non-SMA group, and green represents the SMA group. Confidence ellipses show group distribution and overlap.

3.2.2.2 Screening and Enrolment

The recruitment process followed a screening of malaria parasites and enrollment. Routinely, SMA is defined by WHO as Hb<5 g/dL (WHO, 2014). However, a previous study performed on a longitudinal cohort in Asembo Bay, western Kenya, recorded 14,317 repeated measurements, defined SMA as Hb<6.0 g/dL and non-SMA as Hb≥6.0 g/dL (McElroy *et al.*, 1999). To determine the local (Siaya, western Kenya) definition of SMA using a data-driven approach, data from a Siaya paediatric cohort (i.e., n=1,640 with >19,000 Hb measurements) was used to determine average Hb concentrations across 36 months of follow-up using a dynamic programming algorithm. The modelling revealed three distinct Hb categories at $p<0.001$: ≤5.9 g/dL, 5.91-8.098 g/dL, and >8.098 g/dL (Anyona *et al.*, 2023). Thus, study participants were stratified into non-SMA (Hb≥6.0 g/dL) and SMA (Hb<6.0 g/dL) groups based on the local

definition of SMA. Female and male (sex at birth) febrile children (≥37.5°C axillary) presenting at SCRH were enrolled in the study. At enrollment, demographic and clinical data were collected, and a physical examination was performed. Before treatment with antimalarials or other medications, venipuncture blood samples (3 mL) were collected for laboratory measures. Pre- and post-HIV test counselling was provided to the parents/guardians of all participants. All patients were treated as per the Ministry of Health-Kenya guidelines.

3.2.2.3 Inclusion and Exclusion Criteria

Inclusion criteria included: *P. falciparum* parasitemia (any density), febrile (≥37.5°C axillary), age <5 years, distance to hospital ≤25 km, written informed consent from parent/guardian and intention to attend a follow-up visit on day 14 (well-visit). Exclusion criteria included: previous hospitalization (for any reason), an episode of malaria within the past month, positive cases of HIV-1 and bacteraemia or clinical signs consistent with cerebral malaria (rare in this setting).

3.2.2.4 Sample Size Calculation

The sample size for Specific Objectives 2 and 3, which involved RNA sequencing and downstream analyses (e.g., differential expression and network/pathway analysis), was estimated using the RnaSeqSampleSize package (Zhao *et al.*, 2018) via a web interface https://cqsvumc.shinyapps.io/rnaseqsamplesizeweb/. Assuming 20,000 genes tested, with 500 prognostic genes, a minimum desired fold-change of 3, and 90% statistical power, the minimum required sample size was 44 total samples (22 per group) (**Figure 3.6**).

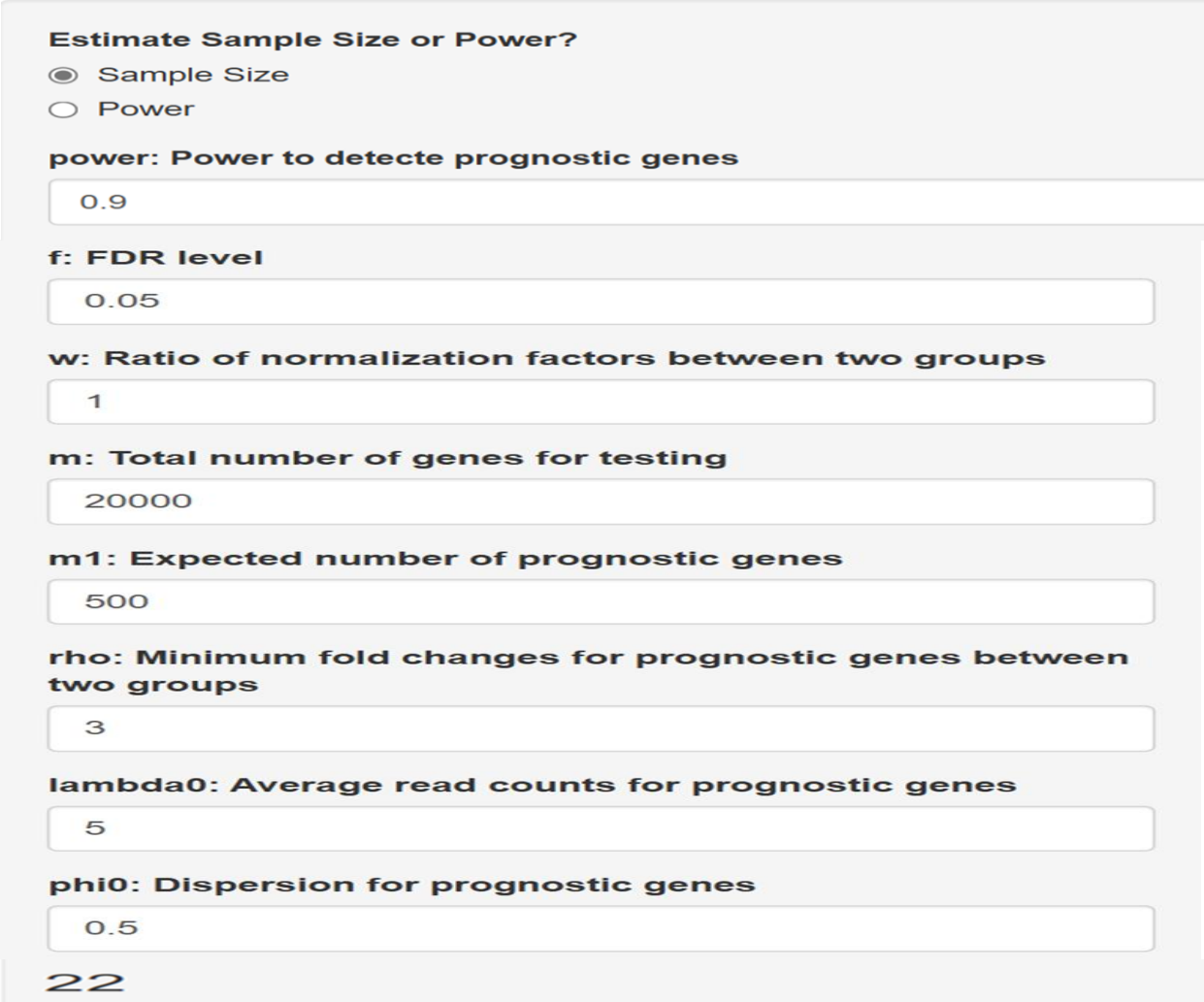


Figure 3.5: Sample size calculation for objective two.

3.2.2.5 Sample Collection and Laboratory Procedures

Venipuncture peripheral blood (3-4 mL) was obtained from each study participant who met the inclusion criteria for laboratory tests on day 0, before antimalarial therapy. Thick and thin Giemsa-stained blood smears were prepared and examined under 100x oil immersion microscopy to determine the presence/absence of *Plasmodium* parasites, and parasite density (Ong'echa *et al.*, 2006). CBCs were assessed using the Beckman Coulter ACT diff2™. To determine additional common causes of severe anaemia, HIV-1 infection, bacteraemia and HbAS status were determined as described above. An aliquot of peripheral blood (~500 µL) was mixed with an equal volume of Trizol® Reagent (Thermo Fisher Scientific) and stored at -80 °C.

3.2.2.6 RNA Isolation

Peripheral blood from study participants, matched for age, sex, and peripheral malaria parasitemia, was used to isolate total RNA for RNA sequencing. Total RNA was isolated using E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek Inc., Norcross, GA, USA) from the peripheral blood (500 µL) preserved in Trizol® for the 66 children who were selected for RNA sequencing (RNA-Seq): non-SMA (n=41) and SMA (n=25). Total RNA was then treated with RNase-free DNase I (New England Biolabs, Ipswich, MA, USA) to remove any contaminating DNA. RNA quantity was measured using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific), while the quantity and integrity of the RNA were evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

3.2.2.7 RNA Sequencing

3.2.2.7.1 Library Construction

A total of 1 µg of RNA [RNA integrity number (RIN) >8], subjected to a post-globin mRNA depletion step to remove globin transcripts after RNA extraction, was used as the input for library construction. The sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (New England BioLabs Inc., Massachusetts, USA), following the manufacturer's recommendations. Briefly, mRNA enrichment was performed using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesised using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Each cDNA was ligated to a NEBNext adaptor, followed by PCR enrichment of adaptor-ligated DNA. The PCR products were then purified, and the sequencing library quality was assessed using Agilent 2100 Bioanalyzer.

3.2.2.7.2 Clustering, Sequencing and Quality Control

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina Inc., San Diego, CA, USA), according to the manufacturer's instructions. Paired-end sequencing of library preparations was performed using Illumina® NovaSeq S4 platform (Illumina, California, USA) with a depth of >20 million high-quality mappable reads. Quality control and preprocessing of sequencing data (raw reads) in the FASTQ format were conducted using fastp (Chen *et al.*, 2018). Clean reads were obtained by trimming adapters, poly-N sequences, and low-quality bases from the raw data, resulting in high-quality sequences used for all downstream analyses. All the subsequent analyses were based on clean reads.

3.2.2.7.3 Mapping to Reference Genomes and Quantification

3.2.2.7.3.1 Mapping to the Human Reference Genome

Reference genome and gene model annotation files were downloaded from the genome browser websites (NCBI/UCSC/Ensembl). Paired-end clean reads were mapped to the reference genome (GRCh38.p13) using the Spliced Transcripts Alignment to a Reference (STAR) software version 2.5 (Dobin *et al.*, 2013). HTSeq v0.6.1 was used to count the read numbers mapped to each gene (Anders *et al.*, 2015). The read counts were converted to Fragments Per Kilobase of transcript per Million mapped reads (FPKM) based on the length of the gene. Differential gene expression analysis was then performed using the R package edgeR (3.16.5) (Robinson *et al.*, 2010). Each false discovery rate (FDR) adjusted *P*-value (*P*adj) was calculated using the Benjamini & Hochberg method (Benjamini & Hochberg, 1995) and *P*adj of 0.050 was set as the threshold for significance.

3.2.2.7.3.2 Mapping to the *pf*KE01 Reference Genome

A *P. falciparum* Kenyan isolate (*pf*KE01) reference genome (Otto *et al.*, 2018), downloaded from PlasmoDB, was used for read mapping. Transcriptome data were mapped to the *pf*KE01 references using Bowtie 2 (Langmead & Salzberg, 2012). There were 6 samples excluded from downstream analyses since they did not meet the quality threshold and/or read counts required for robust interpretation (1 non-SMA and 5 SMA). This yielded 60 samples for downstream analyses: non-SMA (Hb≥6.0 g/dL, n=40) and SMA (n=20). Mapped reads were assigned to genes with HTSeq v0.6.1 to count read numbers mapped to each gene (Anders *et al.*, 2015). The read counts were converted to Transcript per Million mapped reads (TPM) to account for gene length and sequencing depth. Differential gene expression between the SMA and non-SMA groups was then performed using the Welch t-test to account for unequal variances and differing sample sizes in the two groups. The threshold for significance was set at $P \leq 0.050$.

3.2.2.8 Data Analysis

3.2.2.8.1 Host Transcriptomics

Demographic, clinical and laboratory characteristics of the study participants at enrolment were analyzed using SPSS® version 23.0 (IBM SPSS Inc., Chicago, IL, USA). Data across the study groups were compared using Pearson's Chi-square (χ^2) test and the Mann-Whitney U test. Statistical significance was set at $P \leq 0.050$. A volcano plot was used to visualize the distribution of DEGs in GraphPad Prism version 9.0 (GraphPad Software, Inc., Boston, Massachusetts, USA). The relative percentage of different immune cell types/subtypes in peripheral blood was determined using deconvolution analysis with CIBERSORTx (Newman *et al.*, 2019; Steen *et al.*, 2020), an R package that processes gene expression data from a bulk admixture of various cell

types to estimate the abundance of each immune cell type in the sample (Steen *et al.*, 2020). Briefly, a curated signature matrix file, LM22, was used as a reference to deconvolute the relative fraction of different immune cell types in whole blood, identifying 22 types/subtypes of leukocytes. Cell type-specific gene expression levels were imputed at the sample level, with the output presented as the fractional proportions in whole blood for each study participant. A two-sample *t*-test with Welch's correction was used to determine whether the relative proportions (%) of immune cell types, presented as mean (SEM), differed between the non-SMA (n=41) and SMA (n=25) groups. GraphPad Prism version 9.0 (GraphPad Software) was employed to generate a heatmap, as well as the relative proportions (%) of the 22 leukocyte cell types/subtypes at the individual patient level for both the non-SMA and SMA groups.

Gene identifier, $\log_2$fold change and FDR-adjusted *P*-values ≤0.050 of significantly differentially expressed genes (DEGs) were incorporated into GeneGO (MetaCore™), a web-based knowledge database and software suite for pathway and network analysis (Thomson Reuters, New York, USA). To visualise the gene expression patterns, next-generation clustered heatmaps (NG-CHMs) of DEGs were generated using hierarchical clustering with Euclidean and Ward clustering distance, respectively (Ryan *et al.*, 2019). The BioRender web server https://app.biorender.com/ was utilised to construct a model to illustrate molecules involved in the transport and synthesis/catabolism of GLN in leukocytes, categorised as DEGs in SMA vs. non-SMA.

3.2.2.8.2 Parasite Transcriptomics

The demographic, clinical and laboratory characteristics of the study participants were analysed using SPSS® version 23.0 (IBM SPSS Inc., Chicago, IL, USA) at enrolment. The distribution of DEGs was visualised using a volcano plot in GraphPad Prism version 9.0 (GraphPad Software). Since there is no *Pf*KE01 reference database, the KE01 gene identifiers of DEGs were converted to those of *Pf*3D7 to isolate homologs for downstream analysis.

P. falciparum gene expression deconvolution analysis was conducted using CIBERSORTx (Newman *et al.*, 2019). Briefly, reference transcriptome profiles for the different *P. falciparum* developmental stages were obtained from a single-cell RNA-seq study (Tebben *et al.*, 2022) and were uploaded into CIBERSORTx. These include a signature matrix (i.e., a unique gene expression profile corresponding to each parasite developmental stage) and a single-cell RNA expression matrix reference file for batch correction. The signature matrix was used to impute the proportions of each parasite developmental stage in the bulk RNA-seq data of 566 DEGs in the 60 samples. Batch correction was performed in S-mode using the single-cell RNA reference file with 500 permutations to determine significance, correcting for sequencing differences

between single-cell and bulk datasets (Tebben *et al.*, 2022). The proportions of each parasite developmental stage in a sample were plotted using GraphPad Prism version 9.0 (GraphPad Software). The relative proportions of *P. falciparum* developmental stages in the non-SMA (n=40) and SMA (n=20) groups, as well as relative proportions of *P. falciparum* male and female gametocytes across all samples (n=60), were compared using a Mann-Whitney U test using SPSS® version 23.0.

Principal component analysis was conducted in R software version 4.4.1 using the 'prcomp()' function to reduce the high dimensionality of gene expression data, visualize patterns and identify clusters within the data. Weighted Gene Co-expression Network Analysis (WGCNA) of DEGs was performed using integrated Differential Expression and Pathway analysis (iDEP) version 2.0 (Ge *et al.*, 2018) with a soft threshold of 5 and a minimum module size of 20. The network files of the top 10 genes in the WGCNA entire network and sub-networks (modules) were generated with an edge threshold of 0.4 and exported to Cytoscape software (Szklarczyk *et al.*, 2019) for visualization and further analysis. Molecular Complex Detection (MCODE) version 2.0.3 (Bader & Hogue, 2003), a Cytoscape plug-in, was used to analyze the module networks to identify the highest scoring node in each module (seed node), i.e., most interconnected, based on the local neighbourhood density. Functional enrichment analysis was then performed for each WGCNA module using Cytoscape stringApp version 2.1.1(Doncheva *et al.*, 2019).

The protein-protein interaction (PPI) network of *P. falciparum* DEGs was generated using STRING Version 12 database by mapping parasite DEGs to PPI networks, setting the minimum required interaction score at 0.700 (high confidence) with the network viewed in Cytoscape software (Szklarczyk *et al.*, 2019). MCODE version 2.0.3 (Bader & Hogue, 2003), a Cytoscape plugin, was used to analyse the PPI network to identify highly interconnected regions (clusters) with a node size score cut-off standard of >4. The identified clusters were ranked based on the cluster's computed MCODE score. To explore the role of the generated clusters, a STRING functional enrichment analysis was performed and the results were plotted using the Scientific and Research plot (SRplot) tool (Tang *et al.*, 2023).

In addition, the hub gene of the entire PPI network, as well as that of each cluster, was selected using the CytoHubba plugin in Cytoscape which ranked nodes in the cluster networks through 11 topological analysis methods including Degree, Edge Percolated Component, Maximum Neighborhood Component, Density of Maximum Neighborhood Component, Maximal Clique Centrality and six centralities (Bottleneck, EcCentricity, Closeness, Radiality, Betweenness, and Stress) based on shortest paths. Briefly, scores from all 11 methods for a node were computed

and assigned to each node in the clusters. Next, the nodes were ranked considering their scores from all the scoring methods. The sub-graph of the hub nodes was extended to include nodes that directly interacted with these top-ranked nodes (Chin *et al.*, 2014). To analyse the associations between the identified hub genes and severity of disease (non-SMA vs. SMA), a logistic regression analysis was performed with several covariates: age, sex and sickle-cell genotypes in the model in SPSS® (version 23.0).

3.3 Ethical Considerations

The study was performed with ethical approval from the Maseno University Scientific and Ethics Review Committee (**MSU/DRPI/MUERC/00510/18**) and the Institutional Review Board of the University of New Mexico (17-445) (**Appendix 1**). Parents or legal guardians of all participants provided written informed consent prior to enrollment (**Appendix 2**).

3.3.1 Inclusion of Children as a Vulnerable Group

Children under 5 years of age were recruited for this study due to their disproportionate risk of developing severe malarial anemia (SMA), which warranted focused investigation. Including this vulnerable group was ethically justified as the research directly addressed a major cause of morbidity and mortality in this age range, intending to understand the effect of genetic variations and gene expression patterns on the pathogenesis of SMA and improve clinical outcomes.

3.3.2 Ethical Handling of Samples and Clinical Follow-up

To minimize risk and ensure dignity, blood collection (~3–4 mL per timepoint) was conducted by trained clinicians using sterile, single-use equipment. The volume of blood drawn was kept within pediatric safety limits based on age, weight, and anemia status. Children were followed clinically for up to 36 months. Those requiring hospitalization were managed by Ministry of Health staff, while non-hospitalized cases were monitored through scheduled and unscheduled follow-ups to track recovery and allow timely intervention. Strict confidentiality protocols were implemented. Each participant was assigned a unique study ID, with all clinical and laboratory data coded accordingly, complying with data security standards. Clinical and laboratory information was entered into the computers in real-time and verified by a combined system of dual entry and manual checking. HIV test results were password-protected and accessible only to authorized personnel who maintained a high degree of confidentiality when handling this data. All laboratory samples were bar-coded to add to the confidentiality and sample tracking integrity.

3.3.3 Consent Process and Counselling Protocols

Enrollment involved comprehensive consent procedures. Parents/guardians received detailed study information in English or their preferred local language (Kiswahili or Dholuo). They were

informed of their rights, potential risks and benefits, and that participation was voluntary, with no effect on access to care. HIV counseling and testing were offered per national guidelines, delivered by trained counselors in a confidential setting.

3.3.4 Protections against Risk

All venipunctures were performed by trained, qualified clinicians and laboratory technicians to minimize discomfort during blood drawing. Only sterile disposable lancets, syringes, and needles were used, and all sharps were stored in the appropriate biohazard sharps containers before disposal. The amount of blood drawn by venipuncture from all children was in the safe range based on age, weight, gender, and anemia status. In addition, all children in the study were given vitamins supplemented with iron to prevent the initiation and/or exacerbation of anaemia due to vitamin and/or iron deficiency.

3.3.5 Potential Benefits to the Participants and the Community

Study participants had standard intensive clinical management. The potential long-term benefits of the study are improved understanding of the host and parasite genetic contribution to SMA, as well as improved clinical outcomes for children with SMA. The risk to participants was minimal since the study adhered to all measures to minimise risks. Appropriate antimalarial treatment plus appropriate patient care was provided at no cost to the study participants. Per the Ministry of Health (MOH), Kenya guidelines, all children with a clinical diagnosis of malaria received appropriate antimalarial therapy. The study participants also had access to high-quality HIV testing and counselling. In addition, children were screened for bacteremia, mycobacterium tuberculosis, and diarrheal diseases, hookworm, and if a disease was detected, they were referred to the MOH staff for appropriate medical treatment. Children residing in this area would not normally be investigated extensively when they present to the hospital.

3.3.6 Compensation for Research-Related Injury

This study did not involve greater than minimal risk. However, if any adverse events were identified, the study participants were immediately referred to the Ministry of Health Staff to receive appropriate treatment according to the Kenya Ministry of Health Standards. The costs incurred for any referral case management were covered by the project funds.

3.3.7 Economic Burden to Subjects

No cost was incurred by the study participants for participation in the study. The cost of transport for coming to the hospital was reimbursed according to the geographic location of the study participants' residences. Since the study radius was predetermined as 25 km from the study site (SCRH), a transport reimbursement to and from hospital visits ranged from US$2 – US$8 (KES 258 – KES 1,033).

CHAPTER FOUR
RESULTS

4.1 Objective One

4.1.1 Demographic, Clinical and Laboratory Characteristics of Study Participants

The enrollment characteristics of the cohort, stratified according to the presence of malaria parasitaemia and anaemia status [aparasitaemic (n=289, any level of Hb), non-SMA (n=962, Hb≥6.0 g/dL) and SMA (n=264, Hb<60 g/dL)], are presented in **Table 4.1**. The ratio of females and males was comparable among the three groups (*P*=0.752), while age differed across the groups (*P*=0.003), with SMA patients being the youngest. Children with SMA exhibited the lowest hematocrit levels as well as RBC counts (*P*=8.075E-144 and *P*=3.178E-134, respectively). White blood cell counts progressively increased across the groups (*P*=3.676E-09) and were highest in children with SMA. The non-SMA group presented with a higher parasite density than the SMA group (*P*=0.043).

HIV-1 status and bacteraemia were assessed in the cohort since it can enhance the risk of SMA (Otieno *et al.*, 2006; Were *et al.*, 2011). The distribution of HIV-1 was comparable across the groups (*P*=0.116), whereas bacteraemia significantly differed (*P*=0.036) and was highest in the aparasitemic group. Examination of genetic factors known to influence the development of severe malaria (Richer & Chudley, 2005; Taylor *et al.*, 2012; Weatherall, 2018) revealed that the distribution of $\alpha^{3.7}$-thalassaemia variants and sickle cell trait (HbAS) differed across the groups (*P*=0.014 and *P*=7.072E-05, respectively). Children with SMA had the highest carriage of heterozygous and homozygous $a^{3.7}$-thalassemia variants, the lowest inheritance of HbAS, and the highest proportion of HbSS. The distribution of glucose-6-phosphate dehydrogenase (G6PD) deficiency was comparable across the groups (*P*=0.387).

Table 4.1: Admission demographic, clinical and laboratory characteristics of study participants

Characteristics	Total	Aparasitemic (MPS Negative)	Non-SMA (Hb≥5.0 g/dL)	SMA (Hb<5.0 g/dL)	*P*-value
No. of participants	**1,515**	**289**	**962**	**264**	
Sex [n, (%)]: Male	760	145 (50.2)	488 (50.7)	137 (51.9)	0.752[a]
Female	755	144 (49.8)	474 (49.3)	127 (48.1)	
Age, months	1,515	11.0 (13.4)	12.7 (10.4)	10.1 (10.7)	**0.003**[b]
Axillary temperature, °C	1,503	37.0 (1.5)	38.0 (1.5)	38.0 (1.1)	**4.135E-15**[b]
Haematological parameters					
Hemoglobin, g/dL	1,508	10.3 (2.8)	7.7 (2.8)	4.3 (1.1)	N/A
Hematocrit, %	1,507	32.9 (8.2)	25.2 (9.1)	14.1 (3.8)	**8.075E-144**[b]
Red Blood Cells, $\times 10^{6}$/µL	1,504	4.7 (1.1)	3.8 (1.4)	1.9 (0.6)	**3.178E-134**[b]
White Blood Cells, $\times 10^{3}$/µL	1,503	11.0 (7.3)	11.7 (6.5)	14.9 (9.8)	**3.676E-09**[b]
Parasitological Indices					
Parasite density, MPS/µL	1,226	0.0 (0.0)	28,595 (79,256)	24,831 (66,014)	**0.043**[c]
Co-infections					
HIV-1: Negative [n, (%)]	1,457	275 (95.8)	933 (97.2)	249 (94.7)	0.116[a]
Positive [n, (%)]	53	12 (4.2)	27 (2.8)	14 (5.3)	
Bacteraemia: Negative [n, (%)]	1,402	257 (89.9)	902 (94.2)	243 (92.0)	**0.036**[a]
Positive [n, (%)]	106	29 (10.1)	56 (5.8)	21 (8.0)	
Genetics Variants					
$\alpha^{3.7}$-thalassaemia: αα/αα [n, (%)]	554	105 (42.9)	355 (42.4)	94 (39.7)	**0.014**[a]
-α/αα [n, (%)]	505	75 (30.6)	329 (39.3)	101 (42.6)	
-α/-α [n, (%)]	261	65 (26.5)	154 (18.4)	42 (17.7)	
G6PD:					
Normal [n, (%)]	1,078	194 (75.8)	678 (75.8)	206 (80.5)	0.387[a]
Intermediate [n, (%)]	266	51 (19.9)	178 (19.9)	37 (14.5)	
Deficiency [n, (%)]	63	11 (4.3)	39 (4.4)	13 (5.1)	
Sickle cell trait:					
HbAA [n, (%)]	1,241	223 (79.1)	785 (82.5)	233 (90.7)	**7.072E-05**[a]
HbAS [n, (%)]	239	55 (19.5)	164 (17.2)	20 (7.8)	
HbSS [n, (%)]	10	4 (1.4)	2 (0.2)	4 (1.6)	

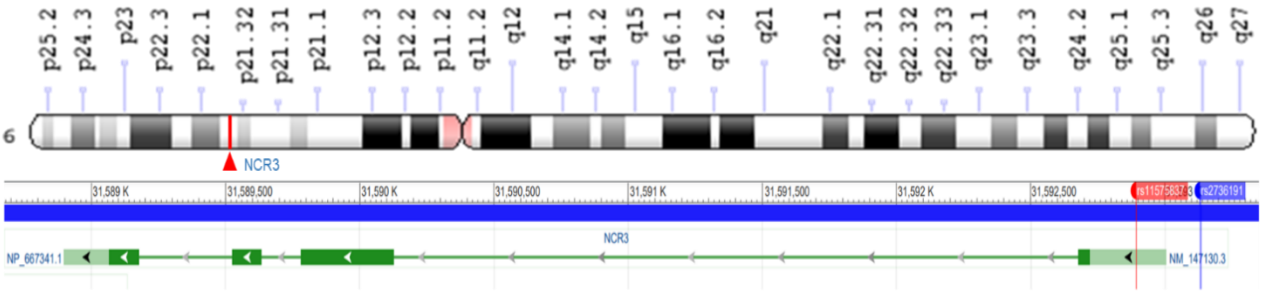
Data are presented as median (interquartile range, IQR) and [n (%)] unless stated otherwise. Children (n=1,515) were categorised into aparasitemic controls (n=289; no parasitemia), and either non-SMA (n=962; Hb≥5.0 g/dL) or SMA (n=264; Hb<5.0 g/dL). Non-SMA and SMA patients are collectively categorised as parasitaemia (n=1,226). Abbreviations: MPS- malaria parasites; HIV, human immunodeficiency virus; G6PD, glucose-6-phosphate dehydrogenase deficiency; HbAA-hemoglobin AA, HbAS- haemoglobin AS, HbSS- haemoglobin SS. [a]Statistical significance was determined by Chi-square analysis. [b]Statistical significance determined by the Kruskal-Wallis test. [c]Statistical significance determined by Mann-Whitney U test. *P*-values ≤0.050 were considered significant and are indicated in bold.

4.1.2 Characteristics of the *NCR3* Variants in the Study Population

The *NCR3* is located on chromosome 6p21.33 (GRCh38.p12) (**Figure 4.1A**). Genotyping of the two SNPs in the population revealed a minor allele frequency (MAF) for rs2736191 at 0.30 and rs11575837 at 0.03 (**Figure 4.1B**). Transcription Factor Binding Sites (TFBS) analysis showed that the conversion of C to G in rs2736191 caused ablation of the binding site for AP-2 alpha (i.e., TFAP2A or AP2) and creation of binding sites for transcription factor 3 (i.e., TCF3 or E47)

and zinc finger and BTB domain containing 6 (i.e., ZBTB6 or ZID, **Figure 4.1B**). TFBS analysis of rs11575837 revealed that conversion of C to T results in the loss of the TFAP2A binding site and the gain of a hepatocyte nuclear factor 4 (HNF4A) binding site (**Figure 4.1B**). LD analysis between the selected SNPs yielded D′ of 0.882, LOD score of 6.61, and r^2 of 0.011 (**Figure 4.1C**).

A



B

Gene variants	Minor Allele Frequency		TFBS	
	dbSNP LWK	Study Population	Major allele	Minor allele
rs2736191:C>G	0.23	0.30	TFAP2A (AP2)	TCF3 ZBTB6
rs11575837:C>T	0.01	0.03	TFAP2A (AP2)	HNF4A

C

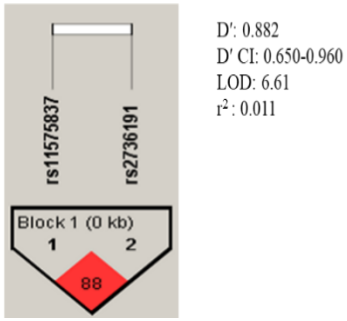


Figure 4.1: NCR3 variants investigated.
A) Chromosome location of NCR3 and the variants under investigation. B) Selection criteria for rs2736191:C>G and rs11575837:C>T variants. C) Linkage disequilibrium.

The distribution of genotypes and haplotypes for rs2736191 and rs11575837 is shown in **Table 4.2.** The observed frequencies of the rs2736191 genotypes in the overall population were 0.503 for CC, 0.386 for CG, and 0.111 for GG, displaying a significant departure from HWE (χ^2=11.58, P<0.001). The observed frequencies of the rs11575837 genotypes in the overall population were 0.941 for CC, 0.054 for CT, and 0.005 for TT, displaying a significant departure from HWE (χ^2=28.57, P<0.001). The distribution of the genotypes and the four haplotypes was comparable across the three clinical groups.

Table 4.2: Distribution of rs2736191: C>G and rs11575837:C>T genotypes and haplotypes

Genotype/haplotype	Total	Aparasitemic	Non-SMA (Hb≥5.0g/dL)	SMA (Hb<5.0g/dL)	*P*-value
No. of participants	**1,515**	**289**	**962**	**264**	
rs2736191					
CC, n (%)	762 (50.3)	153 (52.9)	490 (50.9)	119 (45.1)	0.280
CG, n (%)	585 (38.6)	101 (35.0)	372 (38.7)	112 (42.4)	
GG, n (%)	168 (11.1)	35 (12.1)	100 (10.4)	33 (12.5)	
HWE (χ^2, *P*)	11.58, <**0.001***				
rs11575837					
CC, n (%)	1,426 (94.1)	272 (94.1)	908 (94.4)	246 (93.2)	0.169
CT, n (%)	81 (5.4)	17 (5.9)	46 (4.8)	18 (6.8)	
TT, n (%)	8 (0.5)	0 (0.0)	8 (0.8)	0 (0.0)	
HWE (χ^2, *P*)	28.57, <**0.001***				
rs2736191/rs11575837					
Non-CC, n (%)	196 (12.9)	38 (13.1)	121 (12.6)	37 (14.0)	0.821
CC, n (%)	1,319 (87.1)	251 (86.9)	841 (87.4)	227 (86.0)	
Non-CT, n (%)	1,426 (94.1)	272 (94.1)	908 (94.4)	246 (93.2)	0.762
CT, n (%)	89 (5.9)	17 (5.9)	54 (5.6)	18 (6.8)	
Non-GC, n (%)	764 (50.4)	153 (52.9)	492 (51.1)	119 (45.1)	0.139
GC, n (%)	751 (49.6)	136 (47.1)	470 (48.9)	145 (54.9)	
Non-GT, n (%)	1,513 (99.9)	289 (100.0)	960 (99.8)	264 (100.0)	0.562
GT, n (%)	2 (0.1)	0 (0.0)	2 (0.2)	0 (0.0)	

Data are presented as proportions [n, (%)] unless otherwise stated for *NCR3* variants. Children were categorised into aparasitemic (n=289), non-SMA (Hb≥5.0g/dL, n=962) and SMA (Hb<5.0g/dL, n=264). Non-SMA and SMA patients are collectively categorised as parasitaemia (n=1,226). Statistical significance was determined by Chi-square analysis across groups. *P*-values ≤0.050 were considered significant and are indicated in bold. HWE, Hardy-Weinberg Equilibrium. χ^2 - Hardy-Weinberg Equilibrium Chi-square. * represents a significant *P*-value after the exact test for HWE.

4.1.3 Longitudinal Risk of Malaria and SMA Episodes for the *NCR3* Genotypes/Haplotypes

The impact of the genotypes and haplotypes on malaria and SMA episodes across the 36-mos. follow-up period was determined by fitting a Poisson regression model selecting for covariates [i.e., age, sex, co-infections (HIV-1 and bacteremia), G6PD deficiency, sickle-cell trait and alpha-thalassemia] known to influence susceptibility to malaria and SMA. The results of the regression modelling and the occurrence of events are shown in **Table 4.3**. There were 7,228 malaria episodes, for which 406 SMA events occurred during the cohort observational period. Significant effects on susceptibility to malaria were observed for rs11575837, in which carriage of the homozygous recessive genotype (TT) increased the risk of malaria (IRR=1.540, 95% CI=1.114–2.129, *P*=0.009, significant after Holm-Bonferroni correction). Neither rs2736191 nor any haplotypes derived from the two SNPs significantly impacted the longitudinal susceptibility to malaria.

A similar Poisson regression selecting for the same covariates revealed an increased risk of SMA over 36-mos. in carriers of rs2736191 genotypes CG (IRR=1.269, 95% CI=1.009-1.597, *P*=0.041), and in the additive model (IRR=1.198, 95% CI=1.030-1.393, *P*=0.019). There was a

trend of increased risk of SMA in carriers of genotype GG without significance (IRR=1.378, 95% CI=0.991-1.915, *P*=0.057). However, none of the results remained significant after correction for multiple comparisons. Carriage of CT or TT genotypes for rs11575837 showed a non-significant increased risk of developing SMA [(IRR=1.294, 95% CI=0.828-2.020, *P*=0.258) and (IRR=1.911, 95% CI=0.609-6.000, *P*=0.267), respectively]. Analysis of the impact of the haplotypes on susceptibility to SMA revealed an additive protective effect for individuals who inherited the CC haplotype (IRR=0.823, 95% CI=0.711-0.952, *P*=0.009, significant after Holm-Bonferroni adjustment). Conversely, there was an enhanced risk of SMA in individuals who inherited the GC haplotype (IRR=1.276, 95% CI=1.030-1.581, *P*=0.026), and in the additive model (IRR=1.182, 95% CI=1.018-1.372, *P*=0.029), but neither retained significance after Holm-Bonferroni adjustment. There was no association between an altered risk profile of SMA for either the CT or GT haplotypes (**Table 4.3)**.

Table 4.3: Susceptibility to malaria and SMA over 36 mos. of follow-up

Genotype/ haplotype		Malaria episodes				SMA episodes			
		n	**IRR**	**95% CI**	***P*-value**	**n**	**IRR**	**95% CI**	***P*-value**
rs2736191	CC	3,575	Ref			184	Ref		
	CG	2,832	1.011	0.957-1.067	0.700	170	1.269	1.009-1.597	**0.041**
	GG	821	1.014	0.936-1.100	0.731	52	1.378	0.991-1.915	0.057
	Additive	7,228	1.010	0.974-1.048	0.590	406	1.198	1.030-1.393	**0.019**
rs11575837	CC	6,809	Ref			379	Ref		
	CT	370	0.957	0.853-1.074	0.454	24	1.294	0.828-2.020	0.258
	TT	49	1.540	1.114-2.129	**0.009***	3	1.911	0.609-6.000	0.267
	Additive	7,228	1.031	0.935-1.136	0.540	406	1.330	0.929-1.904	0.119
rs2736191/rs11575837									
CC	Non-CC	978	Ref			61	Ref		
	CC	2,971	0.976	0.908-1.048	0.503	181	0.850	0.637-1.135	0.271
	Additive	6,250	0.990	0.955-1.026	0.571	345	0.823	0.711-0.952	**0.009***
CT	Non-CT	6,809	Ref			379	Ref		
	CT	385	0.995	0.893-1.108	0.920	25	1.271	0.839-1.925	0.257
	Additive	419	1.015	0.918-1.123	0.769	27	1.295	0.886-1.894	0.182
GC	Non-GC	3,590	Ref			185	Ref		
	GC	2,817	1.009	0.960-1.061	0.726	169	1.276	1.030-1.581	**0.026**
	Additive	3,638	1.007	0.971-1.044	0.709	221	1.182	1.018-1.372	**0.029**
GT	Non-GT	7,213	Ref			405	Ref		
	GT	15	1.574	0.947-2.619	0.080	1	1.196	0.166-8.627	0.859
	Additive	15	1.574	0.947-2.619	0.080	1	1.196	0.166-8.627	0.859

Data are presented as an incidence rate ratio (IRR) with 95% confidence intervals (CI) using log-linear regression with the following covariates in the models: age at enrollment, sex, HIV-1, bacteraemia, sickle cell trait, $a^{3.7}$-thalassaemia and G6PD deficiency. The longitudinal relationship between the genetic variants and susceptibility to malaria and SMA (Hb<5.0g/dL) was determined throughout the 36 mos. follow-up period. *P*-values ≤0.050 were considered significant and are indicated in bold. **P*-value remained significant after Holm-Bonferroni correction for multiple comparisons.

A Cox proportional hazard model was fitted to examine the relationship between the *NCR3* genotypes/haplotypes and the total number of deaths from any cause within the study participants over the follow-up period (all-cause mortality). No significant relationships with all-cause mortality were observed for either rs2736191, rs11575837, or their haplotypes (**Table 4.4**).

Table 4.4: Survival analysis for all-cause mortality

Genotype/Haplotype		Mortality			
		n	HR	95% CI	*P*-value
rs2736191	CC	46	Ref		
	CG	35	1.480	0.865-2.520	0.153
	GG	7	0.934	0.354-2.460	0.890
	Additive	88	1.140	0.783-1.650	0.502
rs11575837	CC	83	Ref		
	CT	3	1.130	0.348-3.650	0.842
	TT	2	2.860	0.371-22.10	0.313
	Additive	88	1.430	0.630-3.260	0.390
rs2736191/rs11575837					
CC	Non-CC	9	Ref		
	CC	37	1.410	0.604-3.280	0.428
	Additive	79	0.871	0.608-1.250	0.452
CT	Non-CT	83	Ref		
	CT	4	1.310	0.472-3.630	0.605
	Additive	5	1.240	0.467-3.310	0.664
GC	Non-GC	47	Ref		
	GC	34	1.300	0.778-2.170	0.318
	Additive	41	1.090	0.753-1.590	0.641
GT	Non-GT	88			
	GT	1	5.430	0.688-42.80	0.108
	Additive	1	5.430	0.688-42.80	0.108

Data are presented as hazard ratios (HR) with 95% confidence intervals (CI). Analysis was performed using Cox regression modelling with the following covariates: age at enrollment, sex, HIV-1 and bacteraemia status, sickle cell trait status, $a^{3.7}$-thalassaemia, and G6PD deficiency. Homozygous wild-type genotypes and non-carriers of haplotypes were used as reference groups in the analyses. Statistical significance was set at $P \leq 0.050$. *Significant after Holm-Bonferroni correction for multiple comparisons.

4.2 Objective Two

4.2.1 Admission Demographic, Clinical and Laboratory Characteristics of Study Participants

Age- and sex-matched children without coinfections (i.e., HIV-1 and bacteremia) with peripheral *P. falciparum* parasitemia (any density) were stratified into two groups based on Hb levels: Hb≥6.0 g/dL (non-SMA, n=41) and Hb<6.0 g/dL (SMA, n=25). The demographic, clinical, and laboratory characteristics of these study participants are shown in **Table 4.5**. Children with SMA presented at the hospital with comparable glucose levels (*P*=0.967) and marginally lower axillary temperatures (*P*=0.051). Consistent with the *a priori* classification based on Hb concentrations, children with SMA had lower levels of hematocrit (*P*=1.242E-11) and RBCs (*P*=1.790E-11). Conversely, SMA was characterised by elevated red blood cell distribution width (*P*=4.050E-04), mean corpuscular volume (*P*=0.002), and mean corpuscular haemoglobin concentrations (*P*=0.022). Elevations in the SMA group were also witnessed for white blood cells (*P*=1.393E-04), lymphocytes (*P*=5.509E-06) and monocytes (*P*=0.022). Comparable levels for additional haematological variables were observed: platelets (*P*=0.615), neutrophil (*P*=0.438) and granulocyte (*P*=0.373) counts. Parasitological indices (i.e., parasite density and stratification

based on levels) did not significantly vary across the two groups (*P*=0.155 and *P*=0.134, respectively). However, the distribution of sickle-cell trait status differed between the groups, with SMA characterised by a lower proportion of HbAA and HbAS and a higher proportion of HbSS (*P*=0.029).

Table 4.5: Admission demographic, clinical and laboratory characteristics of study participants

Characteristics	Non-SMA (Hb≥6.0g/dL)	SMA (Hb<6.0g/dL)	*P*-value
No. of participants (n=66)	41	25	
Sex, n (%)			
Male	20 (48.8)	13 (52.0)	0.800[a]
Female	21 (51.2)	12 (48.0)	
Age, months	24.0 (22.0)	25.0 (28.5)	0.797[b]
0 - 12.9	7 (17.1)	5 (20.0)	
13 - 24.9	14 (34.1)	7 (28.0)	
25 - 35.9	9 (22.0)	5 (20.0)	0.461[a]
36 - 48.9	11 (26.8)	6 (24.0)	
>49	0 (0.0)	2 (8.0)	
Blood glucose, mmol/L	5.0 (2.3)	5.0 (1.7)	0.967[b]
Body temperature °C	38.0 (1.2)	37.7 (0.8)	0.051[b]
Hematological Parameters			
Hemoglobin, g/dL	9.9 (1.4)	4.6 (1.2)	NA
Hematocrit, %	29.8 (5.9)	14.4 (2.9)	**1.242E-11**[b]
Red blood cells, × 10^b/µL	4.3 (1.0)	1.9 (0.9)	**1.790E-11**[b]
Red blood cell distribution width, %	18.7 (3.4)	22.3 (8.9)	**4.050E-04**[b]
Mean corpuscular volume, fL	69.5 (9.2)	78.6 (29.9)	0.002[b]
Mean corpuscular haemoglobin, pg	22.9 (4.8)	26.7 (9.4)	0.022[b]
Platelets, ×10^3/µL	124.4 (85.7)	134.0 (139.7)	0.615[b]
Platelet distribution width, %	16.5 (1.3)	17.3 (0.9)	0.370[b]
Mean platelet volume, fL	8.5 (1.6)	8.9 (1.9)	0.124[b]
WBCs, ×10^3/µL	11.3 (6.9)	19.8 (11.5)	**1.393E-04**[b]
Lymphocytes, ×10^3/µL	3.7 (1.9)	10.0 (9.2)	**5.509E-06**[b]
Monocytes, ×10^3/µL	1.2 (1.3)	1.7 (1.4)	**0.022**[b]
Neutrophils, ×10^3/µL	5.3 (4.2)	6.0 (6.9)	0.438[b]
Granulocytes, ×10^3/µL	6.7 (3.0)	9.1 (5.8)	0.373[b]
Parasitological Indices			
Parasite density, MPS/µL	57,915 (81,568)	14,191 (68,728)	0.155[b]
Low (1 – 5,000)	6 (14.6)	8 (32.0)	
Moderate (5001 – 50,000)	13 (31.7)	10 (40.0)	0.134[a]
High (50,001 – 100,000)	14 (34.1)	3 (12.0)	
Hyper (>100,001)	8 (19.5)	4 (16.0)	
Genetic Variants			
Sickle-cell genotypes, n (%)			
HbAA	35 (85.3)	16 (64.0)	
HbAS	4 (9.8)	2 (8.0)	**0.029**[a]
HbSS	2 (4.9)	7 (28.0)	

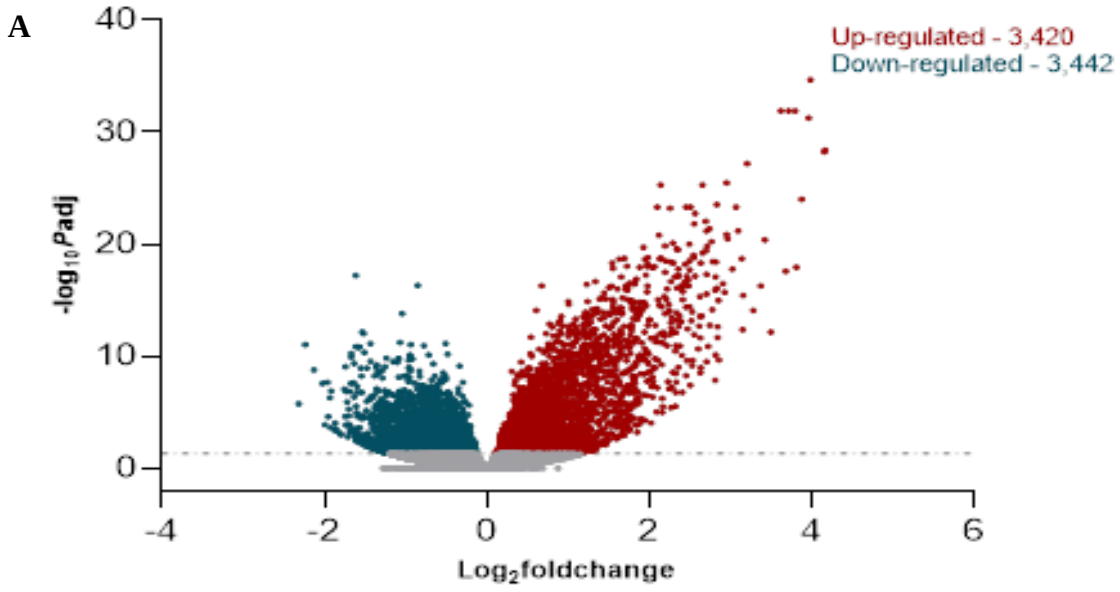
Data are the median (interquartile range; IQR) unless otherwise noted. Children (n=66) presenting with malaria at SCRH were recruited. Based on haemoglobin (Hb) levels, children were categorised into either non-severe malaria anaemia (non-SMA; Hb≥6.0 g/dL, n=41) or severe malarial anaemia (SMA; Hb<6.0 g/dL, n=25). [a]Fisher's exact test with exact *P*-values for homogeneity and [b]Mann-Whitney U test were used to compare the non-SMA and SMA groups. Statistical significance was set at *P*≤0.050, and significant *P*-values were indicated in bold. Abbreviations: MPS - malaria parasites. HbAA-haemoglobin AA, HbAS-haemoglobin AS, HbSS-haemoglobin SS.

