Using the Controlled Human Malaria Infection Model to Investigate Immunity to Malaria

Thesis submitted for the degree of Doctor of Philosophy
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Susanne H. Hodgson (nee Sheehy)

Balliol College

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ABSTRACT

Controlled human malaria infection (CHMI) studies, where healthy volunteers are infected with *Plasmodium falciparum* have become a vital tool to accelerate vaccine and drug development. As CHMI trials are carried out in a controlled environment, they allow unprecedented, detailed evaluation of parasite growth dynamics and immunological responses to infection. Though commonly performed in malaria-naïve populations, CHMI trials have rarely been conducted in malaria-endemic regions and to date, have not been used to investigate naturally acquired immunity (NAI) to *P. falciparum* infection.

This thesis describes the first CHMI study in Kenya and the first attempt to use the modern CHMI model to explore the dynamics and mechanisms of NAI. Using samples collected post-CHMI from both UK volunteers and Kenyan subjects with varying prior exposure to *P. falciparum,* this work reports and compares the findings of key *in vitro* assays including GIA, ADRB activity and changes in gene expression in order to understand the effect of NAI on these measures.

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ABBREVIATIONS

β-HCG	β human chorionic gonadotrophin
ADCI	Antibody-dependent cellular inhibition
ADRB	Antibody-dependent respiratory burst
AE	Adverse event
AIM2	IFN-inducible protein / absent in melanoma 2
ALT	Alanine transaminase
AMA1	Apical membrane antigen 1
BCR	B cell receptor
BCS	Biological safety cabinet
BIP	Backward inner primer
C-1	Day before CHMI
C+7.0	Seven days post-CHMI
CF11	Cellulose powder
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GM-AMP synthase
ChAd63	Chimpanzee adenovirus 63
CHMI	Controlled human malaria infection
CMV	Cytomegalovirus
CRP	C-reactive protein
CS	Circumsporozoite
CSC	Centre Scientific Committee
D-5	Five days prior to diagnosis in CHMI
DAMPs	Damage associated molecular patterns
DB	Development buffer
DBS	Dried blood spot
DC	Dendritic cell
DefExp	Definite prior exposure to *P. falciparum*
DTD	Delay to diagnosis
DVI	Direct venous inoculation
EBA175	Erythrocyte-binding antigen 175
EBV	Epstein barr virus
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERC	KEMRI Ethics review committee
FBC	Full blood count
FC	Fold change
FDA	US Food and Drug Administration
FDR	False discovery rate
FIP	Forward inner primer
fPCA	Functional principal component analysis
GCP	Good clinical practice
GEO	Gene Expression Omnibus
GIA	Growth inhibition activity
GLURP	Glutamate rich protein

GM	Geometric mean
GMP	Good manufacturing practice
GPI	Glycosylphosphatidylinositol
HSA	Human serum albumin
Hb	Haemoglobin
HbC	Haemoglobin C
HbF	Haemoglobin F (Fetal haemoglobin)
HbS	Haemoglobin S
HbSAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
IκB	Inhibitor of NF-κB
ID	Intradermal
IFA	Immunofluorescence assay
IFN	Interferon
IFN-γ	Interferon gamma
IFNAR	IFN-α receptor
IFNR	Interferon regulatory factors
IKK	IκB kinase
IL	Interleukin
IM	Intramuscular
IQR	Interquartile range
IRFs	Interferon Regulatory Factors
IRAK	IL-1 receptor-associated kinase
iRBC	Infected red blood cell
IRF3	IFN-regulatory factor 3
ISG	Interferon-stimulated genes
ISRE	Interferon-stimulated response elements
IV	Intravenous
JAK	Janus activated kinases
KCS	Kenyan Challenge Study using PfSPZ Challenge
KEMRI	Kenya Medical Research Institute
LAMP	Loop mediated isothermal amplification
LBI	Liver to blood inoculum
LIMMA	Linear models for microarray analysis
LLD	Lower limit of detection
LLQ	Lower limit of quantification
LNVP	Liquid nitrogen vapour phase
LP	Lymphoprep™/Plasmodipur
MAL	MYD88 adaptor-like protein
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
mBC	Memory B cell
MDA5	Melanoma differentiation-associated protein 5
MinExp	Minimal prior exposure to *P. falciparum*
MSP	Merozoite surface protein
MVA	Modified vaccinia Ankara virus
MYD88	Myeloid differentiation primary response protein 88
Mz	Merozoite
NAI	Naturally acquired immunity

NASBA	Nucleic acid sequence-based amplification
NAT	Nucleic acid test
ND	Not detected
NDTD	No delay to diagnosis
NF-κB	Activation nuclear factor-κB
NGO	Non-governmental organisation
NK	Natural killer cells
N/K	Not known
NLRP3	NOD-, LRR- and pyrin domain-containing 3
NOS	Nitric oxide synthase
OD	Optical density
OXTREC	Oxford Tropical Research Ethics Committee
p/mL	Parasites/mL
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with Tween
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEMS	Parasitophorous vacuolar membrane-enclosed merozoite structures
Pf	Plasmodium falciparum
PfEMP1	P. falciparum erythrocytic membrane protein 1
PGD	Parasite growth dynamics
PMN	Polymorphonucleocytes
PMR	Parasite multiplication rate
PRR	Pattern recognition receptor
PVM	Parasitophorous vacuolar membrane
QC	Quality control
QIMR	Queensland Institute for Medical Research
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription PCR
RBC	Red blood cell
RBCCS	Red blood cell counting solution
RH5	Reticulocyte-binding protein homologue 5
RIF	Repetitive interspersed families of polypeptides
RIN	RNA integrity number
RLT	RLT-βME buffer
RLU	Relative light unit
ROI	Reactive oxygen intermediates
RT	Room temperature
RUNMC	Radboud University Nijmegen Medical Centre
qPCR	Quantitative PCR
SAE	Serious adverse event
SC	Subcutaneous
SMC	Safety monitoring committee
SmPC	Summary of product characteristics
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signaling

SOP	Standard operating procedure
SPZ	Sporozoites
SRA	Surface reactivity assay
SSC	Scientific Steering Committee
STAT	Signal transducer and activator of transcription
STING	Stimulatory of IFN genes protein
TAE	Tris-acetate-EDTA buffer
TBK1	Serine/threonine-protein kinase
TBV	Transmission blocking vaccine
TGF-β	Transforming growth factor-β
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRAP	Thombospondin related adhesion protein
Treg	Regulatory T cells
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TTD	Time to diagnosis
Tstat	Transferrin saturation
TYK	Tyrosine kinase
UW	University of Washington
VAC045	UK CHMI study using mosquito-bite
VAC049	UK CHMI study using PfSPZ Challenge
VAC052	UK CHMI study using mosquito-bite
VIS	Volunteer information sheet
VSN	Variance stabilization and normalisation
WB	Whole blood
WBC	White blood count
WF	Whatman Filter
WHO	World Health Organisation

PUBLICATIONS RESULTING FROM THIS WORK

Hodgson SH, Juma E, Salim A, Magiri C, Njenga D, Molyneux S, Njuguna P, Awuondo K, Lowe B, Billingsley PF, Osier F, Chilengi R, Hoffman SL, Draper SJ, Ogutu B & Marsh K. Lessons Learnt from the First Controlled Human Malaria Infection Study Conducted in Nairobi, Kenya. *Malar J. 2015 Apr 28;14(1):182.*

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CHAPTER 1: INTRODUCTION

1.1 Rationale for thesis

The controlled human malaria infection (CHMI) model is an established, powerful tool to evaluate the efficacy of novel vaccines and drugs targeting *Plasmodium falciparum* malaria. The CHMI model could be applied to better understand the dynamics and mechanisms of immune responses to *P. falciparum* infection in malaria-naïve and exposed adults.

1.2 Plasmodium malaria

Malaria, a parasitic infection of red blood cells (RBCs) is major global health problem, killing approximately 2,000 people a day, most of whom are children in Africa[1]. Although human disease is caused by five species of the plasmodium parasite (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*), *P. falciparum* is responsible for most mortality[1]. The work in thesis is undertaken with the aim of accelerating the development of a vaccine against *P. falciparum*.

1.3 Plasmodium falciparum malaria

1.3.1 Burden of disease

Although encouraging evidence suggests that the epidemiology of *P. falciparum* malaria is changing across certain parts of Africa[2], the worldwide burden of disease from malaria remains a major public health problem, with approximately 198 million cases and 584,000 deaths worldwide in 2013[1,3], (a potentially

conservative estimate)[4]. The enormous economic and social consequences of malaria have been well documented[5]. The development of resistance both in *Anopheles* mosquitoes to certain insecticides and of malaria parasites to chemotherapeutic agents has contributed to an increasing need for new, effective interventions for the prevention or treatment of *P. falciparum* malaria, especially if elimination is to become a reality[1,6-8].

1.3.2 The lifecycle of *Plasmodium falciparum*

The lifecycle of *P. falciparum* is complex, with stages in both human and mosquito hosts (Figure 1-1)[1,9]. The bites of infected female *Anopheles* mosquitoes inject sporozoites (typically about 15)[10] into the skin where they can remain for up to 6 hours (*skin-stage*)[11]. One third of sporozoites leaving the injection site may enter lymphatics and drain to the regional lymph nodes[12]. The others travel via the bloodstream to the liver where they migrate through Kupffer cells and several hepatocytes before establishing infection in a hepatocyte (*liver-stage*). The sporozoites then undergo a cycle of asexual amplification (schizogony) lasting 6-7 days to create exoerythrocytic schizonts, which contain up to 30,000 merozoites[9,13].

The infected hepatocyte buds off vesicles of several thousand infective merozoites (merosomes) into the blood. The merozoites quickly invade RBCs (*blood-stage*)[14]. Within a parasitophorous vacuole the parasite commences further asexual development, maturing from the ring stage (immature trophozoite) to a trophozoite and then an erythrocytic schizont containing merozoites. After 48 hours, the schizonts rupture, releasing merozoites into the bloodstream. Some of these invade uninfected erythrocytes and repeat the cycle

of blood schizogony.

A small percentage of merozoites differentiate into male or female gametocytes 7-15 days after initial invasion of RBCs and circulate in the peripheral blood stream (*sexual-stage*)[15-17]. When ingested by a feeding *Anopheles* mosquito, following a change in temperature and pH, male and female gametocytes unite in the mosquito gut to create a diploid zygote[18]. The zygote matures to an ookinete that penetrates the gut wall and attaches to the outer aspect of the mosquito gut. The ookinete differentiates into an oocyst, which undergoes schizogony to produce thousands of haploid sporozoites. The mature oocyst ruptures, and the sporozoites migrate actively to the mosquito salivary glands. The sporozoites penetrate the glands and rest in the channels bearing saliva, to be injected into the human when the mosquito feeds.

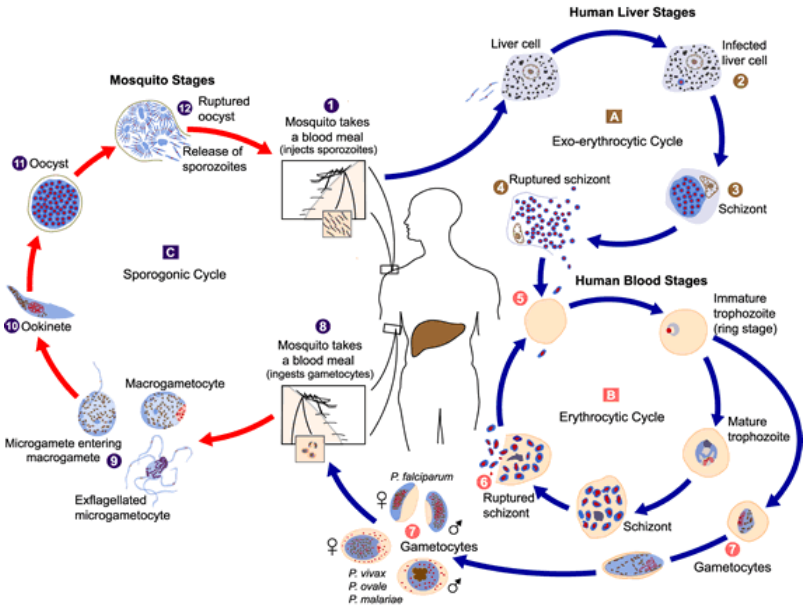


Figure 1-1: Lifecycle of Plasmodium falciparum

Adapted from the CDC website[19].

1.3.3 Pathogenesis of *P. falciparum* infection

Infection by sporozoites and the liver-stage of malaria is asymptomatic and not associated with pathology. The blood-stage of infection is associated with symptoms and disease[1].

Humans with no previous experience of *P. falciparum* usually become ill on their first exposure to the parasite[20,21]. Individuals develop a febrile illness, which may become severe (as defined by clinical or laboratory evidence of vital organ dysfunction), and in some cases leads to death[22]. More than 85% of cases and 90% of deaths attributed to *P. falciparum* occur in sub-Saharan Africa, mainly in young children (aged < 5 years), who are particularly susceptible[23]. The manifestations of severe malaria depend on age. Severe anaemia and hypoglycaemia are more common in children, acute pulmonary oedema, acute kidney injury and jaundice more common in adults, whilst acidosis and coma (cerebral malaria) occur in all age groups[1]. Although complex and incompletely understood, malaria pathogenesis is thought to be driven by two distinct processes: sequestration and inflammation[24].

In *P. falciparum* malaria, protuberances appear on the infected red blood cells' (iRBC) surface 12–15 hours after parasite invasion. These protuberances extrude high-molecular-weight, antigenically variant, strain-specific adhesive proteins (*P. falciparum* erythrocytic membrane protein 1; *Pf*EMP1) that mediate cytoadherence (i.e. attachment to endothelial surface receptors in veins and capillaries)[1,21,25]. Each *P. falciparum* parasite genome includes approximately 60 var genes encoding different PfEMP1 types, enabling the parasite to attach iRBCs to different receptors on the vascular lining[26]. Of the potential receptors

identified, ICAM1 is the most important in the brain, chondroitin sulphate A in the placenta, and CD36 in most other organs[27]. Host endothelial protein C receptor has been associated with severe malaria in children[28].

iRBCs adhere to the microvasculature and to each other (by platelet mediated agglutination) or uninfected RBCs (rosetting; mediated by *P. falciparum*-encoded repetitive interspersed families of polypeptides (RIF) and PfEMP1 expressed on the surface of iRBC)[29,30]. Sequestration resulting from cytoadherence, agglutination and rosetting causes microvascular obstruction, which correlates with clinical severity, and established prognostic measures such as plasma lactate and base deficit[31].

Single nucleotide polymorphisms (SNPs) in genes encoding key elements of the innate immune system (such as tumour necrosis factor (TNF)[32], interleukin 12 (IL-12)[33], interferon γ (IFN-γ)[34,35], and inducible nitric oxide synthase (NOS)[36]) have been associated with protection against severe disease, implying that an excessive pro-inflammatory response is important in the pathogenesis of severe malaria[37]. In addition, cross-sectional studies have shown that higher ratios of pro- to anti-inflammatory cytokines in serum during acute malaria are associated with more severe disease[38,39], although this is not a consistent finding[40].

Severe anaemia, a particularly important cause of mortality in children under 5 years old[41], is thought to result not only from lysis of iRBC but from haemolysis of uninfected RBCs[42], mediated by a number of mechanisms including impairment in erythropoiesis driven by an increase in cytokines (such as TNFα) and IFN-γ[43] and hepcidin up regulation[44,45].

1.3.4 Epidemiology of naturally acquired immunity to *P. falciparum*

In children, the risk of clinical disease increases from birth to 6 months of age, possibly due to a reduction in maternal antibodies, parasite inhibitory factors (such as lactoferrin) and haemoglobin F[9,46]. Beginning around 3 to 4 months of age, children become susceptible to severe disease and death. At about 2 to 5 years of age, the frequency of clinical disease and risk of mortality begins to reduce[9]. Dependent on the intensity of transmission, children become resistant to severe malaria by the age of 5, however, they remain susceptible to uncomplicated episodes of febrile infection until late childhood or early adolescence (Figure 1-2)[21].

With repeated exposure to infection individuals in malaria-endemic regions develop naturally acquired immunity (NAI) first to severe malaria and death and then more slowly to infection itself[1]. This immunity is strain-transcending and acquired relatively quickly, (after as few as three or four exposures), providing protection from high density parasitaemia and severe disease[9]. However, resistance to infection is rarely achieved, and adults living in endemic areas frequently have asymptomatic blood-stage infections[1,20,21]. NAI is known to be compromised in pregnant women (indicated by increased parasite densities and risk of severe malaria especially during first and second pregnancies)[47], and adults removed from an endemic region are known to lose NAI, at least temporarily[9].

In summary, various types of immunity to *P. falciparum* have been described,

which are likely to coexist (Figure 1-2)[9]:

(i) *Anti-disease immunity*; conferring protection against clinical disease. This affects the risk and degree of morbidity associated with a given parasite density and is quickly acquired as seen in young children in malaria-endemic regions.

(ii) A*nti-parasite immunity*; conferring protection against parasitaemia. This affects the density of parasites during an infection and is more slowly acquired.

(iii) *Premunition*; providing protection against new infections by maintaining a low-grade and generally asymptomatic parasitaemia. This is slowly acquired and seen in adolescents and adults[9].

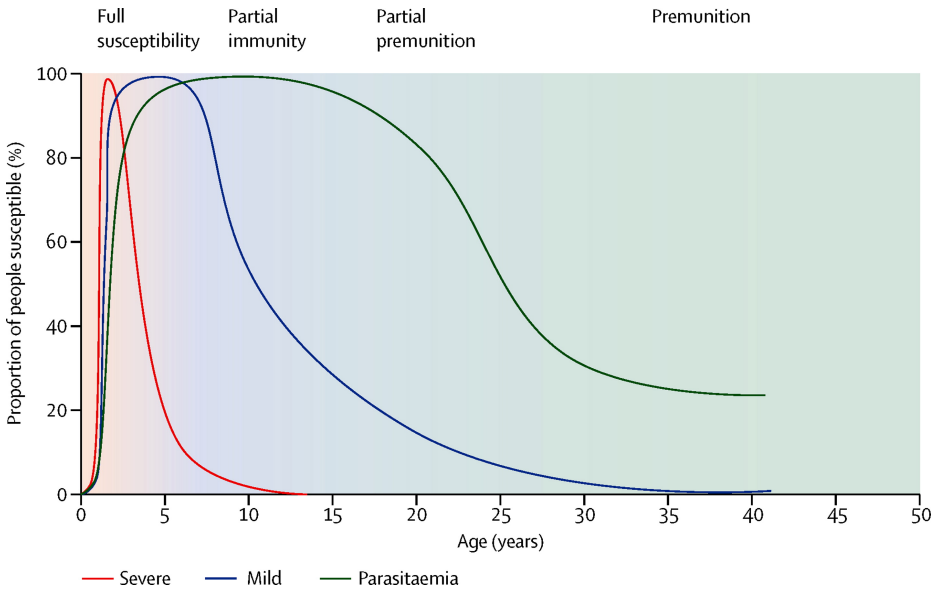


Figure 1-2: Relationship between age and malaria severity in an area of moderate transmission intensity

Adapted from *White et al*[1].