4.2.2 Distribution of Differential Gene Expression in SMA Relative to Non-SMA

RNA-seq of the entire expressed transcriptome in whole blood revealed 6,862 differentially expressed genes (DEGs) in SMA relative to non-SMA after applying a false discovery rate (FDR) correction (*P*adj<0.050). Of these DEGs, 3,420 exhibited up-regulation, while 3,442 displayed downregulation (**Figure 4.2A**).

4.2.3 Leukocytic Immune Cell Profiling

Gene expression deconvolution in the CIBERSORTx platform was used to determine the proportions of the different white blood cell subsets inferred from the RNA-seq data (**Figure 4.2B**). 10 out of 22 leukocytic cell types in whole blood differed significantly between SMA and non-SMA individuals (**Figure 4.2C**). Children with SMA had increased naïve B cells (*P*=9.741E-05), CD8 T cells (*P*=0.009), CD4 memory resting T cells (*P*=0.001), resting NK cells (*P*=0.002), monocytes (*P*=0.039 and M2 macrophages (*P*=0.002) (**Figure 4.2C**). In contrast, the SMA group had a lower proportion of activated dendritic cells (*P*=0.001), activated mast cells (*P*=0.014) and neutrophils (*P*=4.826E-04), along with marginally reduced naïve CD4 T cells (*P*=0.053) (**Figure 4.2C**).



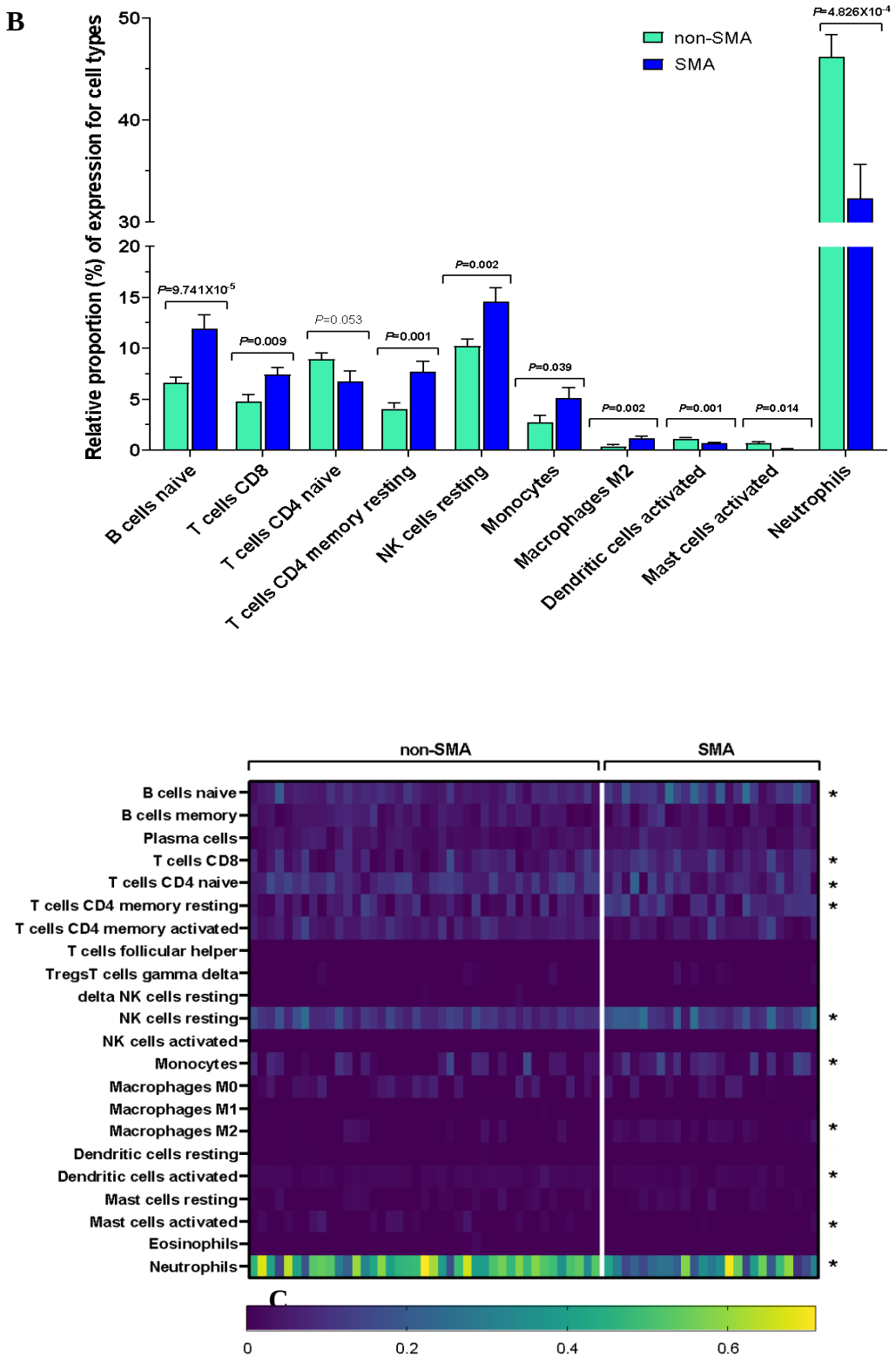


Figure 4.2: Differential gene expression analysis and leukocytic immune profiling in children with severe malarial anaemia

A) The volcano plot shows 3,420 up-regulated (red) and 3,442 down-regulated (turquoise) genes in children with SMA. The horizontal axis shows the Log_2fold change, while the vertical axis shows the -log10 Padj values. The horizontal dotted line corresponds to 1.301 (i.e., $-\log_{10}$ Padj significance threshold at 0.050). B) The composition of different blood cell types was analysed using CIBERSORTx for deconvolution. The LM22 signature matrix file was used to estimate cellular frequencies. A heatmap illustrates the expression levels of 22 leukocyte cell types/subtypes at the individual patient level for both non-SMA (Hb≥6.0 g/dL, n=41) and SMA (Hb<6.0 g/dL, n=25) groups. Significant differences in immune cell proportions between the groups are marked with an asterisk (*), determined by two-sided, two-sample t-tests with Welch correction (P<0.050). C) The relative proportions (%) of immune cell types that differ between the non-SMA (n=41) and SMA (n=25) groups are shown as mean (SEM), based on bivariate analysis using two-sided, two-sample t-tests with Welch correction.

4.2.4 Process Network Enrichment Analysis in SMA

Process network enrichment analysis, performed to determine if NK cell networks were enriched in SMA patients, revealed perturbation in the Inflammation_NK cell cytotoxicity process network (FDR= 3.628E-04, 69/164 network objects) (**Figure 4.3**). The significantly differentially expressed active hubs in the network, localised in the NK cell membrane, cytoplasm and nucleus, were identified. NK cell membrane: DAP12 (log_2FC= -0.49, FDR= 0.022), CD3 zeta (CD 247) (log_2FC= 0.36, FDR= 0.049) and CD244 (log_2FC= 0.50, FDR= 0.003); NK cell cytoplasm: Caspase-3 (log_2FC= -0.21, FDR= 0.012), ERK2 (MAPK1) (log_2FC= 0.60, FDR= 2.30E-06), Slp76 (LCP2) (log_2FC= -0.66, FDR= 4.97E-10), Caspase-8 (log_2FC= -0.23, FDR= 0.023), PAK1 (log_2FC= -0.44, FDR= 0.002) and SHP-1 (PTPN6) (log_2FC= -0.29, FDR= 0.027), GRB2 (log_2FC= -0.26, FDR=0.019), and NK cell nucleus: STAT1 (log_2FC= -0.33, FDR= 0.003), NF-κB [(NFKB1, log_2FC= -0.40, FDR= 5.575E-05) and (NFKB2, log_2FC= -0.66, FDR= 5.057E-05)] and NF-AT1(NFATC2) (log_2FC= 0.67, FDR= 1.426E-07).

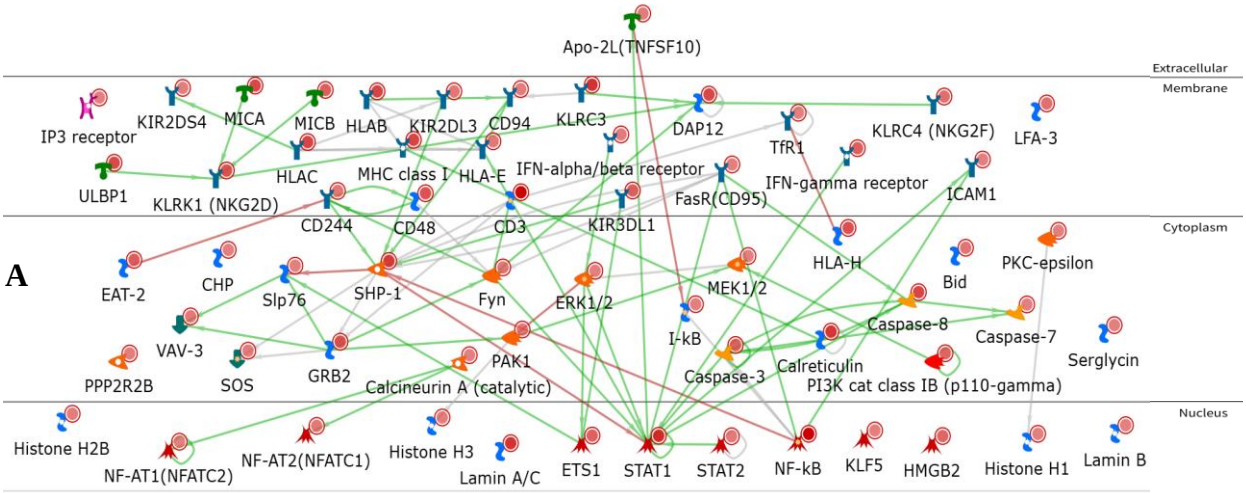
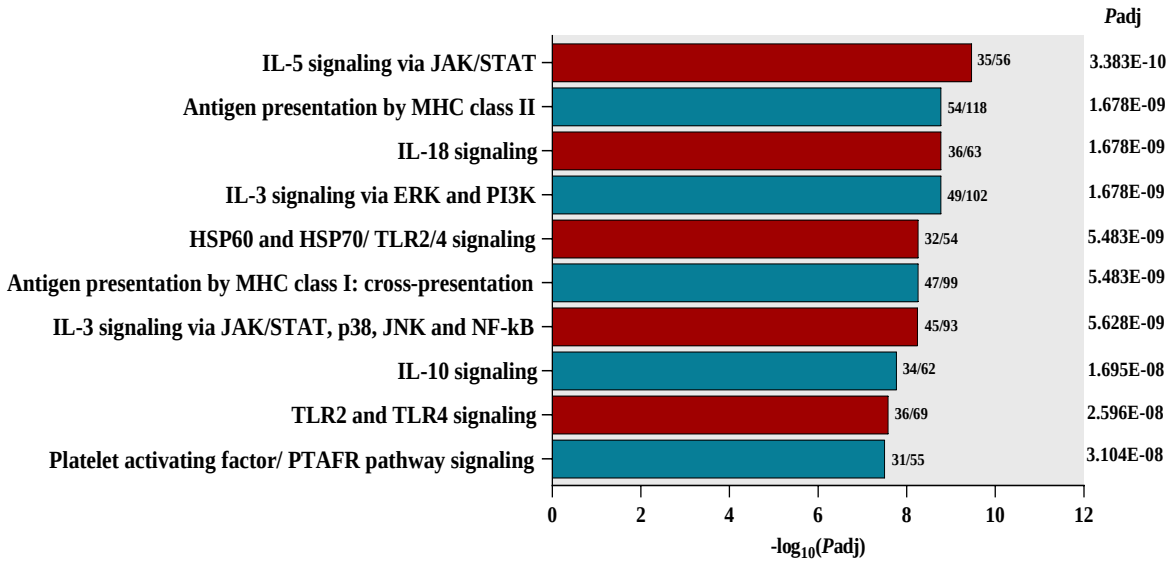


Figure 4.3: Inflammation- NK cell cytotoxicity process network.
Enrichment analysis of significant DEGs on MetaCore™ to determine the NK cell networks enriched in SMA identified the Inflammation_NK cell cytotoxicity process network. The details of symbols used in these figures are available at: https://portal.genego.com/legends/MetaCoreQuickReferenceGuide.pdf.

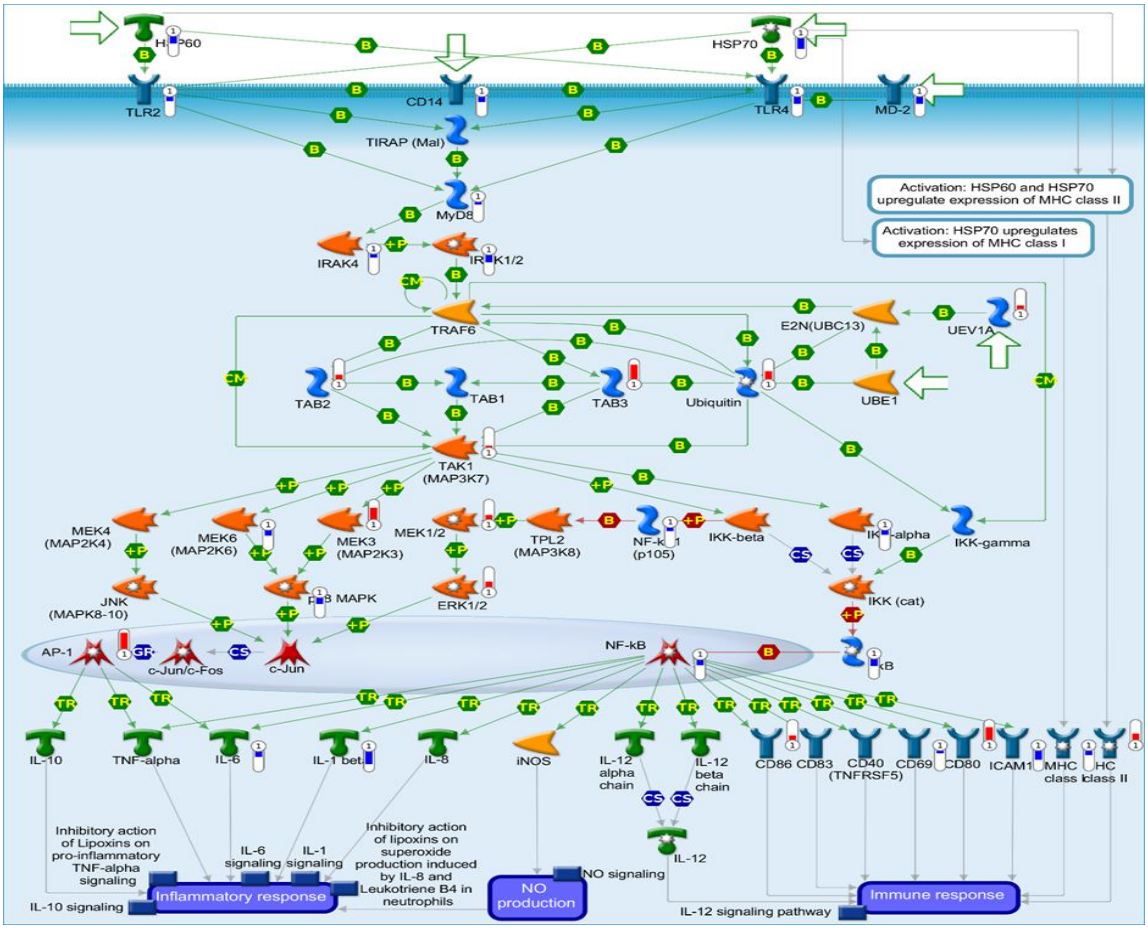
4.2.5 Immune Response Canonical Signalling Pathways in Children with SMA

To identify biological networks associated with the pathogenesis of SMA, canonical pathway maps were generated with Metacore™. The top 10 emergent pathways for the DEGs included a signalling network enriched for HSPs and TLRs, i.e., HSP60-HSP70-TLR2/4 signalling pathway (*P*adj=5.483E-09, 3rd ranked according to *P*adj, **Figure 4.4A**). The DEGs from the RNA-seq represented 32 out of 54 protein nodes for the HSP60-HSP70-TLR2/4 signalling pathway (**Figure 4.4B**).

The 32 proteins (nodes) in the pathway map correspond to 47 human genes, of which 17 were up-regulated and 30 were down-regulated. Below is the description of known signalling actions of the proteins in the pathway in the context of observed changes for each of the 47 genes (+/-) in children with SMA (**Figure 4.4B**). For example, while the known action of a signalling molecule can be the activation of its downstream target, some targets may have reduced transcript expression. The pathogenesis of SMA can be unravelled by interpreting the known action in the setting of transcript expression profiles in the pathway.


Padj
IL-5 signaling via JAK/STAT
35/56
3.383E-10
Antigen presentation by MHC class II
54/118
1.678E-09
IL-18 signaling
36/63
1.678E-09
IL-3 signaling via ERK and PI3K
49/102
1.678E-09
HSP60 and HSP70/ TLR2/4 signaling
32/54
5.483E-09
Antigen presentation by MHC class I: cross-presentation
47/99
5.483E-09
IL-3 signaling via JAK/STAT, p38, JNK and NF-kB
45/93
5.628E-09
IL-10 signaling
34/62
1.695E-08
TLR2 and TLR4 signaling
36/69
2.596E-08
Platelet activating factor/ PTAFR pathway signaling
31/55
3.104E-08
0
2
4
6
8
10
12
-log10(Padj)

B


HSP60
HSP70
TLR2
CD14
TLR4
MD-2
TIRAP (Mal)
MyD88
Activation: HSP60 and HSP70 upregulate expression of MHC class II
Activation: HSP70 upregulates expression of MHC class I
IRAK4
IRAK1/2
TRAF6
E2N(UBC13)
UEV1A
TAB2
TAB1
TAB3
Ubiquitin
UBE1
TAK1 (MAP3K7)
MEK4 (MAP2K4)
MEK6 (MAP2K6)
MEK3 (MAP2K3)
MEK1/2
TPL2 (MAP3K8)
NF-kB1 (p105)
IKK-beta
IKK-alpha
IKK-gamma
JNK (MAPK8-10)
p38 MAPK
ERK1/2
IKK (cat)
AP-1
c-Jun/c-Fos
c-Jun
NF-kB
IL-10
TNF-alpha
IL-6
IL-1 beta
IL-8
iNOS
IL-12 alpha chain
IL-12 beta chain
CD86
CD83
CD40 (TNFRSF5)
CD69
CD80
ICAM1
MHC class I
HC class II
Inhibitory action of Lipoxins on pro-inflammatory TNF-alpha signaling
IL-6 signaling
IL-1 signaling
Inhibitory action of lipoxins on superoxide production induced by IL-8 and Leukotriene B4 in neutrophils
IL-12
NO signaling
IL-10 signaling
Inflammatory response
NO production
IL-12 signaling pathway
Immune response

C

The Log_2foldchange (log_2FC) and *P*adj for each of the genes are shown in **Table 4.6**. Of relevance for the role of HSPs in human malaria pathogenesis, transcripts for HSP60 (*HSPD1*) were down-regulated in SMA (log_2FC= -0.81), as were HSP70 family members: *HSPA1A* (-1.31), *HSPA1B* (-0.99), *HSPA4* (-0.33), *HSPA4L* (-0.68), *HSPA5* (-0.51), and *HSPA6* (-0.81). Although endogenous HSP60 and HSP70, which were reduced, can up-regulate the surface expression of MHC class II, the following were up-regulated: HLA-DMA (+0.38), HLA-DOA (+0.70), HLA-DOB (+0.42), HLA-DPB1(+0.56), and HLA-DPB2 (+0.75). Consistently, downregulation in HSP70, known to up-regulate MHC class I, had observed reductions for B2M (-0.37), HLA-B (-0.33), HLA-C (-0.54), and HLA-E (-0.39) (**Table 4.6**).

Downregulation was also observed for TLR2 (-0.53) and TLR4 (-0.83). HSP60 and HSP70 can bind to TLR2 and TLR4 to form a complex with CD14 (-0.60). MD-2 (-0.89) is required for TLR4-mediated responses to HSP60 and HSP70. TLR2 and TLR4 then bind to TIRAP (Mal) for recruitment of MyD88 (-0.32), which binds to IRAK4 (-0.36), subsequently phosphorylating/activating IRAK1 (+0.23) and IRAK2 (-0.81) (**Table 4.6**).

UEV1A (+0.45) undergoes autoactivation, as well as activation of TAB2 (+0.57) and TAB3 (+1.82) by ubiquitination [Ubiquitin B (UBB), +0.94]. TAB2/3 can form a complex with TAK1(MAP3K7, +0.18) for activation of IKK-alpha (-0.49), which once phosphorylated in the IKK complex, can degrade I-kB (-0.91), resulting in translocation of NF-kB1 (-0.40) for subsequent signalling in the nucleus of IL-6 (-0.60) IL-1b (-1.51), ICAM1 (-1.04), CD69 (-0.29), CD80 (+1.54), and CD86 (+0.54). The latter two are increased despite their upstream signalling molecules being down-regulated (**Table 4.6**).

Degradation of NF-kB1 (-0.40) liberates TPL2(MAP3K8) for activation of MEK1/2 (+0.55), which phosphorylates ERK1/2 (+0.60) and subsequently phosphorylates c-Jun, which then forms a heterodimer with c-Fos for activation of AP-1[(JUNB, -0.57) and (JUND, +1.95)], a process that induces IL-6 (-0.60). Convergent signalling for activation of AP-1[(JUNB, -0.57) and (JUND, +1.95)] also occurs by signalling through TAK1(MAP3K7, +0.18), which phosphorylates MEK3(MAP2K3, +1.44) and MEK6(MAP2K6, -0.68), which then phosphorylate p38 MAPK (-0.78) (**Table 4.6**). Overall, the pathway analysis revealed significant dysregulation in the HSP60-HSP70-TLR2/4 signalling pathway, characterised by both up- and down-regulation of key genes and proteins involved in the immune response and immune signalling.

Table 4.6: Differentially expressed transcripts in the HSP60-HSP70-TLR2/4 signalling pathway in children with SMA

Gene	Log_2foldchange	*P*adj
HSP60		
HSPD1	-0.81	**1.37E-05**
HSP70		
HSPA1A	-1.31	**1.99E-04**
HSPA1B	-0.99	**9.23E-03**
HSPA4	-0.33	**2.27E-02**
HSPA4L	-0.68	**3.49E-02**
HSPA5	-0.51	**1.33E-03**
HSPA6	-0.81	**1.88E-03**
TLR2	-0.53	**1.94E-02**
TLR4	-0.83	**1.29E-04**
CD14	-0.60	**1.07E-02**
MD-2 (LY96)	-0.89	**1.13E-05**
TIRAP(Mal)	0.06	0.670
MYD88	-0.32	**4.60E-02**
IRAK1	0.23	**2.31E-02**
IRAK2	-0.81	**1.08E-04**
IRAK4	-0.36	**1.66E-05**
TRAF6	-0.05	0.581
E2N (UBC13, UBE2N)	-0.10	0.257
UEV1A (UBE2V1)	0.45	**7.99E-03**
TAB1	0.20	0.122
TAB2	0.57	**1.51E-05**
TAB3	1.82	**3.70E-17**
Ubiquitin B (UBB)	0.94	**1.29E-03**
UBE1 (UBA1)	-0.16	0.156
TAK1(MAP3K7)	0.18	**4.97E-02**
MEK1 (MAP2K1)	-0.21	0.057
MEK2 (MAP2K2)	0.55	**1.36E-05**
MEK3 (MAP2K3)	1.44	**7.36E-09**
MEK4 (MAP2K4)	0.11	0.360
MEK6 (MAP2K6)	-0.68	**3.08E-03**
TPL2 (MAP3K8)	-0.13	0.322
NF-kB1 (p105) [NFKB1]	-0.40	**6.97E-04**
IKK-alpha (CHUK)	-0.49	**1.24E-06**
IKK-beta (IKBKB)	-0.08	0.230
IKK-gamma (IKBKG)	0.18	0.211
JNK (MAPK8)	-0.01	0.980
p38 MAPK (MAPK14)	-0.78	**7.52E-05**
ERK1 (MAPK3)	-0.16	0.426
ERK2 (MAPK1)	0.60	**2.30E-06**
AP-1		
JUNB	-0.57	**6.93E-03**
JUND	1.95	**2.31E-19**
c-Jun (JUN)	-0.12	0.677
c-Fos (FOS)	-0.19	0.537
NFKB2	-0.66	**6.40E-04**
RELB	-0.32	**4.98E-02**
I-κB (NFKBIA)	-0.91	**1.08E-04**
IL-1β	-1.51	**4.54E-11**

IL-6	-0.60	**3.56E-02**
IL-8 (CXCL8)	-0.55	0.262
IL-10	-0.23	0.513
IL-12 alpha chain (IL12A)	-0.15	0.535
IL-12 beta chain (IL12B)	-0.26	0.621
TNF-alpha (TNF)	-0.35	0.100
iNOS (NOS2)	-0.48	0.485
CD40	-0.08	0.702
CD69	-0.29	**3.78E-02**
CD80	1.54	**1.88E-13**
CD83	-0.32	0.084
CD86	0.54	**6.41E-03**
ICAM1	-1.04	**6.68E-07**
MHC Class I		
HLA-B	-0.33	**2.34E-02**
HLA-C	-0.54	**7.71E-04**
HLA-E	-0.39	**2.43E-03**
B2M	-0.37	**6.93E-03**
MHC Class II		
HLA-DMA	0.38	**2.94E-03**
HLA-DOA	0.70	**1.11E-04**
HLA-DOB	0.42	**2.06E-02**
HLA-DPB1	0.56	**2.00E-03**
HLA-DPB2	0.75	**2.03E-02**

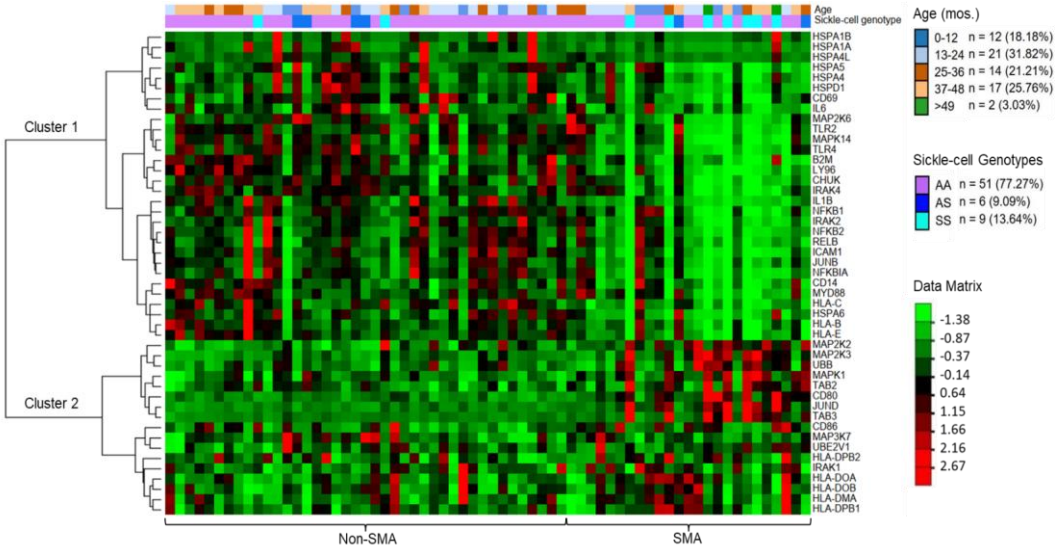
Student's t-test was used to compare the mean gene expression level between non-SMA (Hb≥6.0 g/dL, n=41) and SMA (Hb<6.0 g/dL, n=25). The Log_2foldchange was defined as the ratio of the mean gene expression level in SMA to that of non-SMA. Bold indicates statistical significance at *P*adj≤0.050.

4.2.6 Unsupervised Hierarchical Clustering Analysis of Gene Expression Profiles in the HSP60-HSP70-TLR Signaling Pathway

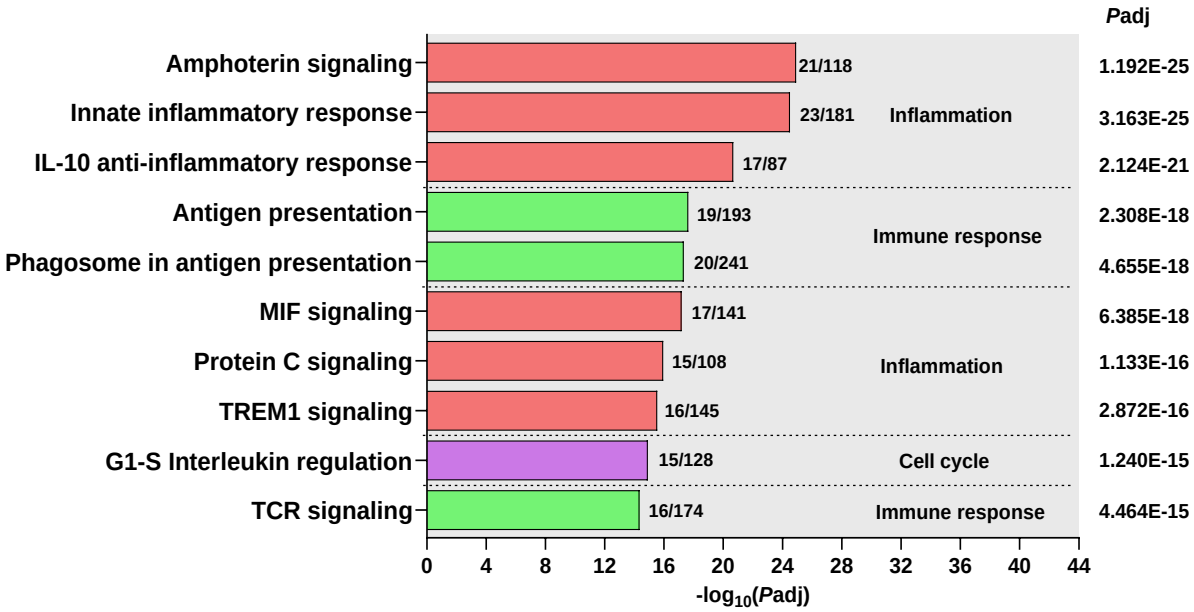
An unsupervised hierarchical clustering heatmap was generated to visualize individual gene expression patterns in the HSP60-HSP70-TLR signalling pathway in children with non-SMA and SMA, along with age and sickle-cell genotype for each participant (**Figure 4.5A**). This analysis revealed two distinct (major) clusters, with cluster 1 composed of 30 down-regulated genes in SMA and cluster 2 containing 17 up-regulated genes. To further explore the biological functions of the DEGs in clusters 1 and 2, MetaCore^{TM} was used to generate the top 10 ranked process networks. The top-ranked process networks for cluster 1 (down-regulated) included Inflammation_Amphoterin Signaling (*P*adj=1.192E-25) and Inflammation-Innate Inflammatory Response (*P*adj=3.163E-25, **Figure 4.5B**).

The top-ranked process networks for cluster 2 (up-regulated) contained Immune Response-T Helper Cell Differentiation (*P*adj=1.061E-10) and Immune Response-TCR signalling (*P*adj=3.163E-25, **Figure 4.5C)**.

A


Age
Sickle-cell genotype
Cluster 1
Cluster 2
HSPA1B
HSPA1A
HSPA4L
HSPA5
HSPA4
HSPD1
CD69
IL6
MAP2K6
TLR2
MAPK14
TLR4
B2M
LY96
CHUK
IRAK4
IL1B
NFKB1
IRAK2
NFKB2
RELB
ICAM1
JUNB
NFKBIA
CD14
MYD88
HLA-C
HSPA6
HLA-B
HLA-E
MAP2K2
MAP2K3
UBB
MAPK1
TAB2
CD80
JUND
TAB3
CD86
MAP3K7
UBE2V1
HLA-DPB2
IRAK1
HLA-DOA
HLA-DOB
HLA-DMA
HLA-DPB1
Non-SMA
SMA
Age (mos.)
0-12 n = 12 (18.18%)
13-24 n = 21 (31.82%)
25-36 n = 14 (21.21%)
37-48 n = 17 (25.76%)
>49 n = 2 (3.03%)
Sickle-cell Genotypes
AA n = 51 (77.27%)
AS n = 6 (9.09%)
SS n = 9 (13.64%)
Data Matrix
-1.38
-0.87
-0.37
-0.14
0.64
1.15
1.66
2.16
2.67

B


Padj
Amphoterin signaling
21/118
1.192E-25
Innate inflammatory response
23/181
Inflammation
3.163E-25
IL-10 anti-inflammatory response
17/87
2.124E-21
Antigen presentation
19/193
Immune response
2.308E-18
Phagosome in antigen presentation
20/241
4.655E-18
MIF signaling
17/141
6.385E-18
Protein C signaling
15/108
Inflammation
1.133E-16
TREM1 signaling
16/145
2.872E-16
G1-S Interleukin regulation
15/128
Cell cycle
1.240E-15
TCR signaling
16/174
Immune response
4.464E-15
0
4
8
12
16
20
24
28
32
36
40
44
-log10(Padj)

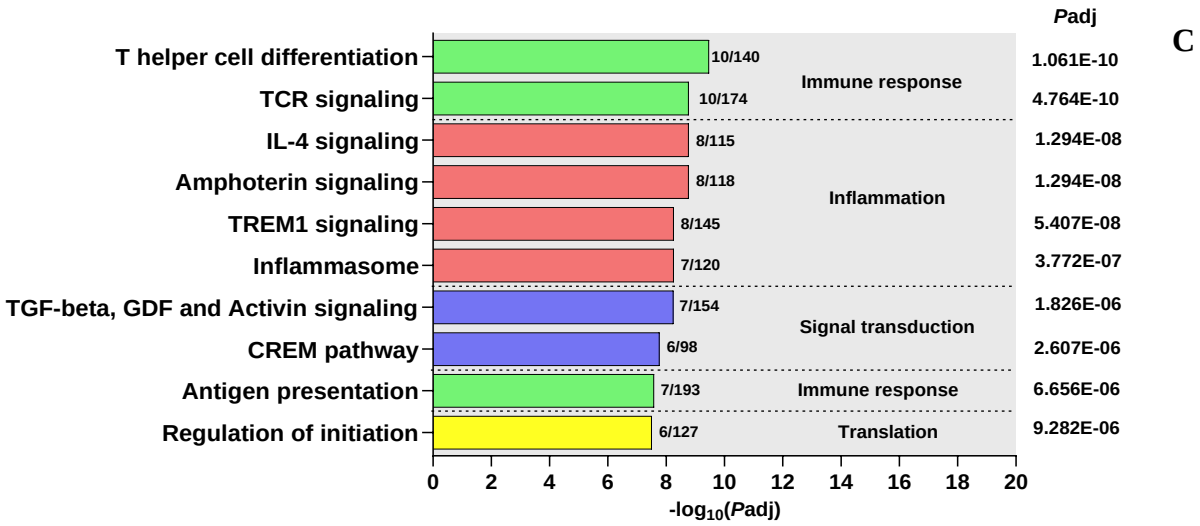


Figure 4.4: Unsupervised hierarchical clustering of RNA-seq data showing differentially expressed genes in the HSP60-HSP70-TLR2/4 pathway.
A) Heatmap showing expression values for each of the 47 DEGs selected based on Padj≤0.050 (rows) were normalised across all samples (columns). Dendrogram of hierarchical clustering of genes based on Euclidean distance of z-score data. Age distributions and sickle cell trait status are shown, along with the data matrix indicating the magnitude of up-regulated (red) and down-regulated (green) genes. Top 10-ranked process networks generated in MetaCore™ for (B) cluster 1 (down-regulated) DEGs and (C) cluster 2 (up-regulated) DEGs. Process networks are represented on the left Y-axis. The number of DEGs in the dataset is shown relative to the total number in the process network. The right Y-axis indicates the Padj value, and the X-axis shows the -log10(Padj)] values.

Common genes in multiple pathways were TLR4 and TLR2, MyD88, IRAK1/2, IKK-alpha (I-kappa-B kinase alpha), NF-kB (various forms including p100, p105, p52, and p50), NFKBIA (I-kappa-B alpha), AP-1 (Activator Protein 1), ICAM1, and IL-1 beta (**Table 4.7**), suggesting that children with SMA have an impaired immune response, weakened TLR-signaling, and a dysregulated inflammatory balance.

An overrepresentation of up-regulation of CD86, ERK1/2 (MAPK1), MEK1/2 (MAP2K1/2), TAK1 (MAP3K7), IRAK1/2, and MHC class II (**Table 4.7**), indicating an enhanced response to specific immune and inflammatory pathways associated with differentiation and activation of T helper cells and pathways associated with T cell receptors.

Table 4.7: Top process networks of DEGs in clusters 1 and 2 of the heatmap

Rank		Networks	Total	In Data	*P*adj	Network Objects from Active Data
Cluster 1 (down-regulated)	1	Inflammation-Amphoterin Signalling	118	21	1.192E-25	IL-1 beta, TLR2, NF-kB2 (p100), IRAK2, I-kB, NF-kB, NF-kB1 (p105), NFKBIA, IL-6, IRAK1/2, IKK-alpha, TLR4, NF-kB2 (p52), ICAM1, AP-1, p38alpha (MAPK14), p38 MAPK, NF-kB1 (p50), NF-kB p50/p50, MyD88, NF-kB p52/RelB
	2	Inflammation-Innate Inflammatory Response	181	23	3.163E-25	IL-1 beta, TLR2, sCD14, NF-kB2 (p100), IRAK2, I-kB, NF-kB, IRAK4, NFKBIA, IL-6, IRAK1/2, IKK-alpha, TLR4, NF-kB2 (p52), p38alpha (MAPK14), p38 MAPK, NF-kB1 (p50), NF-kB p50/p50, MyD88, NF-kB p52/RelB, CD14, MD-2, HSPA1A
	3	Inflammation-IL-10 anti-Inflammatory Response	87	17	2.124E-21	IL-1 beta, NF-kB2 (p100), I-kB, NF-kB, NFKBIA, IL-6, IRAK1/2, IKK-alpha, TLR4, NF-kB2 (p52), p38alpha (MAPK14), MEK6(MAP2K6), p38 MAPK, NF-kB1 (p50), NF-kB p50/p50, MyD88, NF-kB p52/RelB
	4	Immune Response-Antigen Presentation	193	19	2.308E-18	NF-kB2 (p100), HLA-E, I-kB, Beta-2-microglobulin, NF-kB, NF-kB1 (p105), NFKBIA, IKK-alpha, GRP78, NF-kB2 (p52), HLAB, ICAM1, HSP70, NF-kB1 (p50), RelB (NF-kB subunit), NF-kB p50/p50, NF-kB p52/RelB, HLAC, MHC class I
	5	Immune Response-Phagosome in Antigen Presentation	241	20	4.655E-18	NF-kB2 (p100), I-kB, Beta-2-microglobulin, NF-kB, NF-kB1 (p105), NFKBIA, IKK-alpha, GRP78, TLR4, NF-kB2 (p52), HLAB, p38alpha (MAPK14), p38 MAPK, HSP70, NF-kB1 (p50), NF-kB p50/p50, NF-kB p52/RelB, HLAC, MHC class I, CD14
	6	Inflammation-MIF Signalling	141	17	6.385E-18	IL-1 beta, NF-kB2 (p100), IRAK2, I-kB, NF-kB, NFKBIA, IL-6, IRAK1/2, IKK-alpha, TLR4, NF-kB2 (p52), ICAM1, NF-kB1 (p50), NF-kB p50/p50, MyD88, NF-kB p52/RelB, CD14
	7	Inflammation-Protein C Signalling	108	15	1.133E-16	IL-1 beta, NF-kB2 (p100), IRAK2, I-kB, NF-kB, NFKBIA, IL-6, IRAK1/2, IKK-alpha, TLR4, NF-kB2 (p52), NF-kB1 (p50), NF-kB p50/p50, MyD88, NF-kB p52/RelB
	8	Inflammation-TREM1 Signalling	145	16	2.872E-16	IL-1 beta, TLR2, IRAK2, I-kB, NF-kB, NFKBIA, IL-6, IRAK1/2, IKK-alpha, TLR4, ICAM1, p38alpha (MAPK14), p38 MAPK, MyD88, CD14, MD-2
	9	Cell Cycle-G1-S Interleukin Regulation	128	15	1.240E-15	IL-1 beta, NF-kB2 (p100), I-kB, NF-kB, NFKBIA, IRAK1/2, IKK-alpha, NF-kB2 (p52), AP-1, p38alpha (MAPK14), NF-kB1 (p50), RelB (NF-kB subunit), NF-kB p50/p50, NF-kB p52/RelB, JunB

Rank		Networks	Total	In Data	*P*adj	Network Objects from Active Data
	10	Immune Response-TCR Signalling	174	16	4.464E-15	NF-kB2 (p100), I-kB, NF-kB, NF-kB1 (p105), NFKBIA, IKK-alpha, NF-kB2 (p52), ICAM1, AP-1, p38alpha (MAPK14), MEK6(MAP2K6), p38 MAPK, NF-kB1 (p50), NF-kB p50/p50, NF-kB p52/RelB, MHC class I
Cluster 2 (up-regulated)	1	Immune Response-T Helper Cell Differentiation	140	10	1.061E-10	CD86, MEK3(MAP2K3), ERK1/2, MEK2(MAP2K2), TAB2, TAK1(MAP3K7), CD80, IRAK1/2, IRAK1, MHC class II
	2	Immune Response-TCR Signalling	174	10	4.764E-10	AP-1, CD86, MEK3(MAP2K3), ERK1/2, MEK2(MAP2K2), ERK2 (MAPK1), CD80, MEK1/2, Ubiquitin, MHC class II
	3	Inflammation-IL-4 Signalling	115	8	1.294E-08	CD86, ERK1/2, MEK2(MAP2K2), HLA-DPB1, ERK2 (MAPK1), CD80, MEK1/2, MHC class II
	4	Inflammation-Amphoterin Signalling	118	8	1.294E-08	AP-1, ERK1/2, MEK2(MAP2K2), ERK2 (MAPK1), TAK1(MAP3K7), IRAK1/2, IRAK1, MEK1/2
	5	Inflammation-TREM1 Signalling	145	8	5.407E-08	CD86, ERK1/2, MEK2(MAP2K2), ERK2 (MAPK1), TAK1(MAP3K7), IRAK1/2, IRAK1, MEK1/2
	6	Inflammation-Inflammasome	120	7	3.772E-07	AP-1, MEK3(MAP2K3), ERK1/2, TAB2, ERK2 (MAPK1), TAK1(MAP3K7), IRAK1
	7	Signal Transduction-TGF-beta, GDF and Activin Signalling	154	7	1.826E-06	AP-1, MEK3(MAP2K3), ERK1/2, TAB2, ERK2 (MAPK1), TAK1(MAP3K7), JunD
	8	Signal Transduction-CREM Pathway	98	6	2.607E-06	AP-1, ERK1/2, MEK2(MAP2K2), ERK2 (MAPK1), MEK1/2, Ubiquitin
	9	Immune Response-Antigen Presentation	193	7	6.656E-06	CD86, MHC class II beta chain, HLA-DPB1, CD80, HLA-DOA, HLA-DMA, MHC class II
	10	Translation-Regulation of Initiation	127	6	9.282E-06	MEK3(MAP2K3), ERK1/2, MEK2(MAP2K2), ERK2 (MAPK1), TAK1(MAP3K7), MEK1/2

Top 10-ranked process networks generated in MetaCore™ for cluster 1 (down-regulated) and cluster 2 (up-regulated) DEGs. Process networks are represented, along with the total number of genes in the network (total), DEGs in the dataset for SMA (in data), the rank of the network (*P*adj), and actual genes in the network (network objects from active data).

4.2.7 Differential Expression of Glutamine Transporters and Glutamine Synthetase in SMA

Since GLN is a critical regulator of HSP responses and must be actively taken up by cells to counter the stress response induced by conditions such as SMA, GLN transporters were explored. Of the 18 characterised human GLN transporter genes, 7 were differentially regulated between the non-SMA and SMA groups. SMA was characterised by up-regulation of 5 transporter genes: SLC6A19 ($\log_2$FC=4.17), SLC7A5 (2.54), SLC1A5 (2.49), SLC7A8 (0.69)

and SLC38A1 (0.35), and downregulation of two transporter genes: SLC38A2 (-0.28) and SLC38A3 (-1.38) (**Figure 4.6**).

It is known that GLN levels in leukocytes are also impacted by the levels and cellular activities of the glutamine synthetase (i.e., the glutamate-ammonia ligase, encoded by *GLUL*) and glutaminase 1 (GLS1) and GLS2. *GLUL* was significantly up-regulated in children with SMA (log_2FC=1.26, *P*adj=1.82E-10). However, differing gene expression profiles were not witnessed for either *GLS1* (log_2FC=-0.23, *P*adj=0.062) or *GSL2* (log_2FC=0.14, *P*adj=0.778) (**Figure 4.6**). Differential expressions of all glutamine transporter genes are shown in **Table 4.8**.

Collectively, the findings indicate that children with SMA exhibit upregulation of GLN transporters to boost cellular GLN absorption and increased transcripts for a critical enzyme (GLUL) that increases GLN production.

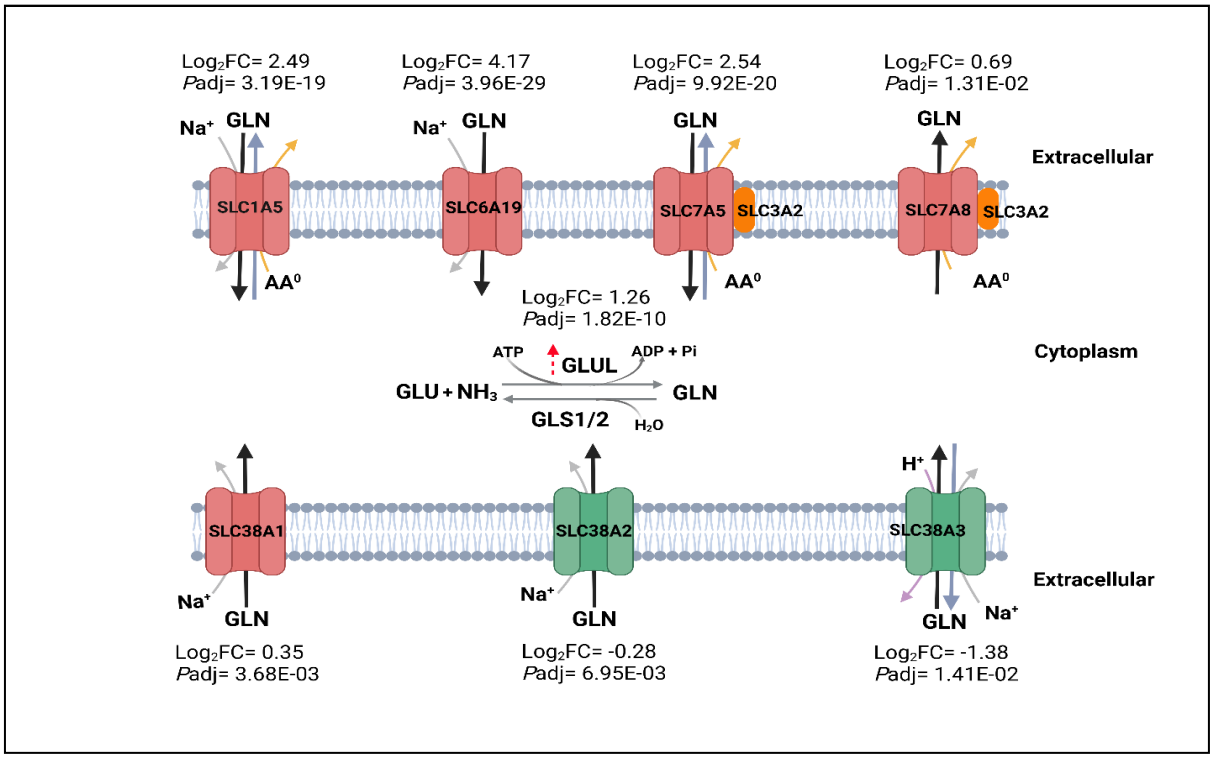


Figure 4.5 Model showing significantly dysregulated glutamine transporters and glutamine synthetase.
The RNA-seq analyses from 66 children [non-SMA (n=41) and SMA (n=25)] containing *P*adj genes at <0.05 revealed dysregulation in seven human GLN transporters in children with SMA, five up-regulated (red) and two down-regulated (green). Functionality of both SLC7A5 and SLC7A8 relies on the formation of a complex with a chaperone, SLC3A2 (orange). Directionality of the arrows indicates flux of GLN (black), Na^+ (gray), neutral amino acids (orange), and H^+ (purple). Conversion of GLU to GLN is catalysed by GLUL (dotted red arrow indicating up-regulation), whereas conversion of GLN to GLU is catalysed by GLS1/2 (not significantly altered). The Log_2FC (for Log_2FoldChange) and *P*adj are shown

for the significant DEGs. Abbreviations and symbols: GLN: glutamine; AA^0: neutral amino acids; Na^+: sodium ion; H^+: hydrogen ion; Glu: glutamate; GLUL: glutamate-ammonia ligase (i.e., glutamine synthetase); GLS1/2: Glutaminase1/2. Created in BioRender.com.

Table 4.8: Differential expression of transcripts of glutamine transporters and glutamine-metabolizing enzymes in children with SMA

	Gene	Log_2foldchange	*P*adj
GLN Transporters	SLC6A19	4.17	**3.96E-29**
	SLC7A5	2.54	**9.92E-20**
	SLC1A5	2.49	**3.19E-19**
	SLC7A8	0.69	**1.31E-02**
	SLC38A1	0.35	**3.68E-03**
	SLC38A2	-0.28	**6.95E-03**
	SLC38A3	-1.38	**1.41E-02**
	SLC6A14	-0.86	0.197
	SLC7A6	0.21	0.068
	SLC7A7	0.11	0.665
	SLC7A9	-0.44	0.320
	SLC38A4	-0.25	0.656
	SLC38A5	-0.14	0.729
	SLC38A6	0.19	0.391
	SLC38A7	0.06	0.699
	SLC38A8	-0.79	0.202
	SLC38A9	-0.15	0.175
	SLC38A10	-0.09	0.513
GLN Enz	GLUL	1.26	**1.82E-10**
	GLS (GLS1)	-0.23	0.062
	GLS2	0.14	0.778

Student's t-test was used to compare the mean gene expression level between non-SMA (Hb≥6.0 g/dL, n=41) and SMA (Hb<6.0 g/dL, n=25). The Log_2foldchange was defined as the ratio of the mean gene expression level in SMA to that in non-SMA. Bold indicates statistical significance at *P*adj≤0.050.

4.3 Objective Three

4.3.1 Demographic, Clinical and Laboratory Characteristics of Study Participants at Enrollment

Age- and sex-matched children without HIV-1 and bacteremia co-infections, presenting with peripheral *P. falciparum* parasitemia (any density) were categorised into two groups based on their Hb levels: Hb≥6.0 g/dL (non-SMA, n=40) and Hb<6.0 g/dL (SMA, n=20). The demographic, clinical and laboratory characteristics of these study participants are shown in **Table 4.9**. Children with SMA presented at the hospital with lower axillary temperature (*P*=0.030) and comparable glucose levels (*P*=0.516). As expected, based on Hb concentrations, children with SMA had lower levels of haematocrit (*P*=3.539E-10) and RBCs (*P*=4.330E-10). Additionally, SMA patients presented with elevated red blood cell distribution width (*P*=0.003) and mean corpuscular volume (*P*=0.003). Similarly, white blood cells (*P*=0.002) and lymphocytes (*P*=1.137E-04) were elevated in SMA. Comparable levels for the other haematological variables were observed: mean corpuscular haemoglobin concentrations (*P*=0.051), platelets (*P*=0.981), monocytes (*P*=0.063), neutrophil (*P*=0.780) and granulocyte (*P*=0.540) counts. Parasitological indices (i.e., parasite density and stratification based on levels) did not show significant variation across the two study groups (*P*=0.520 and *P*=0.377,

respectively). Moreover, the distribution of sickle-cell trait status was also comparable between the groups (*P*=0.079).

Table 4.9: Admission demographic, clinical and laboratory characteristics of study participants

Characteristics	Non-SMA (Hb≥6.0g/dL)	SMA (Hb<6.0g/dL)	*p*-value
No. of participants (n=60)	40	20	
Gender, n (%)			
Male	19 (47.5)	13 (52.0)	1.000[a]
Female	21 (52.5)	12 (48.0)	
Age, months	23.5 (22.3)	22.5 (27.5)	0.724[b]
Glucose, mmol/L	5.0 (2.0)	5.0 (2.0)	0.516[b]
Admission temperature °C	38.0 (1.2)	37.7 (0.8)	**0.030**[b]
Hematological Parameters			
Haemoglobin, g/dL	9.9 (1.4)	4.6 (1.0)	NA
Haematocrit, %	30.0 (5.4)	14.7 (2.7)	**3.539E-10**[b]
Red blood cells, × 10^6/µL	4.3 (0.9)	1.9 (0.8)	**4.330E-10**[b]
Red cell distribution width, %	18.5 (2.8)	21.6 (7.9)	**0.003**[b]
Mean corpuscular volume, fL	69.5 (8.4)	80.0 (28.8)	**0.003**[b]
Mean corpuscular haemoglobin, pg	22.7 (4.2)	25.9 (9.2)	0.051[b]
Platelets, ×10^3/µL	144.0 (99.3)	100.0 (241.0)	0.981[b]
Platelet distribution width, %	16.5 (1.2)	17.2 (1.0)	0.441[b]
Mean platelet volume, fL	8.3 (2.5)	8.0 (2.4)	0.190[b]
WBCs, ×10^3/µL	11.0 (7.0)	21.0 (10.0)	**0.002**[b]
Lymphocytes, ×10^3/µL	3.6 (2.4)	11.5 (14.3)	**1.137E-04**[b]
Monocytes, ×10^3/µL	0.7 (0.8)	1.4 (1.3)	0.063[b]
Neutrophils, ×10^3/µL	5.3 (3.4)	5.2 (8.1)	0.780[b]
Granulocytes, ×10^3/µL	6.7 (3.0)	8.6 (5.1)	0.540[b]
Parasitological Indices			
Parasite density, MPS/µL	59,417 (81,968)	22,570 (72,190)	0.520[b]
Low (1 – 5,000), n (%)	6 (15.0)	5 (25.0)	
Moderate (5001 – 50,000), n (%)	12 (30.0)	8 (40.0)	0.377[a]
High (50,001 – 100,000), n (%)	14 (35.0)	3 (15.0)	
Hyper (>100,001), n (%)	8 (20.0)	4 (20.0)	
Genetic Variants			
Sickle cell genotypes, n (%)			
HbAA	35 (87.5)	14 (70.0)	
HbAS	4 (10.0)	2 (10.0)	0.079[a]
HbSS	1(2.5)	4 (20.0)	

Data are the median (interquartile range; IQR) unless otherwise noted. Children (n=60) presenting with malaria at SCRH were stratified based on haemoglobin (Hb) levels: non-severe malaria anaemia (non-SMA; Hb≥6.0 g/dL, n=40) or severe malarial anaemia (SMA; Hb<6.0 g/dL, n=20). [a]Fisher's exact test with exact *P*-values for homogeneity and [b]Mann-Whitney-U test were used to compare the non-SMA and SMA groups. Statistical significance was set at $P \leq 0.050$, and significant *P*-values were indicated in bold.

Abbreviations: MPS - malaria parasites. HbAA-hemoglobin AA, HbAS- haemoglobin AS, HbSS-haemoglobin SS.

4.3.2 Distribution of Differentially Expressed *P. falciparum* Genes in SMA Relative to Non-SMA

The distribution of 3,155 annotated *P. falciparum* genes was illustrated using a volcano plot (**Figure 4.7**). Among them, 566 genes were DEGs, with 499 up-regulated and 67 down-regulated.

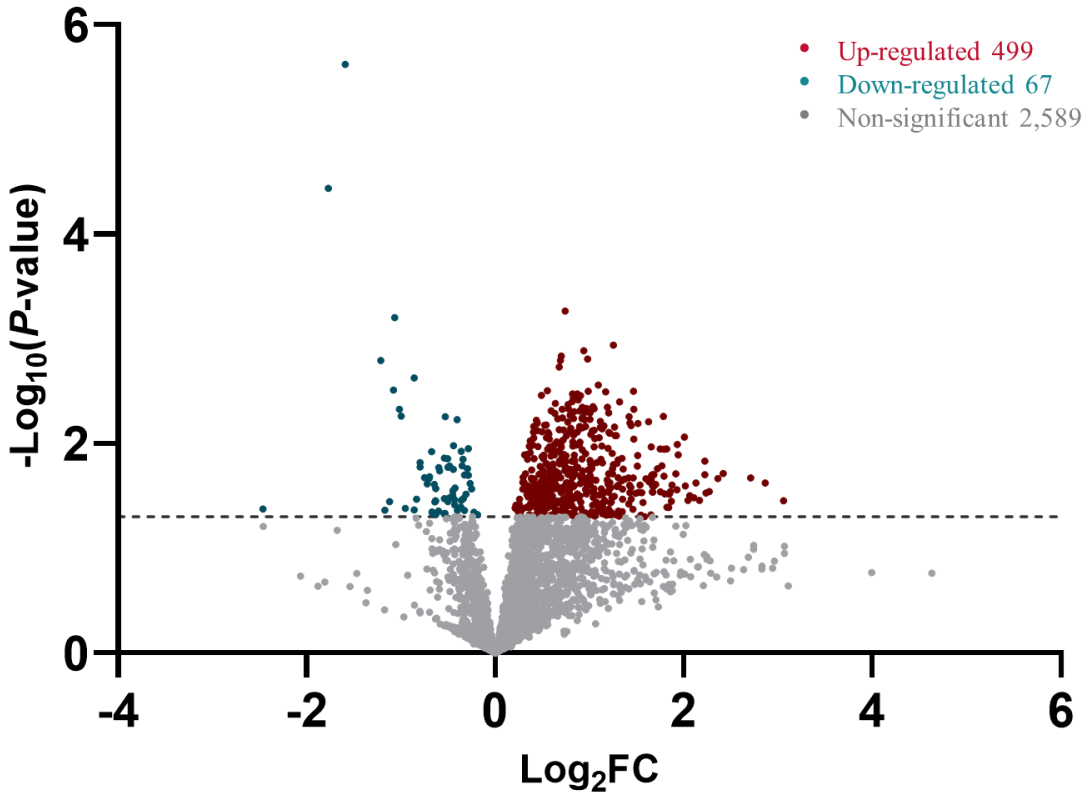


Figure 4.6 Volcano plot showing the magnitude and significance of differentially expressed *P. falciparum* genes in patients with SMA relative to non-SMA.
499 and 67 P. falciparum genes were significantly up- and down-regulated, respectively, while 2,589 genes were not significantly differentially expressed in Kenyan children presenting with non-SMA and SMA. Each dot represents a gene, grey dots indicate no significant difference in genes, red dots indicate up-regulated DEGs and turquoise dots indicate down-regulated DEGs.

The top 3 down-regulated genes include PfKE01_040008500 (surface-associated interspersed protein 4.1), PfKE01_080024800 (perforin-like protein 5) and PfKE01_140017300 (p1/s1 nuclease, putative). In contrast, the most up-regulated genes include PfKE01_120041000 (conserved Plasmodium protein, unknown function), PfKE01_130026100 (type I signal

peptidase) and PfKE01_050018000 (deoxyribodipyrimidine photo-lyase, putative). The rest of the 10 most up- or down-regulated genes are listed in **Table 4.10**.

Table 4.10 10 Most down-regulated and up-regulated P. falciparum genes in SMA vs. non-SMA

Down-regulated	Gene description	Log_2FC
PfKE01_040008500	surface-associated interspersed protein 4.1	-2.464396963
PfKE01_080024800	perforin-like protein 5	-1.769091813
PfKE01_140017300	p1/s1 nuclease, putative	-1.590093646
PfKE01_080020200	conserved *Plasmodium* protein, unknown function	-1.21357049
PfKE01_030005700	acyl-CoA synthetase	-1.171816811
PfKE01_070034000	erythrocyte binding antigen-175	-1.119215846
PfKE01_130062600	protein kinase 5	-1.079821323
PfKE01_050029600	structural maintenance of chromosomes protein 6, putative	-1.066974281
PfKE01_140010000	conserved *Plasmodium* protein, unknown function	-1.015948874
PfKE01_140006200	acidic terminal segments, variant surface antigen of PfEMP1, putative	-0.997865734
Up-regulated	**Gene description**	**Log_2FC**
PfKE01_120041000	conserved *Plasmodium* protein, unknown function	3.057391101
PfKE01_130026100	type I signal peptidase	2.861766756
PfKE01_050018000	deoxyribodipyrimidine photo-lyase, putative	2.710313939
PfKE01_100027300	conserved *Plasmodium* protein (10b antigen), unknown function	2.417452231
PfKE01_080022200	chaperone protein ClpB1	2.36053819
PfKE01_140040600	ataxin-2-like protein, putative	2.268725128
PfKE01_070024100	conserved *Plasmodium* protein, unknown function	2.235476994
PfKE01_050014600	conserved *Plasmodium* protein, unknown function	2.221898286
PfKE01_040023000	glutamyl-tRNA (Gln) amidotransferase subunit A	2.221063246
PfKE01_080026300	2-oxoglutarate dehydrogenase E1 component	2.166511279

The 10 most down-regulated and up-regulated genes in SMA relative to non-SMA and their log_2FC.

4.3.3 Principal Component Analysis (PCA) of DEGs

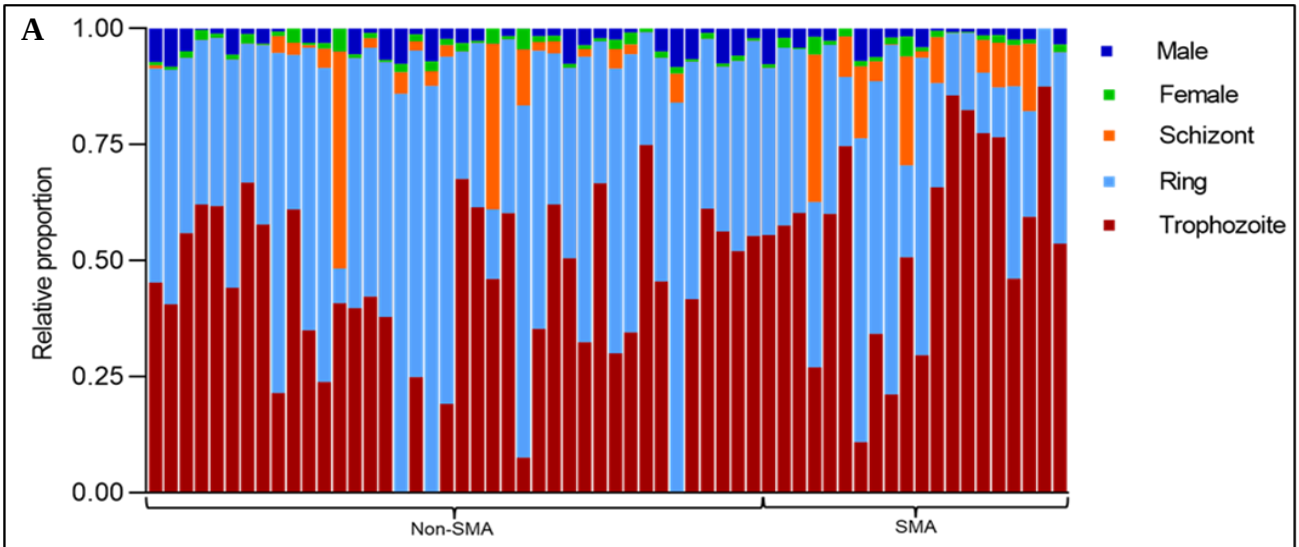
A PCA was performed on the transcriptional profiles of the annotated *P. falciparum* genes (n=3,155) to explore the global structure and variability in the dataset and on DEGs to capture the variance associated with differential expression in the blood of children with SMA and non-SMA (**Figure 4.8**). The PCA revealed distinct clustering patterns between SMA and non-SMA and overlapping confidence ellipses of the two groups in the plot, indicating some similarity between the groups' gene expression profiles. The first two principal components of the PCA of all annotated *P. falciparum* genes and 566 DEGs explained 48.89% and 63.74% of the total variance in the dataset, respectively.

4.3.4 *P. falciparum* Gene Expression Deconvolution Analysis

Gene expression deconvolution revealed relative proportions of parasite developmental stages in peripheral blood from the children in the study (**Figure 4.8A**). The relative proportion of *P. falciparum* trophozoite stage genes was marginally higher in the SMA group (P=0.052), while

that of the ring stage was lower in SMA (*P*=0.008). Schizont, male and female gametocyte stages did not significantly differ between the study groups (**Figure 4.8B**). However, further analysis to compare the relative proportions between male and female gametocytes across all the parasitaemic patients showed a higher proportion of male gametocytes in the study participants (*P*=2.170E-6) (**Figure 4.8C**).

A



B

***P. falciparum* stage**	**non-SMA (Hb≥6.0 g/dL)**	**SMA (Hb<6.0 g/dL)**	***p*-value[a]**
Trophozoite	0.447(0.279)	0.585(0.389)	0.052
Ring	0.472(0.257)	0.355(0.253)	**0.008**
Schizont	0.000(0.026)	0.028(0.098)	0.096
Female gametocyte	0.012(0.012)	0.011(0.008)	0.695
Male gametocyte	0.025(0.048)	0.020(0.029)	0.470

***P. falciparum* gametocytes**		***p*-value[a]**
Female	**Male**	
0.011(0.010)	0.023(0.045)	**2.170E-4**

Figure 4.8: *P. falciparum* gene expression deconvolution analysis and relative proportions of *P. falciparum* developmental stages in non-SMA and SMA.
A) The figure shows the relative proportions of the different P. falciparum developmental stages, imputed using the expression of significant DEGs. B) Relative proportions of P. falciparum developmental stages in non-SMA and SMA. Relative proportions of P. falciparum developmental stages in non-SMA (n=40) and SMA (n=20) presented as median (interquartile range, IQR). [a]Mann-Whitney U test. *P*-values <0.050 were considered significant and are indicated in bold. C) Relative proportions of P. falciparum gametocytes across all samples (n=60) presented as median (interquartile range, IQR). *P*-values <0.050 were considered significant and are indicated in bold.

4.3.5 Weighted gene co-expression network analysis (WGCNA) of P. falciparum DEGs

4.3.5.1 Co-expression Analysis of P. falciparum DEGs

WGCNA of the *P. falciparum* DEGs with a soft threshold of 5 and a minimum module size of 20 revealed a WGCNA network of 559 co-expressed genes in 5 modules: Brown (112 DEGs), Yellow (82 DEGs), Blue (125 DEGs), Turquoise (160 DEGs) and Green (80 DEGs) (**Figure 4.10A**). The entire network consisting of the 5 modules was then enriched for molecular functions, biological processes, cellular components and KEGG pathways (**Figure 4.10B-E**).

The top 10 significant (FDR ≤0.050) *P. falciparum* molecular function pathways include: GO:0004298 threonine-type endopeptidase activity (FDR=3.78E-05, 11/15 genes), GO:0070003 threonine-type peptidase activity (FDR=3.78E-05, 11/15 genes), GO:0046983 protein dimerisation activity (FDR=2.25E-02, 16/46 genes), GO:0004175 endopeptidase activity (FDR=2.59E-02, 27/103 genes), GO:0004812 aminoacyl-tRNA ligase activity (FDR=2.80E-02, 14/40 genes), GO:0016875 ligase activity forming carbon-oxygen bonds (FDR=2.80E-02, 14/40 genes), GO:0008233 peptidase activity (FDR=2.85E-02, 36/158 genes), GO:0016787 hydrolase activity (FDR=4.21E-02, 116/695 genes), GO:0016887 ATP hydrolysis activity (FDR=4.21E-02, 30/128 genes) and GO:0042802 identical protein binding (FDR=4.21E-02, 9/21 genes) (**Figure 4.10B**).

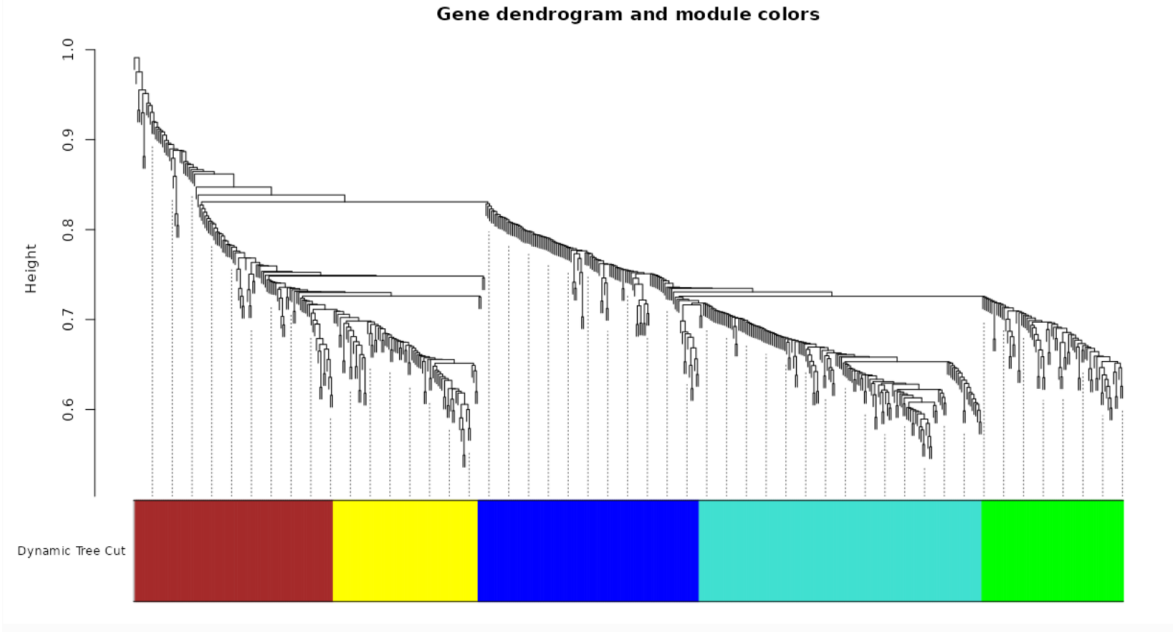
Moreover, the top 10 significant *P. falciparum* biological processes include: GO:0010499 proteasomal ubiquitin-independent protein catabolic process (FDR=4.78E-06, 12/15 genes), GO:0006511 ubiquitin-dependent protein catabolic process (FDR=2.24E-05, 37/121 genes), GO:0010498 proteasomal protein catabolic process (FDR=2.24E-05, 30/86 genes), GO:0019941 modification-dependent protein catabolic process (FDR=3.44E-05, 37/124 genes), GO:0009987 cellular process (FDR=4.02E-05, 356/2670 genes), GO:0043161 proteasome-mediated ubiquitin-dependent protein catabolic process (FDR=4.02E-05, 28/82 genes), GO:0043632 modification-dependent macromolecule catabolic process (FDR=6.08E-05, 37/129 genes), GO:0044257 cellular protein catabolic process (FDR=7.20E-05, 40/147 genes), GO:0051603 proteolysis involved in cellular protein catabolic process (FDR=7.20E-05, 40/147 genes) and GO:0030163 protein catabolic process (FDR=1.15E-05, 42/161 genes) (**Figure 4.10C**).

Enriched *P. falciparum* cellular components included: GO:0000502 proteasome complex (FDR=1.71E-12, 26/43 genes), GO:1905369 endopeptidase complex (FDR=3.28E-11, 26/48 genes), GO:1905368 peptidase complex (FDR=1.69E-10, 27/55 genes), GO:0005622 intracellular anatomical structure (FDR=3.26E-08, 339/2,587 genes), GO:0005737 cytoplasm (FDR=1.41E-06, 261/1,888 genes), GO:0005829 cytosol (FDR=1.41E-06, 73/355 genes), GO:0005839 proteasome core complex (FDR=1.41E-06, 11/15 genes), GO:0032991 protein-

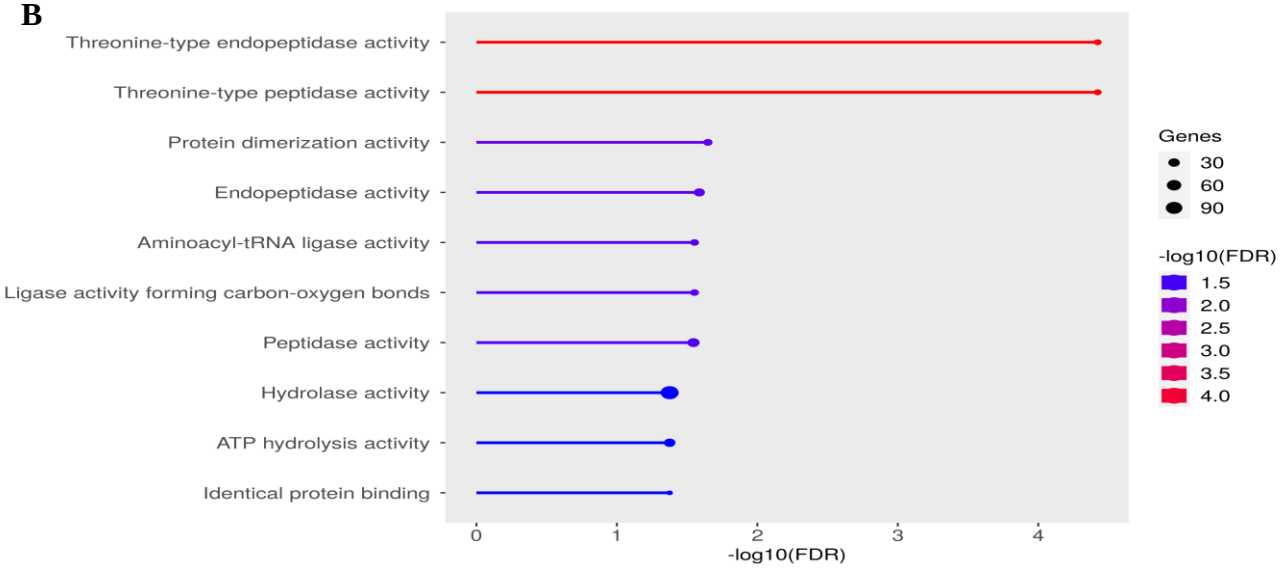
containing complex (FDR=1.04E-05, 170/1,125 genes), GO:0140535 intracellular protein-containing complex (FDR=1.04E-05, 46/195 genes) and GO:0022624 proteasome accessory complex (FDR=3.47E-04, 10/19 genes) (**Figure 4.10D**).

Additional enrichment analyses for the DEGs revealed three significant KEGG pathways: pfa03050 Proteasome (FDR=6.06E-08, 19/34 genes), pfa00970 Aminoacyl-tRNA biosynthesis (FDR=1.39E-03, 14/36 genes) and pfa00564 Glycerophospholipid metabolism (FDR=6.32E-03, 9/20 genes) (**Figure 4.10E**).

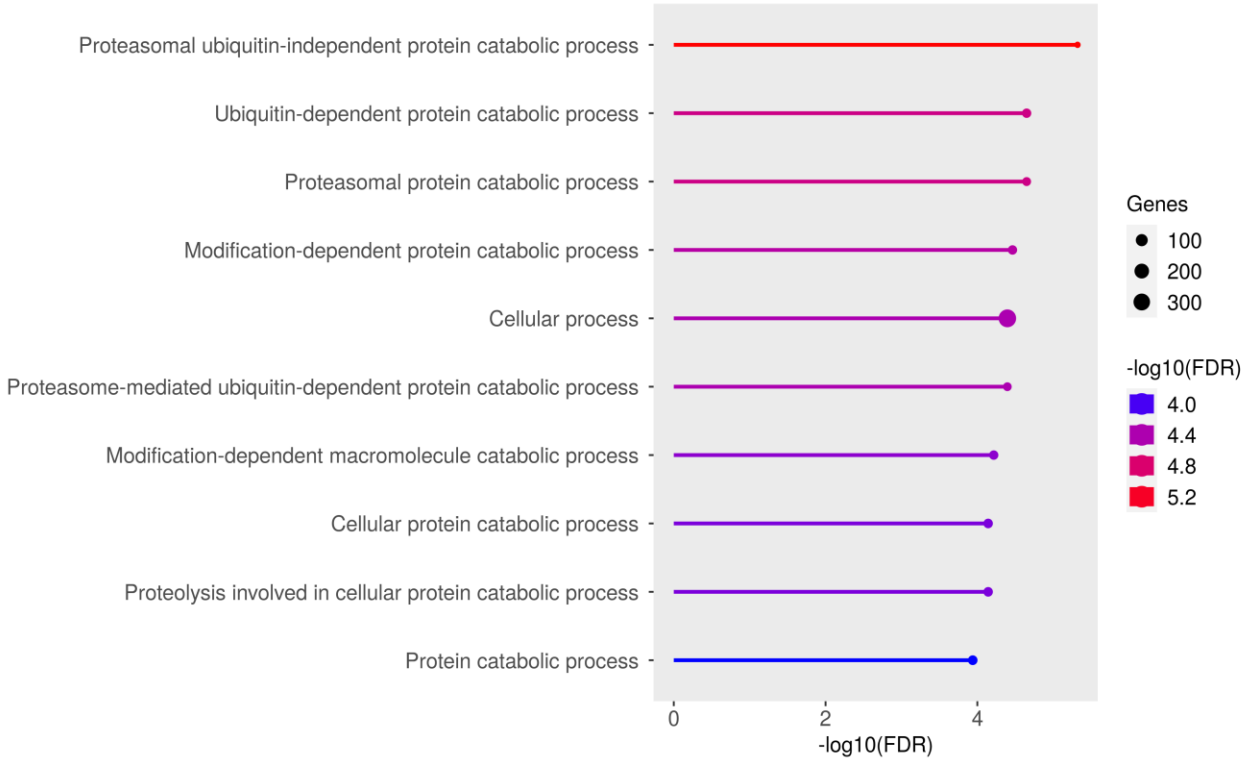
A



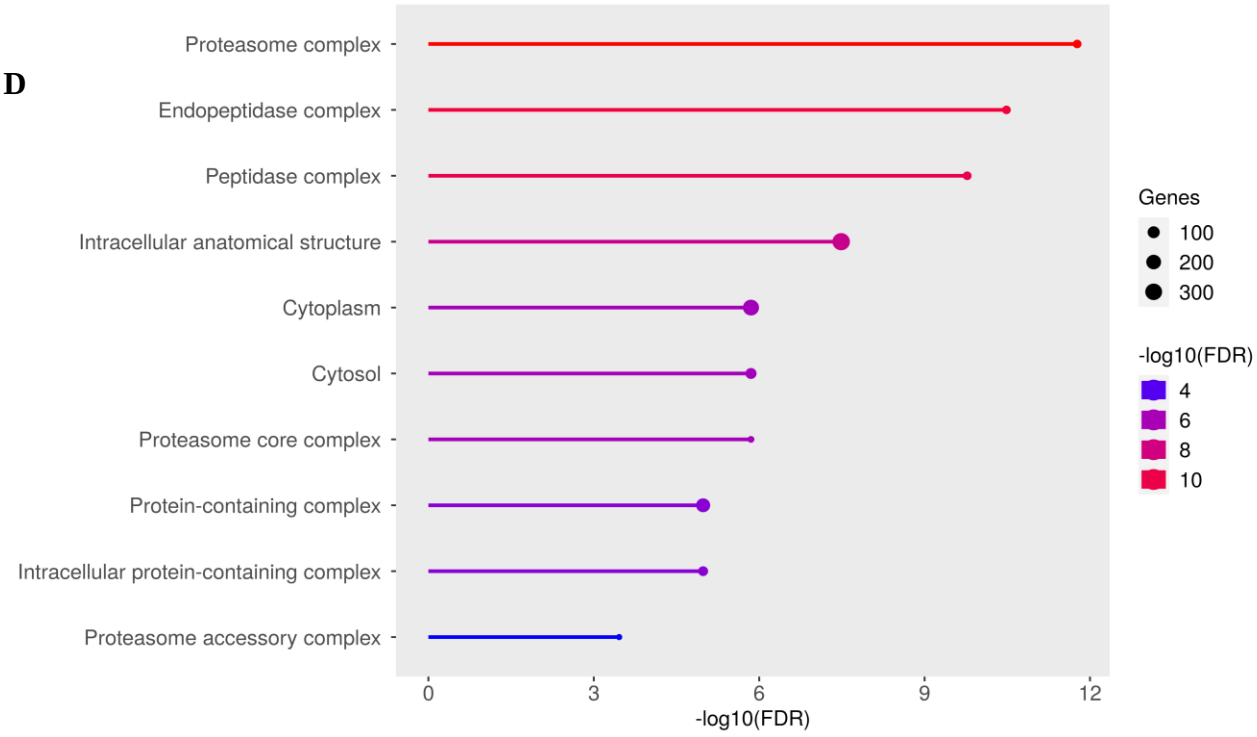
B



C


Proteasomal ubiquitin-independent protein catabolic process
Ubiquitin-dependent protein catabolic process
Proteasomal protein catabolic process
Modification-dependent protein catabolic process
Cellular process
Proteasome-mediated ubiquitin-dependent protein catabolic process
Modification-dependent macromolecule catabolic process
Cellular protein catabolic process
Proteolysis involved in cellular protein catabolic process
Protein catabolic process
0
2
4
-log10(FDR)
Genes
100
200
300
-log10(FDR)
4.0
4.4
4.8
5.2

D


Proteasome complex
Endopeptidase complex
Peptidase complex
Intracellular anatomical structure
Cytoplasm
Cytosol
Proteasome core complex
Protein-containing complex
Intracellular protein-containing complex
Proteasome accessory complex
0
3
6
9
12
-log10(FDR)
Genes
100
200
300
-log10(FDR)
4
6
8
10

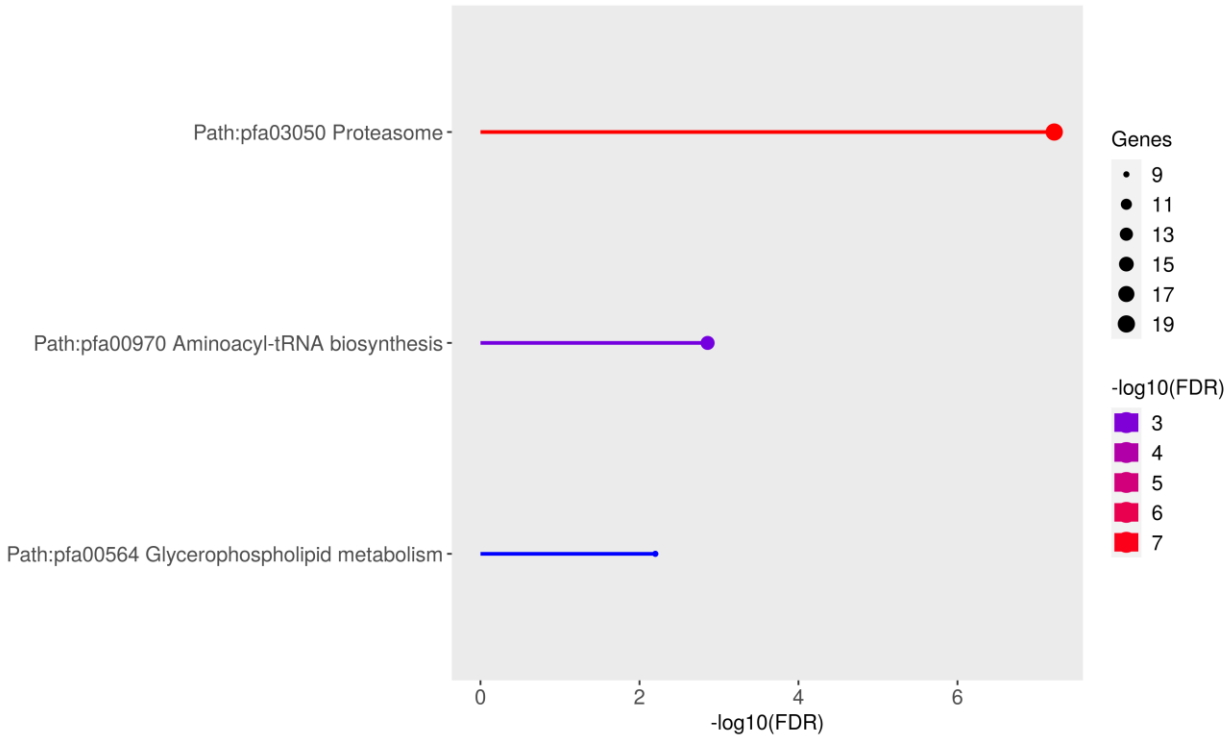


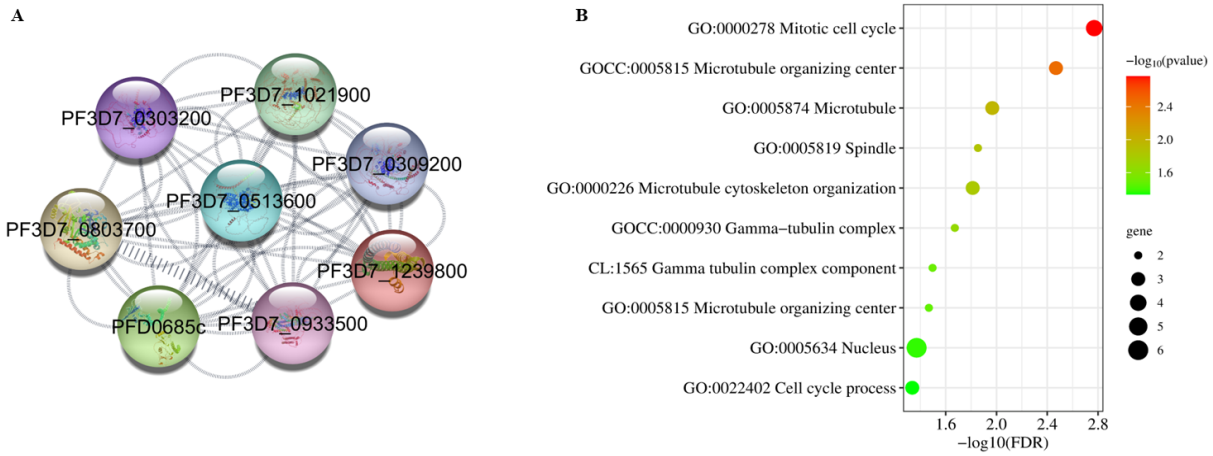
Figure 4.9: Identification of co-expression modules using WGCNA and enrichment analysis of WGCNA networks.
A) Gene cluster dendrogram obtained by hierarchical clustering of co-expressed genes. WGCNA of DEGs yielded 5 gene co-expression network modules represented by colours: brown, yellow, blue, turquoise and green. The branches refer to clusters of genes that are highly connected, and each vertical line represents a single gene. The horizontal bar represents the co-expression modules determined by the Dynamic Tree Cut algorithm, which contains a group of highly correlated genes. **B)** Molecular functions, **C)** Biological processes, **D)** Cellular components and **E)** KEGG pathways.

4.3.5.2 WGCNA Module Analysis

To gain further insight into the gene co-expression networks of each module in the WGCNA network, the network files containing the weight of interaction between genes in a module were imported into CytoScape. Module network construction in Cytoscape showed the interconnected genes (nodes) in the WGCNA modules: 8 nodes and 56 edges for the brown module (**Figure 4.11A**) and 10 nodes and 90 edges for each of the yellow (**Figure 4.12A**), blue (**Figure 4.13A**), turquoise (**Figure 4.14A**) and green modules (**Figure 4.15A**). In addition, the most interconnected node (seed node) in each module was identified: brown (PF3D7_1239800- Conserved *Plasmodium* protein, unknown function, **Figure 4.11C**), yellow (PF3D7_1208900- Protein phosphatase PPM11, putative, **Figure 4.12C**), blue (PF3D7_0322900- 40S ribosomal protein S3A, putative, **Figure 4.13C**), turquoise (PF3D7_0205900- 26S proteasome regulatory

subunit RPN1, putative, **Figure 4.14C**) and green (PF3D7_1332900- Isoleucine--tRNA ligase, putative, **Figure 4.15C**).

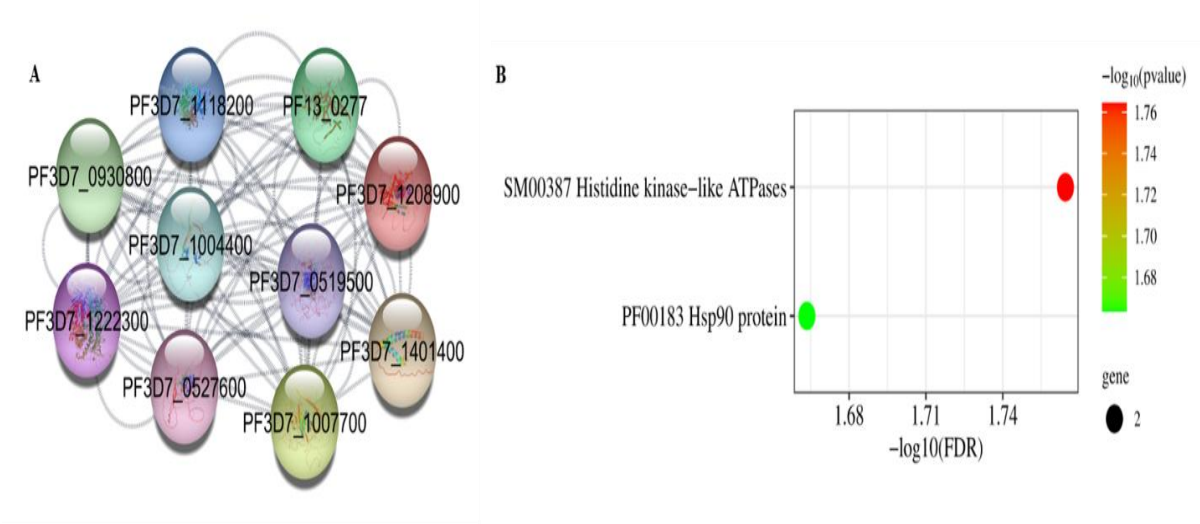
Further analysis revealed that the brown module was enriched for pathways/processes associated with cell cycle/division, microtubule function and nuclear processes (**Figure 4.10B**); the yellow module was enriched for histidine kinase-like ATPases and HSP90 protein pathways (**Figure 4.11B**); the blue module was enriched in pathways involved in ribosomal subunit (40S and 60S) formation and assembly, translational regulation, co-translational protein targeting to the membrane and ribosomal RNA (rRNA) processing (**Figure 4.12B**); the turquoise module was enriched in the parasite proteasome complex (**Figure 4.13B**); and the green module was enriched in pathways associated with energy metabolism, immune evasion and protein synthesis (**Figure 4.14B**).