1.3.5 Importance of host genetics in determining pathogenesis of *P. falciparum*

Malaria has had a profound and extended impact on the evolution of the human genome[48]. Genetic mutations that affect the ability of *P. falciparum* to infect and/or replicate in RBCs such as Haemoglobin S (HbS)[49] and HbC[50], ovalocytosis[51], thalassaemias[52] and glucose-6-phosphate dehydrogenase deficiency[53] have all been shown to confer a survival advantage in the presence of malaria. For example, in the case of HbS, heterozygotes are protected against *P. falciparum* (due to reduced cytoadherence and parasite growth at low oxygen tensions), while homozygotes have sickle cell disease[1]. However, polymorphisms in immune-response-related genes have also been associated with differential outcomes in *P. falciparum* infection[54].

1.4 Immunological responses to *P. falciparum*

1.4.1 Immunological mechanisms of naturally acquired immunity to *P. falciparum* infection

The human immune system mounts a response to *P. falciparum* infection that is incompletely understood. This response varies according to the degree of prior exposure to the parasite and dictates the clinical course of infection in humans[21]. The natural immune response comprises both innate and adaptive components and varies for each stage of the *P. falciparum* lifecycle.

Initially non-specific innate host defense mechanisms control the first wave of parasitaemia, reducing the likelihood of early host death[54]. Subsequent strain transcending and strain-specific blood-stage adaptive immune responses then struggle against parasitic antigenic variation to eliminate the blood-stage infection. Eventually, exposure to sufficient strains confers protection from illness, but not from infection. Both humoral and cellular immunity contribute to NAI and to date, no single molecular signature, key cellular determinant or immune mechanism of NAI, has been unequivocally associated with protection against malaria[9,20].

1.4.1.1 Innate immune response

The innate response has two main functions: to provide an immediate non-specific defense against invading pathogens and to facilitate induction of the adaptive immune response[55]. The innate response to *P. falciparum* occurs in the skin, liver and blood[37,56,57], and comprises of humoral and cellular components, which act immediately following infection or are rapidly induced. In *P. falciparum* infection, the innate response is critically important, not only for host defense and activating the adaptive immune response but for mediating pathogenesis of the disease[58].

1.4.1.2. Activation of the innate immune response

Pathogen associated molecular patterns (PAMPs) are conserved microbial structures that are detected by pattern recognition receptors (PRRs)[59]. For the liver-stage of infection, *Plasmodium* RNA is the only malaria ligand identified to date[60]. Three *P. falciparum* PAMPs for the blood-stage of infection have been

studied in detail; glycosylphosphatidylinositol (GPI) anchors, haemozoin and immunostimulatory nucleic acid motifs (for example CpG and AT-rich immunostimulatory motifs of *Plasmodium* DNA), however many have been identified[61,62]. PAMPs bind to PRRs[63] including Toll-like receptors (TLRs)[64] (at the membrane of endosomes or at the cell surface), RIG-I-like receptors[65] and NOD-like receptors[66] (in the cytosol) to activate distinct transcriptional programmes (including mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB))[67] to induce multiple downstream pathways involved in pathogen clearance (Figure 1-3)[37]. In addition, host-derived damage associated molecular patterns (DAMPs) such as uric acid, microvesicles and haemozin can also trigger a pro-inflammatory response, in part mediated by activation of PRRs[68-73]. A large number of cytokines and chemokines regulate and control the resulting, complex inflammatory response. In some cases the predominant effect of these cytokines is clearly pro-inflammatory, as in the case of TNF, or predominantly anti-inflammatory as in the case of transforming growth factor-β (TGF-β) or IL-10[74].

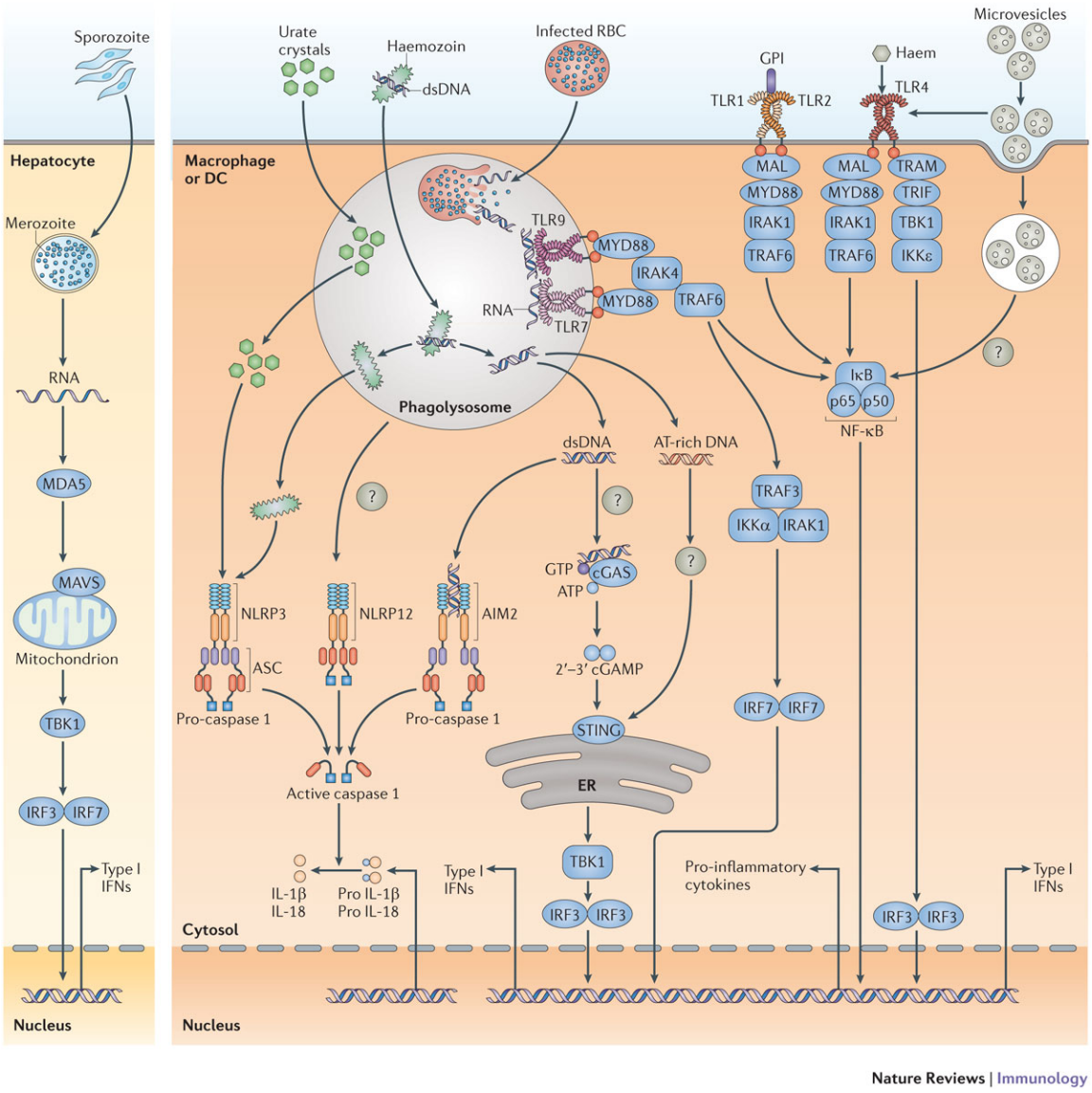


Figure 1-3: Innate sensors of Plasmodium PAMPs and malaria DAMPs

Adapted from *Gazinelli et al*[37].

1.4.1.1.2 Stage specific innate immune responses

In the skin, mosquito saliva, sporozoites and tissue damage resulting from the probing process lead to cutaneous mast cell degranulation. Release of TNF-α and MIP-2 causes local fluid and neutrophil influx to the bite site and subsequent

recruitment of lymphocytes, dendritic cells (DCs) and monocytes. This response is not thought to impede the parasite lifecycle, and the increased blood flow that results may be exploited by the sporozoite as it attempts to enter dermal blood vessels[56]. In addition, the inherent immunoregulatory environment of the skin is exploited by the parasite to evade the immune mechanisms[21]. *Plasmodium*-specific regulatory T cells (Tregs)[75] are induced and DCs are shown to down regulate MHC Class II and CD86[76], in keeping with a tolerogenic response. This immunoregulatory environment may be reinforced by chronic immune activation or immune dysregulation because of repeated exposures to sporozoite and liver-stage antigens[77].

In the liver-stage of infection, sporozoites stimulate the expression of type I IFN-inducible genes in hepatocytes during replication inside a parasitophorous vacuole (Figure 1-3)[57,78]. Type I IFN-induced proteins mediate recruitment of natural killer (NK) cells and NKT cells which produce IFN-γ and trigger the expression of proteins that attempt, unsuccessfully, to inhibit parasite schizogony in the liver. *Plasmodium*-specific mechanisms that disable innate immune defenses, such as sporozoite-mediated disruption of Kupffer cells' respiratory burst in the liver[79], may also limit effectiveness of the immune response in the liver.

Innate immune responses in the blood-stage of infection are critical for sensing early blood-stage infection, promoting inflammation, inhibiting parasite growth, and shaping adaptive immune responses[21,54]. These innate responses are density dependent, triggered when parasitaemia crosses a certain threshold. Parasite densities therefore oscillate between a lower level (at which immune effector

responses are not induced) and a higher level (at which innate effector mechanisms are triggered and partial clearance of infected cells ensues)[54]. These mechanisms limit the growth of all parasites, irrespective of species or strain[54], however some differences in innate response pathways have been shown to correspond to strain-specific disease phenotypes in mice[80].

Macrophages and DCs phagocytose either free haemozoin or iRBCs (Figure 1-3)[81]. Haemozoin or nucleic acids released from the resulting phagolysomes activate TLRs causing the release of transcription factors such as NF-κB[37]. This promotes production of pro-inflammatory cytokines and chemokines[82] including: IL-12, IL-6, IL-8, IL-1β, TNF, nitric oxide and IFN-γ[54,83-87].

The serum complement system is another important component of the innate immune system's response to *P. falciparum*[88]. Activated by PRRs or adaptive immune response-induced antibody, the complement system induces a pro-inflammatory state and contributes to NAI via a number of mechanisms including opsonisation[89-91].

Hyper responsiveness of the innate immune system is thought to be a key factor in the pathogenesis of severe malaria[37,54]. IFN-γ produced early in *P. falciparum* infection pre-activates innate immune cells to respond to minute amounts of microbial products[83,92,93]. This 'pro-inflammatory priming' means the innate immune system can overreact to PRR agonists, causing over production of pro-inflammatory cytokines and the sepsis-like symptoms of acute malaria[37,83]. Indeed, longitudinal studies indicate that children undergoing malaria episodes have increased susceptibility to bacterial infections[94,95].

However, it has been suggested that clinical immunity to malaria may be due in part to immune tolerance of the parasite, via a blunting of the innate immune response, rather than resulting entirely from the development of a protective, adaptive immune response[96,97]. In fact, the ability to control excessive *P. falciparum*–induced inflammation in early life may be the key adaptation that confers protection from potentially life-threatening disease in young children yet to acquire protective antibodies[21]. Mouse models that have shown *Plasmodium*-induced inflammation (indicated by concentrations of TNF and IFN-γ) peaks 5–10 days after infection and then diminishes, before parasitaemia decreases, as a result of the regulatory cytokines TGF-β and IL-10[98,99]. In humans, one possible mechanism of 'malaria tolerance' has been highlighted in a recent study where a subset of innate-like lymphocytes ($V\delta2^+$ γδ T cells) were lost in patients with repeated malaria exposure. In addition, the T cells that remained were found to produce lower amounts of pro-inflammatory cytokines and have up-regulated expression of immunoregulatory genes[100].

However, many questions regarding the innate immune response to malaria remain, including understanding of the immunoregulatory mechanisms and cells that mediate control of *P. falciparum*–induced inflammation and the longevity of immunological responses in the absence of ongoing *P. falciparum* exposure[21,101,102].

1.4.1.2 Adaptive immune response

The adaptive immune system is activated when the innate immune response is unable to overcome an invading pathogen. Adaptive immunity consists of cellular and humoral components, is highly specific and capable of producing

long-lasting memory.

Whilst antibody and T cell responses are generated against skin and liver-stage antigens, there is no convincing evidence of NAI capable of neutralising the parasite at the skin or liver-stages[21,103]. Indeed, adults in malaria-endemic regions are at the same risk of infection as young children[104].

The adaptive immune response against the blood-stage of infection is thought to be responsible for NAI to *P. falciparum*[20,21]. Passive transfer experiments have shown this response is mediated by antibody[105], and it is known to be highly specific and capable of producing long lasting memory[20,106]. Accumulation of high-titre antibody responses (especially subclass IgG), with increasing age and exposure has been shown to be the main correlate of protective NAI in endemic human populations[107-109]. Antibody specific for a number of merozoite antigens and combinations of antigens have been associated with NAI, including PfEMP1, apical membrane antigen 1 (AMA1), merozoite surface proteins (MSP) 1, 2, 3, erythrocyte-binding antigen 175 (EBA175) and glutamate rich protein (GLURP)[110,111]. However, it is unclear which of the more than 5,000 *P. falciparum* proteins[112] are targets of protective antibodies. It is also not known whether immunity is conferred by numerous, highly specific immune responses, by a small number of extensively cross-reactive immune responses, by the development of immune responses targeting highly conserved proteins or by antibody responses mediated by limited combinations of specific antibodies[21,111,113].

The slow rate of acquisition of protective antibodies and inconsistent boosting post-infection has been attributed in part to the genetic diversity of many *P.*

falciparum proteins and the parasite's ability to vary the proteins it expresses on the surface of iRBCs[114]. As a result, it may take years living in an endemic area for an individual to be exposed to a sufficient number of parasite clones to generate a protective repertoire of antibodies[21]. Even when NAI is achieved this is non-sterilising, possibly due to the ability of *P. falciparum* to evade adaptive immune responses through dysregulation of $CD4^+$ T cell and B cell function[21].

The mechanisms by which antibody mediates NAI is debated but likely includes prevention of invasion of new RBCs by attachment to merozoite antigens, opsonisation of surface antigens on iRBC and/or merozoites, activation of different cell types via Fc receptor mediated pathways and activation of the complement system[110,115].

1.4.1.2.1 Production of adaptive immune responses

Development of the antibodies necessary for NAI requires activated, antigen-specific B and T cells. T lymphocytes ($CD4^+$ or $CD8^+$) are activated by the presentation of antigen, by antigen presenting cells (APCs) on MHC molecules and co-stimulatory molecules. Exogenous particles that are internalised by APCs are broken down into peptide fragments and presented on MHC class I or II molecules to stimulate $CD4^+$ or $CD8^+$ T cells respectively[116]. Given that mature RBCs do not express MHC molecules, T cells are not activated by, or directly target iRBC.

In malaria, following activation by APCs and in the presence of IL-12 and IFN-γ, naïve $CD4^+$ T cells develop into Th1 cells, which in turn secrete cytokines (IFN-γ, IL-2, TNF-α and TNF-β) and facilitate the production of antibody[113,117].

Inflammation is limited by Treg cells[113].

Upon binding antigen specific to their functional B cell receptors (BCR) in peripheral lymphoid organs, immature B cells migrate to the T cell zone of secondary lymphoid organs. Here, following interaction with antigen-primed T cells[118], the antigen-BCR complex is internalised, cleaved into peptides and presented on the B cell surface on MHC II molecules. $CD4^+$ T cells recognise the antigen and signaling encourages both the B and T cells to proliferate. Upon activation, B cells undergo somatic hypermutation and class switching, which leads to the introduction of point mutations in the variable regions of Ig genes to ensure that antibodies have high affinity for the antigens they recognise. During proliferation, B cells differentiate into short-lived plasma cells or enter the germinal centre reaction to form long-lived plasma cells and memory B cells (mBCs).[118] mBCs are capable of differentiating into plasma cells upon re-encountering antigen and constitute an important part of long-term immunological memory. Plasma cells, or antibody secreting cells, produce and secrete large quantities of immunoglobulin (secreted BCR) detected in serum.

1.4.1.3 Interactions between the innate and adaptive immune responses

Innate and adaptive immunity are inextricably linked as the cytokines produced by cells of the innate system modify the outcome of the adaptive response (Figure 1-4). Therefore, manipulation of cells of the innate immune system could affect downstream cellular activation, and this relationship could be exploited to aid malaria vaccine design[54]. In addition, aspects of the adaptive immune response can utilise cells of the innate response for pathogen clearance (for example phagocytosis of antibody opsonized iRBC or merozoites by

neutrophils)[119].

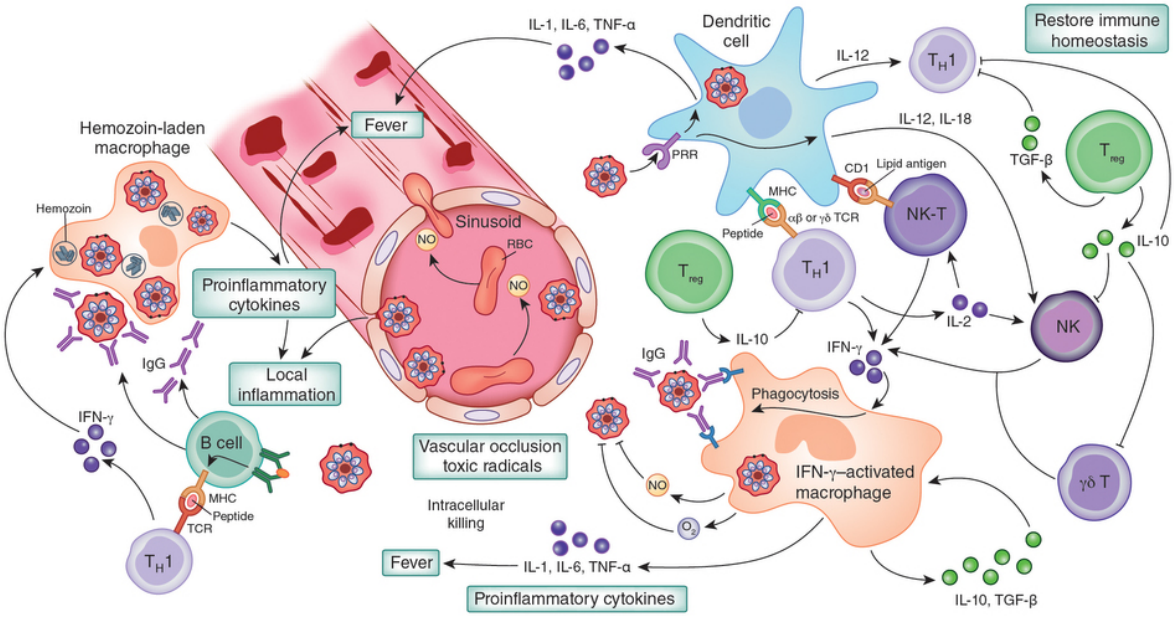


Figure 1-4: Interaction between the innate and adaptive immune systems in the response to malaria

Adapted from *Riley et al*[113].