C

Node	PlasmoDB description	Log2FC	*p*-value
PF3D7_0933500	Gamma-tubulin complex component, putative	1.79	**2.32E-02**
PF3D7_0303200	Lipin, putative	1.67	**1.99E-02**
PF3D7_0309200	Aurora-related kinase ARK2, putative	1.68	**2.56E-02**
PF3D7_0513600	Deoxyribodipyrimidine photo-lyase, putative	2.71	**2.14E-02**
PF3D7_1021900	PHAX domain-containing protein, putative	2.42	**1.94E-02**
PFD0685c (PF3D7_0414000)	Structural maintenance of chromosomes protein 3	1.15	**4.55E-02**
PF3D7_0803700	Tubulin gamma chain	1.52	**2.93E-02**
PF3D7_1239800	Conserved Plasmodium protein, unknown function	1.79	**1.14E-02***

Figure 4.10: Brown module
A) Network showing the 8 nodes and 56 edges of the brown WGCNA module. B) Functional enrichment analysis of the co-expressed genes in the brown module performed in CytoScape using STRING. The x-axis represents the -log10 of the FDR-adjusted p-value, while the y-axis shows the enriched terms. C) Module node description based on PlasmoDB and their differential expression in SMA relative to non-SMA. * Indicates the most interconnected node in the module (seed node). Enrichment identifiers: GO- gene ontology, GOCC- gene ontology compartments and CL- STRING clusters databases.

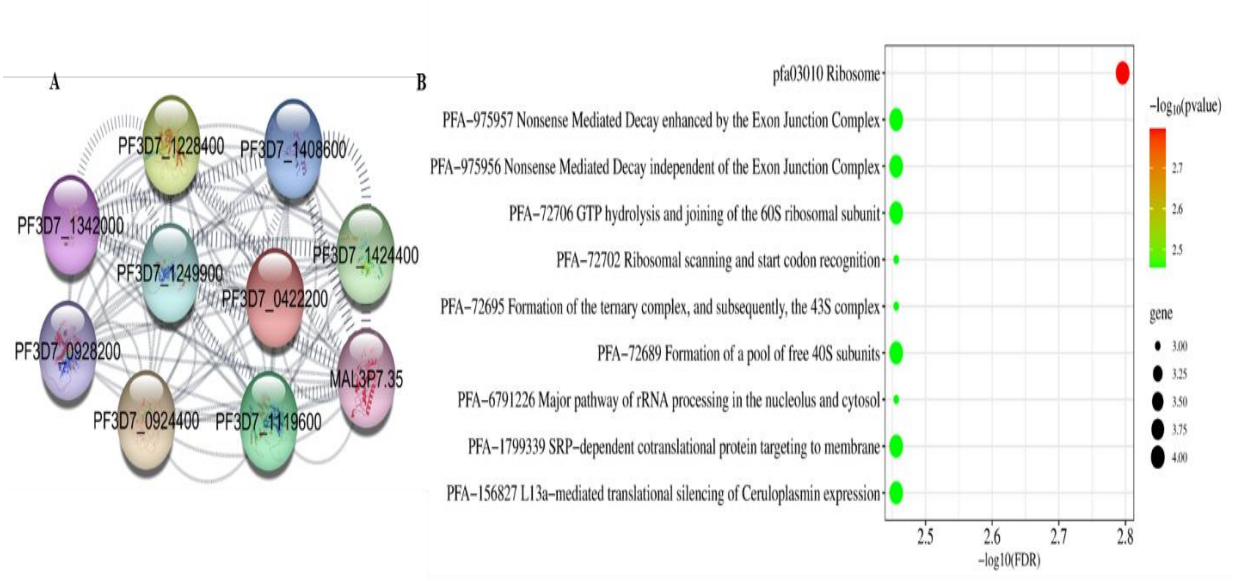


C

Node	PlasmoDB description	Log2FC	*p*-value
PF3D7_0527600	Conserved Plasmodium protein, unknown function	1.01	**4.79E-02**
PF3D7_1222300	Endoplasmin, putative	1.34	**2.25E-02**
PF3D7_0519500	CCR4 domain-containing protein 1, putative	1.70	**2.46E-02**
PF3D7_1118200	Heat shock protein 90, putative	1.15	**2.89E-02**
PF3D7_1004400	RNA-binding protein, putative	1.68	**1.99E-02**
PF13_0277 (PF3D7_1353300)	Uncharacterized protein PF13_0277	1.18	**4.85E-02**
PF3D7_0930800	Regulator of chromosome condensation, putative	1.42	**3.77E-02**
PF3D7_1007700	AP2 domain transcription factor AP2-I	1.67	**2.52E-02**
PF3D7_1208900	Protein phosphatase PPM11, putative	1.37	**2.18E-02***
PF3D7_1401400	Early transcribed membrane protein 14.1	-0.54	**3.37E-02**

Figure 4.11: Yellow module

A) Network showing the yellow WGCNA module consisting of 10 nodes and 90 edges. B) Functional enrichment analysis of the co-expressed genes in the yellow module was performed in CytoScape using STRING. The x-axis represents the -log10 of the FDR-adjusted *p*-value, while the y-axis shows the enriched terms. C) Module node description based on PlasmoDB and their differential expression in SMA relative to non-SMA. * Indicates the most interconnected node in the module (seed node). Enrichment identifiers: SM- Simple Modular Architecture Research Tool (SMART) and PF- Protein FAMilies databases.

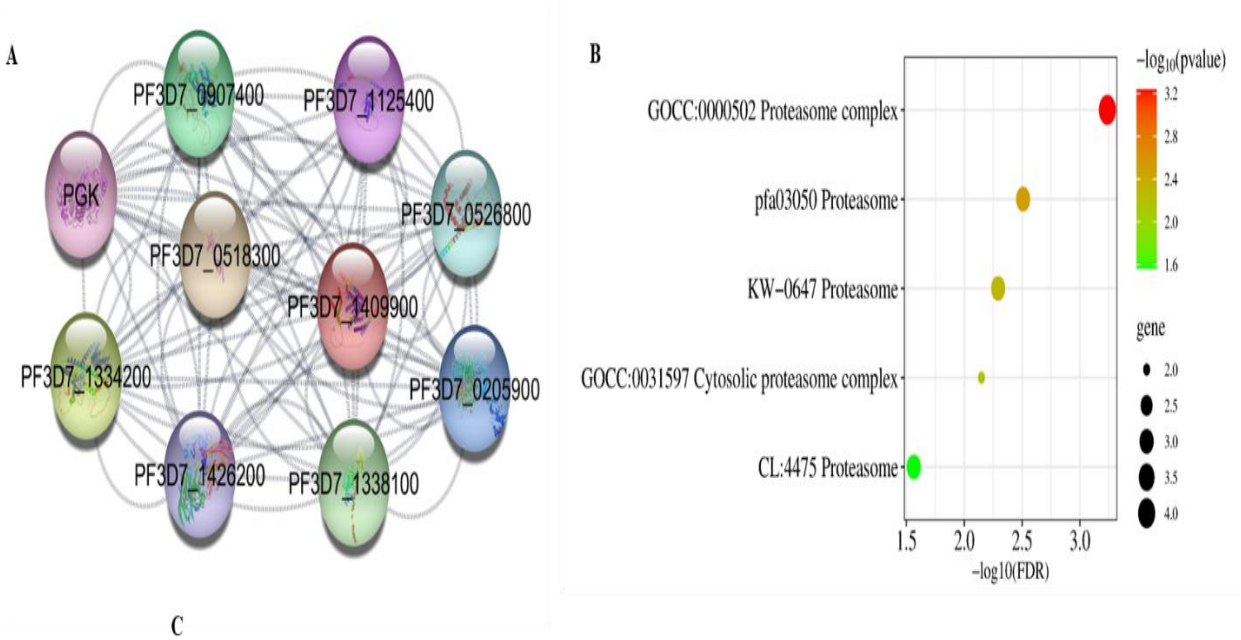


C

Node	PlasmoDB description	Log2FC	p-value
PF3D7_1249900	Ribosomal RNA small subunit methyltransferase A2, putative	0.66	**1.19E-02**
PF3D7_1228400	Conserved Plasmodium protein, unknown function	0.52	**6.69E-03**
PF3D7_1119600	ATP-dependent zinc metalloprotease FTSH, putative	0.87	**3.35E-03**
PF3D7_1408600	40S ribosomal protein S8e, putative	0.35	**3.23E-02**
PF3D7_1342000	40S ribosomal protein S6	0.44	**6.01E-03**
PF3D7_0924400	Conserved Plasmodium protein, unknown function	0.57	**8.13E-03**
MAL3P7.35 (PF3D7_0322900)	40S ribosomal protein S3A, putative	0.31	**1.28E-02***
PF3D7_0928200	Conserved Plasmodium protein, unknown function	0.71	**4.73E-03**
PF3D7_1424400	60S ribosomal protein L7-3, putative	0.36	**2.74E-02**
PF3D7_0422200	Erythrocyte membrane-associated antigen	1.08	**1.52E-02**

Figure 4.12: Blue module

A) Network showing the 10 nodes and 90 edges of the blue WGCNA module. B) Functional enrichment analysis depicting biological pathways and processes associated with the co-expressed genes in the brown module performed in CytoScape using STRING. The x-axis represents the -log10 of the FDR-adjusted p-value, while the y-axis shows the enriched terms. C) Module node description based on PlasmoDB and their differential expression in SMA relative to non-SMA. * Indicates the most interconnected node in the module (seed node). Enrichment identifiers: pfa- KEGG and PFA- Reactome databases.

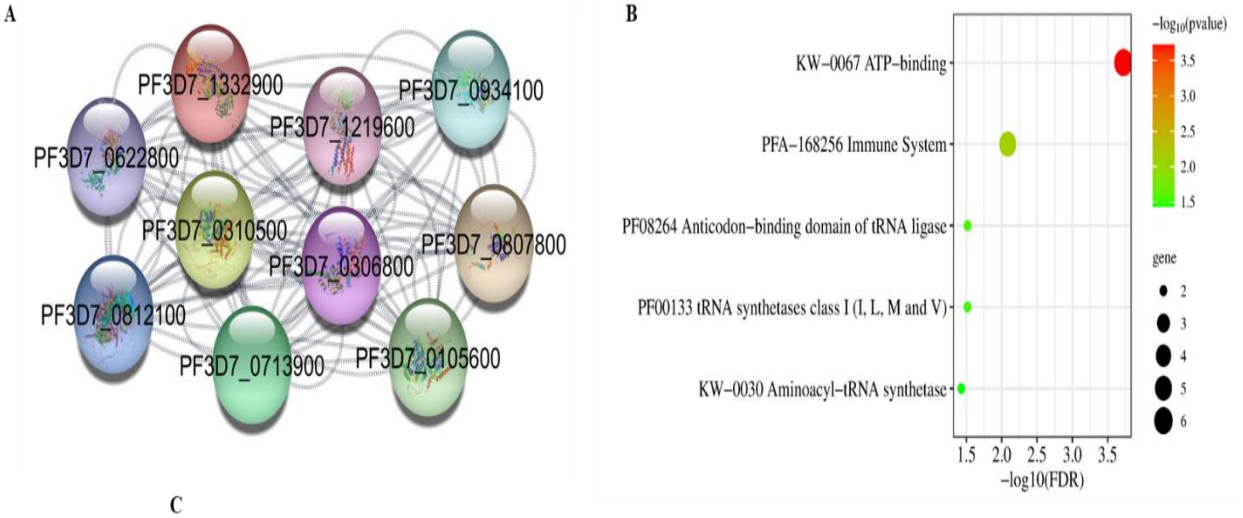


C

Node	PlasmoDB description	Log2 FC	*p*-value
PF3D7_1409900	Cytidine diphosphate-diacylglycerol synthase	1.05	**8.96E-03**
PF3D7_0518300	Proteasome subunit beta type-1, putative	0.90	**2.42E-02**
PF3D7_1426200	Protein arginine N-methyltransferase 1	1.04	**4.46E-03**
PF3D7_0907400	ATP-dependent protease ATPase subunit ClpY	1.05	**7.48E-03**
PF3D7_1338100	26S proteasome regulatory subunit RPN3, putative	1.01	**8.20E-03**
PGK (PF3D7_0922500)	Phosphoglycerate kinase	0.65	**1.84E-02**
PF3D7_0205900	26S proteasome regulatory subunit RPN1, putative	0.72	**1.27E-02***
PF3D7_1334200	Chaperone binding protein, putative	0.74	**4.52E-02**
PF3D7_0526800	Conserved Plasmodium protein, unknown function	0.82	**3.41E-02**
PF3D7_1125400	Mitochondrial import inner membrane translocase subunit TIM44	0.64	**4.30E-02**

Figure 4.13: Turquoise module

A) Network showing 10 nodes and 90 edges of the turquoise WGCNA module. B) Functional enrichment analysis of the coexpressed genes in the turquoise module performed in Cytoscape using STRING. The x-axis represents the -log10 of the FDR-adjusted p-value, while the y-axis shows the enriched terms. C) Module node description based on PlasmoDB and their differential expression in SMA relative to non-SMA. * Indicates the most interconnected node in the module (seed node). Enrichment identifiers: GOCC- gene ontology compartments, pfa- KEGG, KW- UniProt Keywords and CL- STRING cluster databases.



C

Node	PlasmoDB description	Log2FC	*p*-value
PF3D7_1219600	Phospholipid-transporting ATPase	0.44	**6.39E-03**
PF3D7_0306800	T-complex protein 1 subunit beta	0.65	**9.66E-03**
PF3D7_0622800	Leucine--tRNA ligase, putative	0.39	**2.79E-02**
PF3D7_0812100	Proteasome activator complex subunit 4, putative	0.81	**7.66E-03**
PF3D7_0934100	TFIIH basal transcription factor complex helicase XPD subunit	0.61	**1.17E-02**
PF3D7_0713900	Conserved Plasmodium protein, unknown function	0.51	**1.40E-02**
PF3D7_0105600	FtsJ-like methyltransferase, putative	0.72	**7.43E-03**
PF3D7_0310500	ATP-dependent RNA helicase DHX57, putative	0.72	**9.29E-03**
PF3D7_0807800	26S proteasome regulatory subunit RPN10, putative	0.55	**3.13E-03**
PF3D7_1332900	Isoleucine--tRNA ligase, putative	0.46	**4.95E-02***

Figure 4.14: Green module
A) Network showing the green WGCNA module with 10 nodes and 90 edges. B) Functional enrichment analysis of the co-expressed genes in the yellow module was performed in CytoScape using STRING. The x-axis represents the -log10 of the FDR-adjusted p-value, while the y-axis shows the enriched terms. C) Module node description based on PlasmoDB and their differential expression in SMA relative to non-SMA. * Indicates the most interconnected node in the module (seed node). Enrichment identifiers: KW- UniProt Keywords, PFA- Reactome and PF- Protein FAMilies databases.

4.3.6 Protein-Protein Interaction (PPI) Network Analysis

4.3.6.1 PPI Network of *P. falciparum* DEGs

The *P. falciparum* DEGs were uploaded onto the STRING platform, revealing a PPI network of 542 nodes and 1,122 edges (PPI enrichment *P*-value=4.06E-10), reflecting biologically connected proteins (**Figure 4.15**). Additionally, protein clusters in the PPI network that are highly connected (coordinately regulated), as well as the hub node in each cluster, were identified. A total of 17 protein clusters (i.e., sub-networks) emerged from the network analysis, with clusters 1-3 shown in **Figure 4.15**.

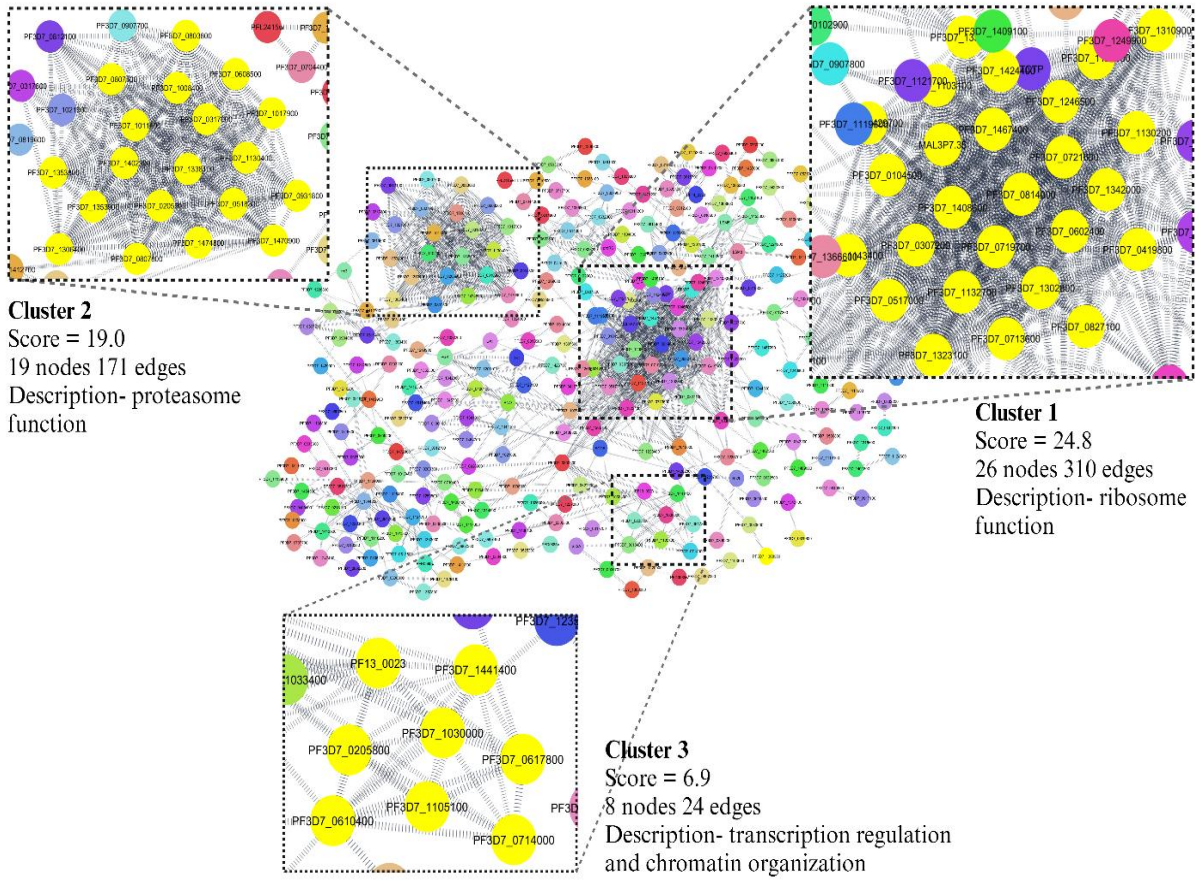


Figure 4.15: Visualization of PPI network of *P. falciparum* DEGs.
A dense PPI network of the P. falciparum DEGs with 3 out of 17 network clusters highlighted. The network consists of 542 nodes and 1,122 edges (PPI enrichment P-value=4.06E-10). Coloured circles represent nodes, while their interactions represent the edges. Yellow nodes in the cluster show the PPI network nodes that form the clusters. The description of the clusters is based on the enrichment terms associated with the PPI network cluster.

The MCODE scores, nodes, edges and hub node for each cluster are shown in **Table 4.11**. The top 5 out of 17 sub-networks included: cluster 1 (MCODE score at 24.80, with 26 nodes and 310 edges. The hub node is PF3D7_0322900- 40S ribosomal protein S3A, putative), cluster 2 (MCODE score at 19.00, 19 nodes and 171 edges and the hub node is PF3D7_1353800- Proteasome subunit alpha type-4, putative), cluster 3 (MCODE score at 6.86, harboring 8 nodes and 24 edges, and PF3D7_1441400- FACT complex subunit SSRP1, putative acts as the hub node), cluster 4 (MCODE score at 5.60, containing 6 nodes and 14 edges, and the hub node is PF3D7_0618700- Trafficking protein particle complex subunit 6A, putative) and cluster 5 (MCODE score at 5.00, with 5 nodes and 10 edges, and the hub node is PF3D7_1352700- Intron-binding protein aquarius, putative). Moreover, the hub node for the entire PPI network was also identified: PF3D7_1331700- Glutamine--tRNA ligase, putative (**Table 4.11)**.

Table 4.11: List of PPI network clusters identified in the PPI analysis of P. falciparum DEGs

Cluster	MCODE Score	Nodes	Edges	Hub Node	PlasmoDB Description
Cluster 1	24.80	26	310	PF3D7_0322900	40S ribosomal protein S3A, putative
Cluster 2	19.00	19	171	PF3D7_1353800	Proteasome subunit alpha type-4, putative
Cluster 3	6.86	8	24	PF3D7_1441400	FACT complex subunit SSRP1, putative
Cluster 4	5.60	6	14	PF3D7_0618700	Trafficking protein particle complex subunit 6A, putative
Cluster 5	5.00	5	10	PF3D7_1352700	Intron-binding protein aquarius, putative
Cluster 6	5.00	7	15	PF3D7_1213800	Proline--tRNA ligase
Cluster 7	5.00	9	20	PF3D7_1134000	Heat shock protein 70
Cluster 8	4.67	10	21	PF3D7_0406100	V-type proton ATPase subunit B
Cluster 9	4.50	5	9	PF3D7_1455300	Conserved protein, unknown function
Cluster 10	4.00	4	6	PF3D7_0409200	Protein SOF1, putative
Cluster 11	4.00	8	14	PF3D7_0710400	DNA repair protein RAD14, putative
Cluster 12	3.33	4	5	PF3D7_0303200	Lipin, putative
Cluster 13	3.00	3	3	PF3D7_1015600	Heat shock protein 60
Cluster 14	3.00	3	3	PF3D7_1110500	Vacuolar protein sorting-associated protein 35, putative
Cluster 15	3.00	3	3	PF3D7_1025300	Conserved protein, unknown function
Cluster 16	3.00	3	3	PF3D7_0820700	2-oxoglutarate dehydrogenase E1 component
Cluster 17	2.67	4	4	PF3D7_1363400	Polyubiquitin binding protein, putative
Entire network		542	1,122	PF3D7_1331700	Glutamine--tRNA ligase, putative

The table shows a list of PPI network clusters consisting of highly connected DEGs. Clusters are ranked based on their MCODE-assigned scores. Cluster membership (nodes) and node interactions are also shown. The Hub genes of these densely connected regions and the entire PPI network identified using CytoHubba through 11 topological analysis methods are shown. The differential expression of all the hub node genes is shown in **Table 4.12.**

Table 4.12: Differential expression of genes encoding the hub nodes of the PPI network clusters

Network	Gene	PlasmoDB Description	Log_2FC	*P*-value
Cluster 1	PF3D7_0322900	40S ribosomal protein S3A, putative	0.312	**0.013**
Cluster 2	PF3D7_1353800	Proteasome subunit alpha type-4, putative	0.487	**0.027**
Cluster 3	PF3D7_1441400	FACT complex subunit SSRP1, putative	0.868	**0.008**
Cluster 4	PF3D7_0618700	Trafficking protein particle complex subunit 6A, putative	0.814	**0.039**
Cluster 5	PF3D7_1352700	Intron-binding protein aquarius, putative	0.412	**0.022**
Cluster 6	PF3D7_1213800	Proline--tRNA ligase	0.371	**0.009**
Cluster 7	PF3D7_1134000	Heat shock protein 70	1.023	**0.022**
Cluster 8	PF3D7_0406100	V-type proton ATPase subunit B	0.625	**0.026**
Cluster 9	PF3D7_1455300	Conserved protein, unknown function	1.229	**0.036**
Cluster 10	PF3D7_0409200	Protein SOF1, putative	-0.224	**0.045**
Cluster 11	PF3D7_0710400	DNA repair protein RAD14, putative	0.771	**0.048**
Cluster 12	PF3D7_0303200	Lipin, putative	1.668	**0.020**
Cluster 13	PF3D7_1015600	Heat shock protein 60	2.063	**0.032**
Cluster 14	PF3D7_1110500	Vacuolar protein sorting-associated protein 35, putative	1.228	**0.045**
Cluster 15	PF3D7_1025300	Conserved protein, unknown function	1.650	**0.048**
Cluster 16	PF3D7_0820700	2-oxoglutarate dehydrogenase E1 component	2.167	**0.035**
Cluster 17	PF3D7_1363400	Polyubiquitin binding protein, putative	0.911	**0.041**
Entire network	PF3D7_1331700	Glutamine--tRNA ligase, putative	0.612	**0.016**

Data represents the differential expression values of genes encoding identified hubs. Welch's t-test compared the mean expression levels of genes encoding identified hubs between SMA and non-SMA. The fold change was defined as the mean gene expression level ratio in SMA to that in non-SMA. Statistical significance was set at *P*-value≤0.050 and indicated in bold.

4.3.6.2 Functional Enrichment Analysis of PPI Network Clusters

Functional enrichment analyses were performed to explore the potential biological roles of the PPI network clusters. The top 10 significantly enriched terms in clusters 1-5 are presented in **Figure 4.16**. The central encompassing enrichment term for biological functions in each of the 5 clusters was as follows: cluster 1- ribosome function and translation process; cluster 2- proteasome function, immune evasion, regulation of DNA replication and antioxidant response; cluster 3- DNA transcription, chromatin structure and function, and protein dimerisation; cluster 4- vesicle transport from the endoplasmic reticulum to the Golgi apparatus, and cluster 5- RNA splicing and spliceosomal.

Furthermore, the top 10 significantly enriched terms in clusters 6-10 are presented in **Figure 4.17**. Enrichment terms related to the following biological functions in clusters 6-10 were cluster 6- the biosynthesis of aminoacyl-tRNAs and protein translation; cluster 7- heat stress and HSP90 function; cluster 8- glycolysis, ribose phosphate metabolism, ATP metabolism, vacuole function and vacuolar proton transport; cluster 9- mRNA splicing and Spliceosome-snRNP complex and cluster 10- processing and maturation of small subunit ribosomal RNA (rRNA).

The top 10 significantly enriched terms in clusters 11-17 are presented in **Figure 4.18**. Central themes for each of the clusters included: cluster 11- DNA repair processes and components; cluster 12- metabolism and biosynthesis of lipids (glycerophospholipids, glycerolipids, and phosphatidic acid); cluster 13- molecular chaperones especially ATP-dependent chaperones; cluster 14- endocytosis and retrograde transport (movement of protein and membrane from endosomes back to the Golgi apparatus); cluster 15- TCA cycle, carbon metabolism and Oxidoreductase activity; cluster 16- protein sumoylation (a post-translational modification process involving the covalent attachment of small ubiquitin-like modifier (SUMO) proteins to target proteins) and cluster17- ubiquitin binding process and the ubiquitin-proteasome system.

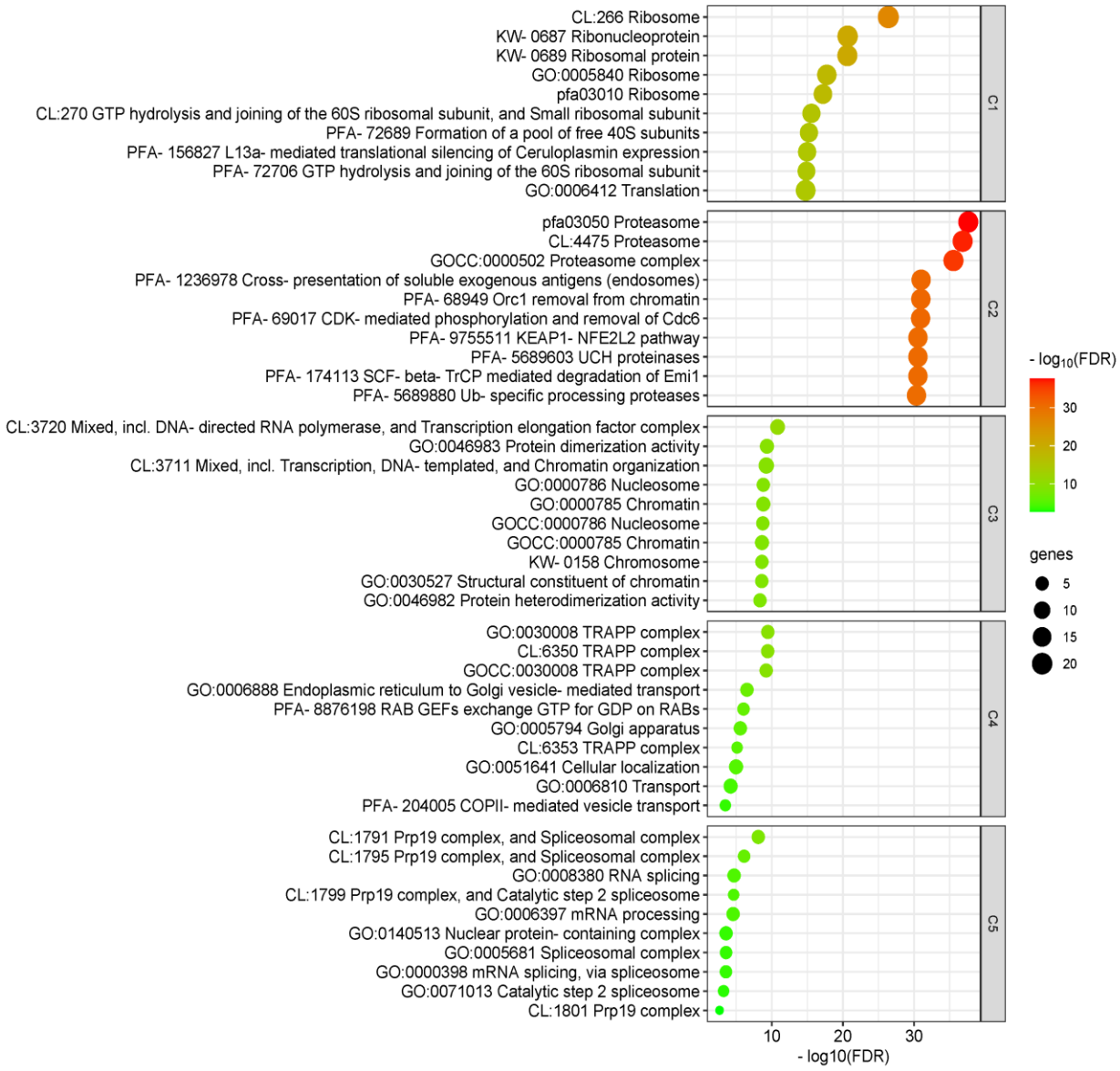


Figure 4.16: Functional enrichment terms of clusters 1-5 in the *P. falciparum* PPI network. The x-axis represents the -log10 of the FDR-adjusted P-value, while the y-axis shows the enriched terms. The top 10 significant enrichment terms for each cluster are shown. Enrichment identifiers: GO- gene ontology, GOCC- gene ontology compartments and CL- STRING clusters databases.

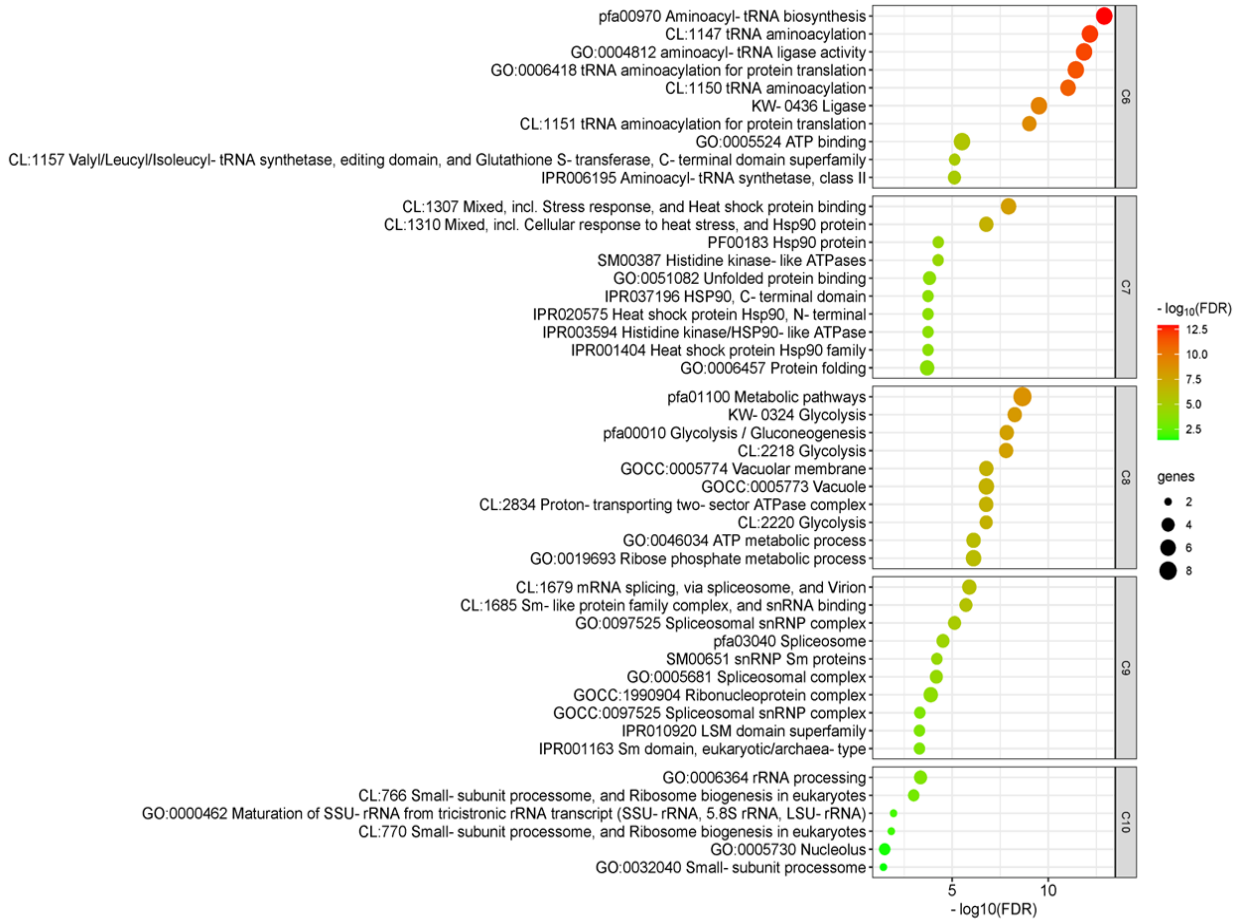


Figure 4.17: Functional enrichment terms of clusters 6-10 in the *P. falciparum* PPI network.

The x-axis represents the -log10 of the FDR-adjusted P-value, while the y-axis shows the enriched terms. The top 10 significant terms for each cluster are shown, unless 10 failed to emerge. Enrichment identifiers: GO- gene ontology, GOCC- gene ontology compartments and CL- STRING clusters databases.

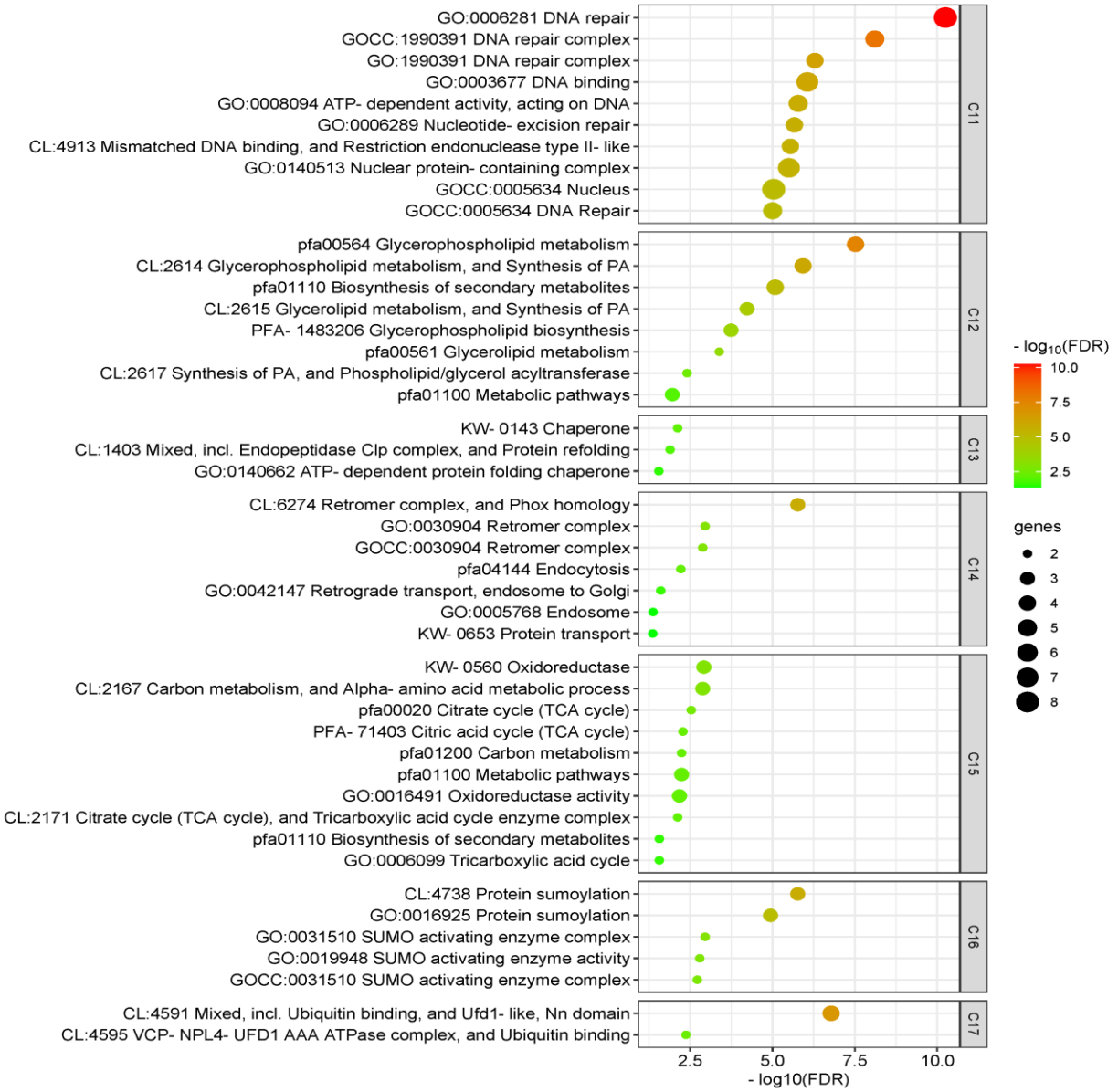


Figure 4.18: Functional enrichment terms of clusters 11-17 in the *P. falciparum* PPI network.

The x-axis represents the -log10 of the FDR-adjusted P-value, while the y-axis shows the enriched terms. The top 10 significant terms for each cluster are shown, or any top significant terms. Enrichment identifiers: GO- gene ontology, GOCC- gene ontology compartments and CL- STRING clusters databases.

4.3.6.3 Relationship between transcript levels of hub genes of blood-stage *P. falciparum* and malaria severity

Since the hub/seed proteins could act as key regulators of signalling in a sub-network, the relationship between transcript levels of hub genes central to the identified clusters/modules and

the risk of SMA was investigated using logistic regression modelling, controlling for the effect of age, sex and sickle-cell genotypes. There was an increased risk of SMA for cluster 1 [PF3D7_0322900-40S ribosomal protein S3A, putative (OR=1.001, *P*-value=0.042)], cluster 2 [PF3D7_1353800-Proteasome subunit alpha type-4, putative (OR=1.009, *P*-value=0.049)], cluster 3 [PF3D7_1441400-FACT complex subunit SSRP1, putative (OR=1.014, *P*-value=0.025)], cluster 4 [PF3D7_0618700-Trafficking protein particle complex subunit 6A, putative (OR=1.046, *P*-value=0.048)], cluster 6 [PF3D7_1213800-Proline--tRNA ligase (OR=1.018, *P*-value=0.005)] and the entire network [PF3D7_1331700- Glutamine--tRNA ligase, putative (OR=1.006, *P*-value=0.041)] (**Table 4.13**). There was also enhanced susceptibility to developing SMA associated with the brown module [PF3D7_1239800-Conserved Plasmodium protein, unknown function (OR=1.208, *P*-value=0.041)], blue module [PF3D7_0322900-40S ribosomal protein S3A, putative (OR=1.001, *P*-value=0.042)] and turquoise module [PF3D7_0205900- 26S proteasome regulatory subunit RPN1, putative (OR=1.013, *P*-value=0.039)] (**Table 4.13**).

Table 4.13 Relationship between transcript levels of P. falciparum hub genes and malaria severity

Network	Hub	OR	95% CI	*P*-value
Cluster 1	PF3D7_0322900	1.001	1.000-1.002	**0.042**
Cluster 2	PF3D7_1353800	1.009	1.000-1.019	**0.049**
Cluster 3	PF3D7_1441400	1.014	1.002-1.027	**0.025**
Cluster 4	PF3D7_0618700	1.046	1.000-1.094	**0.048**
Cluster 5	PF3D7_1352700	1.029	0.998-1.060	0.070
Cluster 6	PF3D7_1213800	1.018	1.005-1.030	**0.005**
Cluster 7	PF3D7_1134000	1.002	0.999-1.005	0.117
Cluster 8	PF3D7_0406100	1.003	0.999-1.007	0.060
Cluster 9	PF3D7_1455300	1.022	0.995-1.050	0.117
Cluster 10	PF3D7_0409200	0.994	0.987-1.002	0.146
Cluster 11	PF3D7_0710400	1.022	0.996-1.050	0.098
Cluster 12	PF3D7_0303200	1.028	0.993-1.065	0.121
Cluster 13	PF3D7_1015600	1.031	0.999-1.063	0.058
Cluster 14	PF3D7_1110500	1.027	0.976-1.080	0.306
Cluster 15	PF3D7_1025300	1.059	0.992-1.131	0.083
Cluster 16	PF3D7_0820700	1.033	0.992-1.076	0.118
Cluster 17	PF3D7_1363400	1.040	0.998-1.083	0.061
Entire PPI network	PF3D7_1331700	1.006	1.000-1.011	**0.041**
Brown	PF3D7_1239800	1.208	1.008-1.447	**0.041**
Yellow	PF3D7_1208900	1.021	0.999-1.044	0.074
Blue	PF3D7_0322900*	1.001	1.000-1.002	**0.042**
Turquoise	PF3D7_0205900	1.013	1.001-1.025	**0.039**
Green	PF3D7_1332900	1.005	0.999-1.011	0.069

Data are presented as an odds ratio (OR) with 95% confidence intervals (CI) using logistic regression with the following covariates in the models: age at enrollment, sex and sickle-cell genotypes. *P*-values ≤0.050 were considered significant and are indicated in bold.

CHAPTER FIVE
DISCUSSION

5.1 Relationship between Genetic Polymorphisms in NCR3 and Longitudinal Susceptibility to Childhood SMA

Nearly every child living in the holoendemic *P. falciparum* transmission areas, such as western Kenya, experiences repeated episodes of malaria (Hollowell *et al.*, 2023; Okoyo *et al.*, 2021). However, only a subset of individuals develop SMA, typically within the first year of life (Hollowell *et al.*, 2023). Young children under five years old are especially susceptible to severe malaria as maternal immunity wanes and adaptive immunity begins to develop through repeated malaria episodes (CDC, 2021). Since childhood, immunity to malaria gradually develops over successive episodes (Griffin *et al.*, 2015), the impact of host genetic variants on the development of malaria and SMA is best captured through longitudinal studies during the development of naturally acquired malarial immunity (Kwiatkowski, 2005). This study reports, for the first time, the longitudinal risk of clinical malaria and SMA in carriers of mutant alleles of rs2736191 and rs11575837.

The SNPs, rs2736191 and rs11575837, have been linked to functional changes in gene expression (Auton *et al.*, 2015; Baaklini *et al.*, 2017; Delahaye *et al.*, 2007; Rusakiewicz *et al.*, 2013). Notably, substitution of C to G in rs2736191 creates TFBSs for TCF3 and ZBTB6, while ablating a binding site for TFAP2A (AP2). Previous studies show that TFAP2A (AP2) regulates the transcription of IFN-γ Receptor 1, and that elevated IFN-γ production is protective against malaria infections (Ahmed & Johnson, 2006; Chen *et al.*, 2012; King & Lamb, 2015). In addition, TCF3 was shown to promote the development of γδ T cells, differentiation of memory CD8 T cells, and increase γδ T cells and CD8+ T cell responses in children with SMA (Hviid *et al.*, 1996; Roels *et al.*, 2022; Safeukui *et al.*, 2015; Schauder *et al.*, 2021). Although not studied in the context of malaria, human *ZBTB6* was one of the most differentially expressed genes in the whole blood of patients with Crohn's disease (Mamoor, 2020). Conversion of C to T in rs11575837 results in the loss of a TFAP2A (AP2) binding site and the creation of an HNF4A binding site. Although HNF4A was shown to promote erythropoiesis during embryonic development in a murine model, it remains to be determined if children carrying the T-allele exhibit HNF4A binding and subsequent alterations in erythropoiesis (Makita *et al.*, 2001). Functionally, these TFBS changes could influence SMA outcome through immune or hematopoietic pathways. The loss of TFAP2A binding may reduce IFN-γ receptor transcription, weakening Th1 responses important for parasite clearance. Conversely, gain of TCF3 binding could enhance γδ T cell and CD8+ T cell responses, potentially improving immunity in SMA.

The gain of HNF4A binding in rs11575837 could alter erythropoiesis and contribute to anaemia severity. Collectively, these transcription factor interactions suggest possible mechanistic roles in shaping susceptibility or resilience to SMA.

The MAFs of the variants under investigation were higher in the study participants than in the LWK ethnic group from the 1000 Genome Project (Auton *et al.*, 2015). Additionally, the overall distribution of rs2736191 and rs11575837 genotypes in the current study population significantly deviated from HWE, indicative of an influence of evolutionary forces on the human genome in the study population. There is increasing evidence that natural selection affects the frequencies of disease-associated genetic variants in different populations (Gorlov *et al.*, 2015). For instance, malaria exerts a strong evolutionary force on risk-associated alleles (Kwiatkowski, 2005; Tishkoff & Williams, 2002) and may, at least in part, underlie the observed allelic distribution. LD analysis revealed that the two SNPs are co-inherited (D′=0.882) because they are rarely separated during recombination. Since D′ values tend to be inflated when there is a low allele frequency (as is the case with rs11575837), additional metrics like the D′ confidence intervals and r^2 value (Mueller, 2004) were also examined. D′ measures the non-random association of alleles at two loci, indicating the likelihood of their co-inheritance, while r^2 is a statistical measure of the correlation in allele frequencies and the predictive power between the SNPs (Bush & Moore, 2012). Despite the D′ being high, the r^2 value was notably low (r^2=0.011), suggesting that while the SNPs are rarely separated by recombination. Importantly, the LOD score, used to assess the strength of evidence of linkage, was 6.61, which supports non-random linkage between the SNPs. Thus, the combination of high D′, strong LOD, and low r^2 indicates that while the SNPs are physically linked and co-inherited, they do not predict each other well. This can occur when one allele is rare, reducing the statistical correlation despite physical linkage.

Previous studies found an association between carriage of the mutant G allele for rs2736191 and increased susceptibility to mild malaria (Baaklini *et al.*, 2017; Delahaye *et al.*, 2007). In a former investigation in Congolese children, Baaklini and colleagues revealed that the relationship between the mutant allele and mild malaria was only significant in children greater than five years of age but not in younger children (Baaklini *et al.*, 2017). Moreover, in the parent-sib study conducted in Burkina Faso, the mean age of the sibs was 12.1±6.2 years (Delahaye *et al.*, 2007). Collectively, these studies suggest an age-specific relationship between carriage of the mutant allele and susceptibility to malaria, which may not be detectable in the current study since the population was considerably younger (primarily <5 years). Although this hypothesis remains to be tested, a significant relationship was observed between carriage of the G-allele and increased

susceptibility to SMA in individuals with the GG genotype, and in the additive model. However, this relationship did not remain significant after correcting for multiple comparisons.

Although the previous family-based association study in Burkina Faso did not include rs11575837 in the analyses due to a low MAF (<0.01) in the study population, the current study investigated this SNP based on its association with functional properties and susceptibility to pSS (Auton *et al.*, 2015; Rusakiewicz *et al.*, 2013). The MAF in the current study for rs11575837 was 0.03, with carriers of the TT genotype exhibiting a significantly increased susceptibility to acute malaria throughout the follow-up period. The mutant allele T has been associated with lowered *NCR3* expression (Rusakiewicz *et al.*, 2013). Since NCR3 can activate NK cells to clear malaria parasites by directly recognizing pRBCs (Baratin *et al.*, 2007; Mavoungou *et al.*, 2007; Wang *et al.*, 2012b), decreased NCR3 expression in TT carriers could reduce antiparasitic effects and result in increased susceptibility to malaria. Thus, even though there was a low MAF for rs11575837 in the study cohort, carriage of homozygous T-alleles did impact susceptibility to malaria, consistent with results found for other diseases in European populations in which the MAF was also low (0.02) (Auton *et al.*, 2015; Rusakiewicz *et al.*, 2013).

While there was also an increased risk for SMA (RR=1.911) in children who inherited TT for rs11575837, the results were non-significant (P=0.267), likely due to the low number of events for the rare variant. Although further evidence is required, it is possible that the rarity of the TT genotype maintained in the study population could be explained by increased susceptibility to malaria, SMA and subsequent mortality. There was a progressive increase in the risk of mortality associated with the carriage of the T-allele, but the low carriage rate in the context of a low mortality rate cannot confirm this hypothesis. In the study region, childhood mortality from malaria has historically been very high (Akech *et al.*, 2019). As such, failure to reach reproductive age has strongly impacted selection.

Investigations on the impact of the co-inheritance of the two SNPs did not elucidate any significant findings for altered susceptibility to longitudinal malaria episodes. However, the additive model revealed that a progressive carriage rate of the CC genotype significantly reduced the risk of SMA. This finding is consistent with selective effects for the variants in the population, particularly for the rarer rs11575837 mutant allele. Additional modelling efforts revealed that inheritance of the CG genotype increased the risk of SMA in carriers versus non-carriers, in the additive model, but the results were not significant after correction for multiple comparisons. Nonetheless, in the context of the significant risk found for carriage of G-allele for rs2736191 in the genotypic model, it appears that carriage of the G-allele in the GC haplotype certainly influences the longitudinal development of SMA.

Secondary analyses were performed to determine if the SNPs (or their haplotypes) predicted all-cause mortality. Inheritance of either of the two SNPs, individually or in combination, was not significantly associated with all-cause mortality. This lack of statistical significance may reflect the overall low number of deaths in the cohort and the reduced frequency of some genotypes, which limited statistical power to detect modest genetic effects. Biologically, it is also possible that the impact of NCR3 variation on malaria susceptibility and SMA pathogenesis does not extend to influencing overall survival, particularly in a setting where mortality can result from multiple competing causes beyond malaria. Thus, while these findings do not support a major role for the selected NCR3 variants in determining childhood mortality, they underscore the need for larger, multi-site studies with sufficient mortality events to fully explore potential genetic contributions to survival outcomes. Collectively, this longitudinal investigation in a holoendemic malaria region reveals that genetic factors, such as the SNPs rs2736191 and rs11575837, modulate disease susceptibility. Specifically, the TT genotype of rs11575837 increased the risk of malaria episodes while the CC haplotype was protective against SMA, likely through mechanisms involving dysregulated innate immune responses, including NK cell function. However, while innate immunity plays a critical early role in parasite control, these findings do not exclude the contribution of other immune mechanisms. In malaria-exposed children, since acquired immunity develops progressively with age and repeated exposure, susceptibility to disease outcomes likely reflects a complex interplay between genetic variation and both innate and acquired immunity, with the timing of acquired immunity shaped by both age and repeated exposure to infection.

5.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA

NK cells are activated in malaria-naïve patients experimentally infected with *P. falciparum* (3D7 strain) (Walk & Sauerwein, 2019). Activation of NK cells was attributed, in part, to NCR3 expression, which was elevated in the experimentally infected volunteers, leading to the increased production of IFN-γ and granzyme B (Walk & Sauerwein, 2019). NK cells induce eryptosis (programmed cell death) of iRBCs in falciparum malaria (Wolf *et al.,* 2017). Overproduction of pro-inflammatory mediators by NK has been linked to an exacerbated innate immune response, leading to immunopathology (Hviid *et al.*, 1996; Wolf *et al.*, 2017). In the current study, although parasitaemia was comparable between the groups, *NCR3* mRNA expression was up-regulated in pediatric patients with SMA relative to those with non-SMA, but the difference did not reach statistical significance (i.e., $Log_2FC=0.285$, FDR=0.112). However, gene expression deconvolution analysis showed that the proportion of NK cell transcripts was significantly higher in SMA, pointing to an increased immune response that could contribute to the pathogenesis of SMA (Walk & Sauerwein, 2019). Actived nodes within Inflammation_NK

cell cytotoxicity process network were differentially expressed in SMA, with reduced transcription of the nuclear factors NF-κB and STAT1, and increased expression of NFATC2 in SMA. NF-κB upregulates the expression of inflammatory cytokines (Liu *et al.*, 2017), while NFATC2 is a negative regulator of NK cell function (Rothfelder *et al.*, 2016). Persistent activation of STAT1 due to a STAT1 gain-of-function mutation causes reduced cytotoxicity and IFN-γ production (Tabellini *et al.*, 2017).

Gene transcripts for NK cell membrane proteins DAP12, CD3ζ, and CD244 were differentially expressed (*DAP12* downregulated while *CD3ζ* and *CD244* upregulated). Adaptor protein DAP12 is used by NK activating receptors lacking intrinsic signalling domains for signal transduction. CD3ζ also promotes downstream signalling as the activating receptor component with tyrosine-based activation motifs (Barrow *et al.*, 2019; Koch *et al.*, 2013). Analysis of NK cell signaling in SMA revealed altered inflammatory and cytotoxic pathways. The CD244 receptor, which can either amplify NK cell activation or deliver inhibitory signals in the absence of strong activating cues (Agresta *et al.*, 2018), showed expression patterns consistent with a mixed activation state. Among cytoplasmic enzymes, the kinase ERK2 mRNA was upregulated, which is known to promote perforin and granzyme B production, whereas PAK1 mRNA was downregulated, a change associated with suppressed NK cytotoxicity (Chen *et al.*, 2007; Jiang *et al.*, 2000). Transcripts for caspase 3 and 8, key mediators of target-cell apoptosis, and the pro-apoptotic ligand Apo-2L were also reduced (Prager *et al.*, 2019; Sordo-Bahamonde *et al.*, 2020), suggesting an impaired ability to induce programmed cell death. In addition, tyrosine phosphatase SHP-1, which normally terminates activating signals, was downregulated (Lanier, 1997), as were transcripts for adaptor proteins Slp76 and GRB2, both required for propagating activation signals. Taken together, these patterns point to a dysregulated NK cell response in SMA: signaling components that initiate or sustain activation (e.g., ERK2) are elevated, yet key downstream cytotoxic and apoptotic machinery (e.g., PAK1, caspases, Apo-2L) are suppressed. This imbalance could sustain pro-inflammatory signaling while limiting effective killing of infected cells, potentially prolonging parasite survival and amplifying immune-mediated damage to uninfected erythrocytes. Such a scenario would be consistent with a hyperinflammatory yet functionally impaired NK cell state that contributes to SMA pathogenesis.

Previous studies in the Siaya community have shown that children with SMA have imbalances in circulating cytokines and chemokines that are associated with inefficient erythropoiesis and severe anaemia [e.g., IFN-γ, IL-6, IL-10, IL-12, IL-13, IL-21, IL-23, MIF, MIP-1α, MIP-1β, RANTES (CCL5), TGF-β1 and TNF-α] (Awandare *et al.*, 2007; Keller *et al.*, 2006; Ong'echa *et al.*, 2011; Ong'echa *et al.*, 2008; Ouma *et al.*, 2008a; Ouma *et al.*, 2008b; Were *et al.*, 2009).

Heat shock proteins (HSP) are immunomodulators that regulate the production of cytokines (Lancaster & Febbraio, 2005; Vega *et al.*, 2008), including those that are dysregulated in children with SMA. Previous investigations by our study team showed that HSP70 transcripts were down-regulated in SMA children (Kempaiah *et al.*, 2016). Additionally, an important up-regulator of HSP70 expression and the heat shock response, GLN, was significantly reduced in children with SMA (Kempaiah *et al.*, 2016; Leite *et al.*, 2016). Host HSP70 was present on the surface of infected and uninfected senescent RBCs and contact with membrane HSP70 on RBCs promotes production of granzyme B in NK cells (Banumathy *et al.*, 2002; Böttger *et al.*, 2012). Extracellular HSPs act as danger-associated molecular patterns, activating signalling cascades upon release into circulation by both necrotic and stressed cells (Asea *et al.*, 2000; Zininga *et al.*, 2018).

Enrichment for canonical pathways in the cohort identified dysregulation of the HSP60-HSP70-TLR2/4 signalling pathway in SMA. Specifically, genes for multiple components of the HSP60-HSP70-TLR2/4 signaling pathway were down-regulated in children with SMA such as HSP70 family members, HSP60, TLR2, TLR4, CD14, MD-2/LY96, MyD88, IRAK4, IRAK2, MEK6/MAP2K6, MAPK14, NF-kB1, NFKB2, IKKa/CHUK, I-κB/NFKBIA, JUNB, IL-6, NF-κB1/NFKB1, IL-1β, CD69, ICAM1 and MHC class I molecules. Conversely, other genes within the pathway were up-regulated including IRAK1, TAB2, TAB3, ubiquitin B/UBB, UEV1A/UBE2V1, TAK1/MAP3K7, MEK2/MAP2K2, MEK3/MAP2K3, ERK2/MAPK1, JUND, CD86, CD80 and MHC class II molecules. The pattern of downregulation of genes in the HSP60-HSP70-TLR2/4 signalling pathway (e.g., HSP70 family members, TLR2, TLR4, CD14, and NF-κB1) suggests that children with SMA may have reduced pattern recognition and inflammatory responses, while up-regulation of other components (e.g., IRAK1, CD86, and MHC class II molecules) indicates an attempt to compensate through alternative immune activation and antigen presentation mechanisms (Hodgson *et al.*, 2019; Sobota *et al.*, 2016). The current results differ from those obtained in adults, both in malaria-naïve volunteers experimentally infected with *P. falciparum* and in naturally infected Cameroonian individuals with clinically apparent falciparum malaria, where GeneChip analysis (Affymetrix U133A) of PBMCs showed up-regulation of genes such as HSP60, HSPA1A, HSPA1B, HSPA4, HSPA5, HSPA9B, TLR2, TLR4, CD14, MYD88, IRAK1, IKKα, NF-κB1, IL-1β and ICAM1 (Ockenhouse *et al.*, 2006). The differences between gene expression patterns in children with SMA and adults with malaria may be due to variations in immune system maturity, disease severity, experimental conditions, pathogen strain differences and methodological approaches.

Leukocytic HSP70 (HSPA1A and HSPA1B) transcript levels were significantly positively correlated with both the reticulocyte production index and Hb concentrations in children with SMA (Kempaiah *et al.*, 2016). The current study extends these findings by linking the suppression of HSP70 family members with TLR 2 and 4, as well as HSP60 and associated signalling cascades in malaria-infected children with severe anaemia. HSP70 plays an essential role in erythropoiesis by protecting an erythroid transcription factor, GATA-1, from caspase-3-mediated proteolysis at later stages of erythroblast maturation (Ribeil *et al.*, 2007). Moreover, HSP70 may play a role in the severe anaemia witnessed in individuals with β-Thalassemia (β-TM) since HSP70 directly interacts with free α-globin chains in human β-TM erythroblasts, resulting in sequestration of HSP70 in the cytoplasm, a process that promotes GATA-1 degradation (Arlet *et al.*, 2014). HSP70, as well as HSP60, are also essential for appropriate erythropoietic responses by aiding in protein folding, protecting erythroid precursors from stress-induced apoptosis during hypoxia and iron deficiency, supporting mitochondrial function, and regulating the differentiation and proliferation of the erythroid lineage (Echeverria & Picard, 2010; Kampinga & Craig, 2010; Quintana & Cohen, 2005). Thus, the previous and current findings offer novel insight into how dysregulation in the HSP60-HSP70-TLR2/4 signalling pathway may contribute to inefficient erythropoiesis and the profound, life-threatening anaemia witnessed in children with SMA. It has been shown that HbSS individuals experience elevated stress in red blood cells, potentially contributing to increased expression of modulators of cellular stress like the HSPs (Olayanju *et al.*, 2022; Umapathy *et al.*, 2013). However, in our study, the vast majority of children in both the SMA and non-SMA groups were HbAA (ranging from 79.1% to 91% across the genotyping and transcriptomics cohorts). Children with HbSS represented a small proportion—1.6% in the genotyping cohort and 20–28% in the transcriptomics cohorts. Thus, while it is plausible that sickling may contribute to stress responses in a subset of individuals, the increased expression of HSP60/70 observed in the SMA group is more likely to reflect general pathophysiological processes associated with SMA, including hemolysis, immune activation, and oxidative stress, rather than sickling alone.

Moreover, the current study uncovered the overall upregulation of transcripts for GLN transporters and glutamine synthetase in SMA. Although GLN has been demonstrated as an important molecule capable of overcoming the parasite pigment, haemozoin (*Pf*Hz)-induced suppression of HSP70 in human PBMCs from malaria-naïve donors, and reduced circulating GLN levels are a significant predictor of SMA, the aetiology of reduced GLN levels remains undetermined (Kempaiah *et al.*, 2016). Multiple functions have been documented for GLN in mammalian cells, including leukocytes, through the following: 1) immune signalling by activating heat shock factor 1 (HSF1) and suppressing NF-κB activity, 2) a crucial energy source

through its involvement in the TCA cycle, 3) synthesis of biomolecules including nucleotides, non-essential amino acids (NEAA) such as glutamate, asparagine, aspartate, and alanine, as well as proteins and uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is important for protein post-translational modifications (Cruzat *et al.*, 2018; Hensen *et al.*, 2012; Pacifico *et al.*, 2023; Xue *et al.*, 2012; Yoo *et al.*, 2020). Furthermore, GLN has been shown to facilitate erythropoiesis by providing succinyl-CoA for heme synthesis (Burch *et al.*, 2018). Potential reasons for reduced GLN in children with SMA, inferred from findings in other non-malarial studies, include increased metabolic demands, where GLN is rapidly consumed via the TCA cycle, and overactivation of the immune response, which significantly consumes GLN for the proliferation and function of immune cells (Cruzat *et al.*, 2018). Children with SMA also experience enhanced oxidative stress, necessitating the utilization of GLN for the synthesis of glutathione (GSH) and NADPH, which are essential for maintaining cellular redox balance (Cruzat *et al.*, 2018). Lastly, children with SMA often suffer from acute kidney injury, which impairs glutamine (GLN) metabolism, leading to increased oxidative stress and apoptosis in tubular epithelial cells (Conroy *et al.*, 2019; Thomas *et al.*, 2022).

The uptake of GLN into immune cells, including NK cells, to facilitate its increased demand, requires transporters on the cell surface (Song *et al.*, 2020). The primary difference in GLN transporters lies in their specific functions, substrate specificities, and tissue distributions (Newsholme, 2021; Pochini *et al.*, 2014; Scalise *et al.*, 2018). The significant up-regulation of SLC1A19, SLC7A5, SLC1A5, and SLC7A8 may be related to an increased metabolic demand by immune cells, in the high-stress state of SMA in which enhanced energy metabolism and synthesis of nucleotides are required (Cruzat *et al.*, 2018; Hensen *et al.*, 2012; Pacifico *et al.*, 2023; Xue *et al.*, 2012; Yoo *et al.*, 2020). The increase in these specific transporters in SMA is also likely related to an increased need for GLN to support the proliferation and activity of immune cells, and an enhanced requirement for cellular repair, antioxidant defence and cellular survival mechanisms (Cruzat *et al.*, 2018; Pérez-Escuredo *et al.*, 2016; Pochini *et al.*, 2014; Scalise *et al.*, 2018). Consistent with the current findings, a previous study in Gabonese children revealed that SLC6A19 transcripts were highly up-regulated in SMA relative to those with uncomplicated malaria (Boldt *et al.*, 2019). In addition to the up-regulation of specific GLN transporters, two transporters were significantly down-regulated: SLC38A2 and SLC38A3. It is expected that down-regulation of these GLN transporters, in the context of decreased GLN levels in children with SMA, would compromise GLN uptake into cells, impairing immune cell function, antioxidant defence, energy metabolism and nitrogen balance (Cruzat *et al.*, 2018; Pochini *et al.*, 2014; Scalise *et al.*, 2018). Collectively, this could weaken the ability to respond to and recover from the malarial infection. Apart from GLN uptake through transporters,

intracellular GLN levels are also controlled by the combined actions of GLUL and GLS/GLS2 (Katt *et al.*, 2017; Kim *et al.*, 2021; Masisi *et al.*, 2020). While GLUL was significantly up-regulated in SMA, GLS1/2 did not differ between the groups. Up-regulation of *GLUL* transcription likely indicates a compensatory mechanism to enhance leukocytic GLN levels in SMA.

In summary, children with SMA have alterations in NK cell activation and up-regulation of a key NK cell receptor that aids in fighting pathogens, NCR3, suggesting a heightened immune response. Dysregulation in the HSP60-HSP70-TLR2/4 signalling pathway and decreased GLN levels, coupled with shifts in GLN transporter expression, indicate further exacerbation of immune dysfunction, inefficient erythropoiesis and oxidative stress, a milieu that likely drives severe anemia in these patients.

5.3 Transcriptome Profile of Blood-Stage *P. falciparum* in Children with SMA

RNA-seq offers insight into the transcriptomic landscape of *P. falciparum* during blood-stage disease. For instance, seven-fold more parasite DEGs were up-regulated in SMA patients compared to down-regulated genes, indicative of active gene expression. This observation agrees with previous studies that blood-stage *P. falciparum* exhibits elevated and tightly regulated gene expression (Bozdech *et al.*, 2003; Painter *et al.*, 2018). Six of the ten most up-regulated genes have been characterised as encoding type I signal peptidase (which cleaves signal peptides from nascent proteins to allow protein processing in the ER) (Sharma *et al.*, 2005); deoxyribodipyrimidine photo-lyase, putative (which repairs pyrimidine dimers in the DNA) (Kavakli *et al.*, 2019); chaperone protein ClpB1 (present in the apicoplast and involved in proteostasis) (AhYoung *et al.*, 2015); ataxin-2 like protein, putative (probably involved in stress granule formation and RNA metabolism) (Kaehler *et al.*, 2012); glutamyl-tRNA(Gln) amidotransferase subunit A (localised in the apicoplast, that is critical for proper acylation of GLN-tRNA) (Mailu *et al.*, 2015) and 2-oxoglutarate dehydrogenase E1 component (which is key to the parasite's energy metabolism through the TCA cycle) (Chang *et al.*, 2022), while the rest encode conserved *Plasmodium* proteins of unknown function. Collectively, over-expression of these genes could enhance the parasite's response to stress, enhance proteostasis, efficient energy production and accurate protein synthesis during the asexual blood stage.

Contrastingly, eight of the top 10 down-regulated genes have been characterised to encode: surface-associated interspersed protein 4.1 (SURFIN 4.1), a schizont-merozoite-associated protein (Moseley, 1998); perforin-like protein 5 facilitating parasite egress (Sassmannshausen *et al.*, 2020); p1/s1 nuclease, putative which could be involved in nucleic acid degradation (Koval & Dohnálek, 2018); acyl-CoA synthetase facilitating lipid metabolism (Bopp *et al.*, 2023);

erythrocyte binding antigen-175, a ligand mediating RBC invasion (Koch *et al.*, 2017); a serine/threonine kinase, protein kinase 5 (Le Roch *et al.*, 2000); structural maintenance of chromosomes protein 6, putative involved in maintaining chromosome structure and stability (Jeppsson *et al.*, 2014) and acidic terminal segments, variant surface antigen of PfEMP1, putative located at the carboxyl terminus of all *Pf*EMP1 proteins (Wahlgren *et al.*, 2017). Collectively, reduced expression of these genes could likely be a regulatory mechanism of down-regulating genes for metabolic efficiency (Machado *et al.*, 2021). Additionally, some genes could be critical during other stages of the parasites' life cycle. For instance, perforin-like 5 has been reported to be crucial during the *Plasmodium* ookinete stage (Sassmannshausen *et al.*, 2020). The majority of the annotated parasite genes, such as the genes for histidine-rich protein II (*Pf*KE01_080037300), histidine-rich protein III (*Pf*KE01_130077300) and skeleton-binding protein 1 (*Pf*KE01_050006000), displayed no significant difference in transcript levels between the SMA and non-SMA groups, potentially owing to their essential cellular functions through the life cycles of blood-stage *P. falciparum* within the human host.

The PCA analysis revealed that the parasite gene expression in SMA and non-SMA patients displays distinct and overlapping clustering patterns. This suggests that while unique *P. falciparum* transcriptome profiles are associated with SMA, there are also shared parasite gene expression patterns, regardless of disease severity—for instance, those facilitating core parasite biological processes such as survival and replication, e.g., genes encoding histidine-rich proteins II and III, apical membrane antigen 1 and chloroquine resistance transporter. The estimation of the developmental stages of *P. falciparum* in natural infections is challenging since the ring stages will be commonly observed in peripheral blood, but iRBCs with trophozoite and schizont stages tend to be sequestered in the vascular endothelium, rendering them less likely to be detected in circulation (Meibalan & Marti, 2017). In addition, the early stages of gametocytes (stages 1-4) get sequestered in the bone marrow, while mature gametocytes (stage 5) circulate in the peripheral blood (Chawla *et al.*, 2021). Despite these biological constraints, the study was able to identify different developmental stages of *P. falciparum* using expression data of parasite DEGs obtained from peripheral blood. The results showed a lower proportion of ring stages but an elevated proportion of trophozoites in SMA, suggesting an accelerated transition from the ring stage to the trophozoite stage. The trophozoite stage is the primary phase where the parasite metabolizes Hb, producing toxic heme, which the parasite then detoxifies to haemozoin (Moore *et al.*, 2006). Enhanced levels of *Pf*Hz in leukocytes have been implicated in the pathogenesis of SMA (Perkins *et al.*, 2011). Consistently, a trend toward a higher fraction of schizont-stage parasites in SMA individuals was observed, indicating increased iRBC rupture and subsequent

invasion of new RBCs by *P. falciparum* parasites, one of the pathophysiological processes contributing to SMA (Perkins *et al.*, 2011).

The observation that there was no difference in gametocyte proportions between the study groups indicates that gametocyte development may not be impacted by malarial severity. However, analysis of the proportion of male and female gametocytes across all parasitaemic patients (both SMA and non-SMA) found significantly more male gametocytes than female gametocytes. The sex ratio (the proportion of male gametocytes) is typically low (female-biased) in natural *P. falciparum* infection and has been shown to increase (male-biased) in stress conditions, i.e., severe disease, host immune response, and antimalarial therapy (Carter *et al.*, 2013). Previously, children in a malaria-endemic region, with *P. falciparum* malaria-associated anaemia at enrollment were associated with a high sex ratio relative to non-anaemic children (Gbotosho *et al.*, 2011). A male gametocyte develops into 8 gametes, while a female gametocyte develops into a single gamete (Paul *et al.*, 2000); therefore, a high sex ratio could be an effort by the parasite to promote enhanced transmission efficiency by maximizing reproductive success under adverse conditions (Henry *et al.*, 2019).

Erythrocytic schizogony is marked by rapid growth and replication, driven by increased specific cellular activities (Cowman *et al.*, 2012; Miller *et al.*, 2002). Enrichment for WGCNA modules and PPI clusters identified key parasite cellular processes that could facilitate exponential multiplication, particularly in SMA. Terms (attributes to represent genes) for parasite ribosomal assembly and protein synthesis were enriched in the WGCNA modules (blue and green), and PPI clusters (1, 6 and 10). Essential events such as rRNA processing and ribosome assembly are critical for ribosome biosynthesis to meet the high demand for protein synthesis (Jackson *et al.*, 2011). In *P. falciparum*, the formation of free 40S subunits is essential for the initiation of translation, while GTP hydrolysis and joining of the 60S subunit are critical for the assembly of the functional 80S ribosome (Sheridan *et al.*, 2018). The involvement of ATP-binding and tRNA-associated enzymes indicated increased energy and protein synthesis demands (Bhatt *et al.*, 2009). Aminoacyl-tRNA synthetases (aaRSs) catalyze the binding of ATP to an amino acid and then combine the resulting aminoacyl-AMP to a cognate tRNA molecule, ensuring fidelity in protein translation, offering therapeautic opportunities to disrupt protein synthesis (Bhatt *et al.*, 2009; Khan, 2016). Additionally, regulatory mechanisms such as L13a-mediated translational silencing for selective inhibition of proteins to be translated (Mazumder *et al.*, 2003) and Signal Recognition Particles for sorting and trafficking nascent proteins (Kellogg *et al.*, 2021) are crucial. The nonsense-mediated decay (NMD) surveillance pathway also facilitates the degradation of mRNAs and nascent peptides when the ribosome encounters a premature termination codon, preventing their translation (Arthur & Djuranovic, 2018). Although the

NMD pathway is non-essential for the parasites' growth *ex vivo* (McHugh *et al.*, 2023), up-regulation of genes encoding core components of the NMD pathway, i.e., *Pf*UPF1 (PF3D7_1005500 or *Pf*KE01_100010900) and *Pf*UPF2 (PF3D7_0925800 or *Pf*KE01_090030100) in the current study may be involved in post-translational regulation, albeit not crucial in degrading truncated transcripts. These gene attributes collectively emphasize the significance of protein synthesis during the parasite's asexual blood stages.

Transcript levels of three out of four hub nodes in the networks were associated with increased risk for SMA: PF3D7_1331700 (Glutamine--tRNA ligase, entire PPI network), PF3D7_1213800 (Proline--tRNA ligase, cluster 6), and PF3D7_0322900 (40S ribosomal protein S3A, cluster 1 and blue module). PF3D7_0409200 (Protein SOF1, cluster 10) and PF3D7_1332900 (Isoleucine--tRNA ligase) did not significantly change in SMA. Previous studies have shown that glutamine, proline and isoleucine, essential amino acids, are obtained exogenously from the host serum by increasing the permeability of the erythrocyte membrane (Counihan *et al.*, 2021; Liu *et al.*, 2006). PF3D7_0322900 (40S ribosomal protein S3A, putative) is a small ribosomal subunit protein vital for mRNA decoding (Wong *et al.*, 2014). These findings highlight the regulatory roles of these parasite tRNA ligases and ribosomal proteins in children with SMA.

The spliceosome complex, RNA splicing and post-translation modifications (SUMOylation) were also enriched in clusters 5, 9 and 16. More than half of *P. falciparum* 3D7 genes contain introns (Gardner *et al.*, 2002), and splicing events occur in the parasite's transcripts of these genes (Sorber *et al.*, 2011). Furthermore, alternative splice sites were also identified, but the biological relevance of alternative splicing in the parasite is still being debated (Otto *et al.*, 2010; Sorber *et al.*, 2011; Yeoh *et al.*, 2019). The *P. falciparum* spliceosome complex is responsible for splicing pre-mRNA. Its core complex consists of small nuclear ribonucleoprotein (snRNP) U1, U2, U4, U5 and U6 that recognize splice sites, assemble the splicing machinery, and catalyse the splicing reaction (Hossain *et al.*, 2013). Although the function of Intron-binding protein Aquarius, putative (encoded by cluster 5's hub gene PF3D7_1352700 or its homolog *Pf*KE01_130058300) is unclear, studies in eukaryotes show that it is a spliceosome RNA helicase that binds introns in pre-mRNA (De *et al.*, 2015). Although RNA interference of Aquarius moderately inhibited splicing in human spliceosomes, it could play a more significant regulatory role in splicing the parasite's pre-mRNA.

Protein SUMOylation is a post-translational modification process involving the covalent attachment of small ubiquitin-like modifier (SUMO) proteins to target proteins (Sumam de Oliveira *et al.*, 2021). The SUMO enzyme complex has been reported to modify blood-stage parasite proteins in the nucleus, cytoplasm and Maurer's clefts (Issar *et al.*, 2008). In *P.*

falciparum trophozoites and schizonts, SUMOylation is involved in pathways related to transcriptional regulation, oxidative stress responses induced by haemoglobin degradation, ubiquitination and proteasome (Reiter *et al.*, 2013; Sumam de Oliveira *et al.*, 2021). Additionally, inhibition of SUMOylation alters schizont morphology, preventing the rupture of iRBCs (Sumam de Oliveira *et al.*, 2021), highlighting its role in the progression of the parasite life cycle. In cluster 16, the hub node was identified as PF3D7_0820700: 2-oxoglutarate dehydrogenase E1 component (OGDC), also known as α-ketoglutarate dehydrogenase. OGDC is a crucial enzyme in the Krebs cycle that catalyzes the conversion of 2-oxoglutarate to succinyl-CoA, fundamental for energy metabolism and amino acid degradation (Günther *et al.*, 2005). This enzyme is located in the mitochondria and is expressed during the erythrocytic stages of the parasites, with increased expression levels in gametocytes compared to asexual erythrocytic stages (McMillan *et al.*, 2005). The observation that metabolic pathway proteins can be targets of SUMOylation suggests that the activity of the E1 component of the 2-oxoglutarate dehydrogenase complex could be regulated by this mechanism (Issar *et al.*, 2008).

Moreover, the brown module showed enrichment for biological pathways and processes associated with the mitotic cell cycle, microtubule function and nuclear processes. In the parasite's life cycle, a single merozoite develops into a trophozoite, which undergoes multiple cycles of DNA synthesis, mitosis and nuclear division, ultimately forming a multi-nucleated parasite (schizont) (Gerald *et al.*, 2011). Microtubules play a vital role in this process, forming the mitotic spindle, which is necessary for chromosome segregation and distribution during the rapid replication and multiplication of the parasite within the human host (Gerald *et al.*, 2011; Morrissette *et al.*, 2023). Microtubules are also crucial components of the cytoskeleton of *P. falciparum*, providing structural support for its distinct shapes and facilitating parasite motility (Ferreira *et al.*, 2022). Although the specific function of the most interconnected node in the brown module remains undetermined, it could facilitate important parasite biological processes during the blood stage.

The PF3D7_1441400 (FACT complex subunit SSRP1, putative) emerged as a cluster 3 hub node. Structure-Specific Recognition Protein 1 (SSRP1) is a small subunit of histone chaperone complex, Facilitates Chromatin Transcription (FACT), which is involved in the assembly and reassembly of nucleosomes, which is critical for DNA transcription, replication and repair (Laurentino *et al.*, 2011; Winkler & Luger, 2011). The histone chaperone FACT is abundant in male gametocytes during the rapid generation of eight haploid gametes by a single male gametocyte (Laurentino *et al.*, 2011), suggesting that this hub node could regulate other nodes associated with nucleosome remodelling to mediate the rapid multiplication of the parasite during the blood stage. Repair of DNA damage is crucial for genomic integrity during DNA replication

and under stress conditions (Lee *et al.*, 2014). Enrichment of DNA repair terms in the dataset highlights the up-regulation of genes associated with DNA repair processes as a strategy for ensuring the parasite's genomic stability and survival within the human host. Specifically, cluster 13 hub node PF3D7_0710400 (DNA repair protein RAD14, putative) is anticipated to play an important role in nucleotide excision repair and DNA damage recognition to guard the stability of the *P. falciparum* genome for optimal growth of asexual blood-stage parasites (Zhang *et al.*, 2020). Further, a missense mutation in PF3D7_0710400 was found to be associated with mutations in the Kelch propeller domain (K13 mutations), which underlies the establishment and transmission of artemisinin resistance in the human population (Xiong *et al.*, 2020).

Stress response and molecular chaperones associated terms were enriched in the yellow module and clusters 7 and 13. *P. falciparum* expresses molecular chaperones, including HSPs, constitutively as well as during stress, with 2% of the genome encoding these molecules. Parasite HSPs facilitate protein homeostasis and trafficking to the erythrocyte surface (Blatch, 2022; Shonhai, 2010). Terms relating to the parasite HSP90 family and their N- and C- terminals were enriched in both the blue module and cluster 7. The *P. falciparum* genome contains four HSP90 genes encoding cytosolic, mitochondrial, endoplasmic reticulum and apicoplast *Pf*HSP90s (Dutta *et al.*, 2022; Elnatan & Agard, 2018). These *Pf*Hsp90s have N-terminal ATP-binding domains essential for ATPase activity (Barth *et al.*, 2022) and a C-terminal Domain (CTD) crucial for binding client proteins, dimerization and interaction with co-chaperones (Singh *et al.*, 2024). Inhibition of parasite HSP90 disrupted the 26S proteasome and arrested intraerythrocytic parasite growth *in vitro* (Banumathy *et al.*, 2003; Dutta *et al.*, 2022; Mansfield *et al.*, 2024; Pallavi *et al.*, 2010). Among the yellow module nodes was mitochondrial *Pf*HSP90 (PF3D7_1118200), crucial for mitochondrial proteostasis and maintaining its antioxidant activity against reactive oxygen species (Stofberg *et al.*, 2021). Similarly, cluster 7 hub node PF3D7_1134000 encodes one of the *Pf*HSP70 family members, i.e., *Pf*HSP70-3, which was shown to play roles in the import of pre-proteins into the mitochondria, just as other eukaryotic Hsp70 proteins (Shonhai, 2010). Moreover, a *Pf*HSP60 encoded by the cluster 13 hub node PF3D7_1015600 is also localised to the parasite mitochondria (Sato *et al.*, 2003). Recently, the interplay between *Pf*HSP70 family members (including *Pf*HSP70-3) and co-chaperones, as well as the functions of these parasite protein complexes in mediating proteolysis and antioxidant defence in the mitochondria, were summarised in a review paper (Barth *et al.*, 2022).

The cluster seed in the yellow module was a putative protein phosphatase PPM11 (encoded by PF3D7_1208900). PPM11 belongs to the Mn^{2+} or Mg^{2+}-dependent serine/threonine phosphatases family (PPM1-13) in *P. falciparum* (Fréville *et al.*, 2022). These holoenzymes are associated with modulating stress responses in various species (Wilkes & Doerig, 2008).

Saturation mutagenesis in the coding sequence of *P. falciparum* NF54 isolate genes showed that, unlike some of the PPM family members (PfPPM2, 6, 8, and 9), *Pf*PPM11 was not essential for the parasite blood development stage *in vitro* (Zhang *et al.*, 2018). This difference might be due to varying *in vitro* and *in vivo* stress conditions that may prompt the up-regulation of the cluster node. Notably, PF3D7_1401400 (encoding ETRAMP14.1) was the only down-regulated gene in the yellow module. ETRAMP14.1 is a developmentally regulated membrane protein, i.e., it is expressed through the early ring stage until the late ring stage starts (18 hours postinfection) (Spielmann *et al.*, 2003), suggesting prioritizing the expression of proteins involved in protein folding and ATP-dependent processes under certain stress conditions to optimize adaptation, growth and survival of the parasites in host.

The genes in the turquoise module, clusters 2 and 17, were enriched for terms relating to the proteasome complex, ubiquitin-proteasome system (UPS) and UPS-dependent pathways. The *P. falciparum* proteasome is a multi-subunit enzyme complex that recognizes and degrades ubiquitinated cellular proteins (Li *et al.*, 2016). Various protease complexes of the *Plasmodium* parasite have been identified, including a eukaryotic proteasome (26S) in the cytoplasm and nucleus, a prokaryotic proteasome homolog (ClpQ) in the mitochondria and a caseinolytic protease complex (ClpP) in the apicoplast (Krishnan & Williamson, 2018). The α- and β-proteasome subunits are expressed in all blood stages, highlighting the need for protein degradation during development (Aminake *et al.*, 2011). The turquoise module seed node, 26S proteasome regulatory subunit RPN1, putative (encoded by PF3D7_0205900), is part of the 26S proteasome. The RPN1 subunit forms the base of the 19S regulatory particle of the 26S proteasome and is responsible for the ATP-dependent translocation of deubiquitinated proteins into the core particle of the proteasome for degradation (Aminake *et al.*, 2012). Increased PF3D7_0205900 transcript levels were associated with a higher risk of SMA, indicating a greater need for proteolysis and protein turnover in these patients. Similarly, increased transcript levels of cluster 2 hub node PF3D7_1353800 (encoding putative proteasome subunit alpha type-4) were significantly associated with a higher risk of SMA, and increased expression of PF3D7_1353800 in SMA may reflect the parasite's response to high protein turnover during its growth in iRBCs. The putative proteasome subunit alpha type-4 is a component of the 20S core particle of the proteasome involved in protein degradation (Aminake *et al.*, 2012), and targeting the proteolytic core 20S proteasome with antimalarials was proposed to be capable of inhibiting parasite growth at all stages (Krishnan & Williamson, 2018). Moreover, PF3D7_1363400 (Polyubiquitin binding protein, putative) emerged as the hub node of cluster 17. Polyubiquitin protein was identified in ring, trophozoite and schizont stages, with its abundance peaking in trophozoites (Ponts *et al.*, 2011), and polyubiquitination targets protein substrates for degradation by the proteasome

(Hamilton *et al.*, 2014). Although less studied, polyubiquitin-binding proteins could recognize and bind polyubiquitinated chains attached to substrate proteins, targeting proteins for degradation (Hamilton *et al.*, 2014). Overall, the parasite UPS is essential for protein quality control and regulation of cellular protein levels during rapid growth and replication.

Enriched terms in cluster 4 were involved in vesicle transport from the endoplasmic reticulum (ER) to the Golgi apparatus, while cluster 14 terms were related to endocytosis and retrograde transport. Parasite proteins are co-translationally transported into the ER, where they are modified to become functional or partially functional (Cooke *et al.*, 2004). Newly synthesised molecules in the ER are packaged into ER-derived transport vehicles and sent to the Golgi apparatus to be modified further and sorted before being trafficked to their final locations within the cell or beyond the cell membrane, such as the parasitophorous vacuole, host RBC cytoplasm and RBC plasma membrane (Cooke *et al.*, 2004; Deponte *et al.*, 2012; Krai *et al.*, 2014). The Transport Protein Particle (TRAPP) complex, identified in multiple enriched terms, aids in tethering the ER-derived vesicles, facilitating their docking and fusion to the Golgi and plasma membrane (Mayer, 2021; Whyte & Munro, 2002). Cluster 4's hub node, PF3D7_0618700, encodes a putative TRAPP complex subunit 6A (TRAPPC6A) protein (Kümmel *et al.*, 2005). While TRAPPC6A's specific role in *P. falciparum* is unclear, it likely plays a significant role in TRAPP-mediated vesicle tethering in the secretory pathway. On the other hand, malaria parasites rely on endocytosis to take up host cell cytosol components as nutrients into their digestive vacuoles during intra-erythrocytic development (Spielmann *et al.*, 2020). It was shown that protein translocation from the endosome to the Golgi body in yeast cells relies on a retrograde transport process mediated by the retromer complex, composed of three core sub-units: vacuolar protein sorting-associated protein (VPS) 26, 29, and 35 (Burd & Cullen, 2014). In the current study, transcript levels of three parasite retromer complex components, *Pf*VPS26 (encoded by PF3D7_1250300), *Pf*VPS35 (encoded by PF3D7_1110500), and *Pf*VPS52 (encoded by PF3D7_1323800) were all significantly up-regulated in children with SMA. Interestingly, PF3D7_1110500 is the hub node of cluster 14.

Cluster 8 was enriched for metabolic pathways, i.e., glycolysis, ribose phosphate metabolism, ATP metabolism, vacuole function and vacuolar proton transport. Erythrocyte stage *P. falciparum* lacks a fully functional TCA cycle and primarily depends on anaerobic glycolysis to generate two ATPs (in addition to two each of pyruvate and NADH molecules) from each glucose molecule for the energy needs of the parasite (van Niekerk *et al.*, 2023). To sustain glycolysis, NADH is recycled back to NAD^+ during the conversion of pyruvate to lactate, ensuring a continuous supply of NAD+ for glycolysis (van Niekerk *et al.*, 2023). The rate of glucose utilisation by iRBCs was reported to be 100-fold compared to that of healthy RBCs

(Roth *et al.*, 1982). Notably, some of the life-threatening clinical manifestations in patients with severe malaria include hypoglycaemia and hyperlactatemia (Marsh *et al.*, 1995), which could be attributed in part to the metabolic disturbances caused by the intracellular parasite. The *P. falciparum* food vacuole function was also enriched in this cluster. The parasite utilises this organelle to digest erythrocyte haemoglobin for amino acids and to reduce the osmotic pressure in host RBCs (Krugliak *et al.*, 2002; Lew *et al.*, 2004). V-type proton ATPase subunit B, encoded by the hub node PF3D7_0406100 of this cluster, is a component of the vacuolar-type H^+-ATPase (V-ATPase) complex, crucial for maintaining the acidic environment of the digestive vacuole, with ablation of this protein causing deacidification of the digestive vacuole and halted parasite growth in RBCs (Alder *et al.*, 2023).

Lipid (glycerophospholipids, glycerolipids and phosphatidic acid) biosynthesis emerged in cluster 12. Apicomplexa parasites such as the *Plasmodium* and *Toxoplasma* parasites require lipids for membrane biogenesis and signalling during parasite invasion and egress from the iRBCs (Shunmugam *et al.*, 2022). Upon infection by *Plasmodium* parasites, total phospholipids, which are composed of the glycerophospholipid (GPLs) [including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and lysophosphatidylcholine (LysoPC)] and sphingomyelin (SM), increased six-fold in infected erythrocytes (i.e. iRBC) relative to non-infected erythrocytes (Déchamps *et al.*, 2010). During the intraerythrocytic stage, GPLs are the main membrane constituents, and their levels remain constant across the asexual stages (Gulati *et al.*, 2015). Moreover, the major parasite glycerolipids (diacylglycerol and triacylglycerol) levels increased as blood-stage parasites mature, while gametocytes have lower glycerolipid levels (Gulati *et al.*, 2015). When the parasite synthesizes GPLs, it acquires fatty acid precursors from serum or *de novo* synthesis in the apicoplast (Mazumdar & Striepen, 2007; Shears *et al.*, 2015). Cluster 12's hub node PF3D7_0303200 encodes lipin, an enzyme that dephosphorylates phosphatidic acid to diacylglycerol, a precursor of triacylglycerol and phospholipids, preventing lipotoxicity (Dass *et al.*, 2021). Disruption of lipin leads to the accumulation of elevated levels of ceramides and lysophosphatidic acid lipid species in iRBC, significantly inhibiting parasite growth (Cobbold *et al.*, 2021).

Other enriched metabolic terms included the TCA cycle, carbon metabolism and oxidoreductase activity (cluster 15). The TCA cycle, although not essential for asexual blood stage parasite survival (van Niekerk *et al.*, 2023), likely generates ATP in sporozoites and ookinetes (van Dooren *et al.*, 2006). However, the TCA cycle supports electron chain transport, crucial for generating a proton gradient for both ATP production and biosynthetic pathways (Painter *et al.*, 2007; van Dooren *et al.*, 2006). As such, enrichment in the TCA cycle during asexual stages

suggests a biosynthetic rather than ATP-producing role. In addition, oxidoreductase activity is catalysed by malate-quinone oxidoreductase in the mitochondria, facilitating electron chain transport, which is linked to pyrimidine biosynthesis (Ito *et al.*, 2023). Collectively, TCA metabolism's ability to utilise multiple carbon sources allows metabolic flexibility during the parasite's lifecycle (Ke *et al.*, 2015).