1.4.1.3.1 Central role of interferons (IFN) in coordinating the innate and adaptive immune responses

Interferons (IFN) are key cytokines that regulate and control the immune response, acting to either support or suppress inflammation, and have important roles in both the innate and adaptive immune responses. Based on their evolution, structure and interactions with distinct receptor complexes, IFN are subdivided into three distinct types; Type I (13 types of IFNα and IFNβ) produced by most tissue and cell types in response PAMPs or DAMPs; Type II (IFN-γ) produced by various T cell and NK cells; and Type III (IFN-λ 1-3 or

synonymously IL-29, IL-28A and IL-28B). All IFN share a secondary structure, however each class of IFN binds to distinct multi-chain receptor complexes which have a highly restricted tissue expression[74].

Whilst receptors of all IFN types employ the janus activated kinases – signal transducer and activator of transcription (JAK-STAT) pathway for nuclear signaling (Figure 1-5)[120], each IFN engages different JAK-STAT molecules, drives the expression of different interferon-stimulated response elements (ISRE) and induces various interferon-stimulated genes (ISGs)[62].

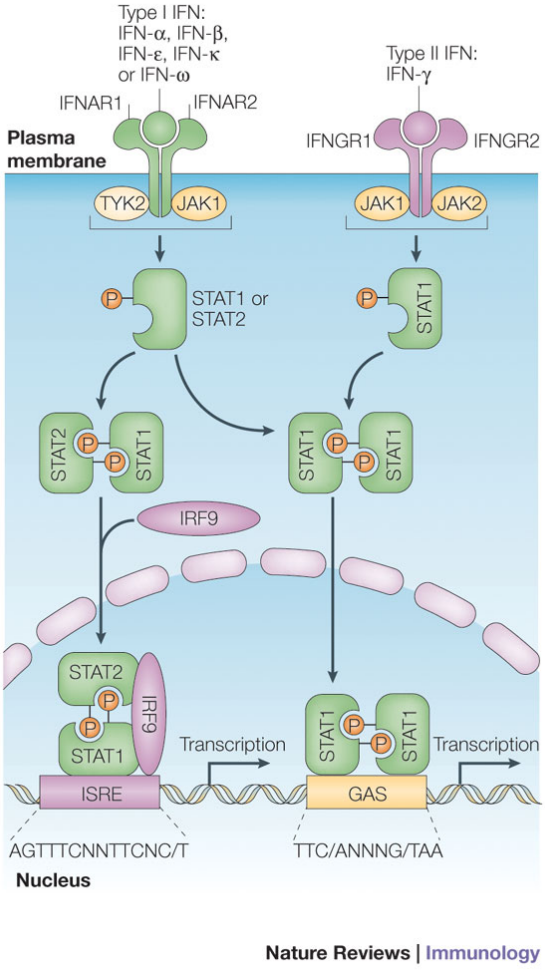


Figure 1-5: JAK-STAT pathway signaling for type I and type II interferons

JAK; Janus activated kinases, TYK2; tyrosine kinase 2, STAT; signal transducer and activator of transcription, ISGF3; IFN-stimulated gene factor 3, ISRE; IFN-stimulated response elements in DNA, GAS; IFN-γ activated site. Adapted from *Platanias et al*[120].

The JAK-STAT signaling pathways are regulated by a vast array of intrinsic and environmental stimuli. Diverse protein kinases, including several MAPKs, phosphorylate STATs on serine residues, allowing additional cellular signaling

pathways to potentiate the primary STAT-activating stimulus[121]. Negative regulation of the JAK-STAT pathway is accomplished by receptor degradation and specific inhibition signals from protein tyrosine phosphatases acting at the level of the membrane-associated receptor-kinase complex or, by de-phosphorylation of activated STAT dimers in the nucleus[122]. JAKs also have their own inhibitors; suppressor of cytokine signaling (SOCS) proteins[123] and interferon regulatory factors (IRF1-9) are transcription factors to regulate IFN production[62].

IFN and their STATs are crucial in mediating inflammation via a number of mechanisms including; regulating cell survival[74], mediating cell trafficking via chemokine expression[124], activation of macrophages, resulting in cytokine, NO and reactive oxygen intermediates (ROI) synthesis[125] and mediating pathogenesis of endotoxin-related shock[126].

1.3.1.3.1.1 Type I interferons (IFN-α/β)

Originally described as central to effective antiviral immune responses[127], Type I IFN has been shown to have an important role in the immune response to both liver and blood-stage malaria. In the liver-stage of infection, early production of Type I IFN prior to infection can impair parasite establishment, whilst production during the very late phase is capable of partially limiting parasite growth in the liver[60,62].

In the blood-stage of malaria, genetic studies of malaria-infected patients have shown an association between mutations within IFN-α receptor (IFNAR) and susceptibility to severe malaria[128,129], and IFN-α has been identified in sera from

a subset of malaria-infected patients[130,131]. Analysis of paired whole blood human transcription profiles from children with severe and mild malaria showed mild malaria to be associated with enrichment of pathways associated with Type I IFN-mediated signaling[132].

However, mouse studies have provided conflicting results regarding the role of Type I IFN in blood-stage infection[132]. *Plasmodium berghei* ANKA-infected mice treated with IFN-β demonstrated a 60% improvement in survival compared to untreated mice, without any differences in parasitaemia[133], whilst 25 days of IFN-α reduced mortality from cerebral malaria in C57BL/6 *P. berghei* ANKA-infected mice from 87% to 6%[134]. However, in contrast, mice lacking IFNAR have been shown to be protected against cerebral malaria in the *P. berghei* ANKA model[61,135]. These conflicting data may suggest that particular concentrations and duration of action of type I IFNs are needed to generate an immune response that can control parasite growth.[62]

1.3.1.3.1.2 Type II Interferon (IFN-γ)

IFN-γ is a key cytokine against malaria at all stages in the life-cycle as part of both the innate and adaptive immune responses (Table 1-1)[136].

Innate immune responses
• Increased production of reactive nitrogen species by liver cells against intrahepatic parasites[137-139]. • Increased production of reactive oxygen and nitrogen species by monocytes against blood-stage parasites[140-143]. • Enhanced phagocytosis of merozoites and iRBC[141,144-146]. • Inhibition of gametocyte infectivity to mosquitoes[147].
Interplay between innate and adaptive systems
• Pro-inflammatory priming of TLR responses[83,92,93,148]. • Up-regulation of MHC class I and II expression[149]. • Enhanced antibody dependent cellular inhibition (ADCI) against blood-stage parasites[150].
Adaptive immune responses
• Increased Th1/Th2 ratio amongst T cells[151]. • Class-switching by B cells to cytophilic antibody isotypes[152]. • Enhanced induction of cellular (central) memory responses[153].

Table 1-1: Effects of IFN-γ on innate and adaptive immune responses against malaria

Adapted from *McCall et al* [136].

IFN-γ is produced by a number of cell types (Figure 1-6);[54,136,154,155] γδ T cells (innate) recognise malaria phosphoantigens by MHC class II independent pathways via γδ T cell receptors, and as a result produce IFN-γ which in turn activates DCs to mature. In response to activation of PRRs or inflammatory cytokines including IFN-γ, DCs mature, up-regulating expression of MHC class II molecules, adhesion molecules, and the production of cytokines including IL-12 (DCs may themselves produce IFN-γ). DCs then migrate to the spleen, where the

IL-12 they produce activates NK cells to produce IFN-γ. DCs present antigen to classical, adaptive $CD4^+$ and $CD8^+$ αβ T cells, which, together with IL-12, induces the differentiation and clonal expansion of antigen-specific Th1 cells, which in turn produce further IFN-γ. Activated αβ T cells also produce IL-2, resulting in the proliferation of γδ T cells, NK cells and αβ T cells themselves. IL-2 also further activates NK cells to produce IFN-γ, which induces DC maturation and activates macrophages, further amplifying the immune response. Cytokines such as IL-10 and TGF-β negatively regulate both innate and adaptive responses. The relative contribution of different cell types *in vivo* to total IFN-γ during an acute infection is unclear. However blood samples from malaria-naïve volunteers undergoing CHMI suggest an early role for NK cells[156] followed by a contribution from αβ T cells, γδ T cells and NK cells[136].

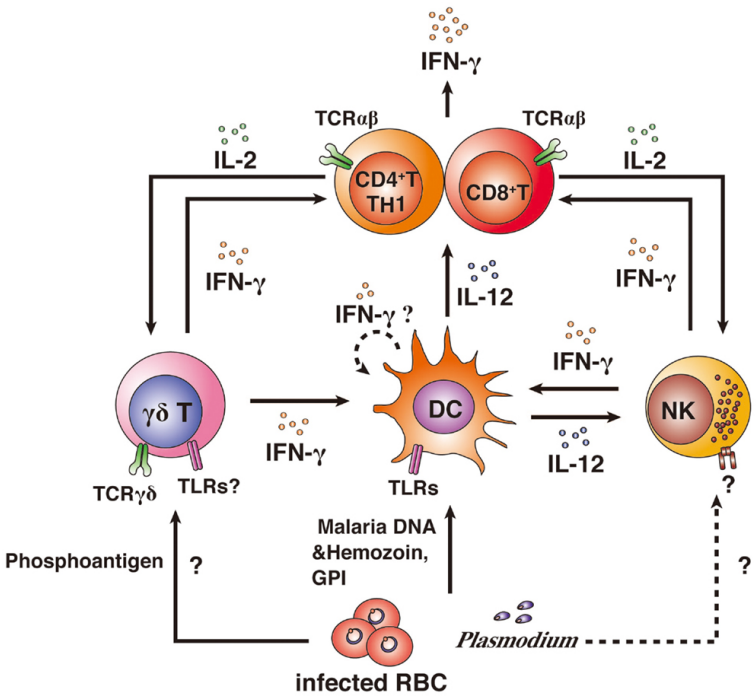


Figure 1-6: IFNγ producing cells and their activators in malaria infection

Adapted from *Inoue et al*[154].

Evidence from numerous animal studies has shown that IFN-γ is a key cytokine in the immune response to blood-stage malaria. For example, IFN-γ -/- mice fail to control the initial wave of parasite multiplication and succumb rapidly to hyperparasitaemia[142,157,158], whilst a similar outcome is seen in immunocompetent animals in which IFN-γ is depleted during infection[159,160].

With the exception of one retrospective study,[161] field studies have shown iRBC-induced IFN-γ responses to be associated with reduced risk of fever and clinical malaria episodes[39,162-164]. However, one study has shown IFN-γ responses, specifically to blood-stage antigens, to be associated with exposure but not protection against malaria[165], suggesting broad anti-parasite IFN-γ responses but not necessarily responses to individual blood-stage antigens are important for protection against clinical malaria episodes[164]. Studies have also shown an association between SNPs in the genomic region of IFN-γ and susceptibility to malaria[166-168]. Further evidence for a central role of IFN-γ in NAI to malaria comes from a landmark controlled human malaria infection (CHMI) study by *Pombo et al*[169]. Malaria naïve, Caucasian volunteers were repeatedly infected with ultra-low doses of blood-stage parasites and drug cleared before becoming symptomatic. These volunteers were protected against subsequent blood-stage infection with immunity associated with strong $CD4^+$ T cell mediated responses against iRBCs (including IFN-γ production) but not antibody responses. However, the study results may have been confounded by residual Atovaquone-Proguanil detectable at the time of the final infection[170].

To date, four CHMI studies have prospectively characterised cytokine changes during an acute malaria infection[82,156,171,172]. The first involving 4 non-

immunised malaria-naïve adults showed progressive up-regulation of IFN-γ during blood-stage infection[171]. The second, examining changes in key cytokines in 18 UK malaria-naïve adults, showed different cytokines up-regulated at different time-points post-infection[82]. Initially, TGF-β levels increased, peaking 6-18 hours prior to the first detection of parasitaemia > 50 p/ml and then oscillating with a peak every 48 hours. This first wave of cytokine production was followed by a second wave in which IFN-γ, along with TNF-α, IL-6, IL-12 and IL-10 were all up-regulated 2-3 days after qPCR monitoring became positive for blood-stage parasites, with IFN-γ up-regulation observed approximately one day earlier than IL-12 or IL-10. However, marked heterogeneity was seen in subjects' cytokine responses, and not all individuals up-regulated IFN-γ production in response to infection. In the third study, which involved five Dutch malaria-naïve adults, IFN-γ was up-regulated 1-2 days prior to diagnosis and decreased sharply one-day post-treatment[156]. The final study of six UK malaria-naïve subjects showed a trend for an increase IFN-γ at diagnosis in comparison to baseline[172].

The magnitude of IFN-γ responses to malaria infection is determined by the degree of prior exposure. Adults in highly endemic regions produce markedly lower IFN-γ responses against iRBC than those in low-endemic regions or malaria-naïve volunteers[173] and plasma IFN-γ levels during clinical malaria episodes are relatively lower in semi-immune than in non-immune patients[174-176]. This paradoxical effect could be a protective mechanism, possibly mediated by Treg cells[101], to limit the pathological effects of the immune response to malaria, however, evidence for this is limited[136]. It is also postulated that suppression of IFN-γ and pro-inflammatory responses could be actively

employed by the malaria parasite to evade the immune system and promote its survival[136,177]. Of note, low-level responses to iRBC have been demonstrated in malaria-naïve individuals[178-180] and are thought to be as a result of innate responses, non-specific polycolonal responses to super antigens or cross-reactive responses by T cells primed by environmental antigens[181]. Whilst IFN-γ responses to individual blood-stage antigens are relatively short-lived or unstable, cellular responses to whole parasite antigen are more long-lived, practically undiminished at 14 months post-infection in previously naïve subjects[136].

When considering the role of IFN-γ in clinical and severe malaria, it is difficult to distinguish cause from effect, as although IFN-γ responses have been shown to correlate with symptomatic infection, the relationship between IFN-γ and manifestations of severe disease is much more complex and requires further research[136,182].

1.3.3.1.3 Type III interferons (IFN-λs)

There are three Type III interferons (IFN-λ): IFN-λ1, also called IL-29, IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). Type III IFNs are similar to Type I IFNs in their expression patterns and biological activities, however in contrast to Type I IFNs, expression of Type III IFNs can be induced by a broader spectrum of stimuli, which include diverse viruses and various TLR agonists[183,184].

The main cells to produce IFN-λ are DCs, monocytes, mast cells, and epithelial cells[185-187]. The IFN-λ receptor is only expressed by epithelial-like cells and some immune cells in the skin, respiratory tract and gastrointestinal tract and the

intact gene is not present in mice[188-190]. However, despite receptor expression, there remains controversy as to the responsiveness of immune cells to IFN-λs, and it may be that only certain small subpopulations of immune cells are responsive to IFN-λs in particular circumstances[191].

DCs are the main source of IFN-λs after specific stimulation of TLRs[192]. IFN-λs can act on many cells of both the innate and adaptive immune systems (Table 1-2)[193,194]. IFN-λs are known to have a key role in the immune response to viral infections, especially Hepatitis B and C[195,196]. They are also thought to have an important role in immunomodulation and play a significant role in autoimmune diseases such as systemic lupus erythematosis and asthma, as well as cancer[190,193]. However, there is no published literature regarding a role for IFN-λs in the immune response to malaria.

Subtype	Target cell	Effect and possible mechanism	Overall impression
λ1	pDC	increase the expression of the homing molecules CCR7 and CD62L; upregulate co-stimulatory molecules CD80 and ICOS-L, and reduce stimulation of IL-10, IL-13, and IFN-γ production from allogeneic T cells	contribute to pDC activity and prolong pDC survival
λ1, λ2	mDC (without LPS)	induce partial maturation of DCs; high levels of major MHC class I and MHC class II; express CCR7; low levels of costimulatory molecules, and retained phagocytic ability	induce partial maturation of mDCs
λ1, λ2	mDC	profoundly inhibit the generation of Th2 and Th17 responses; enhance Th1 polarization, and augment IL-12 secretion	promote mDC maturation
λ1	CD4+ T cell	alter the Th1/Th2 development of naive human T cells; diminish the development of Th2 cells and lower the secretion of IL-13, and induce CD3+ T cell apoptosis	inhibit Th2, promote Th1, and induce apoptosis
λ1, λ2	Treg	IL-2-dependent proliferation of CD4+CD25+Foxp3+ T cells	induce proliferation of Treg
λ3	CD8+ T cell	increase CTL killing activity, and enhance antigen-specific cytolytic degranulation	promote killing activity
λ1, λ2/3	NK	do not enhance NK cytotoxic activity and chemotaxis directly	no direct influence on NK
λ1	PBMC	elevate chemokines mRNA levels of CXCL9, CXCL10, and CXCL11, and upregulate IL-6, IL-8, and IL-10	activate PBMC
λ1	monocyte	upregulate IL-6, IL-8, and IL-10; change morphology, and more motile	activate monocyte
λ1	macrophage	upregulate IL-6, IL-8, and IL-10; increase TLR-induced IL-12p40 production, and upregulate IFN-γR1	activate macrophage
λ2/3	B cell	enhance TLR7 on B cells	unclear

Table 1-2: Effect and possible mechanisms of differing subtype of type III IFNs

pDC = plasmacytoid DCs, mDC = myeloid DCs, LPS = lipopolysaccharide. Adapted from Zheng et al[193].

1.3.3.1.4 Interferon regulatory factors (IRFs)

IRFs are transcription factors important for mediating IFN signaling. Of the nine IRFs, there is the most evidence for the importance of IRF-1, an activator of Type I IFNs, in the immune response to blood-stage *P. falciparum* infection[62]. In humans the IRF-1 gene is located in chromosome region 5q31-33. In one study, variation in this region accounted for approximately 45% of the variance in parasite density seen[197]. Polymorphisms in the IRF-1 gene have also been

associated with differences in parasite density and severity of disease[198]. Whilst one mouse study has shown a role for IRF-1 in limiting parasite growth[199], others have shown IRF-1 to be important in mediating the pathogenesis of cerebral malaria[200,201].

1.3.3.2 Neutrophils – innate effectors of the adaptive immune response

Neutrophils, or polymorphonuclear cells (PMN), are the most numerous blood leukocyte (50-75%) and the best circulatory phagocyte[202]. Neutrophils can destroy microbes in four major ways, mediated by antibody opsonisation: phagocytosis; release of granules; respiratory burst, and the production of neutrophil extracellular traps. Attracted to sites of inflammation by inflammatory signals, neutrophils have an important role in recruiting and activating other cells types including DCs, monocytes and NK cells. They can also activate the proliferation and maturation of T cells and B cells[203,204].

The role of neutrophils in NAI is debated. Opsonised merozoites can be phagocytosed by neutrophils *in vitro*[205,206] and *in vivo*[207] and neutrophil phagocytosis of free merozoites and iRBCs can inhibit parasite growth *in vitro*[206,208], however its relevance *in vivo* is unclear. Reactive oxygen species (ROS) are known to have a toxic effect on intra-erythrocytic *P. falciparum* parasites[209] and neutrophils are effective producers of ROS via respiratory burst[210]. Indeed, high ROS production by neutrophils has correlated with increased parasite clearance in Gabonese children with *P. falciparum*[210,211] and neutrophil antibody-dependent respiratory burst (ADRB) activity has been shown to correlate with clinical protection against malaria in Senegalese children[202].

1.4.2 Vaccine-induced immunity to *P. falciparum* infection

An effective malaria vaccine is widely viewed as a much-needed tool for reducing malaria risk in endemic areas[212]. The pre-erythrocytic *P. falciparum* vaccine, RTS,S, formed from fusion of the circumsporozoite (CS) protein to the surface-antigen of Hepatitis B, is the most advanced and efficacious malaria vaccine in development. However, it confers only limited relatively short-lived protection: in a large Phase 3 trial, vaccine efficacy for clinical malaria was 50% in older children[213] but only 30% in infants, the target population[214]. In addition, no significant protection against severe malaria was seen 18 months following a booster in older children[215,216]. Analysis of the immunological correlates of immunity induced by the RTS,S vaccine suggests that high levels of antibodies against CS correlate with protection, with a possible minor contribution from low levels of induced $CD4^+$ T cells[213,217,218]. Whilst these clinical results are the most effective to date, there remains a need to improve on this limited clinical efficacy[113].

Development of an effective malaria vaccine has been hampered by the parasite's complex lifecycle and extensive polymorphisms[219]. To date, most vaccine strategies in advanced development have focused on the pre-erythrocytic stage of infection and utilised immunological pathways not induced in NAI[220,221]. There are, however important arguments for pursing the development of a vaccine targeting the blood-stage of infection, including the ability to reduce mortality and morbidity whilst allowing on-going acquisition of NAI[222]. Recently, the conserved blood-stage antigen reticulocyte-binding protein homologue 5 (RH5) has emerged as an extremely promising target antigen[223]

and is currently being assessed in early-stage clinical trials[221].

Work is also underway to design a vaccine capable of interrupting transmission of malaria. Animal models have shown that antibodies targeting the sexual-stage of the parasite life cycle are capable of inhibiting parasite survival in the mosquito mid gut. So called transmission blocking vaccines (TBVs) could therefore play an important role in malaria elimination, however, clinical testing has been limited to date[224].

Greater understanding of the immune response to malaria and the mechanisms of NAI would greatly inform vaccine design. As well as identifying novel antigenic targets[111] and mechanisms of anti-parasitic pathways, understanding of the role of tolerising or regulatory pathways that control the inflammatory response to the parasite will be essential for the safe use of vaccines that seek to induce pro-inflammatory, anti-parasite effector mechanisms[113].

1.5 Methods of understanding naturally-acquired immunity to *P. falciparum* infection

Anti-malarial antibody responses can be assessed by a variety of laboratory assays. ELISA-based methods typically provide information on the magnitude of an antibody response measured against a recombinant antigen, whilst immunofluorescence assays (IFA) can confirm recognition of native parasite antigen[222]. *In vitro* assays have also been developed to attempt to measure the functional activity of antibodies against the parasite, as opposed to their titre or

magnitude as often determined by ELISA.

The development of any assay that could predict NAI would have extremely important applications, potentially reducing the cost and ethical risks associated with human clinical trials and helping to prioritize progression of the most promising vaccines that mimic NAI to clinical studies. Given the biological and immunological complexity of the *P. falciparum* blood-stage, it seems likely that more than one immunological response is capable of contributing to protection and therefore unlikely that a single assay will predict all mechanisms of blood-stage immunity[222].

It is thought that antibody responses to parasite antigens expressed on the surface of the iRBC and merozoites may be important for NAI[225].

1.5.1 Functional *in vitro* measures of NAI

1.5.1.1 Assessing anti-iRBC responses

1.5.1.1.1 Surface reactivity assay

Individuals in malaria-endemic regions have been shown to develop antibodies targeting variant surface antigens on iRBCs. Surface reactivity assays (SRA), which assess for such antibodies, have been developed[226,227] and studies have shown an association between these and clinical benefit[228-230]. For example, the reduction in complications associated with malaria infection seen in successive pregnancies is known to be mediated by antibodies binding to VAR2CSA, an antigen expressed on the surface of iRBCs[231].

Whilst some antibody responses to domain cassettes of PfEMP1 can provide

broad protection against disease phenotype[232-234], generally anti-iRBC humoral responses are strain-dependent and determined by local circulating strains[227]. SRA-type assays are therefore reliant on access to appropriate parasite isolates *in vitro* and are currently difficult to standardise across multiple cohorts.

1.5.1.2 Assessing anti-merozoite responses

1.5.1.2.1 Growth inhibition activity (GIA) assay

The most widely used and established functional assay, the assay of GIA[235], assesses the ability of diluted serum or purified IgG antibodies to inhibit the invasion and growth of *P. falciparum* parasites in human RBCs *in* vitro (anti-merozoite immunity), and has been historically used to down-select blood-stage vaccine candidates for further clinical development[236]. Importantly this assay measures only cell-independent parasite neutralisation by antibodies. Variations of this assay have been reported that distinguish between invasion inhibition alone versus invasion and/or intracellular growth inhibition combined, with antibodies against different antigens reportedly acting in a different manner[237].

There have been highly conflicting results from immuno-epidemiological studies investigating the relationship between *in vitro* GIA and clinical outcome, where associations with exposure rather than protection have been observed[236]. Considerable difference in assay methodologies and reporting between centres have likely contributed to the lack of consensus regarding the relationship between GIA and NAI[236].

Modern blood-stage vaccines can induce levels of GIA that exceed those seen in NAI[238]. GIA has been closely associated with protective outcome of MSP1 and

AMA1-based vaccine candidates in non-human primate challenge models[239,240]. However, to date in humans, only one small CHMI study has directly assessed this relationship in the context of AMA1 vaccination, and encouragingly the data indicated a possible association between *in vitro* GIA and *in vivo* parasite multiplication rates (PMR) in vaccinees[241]. Overall, however, this vaccine showed no significant efficacy in the vaccinees in comparison to controls, making interpretation of this result difficult at best, and consequently further studies are urgently required.

The establishment of an international reference laboratory at NIH-NIAID to perform standardized assays of GIA[235] has significantly improved the ability of investigators and funders to obtain robust and comparable data[242]. The version of the assay routinely employed at this centre uses purified IgG (avoiding the potential for non-specific effects due to serum constituents, and permitting testing at a wide range of supra- and sub-physiological concentrations); a single complete parasite lifecycle (permitting detection of effects of vaccines upon both invasion and intracellular growth) and a simple colorimetric readout. This assay has been carefully demonstrated to give reproducible results both within and between centres[242,243].

1.5.1.2.2 Antibody-dependent cellular inhibition (ADCI) assay

An assay measuring antibody-dependent cellular inhibition (ADCI) was first described in the late 1980s and measures the antibody-dependent cellular activity of monocytes against *P. falciparum* parasites *in vitro*[244]. This assay was developed from the observation that IgG that failed to demonstrate GIA *in vitro* could demonstrate strain transcending parasite growth inhibition when

monocytes were added[244,245]. ADCI activity relies on IgG recognition of antigen and subsequent engagement of antibody Fc receptors to induce the release of monocyte soluble factors such as TNF-α[150]. Data were used to design an ADCI-inducing vaccine candidate[246,247], but unfortunately the vaccine has had limited success reported to date. Other groups have failed to reproduce the ADCI assay reliably, such that this assay has not been widely adopted[222].

1.5.1.2.3 Antibody-dependent respiratory burst (ADRB) assay

The antibody-dependent respiratory burst (ADRB) assay assesses the ability of antibody to opsonise merozoites and induce the release of ROS from polymorphonuclear neutrophils *in vitro.* Oxidative burst is measured by means of a luminol based chemiluminescent reaction[202] and a standardised assay has now been established[119,225,248]. One field study has shown an association between ADRB and protection against malaria[202] whilst another has shown that malaria-exposed individuals induce higher ADRB activity than malaria-naïve individuals[225]. Whilst promising, these results have yet to be repeated and the assay has not been applied to vaccine studies.

1.5.1.2.4 Phagocytosis assays

The most widely used phagocytosis assay assesses the ability of THP-1 monocytic cell line to phagocytosis merozoites opsonized with antibody using flow cytometry[249]. Using this assay, individuals with NAI have higher rates of phagocytosis compared to non-exposed controls, and this measure has been associated with reduced risk of clinical malaria[250,251]. Other phagocytic assays have been developed but have little or no published data relating to NAI[144,252].

1.5.2 Transcriptomics

Transcriptomics, which allows the expression of thousands of genes to be assessed in parallel for a single RNA sample, is an exciting, expanding area of research with vast potential application in the field of infection[253]. Facilitating a systems biology approach, gene expression data from high-throughput technologies such as microarrays can allow greater understanding of individuals' immune response to infection. To date, expression data has been used to dissect mechanisms of vaccine efficacy[254], inform the design of new vaccines[255,256], predict response to infection and outcome[257], characterise and improve understanding of sepsis[258] and offer a novel approach to the diagnosis of infectious pathogens[259-261].

1.5.2.1 Use of transcriptomics to study clinical P. falciparum infection

In the case of *P. falciparum* infection, it is clear that differences in individuals' immune response to infection determine the clinical outcome[1]. Given the limited understanding of the mechanisms of NAI from traditional immunological studies, a systems approach characterising the gene expression patterns associated with NAI could provide novel and valuable insights[262,263].

Thirteen datasets of transcriptomic data from clinical samples following *P. falciparum* infection are listed on Gene Expression Omnibus (GEO - a public functional genomics data repository supporting MIAME-compliant data submissions) (Table 1-3 A and B). Only 7 (54%) of these have published results and include data from both field and CHMI settings. Whilst significant differences in the datasets in terms of experimental design, microarray platform, cell

population sampling, statistical basis for selection of differentially expressed genes and gene clustering methods make comparisons of results difficult, the reported findings provide key insights into the immunological response against malaria, and have important potential applications.

In both experimental and 'natural' infection, antigen presentation, TLR and interferon signaling pathways have repeatedly been shown to be important[61,83,84]. A gene expression profile has been characterised that can distinguish Kenyan children presenting unwell with acute malaria from other pathogens[264]. One study has shown differentially expressed genes between mild and severe malaria relating to the Type I IFN signaling pathways, T cell activation and immune activation[132], whilst another reports differences in genes regulating induction of apoptosis through MAP kinases and IL-1β between early pre-symptomatic infection seen in a CHMI study and natural infection in the field[84]. Analysis of genotype-by-infection interactions in children with mild malaria implicated the complement system, antigen processing and presentation and T cell activation, highlighting the importance of an individual's genes in determining the transcriptomic changes seen post-infection[265]. Finally, in a recent study, examination of the gene expression of $Vd2^+$ T cells from children with high and low prior malaria exposure supported the finding that repeated malaria infection during childhood results in progressive loss and dysfunction of $Vd2^+$ γδ T cells that may help explain immunological tolerance to the parasite[100]. With the exception of the last study, no published work has examined the impact of prior exposure to *P. falciparum* on gene expression.

1.5.2.2 Use of transcriptomics to study clinical P. vivax infection

Recently published work has compared the transcription profiles over time of semi-immune and malaria-naïve individuals post-mosquito-bite *P.vivax* CHMI[266] using RT-PCR of 9 axes of blood informative transcripts[267] and RNA-seq. Contrasting profiles at baseline with early blood-stage infection showed no evidence for transcriptional changes prior to C+12 or C+13, at which time there was a strong IFN response and, unexpectedly, down-regulation of transcripts related to inflammation. A reduction in T cell signaling and an increase in erythropoiesis was also seen at diagnosis. Despite differences in clinical symptoms between the semi-immune and malaria naïve individuals, only subtle differences in their transcriptomes were observed. Greater changes in gene expression were seen in malaria-naïve at diagnosis, possibly suggesting resistance to disease in exposed individuals was mediated by tolerance.

Series Code	Title of Dataset	Key Finding In Relation To Immune Response to Malaria	'Challenge'	Sampling Time points	Tissue	Age
GSE15221	Malaria primes the innate immune response due to IFN-ϒ induced enhancement of TLR expression and function[61, 83]	Up regulation of TLR signaling, IFN-γ, antigen processing and presentation and cytokine-cytokine receptor interaction. Large % up-regulated genes had sites for IFN-inducible transcription factors.	Natural	(A) Diagnosis acute febrile malaria (B) 3-4 weeks post treatment (paired samples)	PBMC	Adults
GSE18323	Expression data from a malaria vaccine trial (RTS,S)[268]	Differential expression pre-CHMI of genes in the immunoproteasome pathway that impinge on the efficiency of MHC class 1 processing and antigen presentation, distinguish subjects protected by the vaccine.	CHMI - Mosquito bite	(A) Study Entry (B) Pre 3rd Vaccination (3V) (C) 24hr post 3V (D) 72hr post TV (E) 14 days post TV (F) 5 days post CHMI	PBMC	Adults
GSE2900	Host Response Malaria[264]	A neutrophil-related gene region (containing genes predicted to encode mediators of innate and adaptive immunity and neutrophil activation) distinguished convalescent from acutely ill subjects. 22 genes were associated with parasitaemia.	Natural	(A) Acute presentation with febrile illness (n=22; 16 = *P. falciparum*) (B) 2 weeks post discharge (n=6)	PG	Childre
GSE33811	Paired whole blood human transcription profiles from children with severe malaria and mild malaria[132]	Mild malaria associated with differential expression of 68 genes and enrichment of Type I IFN mediated signaling pathway, T cell activation and immune activation. Severe malaria associated with 6 genes including thymidine kinase 1 and carbonic anhydrase.	Natural	(A) Diagnosis severe malaria (B) 1 month later when diagnosed with mild malaria (paired samples)	TR	Childre
GSE34404	The genomic architecture of host whole blood transcriptional response to malaria infection[265]	Genotype-by-infection interactions demonstrated implicating the complement system, antigen processing and presentation, and T cell activation.	Natural	(A) Mild acute malaria (B) Unmatched Control	Tempus	Childre
GSE50957*	Molecular Hallmarks of Experimentally Acquired Immunity to Malaria (Chloroquine Prophylaxis)	Unpublished	CHMI - Mosquito bite	(A) Pre CHMI (B) Post CHMI - paired samples	N/K	Adults
GSE52166*	Molecular Hallmarks of Naturally Acquired Immunity to Malaria	Unpublished	Natural	(A) Pre infection (B) Post Infection	N/K	N/K

Table 1-3 (A): Transcriptomic datasets listed on Gene Expression Omnibus

*Shaded rows = malaria vaccines. * = Datasets using high throughput sequencing, others used arrays. NK = Natural K*
Blood; PG = WB collected in PaxGene tube; TR = WB collected in Tri-Reagent (BD); Tempus = WB collected in Tempu
(Life Technologies); PBMC = Peripheral blood mononuclear cells; TLR = Toll like Receptor; N/K = Not known.

for clinical samples relating to P. falciparum

Series Code	Title of Dataset	Key Finding In Relation To Immune Response to Malaria	'Challenge'	Sampling Time points	Tissue	Age
GSE5418	Gene Expression Analysis in Malaria Infection[84]	Differences were seen between early pre-symptomatic infection (CHMI) and natural infection in genes regulating induction of apoptosis through MAP kinases and IL-1B. TLR signaling through NF-KB, genes associated with phagocytosis and inflammation were up-regulated in both groups	Natural & CHMI – Mosquito bite	CHMI: (A) Pre infection (B) Diagnosis. Natural: (A) Diagnosis	PBMC	Adult
GSE5543	Loss and dysfunction of Vdelta2+ γδ-low T cells is associated with clinical tolerance to malaria[100]	Comparison of gene expression of sorted, un stimulated $Vd2^{+}$ T cells from children with high (n = 4) and low (n = 4) prior malaria incidence identified 48 differentially expressed genes, many with known roles in immunomodulation. For each gene expression was higher in subjects with high prior exposure.	Repeated Natural infection	Sampling at age 4 or 5 (no relation to infection)	Vdelta2+ γδ T cells	Childre
GSE2491	Expression profile of human natural killer cells co-cultured with P. falciparum infected erythrocytes	Unpublished	*In-vitro* incubation NK cells with iRBC	(A) NK incubated with iRBC (B) and without	NK cells	Adult
GSE6493	FCRL5 delineates functionally impaired memory B cells associated with malaria exposure	Unpublished	Natural	N/K	PBMC	Childre
GSE6338	Expression data from whole blood samples of Rwandan adults with mild malaria with matched sample thirty days later (convalescence)	Unpublished	Natural	(A) Diagnosis Mild malaria (B) 30 days later.	TR	Adults

Table 1-3 (B): Transcriptomic datasets listed on Gene Expression Omnibus for clinical samples relating to P. falciparum continued

All datasets used arrays. NK = Natural Killer; WB = Whole Blood; PG = WB collected in PaxGene tube; TR = WB collected in Tri-Reagent (BD); PBMC = Peripheral Blood Mononuclear Cells; TLR = Toll like Receptor; MAP: mitogen-activated protein; N/K = Not Known.

1.6 Models for investigating immune responses to *P. falciparum* infection

1.6.1 Animal models

Non-human *Plasmodium* species, particularly mouse models, have been essential in the study of immune responses to malaria and assessment of candidate vaccines. There are 4 rodent malaria species, *P. berghei*, *P. chabaudi*, *P. vinckei*, and *P. yoelii*, 13 subspecies, and various strains and cloned lines[269]. Depending on the host and parasite combinations, different disease profiles, such as cerebral malaria, can be induced. Together with genetically deficient mice, these models allow in-depth study of experimental infection and greater understanding of the mechanisms underlying NAI and immunopathology, in particular by allowing access to organs and tissues usually inaccessible in clinical trials[62,270].

However, there is currently no established, validated animal model of malaria infection. Fundamental differences in immunological processes limit how far findings from animal models can be extrapolated to humans, for example, mouse models have not always accurately predicted vaccine efficacy in clinical trials[270].

1.6.2 Field studies

The gold standard for assessing immune responses in humans has been sampling from field studies undertaken as part of immunoepidemiological investigations or assessment of the efficacy of candidate vaccines. These studies allow study of variables in relation to the most clinically relevant endpoint: protection against

malaria disease in the field. However, such trials are often costly, can take years to perform and rely on enrolment of large numbers of individuals, a problem set to increase given declining transmission rates[1]. In addition, it is extremely difficult to study immune responses to the pre-erythrocytic and early blood-stage of the lifecycle as infection in the field is sporadic and unpredictable[222].

1.6.3 Controlled human malaria infection studies

The deliberate infection of human volunteers with micro-organisms ('challenge studies') have contributed uniquely to our understanding of the pathogenesis, immune responses and the treatment and prevention of numerous microbial diseases including influenza, cholera, typhoid and hepatitis[271,272]. Microbial challenge studies are recognised as essential for providing proof of concept for therapeutic interventions and can significantly accelerate progress to Phase III studies[271].

P. falciparum is a microbe particularly well suited to challenge studies. It has a relatively short asymptomatic period, a well-established diagnostic laboratory test (thick film microscopy), and no long term sequelae or infectious state following appropriate and timely treatment. More recently CHMI studies using *P. vivax* have also been performed[273-275], however difficulty culturing this parasite *in vitro* has considerably limited CHMI trials to date.

Deliberate infection of humans with *P. falciparum* was first performed in 1917 primarily as a therapy for patients with neurosyphilis[276]. Thousands of patients underwent the treatment (the objective of which was to induce a febrile illness that was thought beneficial for the progress of the disease) administered by the

bites of infectious mosquitoes or by intravenous (IV) or subcutaneous (SC) inoculation of dissected *Plasmodium* sporozoites suspended in media. The practice stopped with the advent of antibiotics. In the 1960s, CHMI trials were used to assess the effects of anti-malaria treatments on healthy non-immune male inmates in the United States[277]. Following the development of protocols for the continuous culture of *P. falciparum* in 1976[278], and for the generation of mature *P. falciparum* gametocytes *in vitro* in 1981[279], it became possible to produce laboratory-reared infectious mosquitoes[280], meaning that CHMI trials could be performed more routinely and without having to infect mosquitoes by feeding on infected volunteers.