Collectively, RNA-seq analysis reveals that *P. falciparum* displays distinct transcriptional profiles in SMA. Significant up-regulation of genes involved in protein synthesis, stress responses and energy metabolism likely contributes to the parasite's rapid growth and survival. Additionally, down-regulation of genes related to erythrocyte invasion and metabolic efficiency in SMA suggests a complex regulatory mechanism that enables the parasite to adapt to the host environment while also contributing to the pathophysiology of the disease.

5.4 Limitations of the Study

Limitations for this comprehensive examination of host-parasite dynamics include: 1) for the genetic study, the low minor allele frequency for one of the SNPs limited the ability to achieve statistical significance in some analyses despite a large sample size with 1000s of follow-up events. In addition, covariates in the statistical models for determining the relationship between *NCR3* polymorphisms and clinical outcomes did not include nutritional status as a potential anaemia-promoting factor. 2) Although the RNA-seq study was the largest performed to date and yielded novel information about the pathogenesis of SMA, including important information about signalling networks for NK cells and others, single-cell RNA-seq was not performed. Thus, while the study revealed what happens at the bulk cellular aggregation scale, granularity could not be achieved at the individual cellular level. 3) For the *P. falciparum* RNA-seq analyses, the natural biological process in human infections is the difficulty in detecting certain parasite stages due to sequestration in tissues. Thus, while there are novel and important parasitic networks that were identified in children with SMA, additionally important pathological processes cannot be defined from the full transcriptomic profile obtained from peripheral blood.

CHAPTER SIX
SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

6.1.1 Relationship between Genetic Polymorphisms in *NCR3* and Longitudinal Susceptibility to Childhood Malaria, SMA and All-cause mortality

The SNPs rs2736191 and rs11575837 had an MAF of 0.30 and 0.03, respectively, in the study population. TFBS analysis showed that the C to G conversion in rs2736191 caused the ablation of the binding site for transcription factor TFAP2A and the creation of a binding site for TCF3 and ZBTB6. Conversely, the conversion of C to T in rs11575837 resulted in the loss of TFAP2A binding site and the gain of HNF4A binding site. LD analysis between these SNPs revealed a strong correlation between the SNPs ($D'=0.882$). The observed frequencies of the rs2736191 and rs11575837 genotypes in the overall population displayed a significant departure from HWE. Carriage of the rs11575837 genotype (TT) increased the risk of malaria, while inheritance of CC haplotype conferred an additive protective effect against SMA during the follow-up period. However, there was no relationship between the genotypes/haplotypes of rs2736191 and rs11575837 and all-cause mortality.

6.1.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA

RNA-seq of the entire expressed transcriptome in whole blood revealed 6,862 DEGs in SMA relative to non-SMA (3,420 up-regulated and 3,442 down-regulated). Children with SMA showed increased expression of resting NK cells. Process network enrichment analysis identified dysregulation in the Inflammation_NK cell cytotoxicity process network in SMA patients with differential expression of crucial components such as transcription factors, enzymes and adaptor proteins affecting proper NK cell function. Enrichment for immune response canonical pathways revealed the HSP60-HSP70-TLR2/4 signalling pathway as one of the top 10 dysregulated pathways in SMA. In children with SMA, key immune response genes such as HSP70, HSP60, TLR2, TLR4, and various cytokines (e.g., IL-6, IL-1β) were significantly down-regulated, suggesting impaired immune signalling. Conversely, genes like IRAK1, TAK1, and MHC class II molecules were up-regulated, indicating a potentially compensatory immune response. These expression changes reflect a complex immune dysregulation in SMA that may affect disease severity and progression.

An unsupervised hierarchical clustering of DEGs in the HSP60-HSP70-TLR signalling pathway revealed two distinct clusters. Cluster 1 DEGs were enriched for process networks related to Inflammation_Amphoterin Signaling and Inflammation-Innate Inflammatory Response. In contrast, cluster 2 analysis indicated enrichment in Immune Response_T Helper Cell

Differentiation and Immune Response_TCR signalling process networks. Moreover, seven genes encoding transporters of GLN were differentially expressed (Up-regulated: SLC6A19, SLC7A5, SLC1A5, SLC7A8, and SLC38A1, and down-regulated: SLC38A2 and SLC38A3) in SMA vs non-SMA. Furthermore, the gene encoding glutamine synthetase was up-regulated in children with SMA.

6.1.3 Transcriptome Profile of Blood-stage *P. falciparum* in Children with SMA

Transcriptomic analysis of blood-stage *P. falciparum* in paediatric malaria patients uncovered 566 parasite DEGs (499 up-regulated, while 67 down-regulated in SMA relative to non-SMA) out of 3,155 annotated parasite gene transcripts. The top up-regulated genes encoded mitochondrial and ribosomal proteins, DNA methyltransferases, proteasome subunits and trafficking proteins, underscoring these functions' importance in sustaining the parasite's rapid growth and replication within red blood cells. The imputation of different *P. falciparum* developmental stages from peripheral blood suggest an accelerated transition from the ring stage to the trophozoite stage in SMA patients, suggesting stage-specific expression patterns. This transition resulted in higher proportions of schizonts, potentially leading to more frequent rupture and invasion of new red blood cells. Furthermore, a higher male-to-female gametocyte ratio across all patients highlights a possible adaptive response for enhanced transmission efficiency under stress conditions.

Enrichment analyses of WGCNA modules and PPI clusters identify key parasite cellular processes such as cell division, RNA splicing, ribosomal assembly, protein synthesis, post-translational modifications, protein transport, metabolic pathways and molecular chaperoning, which are crucial for the exponential multiplication of the parasite in SMA. Hub nodes, including PF3D7_1331700 (glutamine–tRNA ligase), PF3D7_0322900 (40S ribosomal protein S3A), PF3D7_1353800 (proteasome subunit alpha type-4), PF3D7_0205900 (26S proteasome regulatory subunit RPN1), PF3D7_1441400 (FACT complex subunit SSRP1), PF3D7_0618700 (trafficking protein particle complex subunit 6A), and PF3D7_1213800 (proline–tRNA ligase), were identified as potential risk factors for developing severe malarial anemia (SMA) in pediatric malaria patients.

6.2 Conclusions

6.2.1 Relationship between Genetic Polymorphisms in *NCR3* and Longitudinal Susceptibility to Childhood malaria, SMA and All-cause Mortality

The study presents the first evidence that carriage of the TT genotype for rs11575837 is linked to an increased number of malaria episodes over time. Additionally, the study provides evidence that the carriage of CC haplotype confers a protective effect against SMA in an additive model

and, conversely, the additive effect of GC haplotype carriage on enhanced susceptibility to SMA. Collectively, these findings show the relationship between genetic variations in *NCR3* and disease outcomes in pediatric malaria patients in holoendemic regions, thus facilitating more investigations to unveil the impacts of additional genetic factors on susceptibility to malaria and the development of childhood SMA.

6.2.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA

The current study provides evidence of NK cell activation and subsequent heightened immune response in malaria-naïve children who develop SMA. Enhanced immune response contributes to SMA due to altered NK cell-mediated cytotoxicity, suppressed apoptotic ability, and altered inflammatory signalling. The study also highlights the dysregulation of immune response and inflammatory pathways, including the HSP60-HSP70-TLR2/4 signalling pathway. Downregulation of gene products in the pathway (e.g., HSP70 family members, TLR2, TLR4, CD14, and NF-κB1) suggests that children with SMA may have reduced pattern recognition and inflammatory response. At the same time, up-regulation of other components (e.g., IRAK1, CD86, and MHC class II molecules) indicates an attempt of leukocytes to compensate through alternative immune activation and antigen presentation mechanisms. Furthermore, the enrichment of DEGs in the pathway for process networks underscored the dysregulation of inflammation and immune response pathways in SMA patients. Lastly, the overall up-regulation of GLN transporters and up-regulated GLN synthesis enzyme GLUL indicates a compensatory mechanism by leukocytes to raise intracellular GLN levels and regulate the immune response and metabolic requirements in SMA. These findings enhance our understanding of the molecular mechanisms of SMA pathogenesis in children.

6.2.3 Transcriptome Profile of Blood-stage *P. falciparum* in Children with SMA

The transcriptome profiling of blood-stage *P. falciparum* in children with SMA revealed insights into the parasite's gene expression and adaptive strategies during the blood stage. Notably, there are approximately sixfold more upregulated DEGs than down-regulated DEGs in SMA. The accelerated transition from the ring stage to the trophozoite stage in SMA patients and higher proportions of schizonts could contribute to the pathophysiology of SMA through increased haemoglobin metabolism, *Pf*Hz formation, and a more frequent rupture and invasion of new RBCs. Furthermore, a high parasite sex ratio in SMA patients highlights a potential adaptive strategy by the parasite to facilitate increased transmission efficiency by maximizing reproductive success under adverse conditions. The study also revealed the upregulation of genes involved in the parasite's biological processes, for instance, cell division, protein synthesis and degradation, RNA splicing and post-translational modification, metabolic pathways, molecular chaperones and cellular transport. These biological processes support the parasite's rapid growth and

increased metabolism, mediating its adaptation and survival in the host. The study identified the hub nodes as potential predictors for the risk of developing SMA in children, indicating that they may play a critical role in the pathogenesis of SMA and suggesting their potential as therapeutic targets.

6.3 Recommendations from the Current Study

6.3.1 Relationship between Genetic polymorphisms in *NCR3* and Longitudinal Susceptibility to Childhood Malaria, SMA and All-cause mortality

It is recommended that the study findings inform the genomic epidemiology of SMA by adding knowledge about genetic variants in immune response genes associated with susceptibility to SMA and other disease outcomes in holoendemic malaria transmission areas like western Kenya. This information could be used to determine the polygenic risk score of an individual to estimate their genetic predisposition to developing SMA, enabling the identification of high-risk individuals for targeted prevention and treatment strategies.

6.3.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA

The current study recommends the identification and evaluation of small-molecule therapeautic compounds that target dysregulated genes/gene pathways in SMA to modulate the impaired immune response, fostering the development of novel therapies to improve clinical outcomes in individuals with SMA. Additionally, the study recommends the exploration of the potential effect of GLN supplementation on immune response and metabolism in children with SMA.

6.3.3 Transcriptome Profile of Blood-stage *P. falciparum* in Children with SMA

The study recommends the development of targeted therapeautics targeting *P. falciparum* genes and pathways that are active in blood-stage parasites within the human host, i.e., those involved in mitochondrial function, protein synthesis, and the ubiquitin-proteasome system to disrupt parasite growth. Moreover, the study recommends the combination of therapies targeting multiple significant pathways in *P. falciparum* simultaneously to enhance the efficacy of anti-parasite treatment and reduce the risk of drug resistance.

6.4 Recommendations for Future Studies

6.4.1 Relationship between Genetic Polymorphisms in *NCR3* and Longitudinal Susceptibility to Childhood Malaria, SMA and All-cause Mortality

This study recommends the following:

i. Mechanistic studies on TFBS affected by rs2736191 and rs11575837 SNPs to investigate how the changes in transcription factor binding sites affect NCR3 gene transcription and subsequent NCR3 production.

ii. Functional analysis to investigate the underlying molecular mechanisms by which NCR3 expressed by NK cells impacts the development of SMA in paediatric malaria patients.

6.4.2 NK Cell Networks, and Other Immune signalling Pathways in Children with SMA

The study recommends the following:

i. Investigation into the cytotoxic activity of NK cells in SMA patients given the increased expression of resting NK cells and dysregulated Inflammation_NK cell cytotoxicity process network.
ii. Measurement of the protein levels of the key genes identified in the study to establish whether their expression levels correspond to their protein levels.
iii. Validation of the observed findings with both an independent RNA-seq dataset of children with SMA and protein abundance levels in the plasma of children with SMA to corroborate the current transcriptional data.
iv. Identifying and evaluating small molecule therapeautic compounds (*in vitro*) that target dysregulated genes/gene pathways in SMA to foster the development of novel therapies to improve clinical outcomes in individuals with SMA

6.4.3 Transcriptome Profile of Blood-stage *P. falciparum* in Children with SMA

The study recommends the following:

i. Functional characterisation of top up-regulated genes and hub genes with unknown functions to understand their precise roles in the parasite's lifecycle and SMA pathogenesis. Additionally, gene knockout or knockdown studies should be carried out on the identified key genes to assess the effect of gene disruption on parasite growth, survival, and virulence.
ii. Host-parasite co-expression analysis to understand host-parasite interactions, pathophysiological changes in both the host and parasite during infection and the host's immune response against the parasite.
iii. Longitudinal transcriptional studies to profile the expression dynamics of parasite genes to provide insights into the temporal regulation of gene expression.
iv. Integrating transcriptomic data with other omics data, e.g., proteomics and metabolomics, to uncover the complex interactions and regulatory mechanisms necessary for parasite survival.

REFERENCES

Abel, A. M., Yang, C., Thakar, M. S., & Malarkannan, S. (2018). Natural Killer Cells: Development, Maturation, and Clinical Utilization. *Frontiers in Immunology, 9*(1869). doi:10.3389/fimmu.2018.01869

Achieng', A. O., Guyah, B., Cheng, Q., Ong'echa, J. M., Ouma, C., Lambert, C. G., & Perkins, D. J. (2019a). Molecular Basis of Reduced Lair1 Expression in Childhood Severe Malarial Anaemia: Implications for Leukocyte Inhibitory Signalling. *EBioMedicine, 45*, 278-289. doi:10.1016/j.ebiom.2019.06.040

Achieng', A. O., Hengartner, N. W., Raballah, E., Cheng, Q., Anyona, S. B., Lauve, N., . . . Perkins, D. J. (2019b). Integrated Omics Platforms Identify Lair1 Genetic Variants as Novel Predictors of Cross-Sectional and Longitudinal Susceptibility to Severe Malaria and All-Cause Mortality in Kenyan Children. *EBioMedicine, 45*, 290-302. doi:10.1016/j.ebiom.2019.06.043

Agamah, F. E., Damena, D., Skelton, M., Ghansah, A., Mazandu, G. K., & Chimusa, E. R. (2021). Network-Driven Analysis of Human-*Plasmodium Falciparum* Interactome: Processes for Malaria Drug Discovery and Extracting in Silico Targets. *Malaria Journal, 20*(1), 421. doi:10.1186/s12936-021-03955-0

Agresta, L., Hoebe, K. H. N., & Janssen, E. M. (2018). The Emerging Role of Cd244 Signaling in Immune Cells of the Tumor Microenvironment. *Frontiers in Immunology, 9*, 2809. doi:10.3389/fimmu.2018.02809

Agudelo, O., Bueno, J., Villa, A., & Maestre, A. (2012). High Ifn-Gamma and Tnf Production by Peripheral Nk Cells of Colombian Patients with Different Clinical Presentation of *Plasmodium Falciparum*. *Malar J, 11*, 38. doi:10.1186/1475-2875-11-38

Aguiar, J. C., Bolton, J., Wanga, J., Sacci, J. B., Iriko, H., Mazeika, J. K., . . . Richie, T. L. (2015). Discovery of Novel *Plasmodium Falciparum* Pre-Erythrocytic Antigens for Vaccine Development. *PLoS One, 10*(8), e0136109. doi:10.1371/journal.pone.0136109

Ahmed, C. M., & Johnson, H. M. (2006). Ifn-Gamma and Its Receptor Subunit Ifngr1 Are Recruited to the Ifn-Gamma-Activated Sequence Element at the Promoter Site of Ifn-Gamma-Activated Genes: Evidence of Transactivational Activity in Ifngr1. *Journal of Immunology (Baltimore, Md. : 1950), 177*(1), 315-321. doi:10.4049/jimmunol.177.1.315

AhYoung, A. P., Koehl, A., Cascio, D., & Egea, P. F. (2015). Structural Mapping of the Clpb Atpases of *Plasmodium Falciparum*: Targeting Protein Folding and Secretion for Antimalarial Drug Design. *Protein Science : a Publication of the Protein Society, 24*(9), 1508-1520. doi:10.1002/pro.2739

Aidoo, M., Terlouw, D., Kolczak, M., McElroy, P., Kuile, F., Kariuki, S., . . . Udhayakumar, V. (2002). Protective Effects of the Sickle Cell Gene against Malaria Morbidity and Mortality. *Lancet, 359*(9314), 1311-1312.

Akech, S., Chepkirui, M., Ogero, M., Agweyu, A., Irimu, G., English, M., & Snow, R. W. (2019). The Clinical Profile of Severe Pediatric Malaria in an Area Targeted for Routine Rts,S/As01 Malaria Vaccination in Western Kenya. *Clinical Infectious Diseases, 71*(2), 372-380. doi:10.1093/cid/ciz844

Alder, A., Sanchez, C. P., Russell, M. R. G., Collinson, L. M., Lanzer, M., Blackman, M. J., . . . Matz, J. M. (2023). The Role of <I>Plasmodium</I> V-Atpase in Vacuolar Physiology and Antimalarial Drug Uptake. *Proceedings of the National Academy of Sciences, 120*(30), e2306420120. doi:doi:10.1073/pnas.2306420120

Aminake, M. N., Arndt, H.-D., & Pradel, G. (2012). The Proteasome of Malaria Parasites: A Multi-Stage Drug Target for Chemotherapeutic Intervention? *International Journal for Parasitology: Drugs and Drug Resistance, 2*, 1-10. doi:https://doi.org/10.1016/j.ijpddr.2011.12.001

Aminake, M. N., Schoof, S., Sologub, L., Leubner, M., Kirschner, M., Arndt, H. D., & Pradel, G. (2011). Thiostrepton and Derivatives Exhibit Antimalarial and Gametocytocidal Activity by Dually Targeting Parasite Proteasome and Apicoplast. *Antimicrobial Agents and Chemotherapy, 55*(4), 1338-1348. doi:10.1128/aac.01096-10

Anders, S., Pyl, P. T., & Huber, W. (2015). Htseq—a Python Framework to Work with High-Throughput Sequencing Data. *Bioinformatics, 31*(2), 166-169.

Anyona, S., Cheng, Q., Guo, Y., Raballah, E., Hurwitz, I., Onyango, C., . . . Perkins, D. (2023). Entire Expressed Peripheral Blood Transcriptome in Pediatric Severe Malarial Anemia. *Research Square*. doi:10.21203/rs.3.rs-3150748/v1

Anyona, S. B., Hengartner, N. W., Raballah, E., Ong'echa, J. M., Lauve, N., Cheng, Q., . . . Perkins, D. J. (2020). Cyclooxygenase-2 Haplotypes Influence the Longitudinal Risk of Malaria and Severe Malarial Anemia in Kenyan Children from a Holoendemic Transmission Region. *Journal of Human Genetics, 65*(2), 99-113. doi:10.1038/s10038-019-0692-3

Anyona, S. B., Raballah, E., Cheng, Q., Hurwitz, I., Ndege, C., Munde, E., . . . Perkins, D. J. (2021). Differential Gene Expression in Host Ubiquitination Processes in Childhood Malarial Anemia. *Frontiers in Genetics, 12*, 764759. doi:10.3389/fgene.2021.764759

Arlet, J. B., Ribeil, J. A., Guillem, F., Negre, O., Hazoume, A., Marcion, G., . . . Courtois, G. (2014). Hsp70 Sequestration by Free A-Globin Promotes Ineffective Erythropoiesis in B-Thalassaemia. *Nature, 514*(7521), 242-246. doi:10.1038/nature13614

Arnon, T. I., Markel, G., & Mandelboim, O. (2006). Tumor and Viral Recognition by Natural Killer Cells Receptors. *Seminars in Cancer Biology, 16*(5), 348-358. doi:10.1016/j.semcancer.2006.07.005

Arora, G., Hart, G. T., Manzella-Lapeira, J., Doritchamou, J. Y., Narum, D. L., Thomas, L. M., . . . Long, E. O. (2018). Nk Cells Inhibit *Plasmodium Falciparum* Growth in Red Blood Cells Via Antibody-Dependent Cellular Cytotoxicity. *eLife, 7*. doi:10.7554/eLife.36806

Artavanis-Tsakonas, K., Eleme, K., McQueen, K. L., Cheng, N. W., Parham, P., Davis, D. M., & Riley, E. M. (2003a). Activation of a Subset of Human Nk Cells Upon Contact with *Plasmodium Falciparum*-Infected Erythrocytes. *Journal of Immunology (Baltimore, Md. : 1950), 171*(10), 5396-5405. doi:10.4049/jimmunol.171.10.5396

Artavanis-Tsakonas, K., & Riley, E. M. (2002). Innate Immune Response to Malaria: Rapid Induction of Ifn-Gamma from Human Nk Cells by Live *Plasmodium Falciparum*-Infected Erythrocytes. *Journal of Immunology (Baltimore, Md. : 1950), 169*(6), 2956-2963. doi:10.4049/jimmunol.169.6.2956

Artavanis-Tsakonas, K., Tongren, J. E., & Riley, E. M. (2003b). The War between the Malaria Parasite and the Immune System: Immunity, Immunoregulation and Immunopathology. *Clinical and Experimental Immunology, 133*(2), 145-152. doi:10.1046/j.1365-2249.2003.02174.x

Arthur, L. L., & Djuranovic, S. (2018). Polya Tracks, Polybasic Peptides, Poly-Translational Hurdles. *Wiley Interdisciplinary Reviews. RNA, 9*(5), e1486. doi:10.1002/wrna.1486

Asea, A., Kraeft, S. K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., . . . Calderwood, S. K. (2000). Hsp70 Stimulates Cytokine Production through a Cd14-Dependant Pathway, Demonstrating Its Dual Role as a Chaperone and Cytokine. *Nature Medicine, 6*(4), 435-442. doi:10.1038/74697

Auton, A., Abecasis, G. R., Altshuler, D. M., Durbin, R. M., Abecasis, G. R., Bentley, D. R., . . . National Eye Institute, N. I. H. (2015). A Global Reference for Human Genetic Variation. *Nature, 526*(7571), 68-74. doi:10.1038/nature15393

Awandare, G. A., Hittner, J. B., Kremsner, P. G., Ochiel, D. O., Keller, C. C., Weinberg, J. B., . . . Perkins, D. J. (2006). Decreased Circulating Macrophage Migration Inhibitory Factor (Mif) Protein and Blood Mononuclear Cell Mif Transcripts in Children with *Plasmodium Falciparum* Malaria. *Clinical Immunology (Orlando, Fla.), 119*(2), 219-225. doi:10.1016/j.clim.2005.12.003

Awandare, G. A., Martinson, J. J., Were, T., Ouma, C., Davenport, G. C., Ong'echa, J. M., . . . Perkins, D. J. (2009). Mif (Macrophage Migration Inhibitory Factor) Promoter Polymorphisms and Susceptibility to Severe Malarial Anemia. *The Journal of Infectious Diseases, 200*(4), 629-637. doi:10.1086/600894

Awandare, G. A., Ouma, Y., Ouma, C., Were, T., Otieno, R., Keller, C. C., . . . Perkins, D. J. (2007). Role of Monocyte-Acquired Hemozoin in Suppression of Macrophage Migration

Inhibitory Factor in Children with Severe Malarial Anemia. *Infection and Immunity, 75*(1), 201-210. doi:10.1128/iai.01327-06
Baaklini, S., Afridi, S., Nguyen, T. N., Koukouikila-Koussounda, F., Ndounga, M., Imbert, J., . . . Rihet, P. (2017). Beyond Genome-Wide Scan: Association of a Cis-Regulatory Ncr3 Variant with Mild Malaria in a Population Living in the Republic of Congo. *PloS one, 12*(11), e0187818-e0187818. doi:10.1371/journal.pone.0187818
Bader, G. D., & Hogue, C. W. V. (2003). An Automated Method for Finding Molecular Complexes in Large Protein Interaction Networks. *BMC Bioinformatics, 4*(1), 2. doi:10.1186/1471-2105-4-2
Banumathy, G., Singh, V., Pavithra, S. R., & Tatu, U. (2003). Heat Shock Protein 90 Function Is Essential for <Em>*Plasmodium Falciparum*</Em> Growth in Human Erythrocytes *. *Journal of Biological Chemistry, 278*(20), 18336-18345. doi:10.1074/jbc.M211309200
Banumathy, G., Singh, V., & Tatu, U. (2002). Host Chaperones Are Recruited in Membrane-Bound Complexes by *Plasmodium Falciparum*. *The Journal of Biological Chemistry, 277*(6), 3902-3912. doi:10.1074/jbc.M110513200
Baratin, M., Roetynck, S., Lépolard, C., Falk, C., Sawadogo, S., Uematsu, S., . . . Ugolini, S. (2005). Natural Killer Cell and Macrophage Cooperation in Myd88-Dependent Innate Responses to *Plasmodium Falciparum*. *Proc Natl Acad Sci U S A, 102*(41), 14747-14752. doi:10.1073/pnas.0507355102
Baratin, M., Roetynck, S., Pouvelle, B., Lemmers, C., Viebig, N. K., Johansson, S., . . . Ugolini, S. (2007). Dissection of the Role of Pfemp1 and Icam-1 in the Sensing of Plasmodium-Falciparum-Infected Erythrocytes by Natural Killer Cells. *PLoS One, 2*(2), e228. doi:10.1371/journal.pone.0000228
Barrow, A. D., Martin, C. J., & Colonna, M. (2019). The Natural Cytotoxicity Receptors in Health and Disease. *Frontiers in Immunology, 10*(909). doi:10.3389/fimmu.2019.00909
Barth, J., Schach, T., & Przyborski, J. M. (2022). Hsp70 and Their Co-Chaperones in the Human Malaria Parasite P. Falciparum and Their Potential as Drug Targets. *Frontiers in Molecular Biosciences, 9*. doi:10.3389/fmolb.2022.968248
Becknell, B., & Caligiuri, M. A. (2005). Interleukin-2, Interleukin-15, and Their Roles in Human Natural Killer Cells. *Advances in Immunology, 86*, 209-239. doi:10.1016/s0065-2776(04)86006-1
Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of he Royal Statistical Society: Series B (Methodological), 57*(1), 289-300.
Bhatt, T. K., Kapil, C., Khan, S., Jairajpuri, M. A., Sharma, V., Santoni, D., . . . Sharma, A. (2009). A Genomic Glimpse of Aminoacyl-Trna Synthetases in Malaria Parasite *Plasmodium Falciparum*. *BMC Genomics, 10*(1), 644. doi:10.1186/1471-2164-10-644
Björkström, N. K., Ljunggren, H.-G., & Michaëlsson, J. (2016). Emerging Insights into Natural Killer Cells in Human Peripheral Tissues. *Nature Reviews Immunology, 16*(5), 310-320. doi:10.1038/nri.2016.34
Blatch, G. L. (2022). *Plasmodium Falciparum* Molecular Chaperones: Guardians of the Malaria Parasite Proteome and Renovators of the Host Proteome. *Frontiers in Cell and Developmental Biology, 10*. doi:10.3389/fcell.2022.921739
Bloland, P. B., Boriga, D. A., Ruebush, T. K., McCormick, J. B., Roberts, J. M., Oloo, A. J., . . . Campbell, C. C. (1999). Longitudinal Cohort Study of the Epidemiology of Malaria Infections in an Area of Intense Malaria Transmission Ii. Descriptive Epidemiology of Malaria Infection and Disease among Children. *American Journal of Tropical Medicine & Hygiene, 60*(4), 641-648.
Boldt, A. B. W., van Tong, H., Grobusch, M. P., Kalmbach, Y., Dzeing Ella, A., Kombila, M., . . . Velavan, T. P. (2019). The Blood Transcriptome of Childhood Malaria. *EBioMedicine, 40*, 614-625. doi:10.1016/j.ebiom.2018.12.055
Bopp, S., Pasaje, C. F. A., Summers, R. L., Magistrado-Coxen, P., Schindler, K. A., Corpas-Lopez, V., . . . Wirth, D. F. (2023). Potent Acyl-Coa Synthetase 10 Inhibitors Kill

Plasmodium Falciparum by Disrupting Triglyceride Formation. *Nature Communications, 14*(1), 1455. doi:10.1038/s41467-023-36921-2
Böttger, E., Multhoff, G., Kun, J. F., & Esen, M. (2012). *Plasmodium Falciparum*-Infected Erythrocytes Induce Granzyme B by Nk Cells through Expression of Host-Hsp70. *PLoS One, 7*(3), e33774. doi:10.1371/journal.pone.0033774
Bozdech, Z., Llinás, M., Pulliam, B. L., Wong, E. D., Zhu, J., & DeRisi, J. L. (2003). The Transcriptome of the Intraerythrocytic Developmental Cycle of *Plasmodium Falciparum*. *PLoS Biology, 1*(1), e5. doi:10.1371/journal.pbio.0000005
Bradwell, K. R., Coulibaly, D., Koné, A. K., Laurens, M. B., Dembélé, A., Tolo, Y., . . . Serre, D. (2020). Host and Parasite Transcriptomic Changes Upon Successive *Plasmodium Falciparum* Infections in Early Childhood. *mSystems, 5*(4). doi:10.1128/mSystems.00116-20
Brisebarre, A., Kumulungui, B., Sawadogo, S., Atkinson, A., Garnier, S., Fumoux, F., & Rihet, P. (2014). A Genome Scan for *Plasmodium Falciparum* Malaria Identifies Quantitative Trait Loci on Chromosomes 5q31, 6p21.3, 17p12, and 19p13. *Malaria Journal, 13*, 198. doi:10.1186/1475-2875-13-198
Burch, J. S., Marcero, J. R., Maschek, J. A., Cox, J. E., Jackson, L. K., Medlock, A. E., . . . Dailey, H. A., Jr. (2018). Glutamine Via A-Ketoglutarate Dehydrogenase Provides Succinyl-Coa for Heme Synthesis During Erythropoiesis. *Blood, 132*(10), 987-998. doi:10.1182/blood-2018-01-829036
Burd, C., & Cullen, P. J. (2014). Retromer: A Master Conductor of Endosome Sorting. *Cold Spring Harbor Perspectives in Biology, 6*(2). doi:10.1101/cshperspect.a016774
Burrack, K. S., Hart, G. T., & Hamilton, S. E. (2019). Contributions of Natural Killer Cells to the Immune Response against Plasmodium. *Malaria Journal, 18*(1), 321. doi:10.1186/s12936-019-2953-1
Burshtyn, D. N., Scharenberg, A. M., Wagtmann, N., Rajagopalan, S., Berrada, K., Yi, T., . . . Long, E. O. (1996). Recruitment of Tyrosine Phosphatase Hcp by the Killer Cell Inhibitor Receptor. *Immunity, 4*(1), 77-85. doi:10.1016/s1074-7613(00)80300-3
Bush, W. S., & Moore, J. H. (2012). Chapter 11: Genome-Wide Association Studies. *PLOS Computational Biology, 8*(12), e1002822. doi:10.1371/journal.pcbi.1002822
Cabantous, S., Poudiougou, B., Bergon, A., Barry, A., Oumar, A. A., Traore, A. M., . . . Marquet, S. (2020). Understanding Human Cerebral Malaria through a Blood Transcriptomic Signature: Evidences for Erythrocyte Alteration, Immune/Inflammatory Dysregulation, and Brain Dysfunction. *Mediators of Inflammation, 2020*, 3280689. doi:10.1155/2020/3280689.
Carter, L. M., Kafsack, B. F., Llinás, M., Mideo, N., Pollitt, L. C., & Reece, S. E. (2013). Stress and Sex in Malaria Parasites: Why Does Commitment Vary? *Evolution, Medicine, and Public Health, 2013*(1), 135-147. doi:10.1093/emph/eot011
CDC. (2021). Malaria's Impact Worldwide. Retrieved from https://www.cdc.gov/malaria/malaria_worldwide/impact.html
Chang, L. C., Chiang, S. K., Chen, S. E., & Hung, M. C. (2022). Targeting 2-Oxoglutarate Dehydrogenase for Cancer Treatment. *American Journal of Cancer Research, 12*(4), 1436-1455.
Chawla, J., Oberstaller, J., & Adams, J. H. (2021). Targeting Gametocytes of the Malaria Parasite *Plasmodium Falciparum* in a Functional Genomics Era: Next Steps. *Pathogens (Basel, Switzerland), 10*(3). doi:10.3390/pathogens10030346
Cheaveau, J., Mogollon, D. C., Mohon, M. A. N., Golassa, L., Yewhalaw, D., & Pillai, D. R. (2019). Asymptomatic Malaria in the Clinical and Public Health Context. *Expert Review of Anti-infective Therapy, 17*(12), 997-1010. doi:10.1080/14787210.2019.1693259
Chen, C., Guo, L., Shi, M., Hu, M., Hu, M., Yu, M., . . . Guo, N. (2012). Modulation of Ifn-Γ Receptor 1 Expression by Ap-2α Influences Ifn-Γ Sensitivity of Cancer Cells. *The American journal of pathology, 180*(2), 661-671. doi:10.1016/j.ajpath.2011.10.040
Chen, Q., Amaladoss, A., Ye, W., Liu, M., Dummler, S., Kong, F., . . . Chen, J. (2014). Human Natural Killer Cells Control *Plasmodium Falciparum* Infection by Eliminating Infected

Red Blood Cells. *Proceedings of the National Academy of Sciences of the United States of Americad Sci U S A, 111*(4), 1479-1484. doi:10.1073/pnas.1323318111
Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). Fastp: An Ultra-Fast All-in-One Fastq Preprocessor. *Bioinformatics, 34*(17), i884-i890. doi:10.1093/bioinformatics/bty560
Chen, X., Trivedi, P. P., Ge, B., Krzewski, K., & Strominger, J. L. (2007). Many Nk Cell Receptors Activate Erk2 and Jnk1 to Trigger Microtubule Organizing Center and Granule Polarization and Cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of Americad Sci U S A, 104*(15), 6329-6334. doi:10.1073/pnas.0611655104
Chin, C.-H., Chen, S.-H., Wu, H.-H., Ho, C.-W., Ko, M.-T., & Lin, C.-Y. (2014). Cytohubba: Identifying Hub Objects and Sub-Networks from Complex Interactome. *BMC Systems Biology, 8*(4), S11. doi:10.1186/1752-0509-8-S4-S11
Cobbold, S. A., M, V. T., Frasse, P., McHugh, E., Karnthaler, M., Creek, D. J., . . . McConville, M. J. (2021). Non-Canonical Metabolic Pathways in the Malaria Parasite Detected by Isotope-Tracing Metabolomics. *Molecular Systems Biology, 17*(4), e10023. doi:10.15252/msb.202010023
Conroy, A. L., Opoka, R. O., Bangirana, P., Idro, R., Ssenkusu, J. M., Datta, D., . . . John, C. C. (2019). Acute Kidney Injury Is Associated with Impaired Cognition and Chronic Kidney Disease in a Prospective Cohort of Children with Severe Malaria. *BMC Medicine, 17*(1), 98. doi:10.1186/s12916-019-1332-7
Cooke, B. M., Lingelbach, K., Bannister, L. H., & Tilley, L. (2004). Protein Trafficking in *Plasmodium Falciparum*-Infected Red Blood Cells. *Trends in Parasitology, 20*(12), 581-589. doi:10.1016/j.pt.2004.09.008
Cooper, M. A., Fehniger, T. A., & Caligiuri, M. A. (2001). The Biology of Human Natural Killer-Cell Subsets. *Trends in Immunology, 22*(11), 633-640. doi:10.1016/s1471-4906(01)02060-9
Counihan, N. A., Modak, J. K., & de Koning-Ward, T. F. (2021). How Malaria Parasites Acquire Nutrients from Their Host. *Frontiers in Cell and Developmental Biology, 9*. doi:10.3389/fcell.2021.649184
Cowman, A. F., Berry, D., & Baum, J. (2012). The Cellular and Molecular Basis for Malaria Parasite Invasion of the Human Red Blood Cell. *Journal of Cell Biology, 198*(6), 961-971. doi:10.1083/jcb.201206112
Cruzat, V., Macedo Rogero, M., Noel Keane, K., Curi, R., & Newsholme, P. (2018). Glutamine: Metabolism and Immune Function, Supplementation and Clinical Translation. *Nutrients, 10*(11). doi:10.3390/nu10111564
Dass, S., Shunmugam, S., Berry, L., Arnold, C. S., Katris, N. J., Duley, S., . . . Botté, C. Y. (2021). Toxoplasma Lipin Is Essential in Channeling Host Lipid Fluxes through Membrane Biogenesis and Lipid Storage. *Nature Communications, 12*(1), 2813. doi:10.1038/s41467-021-22956-w
De, I., Bessonov, S., Hofele, R., dos Santos, K., Will, C. L., Urlaub, H., . . . Pena, V. (2015). The Rna Helicase Aquarius Exhibits Structural Adaptations Mediating Its Recruitment to Spliceosomes. *Nature Structural & Molecular Biology, 22*(2), 138-144. doi:10.1038/nsmb.2951
Déchamps, S., Shastri, S., Wengelnik, K., & Vial, H. J. (2010). Glycerophospholipid Acquisition in Plasmodium – a Puzzling Assembly of Biosynthetic Pathways. *International Journal for Parasitology, 40*(12), 1347-1365. doi:https://doi.org/10.1016/j.ijpara.2010.05.008
Delahaye, N. F., Barbier, M., Fumoux, F., & Rihet, P. (2007). Association Analyses of Ncr3 Polymorphisms with P. Falciparum Mild Malaria. *Microbes Infect, 9(2)*, 160-166. doi:S1286-4579(06)00385-6 [pii]
10.1016/j.micinf.2006.11.002
Depinay, N., Franetich, J. F., Grüner, A. C., Mauduit, M., Chavatte, J. M., Luty, A. J., . . . Rénia, L. (2011). Inhibitory Effect of Tnf-A on Malaria Pre-Erythrocytic Stage Development: Influence of Host Hepatocyte/Parasite Combinations. *PLoS One, 6*(3), e17464. doi:10.1371/journal.pone.0017464

Deponte, M., Hoppe, H. C., Lee, M. C., Maier, A. G., Richard, D., Rug, M., . . . Przyborski, J. M. (2012). Wherever I May Roam: Protein and Membrane Trafficking in P. Falciparum-Infected Red Blood Cells. *Molecular and Biochemical Parasitology, 186*(2), 95-116. doi:10.1016/j.molbiopara.2012.09.007

DNMP., & ICF. (2021). *Kenya Malaria Indicator Survey 2020. Nairobi, Kenya and Rockville, Maryland, USA: Division of National Malaria Programme (Dnmp) Kenya and Icf International.* Retrieved from https://dhsprogram.com/pubs/pdf/MIS36/MIS36.pdf

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., . . . Gingeras, T. R. (2013). Star: Ultrafast Universal Rna-Seq Aligner. *Bioinformatics (Oxford, England), 29*(1), 15-21. doi:10.1093/bioinformatics/bts635

Dodé, C., Krishnamoorthy, R., Lamb, J., & Rochette, J. (1993). Rapid Analysis of -A3.7 Thalassaemia and Aααanti 3.7 Triplication by Enzymatic Amplification Analysis. *British Journal of Haematology, 83*(1), 105-111. doi:https://doi.org/10.1111/j.1365-2141.1993.tb04639.x

Doncheva, N. T., Morris, J. H., Gorodkin, J., & Jensen, L. J. (2019). Cytoscape Stringapp: Network Analysis and Visualization of Proteomics Data. *Journal of Proteome Research, 18*(2), 623-632. doi:10.1021/acs.jproteome.8b00702

Dooley, N. L., Chabikwa, T. G., Pava, Z., Loughland, J. R., Hamelink, J., Berry, K., . . . Boyle, M. J. (2023). Single Cell Transcriptomics Shows That Malaria Promotes Unique Regulatory Responses across Multiple Immune Cell Subsets. *Nature Communications, 14*(1), 7387. doi:10.1038/s41467-023-43181-7

Dushek, O., Goyette, J., & van der Merwe, P. A. (2012). Non-Catalytic Tyrosine-Phosphorylated Receptors. *Immunological Reviews, 250*(1), 258-276. doi:10.1111/imr.12008

Dutta, T., Singh, H., Edkins, A. L., & Blatch, G. L. (2022). Hsp90 and Associated Co-Chaperones of the Malaria Parasite. *Biomolecules, 12*(8). doi:10.3390/biom12081018

Echeverria, P. C., & Picard, D. (2010). Molecular Chaperones, Essential Partners of Steroid Hormone Receptors for Activity and Mobility. *Biochimica et Biophysica Acta, 1803*(6), 641-649. doi:10.1016/j.bbamcr.2009.11.012

Elnatan, D., & Agard, D. A. (2018). Calcium Binding to a Remote Site Can Replace Magnesium as Cofactor for Mitochondrial Hsp90 (Trap1) Atpase Activity. *The Journal of Biological Chemistry, 293*(35), 13717-13724. doi:10.1074/jbc.RA118.003562

Erath, J., Djuranovic, S., & Djuranovic, S. P. (2019). Adaptation of Translational Machinery in Malaria Parasites to Accommodate Translation of Poly-Adenosine Stretches Throughout Its Life Cycle. *Frontiers in Microbiology, 10*. doi:10.3389/fmicb.2019.02823

Fauriat, C., Long, E. O., Ljunggren, H. G., & Bryceson, Y. T. (2010). Regulation of Human Nk-Cell Cytokine and Chemokine Production by Target Cell Recognition. *Blood, 115*(11), 2167-2176. doi:10.1182/blood-2009-08-238469

Ferreira, J. L., Pražák, V., Vasishtan, D., Siggel, M., Hentzschel, F., Pietsch, E., . . . Grünewald, K. (2022). Form Follows Function: Variable Microtubule Architecture in the Malaria Parasite. *bioRxiv*, 2022.2004.2013.488170. doi:10.1101/2022.04.13.488170

Flint, J., Harding, R. M., Boyce, A. J., & Clegg, J. B. (1998). The Population Genetics of the Haemoglobinopathies. *Bailliere's Clinical Haematology, 11*(1), 1-51.