The first well-documented CHMI with laboratory-reared infectious mosquitoes fed on *P. falciparum* culture-produced gametocytes was performed in 1986 in the US. Six volunteers were infected with *P. falciparum* sporozoites by the bites of infectious *A. freeborni* or *A.* mosquitoes[281]. The following year, the efficacies of the first recombinant protein and synthetic peptide *P. falciparum* vaccines were tested in experimentally infected volunteers[282,283]. Since the late 1980s, the number of institutions performing CHMI with *P. falciparum* has grown. In 2007, data were published from a total of 532 volunteers[281,284]. So far, unpublished analysis shows that 1,343 volunteers were experimentally infected with *P. falciparum* between 1985 and 2009[285] and the figure today is likely to be considerably higher.

In modern CHMI studies, volunteers are infected with *P. falciparum* under carefully controlled conditions and treated (in the majority of cases) when thick blood-film positive by microscopy (the clinical gold standard for diagnosis)[286-

[288]. As CHMI trials are performed in a controlled environment, they allow unprecedented detailed evaluation of parasite growth and immunological responses, providing essential information for vaccine and drug development[287]. As a result, the CHMI model has become established as a routine investigation that is the most rapid and relatively cost-effective method of assessing vaccine and drug efficacy *in vivo* without a field trial[287], however to date the CHMI model has not been used to assess the dynamics and mechanisms of NAI[222].

In the great majority of Phase IIa CHMI studies performed to date, group sizes have been in single figures reflecting both the practical and ethical limitations on the numbers of individuals that can be recruited and challenged safely. For this reason, small CHMI studies usually seek to assess proof-of-concept in the first instance, with a view to validating observed efficacy results in repeat studies or progressing to a field study if an encouraging signal is seen. Nevertheless, given all vaccinees are exposed to infection, and all non-vaccinees almost without exception become infected, CHMI studies can provide sufficient statistical power both to detect and to rule out meaningful vaccine effects using small sample sizes (often twelve or fewer volunteers per group)[289]. Indeed "*large-scale malaria vaccine trials in the absence of evidence of efficacy in studies of controlled human infection would now be thought unethical by many*"[290].

1.6.2.1 Controlled human malaria infection studies in malaria exposed populations

Though commonly performed in malaria-naïve populations[287], CHMI trials have rarely been conducted in malaria-endemic regions[222]. In 1957 in Lagos, 22 adults with prior exposure to malaria were challenged with "*an intravenous injection of*

blood heavily parasitized with P. falciparum"[291,292]. Whilst infection was successfully induced in 11 volunteers, only 3 of these individuals demonstrated any clinical symptoms, and these "*disappeared within two to three days without any specific treatment*." Examination of parasite load measured by blood film from day of CHMI for up to 8 weeks clearly showed individuals controlling and in some cases, clearing parasitaemia.

In a study published in 1954, conducted in Nairobi, 30 African adults were challenged with *P. falciparum* malaria administered by either injection of blood infected with trophozoites or mosquito-bite[49]. The study clearly demonstrated a protective effect of sickle cell trait in the 15 individuals with this condition. A further study involving the '*inoculation of semi-immune Africans with P. falciparum'* was also conducted in Liberia in the 1960s[293].

In fact, since the advent of the modern era of CHMI trials in which mosquitoes are fed on cultures containing *P. falciparum* gametocytes[281], there have not been any studies of mosquito-bite induced CHMI in Africa and the CHMI model has not been used to examine the kinetics or mechanisms of NAI[222] in *P. falciparum* infection. One recent *P. vivax* mosquito-bite CHMI study Columbian volunteers showed no difference in time to diagnosis between malaria-naïve and semi-immune volunteers [273]. However, whilst malaria-naïve volunteers in this study developed classical malaria signs and symptoms, semi-immune volunteers displayed minor or no symptoms at the day of diagnosis.

1.6.2.2 Methods of inducing *P. falciparum* infection for CHMI Trials

Currently, there are three methods of inducing experimental human malaria infections: (i) injection of blood-stage parasite via iRBCs; (ii) administration of sporozoites by mosquito-bite; or (iii) injection of sporozoites by needle and syringe (Table 1-4).

	Sporozoite – Mosquito bite	**Sporozoite – Injection**	**Blood-Stage (iRBC)**
Mimicking natural route of infection	Yes	No	No
Ability to control inoculum size	+/-	++	+++
Life-cycle stages amenable to study	Sporozoite (skin and blood) Liver-Stage Blood-Stage	Sporozoite (blood) Liver-Stage Blood-Stage	Blood-Stage
Availability	Limited trial centres	Potential for widespread use	Potential for widespread use
Duration of blood-stage parasite exposure	Short (2-4 days)	Variable (2-8 days)	Longer (8-9 days)
Reliably achieves 100% infection	+++	++	+++

Table 1-4: Characteristics of various methods of inducing experimental human malaria infection in controlled human malaria infection studies

Adapted from Sheehy et al[222].

1.6.2.2.1 Experimental blood-stage infection

One method of performing CHMI trials is to administer intravenous (IV) iRBCs from a cryopreserved inoculum originally obtained from an infected parasitaemic donor[169,286,288,294]. Since the liver-stage of infection is circumvented, this method can only be used to assess blood-stage immunity, and is particularly

suited to this application. The thawed inoculum is prepared and administered, and a subsequent limiting dilution parasite viability assay is performed to ascertain the infectious dose. Consequently, a very low dose of parasites (30-6000 parasites in studies to date)[286] can be uniformly administered to all volunteers, allowing accurate modelling of parasite growth[285-287,295]. Importantly, the time-frame of blood-stage parasite exposure prior to thick-film microscopy diagnosis is 8-9 days (unlike 2-4 days following mosquito-bite CHMI), and infection is initiated by much lower parasite densities – providing improved conditions to assess partial blood-stage immunity. For vaccines demonstrating efficacy in a CHMI trial initiated by sporozoites, the blood-stage CHMI model can also provide a useful method of confirming the life-cycle stage specificity of the protective response[296]. However, one potential confounder of the model is that there is no possibility of detecting a protective antibody response against the merosomes released from the liver[285,297], although no data exist to argue whether such a factor would be an important contributor to anti-merozoite vaccine efficacy or component of NAI. To date only approximately 230 volunteers have taken part in blood-stage CHMI trials[288] (unlike >1,300 for mosquito bite challenge)[287], and only three vaccine candidates have undergone efficacy testing in this manner (*Payne et al. Manuscript in preparation*)[241,298].

Several factors have limited the widespread use of blood-stage CHMI studies. Firstly, for many years the sole source of the inoculum came from a study performed at the Queensland Institute for Medical Research (QIMR) in 1994[169,294]. Two malaria-naïve individuals were infected with *P. falciparum* (3D7 clone) by the bite of laboratory-reared *A stephensi* mosquitoes. 13-14 days later,

when pyrexial and blood-film positive, 500 mL of blood was collected from each volunteer and iRBCs cryopreserved at known parasite concentrations. Both individuals were seropositive for Epstein-Barr virus (EBV) and cytomegalovirus (CMV)[294]. As a result, participants in some blood-stage CHMI studies have been restricted to those that are seropositive for EBV and CMV, considerably limiting recruitment and leading to small and sub-optimal group sizes[241,295]. This, in combination with volunteer and regulatory body concerns regarding the potential risks of administering a blood product to healthy volunteers has considerably restricted application of the model, meaning that blood-stage CHMI trials have not been performed in the US[286].

However, to date, three centres (QIMR, Radboud University Nijmegen Medical Centre (RUNMC) and the University of Oxford) have performed blood-stage CHMI studies in healthy volunteers sero-negative for EBV & CMV, with no evidence of seroconversion post-CHMI (*personal communication R. Sauerwein*)[288,296]. As a result, other national regulatory bodies, including the UK have removed EBV/CMV seropositivity as eligibility criteria, increasing the likelihood of an increasing number of blood-stage CHMI trials in the future.

The recent production of NF54, 3D7 and 7G8 *P. falciparum* parasites to good manufacturing practice (GMP) grade standards should also make blood-stage CHMI studies easier to perform as well as allowing different parasite strains to be used[299].

1.6.2.2.2 Experimental sporozoite infection – mosquito-bite

The delivery of sporozoite-stage malaria parasites by mosquito-bite has traditionally been used as a model to test pre-erythrocytic stage vaccines[287]. Such infections are currently routinely carried out at a number of centres in Europe and the US[285]. All centres use *A. stephensi* mosquitoes that feed on either the chloroquine-sensitive NF54 strain of *P. falciparum* or the 3D7 clone of NF54. In addition, limited numbers of volunteers have been challenged with the 7G8 and NF135.C10 strains of *P. falciparum*[300,301].

Approximately 14–21 days after feeding, mosquitoes are checked for infection by microscopic examination of salivary glands. Volunteers are subsequently exposed to the bites of five infectious mosquitoes for either 5 or 10 minutes. Almost 100% of volunteers bitten by five infected mosquitoes develop patent parasitaemia, with rare exceptions[284,302,303]. Importantly, trials using the pre-erythrocytic malaria vaccine RTS,S showed efficacy estimates to be comparable when measured by mosquito-bite CHMI (number of participants sterilely protected) or field studies (number of cases of clinical malaria prevented)[213,304].

While mosquito-bite CHMI studies have the benefit of representing the natural route of infection and an established protocol that reliably infects 100% of volunteers[287], some have questioned its utility in the assessment of blood-stage immunity[285,289,298]. Primarily, this model only provides a small window of opportunity in which to observe a protective impact of immune responses on blood-stage *P. falciparum* parasites. Typically following five infectious mosquito-bites, volunteers display parasitaemia detectable by nucleic acid test (NAT) in the first blood-stage parasite growth cycle (peaking on day 7-8 post-CHMI). Among unvaccinated infectivity controls, a 25-fold variation between trials in the

median level of parasitaemia in this first wave (range of medians 30-750) has been observed, and 75-fold variation among the controls in a single trial (range 27-2000)[305]. Such levels may be dependent on a number of variables (CHMI centre, *P. falciparum* parasite strain (NF54 versus 3D7), intensity of mosquito infection, mosquito feeding exposure time), but all lead to diagnosis by thick film microscopy within a median time frame of 2-4 days[305]. It is impossible to observe potential blood-stage immunity at levels of blood-stage parasitaemia that exceed the typical peak levels observed in CHMI studies (approximately 8,000-16,000 p/mL). Such a window of opportunity may thus be potentially too short for protective immune mechanisms to have a significant or measurable impact.

Secondly, it has been noted that CHMI trials are unlikely to fully replicate field conditions where prior exposure to malaria and other infections or immunisations may impact on results[305,306]. Whilst it has been reported in malaria-endemic regions that an individual may be subjected to 35-90 mosquito bites a night (where 10% of mosquitos may be infected with *P. falciparum)*, the administration of bites from five heavily infected mosquitos almost instantaneously in the CHMI model is considered unnatural and is likely to represent a stringent test of immune responses[305]. However, five mosquito bites continue to be used as a standard infectious dose across centres in order to ensure infection of all volunteers, and this factor likely accounts for the large variability in the infectious dose mosquitoes administer[287,305].

A further benefit of the sporozoite CHMI model is the ability to assess protective immunity against the pre-erythrocytic- and blood-stages of the *P. falciparum*

lifecycle. This can be especially useful given that the traditional 'blood-stage' antigens such as AMA1 and MSP1 are essentially multi-stage antigens, expressed in the sporozoite[307] and late liver-stages[308] as well as on the invasive blood-stage merozoite. For example, in a recent mosquito-bite sporozoite CHMI study assessing the efficacy of viral vectored vaccines encoding AMA1 and MSP1, modelling of parasite dynamics post-CHMI was able to demonstrate that delay in time to diagnosis seen in some vaccinees was likely attributable to a vaccine effect at the pre-erythrocytic rather than blood-stage of the parasite life-cycle[309].

1.6.2.2.3 Experimental sporozoite infection – injection

To date, the need for an insectary and skilled entomology staff have precluded the conduct of mosquito-bite CHMI trials outside Europe and the US[287]. However, the recent production of aseptic cryopreserved *P. falciparum* sporozoites (NF54) for CHMI via injection by the biotechnology company Sanaria Inc offers a practical alternative for the conduct of sporozoite CHMI trials, particularly in malaria-endemic countries[310].

The salivary glands of aseptic *A. stephensi* mosquitoes infected with *P. falciparum* sporozoites are removed by dissection and triturated to release the sporozoites. The sporozoites are purified, counted and cryopreserved at a specified concentration to produce the challenge inoculum: PfSPZ Challenge. PfSPZ Challenge, which is stored and transferred in liquid nitrogen vapour phase (LNVP), allows the administration of a known concentration of sporozoites simply by needle and syringe.

As well as reducing variability in infectious dose between trials, PfSPZ Challenge could be a key product for assessing blood-stage immunity in particular. If PfSPZ Challenge[311] can be administered in a regimen that leads to a lower burden of liver infection than that seen in mosquito-bite CHMI studies, the resulting longer interval between infection and microscope-patent parasitaemia could allow for more subtle signals of partial blood-stage immunity to be detected[285].

The first CHMI trial using aseptic cryopreserved *P. falciparum* sporozoites administered by needle injection was performed by RUNMC in 2010/11[311]. This was a dose finding study in malaria-naïve volunteers to establish which dose of PfSPZ Challenge administered intradermally (ID) would infect 100% of inoculated volunteers. In all 3 groups (2,500, 10,000 or 25,000 sporozoites ID, 6 volunteers in each group), 5 out of 6 inoculated volunteers were successfully infected with *P. falciparum* (an infectivity rate of 83%) that was independent of PfSPZ Challenge dose. The first positive blood slides were found between day 11 to 14.3 after administration of PfSPZ Challenge. The geometric mean pre-patent period was similar for all dose groups (i.e. 13.0, 12.7, and 13.0 days for the groups receiving 2,500, 10,000, and 25,000 PfSPZ respectively). Comparison with data from this site's CHMI studies using mosquito-bites showed that the pre-patent period was longer and PMRs higher with PfSPZ Challenge compared to CHMI administered by mosquito-bite (*R. Sauerwein personal communication*)[305]. Parasite densities at the time of diagnosis were not significantly different among the groups (12.4, 11.2, and 23.4 parasites/μL in the three groups respectively) and volunteers developed symptoms of *P. falciparum*

infection that were similar to those seen following CHMI administered by mosquito bite at this centre[305].

It is important that any CHMI model reliably infects 100% of control volunteers. Why 100% infectivity was not achieved is this study is unclear. Murine data using *in vivo* imaging techniques have shown that the route of administration of sporozoites is a key determinant of infectivity and clearly demonstrated that IV administration was associated with the greatest infectivity. In turn, intramuscular (IM) administration was associated with greater infectivity than subcutaneous (SC) or ID injection. Murine studies have also shown that reducing the volume of injection and increasing the number of injection sites for a given number of sporozoites is associated with increased infectivity rates[312]. As a result, numerous other CHMI studies[313-315], including those outlined in this thesis[316,317], have been performed in US, European and African sites assessing the effect of varying dose, route and volume of administration of PfSPZ Challenge on infectivity in humans. As a result, administration of PfSPZ Challenge IV by direct venous inoculation (DVI) in 0.5ml has been adopted as the preferred dose and route to be used for future PfSPZ Challenge CHMI trials.[313,315]

1.6.2.3 Conduct of CHMI trials

Following a collaborative process involving international investigators, a consensus document; *"Standardization of Design and Conduct of P. falciparum Sporozoite Challenge Trials"* was developed, and provides a comprehensive guide to the appropriate conduct of CHMI studies[318]. Although there remain minor differences between centres in follow-up procedures post-infection, there is consensus on key points regarding end-points for treatment, grading of adverse

events (AEs), frequency of blood sampling and clinical review and safety monitoring.

For any clinical trial, the risk for potential volunteers should be weighed against the benefit. As often no direct benefit accrues to CHMI trial participants, benefits are considered in the context of possible public health gains that may occur as a result of scientific advances made through CHMI studies. This places a burden on the CHMI trial investigators both to exercise all possible safeguards for volunteer safety (primary consideration) and to ensure that maximal scientific benefit accrues from each CHMI trial (secondary consideration). Key ethical considerations agreed by consensus of the field include[319];

(i) Safety is the paramount consideration in conduct of CHMI trials. When CHMI trials are conducted at existing and new centres, the practical considerations should always be focused on volunteer safety.

(ii) Investigators are required to follow both international and local guidelines with respect to ethical considerations and work in accordance with the Declaration of Helsinki.

(iii) CHMI trials should be conducted according to Good Clinical Practice (GCP) Guidelines. It is important that the results of CHMI trials enable comparative evaluation and collect as much information as is reasonably possible. The raw data (both microscopy and qPCR where available) should be made publicly available to facilitate scientific benefit to the community.

(iv) If an unexpected serious AE (SAE) which is possibly related to CHMI occurs at a trial centre, every effort should be made to communicate information on this SAE to the community of CHMI trial centres within 90 days of the occurrence of the SAE. This is because safety of participants at other centres may be affected by occurrence of an SAE at one centre.

1.6.2.4 Clinical presentation post-CHMI

To date, the vast majority of data regarding AEs in CHMI studies is derived from malaria-naïve individuals. Nearly all volunteers in CHMI studies develop symptoms of clinical malaria infection. Approximately one-fifth of volunteers temporarily develop symptoms graded as severe (symptoms that prevent daily activities), but severe (as classified by the WHO) or life-threatening malaria has never occurred[284]. The most common symptoms are fatigue and headache, and severe symptoms can include headache, fatigue, malaise, chills, myalgia, rigors, nausea and vomiting. Clinical symptoms generally coincide with the detection of blood-stage parasites at densities of 10–20 parasites per μl of blood by microscopy of thick film microscopy smears[284]. This corresponds to a parasitaemia of approximately 0.0004%. Severe malaria is generally diagnosed when parasitaemia is 3 to 4 logs greater than the peak parasitaemia in CHMI trials. After the start of malaria treatment, symptoms can temporarily increase in severity but subside quickly with an average duration of approximately 2–3 days[287].

Routine laboratory checks generally show a moderate decrease in leukocyte and platelet numbers during infection, with no change in haemoglobin

concentration[302]. Bleeding or thrombogenic complications have never been described[284,302]. Abnormalities of liver enzymes have been observed, but these abnormalities did not result in clinical manifestations and they resolved after a few days[284,302]. Immediate treatment of volunteers at the earliest phase of microscopically detectable blood-stage infection ensures that the potential risks of complications associated with severe malaria are minimised to the greatest extent possible. Indeed, CHMIs have been shown to be safe in published data for 1,343 volunteers[284,300,302].

In 2008 safety concerns were raised following a cardiac event in a 20 year old volunteer participating in a malarial vaccine and CHMI trial in the Netherlands. The individual received a recombinant protein vaccine (PfLSA-3) adjuvanted with aluminum hydroxide followed several months later by CHMI administered by mosquito bite (NF54). Shortly after completing treatment for malaria with artemether/lumefantrine the individual was hospitalised with retrosternal chest pain and a diagnosis of acute coronary syndrome. The subject made a full recovery and was discharged with no long-term sequelae. A definite relationship between the cardiac event and the experimental malaria infection, vaccinations or anti-malarial medication was not established[320].