Flori, L., Sawadogo, S., Esnault, C., Delahaye, N. F., Fumoux, F., & Rihet, P. (2003). Linkage of Mild Malaria to the Major Histocompatibility Complex in Families Living in Burkina Faso. *Human Molecular Genetics, 12*(4), 375-378. doi:10.1093/hmg/ddg033

Forconi, C. S., Cosgrove, C. P., Saikumar-Lakshmi, P., Nixon, C. E., Foley, J., Ong'echa, J. M., . . . Moormann, A. M. (2018). Poorly Cytotoxic Terminally Differentiated Cd56 (Neg) Cd16 (Pos) Nk Cells Accumulate in Kenyan Children with Burkitt Lymphomas. *Blood Advances, 2*(10), 1101-1114. doi:10.1182/bloodadvances.2017015404

Fréville, A., Gnangnon, B., Khelifa, A. S., Gissot, M., Khalife, J., & Pierrot, C. (2022). Deciphering the Role of Protein Phosphatases in Apicomplexa: The Future of Innovative Therapeutics? *Microorganisms, 10*(3). doi:10.3390/microorganisms10030585

Gallego, D., Serrano, M., Cordoba-Caballero, J., Gámez, A., Seoane, P., Perkins, J. R., . . . Pérez, B. (2024). Transcriptomic Analysis Identifies Dysregulated Pathways and Therapeutic

Targets in Pmm2-Cdg. *Biochimica et Biophysica Acta. Molecular Basis of Disease, 1870*(5), 167163. doi:10.1016/j.bbadis.2024.167163
Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., . . . Barrell, B. (2002). Genome Sequence of the Human Malaria Parasite *Plasmodium Falciparum*. *Nature, 419*(6906), 498-511. doi:10.1038/nature01097
Gaunt, T. R., Rodriguez, S., Zapata, C., & Day, I. N. M. (2006). Midas: Software for Analysis and Visualisation of Interallelic Disequilibrium between Multiallelic Markers. *BMC Bioinformatics, 7*(1), 227. doi:10.1186/1471-2105-7-227
Gbotosho, G. O., Sowunmi, A., Okuboyejo, T. M., Happi, C. T., Michael, O. S., Folarin, O. A., & Adewoye, E. O. (2011). *Plasmodium Falciparum* Gametocyte Carriage, Emergence, Clearance and Population Sex Ratios in Anaemic and Non-Anaemic Malarious Children. *Memorias do Instituto Oswaldo Cruz, 106*(5), 562-569. doi:10.1590/s0074-02762011000500008
Ge, S. X., Son, E. W., & Yao, R. (2018). Idep: An Integrated Web Application for Differential Expression and Pathway Analysis of Rna-Seq Data. *BMC Bioinformatics, 19*(1), 534. doi:10.1186/s12859-018-2486-6
Gerald, N., Mahajan, B., & Kumar, S. (2011). Mitosis in the Human Malaria Parasite *Plasmodium Falciparum*. *Eukaryot Cell, 10*(4), 474-482. doi:10.1128/ec.00314-10
Gorlov, I. P., Gorlova, O. Y., & Amos, C. I. (2015). Allelic Spectra of Risk Snps Are Different for Environment/Lifestyle Dependent Versus Independent Diseases. *PLoS Genetics, 11*(7), e1005371. doi:10.1371/journal.pgen.1005371
Griffin, J. T., Hollingsworth, T. D., Reyburn, H., Drakeley, C. J., Riley, E. M., & Ghani, A. C. (2015). Gradual Acquisition of Immunity to Severe Malaria with Increasing Exposure. *Proceedings. Biological Sciences, 282*(1801), 20142657. doi:10.1098/rspb.2014.2657
Guillochon, E., Fraering, J., Joste, V., Kamaliddin, C., Vianou, B., Houzé, L., . . . group, N. (2022). Transcriptome Analysis of *Plasmodium Falciparum* Isolates from Benin Reveals Specific Gene Expression Associated with Cerebral Malaria. *The Journal of Infectious Diseases, 225*(12), 2187-2196. doi:10.1093/infdis/jiac086
Gulati, S., Ekland, E. H., Ruggles, K. V., Chan, R. B., Jayabalasingham, B., Zhou, B., . . . Fidock, D. A. (2015). Profiling the Essential Nature of Lipid Metabolism in Asexual Blood and Gametocyte Stages of *Plasmodium Falciparum*. *Cell Host & Microbe, 18*(3), 371-381. doi:10.1016/j.chom.2015.08.003
Günther, S., McMillan, P. J., Wallace, L. J., & Müller, S. (2005). *Plasmodium Falciparum* Possesses Organelle-Specific Alpha-Keto Acid Dehydrogenase Complexes and Lipoylation Pathways. *Biochemical Society Transactions, 33*(Pt 5), 977-980. doi:10.1042/bst20050977
Gunturi, A., Berg, R. E., & Forman, J. (2004). The Role of Cd94/Nkg2 in Innate and Adaptive Immunity. *Immunologic Research, 30*(1), 29-34. doi:10.1385/ir:30:1:029
Hamilton, M. J., Lee, M., & Le Roch, K. G. (2014). The Ubiquitin System: An Essential Component to Unlocking the Secrets of Malaria Parasite Biology. *Molecular bioSystems, 10*(4), 715-723. doi:10.1039/c3mb70506d
Hansen, D. S., Bernard, N. J., Nie, C. Q., & Schofield, L. (2007). Nk Cells Stimulate Recruitment of Cxcr3+ T Cells to the Brain During Plasmodium Berghei-Mediated Cerebral Malaria1. *The Journal of Immunology, 178*(9), 5779-5788. doi:10.4049/jimmunol.178.9.5779
Hart, G. T., Tran, T. M., Theorell, J., Schlums, H., Arora, G., Rajagopalan, S., . . . Long, E. O. (2019). Adaptive Nk Cells in People Exposed to *Plasmodium Falciparum* Correlate with Protection from Malaria. *Journal of Experimental Medicine, 216*(6), 1280-1290. doi:10.1084/jem.20181681
Henry, N. B., Sermé, S. S., Siciliano, G., Sombié, S., Diarra, A., Sagnon, N., . . . Alano, P. (2019). Biology of *Plasmodium Falciparum* Gametocyte Sex Ratio and Implications in Malaria Parasite Transmission. *Malaria Journal, 18*(1), 70. doi:10.1186/s12936-019-2707-0

Hensen, S. M., Heldens, L., van Enckevort, C. M., van Genesen, S. T., Pruijn, G. J., & Lubsen, N. H. (2012). Heat Shock Factor 1 Is Inactivated by Amino Acid Deprivation. *Cell Stress & Chaperones, 17*(6), 743-755. doi:10.1007/s12192-012-0347-1

Hodgson, S. H., Muller, J., Lockstone, H. E., Hill, A. V. S., Marsh, K., Draper, S. J., & Knight, J. C. (2019). Use of Gene Expression Studies to Investigate the Human Immunological Response to Malaria Infection. *Malaria Journal, 18*(1), 418. doi:10.1186/s12936-019-3035-0

Hollowell, T., Sewe, M. O., Rocklöv, J., Obor, D., Odhiambo, F., & Ahlm, C. (2023). Public Health Determinants of Child Malaria Mortality: A Surveillance Study within Siaya County, Western Kenya. *Malaria Journal, 22*(1), 65. doi:10.1186/s12936-023-04502-9

Horowitz, A., Newman, K. C., Evans, J. H., Korbel, D. S., Davis, D. M., & Riley, E. M. (2010). Cross-Talk between T Cells and Nk Cells Generates Rapid Effector Responses to *Plasmodium Falciparum*-Infected Erythrocytes. *Journal of Immunology (Baltimore, Md. : 1950), 184*(11), 6043-6052. doi:10.4049/jimmunol.1000106

Hossain, M., Sharma, S., Korde, R., Kanodia, S., Chugh, M., Rawat, K., & Malhotra, P. (2013). Organization of *Plasmodium Falciparum* Spliceosomal Core Complex and Role of Arginine Methylation in Its Assembly. *Malaria Journal, 12*(1), 333. doi:10.1186/1475-2875-12-333

Hviid, L., Kurtzhals, J. A., Dodoo, D., Rodrigues, O., Rønn, A., Commey, J. O., . . . Theander, T. G. (1996). The Gamma/Delta T-Cell Response to *Plasmodium Falciparum* Malaria in a Population in Which Malaria Is Endemic. *Infection and Immunity, 64*(10), 4359-4362. doi:doi:10.1128/iai.64.10.4359-4362.1996

Issar, N., Roux, E., Mattei, D., & Scherf, A. (2008). Identification of a Novel Post-Translational Modification in *Plasmodium Falciparum*: Protein Sumoylation in Different Cellular Compartments. *Cellular Microbiology, 10*(10), 1999-2011. doi:https://doi.org/10.1111/j.1462-5822.2008.01183.x

Ito, T., Kajita, S., Fujii, M., & Shinohara, Y. (2023). Plasmodium Parasite Malate-Quinone Oxidoreductase Functionally Complements a Yeast Deletion Mutant of Mitochondrial Malate Dehydrogenase. *Microbiology Spectrum, 11*(3), e0016823. doi:10.1128/spectrum.00168-23

Jackson, K. E., Habib, S., Frugier, M., Hoen, R., Khan, S., Pham, J. S., . . . Ralph, S. A. (2011). Protein Translation in Plasmodium Parasites. *Trends in Parasitology, 27*(10), 467-476. doi:10.1016/j.pt.2011.05.005

Jeppsson, K., Kanno, T., Shirahige, K., & Sjögren, C. (2014). The Maintenance of Chromosome Structure: Positioning and Functioning of Smc Complexes. *Nature reviews. Molecular Cell Biology, 15*(9), 601-614. doi:10.1038/nrm3857

Jepson, A., Sisay-Joof, F., Banya, W., Hassan-King, M., Frodsham, A., Bennett, S., . . . Whittle, H. (1997). Genetic Linkage of Mild Malaria to the Major Histocompatibility Complex in Gambian Children: Study of Affected Sibling Pairs. *BMJ (Clinical research ed.), 315*(7100), 96-97. doi:10.1136/bmj.315.7100.96

Jiang, K., Zhong, B., Gilvary, D. L., Corliss, B. C., Hong-Geller, E., Wei, S., & Djeu, J. Y. (2000). Pivotal Role of Phosphoinositide-3 Kinase in Regulation of Cytotoxicity in Natural Killer Cells. *Nature Immunology, 1*(5), 419-425. doi:10.1038/80859

Kaehler, C., Isensee, J., Nonhoff, U., Terrey, M., Hucho, T., Lehrach, H., & Krobitsch, S. (2012). Ataxin-2-Like Is a Regulator of Stress Granules and Processing Bodies. *PLoS One, 7*(11), e50134. doi:10.1371/journal.pone.0050134

Kaifu, T., Escalière, B., Gastinel, L. N., Vivier, E., & Baratin, M. (2011). B7-H6/Nkp30 Interaction: A Mechanism of Alerting Nk Cells against Tumors. *Cellular and Molecular Life Sciences : CMLS, 68*(21), 3531-3539. doi:10.1007/s00018-011-0802-7

Kampinga, H. H., & Craig, E. A. (2010). The Hsp70 Chaperone Machinery: J Proteins as Drivers of Functional Specificity. *Nature reviews. Molecular Cell Biology, 11*(8), 579-592. doi:10.1038/nrm2941

Kapelski, S., de Almeida, M., Fischer, R., Barth, S., & Fendel, R. (2015). Antimalarial Activity of Granzyme B and Its Targeted Delivery by a Granzyme B-Single-Chain Fv Fusion

Protein. *Antimicrobial Agents and Chemotherapy, 59*(1), 669-672. doi:10.1128/aac.04190-14
Kapesa, A., Kweka, E. J., Atieli, H., Afrane, Y. A., Kamugisha, E., Lee, M. C., . . . Yan, G. (2018). The Current Malaria Morbidity and Mortality in Different Transmission Settings in Western Kenya. *PLoS One, 13*(8), e0202031. doi:10.1371/journal.pone.0202031
Karikari, A. A., Wruck, W., & Adjaye, J. (2021). Transcriptome-Based Analysis of Blood Samples Reveals Elevation of DNA Damage Response, Neutrophil Degranulation, Cancer and Neurodegenerative Pathways in *Plasmodium Falciparum* Patients. *Malaria Journal, 20*(1), 383. doi:10.1186/s12936-021-03918-5
Kärre, K. (2008). Natural Killer Cell Recognition of Missing Self. *Nature Immunology, 9*(5), 477-480. doi:10.1038/ni0508-477
Katt, W. P., Lukey, M. J., & Cerione, R. A. (2017). A Tale of Two Glutaminases: Homologous Enzymes with Distinct Roles in Tumorigenesis. *Future Medicinal Chemistry, 9*(2), 223-243. doi:10.4155/fmc-2016-0190
Kavakli, I. H., Ozturk, N., & Gul, S. (2019). DNA Repair by Photolyases. *Advances in protein chemistry and structural biology, 115*, 1-19. doi:10.1016/bs.apcsb.2018.10.003
Ke, H., Lewis, I. A., Morrisey, J. M., McLean, K. J., Ganesan, S. M., Painter, H. J., . . . Vaidya, A. B. (2015). Genetic Investigation of Tricarboxylic Acid Metabolism During the *Plasmodium Falciparum* Life Cycle. *Cell Reports, 11*(1), 164-174. doi:10.1016/j.celrep.2015.03.011
Keller, C. C., Yamo, O., Ouma, C., Ong'echa, J. M., Ounah, D., Hittner, J. B., . . . Perkins, D. J. (2006). Acquisition of Hemozoin by Monocytes Down-Regulates Interleukin-12 P40 (Il-12p40) Transcripts and Circulating Il-12p70 through an Il-10-Dependent Mechanism: In Vivo and in Vitro Findings in Severe Malarial Anemia. *Infection and Immunity, 74*(9), 5249-5260. doi:10.1128/iai.00843-06
Kellogg, M. K., Miller, S. C., Tikhonova, E. B., & Karamyshev, A. L. (2021). Srpassing Co-Translational Targeting: The Role of the Signal Recognition Particle in Protein Targeting and Mrna Protection. *International Journal of Molecular Sciences, 22*(12). doi:10.3390/ijms22126284
Kempaiah, P., Dokladny, K., Karim, Z., Raballah, E., Ong'echa, J. M., Moseley, P. L., & Perkins, D. J. (2016). Reduced Hsp70 and Glutamine in Pediatric Severe Malaria Anemia: Role of Hemozoin in Suppressing Hsp70 and Nf-Kb Activation. *Molecular medicine (Cambridge, Mass.), 22*, 570-584. doi:10.2119/molmed.2016.00130
Khan, S. (2016). Recent Advances in the Biology and Drug Targeting of Malaria Parasite Aminoacyl-Trna Synthetases. *Malaria Journal, 15*(1), 203. doi:10.1186/s12936-016-1247-0
Kim, G. W., Lee, D. H., Jeon, Y. H., Yoo, J., Kim, S. Y., Lee, S. W., . . . Kwon, S. H. (2021). Glutamine Synthetase as a Therapeutic Target for Cancer Treatment. *International Journal of Molecular Sciences, 22*(4). doi:10.3390/ijms22041701
King, T., & Lamb, T. (2015). Interferon-Γ: The Jekyll and Hyde of Malaria. *PLoS Pathog, 11*(10), e1005118. doi:10.1371/journal.ppat.1005118
Kisia, L. E., Cheng, Q., Raballah, E., Munde, E. O., McMahon, B. H., Hengartner, N. W., . . . Anyona, S. B. (2022). Genetic Variation in Csf2 (5q31.1) Is Associated with Longitudinal Susceptibility to Pediatric Malaria, Severe Malarial Anemia, and All-Cause Mortality in a High-Burden Malaria and Hiv Region of Kenya. *Tropical Medicine and Health, 50*(1), 41. doi:10.1186/s41182-022-00432-5
Kisia, L. E., Kempaiah, P., Anyona, S. B., Munde, E. O., Achieng, A. O., Ong'echa, J. M., . . . Raballah, E. (2019). Genetic Variation in Interleukin-7 Is Associated with a Reduced Erythropoietic Response in Kenyan Children Infected with *Plasmodium Falciparum*. *BMC Medical Genetics, 20*(1), 140. doi:10.1186/s12881-019-0866-z
Koch, J., Steinle, A., Watzl, C., & Mandelboim, O. (2013). Activating Natural Cytotoxicity Receptors of Natural Killer Cells in Cancer and Infection. *Trends in Immunology, 34*(4), 182-191. doi:10.1016/j.it.2013.01.003

Koch, M., Wright, K. E., Otto, O., Herbig, M., Salinas, N. D., Tolia, N. H., . . . Baum, J. (2017). *Plasmodium Falciparum* Erythrocyte-Binding Antigen 175 Triggers a Biophysical Change in the Red Blood Cell That Facilitates Invasion. *Proc Natl AcaProceedings of the National Academy of Sciences of the United States of Americad Sci U S A, 114*(16), 4225-4230. doi:10.1073/pnas.1620843114

Korbel, D. S., Finney, O. C., & Riley, E. M. (2004). Natural Killer Cells and Innate Immunity to Protozoan Pathogens. *International Journal for Parasitology, 34*(13-14), 1517-1528. doi:S0020-7519(04)00211-5 [pii]
10.1016/j.ijpara.2004.10.006

Korbel, D. S., Newman, K. C., Almeida, C. R., Davis, D. M., & Riley, E. M. (2005). Heterogeneous Human Nk Cell Responses to *Plasmodium Falciparum*-Infected Erythrocytes1. *The Journal of Immunology, 175*(11), 7466-7473. doi:10.4049/jimmunol.175.11.7466

Korbel, D. S., Norman, P. J., Newman, K. C., Horowitz, A., Gendzekhadze, K., Parham, P., & Riley, E. M. (2009). Killer Ig-Like Receptor (Kir) Genotype Predicts the Capacity of Human Kir-Positive Cd56dim Nk Cells to Respond to Pathogen-Associated Signals. *Journal of Immunology (Baltimore, Md. : 1950), 182*(10), 6426-6434. doi:10.4049/jimmunol.0804224

Kouriba, B., Daou, M., Arama, C., Ouédraogo, N., Niaré, K., Keita, Y., . . . Scholzen, A. (2023). *Plasmodium Falciparum* Malaria Exposure and Carriage Associate with Reduced Γδ T-Cells and Nk Cell Responses to Infected Red Blood Cells in Vitro. Https://Doi.Org/10.21203/Rs.3.Rs-2771048/V1.

Koval, T., & Dohnálek, J. (2018). Characteristics and Application of S1–P1 Nucleases in Biotechnology and Medicine. *Biotechnology Advances, 36*(3), 603-612. doi:https://doi.org/10.1016/j.biotechadv.2017.12.007

Krai, P., Dalal, S., & Klemba, M. (2014). Evidence for a Golgi-to-Endosome Protein Sorting Pathway in *Plasmodium Falciparum*. *PLoS One, 9*(2), e89771. doi:10.1371/journal.pone.0089771

Krishnan, K. M., & Williamson, K. C. (2018). The Proteasome as a Target to Combat Malaria: Hits and Misses. *Translational Research : The Journal of Laboratory and Clinical Medicine, 198*, 40-47. doi:10.1016/j.trsl.2018.04.007

Krugliak, M., Zhang, J., & Ginsburg, H. (2002). Intraerythrocytic *Plasmodium Falciparum* Utilizes Only a Fraction of the Amino Acids Derived from the Digestion of Host Cell Cytosol for the Biosynthesis of Its Proteins. *Molecular and Biochemical Parasitology, 119*(2), 249-256. doi:10.1016/s0166-6851(01)00427-3

Kümmel, D., Müller, J. J., Roske, Y., Misselwitz, R., Büssow, K., & Heinemann, U. (2005). The Structure of the Trapp Subunit Tpc6 Suggests a Model for a Trapp Subcomplex. *EMBO Reports, 6*(8), 787-793. doi:https://doi.org/10.1038/sj.embor.7400463

Kwiatkowski, D. P. (2005). How Malaria Has Affected the Human Genome and What Human Genetics Can Teach Us About Malaria. *American Journal of Human Genetics, 77*(2), 171-192. doi:10.1086/432519

Lancaster, G. I., & Febbraio, M. A. (2005). Exosome-Dependent Trafficking of Hsp70: A Novel Secretory Pathway for Cellular Stress Proteins. *The Journal of Biological Chemistry, 280*(24), 23349-23355. doi:10.1074/jbc.M502017200

Langlois, A. C., Marinach, C., Manzoni, G., & Silvie, O. (2018). Plasmodium Sporozoites Can Invade Hepatocytic Cells Independently of the Ephrin Receptor A2. *PLoS One, 13*(7), e0200032. doi:10.1371/journal.pone.0200032

Langmead, B., & Salzberg, S. L. (2012). Fast Gapped-Read Alignment with Bowtie 2. *Nature Methods, 9*(4), 357-359. doi:10.1038/nmeth.1923

Lanier, L. L. (1997). Natural Killer Cells: From No Receptors to Too Many. *Immunity, 6*(4), 371-378. doi:10.1016/s1074-7613(00)80280-0

Lanier, L. L. (2008). Up on the Tightrope: Natural Killer Cell Activation and Inhibition. *Nature Immunology, 9*(5), 495-502. doi:10.1038/ni1581

Laurentino, E. C., Taylor, S., Mair, G. R., Lasonder, E., Bartfai, R., Stunnenberg, H. G., . . . Waters, A. P. (2011). Experimentally Controlled Downregulation of the Histone Chaperone Fact in Plasmodium Berghei Reveals That It Is Critical to Male Gamete Fertility. *Cellular Microbiology, 13*(12), 1956-1974. doi:10.1111/j.1462-5822.2011.01683.x

Le Roch, K., Sestier, C., Dorin, D., Waters, N., Kappes, B., Chakrabarti, D., . . . Doerig, C. (2000). Activation of a *Plasmodium Falciparum* Cdc2-Related Kinase by Heterologous P25 and Cyclin H. Functional Characterization of a P. Falciparum Cyclin Homologue. *The Journal of Biological Chemistry, 275*(12), 8952-8958. doi:10.1074/jbc.275.12.8952

Lee, A. H., Symington, L. S., & Fidock, D. A. (2014). DNA Repair Mechanisms and Their Biological Roles in the Malaria Parasite *Plasmodium Falciparum*. *Microbiology & Molecular Biology Reviews : MMBR, 78*(3), 469-486. doi:10.1128/mmbr.00059-13

Lee, H. J., Georgiadou, A., Otto, T. D., Levin, M., Coin, L. J., Conway, D. J., & Cunnington, A. J. (2018a). Transcriptomic Studies of Malaria: A Paradigm for Investigation of Systemic Host-Pathogen Interactions. *Microbiology and Molecular Biology Reviews, 82*(2), 10.1128/mmbr.00071-00017. doi:doi:10.1128/mmbr.00071-17

Lee, H. J., Georgiadou, A., Walther, M., Nwakanma, D., Stewart, L. B., Levin, M., . . . Cunnington, A. J. (2018b). Integrated Pathogen Load and Dual Transcriptome Analysis of Systemic Host-Pathogen Interactions in Severe Malaria. *Science Translational Medicine, 10*(447). doi:10.1126/scitranslmed.aar3619

Leite, J. S. M., Cruzat, V. F., Krause, M., & Homem de Bittencourt, P. I. (2016). Physiological Regulation of the Heat Shock Response by Glutamine: Implications for Chronic Low-Grade Inflammatory Diseases in Age-Related Conditions. *Nutrire, 41*(1), 17. doi:10.1186/s41110-016-0021-y

Lew, V. L., Macdonald, L., Ginsburg, H., Krugliak, M., & Tiffert, T. (2004). Excess Haemoglobin Digestion by Malaria Parasites: A Strategy to Prevent Premature Host Cell Lysis. *Blood cells, Molecules & Diseases, 32*(3), 353-359. doi:10.1016/j.bcmd.2004.01.006

Li, H., O'Donoghue, A. J., van der Linden, W. A., Xie, S. C., Yoo, E., Foe, I. T., . . . Bogyo, M. (2016). Structure- and Function-Based Design of Plasmodium-Selective Proteasome Inhibitors. *Nature, 530*(7589), 233-236. doi:10.1038/nature16936

Li, Q., & Weina, P. (2010). Artesunate: The Best Drug in the Treatment of Severe and Complicated Malaria. *Pharmaceuticals (Basel, Switzerland), 3*(7), 2322-2332. doi:10.3390/ph3072322

Liu, J., Istvan, E. S., Gluzman, I. Y., Gross, J., & Goldberg, D. E. (2006). *Plasmodium Falciparum* Ensures Its Amino Acid Supply with Multiple Acquisition Pathways and Redundant Proteolytic Enzyme Systems. *Proceedings of the National Academy of Sciences, 103*(23), 8840-8845. doi:doi:10.1073/pnas.0601876103

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). Nf-Kb Signaling in Inflammation. *Signal Transduction and Targeted Therapy, 2*(1), 17023. doi:10.1038/sigtrans.2017.23

Machado, M., Steinke, S., & Ganter, M. (2021). Plasmodium Reproduction, Cell Size, and Transcription: How to Cope with Increasing DNA Content? *Frontiers in Cellular and Infection Microbiology, 11*. doi:10.3389/fcimb.2021.660679

Mailu, B. M., Li, L., Arthur, J., Nelson, T. M., Ramasamy, G., Fritz-Wolf, K., . . . Gardner, M. J. (2015). Plasmodium Apicoplast Gln-Trnagln Biosynthesis Utilizes a Unique Gatab Amidotransferase Essential for Erythrocytic Stage Parasites. *The Journal of Biological Chemistry, 290*(49), 29629-29641. doi:10.1074/jbc.M115.655100

Makita, T., Hernandez-Hoyos, G., Chen, T. H., Wu, H., Rothenberg, E. V., & Sucov, H. M. (2001). A Developmental Transition in Definitive Erythropoiesis: Erythropoietin Expression Is Sequentially Regulated by Retinoic Acid Receptors and Hnf4. *Genes & Development, 15*(7), 889-901. doi:10.1101/gad.871601

Mamoor, S. (2020). Zbtb6 Is Hyper-Methylated and Differentially Expressed in the Blood of Patients with Crohn's Disease. Doi:10.31219/Osf.Io/Xne67. doi:DOI:10.31219/osf.io/xne67

Mansfield, C. R., Quan, B., Chirgwin, M. E., Eduful, B., Hughes, P. F., Neveu, G., . . . Derbyshire, E. R. (2024). Selective Targeting of *Plasmodium Falciparum* Hsp90 Disrupts the 26s Proteasome. *Cell Chem Biol, 31*(4), 729-742.e713. doi:10.1016/j.chembiol.2024.02.008

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., . . . et al. (1995). Indicators of Life-Threatening Malaria in African Children. *The New England Journal of Medicine, 332*(21), 1399-1404. doi:10.1056/nejm199505253322102

Masisi, B. K., El Ansari, R., Alfarsi, L., Rakha, E. A., Green, A. R., & Craze, M. L. (2020). The Role of Glutaminase in Cancer. *Histopathology, 76*(4), 498-508. doi:10.1111/his.14014

Mavoungou, E., Held, J., Mewono, L., & Kremsner, P. G. (2007). A Duffy Binding-Like Domain Is Involved in the Nkp30-Mediated Recognition of *Plasmodium Falciparum*-Parasitized Erythrocytes by Natural Killer Cells. *The Journal of Infectious Diseases, 195*(10), 1521-1531. doi:JID37754 [pii]10.1086/515579

Mayer, D. C. G. (2021). Protein Sorting in *Plasmodium Falciparum*. *Life (Basel, Switzerland), 11*(9). doi:10.3390/life11090937

Mazumdar, J., & Striepen, B. (2007). Make It or Take It: Fatty Acid Metabolism of Apicomplexan Parasites. *Eukaryot Cell, 6*(10), 1727-1735. doi:10.1128/ec.00255-07

Mazumder, B., Sampath, P., Seshadri, V., Maitra, R. K., DiCorleto, P. E., & Fox, P. L. (2003). Regulated Release of L13a from the 60s Ribosomal Subunit as a Mechanism of Transcript-Specific Translational Control. *Cell, 115*(2), 187-198. doi:10.1016/S0092-8674(03)00773-6

McElroy, P. D., Lal, A. A., Hawley, W. A., Bloland, P. B., Kuile, F. O., Oloo, A. J., . . . Nahlen, B. L. (1999). Analysis of Repeated Hemoglobin Measures in Full-Term, Normal Birth Weight Kenyan Children between Birth and Four Years of Age. Iii. The Asemobo Bay Cohort Project. *American Journal of Tropical Medicine & Hygiene, 61*(6), 932-940. doi:10.4269/ajtmh.1999.61.932

McHugh, E., Bulloch, M. S., Batinovic, S., Patrick, C. J., Sarna, D. K., & Ralph, S. A. (2023). Nonsense-Mediated Decay Machinery in *Plasmodium Falciparum* Is Inefficient and Non-Essential. *mSphere, 8*(4), e0023323. doi:10.1128/msphere.00233-23

McMillan, P. J., Stimmler, L. M., Foth, B. J., McFadden, G. I., & Müller, S. (2005). The Human Malaria Parasite *Plasmodium Falciparum* Possesses Two Distinct Dihydrolipoamide Dehydrogenases. *Molecular Microbiology, 55*(1), 27-38. doi:https://doi.org/10.1111/j.1365-2958.2004.04398.x

Meazza, R., Azzarone, B., Orengo, A. M., & Ferrini, S. (2011). Role of Common-Gamma Chain Cytokines in Nk Cell Development and Function: Perspectives for Immunotherapy. *Journal of Biomedicine and Biotechnology, 2011*, 861920. doi:10.1155/2011/861920

Meibalan, E., & Marti, M. (2017). Biology of Malaria Transmission. *Cold Spring Harbor perspectives in medicine, 7*(3). doi:10.1101/cshperspect.a025452

Mikulak, J., Bruni, E., Oriolo, F., Di Vito, C., & Mavilio, D. (2019). Hepatic Natural Killer Cells: Organ-Specific Sentinels of Liver Immune Homeostasis and Physiopathology. *Frontiers in Immunology, 10*(946). doi:10.3389/fimmu.2019.00946

Miller, L. H., Baruch, D. I., Marsh, K., & Doumbo, O. K. (2002). The Pathogenic Basis of Malaria. *Nature, 415*(6872), 673-679. doi:10.1038/415673a

Miller, L. H., Good, M. F., & Milon, G. (1994). Malaria Pathogenesis. *Science (New York, N.Y.), 264*(5167), 1878-1883. doi:doi:10.1126/science.8009217

Minakawa, N., Dida, G. O., Sonye, G. O., Futami, K., & Njenga, S. M. (2012). Malaria Vectors in Lake Victoria and Adjacent Habitats in Western Kenya. *PLoS One, 7*(3), e32725. doi:10.1371/journal.pone.0032725PONE-D-11-18095 [pii]

Moebius, J., Guha, R., Peterson, M., Abdi, K., Skinner, J., Li, S., . . . Crompton, P. D. (2020). Pd-1 Expression on Nk Cells in Malaria-Exposed Individuals Is Associated with Diminished Natural Cytotoxicity and Enhanced Antibody-Dependent Cellular Cytotoxicity. *Infection and Immunity, 88*(3), e00711-00719. doi:10.1128/IAI.00711-19

MoH. (2020). *Guidelines for the Diagnosis, Treatment and Prevention of Malaria in Kenya*. Nairobi: Division of National Malaria Program.

Moore, L. R., Fujioka, H., Williams, P. S., Chalmers, J. J., Grimberg, B., Zimmerman, P. A., & Zborowski, M. (2006). Hemoglobin Degradation in Malaria-Infected Erythrocytes Determined from Live Cell Magnetophoresis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 20*(6), 747-749. doi:10.1096/fj.05-5122fje

Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M. C., . . . Moretta, L. (2001). Activating Receptors and Coreceptors Involved in Human Natural Killer Cell-Mediated Cytolysis. *Annual Review of Immunology, 19*, 197-223. doi:10.1146/annurev.immunol.19.1.197

Moretta, L., Bottino, C., Pende, D., Mingari, M. C., Biassoni, R., & Moretta, A. (2002). Human Natural Killer Cells: Their Origin, Receptors and Function. *European Journal of Immunology, 32*(5), 1205-1211. doi:10.1002/1521-4141(200205)32:5<1205::aid-immu1205>3.0.co;2-y

Morrissette, N., Abbaali, I., Ramakrishnan, C., & Hehl, A. B. (2023). The Tubulin Superfamily in Apicomplexan Parasites. *Microorganisms, 11*(3), 706.

Moseley, P. L. (1998). Heat Shock Proteins and the Inflammatory Response. *Annals of the New York Academy of Sciences, 856*, 206-213. doi:10.1111/j.1749-6632.1998.tb08327.x

Mueller, J. C. (2004). Linkage Disequilibrium for Different Scales and Applications. *Briefings in Bioinformatics, 5*(4), 355-364. doi:10.1093/bib/5.4.355

Munde, E. O., Okeyo, W. A., Raballah, E., Anyona, S. B., Were, T., Ong'echa, J. M., . . . Ouma, C. (2017). Association between Fcγ Receptor Iia, Iiia and Iiib Genetic Polymorphisms and Susceptibility to Severe Malaria Anemia in Children in Western Kenya. *BMC Infectious Diseases, 17*(1), 289. doi:10.1186/s12879-017-2390-0

Newman, A. M., Steen, C. B., Liu, C. L., Gentles, A. J., Chaudhuri, A. A., Scherer, F., . . . Alizadeh, A. A. (2019). Determining Cell Type Abundance and Expression from Bulk Tissues with Digital Cytometry. *Nature Biotechnology, 37*(7), 773-782. doi:10.1038/s41587-019-0114-2

Newsholme, P. (2021). Cellular and Metabolic Mechanisms of Nutrient Actions in Immune Function. *European Journal of Clinical Nutrition, 75*(9), 1328-1331. doi:10.1038/s41430-021-00960-z

Nielsen, C. M., Wolf, A. S., Goodier, M. R., & Riley, E. M. (2016). Synergy between Common Γ Chain Family Cytokines and Il-18 Potentiates Innate and Adaptive Pathways of Nk Cell Activation. *Frontiers in Immunology, 7*, 101. doi:10.3389/fimmu.2016.00101

Obiero, J. M., Shekalaghe, S., Hermsen, C. C., Mpina, M., Bijker, E. M., Roestenberg, M., . . . Scholzen, A. (2015). Impact of Malaria Preexposure on Antiparasite Cellular and Humoral Immune Responses after Controlled Human Malaria Infection. *Infection and Immunity, 83*(5), 2185-2196. doi:10.1128/iai.03069-14

Obonyo, C. O., Vulule, J., Akhwale, W. S., & Grobbee, D. E. (2007). In-Hospital Morbidity and Mortality Due to Severe Malarial Anemia in Western Kenya. *American Journal of Tropical Medicine & Hygiene, 77*(6 Suppl), 23-28. doi:77/6_Suppl/23 [pii]

Ockenhouse, C. F., Hu, W. C., Kester, K. E., Cummings, J. F., Stewart, A., Heppner, D. G., . . . Burke, D. S. (2006). Common and Divergent Immune Response Signaling Pathways Discovered in Peripheral Blood Mononuclear Cell Gene Expression Patterns in Presymptomatic and Clinically Apparent Malaria. *Infection and Immunity, 74*(10), 5561-5573. doi:10.1128/iai.00408-06

Odera, D. O., Tuju, J., Mwai, K., Nkumama, I. N., Fürle, K., Chege, T., . . . Williams, T. N. (2023). Anti-Merozoite Antibodies Induce Natural Killer Cell Effector Function and Are Associated with Immunity against Malaria. *Science Translational Medicine, 15*(682), eabn5993. doi:10.1126/scitranslmed.abn5993

Okeyo, W. A., Munde, E. O., Okumu, W., Raballah, E., Anyona, S. B., Vulule, J. M., . . . Ouma, C. (2013). Interleukin (Il)-13 Promoter Polymorphisms (-7402 T/G and -4729g/a) Condition Susceptibility to Pediatric Severe Malarial Anemia but Not Circulating Il-13 Levels. *BMC Immunology, 14*, 15. doi:10.1186/1471-2172-14-15

Okoyo, C., Githinji, E., Muia, R. W., Masaku, J., Mwai, J., Nyandieka, L., . . . Kanyi, H. M. (2021). Assessment of Malaria Infection among Pregnant Women and Children Below Five Years of Age Attending Rural Health Facilities of Kenya: A Cross-Sectional Survey in Two Counties of Kenya. *PLoS ONE, 16*(9), e0257276. doi:10.1371/journal.pone.0257276

Olayanju, A. O. D., Adeleke, A., Okolo, C. S., Ogunyemi, O. O., & Mary, O. K. (2022). Association between Haematological Values and Heat Shock Protein 70 of Sickle Cell Disease Patients in Ado-Ekiti, Ekiti State, Nigeria. *The Pan African Medical Journal, 43*, 47. doi:10.11604/pamj.2022.43.47.33346

Ong'echa, J. M., Keller, C. C., Were, T., Ouma, C., Otieno, R. O., Landis-Lewis, Z., . . . Perkins, D. J. (2006). Parasitemia, Anemia, and Malarial Anemia in Infants and Young Children in a Rural Holoendemic *Plasmodium Falciparum* Transmission Area. *American Journal of Tropical Medicine & Hygiene, 74*(3), 376-385. doi:74/3/376 [pii]

Ong'echa, J. M., Raballah, E. O., Kempaiah, P. M., Anyona, S. B., Were, T., Davenport, G. C., . . . Perkins, D. J. (2011). Polymorphic Variability in the 3' Untranslated Region (Utr) of Il12b Is Associated with Susceptibility to Severe Anaemia in Kenyan Children with Acute *Plasmodium Falciparum* Malaria. *BMC genetics, 12*, 69. doi:10.1186/1471-2156-12-69

Ong'echa, J. M., Remo, A. M., Kristoff, J., Hittner, J. B., Were, T., Ouma, C., . . . Perkins, D. J. (2008). Increased Circulating Interleukin (Il)-23 in Children with Malarial Anemia: In Vivo and in Vitro Relationship with Co-Regulatory Cytokines Il-12 and Il-10. *Clinical Immunology (Orlando, Fla.), 126*(2), 211-221. doi:10.1016/j.clim.2007.08.007

Otieno, R. O., Ouma, C., Ong'echa, J. M., Keller, C. C., Were, T., Waindi, E. N., . . . Perkins, D. J. (2006). Increased Severe Anemia in Hiv-1-Exposed and Hiv-1-Positive Infants and Children During Acute Malaria. *AIDS, 20*(2), 275-280. doi:10.1097/01.aids.0000200533.56490.b700002030-200601090-00017 [pii]

Otto, T. D., Böhme, U., Sanders, M., Reid, A., Bruske, E. I., Duffy, C. W., . . . Berriman, M. (2018). Long Read Assemblies of Geographically Dispersed *Plasmodium Falciparum* Isolates Reveal Highly Structured Subtelomeres. *Wellcome Open Research, 3*, 52. doi:10.12688/wellcomeopenres.14571.1

Otto, T. D., Wilinski, D., Assefa, S., Keane, T. M., Sarry, L. R., Böhme, U., . . . Llinás, M. (2010). New Insights into the Blood-Stage Transcriptome of *Plasmodium Falciparum* Using Rna-Seq. *Molecular Microbiology, 76*(1), 12-24. doi:10.1111/j.1365-2958.2009.07026.x

Ouma, C., Davenport, G. C., Awandare, G. A., Keller, C. C., Were, T., Otieno, M. F., . . . Perkins, D. J. (2008a). Polymorphic Variability in the Interleukin (Il)-1beta Promoter Conditions Susceptibility to Severe Malarial Anemia and Functional Changes in Il-1beta Production. *The Journal of Infectious Diseases, 198*(8), 1219-1226. doi:10.1086/592055

Ouma, C., Davenport, G. C., Garcia, S., Kempaiah, P., Chaudhary, A., Were, T., . . . Perkins, D. J. (2012). Functional Haplotypes of Fc Gamma (Fcγ) Receptor (Fcγriia and Fcγriiib) Predict Risk to Repeated Episodes of Severe Malarial Anemia and Mortality in Kenyan Children. *Human Genetics, 131*(2), 289-299. doi:10.1007/s00439-011-1076-8

Ouma, C., Davenport, G. C., Were, T., Otieno, M. F., Hittner, J. B., Vulule, J. M., . . . Perkins, D. J. (2008b). Haplotypes of Il-10 Promoter Variants Are Associated with Susceptibility to Severe Malarial Anemia and Functional Changes in Il-10 Production. *Human Genetics, 124*(5), 515-524. doi:10.1007/s00439-008-0578-5

Ouma, C., Keller, C. C., Opondo, D. A., Were, T., Otieno, R. O., Otieno, M. F., . . . Perkins, D. J. (2006). Association of Fcgamma Receptor Iia (Cd32) Polymorphism with Malarial Anemia and High-Density Parasitemia in Infants and Young Children. *American Journal of Tropical Medicine & Hygiene, 74*(4), 573-577.

Oyegue-Liabagui, S. L., Bouopda-Tuedom, A. G., Kouna, L. C., Maghendji-Nzondo, S., Nzoughe, H., Tchitoula-Makaya, N., . . . Lekana-Douki, J. B. (2017). Pro- and Anti-Inflammatory Cytokines in Children with Malaria in Franceville, Gabon. *American Journal of Clinical and Experimental Immunology, 6*(2), 9-20.

Pacifico, F., Leonardi, A., & Crescenzi, E. (2023). Glutamine Metabolism in Cancer Stem Cells: A Complex Liaison in the Tumor Microenvironment. *International Journal of Molecular Sciences, 24*(3). doi:10.3390/ijms24032337

Pahl, J. H. W., Cerwenka, A., & Ni, J. (2018). Memory-Like Nk Cells: Remembering a Previous Activation by Cytokines and Nk Cell Receptors. *Frontiers in Immunology, 9*, 2796. doi:10.3389/fimmu.2018.02796

Painter, H. J., Chung, N. C., Sebastian, A., Albert, I., Storey, J. D., & Llinás, M. (2018). Genome-Wide Real-Time in Vivo Transcriptional Dynamics During *Plasmodium Falciparum* Blood-Stage Development. *Nature Communications, 9*(1), 2656. doi:10.1038/s41467-018-04966-3

Painter, H. J., Morrisey, J. M., Mather, M. W., & Vaidya, A. B. (2007). Specific Role of Mitochondrial Electron Transport in Blood-Stage *Plasmodium Falciparum*. *Nature, 446*(7131), 88-91. doi:10.1038/nature05572

Pallavi, R., Roy, N., Nageshan, R. K., Talukdar, P., Pavithra, S. R., Reddy, R., . . . Tatu, U. (2010). Heat Shock Protein 90 as a Drug Target against Protozoan Infections: Biochemical Characterization of Hsp90 from *Plasmodium Falciparum* and Trypanosoma Evansi and Evaluation of Its Inhibitor as a Candidate Drug. *The Journal of Biological Chemistry, 285*(49), 37964-37975. doi:10.1074/jbc.M110.155317

Paton, R. S., Kamau, A., Akech, S., Agweyu, A., Ogero, M., Mwandawiro, C., . . . Snow, R. W. (2021). Malaria Infection and Severe Disease Risks in Africa. *Science (New York, N.Y.), 373*(6557), 926-931. doi:10.1126/science.abj0089

Paul, R. E., Coulson, T. N., Raibaud, A., & Brey, P. T. (2000). Sex Determination in Malaria Parasites. *Science (New York, N.Y.), 287*(5450), 128-131. doi:10.1126/science.287.5450.128

Pegram, H. J., Andrews, D. M., Smyth, M. J., Darcy, P. K., & Kershaw, M. H. (2011). Activating and Inhibitory Receptors of Natural Killer Cells. *Immunology & Cell Biology, 89*(2), 216-224. doi:https://doi.org/10.1038/icb.2010.78

Pérez-Escuredo, J., Dadhich, R. K., Dhup, S., Cacace, A., Van Hée, V. F., De Saedeleer, C. J., . . . Sonveaux, P. (2016). Lactate Promotes Glutamine Uptake and Metabolism in Oxidative Cancer Cells. *Cell cycle (Georgetown, Tex.), 15*(1), 72-83. doi:10.1080/15384101.2015.1120930

Perkins, D. J., Were, T., Davenport, G. C., Kempaiah, P., Hittner, J. B., & Ong'echa, J. M. (2011). Severe Malarial Anemia: Innate Immunity and Pathogenesis. *International Journal of Biological Sciences, 7*(9), 1427-1442. doi:10.7150/ijbs.7.1427

Pochini, L., Scalise, M., Galluccio, M., & Indiveri, C. (2014). Membrane Transporters for the Special Amino Acid Glutamine: Structure/Function Relationships and Relevance to Human Health. *Frontiers in Chemistry, 2*, 61. doi:10.3389/fchem.2014.00061

Ponts, N., Saraf, A., Chung, D. W., Harris, A., Prudhomme, J., Washburn, M. P., . . . Le Roch, K. G. (2011). Unraveling the Ubiquitome of the Human Malaria Parasite. *Journal of Biological Chemistry, 286*(46), 40320-40330. doi:10.1074/jbc.M111.238790

Prager, I., Liesche, C., van Ooijen, H., Urlaub, D., Verron, Q., Sandström, N., . . . Watzl, C. (2019). Nk Cells Switch from Granzyme B to Death Receptor-Mediated Cytotoxicity During Serial Killing. *Journal of Experimental Medicine, 216*(9), 2113-2127. doi:10.1084/jem.20181454

Preuss, J., Jortzik, E., & Becker, K. (2012). Glucose-6-Phosphate Metabolism in *Plasmodium Falciparum*. *IUBMB Life, 64*(7), 603–611.

Quintana, F. J., & Cohen, I. R. (2005). Heat Shock Proteins as Endogenous Adjuvants in Sterile and Septic Inflammation. *Journal of Immunology (Baltimore, Md. : 1950), 175*(5), 2777-2782. doi:10.4049/jimmunol.175.5.2777

Raballah, E., Anyona, S. B., Cheng, Q., Munde, E. O., Hurwitz, I. F., Onyango, C., . . . Perkins, D. J. (2022a). Complement Component 3 Mutations Alter the Longitudinal Risk of Pediatric Malaria and Severe Malarial Anemia. *Experimental biology and medicine (Maywood, N.J.), 247*(8), 672-682. doi:10.1177/15353702211056272

Raballah, E., Kempaiah, P., Karim, Z., Orinda, G. O., Otieno, M. F., Perkins, D. J., & Ong'echa, J. M. (2017). Cd4 T-Cell Expression of Ifn-Γ and Il-17 in Pediatric Malarial Anemia. *PLoS One, 12*(4), e0175864. doi:10.1371/journal.pone.0175864

Raballah, E., Wilding, K., Anyona, S. B., Munde, E. O., Hurwitz, I., Onyango, C. O., . . . Perkins, D. J. (2022b). Nonsynonymous Amino Acid Changes in the A-Chain of Complement Component 5 Influence Longitudinal Susceptibility to *Plasmodium Falciparum* Infections and Severe Malarial Anemia in Kenyan Children. *Frontiers in Genetics, 13*, 977810. doi:10.3389/fgene.2022.977810

Reiter, K., Mukhopadhyay, D., Zhang, H., Boucher, L. E., Kumar, N., Bosch, J., & Matunis, M. J. (2013). Identification of Biochemically Distinct Properties of the Small Ubiquitin-Related Modifier (Sumo) Conjugation Pathway in *Plasmodium Falciparum*. *Journal of Biological Chemistry, 288*(39), 27724-27736. doi:10.1074/jbc.M113.498410

Ribeil, J. A., Zermati, Y., Vandekerckhove, J., Cathelin, S., Kersual, J., Dussiot, M., . . . Hermine, O. (2007). Hsp70 Regulates Erythropoiesis by Preventing Caspase-3-Mediated Cleavage of Gata-1. *Nature, 445*(7123), 102-105. doi:10.1038/nature05378

Richer, J., & Chudley, A. (2005). The Hemoglobinopathies and Malaria. *Clinical Genetics, 68*(4), 332-336. doi:https://doi.org/10.1111/j.1399-0004.2005.00503.x

Riley, E. M., & Stewart, V. A. (2013). Immune Mechanisms in Malaria: New Insights in Vaccine Development. *Nature Medicine, 19*(2), 168-178. doi:10.1038/nm.3083

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). Edger: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data. *Bioinformatics, 26*(1), 139-140.