In 2013 a further cardiac event was reported in a malarial vaccine and CHMI trial at the same centre in the Netherlands. A 23 year old healthy volunteer who had received PfSPZ-CVac (PfSPZ Challenge (un-irradiated *P. falciparum* sporozoites with chloroquine chemoprophylaxis) followed by CHMI by mosquito-bite (NF54) 60 days after the last day of vaccination, developed elevated cardiac troponins one day after commencing treatment for malaria with Atovaquone/Proguanil.

Cardiac MRI demonstrated myocarditis. A throat smear was positive for rhinovirus and it is possible this was the cause of the myocarditis, since myocarditis has previously only been seen rarely in malaria in the context of high parasitaemias[321-327]. The volunteer recovered uneventfully. Given the temporal relationship to the malaria vaccine, CHMI and anti-malarial medication it was deemed possibly related to study activities[328].

In light of these two cases it has been generally agreed that volunteers with an increased risk of cardiac disease (for example; positive family history, abnormal electrocardiogram (ECG) at screening, hypertension) should be excluded from participating in CHMI studies[319].

1.6.2.5 Outcome measures in CHMI studies

1.6.2.5.1. Microscopy

Post-CHMI, volunteers are diagnosed and treated when thick blood-film positive at most trial centres, meaning microscopy is a key outcome in Phase IIa trials[318]. Whilst most microscopists have considerable experience reading blood-films at the high parasite densities typically seen in symptomatic patients in the field, most lack experience reliably identifying the extremely low level parasitaemias seen in CHMI studies. This is likely to contribute to the considerable variability in parasitaemia at diagnosis (geometric mean 8,000-16,000 p/mL)[305], and is an understandable consequence given the significant role of chance in finding parasites at such low parasitaemias. Conversely, since a number of trial centres process NAT samples retrospectively, a false positive blood-film can lead to the incorrect and early treatment of a volunteer. The 'Consensus SOP for Malaria

Microscopy in the Context of Clinical Challenge Trials'[318] has been helpful to aid standardisation across trial centres, however the lack of a definition of an 'unambiguous' parasite still allows for subjective interpretation of blood-films and variability between microscopists. Whilst the need for two parasites to be identified on a blood-film or 'real-time' contemporaneous processing of NAT samples reduces the likelihood of false positive blood-film results[318], standardised training specifically in the identification of low patent parasitaemia would greatly assist the field.

1.6.2.5.2. Nucleic acid tests

Post-CHMI, highly sensitive NATs are usually performed 1-3 times daily until point of diagnosis (typically 2-3 blood-stage growth cycles following mosquito-bite sporozoite CHMI) in order to detect and quantify pre-microscopy patent blood-stage parasitaemia[318]. The most common nucleic acid target is the 18S rRNA-coding genes (rDNA) or the asexual-type 18S rRNAs themselves, which can be detected by nested polymerase chain reaction (PCR), quantitative PCR (qPCR), nucleic acid sequence-based amplification (NASBA) and quantitative reverse transcription PCR (qRT-PCR).[329] When applied in the post-CHMI setting, blood-stage infections can often be detected by NAT tests 2-5 days earlier than by blood smears, with most sensitive NAT assays becoming positive around day 7 post-CHMI (C+7.0) when the standard CHMI dose of five infectious mosquito bites is used[305,311,316,330,331].

Mathematical modelling of NAT data allows two key measures of parasite growth dynamics to be estimated:

- **Parasite Multiplication Rate (PMR):** This is the fold-change in number of parasites in the blood over one lifecycle (48 hours). *P. falciparum* schizonts usually contain approximately 20 merozoites[1]. If all of these successfully invade a different RBC, the PMR would be 20. In CHMI studies including individuals with no prior exposure to malaria, PMR has been reported to range between 12 and 15[305,309].
- **Liver to Blood Inoculum (LBI):** This is a quantitation of the total number of parasites released from the liver and a useful indicator of the infectious parasite load in the liver. In sporozoite CHMI studies administered by 5 infectious mosquito-bites, LBI, estimated using the geometric mean of parasitaemia in the blood after the first cycle of asexual replication, ranged between 240,000 – 2835,000 parasites, depending on centre[305].

Simple linear[332], sine-wave[333], and normal-cumulative-density-function[334] models have been proposed to calculate PMR and LBI. A recent comparison using a large historical dataset of NAT post-CHMI found that parameters estimated by these models were closely correlated with similar predictive accuracy for omitted data points[335]. The same was the case in a recent vaccine efficacy study where the results of both linear and sine wave modelling were reported and gave comparable results[309]. It has therefore been suggested that the simple and transparent linear model (essentially, linear regression) be used for all future analyses in order to improve comparability between the outcomes of CHMI trials[335].

Although methodology varies[335], considerably limiting direct comparison of NAT data, the typical lower limit of detection (LLD) is 20 parasites per mL (p/mL) across all centres[318]. Whilst this is sensitive, there remains a need to improve this sensitivity further. Parasite release from the liver after CHMI with 3D7 or NF54 sporozoites is thought to mainly start on C+6.0 but not be complete until C+7.5[336,337] with one report indicating detection as early as C+5.0[331]. Given that many samples post-CHMI are NAT negative at these time-points and direct measurement of LBI is therefore not possible, modelling methods are usually employed to estimate LBI[335]. If it were possible to improve NAT sensitivity at these early time-points to allow direct measurement of LBI, the accuracy and confidence of LBI measures would be markedly increased[338].

1.7 Aim of work

The development of PfSPZ Challenge made the conduct of CHMI studies in Africa a practical and realistic possibility for the first time. However, at the start of this thesis, lack of an administration regimen for PfSPZ Challenge that could reliably infect 100% of volunteers limited its application. Knowing that PfSPZ Challenge IM was associated with greater infectivity than ID administration in mice, it was logical to evaluate the safety and infectivity of PfSPZ Challenge IM in humans for the first time. Given the advantages of routinely performing CHMI studies in African populations, this work went on to perform the first CHMI study in Kenya, assessing the safety and infectivity of PfSPZ Challenge IM in Kenyan adults.

To date, CHMI studies have not been used as a method to examine the mechanisms or dynamics of NAI. Evidence from a comparison of PMRs between malaria-naïve volunteers in CHMI studies in Oxford and Gambian adults with NAI exposed to malaria in the field, showed a 7-fold difference in PMRs between the two groups,[339] suggesting that NAI impacts on PMR in a detectable fashion that could be quantified in a modern CHMI study. It should therefore be possible to correlate prior exposure to malaria (a generally accepted surrogate of NAI in the absence of a validated *in vitro* assay) with PMR following CHMI, and use this information to interrogate the validity of proposed assays of NAI, such as GIA and ADRB activity, as predictors of NAI.

Performing a CHMI study in individuals where the exact burden and timing of infection is known provides a unique opportunity to gain an insight in real time into the immunological responses associated with infection. Taking advantage of the necessary multiple blood-sampling time points post-CHMI in both the UK and Kenyan CHMI studies, the work set out to perform time course analyses of changes in RNA expression, measures of innate and adaptive immunity and inflammatory markers to understand the effects of prior exposure to malaria on pre-erythrocytic and early blood-stage infection.

Mindful of the advantages of increasing the sensitivity of NAT tests used in CHMI studies, particularly at the LLD, evaluation of potential methods of improving sensitivity of detection of pre-microscopy patent *P. falciparum* blood-stage parasites was undertaken.

To summarise, the aims of this thesis were therefore:

- To establish and evaluate the safety and infectivity of the PfSPZ Challenge CHMI sporozoite model in malaria-naïve (UK) individuals to identify an administration regimen associated with 100% infectivity.
- To establish and evaluate the safety and infectivity of PfSPZ Challenge CHMI sporozoite model in a Kenyan population with varying prior exposure to malaria.
- To examine correlations between CHMI outcomes and assays of NAI to *P falciparum.*
- To describe changes in RNA expression, antibody, T cell and inflammatory markers associated with pre-erythrocytic and early blood-stage *P. falciparum* infection and NAI.
- To evaluate novel methods to improve NAT sensitivity for *P. falciparum* blood-stage at low, pre-microscopy patent parasite concentrations.

1.8 Overview of Thesis Chapters

Chapter 2 describes materials and methods.

Chapter 3 describes the clinical and parasitological results for the first UK CHMI study using PfSPZ Challenge administered IM (VAC049).

Chapter 4 describes the clinical and parasitological results for the first CHMI study conducted in Kenya using PfSPZ Challenge administered IM (KCS).

Chapter 5 discusses lessons learnt from conducting the first CHMI study in Kenya.

Chapter 6 describes and compares immunological and inflammatory data from VAC049 and KCS.

Chapter 7 summaries results from microarray analysis of changes in RNA expression post-CHMI in the KCS study.

Chapter 8 outlines results from attempts to improve the sensitivity of NAT methods used in CHMI studies.

Chapter 9 provides an overview of the impact of this work in the field with suggestions for possible future avenues of investigation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffers and solutions

Development Buffer (**DB**): 4-Nitrophenylphosphate tablets (Sigma N2765) dissolved in diethanolamine buffer (Fisher 34064) to give final concentration of 1mg/ml.

Carbonate coating buffer: (15mM Na_2CO_3 (Sigma), 35mM $NaHCO_3$ (Sigma), pH 9.3).

Incomplete RPMI culture medium (**yellow RPMI**): RPMI (Sigma R0883) with 5ml L-glutamine (2mM) (Gibco 25030-24), 5ml pen/strep (100U penicillin, 100µg strep) (Gibco 15140-122).

Phosphate buffered saline (**PBS**): 0.01M (Sigma P4417): NaCl 0.138 M, KCl 0.0027 M, pH 7.4: made by dissolving tablets in dH_2O.

PBS/Tween 0.01M (**PBS/T**) (Sigma P3563): NaCl 0.138 M, KCl 0.0027 M, pH 7.4 Tween 0.05%: made by dissolving sachets in dH_2O.

Polymorphonucleocytes Buffer (**PMN Buffer**): 5g D-(+)- Glucose Hybri Max (Sigma) and 0.5g bovine serum albumin (Sigma) in 500mL of hanks buffered salt solution with $NaHCO_3$ (Sigma) without Phenol red (Sigma) passed through sterile filter.

R0: RPMI (Sigma R0883) with 5mL Pen/strep (0.1 mg/mL) (Gibco BRL/Invitrogen) and 5mL L-glutamine) (4mM) (Gibco).

R10: RPMI (Sigma R0883) with 5ml L-glutamine (2mM) (Gibco 25030-24), 5ml

pen/strep (100U penicillin, 100µg strep) (Gibco 15140-122) and batch-tested heat inactivated FCS (10%) (Biotest S1810).

Red Blood Cell Counting Solution (**RBCCS**): 10ml formalin and 32g trisodium citrate made up to 1L in dH_2O.

RLT-βME buffer **(RLT):** 1.98ml RLT lysis buffer (Quiagen) and 20µl β-Mercaptoethanol (Calbiochem).

Tris-acetate-EDTA (TAE) buffer: Made up from 50x concentrate with H_2O (Fisher BP1332-1).

2.2 Methods

2.2.1 Controlled human malaria infection studies: VAC049 & KCS

2.2.1.1 Study designs

The study designs for VAC049 and KCS are shown in Figure 2-1 and 2-2 respectively.

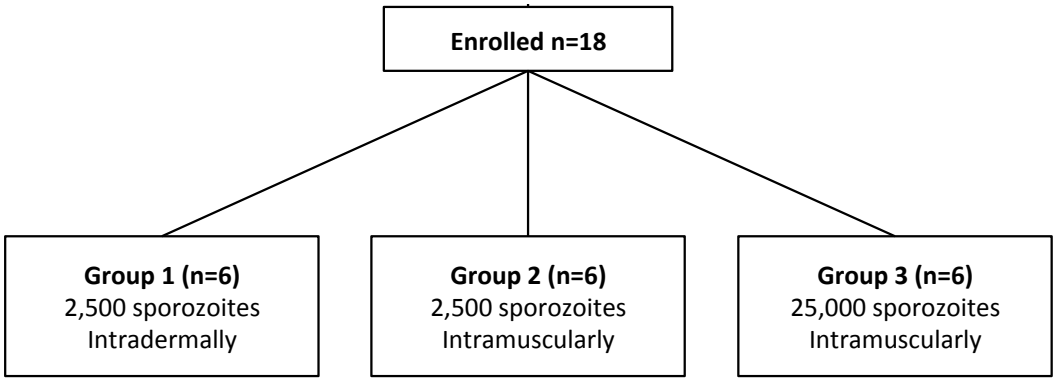


Figure 2-1: Study Design for VAC049

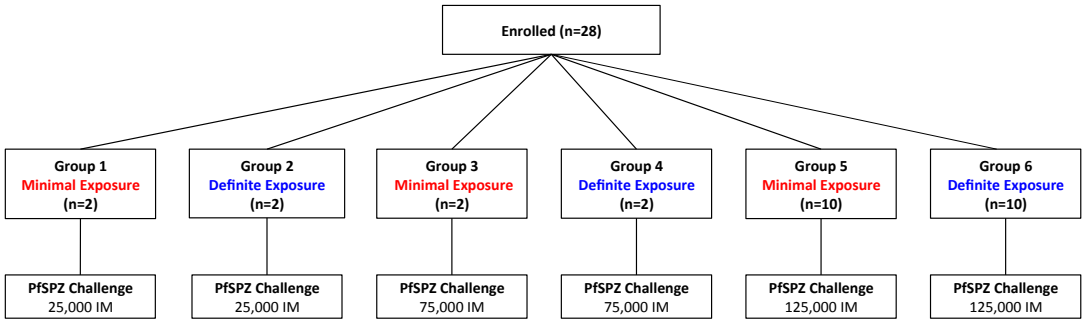


Figure 2-2: Study Design for KCS

2.2.1.2 Ethical & regulatory approval

VAC049: Ethical approval was granted by the UK National Research Ethics Committee South Central Oxford A (Ref: 11/SC/0351). UK regulatory approval of the trial was not necessary as PfSPZ Challenge is considered a non-investigational product in the UK. The study was reviewed and allowed to proceed by the US Food and Drug Administration under IND 14267. The trial was registered with ClinicalTrials.gov (NCT01465048).

KCS: Ethical approval was granted by the Kenya Medical Research Institute (KEMRI) Ethics Review Committee (ERC) (KEMRI/RES/7/3/1) and the Oxford Tropical Research Ethics Committee (OXTREC 161-12). Regulatory approval was provided by the Kenyan Pharmacy and Poisons Board (PPB/ECCT/12/12/01/2013). The study was reviewed and allowed to proceed by the US Food and Drug Administration under IND 14267. The study was

registered with the Pan African Clinical Trial Registry (PACTR20121100033272).

2.2.1.3 Safety overview

Both studies were conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice (GCP).

VAC049: A local safety committee chaired by Professor Brian Angus provided safety oversight. The University of Oxford's department for Clinical Trials and Research Governance independently monitored GCP compliance.

KCS: A safety monitoring committee was convened to overview the conduct and results of the study. Committee members included: Malcolm Molyneux, Walter Jaoko, Fred Were, Gilbert Kokwaro and Ly-Mee Yu. GCP compliance was independently monitored by the Clinical Trials Facility, Monitoring Department, KEMRI and Wellcome Trust Centre for Geographical Medicine Research – Coast, Kilifi, Kenya. A local safety monitor (Dr Charles Chunge) was appointed to provide safety oversight and support to the clinical trial team.

2.2.1.4 Assessment prior to enrolment (screening visit)

All volunteers wishing to participate in the studies attended a screening visit (Appendix 2-1 and 2-2). At this clinical review, following informed consent, a full history and medical examination was undertaken, with ECG, urinalysis for pregnancy (β-HCG), haematuria, proteinuria and glycosuria and venepuncture for full blood count (FBC), clinical biochemistry and serology for blood borne viruses (HIV, Hepatitis B and C) performed. In KCS blood was also collected to

assess for haemoglobinopathies, baseline antibody responses to schizont and MSP2 and NAT for *P. falciparum* infection (assessed by qPCR).

2.2.1.5 Informed consent

In both studies, volunteers were provided with the volunteer information sheet (VIS) at least 24 hours prior to their screening appointment. Before providing consent, individuals were required to pass a written questionnaire assessing their understanding of the study. Volunteers in VAC049 were allowed to take this questionnaire an unlimited number of times. Volunteers in KCS were limited to three attempts. At the screening visit, volunteers were asked to provide written consent following extended discussion of the study. Volunteers in VAC049 signed one consent form that included an optional section consenting for the storage of samples for up to 15 years and their subsequent use in studies understanding malaria. Volunteers in KCS were asked to complete two consent forms: one relating to participation in the study and another giving permission for long term storage and transfer of samples abroad.

2.2.1.6 Inclusion & exclusion criteria

VAC049 Inclusion Criteria:

- Healthy adults aged 18 to 45 years.
- Able and willing (in the investigator's opinion) to comply with all study requirements.
- Willing to allow the investigators to discuss the volunteer's medical history with their General Practitioner.

- Women only: Continuous effective contraception for the duration of the study.
- Agreement to refrain from blood donation during the course of the study and for at least 3 years after the end of their involvement in the study.
- Written informed consent to undergo CHMI.
- Reachable by mobile phone during the whole study period.
- Willingness to take a curative anti-malarial regimen.
- For volunteers not living in Oxford, an agreement to stay in a hotel room close to the trial centre during a part of the study (from at least C+6.5 until 2 days after treatment commenced).
- Answer all questions on the informed consent quiz correctly.

VAC049: Exclusion Criteria:

- History of clinical *P. falciparum* malaria.
- Travel to a malaria endemic region during the study period or within the preceding six months, with positive anti-schizont serology.
- Use of systemic antibiotics with known antimalarial activity within 30 days of study enrolment (e.g. trimethoprim-sulfamethoxazole, doxycycline, tetracycline, clindamycin, erythromycin, fluoroquinolones and azithromycin).
- Receipt of an investigational product in the 30 days preceding enrolment, or planned receipt during the study period.

- Prior receipt of an investigational malaria vaccine.
- Any confirmed or suspected immunosuppressive or immunodeficient state including: HIV infection, asplenia, recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (inhaled and topical steroids are allowed).
- Use of immunoglobulins or blood products within the 3 months prior to enrolment.
- History of sickle cell anaemia, sickle cell trait, thalassemia or thalassemia trait.
- Pregnancy, lactation or intention to become pregnant during the study.
- A history of allergic disease or reactions likely to be exacerbated by malaria infection.
- Contraindications to the use of all three proposed anti-malarial medications: Malarone (Atovaquone and Proguanil), Riamet (Artemether and Lumefantrine) and Chloroquine.
- History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ).
- History of serious psychiatric condition that may affect participation in the study.
- Any other serious chronic illness requiring hospital specialist supervision.
- Suspected or known current alcohol abuse as defined by an alcohol intake of greater than 42 units every week.

- Suspected or known injecting drug abuse in the 5 years preceding enrolment.
- Seropositive for Hepatitis B surface antigen (HbSAg).
- Seropositive for Hepatitis C virus antibodies.
- An estimated, ten-year risk of fatal cardiovascular disease of ≥5%, as estimated by the Systematic Coronary Risk Evaluation system.
- Positive family history in 1st and 2nd degree relatives < 50 years old for cardiac disease.
- Volunteers unable to be closely followed for social, geographic or psychological reasons.
- Any clinically significant abnormal finding on biochemistry or haematology blood tests, urinalysis or clinical examination.
- Any other significant disease, disorder or finding which may significantly increase the risk to the volunteer because of participation in the study, affect the ability of the volunteer to participate in the study or impair interpretation of the study data.

KCS: Inclusion Criteria:

- Healthy adults aged 18 to 40 years.
- Minimum of 4 completed years of secondary education.
- Able and willing, in the investigator's opinion, to comply with all study requirements.

- Informed consent to undergo CHMI.
- Answer all questions on the informed consent questionnaire correctly.
- Willingness to take a course of curative anti-malaria medication.
- Agreement to stay in an in-patient unit during a part of the study (from day of administration of PfSPZ Challenge until completion of curative course of anti-malarial therapy given either at malaria diagnosis or C+21).
- Use of effective method of contraception for duration of study (women only).

KCS: Exclusion criteria

- qPCR positive (>LLD) for *P. falciparum* parasites at screening.
- Use of systemic antibiotics with known antimalarial activity within 30 days of administration of PfSPZ Challenge (e.g. trimethoprim-sulfamethoxazole, doxycycline, tetracycline, clindamycin, erythromycin, fluoroquinolones and azithromycin).
- Receipt of an investigational product in the 30 days preceding enrolment, or planned receipt during the study period.
- Current participation in another clinical trial or recent participation within 12 weeks of enrolment.

- Prior receipt of an investigational malaria vaccine.
- Any confirmed or suspected immunosuppressive or immunodeficient state, including: HIV infection, asplenia, recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (inhaled and topical steroids are allowed).
- Use of immunoglobulins or blood products within 3 months prior to enrolment.
- Sickle cell trait or heterozygous or homozygous α thalassaemia.
- A history of allergic disease or reactions likely to be exacerbated by malaria infection.
- Contraindications to Atovaquone/Proguanil hydrochloride.
- History of cancer (except basal cell carcinoma of the skin and cervical carcinoma *in situ*).
- History of serious psychiatric condition that may affect participation in the study.
- Any other serious chronic illness requiring hospital specialist supervision.
- Women only; pregnancy, intention to become pregnant or breast-feeding during study.
- Suspected or known current alcohol abuse.
- Suspected or known injecting drug abuse.

- Seropositive for HBsAg.
- Seropositive for Hepatitis C virus with PCR positive for Hepatitis C.
- Positive family history in 1st and 2nd degree relatives < 50 years old for cardiac disease.
- Any clinically significant abnormal finding on biochemistry or haematology blood tests, urinalysis or clinical examination.
- Any other significant disease, disorder or finding which may significantly increase the risk to the volunteer because of participation in the study, affect the ability of the volunteer to participate in the study or impair interpretation of the study data.

Both Studies: Exclusion Criterion on Day of CHMI

- Acute disease, defined as moderate or severe illness with or without fever.

2.2.1.7 Group allocation and blinding

VAC049: Initially volunteers were allowed to opt into any group. Once one group had the required number of volunteers, subsequent volunteers were allocated to the other groups.

KCS: Volunteers were allocated to groups according to the criteria in Table 2-1. Subjects for groups 2, 4 and 6 were selected from eligible volunteers starting with those with the highest anti-schizont ELISA OD first. Once participants to be

enrolled in minimal prior exposure (MinExp) and definite prior exposure (DefExp) groups were confirmed, further appropriate group allocation was performed using randomization.

Groups	**Prior Malaria Exposure**	**<u>Primary Criterion:</u> Anti-Pf serology***	**<u>Secondary Criterion:</u> Time Spent in Endemic Region**
1, 3, 5	Minimal (MinExp)	All Negative	Lived majority of life in Nairobi or area where *P. falciparum* is not endemic.
2, 4, 6	Definite (DefExp)	Positive Anti-Schizont +/- Positive Anti-MSP2	Considerable time spent in areas of *P. falciparum* transmission.

Table 2-1: Criteria for determining group allocation for KCS

In both studies, volunteers and clinical investigators were not be blinded to group allocation, however laboratory investigators were unaware of group allocation.

2.2.1.8 Schedule of enrolment

The primary outcome measurement of both studies was safety. Volunteer enrolment was therefore staggered in both studies to allow assessment of safety prior to dose escalation of PfSPZ Challenge.

VAC049: The 6 participants in Group 1 and 2 participants from group 2 were enrolled first, on the same day. In the absence of safety concerns in these participants, 48 hours later the remaining 4 participants in Group 2 and 2 participants from Group 3 were enrolled. In the absence of safety concerns in these participants, 48 hours later the remaining participants in Group 3 were enrolled.

KCS: The 4 volunteers in Groups 1 and 2 were enrolled first. 48 hours later and following SMC review, the 4 volunteers in Groups 3 and 4 were enrolled. 48 hours later and following SMC review the remaining volunteers were enrolled.

2.2.1.9 PfSPZ Challenge manufacture & preparation for administration

PfSPZ Challenge was manufactured according to Good Manufacturing Practice (GMP) standards by Sanaria Inc. Manufacture includes the production, under traditional environmental conditions, of eggs from a colony of *A. stephensi* mosquitoes housed in a controlled environmental chamber. Surface disinfection of the eggs was performed by exposure to chemical agents in a Class II biosafety cabinet (BSC). From this point forward, all materials and product were handled using aseptic methods to ensure that contaminating microorganisms were not introduced to, and carried through the process. Surface-disinfected eggs were inoculated into sterile, vented flasks containing aseptic growth medium. The eggs hatched and developed into pupae, which were transferred to an adult mosquito container where the adult mosquitoes emerged. These adult mosquitoes, which had been raised under aseptic conditions, were fed *P. falciparum* gametocyte-infected blood in a BSC. The *P. falciparum* gametocyte-infected blood was produced from cultures of the *P. falciparum* strain NF54

derived from a master cell bank of the well-characterized *P. falciparum* strain NF54. Infected adult mosquitoes were maintained under aseptic conditions until *P. falciparum* sporozoites (SPZ) migrated to the salivary glands. The salivary glands from the *P. falciparum* SPZ-infected mosquitoes were removed by hand dissection. Salivary glands were then triturated to release the *P. falciparum* SPZ. SPZ were purified, counted, and, at a specified concentration, cryopreserved. Cryopreservation commenced with the addition of cryoprotective additives to the purified SPZ to produce the PfSPZ Challenge product. PfSPZ Challenge was dispensed into screw-cap vials containing 15,000 or 50,000 SPZ in 20 µL aliquots. PfSPZ Challenge was stored in LNVP at -150°C to -196°C.

The diluent for PfSPZ Challenge was composed of PBS and human serum albumin (HSA). PBS that was manufactured in compliance with GMP and according to upstream processing specifications was purchased by Sanaria Inc. The PBS was stored at ambient temperature in a controlled room. HSA (25%), which is approved for parenteral, IV administration to humans was purchased by Sanaria Inc. HSA vials were stored at ambient temperature in a controlled room.

Vials of PfSPZ Challenge were stored and transported to site in LNVP. Immediately prior to use, PfSPZ Challenge was thawed in individual cyrovials by partial submersion of vials for 30 seconds in a 37°C ± 1°C water bath. Designated, trained study staff then prepared, diluted (using PBS and HAS) and dispensed PfSPZ Challenge to clinical staff at the study sites. The maximum time interval allowed between thawing of PfSPZ Challenge and administration to a volunteer was 30 minutes.

2.2.1.10 Administration of PfSPZ Challenge

Volunteers were reviewed the day before CHMI (C-1) to check on-going eligibility, and blood drawn for safety assessment and baseline immunology (Appendix 2-1 and 2-2). For both studies PfSPZ Challenge was administered IM using the BD 1ml syringe (Ref 309628) and BD Microlance 3 (23G 1", 0.6 x 25mm Ref 300800). In the VAC049, PfSPZ Challenge was administered ID using the BD Integra 1ml TB syringe & needle (1ml 26G 3/8 (0.45mm x 10mm) Ref 305279). Study staff administrating PfSPZ Challenge wore gloves and eye protection. During administration of PfSPZ Challenge, advanced life support drugs and resuscitation equipment were immediately available for the management of potential anaphylaxis. For each group the total dose of SPZ was divided into two equal doses and administered as 50μL injections, one in each deltoid. The inoculation sites were immediately covered with a dressing to absorb any PfSPZ Challenge that may have leaked out through the needle track. The dressing was removed from the injection site after 1 hour when the volunteers were reviewed and provided with an emergency contact card detailing the 24-hour telephone number of the on-call study clinician and the sensitivities of the infecting strain of malaria.

2.2.1.11 Clinical follow-up post-CHMI

The follow-up schedule post-CHMI was the same for both studies. Subjects in VAC049 were managed as out-patients throughout the study. In KCS, all participants were admitted as in-patients from C-1 until completion of anti-malarial therapy. All volunteers underwent once daily clinical review on C+1-5 to enquire as to AEs and use of medications. Volunteers were reviewed on C+6 in

the evening (C+6.5) and then twice a day, morning and evening between C+7 and C+14. Undiagnosed volunteers were reviewed once a day in the morning between C+15 and C+21. At each visit physical observations performed and AEs solicited. Volunteers were treated with a 3-day curative course of oral Atovaquone/Proguanil where each dose was directly observed. Volunteers were reviewed 24 and 48 hours post-diagnosis where blood was sampled for microscopy and qPCR. Provided these two blood-films after treatment were negative for parasites, volunteers were not reviewed again in clinic until C+35. If one of these blood films was positive, volunteers continued to be reviewed at 24-hour intervals until two consecutive blood-films were negative. Volunteers were then reviewed at C+35 and C+90 where safety assessments were performed.

2.2.1.12 Blood sampling post-CHMI

VAC049: FBC with differential, platelet count and serum biochemistry (including electrolytes, urea, creatinine, bilirubin, alanine transferase, alkaline phosphatase and albumin) were measured at C+9, within 24 hours of diagnosis, and at C+35 and C+90. Blood was drawn for exploratory assays at C-1, C+7, C+11 and C+21 if persistently undiagnosed with malaria (Appendix 2-1). If diagnosed with malaria, 70mls of blood was drawn within 24 hours of diagnosis (but not if diagnosed on C+7, C+7.5, C+11 or C+11.5) and then no further blood drawn for explorative immunology until C+35, then C+90.

KCS: FBC with differential, platelet count and serum biochemistry (including electrolytes, urea, creatinine, bilirubin, alanine transferase, alkaline phosphatase and albumin) were measured at C+9, within 24 hours of diagnosis, and at C+35 and C+90. Blood was drawn for exploratory assays at C-1, C+7, C+9, C+11, C+13,

C+15, C+17, C+19 and C+21 if persistently undiagnosed with malaria. No further blood was drawn for explorative immunology until C+35 and C+90.

Throughout this thesis, study day refers to the nominal time-point for a group and not the actual day of sampling. All laboratory personnel and microscopists were blinded to group allocation.

2.2.1.13 Assessment of adverse events

In both studies, adverse events (AEs) were graded according to the criteria in Table 2-2 & Table 2-3.

Adverse Event	Grade	Intensity
Pain at injection site	1	Pain that is easily tolerated
	2	Pain that interferes with daily activity
	3	Pain that prevents daily activity
Erythema at injection site*	1	>3 - ≤50 mm
	2	>50 - ≤100 mm
	3	>100 mm
Swelling at injection site	1	>0 - ≤20 mm
	2	>20 - ≤50 mm
	3	>50 mm

Table 2-2: Criteria for grading severity of local adverse events related to PfSPZ Challenge in VAC049 and KCS

** ≤3mm erythema was considered consistent with needle puncture.*

GRADE 0	None
GRADE 1	Mild: Transient or mild discomfort (< 48 hours); no medical

	intervention/therapy required
GRADE 2	Moderate: Mild to moderate limitation in activity - some assistance may be needed; no or minimal medical intervention/therapy required
GRADE 3	Severe: Marked limitation in activity, some assistance usually required; medical intervention/therapy required, hospitalisation possible

Table 2-3: Functional criteria for grading severity of systemic adverse events in VAC049 and KCS

2.2.1.14 Microscopy for *P. falciparum*

VAC049: Blood smears were prepared and interpreted according to Jenner Institute Protocol ML009; '*Collection, preparation and slide reading: Malaria Challenge Studies.'* Briefly, slides were prepared ('one small drop of blood'), stained with Field's stain and 200 fields read at 'high power.' A positive thick film on microscopy was defined as at least *one* morphologically normal, 'unambiguous' malaria parasite seen in 200 high power fields by two or more experienced microscopists. Blood smears were not quantified for parasite density.

KCS: Blood smears were prepared and interpreted according KCS SOP Laboratory 015 based on the "*Consensus SOP for Malaria Microscopy in the Context of Clinical Challenge Trials*" documents[318]. Briefly, slides were prepared using 10µL of whole blood spread over a rectangle measuring 1 x 2 cm. Slides were stained using Giemsa stain solution (10% for slides from symptomatic volunteers, 4% for all other slides). Using a microscope with a high-power field

of 0.22 mm diameter, 5 passes were read for samples from asymptomatic and treated volunteers and 10 passes for samples from symptomatic volunteers. A positive thick film on microscopy was defined as at least two morphologically normal, 'unambiguous' malaria parasites seen in 0.5µL of blood by two or more experienced microscopists, blinded to each other's findings. A parasite was considered 'unambiguous' if all features (including purple red chromatin dot, blue cytoplasm, dark mass of pigment and distinct morphology) were clearly visible. A parasite was considered 'ambiguous' if it did not demonstrate the features described above. All 'positive' slides were quantified for parasite density (parasites/µL). A third microscopist acted as slide coordinator, distributing the slides between microscopists and reconciling any discrepant findings. All microscopists working on the study passed a 2-week refresher-training course at the KEMRI-affiliated Malaria Diagnostics Centre of Excellence, Kisumu, approximately 3 months prior to working on study samples.

2.2.1.15 Criteria for treatment post-CHMI

VAC049 (Table 2-4): Volunteers were treated if one or more of the following criteria were met:

- Positive thick film microscopy with at least one morphologically normal malaria trophozoite in the presence of symptoms consistent with malaria.
- Volunteer describing symptoms or displaying signs or symptoms likely to represent *P. falciparum* malaria infection in the opinion of clinical investigators (such as fever, rigors or severe symptomatology), with negative blood film and at least one qPCR result > 500 p/mL.

- Volunteers with positive blood smear, no symptoms consistent with *P. falciparum* malaria infection, and at least one qPCR result > 500 p/mL.
- Volunteers withdrawn from study post-CHMI but before completion of a course of anti-malarial therapy.
- Volunteers undiagnosed by C+21.

	THICK FILM MICROSCOPY	
MALARIAL SYMPTOMS	**Positive**	**Negative**
Symptomatic	Treatment started	Treatment started if any available PCR result is > 500 p/ml
Asymptomatic	Treatment started if any available PCR result is > 500 p/ml	Treatment not started

Table 2-4: Criteria for starting anti-malarial therapy in VAC049

p/mL = parasites per mL measured by qPCR.

KCS: Volunteers were treated if one or more of the following criteria were met (of note, for KCS qPCR was not performed in real time and so did not contribute to treatment decisions):

- A positive blood smear on microscopy defined as at least two morphologically normal, 'unambiguous' malaria parasites seen in 0.5µL of blood by two or more experienced microscopists blinded to each other's findings.

- Volunteers blood smear negative but describing symptoms or displaying signs likely to represent clinical *P. falciparum* infection in the opinion of clinical investigators (such as fever, rigors or severe symptomatology).
- Volunteers withdrawn from study post-CHMI but before completion of a course of anti-malarial therapy.
- Volunteers undiagnosed by C+21.

2.2.1.16 Treatment of *P. falciparum* malaria

Curative anti-malarial therapies and supportive medications were the same for both studies. The first line treatment for both studies was oral atovaquone/proguanil hydrochloride diagnosis (see summary of product characteristics (SmPC)). Atovaquone/proguanil hydrochloride is a licensed drug for the treatment of acute uncomplicated malaria caused by *P. falciparum*. A treatment course of atovaquone/proguanil hydrochloride for an adult consists of 4 'standard tablets' (containing proguanil hydrochloride hydrochloride 100mg, atovaquone 250mg) once daily, orally for 3 days. All three doses of atovaquone/proguanil hydrochloride were directly observed for both studies. PfSPZ Challenge is known to be fully sensitive to atovaquone/proguanil hydrochloride. Prior to administration of atovaquone/proguanil hydrochloride, volunteers were screened for drug interactions and contraindications and reminded of the potential side effects of atovaquone/proguanil hydrochloride. If a volunteer was unable to tolerate Atovaquone/Proguanil hydrochloride, oral Chloroquine or Artemether & Lumefantrine was prescribed as an alternative treatment (see relevant SmPCs).

Provided there were no contraindications, all volunteers were offered Paracetamol (1g orally up to four times a day) and Cyclizine (50mg orally three times a day) (See relevant SmPCs) to help relieve symptoms associated with malaria if these occurred.

2.2.1.17 Compensation for study participants

Volunteers in both studies were offered financial compensation for transport expenses and time taken away from potential income generation or actual work in order to participate in the study. These amounts took into consideration any lost wages as a result of participating in the study while ensuring that it neither unduly coerced potential participants nor set a difficult precedent for future research within the host centres.

VAC049: Based on local wages and comparison with CHMI studies undertaken at the same centre, volunteers were compensated £15 per hour for each clinic visit, £6 for each venepuncture, £6 travel expenses for each clinic visit and £360 compensation for time spent unwell and unable to work.

KCS: Based on local wages and comparison with a similar CHMI study initiated in Bagamoyo Tanzania in March 2012[314], volunteers were compensated US$50 (Kshs. 4,000) for each overnight stay under observation at the KEMRI Clinical Research Centre, US$12 (Kshs. 1,000) for each scheduled clinic visit and US$6 (Kshs. 500) for transport costs for each attendance at the clinic.

2.2.2 Other CHMI Trials

2.2.2.1. VAC045

VAC045 was a mosquito-bite CHMI trial conducted at the Centre for Clinical Vaccinology & Tropical Medicine (CCVTM), Oxford, UK to assess the efficacy of candidate pre-erythrocytic viral vectors: chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) containing CS or thrombospondin related adhesion protein (ME-TRAP) (Clinicaltrials.gov NCT01623557)[340]. The study consisted of three groups: Group 1 immunized with ChAd63-MVA CSP (n=15), Group 2 immunized with ChAd63-MVA ME-TRAP (n=15) and Group 3 unimmunized infectivity controls (n=6). VAC045 was approved by the National Research Ethics Service Committee South Central – Oxford A, UK and the Western Institutional Review Board, USA. The study was conducted from March – June 2012.

2.2.2.2. VAC052

VAC052 was a mosquito-bite CHMI trial conducted at the CCVTM, Oxford, UK to assess the efficacy of ChAd63 and MVA vectors containing ME-TRAP, CS and AMA1 administered in combination (Clinicaltrials.gov NCT01739036) (*Rampling et al. Manuscript in preparation*). The study consisted of three groups: Group 1 immunized with ChAd63-MVA ME-TRAP+CSP (*n=13*), Group 2 immunized with ChAd63-MVA ME-TRAP+CSP+AMA1 (*n=13*) and Group 3 unimmunized infectivity controls (*n=6*). VAC052 was approved by the National Research Ethics Service Committee South Central – Oxford A, UK and the Western Institutional Review Board, USA. The study was conducted from January – October 2013.