Roels, J., Van Hulle, J., Lavaert, M., Kuchmiy, A., Strubbe, S., Putteman, T., . . . Taghon, T. (2022). Transcriptional Dynamics and Epigenetic Regulation of E and Id Protein Encoding Genes During Human T Cell Development. *Frontiers Immunology, 13*, 960918. doi:10.3389/fimmu.2022.960918

Roth, E. F., Jr., Raventos-Suarez, C., Perkins, M., & Nagel, R. L. (1982). Glutathione Stability and Oxidative Stress in P. Falciparum Infection in Vitro: Responses of Normal and G6pd Deficient Cells. *Biochemical and Biophysical Research Communications, 109*(2), 355-362. doi:10.1016/0006-291x(82)91728-4

Rothfelder, K., Märklin, M., Wild, J., Dörfel, D., Kanz, L., Müller, M. R., & Salih, H. R. (2016). Involvement of Nfat Transcription Factors in Nk Cell Reactivity. *Blood, 128*(22), 1344-1344. doi:10.1182/blood.V128.22.1344.1344

Royo, J., Rahabi, M., Kamaliddin, C., Ezinmegnon, S., Olagnier, D., Authier, H., . . . Aubouy, A. (2019). Changes in Monocyte Subsets Are Associated with Clinical Outcomes in Severe Malarial Anaemia and Cerebral Malaria. *Scientific Reports, 9*(1), 17545. doi:10.1038/s41598-019-52579-7

Rusakiewicz, S., Nocturne, G., Lazure, T., Semeraro, M., Flament, C., Caillat-Zucman, S., . . . Mariette, X. (2013). Ncr3/Nkp30 Contributes to Pathogenesis in Primary Sjogren's Syndrome. *Science Translational Medicine, 5*(195), 195ra196. doi:10.1126/scitranslmed.3005727

Ryan, M. C., Stucky, M., Wakefield, C., Melott, J. M., Akbani, R., Weinstein, J. N., & Broom, B. M. (2019). Interactive Clustered Heat Map Builder: An Easy Web-Based Tool for Creating Sophisticated Clustered Heat Maps. *F1000Res, 8*, ISCB Comm J-1750. Retrieved from https://doi.org/10.12688/f1000research.20590.2 doi:10.12688/f1000research.20590.2

Safeukui, I., Gomez, N. D., Adelani, A. A., Burte, F., Afolabi, N. K., Akondy, R., . . . Haldar, K. (2015). Malaria Induces Anemia through Cd8+ T Cell-Dependent Parasite Clearance and Erythrocyte Removal in the Spleen. *mBio, 6*(1). doi:10.1128/mBio.02493-14

Sassmannshausen, J., Pradel, G., & Bennink, S. (2020). Perforin-Like Proteins of Apicomplexan Parasites. *Frontiers in Cellular and Infection Microbiology, 10*. doi:10.3389/fcimb.2020.578883

Sato, S., Rangachari, K., & Wilson, R. J. (2003). Targeting Gfp to the Malarial Mitochondrion. *Molecular and Biochemical Parasitology, 130*(2), 155-158. doi:10.1016/s0166-6851(03)00166-x

Scalise, M., Pochini, L., Console, L., Losso, M. A., & Indiveri, C. (2018). The Human Slc1a5 (Asct2) Amino Acid Transporter: From Function to Structure and Role in Cell Biology. *Front Cell Dev Biol, 6*, 96. doi:10.3389/fcell.2018.00096

Schauder, D. M., Shen, J., Chen, Y., Kasmani, M. Y., Kudek, M. R., Burns, R., & Cui, W. (2021). E2a-Regulated Epigenetic Landscape Promotes Memory Cd8 T Cell Differentiation. *Proc Natl Acad Sci U S A, 118*(16). doi:10.1073/pnas.2013452118

Scoville, S. D., Freud, A. G., & Caligiuri, M. A. (2017). Modeling Human Natural Killer Cell Development in the Era of Innate Lymphoid Cells. *Frontiers in Immunology, 8*(360). doi:10.3389/fimmu.2017.00360

Sekar, P., Rajagopalan, S., Shabani, E., Kanjee, U., Schureck, M. A., Arora, G., . . . Long, E. O. (2023). Nk Cell-Induced Damage to P.Falciparum-Infected Erythrocytes Requires Ligand-Specific Recognition and Releases Parasitophorous Vacuoles That Are Phagocytosed by Monocytes in the Presence of Immune Igg. *PLoS Pathog, 19*(11), e1011585. doi:10.1371/journal.ppat.1011585

Sharma, S., Pradhan, A., Chauhan, V. S., & Tuteja, R. (2005). Isolation and Characterization of Type I Signal Peptidase of Different Malaria Parasites. *Journal of Biomedicine & Biotechnology, 2005*(4), 301-309. doi:10.1155/jbb.2005.301

Shears, M. J., Botté, C. Y., & McFadden, G. I. (2015). Fatty Acid Metabolism in the Plasmodium Apicoplast: Drugs, Doubts and Knockouts. *Molecular and Biochemical Parasitology, 199*(1), 34-50. doi:https://doi.org/10.1016/j.molbiopara.2015.03.004

Sheridan, C. M., Garcia, V. E., Ahyong, V., & DeRisi, J. L. (2018). The *Plasmodium Falciparum* Cytoplasmic Translation Apparatus: A Promising Therapeutic Target Not yet Exploited by Clinically Approved Anti-Malarials. *Malar J, 17*(1), 465. doi:10.1186/s12936-018-2616-7

Shonhai, A. (2010). Plasmodial Heat Shock Proteins: Targets for Chemotherapy. *FEMS Immunology & Medical Microbiology, 58*(1), 61-74. doi:10.1111/j.1574-695X.2009.00639.x

Shunmugam, S., Arnold, C.-S., Dass, S., Katris, N. J., & Botté, C. Y. (2022). The Flexibility of Apicomplexa Parasites in Lipid Metabolism. *PLoS Pathogens, 18*(3), e1010313. doi:10.1371/journal.ppat.1010313

Singh, M. K., Shin, Y., Ju, S., Han, S., Choe, W., Yoon, K.-S., . . . Kang, I. (2024). Heat Shock Response and Heat Shock Proteins: Current Understanding and Future Opportunities in Human Diseases. *International Journal of Molecular Sciences, 25*(8), 4209.

Sobota, R. S., Dara, A., Manning, J. E., Niangaly, A., Bailey, J. A., Kone, A. K., . . . Doumbo, O. K. (2016). Expression of Complement and Toll-Like Receptor Pathway Genes Is Associated with Malaria Severity in Mali: A Pilot Case Control Study. *Malar J, 15*, 150. doi:10.1186/s12936-016-1189-6

Song, W., Li, D., Tao, L., Luo, Q., & Chen, L. (2020). Solute Carrier Transporters: The Metabolic Gatekeepers of Immune Cells. *Acta Pharmaceutica Sinica. B, 10*(1), 61-78. doi:10.1016/j.apsb.2019.12.006

Sorber, K., Dimon, M. T., & DeRisi, J. L. (2011). Rna-Seq Analysis of Splicing in *Plasmodium Falciparum* Uncovers New Splice Junctions, Alternative Splicing and Splicing of Antisense Transcripts. *Nucleic Acids Research, 39*(9), 3820-3835. doi:10.1093/nar/gkq1223

Sordo-Bahamonde, C., Lorenzo-Herrero, S., Payer Á, R., Gonzalez, S., & López-Soto, A. (2020). Mechanisms of Apoptosis Resistance to Nk Cell-Mediated Cytotoxicity in Cancer. *International Journal of Molecular Sciences, 21*(10). doi:10.3390/ijms21103726

Spielmann, T., Fergusen, D. J., & Beck, H. P. (2003). Etramps, a New *Plasmodium Falciparum* Gene Family Coding for Developmentally Regulated and Highly Charged Membrane Proteins Located at the Parasite-Host Cell Interface. *Molecular Biology of the Cell, 14*(4), 1529-1544. doi:10.1091/mbc.e02-04-0240

Spielmann, T., Gras, S., Sabitzki, R., & Meissner, M. (2020). Endocytosis in Plasmodium and Toxoplasma Parasites. *Trends in Parasitology, 36*(6), 520-532. doi:10.1016/j.pt.2020.03.010

Steen, C. B., Liu, C. L., Alizadeh, A. A., & Newman, A. M. (2020). Profiling Cell Type Abundance and Expression in Bulk Tissues with Cibersortx. *Methods in Molecular Biology (Clifton, N.J.), 2117*, 135-157. doi:10.1007/978-1-0716-0301-7_7

Stevenson, M. M., & Riley, E. M. (2004). Innate Immunity to Malaria. *Nature Reviews Immunology, 4*(3), 169-180. doi:10.1038/nri1311

Stich, A., Oster, N., Abdel-Aziz, I. Z., Stieglbauer, G., Coulibaly, B., Wickert, H., . . . Lanzer, M. (2006). Malaria in a Holoendemic Area of Burkina Faso: A Cross-Sectional Study. *Parasitology Research, 98*(6), 596-599. doi:10.1007/s00436-005-0104-9

Stofberg, M. L., Caillet, C., de Villiers, M., & Zininga, T. (2021). Inhibitors of the *Plasmodium Falciparum* Hsp90 Towards Selective Antimalarial Drug Design: The Past, Present and Future. *Cells, 10*(11), 2849.

Sulica, A., Galatiuc, C., Manciulea, M., Bancu, A. C., DeLeo, A., Whiteside, T. L., & Herberman, R. B. (1993). Regulation of Human Natural Cytotoxicity by Igg. Iv. Association between Binding of Monomeric Igg to the Fc Receptors on Large Granular Lymphocytes and Inhibition of Natural Killer (Nk) Cell Activity. *Cellular Immunology, 147*(2), 397-410. doi:10.1006/cimm.1993.1079

Sumam de Oliveira, D., Kronenberger, T., Palmisano, G., Wrenger, C., & de Souza, E. E. (2021). Targeting Sumoylation in Plasmodium as a Potential Target for Malaria Therapy. *Frontiers in Cellular and Infection Microbiology, 11*. doi:10.3389/fcimb.2021.685866

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., . . . Mering, C. V. (2019). String V11: Protein-Protein Association Networks with Increased Coverage, Supporting Functional Discovery in Genome-Wide Experimental Datasets. *Nucleic Acids Research, 47*(D1), D607-d613. doi:10.1093/nar/gky1131

Tabellini, G., Vairo, D., Scomodon, O., Tamassia, N., Ferraro, R. M., Patrizi, O., . . . Badolato, R. (2017). Impaired Natural Killer Cell Functions in Patients with Signal Transducer and Activator of Transcription 1 (Stat1) Gain-of-Function Mutations. *The Journal of Allergy and Clinical Immunology, 140*(2), 553-564.e554. doi:10.1016/j.jaci.2016.10.051

Tang, D., Chen, M., Huang, X., Zhang, G., Zeng, L., Zhang, G., . . . Wang, Y. (2023). Srplot: A Free Online Platform for Data Visualization and Graphing. *PLOS ONE, 18*(11), e0294236. doi:10.1371/journal.pone.0294236

Taylor, S. M., Parobek, C. M., & Fairhurst, R. M. (2012). Haemoglobinopathies and the Clinical Epidemiology of Malaria: A Systematic Review and Meta-Analysis. *The Lancet. Infectious Diseases, 12*(6), 457-468. doi:10.1016/s1473-3099(12)70055-5

Tebben, K., Dia, A., & Serre, D. (2022). Determination of the Stage Composition of Plasmodium Infections from Bulk Gene Expression Data. *mSystems, 7*(4), e0025822. doi:10.1128/msystems.00258-22

Thiam, A., Sanka, M., Ndiaye Diallo, R., Torres, M., Mbengue, B., Nunez, N. F., . . . Rihet, P. (2019). Gene Expression Profiling in Blood from Cerebral Malaria Patients and Mild Malaria Patients Living in Senegal. *BMC Medical Genomics, 12*(1), 148. doi:10.1186/s12920-019-0599-z

Thomas, K., Zondler, L., Ludwig, N., Kardell, M., Lüneburg, C., Henke, K., . . . Zarbock, A. (2022). Glutamine Prevents Acute Kidney Injury by Modulating Oxidative Stress and Apoptosis in Tubular Epithelial Cells. *JCI insight, 7*(21). doi:10.1172/jci.insight.163161

Tishkoff, S. A., & Williams, S. M. (2002). Genetic Analysis of African Populations: Human Evolution and Complex Disease. *Nature Reviews Genetics, 3*(8), 611-621. doi:10.1038/nrg865nrg865 [pii]

Tonkin-Hill, G. Q., Trianty, L., Noviyanti, R., Nguyen, H. H. T., Sebayang, B. F., Lampah, D. A., . . . Duffy, M. F. (2018). The *Plasmodium Falciparum* Transcriptome in Severe Malaria Reveals Altered Expression of Genes Involved in Important Processes Including Surface Antigen-Encoding Var Genes. *PLoS Biol, 16*(3), e2004328. doi:10.1371/journal.pbio.2004328

Tsunoda, T., & Takagi, T. (1999). Estimating Transcription Factor Bindability on DNA. *Bioinformatics, 15*(7-8), 622-630. doi:10.1093/bioinformatics/15.7.622
Tu, M. M., Mahmoud, A. B., & Makrigiannis, A. P. (2016). Licensed and Unlicensed Nk Cells: Differential Roles in Cancer and Viral Control. *Frontiers in Immunology, 7*, 166-166. doi:10.3389/fimmu.2016.00166
Tukwasibwe, S., Nakimuli, A., Traherne, J., Chazara, O., Jayaraman, J., Trowsdale, J., . . . Colucci, F. (2020). Variations in Killer-Cell Immunoglobulin-Like Receptor and Human Leukocyte Antigen Genes and Immunity to Malaria. *Cellular & Molecular Immunology, 17*(8), 799-806. doi:10.1038/s41423-020-0482-z
Ty, M., Sun, S., Callaway, P. C., Rek, J., Press, K. D., van der Ploeg, K., . . . Jagannathan, P. (2023). Malaria-Driven Expansion of Adaptive-Like Functional Cd56-Negative Nk Cells Correlates with Clinical Immunity to Malaria. *Science Translational Medicine, 15*(680), eadd9012. doi:doi:10.1126/scitranslmed.add9012
Umapathy, N. S., Natrajan, K., Kutlar, A., Meiler, S. E., & Brittain, J. E. (2013). Pivotal Role of Heat Shock Proteins in Monocyte and Endothelial Cell Pathology in Sickle Cell Disease. *Blood, 122*(21), 969-969. doi:10.1182/blood.V122.21.969.969
van Dooren, G. G., Stimmler, L. M., & McFadden, G. I. (2006). Metabolic Maps and Functions of the Plasmodium Mitochondrion. *FEMS Microbiology Reviews, 30*(4), 596-630. doi:10.1111/j.1574-6976.2006.00027.x
van Niekerk, D. D., du Toit, F., Green, K., Palm, D., & Snoep, J. L. (2023). A Detailed Kinetic Model of Glycolysis in *Plasmodium Falciparum*-Infected Red Blood Cells for Antimalarial Drug Target Identification. *The Journal of Biological Chemistry, 299*(9), 105111. doi:10.1016/j.jbc.2023.105111
Vega, V. L., Rodríguez-Silva, M., Frey, T., Gehrmann, M., Diaz, J. C., Steinem, C., . . . De Maio, A. (2008). Hsp70 Translocates into the Plasma Membrane after Stress and Is Released into the Extracellular Environment in a Membrane-Associated Form That Activates Macrophages. *Journal of Immunology (Baltimore, Md. : 1950), 180*(6), 4299-4307. doi:10.4049/jimmunol.180.6.4299
Vivier, E., Nunès, J. A., & Vély, F. (2004). Natural Killer Cell Signaling Pathways. *Science (New York, N.Y.), 306*(5701), 1517-1519. doi:10.1126/science.1103478
Voskoboinik, I., Whisstock, J. C., & Trapani, J. A. (2015). Perforin and Granzymes: Function, Dysfunction and Human Pathology. *Nature Reviews Immunology, 15*(6), 388-400. doi:10.1038/nri3839
Wahlgren, M., Goel, S., & Akhouri, R. R. (2017). Variant Surface Antigens of *Plasmodium Falciparum* and Their Roles in Severe Malaria. *Nature reviews. Microbiology, 15*(8), 479-491. doi:10.1038/nrmicro.2017.47
Walk, J., & Sauerwein, R. W. (2019). Activatory Receptor Nkp30 Predicts Nk Cell Activation During Controlled Human Malaria Infection. *Frontiers in Immunology, 10*(2864). doi:10.3389/fimmu.2019.02864
Wang, F. Q., Shao, L., Dang, X., Wang, Y.-F., Chen, S., Liu, Z., . . . Yang, W. (2024). Unraveling Transcriptomic Signatures and Dysregulated Pathways in Systemic Lupus Erythematosus across Disease States. *Arthritis research & therapy, 26*(1), 99. doi:10.1186/s13075-024-03327-4
Wang, H., Zheng, X., Wei, H., Tian, Z., & Sun, R. (2012a). Important Role for Nkp30 in Synapse Formation and Activation of Nk Cells. *Immunological Investigations, 41*(4), 367-381. doi:10.3109/08820139.2011.632799
Wang, R., Jaw, J. J., Stutzman, N. C., Zou, Z., & Sun, P. D. (2012b). Natural Killer Cell-Produced Ifn-Γ and Tnf-A Induce Target Cell Cytolysis through up-Regulation of Icam-1. *Journal of leukocyte biology, 91*(2), 299-309. doi:10.1189/jlb.0611308
Weatherall, D. J. (2018). The Evolving Spectrum of the Epidemiology of Thalassemia. *Hematology and Oncology Clinics of North America, 32*(2), 165-175. doi:10.1016/j.hoc.2017.11.008

Were, T., Davenport, G. C., Hittner, J. B., Ouma, C., Vulule, J. M., Ong'echa, J. M., & Perkins, D. J. (2011). Bacteremia in Kenyan Children Presenting with Malaria. *Journal of Clinical Microbiology, 49*(2), 671–676.

Were, T., Davenport, G. C., Yamo, E. O., Hittner, J. B., Awandare, G. A., Otieno, M. F., . . . Perkins, D. J. (2009). Naturally Acquired Hemozoin by Monocytes Promotes Suppression of Rantes in Children with Malarial Anemia through an Il-10-Dependent Mechanism. *Microbes and infection, 11*(8-9), 811-819. doi:10.1016/j.micinf.2009.04.021

Were, T., Hittner, J. B., Ouma, C., Otieno, R. O., Orago, A. S., Ong'echa, J. M., . . . Perkins, D. J. (2006). Suppression of Rantes in Children with *Plasmodium Falciparum* Malaria. *Haematologica, 91*(10), 1396-1399.

WHO. (2000). Severe Falciparum Malaria. World Health Organization, Communicable Diseases Cluster. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 94 Suppl 1*, S1-90.

WHO. (2014). Severe Malaria. *Tropical Medicine & International Health, 19*, 7-131.

WHO. (2020). World Malaria Report.

WHO. (2023). World Malaria Report 2023. Geneva: World Health Organization; 2023. Licence: Cc by-Nc-Sa 3.0 Igo. Retrieved from https://www.who.int/publications/i/item/9789240086173

Whyte, J. R. C., & Munro, S. (2002). Vesicle Tethering Complexes in Membrane Traffic. *Journal of Cell Science, 115*(13), 2627-2637. doi:10.1242/jcs.115.13.2627

Wichers, J. S., Tonkin-Hill, G., Thye, T., Krumkamp, R., Kreuels, B., Strauss, J., . . . Bachmann, A. (2021). Common Virulence Gene Expression in Adult First-Time Infected Malaria Patients and Severe Cases. *elife, 10*. doi:10.7554/eLife.69040

Wiesmann, U. K., Boniface; Mwangi, Zachary. (2016). *Socio-Economic Atlas of Kenya: Depicting the National Population Census by County and Sub-Location. Second, Revised Edition. Nairobi and Nanyuki, Kenya and Bern, Switzerland: Kenya National Bureau of Statistics, Centre for Training and Integrated Research in Asal Development, Centre for Development and Environment.*

Wilkes, J. M., & Doerig, C. (2008). The Protein-Phosphatome of the Human Malaria Parasite *Plasmodium Falciparum*. *BMC Genomics, 9*(1), 412. doi:10.1186/1471-2164-9-412

Winkler, D. D., & Luger, K. (2011). The Histone Chaperone Fact: Structural Insights and Mechanisms for Nucleosome Reorganization. *Journal of Biological Chemistry, 286*(21), 18369-18374. doi:10.1074/jbc.R110.180778

Wolf, A. S., Sherratt, S., & Riley, E. M. (2017). Nk Cells: Uncertain Allies against Malaria. *Frontiers in Immunology, 8*, 212. doi:10.3389/fimmu.2017.00212

Wong, W., Bai, X.-c., Brown, A., Fernandez, I. S., Hanssen, E., Condron, M., . . . Scheres, S. H. W. (2014). Cryo-Em Structure of the *Plasmodium Falciparum* 80s Ribosome Bound to the Anti-Protozoan Drug Emetine. *eLife, 3*, e03080. doi:10.7554/eLife.03080

Xiong, A., Prakash, P., Gao, X., Chew, M., Tay, I. J. J., Woodrow, C. J., . . . Preiser, P. R. (2020). K13-Mediated Reduced Susceptibility to Artemisinin in *Plasmodium Falciparum* Is Overlaid on a Trait of Enhanced DNA Damage Repair. *Cell Reports, 32*(5), 107996. doi:10.1016/j.celrep.2020.107996

Xue, H., Slavov, D., & Wischmeyer, P. E. (2012). Glutamine-Mediated Dual Regulation of Heat Shock Transcription Factor-1 Activation and Expression. *Journal of Biological Chemistry, 287*(48), 40400-40413. doi:10.1074/jbc.M112.410712

Ye, W., Chew, M., Hou, J., Lai, F., Leopold, S. J., Loo, H. L., . . . Chen, J. (2018). Microvesicles from Malaria-Infected Red Blood Cells Activate Natural Killer Cells Via Mda5 Pathway. *PLoS Pathogens, 14*(10), e1007298. doi:10.1371/journal.ppat.1007298

Yeoh, L. M., Lee, V. V., McFadden, G. I., & Ralph, S. A. (2019). Alternative Splicing in Apicomplexan Parasites. *mBio, 10*(1). doi:10.1128/mBio.02866-18

Yokoyama, W. M., Kim, S., & French, A. R. (2004). The Dynamic Life of Natural Killer Cells. *Annual Review of Immunology, 22*, 405-429. doi:10.1146/annurev.immunol.22.012703.104711

Yoo, H. C., Yu, Y. C., Sung, Y., & Han, J. M. (2020). Glutamine Reliance in Cell Metabolism. *Experimental and Molecular Medicine 52*(9), 1496-1516. doi:10.1038/s12276-020-00504-8

Zhang, C., Hu, Y., & Shi, C. (2020). Targeting Natural Killer Cells for Tumor Immunotherapy. *Frontiers in Immunology, 11*, 60. doi:10.3389/fimmu.2020.00060

Zhang, M., Wang, C., Otto, T. D., Oberstaller, J., Liao, X., Adapa, S. R., . . . Adams, J. H. (2018). Uncovering the Essential Genes of the Human Malaria Parasite *Plasmodium Falciparum* by Saturation Mutagenesis. *Science (New York, N.Y.), 360*(6388). doi:10.1126/science.aap7847

Zhao, S., Li, C. I., Guo, Y., Sheng, Q., & Shyr, Y. (2018). Rnaseqsamplesize: Real Data Based Sample Size Estimation for Rna Sequencing. *BMC Bioinformatics, 19*(1), 191. doi:10.1186/s12859-018-2191-5

Zhong, H., Zhou, X., Uhm, H., Jiang, Y., Cao, H., Chen, Y., . . . Ip, N. Y. (2024). Using Blood Transcriptome Analysis for Alzheimer's Disease Diagnosis and Patient Stratification. *Alzheimer's & Dementia*, *20*(4), 2469-2484. doi:https://doi.org/10.1002/alz.13691

Zininga, T., Ramatsui, L., & Shonhai, A. (2018). Heat Shock Proteins as Immunomodulants. *Molecules (Basel, Switzerland), 23*(11). doi:10.3390/molecules23112846

APPENDICES

Appendix 1. SGS Approval Letter

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PHD/PH/00068/019

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 19th June, 2023

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR ONYANGO CLINTON ODHIAMBO—PHD/PH/00068/019

The above named is registered in the programme of Doctor of Philosophy in Medical Genetics in the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal titled **"Effect of Genetic Variation and Altered Gene Expression Patterns on Severe Malarial Anaemia Pathogenesis in Children from Siaya County"** has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

SCHOOL OF GRADUATE STUDIES MASENO UNIVERSITY BOX PRIVATE BAG MASENO

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Appendix 2. Ethical Approvals



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221
Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

REF: MSU/DRPI/MUERC/00510/18 Date: 26th November, 2021

TO: Prof. Douglas J. Perkins
University of New Mexico /KEMRI
Centre for Global Health Research
P.O Box 1579
Kisumu, Kenya

Dear Sir,

RE: Defining the Inflammation and Immunity Transcriptome in Severe Malaria Anemia for Immunotherapeutic Discovery

This is to inform you that **Maseno University Ethics Review Committee (MUERC)** has reviewed and approved your above application for study **continuation**. Your application approval number is MUERC/00510/18. The approval period is 29th January 2022 – 28th January, 2023.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by Maseno University Ethics Review Committee (MUERC).
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to Maseno University Ethics Review Committee (MUERC) within 24 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to Maseno University Ethics Review Committee (MUERC) within 24 hours.
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to Maseno University Ethics Review Committee (MUERC).

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely



Prof. Philip O. Owuor, PhD, FAAS, FKNAS
Chairman, MUERC



Human Research Protections Program

May 30, 2024
Douglas Perkins
505-272-6867
DPerkins@salud.unm.edu

Dear Douglas Perkins:

On 5/30/2024, the HRRC reviewed the following submission:

Type of Review:	Continuing Review
Title of Study:	Defining the Inflammation and Immunity Transcriptome in Severe Malaria Anemia for Immunotherapeutic Discovery
Investigator:	Douglas Perkins
Study ID:	17-445
Submission ID:	CR00010684
IND, IDE, or HDE:	None
Submission Summary:	Continuing Review for Study 17-445.
Documents Approved:	All documents remain unchanged and approved as previously determined and stated in the most recent study submission approval letter.
Review Category:	EXPEDITED: CATEGORIES (3) Noninvasive biological specimens(2)(a) Blood samples from healthy, non-pregnant adults(2)(b) Blood samples from others
Determinations/Waivers:	Subpart B 45CFR46.204 Subpart D 45CFR46.404 Requires a signed Consent form. No waivers or alterations of HIPAA.
Submission Approval Date:	5/30/2024
Approval End Date:	5/29/2025
Effective Date:	5/30/2024

The HRRC approved the study from 5/30/2024 to 5/29/2025 inclusive. If modifications were required to secure approval, the effective date will be later than the approval date. The "Effective Date" 5/30/2024 is the date the HRRC approved your modifications and, in all cases, represents the date study activities may begin.

Human Research Protections Program

To initiate a study closure or within 45 days of 5/29/2025, you are required to submit a continuing review. You may submit a continuing review by navigating to the active study and clicking the "Create Modification/CR" button.

Please use the consent documents that were approved by the HRRC. The approved consents are available for your retrieval in the "Documents" tab of the parent study.

If the study meets the definition of an NIH Clinical Trial, the study must be registered in the ClinicalTrials.gov database. Additionally, the approved consent document(s) must be uploaded to the ClinicalTrials.gov database.

This determination applies only to the activities described in this submission and does not apply should you make any changes to these documents. If changes are being considered these must be submitted for review in a study modification to the HRRC for a determination prior to implementation. If there are questions about whether HRRC review is needed, contact the HRPO before implementing changes without approval. A change in the research may disqualify this research from the current review category. You may submit a modification by navigating to the active study and clicking the "Create Modification/CR" button.

If this study is approved for a waiver of HIPAA authorization, the IRB had determined the use or disclosure of protected health information in this study involves no more than a minimal risk to the privacy of individuals because the study contains a plan to protect the identifiers from improper use and disclosure, a process to destroy the identifiers at the earliest opportunity consistent with conduct of the research, and there are written assurances that the protected health information will not be reused or disclosed to any other person or entity, except as required by law, for authorized oversight of the research study, or for other research for which the use or disclosure of protected health information would be permitted. The IRB recognizes that the research could not practicably be conducted without the waiver, and could not practicably be conducted without access to and use of the protected health information.

If your submission indicates you will translate materials post-approval of English materials, you may not recruit or enroll participants in another language, until all translated materials are reviewed and approved.

In conducting this study, you are required to follow the Investigator Manual (HRP-103), which can be found by navigating to the IRB Library.

Sincerely,

505.272.1129 | The University of New Mexico Health Sciences Office of Research Human Research Protections Program
1 University of New Mexico | MSC08 4560 | Albuquerque, NM 87131
hso.unm.edu/research/hrpo

Thomas F. Byrd, MD
HRRC Executive Chair

Abbreviated Investigator Responsibilities

NOTE: For a full unabbreviated version of the **Investigator Manual**, please visit the HRPO website at https://hsc.unm.edu/research/hrpo/.

What will happen after HRRC review?

The HRPO will provide you with a written decision indicating that the HRRC has approved the Human Research, requires modifications to secure approval, or has disapproved the Human Research.

- If the HRRC has approved the Human Research: The Human Research may commence once all other organizational approvals have been met. HRRC approval is usually good for a limited period of time which is noted in the approval letter.
- If the HRRC requires modifications to secure approval and you accept the modifications: Make the requested modifications and submit them to the HRRC. If all requested modifications are made, the HRRC will issue a final approval. Research cannot commence until this final approval is received. If you do not accept the modifications, write up your response and submit it to the HRRC.
- If the HRRC defers the Human Research: The HRRC will provide a statement of the reasons for deferral and suggestions to make the study approvable, and give you an opportunity to respond in writing. In most cases if the HRRC's reasons for the deferral are addressed in a modification, the Human Research can be approved
- If the HRRC disapproves the Human Research: The HRRC will provide a statement of the reasons for disapproval and give you an opportunity to respond in writing.

In all cases, you have the right to address your concerns to the HRRC directly at an HRRC meeting.

What are my obligations after HRRC approval?

1. Do not start Human Research activities until you have the final HRRC approval letter.
2. Do not start Human Research activities until you have obtained all other required institutional approvals, including approvals of departments or divisions that require approval prior to commencing research that involves their resources.
3. Ensure that there are adequate resources to carry out the research safely. This includes, but is not limited to, sufficient investigator time, appropriately qualified research team members, equipment, and space.
    a. Delegate responsibility to the research staff in accordance with the staff's training and qualifications.

Appendix 3. Consent Forms

Consent (Form 1): Written consent for participation in the study "Defining the Inflammation and Immunity Transcriptome in Severe Malarial Anemia for Immunotherapeutic Discovery".

You and your child are being asked to participate in a research study. The purpose of this document is to provide you with information to consider whether you are willing to participate in the research study. The research study will enroll HIV negative pregnant women and their child. After delivery, we will follow your child for three years. We will ask that you bring your child back monthly for a health checkup. We will also ask that you bring your child back immediately if she/he has a fever, or if there are health concern between the monthly visits. Please ask questions if there is anything you do not understand. Your participation and your child's participation in the study is voluntary. If you choose for you or your child to not participate in the study, it will have no effect on the quality of your medical care either of you receive.

Is participation in the research voluntary?
Being in the research study is up to you. If you do not want to be in the research study, you and your child will still get the best possible medical care at the hospital. You can change your mind at any time. There will not be any loss of care or benefits if you or your child refuse to participate. If you want, you can withdraw you or your child at any time.

Who will be conducting the research study?
Maseno University and the University of New Mexico are doing a research study at the Siaya County Referral Hospital to determine how inherited factors (genes) change during a malaria infection. The principal investigator, Dr. Douglas Perkins, of the University of New Mexico, would like you to be in the research study since you have met the inclusion criteria.

The title of the study is "Defining the Inflammation and Immunity Transcriptome in Severe Malarial Anemia for Immunotherapeutic Discovery". If you have questions about the study, you can contact Dr. Douglas Perkins (University of New Mexico, P. O. Box 144, Siaya, Kenya, telephone: 0705399133). You can also contact co-investigators on the study, Dr. Collins Ouma (Maseno University, P. O. Box 333-40105, Maseno, telephone: 0722381214) or Dr. Walter Otieno (Maseno University, P. O. Box 333-40105, Maseno, telephone 0714481488), or Dr. Evans Raballah University of New Mexico Laboratories in Siaya County Referral Hospital (P. O. Box 144-Siaya; telephone 0733792627).

The physician in charge of treatment and care is Dr. Walter Otieno. You can contact Dr. Otieno (Maseno University, P. O. Box 333-40105, Maseno, telephone 0714481488). In the United States, Dr. Perkins address is: University of New Mexico, MSC10-5550, 1 University of New Mexico, Albuquerque, NM, 87131 (telephone 505-272-6867, e-mail dperkins@salud.unm.edu). For questions or problems about your rights as a research subject, please call or write to The Secretary, Maseno University Ethics Review Committee, Private Bag, Maseno; Telephone numbers: 057-51622, 0722203411, 0721543976, 0733230878; Email address: muerc-secretariate@maseno.ac.ke; muerc-secretariate@gmail.com

Who is sponsoring the research study?
The research study is funded National Institutes of Health (NIH) in the United States.

Where will the research study be conducted*?*
The research study will be conducted at the Siaya County Referral Hospital.

What is the purpose of the research?

The purpose of the research study is to understand how inherited factors (genes) change during malaria infections. We want to learn how changes in these genes determine whether children get mild or severe forms or malaria. Following delivery, we will follow your child for three years. This will involve seeing your child at the hospital monthly for the three-year period to check on their health status. We will give you a schedule. If your child gets a fever between the monthly visits, or if you have any health concerns, we ask that you return to the hospital with your child immediately. If we detect malaria in your child, we ask that you bring your child back to the hospital for a follow-up visit in two weeks (day 14 visit) as per the schedule provided. If your child gets a fever before this time or if you have any health concerns, we ask that you return to the hospital with your child immediately. Your child's involvement with the study ends after the three-year follow-up period. We hope to include 750 mother/child pairs in this study. The research study might help learn ways to improve diagnosis and treatment of malaria.

Who is being asked to participate in this research study?
You and your child are being asked to participate since you both meet the requirements for being in the study.

What procedures will be performed for the research purposes?
Prior to your delivery, a small amount of blood (3-6 mL – about a teaspoon) will be drawn from your arm upon enrollment to test for malaria. We will also take a vaginal swab to test for other diseases. In addition, once you have delivered your child, we will examine your placenta to see if you have malaria in your placenta.

Your child will be tested for diseases that are common in this region, such as malaria. If you agree for your child to be involved in this study, you will be asked to return with your child monthly and during all acute febrile episodes for the three-year follow-up period. At each visit, we will test for low blood (anemia) and malaria parasitemia in heel/finger-prick blood. When your child has her/his first episode of malaria, a small amount of blood (2-3 mL – less than a teaspoon) will be drawn from her/his arm. This blood will be used to see what diseases your child may have. A small amount of blood (2-3 mL – less than a teaspoon) will be drawn from your child's arm each day for two days to see how your child is responding to treatment. We also ask that you bring your child back 14 days later for a well visit to make sure that your child is free of disease(s). On the day 14 visit, a small amount of blood (2-3 mL – less than a teaspoon) will be drawn from your child's arm.

Children in this region often get malaria many times within the first three years of life. When we find additional malaria episodes, a small amount of blood (2-3 mL – less than a teaspoon) will be drawn from your child's arm. This blood will be used to see what diseases your child may have. To make sure that your child is free of diseases after treatment, we ask that you bring your child back 14 days later so that we can take a small amount of blood (2-3 mL – less than a teaspoon) from your child's arm.

At each quarterly visit (every 3 months), a small amount of blood (2-3 mL – less than a teaspoon) will be drawn from your child's arm to see how your child's immune system looks when she/he is healthy.

We will also take a urine and stool sample from your child to see if she/he has any diseases that can be found in these samples. This will be done when your child has a fever. This will also be done at a follow up visit (usually two weeks) to make sure your child is healthy if she/he was sick.

A sputum (cough) sample will also be collected to see if your child has any infections in his/her lungs such as tuberculosis. This will be done when your child has a fever.

If your child has had a chest or throat infection for more than two weeks that has not gone away, or other signs of chest or throat infections, we will also take a nasal or throat swab from your child. This will be done when your child has a fever. This will also be done at a follow up visit (usually two weeks) to make sure your child is healthy if she/he was sick.

The samples that we are taking from your child are not different than those that should be done to check on what is making your child ill.

Your child will be in the study for a total of three years.

We may store samples from you or your child long term to see how differences in genes influence disease outcomes. This will only be done if you sign a separate consent form.

What are the possible risks, side effects, and discomforts of the research procedures?
There may be a small bruise, bleeding, or short time of discomfort when we take blood from a vein in your arm or your child's arm. Some infections can happen if the process is not kept sterile (clean). Taking too much blood from an adult or child with low blood could make the low blood problem worse or have unseen outcomes. To avoid this, we will take only an amount of blood that is safe. Taking blood could cause vitamin and iron loss in you or your child. This could make the low blood problem worse. To avoid this, we will give you and your child vitamins that have iron and folate in them. Only a person trained to get blood will take blood from you or your child. If you or your child has no problems with bleeding, taking blood should not cause harm.

Additional risks include the unintentional disclosure (release) of health information. This too could cause problems for you in the community. Participation in this study may also cause a loss of time and potential inconvenience since we need to see your child back at the hospital for a three year follow up time.

What are the possible benefits from taking part in this research study?
There may be no direct benefits for participation in the research study. However, you will be watched closely during hospitalization. Also, your child will be watched closely for three years. In case of any sickness during those periods, you and your child will be given treatment without any cost. Also, participating in this study could help to understand what diseases are in this area and how they can best be treated.

What treatments or procedures are available if I decide I will not participate in this research study?
If you choose to not participate in the research study, you and your child will still receive all of the standard medical tests and procedures available from the Ministry of Health. Your child will also be tested for malaria, blood levels, and hookworm infection.

Will I be told of any new information or new risks that may be found during the course of this study? You will be told of any significant new developments (findings) that may cause you or your child to re-evaluate participation in the research study.

Will my insurance provider or I be charged for any costs of any procedures performed as part of this study?
Neither you nor your insurance provider will be charged for the costs of any of the procedures performed for the purpose of screening.

Who will pay if I am injured as a result of taking part in the research study?

In the unlikely event that you or your child become ill or injured as a direct result of participating in the study, you will receive medical care that will be provided free of charge from the Ministry of Health. The cost of the health care will be paid for by the project.

Will I be paid for participating in this study?
You will not be paid for your participation in the study. However, we will provide reimbursement for travel costswhen you come for the follow-up visit, or if you come for a visit when your child is sick (has a fever). We will also provide you with soap and a basin when you enroll in the research study.

Who will know about my participation and my child's participation in this research study?
Information related to you and your child will be treated in strict confidence to the extent provided by law. Your identity and your child's will be coded and will not be associated with any published results. Your code number and identity, as well as your child's, will be kept in a locked file of the Principal Investigator. In order to monitor this research study, representatives from Maseno University Ethics Review Committee, Private Bag, Maseno; Telephone numbers: 057-51622, 0722203411, 0721543976, 0733230878; Email address: muerc-secretariate@maseno.ac.ke; muerc-secretariate@gmail.com or the University of New Mexico Health Sciences Center Institutional Review Board or federal agencies such as NIH (National Institutes of Health) and OHRP (Office of Human Research Protection) may inspect the research records which may reveal your identity.

Name: _____
(Please Print)

Date: _____ **Study #:_____**

Study Participants Statement:

□ The above research study has been explained to me. The consent form has been read to me or I have read the consent form. My questions have been answered to my satisfaction. I have received a copy of this form. I was told that being in this research study is my choice. I was told that I cannot be in the research study without my consent. I agree to take part in this research study. By signing this form, I give my consent to join this research study.

Signatures:

Study Participants Signature: _____ **Date: _____**

Witness Signature: _____ **Date: _____**

Thumbprint: _____

****

Parent/Guardian's Name: _____ Child's Name:_____
(Please Print) (Please Print)

Date: _____ Study #:_____

Parent/Guardian's Statement:

□ The above research study has been explained to me. The consent form has been read to me or I have read the consent form. My questions have been answered to my satisfaction. I have received a copy of this form. I was told that being in this research study is my choice. I was told that my child cannot be in the research study without my consent. I agree for my child to take part in this research study. By signing this form, I give my consent for my child to join this research study.

Signatures:

Parent/Guardian's Signature: _____ Date: _____

Witness Signature: _____ Date: _____

Parent/Guardian's Thumbprint: _____

****

STUDY EMPLOYEE CERTIFICATION

I certify that the nature and purpose, the potential benefits and possible risks associated with participation in this research study have been explained to the above individual and that any questions about this information have been answered.

Study employee's signature: _____ Date: _____

Consent (Form 2): Written consent to allow use of blood specimens for genetic studies.

As part of the research study explained in the consent form "*Defining the Inflammation and Immunity Transcriptome in Severe Malarial Anemia for Immunotherapeutic Discovery*", Maseno University and the University of New Mexico are studying how inherited factors (genes) affect diseases. Genes are passed on from both parents and the code (language) used for genes is called DNA. Also, the DNA gets turned into RNA. By looking at levels of RNA, we can tell if the genes are turned on or off. How the RNA gets turned on or off tells which genes are active (expressed). These genes can be important for what diseases you and your child get and how you respond to treatment. We also want to know how genes in the pathogens (infectious agents) affect your body. We will test the genes using blood taken from you and your child. We hope that learning about genes will help treat and prevent diseases.

What procedures will be performed for research purposes related to genetics?
Since you agreed to take part in this research study, we will take blood from you and your child and a cheek (buccal) swab. We would like you to let us store some of the blood and facts about you and your child, such as age and health status. We will check your child's G6PD and sickle cell status now. These tests will help us determine how your child responds to some medications and how much anemia your child may get. We will give you the results of your child when you return for the first quarterly follow-up visit. These tests will help us learn what kind of responses your child may have to diseases and treatment, such as malaria. We will test other genes in your child's body later. If we identify genes that are important in your child's health outcomes, we may also test these same genes in samples we have stored for you. We may also test the genes in the pathogens (infectious agent) in you and your child. So, this may need longer storage of your blood.

We can only do some of the tests in hospital now. To do some of the other tests, we will need to store part of your blood and your child's blood. The blood will be stored in the freezers at Siaya County Referral Hospital. We will also store some data (facts) about you and your child, such as age and health status. Later on, we will need to do more tests on the stored blood at Siaya County Referral Hospital and/or at the Maseno University laboratory in Kisumu. If the machines needed for the tests are not in these places, the tests may be done in the United States. We are requesting that you allow us to ship these samples to the United States for these tests. In addition, even if you allow us to ship your/child's samples, we will not ship your samples to the United States without approval from Maseno University Ethics Review Committee (MUERC).

Who will know about my participation in the genetic studies?
Blood and/or genetic material results will be used only for science. No names will ever be used and data will be kept confidential. A small portion of the blood and/or genetic material will be stored for future testing. The investigators may look at inherited factors which are related to diseases by examining DNA and RNA obtained from the stored samples. This DNA and RNA may be used for future analysis of genes that may influence disease.

By signing this form, you are giving consent for future studies of genes that we may perform in the laboratory. The blood sample will remain the property of Maseno University and the University of New Mexico, and may be shared with other researchers as long as confidentiality is maintained. All names will be removed from samples prior to being given to other researchers. The blood samples and the DNA and RNA obtained from the blood or cheek swabs are stored and tested with an identifying number, and your name will not appear on the stored samples.

By signing this form, you understand that there is a possibility that the blood or genetic material, which you are providing under this study, may also be used in other research studies and could

potentially have commercial applicability. You will be unable to participate in receiving any revenues generated by commercial application. Results of studies may be reported in medical journals or at meetings. However, individuals in the study will not be identified in any way.

What are the possible risks of genetic testing?
As has been explained above, genes are inherited from one's parents. Therefore, the risks of the genetic testing may be that we find out that you or certain children from your family develop more severe diseases. This could cause stress for you if you or your children have these genetic (inherited) risks. Finding these risk factors, if present, could also help you by letting you know if you or your child needs closer monitoring for diseases. Since we will not disclose this information or place it in your medical record, there will not be a risk of you losing any health benefits that you are able to get.

What are the possible benefits of genetic testing?
Benefits of the study may include determining which individuals are likely to develop severe diseases that can cause harm and death to children and adults.

Is my participation in this genetic research voluntary?
If you want, you can agree that we store your blood and your child's blood and/or genetic material for these tests. If you refuse, you and your child can still be part of the main study. You can tell us any time during the next 5 years if you don't want us to keep your blood and/or your child's blood or genetic material for these tests. No matter what you decide, you and your child will still get the best possible medical care here at the hospital.

Who will be conducting the genetic studies?
If you have questions about the study, you can contact Dr. Douglas Perkins (University of New Mexico, P. O. Box 144, Siaya, Kenya, telephone: 0705399133). You can also contact co-investigators on the study, Dr. Collins Ouma (Maseno University, P. O. Box 54-40100, Kisumu, telephone: 0722381214) or Dr. Evans Raballah University of New Mexico Laboratories in Siaya County Referral Hospital (P. O. Box 144-Siaya; telephone 0733792627).

Name: _____
(Please Print)

Date: _____ **Study #**:_____

Study Participant's Statement:

□ The above study has been explained to me. The consent form has been read to me and my questions have been answered to my satisfaction. I have been told that taking part is voluntary. **I agree** to allow my blood sample to be stored for genetic tests. I have received a copy of this form.

□ The above study has been explained to me. The consent form has been read to me and my questions have been answered to my satisfaction. I have been told that taking part is voluntary. **I do not agree** to allow storage of my blood sample for genetic testing. I have received a copy of this form.

Signatures:

Study Participant's Signature: _____ **Date**: _____

Witness' Signature: _____ **Date**:_____

Investigator's Signature: _____ **Date**: _____

Study Participant's Thumbprint: _____

Parent/Guardian's Name: _____ Child's Name:_____
(Please Print) (Please Print)

Date: _____ Study #:_____

Parent/Guardian's Statement:

□ The above study has been explained to me. The consent form has been read to me and my questions have been answered to my satisfaction. I have been told that taking part is voluntary. **I agree** to allow my child's blood sample to be stored for genetic tests. I have received a copy of this form.

□ The above study has been explained to me. The consent form has been read to me and my questions have been answered to my satisfaction. I have been told that taking part is voluntary. **I do not agree** to allow storage of my child's blood sample for genetic testing. I have received a copy of this form.

Signatures:

Parent/Guardian's Signature: _____ Date: _____

Witness Signature: _____ Date: _____

Parent/Guardian's Thumbprint: _____

****

STUDY EMPLOYEE CERTIFICATION

I certify that the nature and purpose, the potential benefits and possible risks associated with participation in this research study have been explained to the above individual and that any questions about this information have been answered.

Study employee's signature: _____ Date: _____

Maseno University Ethics Review Committee, Private Bag, Maseno; Telephone numbers: 057-51622, 0722203411, 0721543976, 0733230878; Email address: muerc-secretariate@maseno.ac.ke; muerc-secretariate@gmail.com

Figure 4.4 Top emergent immune response

Appendix 4. Genotyping Primer Sequences

SNPs rs2736191 and rs11575837 were genotyped using the TaqMan® 5' allelic discrimination Assay-By-Design high-throughput method according to the manufacturer's instructions [Assay ID: C_16286876_10 for rs2736191 and C_27834902_10 for rs11575837; Thermofisher Scientific, Carlsbad, CA, USA). The primer sequences for the two SNPs include rs2736191: ACCAGCTTTTACCCAGAACAAGCCT**[C/G]**CAGGAGCCCTCCTTGGCCCAGAAGC
while that of rs11575837:
CAAGGACCACAACTGCCAGGGACCT**[C/T]**GAGCATCAAATGCTTGCCTCCCTGA.