2.2.3 Methods to assess P. falciparum parasitaemia

2.2.3.1 Oxford standard qPCR protocol

qPCR for KCS, VAC049, VAC045 and VAC052 was performed using the standard 'Oxford' qPCR protocol as outlined in the SOPs: KCS SOP Laboratory 012: 'DNA extraction and PCR for Whatman Filtered Samples' and Jenner SOP ML008: 'Malaria qPCR.'

Whole blood samples were collected in EDTA vacutainers (BD Bioscience). For KCS samples were filtered immediately and frozen at -80°C. DNA extraction and qPCR was performed at a later date. For the other CHMI studies, samples were filtered, DNA extracted and qPCR performed in real time. Based upon results obtained using a dilution series of microscopically-counted cultured parasites this method has a lower limit of quantification (LLQ, defined as %CV<20%) of around 20 p/mL. Counted parasite dilution series results suggest that the LLD (i.e. a probability of >50% of ≥1 positive result among three replicate PCR reactions) is in the region of 5 p/mL, while samples at 1 p/mL are consistently negative[332].

Filtration of Whole Blood Samples to Remove Leucocytes (Human DNA): After centrifugation of a 2mL sample, plasma was removed and 1mL sterile PBS added to the tube. The sample was then passed through a 24 well, two-layer Whatman filter plate (WF) (7700-9902) attached to a UniVac3 manifold and PBS added to the sample to return it to its original volume.

DNA Extraction: 0.5mL filtered blood was added to 40mL protease (Qiagen 51106) and 400mL lysis buffer AL (Qiagen 51106) and incubated in a water bath at 56˚C for 10 minutes. 400ml 100% ethanol was then added and mixed by pulse-vortexing for 15 seconds. The sample was then added to a QIAamp spin column attached to a vacuum pump. The column was washed with 750mL of wash buffer AW1 (Qiagen 51106) followed by 750mL wash buffer AW2 (Qiagen 51106), after which it was spun at 13000 rpm for 4 minutes to dry the membrane completely. DNA was eluted by adding 50µL sterile 10mM Tris solution pH 8.0 (Qiagen 51106) to each column.

qPCR: The Taqman probe-based PCR amplified a 133 base pair (bp) PCR product from the multi-copy (3 per parasite) 18S (small subunit) ribosomal RNA genes of *P. falciparum* (Accession number: M19173). A 5' FAM labelled probe with the Applied Biosystems proprietary NFQ quencher plus MGB moiety at the 3' then bound to the PCR product and was hydrolysed on each PCR cycle to release the flurophore that was then detected in a quantitative manner[341].

The sequences of the primers and probe used were[342]:

- *18S Pf Forward Primer:* 5' GTAATTGGAATGATAGGAATTTACAAGGT 3'
- *18S Pf Reverse Primer:* 5' TCAACTACGAACGTTTTAACTGCAAC 3'
- *18S Pf MGB Probe:* 5' FAM- AACAATTGGAGGGCAAG-NFQ-MGB 3'

MicroAmp Fast Optical 96 well plates (Applied Biosystems) were used. The components of each 25ul PCR reaction are outlined in Table 2-5.

Qiagen Nuclease Free water	5.25µL
18S Pf Forw (10mM stock)	1.0µL
18S Pf Rev (10mM stock)	1.0µL
18S MGB (10mM stock)	0.25µL
Universal Probe PCR Master Mix (2x)	12.5µL
Sample/Standard	5µL
Total Volume	25µL

Table 2-5: Components of qPCR reactions for Oxford standard qPCR protocol

The qPCR standards (made by Nick Edwards, The Jenner Institute, University of Oxford) contained the 133bp PCR product cloned into a plasmid (#2231 pCR-Blunt II-TOPO-18s Pf-Herm) (Table 2-6). The negative control was dH_20. All test samples, standards and negative control were tested in triplicate.

PCR Product Copies/reaction	**Parasites/mL equivalent**	**Name**
15000	100000	PT1
1500	10000	PT2
150	1000	PT3
45	300	PT4
15	100	PT5
6	40	PT6
3	20	PT7

Table 2-6: qPCR plasmid standards for Oxford standard qPCR protocol

An Applied Biosystems Step One Plus PCR machine was used with the following programme;

- *AMPerase step:* 50˚C for 2 minutes.
- *Hotstart activation:* 95˚C for 10 minutes.
- *Cycling (repeated 45 times):* 95˚C for 15 seconds (denature) then 60˚C for 1 minute (annealing and extension).

Quantification was performed using Applied Biosystems Step Oneplus software v2.1. The mean of triplicate readings was taken as the final value.

qPCR quality control: Samples were re-tested if:

- Replicates included a mixture of positive and negative (in terms of amplification) results with one or more positive results > 100 p/mL.
- The %CV of qPCR CT for any samples were high outliers.

2.2.3.2 CF11 Filtration

Dilution Series: Two dilution series (A and B) of *P. falciparum* parasites in Tris (Qiagen 51106) were made (0, 5, 10, 20, 40, 100, 1000, 3000, 10000, 100000 p/mL). 2ml of each of these samples were filtered using WFs and DNA eluted from 0.5mL of this filtrate into 50µl of Tris, as per the Oxford standard qPCR protocol. 2mL of each concentration from each series was also filtered using 'CF11 columns,' and DNA eluted from the whole filtrate into 100µl of Tris.

For samples from VAC049, WF filtering, DNA extraction and qPCR occurred on fresh samples within 4 hours of sampling. Frozen blood samples from the same

time points were thawed at a later date and CF11 filtration, DNA extraction and qPCR performed.

CF11 Filtration: A 10mL syringe with lens paper covering the syringe opening was filled with 5mL CF11 cellulose (Whatman), compressed to 2.5mL and pre-wet with 3mL of PBS. The 2mL blood sample was added to the column after centrifugation and removal of plasma. The sample was passed through the column by applying gentle pressure to the syringe plunger, followed by a 5mL PBS wash and finally the filtrate reduced to 2mL by removing excess PBS post centrifugation. DNA was eluted from the whole 2mL sample into 100µl of Tris (Qiagen 51106).

Quantification of Extracted DNA: The total amount of eluted DNA in WF and CF11 samples was measured in triplicate using spectrophotometry (Nanodrop 2000, Thermo-Scientific) and the mean calculated. Readings were excluded and repeated if OD 260/280 was < 1.8.

qPCR: Samples filtered by both WF and CF11 protocols were processed according to the Oxford standard qPCR protocol with differences as highlighted in Table 2-7.

	WF Samples	**CF11 samples**
Volume DNA extracted from	500µl	2mL
Volume DNA eluted in	50µl	100µl
Volume of sample added to PCR reaction	5µl	10µl

Standards	5µl	5µl + 5µl dH_20

Table 2-7: Differences in qPCR protocol for Whatman and CF11 filtered samples

The components of each 25µL qPCR reaction for CF11 filtered samples are outlined in Table 2-8.

Qiagen Nuclease Free water	0.25µL
18S Pf Forw (10mM stock)	1.0µL
18S Pf Rev (10mM stock)	1.0µL
18S MGB (10mM stock)	0.25µL
Universal Probe PCR Master Mix (2x)	12.5µL
Sample/Standard	10µL
Total Volume	25µL

Table 2-8: Components of each qPCR reaction for CF11 filtered samples

2.2.3.3 Zap-Oglobin treatment

In VAC045 and VAC052 for each sample collected at C+7 (VAC045) or C+7.5 (VAC052), two 500µl aliquots of whole blood were made. 5µl of Zap-Oglobin II Lytic Reagent (1:100) (Beckman Coulter) was added to the first aliquot, and none to the second. Both aliquots were then stored at -80°C. At a later date, the aliquots were thawed and DNA extraction and qPCR undertaken according to the Oxford standard qPCR protocol.

2.2.3.4 Loop mediated isothermal amplification

Loop mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification technique. This method relies on auto-cycling strand displacement DNA synthesis (Figure 2-3)[343]. In the reaction, amplification proceeds through repetition of two types of elongation reactions that occur via the loop regions. In this reaction, pairs of inner and outer primers are used which are complementary to the 3-terminal and 5-terminal regions. The elongation reactions are sequentially repeated by DNA polymerase-mediated strand-displacement synthesis using the loop regions as a stage to create stem-loops of DNA. These can then by measured by a variety of methods including turbidity and immunofluorescence.

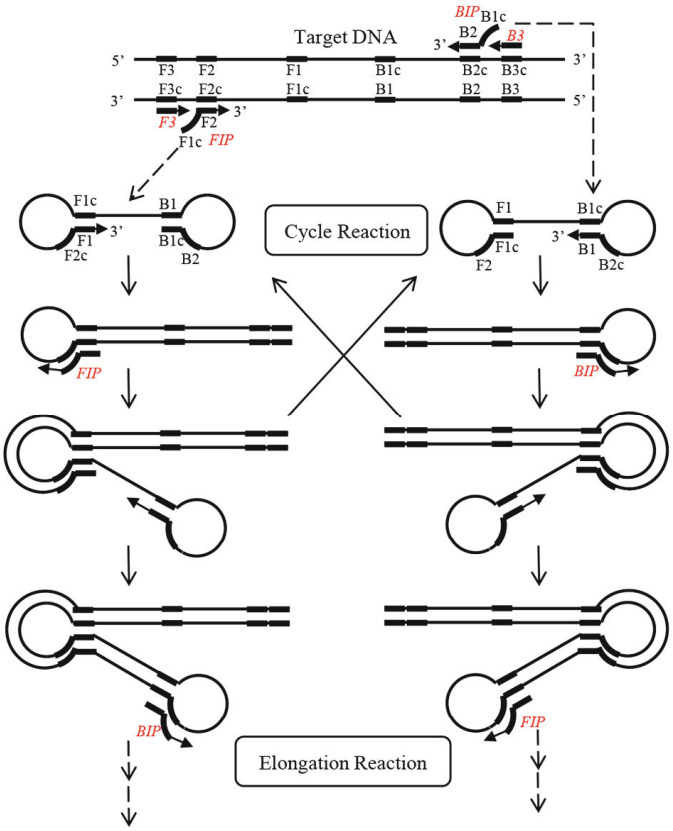


Figure 2-3: Schematic representation of the LAMP mechanism

FIP: Forward inner primer; BIP: Backward inner primer. Outer primers are designed at the regions of F3 and B3. The LAMP method employs a single strand of DNA shaped like a dumbbell with loops at both ends. This starting material is used to initiate a cycle of amplification reactions. DNA having an inverse structure relative to the starting material is produced, and the starting material is formed again by the same reaction. This cycle produces amplified DNA products that are connected to an inverted repeat structure at the amplified region. The amplified products again pass through repeated elongation reactions, which generate amplified DNA products of various stem lengths. Adapted from Notomi et al[343].

Species-specific primers, as described by Han et al[344] were used to amplify the gene coding for the 18s ribosomal RNA. MicroAmp Fast Optical 96 well plates

(Applied Biosystems) were used. The components of each LAMP reaction are outlined in Table 2-9. Sybr Green (Life Technologies S-7563) was added to assess if changes in fluorescence over time could be used to quantify starting target DNA. A low concentration of Sybr green was used given reports that higher concentrations can inhibit LAMP[345].

Primer Mix	
Qiagen Nuclease Free water	5.2µL
FIP (100uM stock)	0.4µL
BIP (100uM stock)	0.4µL
LPF (100uM stock)	0.2µL
LPR (100uM stock)	0.2µL
F3	0.05µL
B3C	0.05µl
2 x Buffer Master Mix	
Tris-HCl pH 9.0 (1M/L stock)	0.5µl
KCl (2M/L stock)	0.125
$MgSO_4$ (1M/L stock)	0.2µl
$(NH_4)_2SO_4$ 1M	0.25µl
Tween 20 1:10 (0.2% final)	0.25µl
Betaine (5M/L)	4µl
dNTPs (100mmol/L)	1.25µl
Qiagen Nuclease Free water	5.93µl
Other	
SYBR green (1:100 of 10,000x stock)	0.25µL
BST Polymerase	1µl
Sample	5µL
Volume	25.25µl

Table 2-9: Components of each LAMP reaction

FIP = forward inner primer; BIP = Backward inner primer; F3 = Forward outer primer; B3C = Backward outer primer. LPF and FPR = other primers; dNTPs = nucleotide triphosphates containing deoxyribose.

For LAMP samples, an Applied Biosystems Step One Plus PCR machine was used with the following programme;

- 60°C for 120 minutes.

- 80°C for 10 minutes.
- Assessment of fluorescence every minute.

Samples tested were a series of whole blood samples collected in EDTA vacutainers (BD bioscience) and spiked with thawed *P. falciparum* blood-stage parasites, diluted in Tris (Qiagen 51106) and frozen at -80°C. Samples were heat treated (5 minutes on 60°C heat block) prior to each dilution. The samples were quantified by qPCR against the same plasmid standards outlined in Table 2-6. Results of spiked samples assessed by qPCR are shown in Table 2-10.

Parasites/mL as measured by qPCR	Name
872071	Q2
80820	S1
6706	S2
590	S3
71	S4
7	S5

Table 2-10: Parasite concentrations of dilution series used for LAMP

As measured by Oxford standard qPCR protocol.

LAMP products were separated on 1% agarose gels with SYBR Safe (1:10,000) (Invitrogen S33102) in Tris-acetate-EDTA (TAE) buffer by electrophoresis (100 V, 55 min). Samples were loaded in loading dye (1:6) and visualised on a transilluminator. Band size was determined against GeneRuler 1kb (Thermo Scientific) DNA ladders, which were run on each gel.

2.2.3.4 qRT PCR

Sample Preparation: For quantitative reverse-transcription PCR (qRT-PCR) 'large volume' samples, 500µl of EDTA anti-coagulated whole blood (BD Bioscience) was added directly into 20mL NucliSens Lysis Buffer (LB) (BioMerieux) in a 50mL tube. For qRT-PCR 'standard volume' samples, 50µl of whole blood were added to 2mL LB in a 3.6mL tube. Samples were vortexed at full speed for 10-15 seconds to ensure homogenous mixture and stored at room temperature for at least 10 minutes. Samples were then stored at -80°C before shipping to the University of Washington (UW) for further processing.

For qRT-PCR from dried blood spot (DBS) cards, 50 µL of EDTA-anticoagulated whole blood (BD Bioscience) was pipetted onto a standard DBS card (Whatman), which was subsequently dried, desiccated and frozen on-site[346]. The cards were dried in a fume hood out of direct sunlight for at least 4 hours. The cards were placed in a gas impermeable bag with a desiccant pack and humidity indicator card and stored at -80°C before shipping to the UW.

Nucleic acid extraction and qRT-PCR (UW protocol): After storage at -80°C, standard volume liquid samples were thawed and extracted as previously described[346]. DBS samples were laser cut, incubated with 2 mL LB with continuous shaking overnight and then the LB was transferred to the m2000sp (Abbott Molecular, Inc.). Large volume samples were completely thawed and 1 mL transferred to the m2000sp instrument for extraction using the same parameters as for standard volume samples. Extraction and qRT-PCR for all specimens were performed as previously reported[346], with the exception that additional standard curve testing was performed to ascertain the linearity of

qRT-PCR for RNA template corresponding to samples in the 1-20 p/mL range. Extraction and amplification was monitored with the previously described internal control[330]. Standard volume and DBS samples were tested in singlet while large volume samples were tested in duplicate (2 x 0.5 mL = 1 mL sampling volume). Results were reported for standard volume and DBS samples to 20p/mL (≥LLQ) with 'low positive below the LLQ' reported in the 10-20p/mL range (≥LLD). Large volume samples were reported to a quantitative limit of 2p/mL (≥LLQ) with 'low positive below the LLQ' reported for samples in the 1-2p/mL range (≥LLD).

2.2.3.5 Modelling of data to estimate parasite growth dynamics

For modelling purposes, in the following circumstances the qPCR value was replaced with the nominal value of 5 p/ml:

- Any qPCR negative time-points immediately followed by a qPCR positive time-point.
- qPCR values <5/ml but > 0p/mL.

Time in hours between CHMI and collection of each blood-sample was calculated using data specific to each volunteer for each of these events and then converted to days.

For VAC049 and KCS, simple linear regression[332] was then used to estimate;

(a) PMR: The fold change in number of parasites in the blood over 48 hours. PMR was only calculated for volunteers with 5 or more positive qPCR values post-CHMI.

$$PMR = 10\text{^}(m\times 2)$$

(b) LBI: The total number of parasites released from the liver into the blood at C+6.5 post-CHMI (BV = blood volume; 70mL per kg).

$$LBI = (m\times 6.5) - y)(BV)$$

In addition, LBI from VAC052 and KCS was calculated using the peak first-cycle parasitaemia method (highest qPCR value between C+6.5-7.5 multiplied by the blood volume[335]).

2.2.4 Immunology

2.2.4.1 Blood separation

Blood was collected in both serum and lithium heparinised vacutainer tubes (BD bioscience) and initiation of the relevant separation protocol(s) begun within 4 hours of blood collection.

Serum tubes were centrifuged at 755 *x g* for 5 minutes to separate serum, which was then removed by pipette and stored in 1-2 mL aliquots at -80°C.

Blood from lithium heparinised tubes was poured into pre-prepared Leucosep tubes (Greiner) and centrifuged at 1000 *x g* for 13 minutes (brake off) to allow separation of PBMC into a distinct layer. 1 mL aliquots of plasma were taken and stored at -80°C before the excess plasma containing PBMC was transferred to a new tube. This volume was then topped up and washed with R0 media twice, before PBMC were re-suspended in 10mL R10 media. After resting for 1 hour at 37°C, PBMC were counted, using a CasyCounter in VAC049 and Tryphan blue

(Sigma) in KCS.

1 million PBMCs were then added to 350µl of RLT-βME buffer. These cells were stored immediately at -20°C and transferred to -80°C within a week. Remaining PBMCs were stored in aliquots of 5-10 million cells/vial in filtered, heat treated fetal calf serum (FCS) and 20% DMSO. These cells were stored initially in 'Mr. Frosty' containers at -80°C and transferred to liquid nitrogen the following day.

2.2.4.2 ELISAs

2.2.4.2.1 Anti-AMA1, Anti-MSP1 and Anti-RH5 ELISAs

Sera from participants were analysed for antibody responses according to published standardised ELISA methodology[347] (Jenner protocol ML023 and ML011). Recombinant ETSR-MSP1$_{19}$ fusion protein (3D7 allelle)[348], GST control protein[348], 3D7 AMA1 protein and RH5 protein were diluted in PBS to a previously optimised concentration (2µg/mL). 50µL protein/well was adsorbed to 96-well Nunc-Immuno Maxisorp plates (Fisher Scientific) and stored at 4°C for approximately 16 hours. Plates were washed 6x in PBS/T and blocked with Caesin blocker (Pierce) for at least 1 hour at room temperature (RT). Test sera were diluted in PBS/T (1:300, 1:3,000 and 1:30,000) and added to wells in duplicate.

To make a standard curve, sera from hyperimmune individuals were diluted in PBS/T to 1:900 for MSP1 and GST ELISAs, 1:1000 for AMA1 ELISAs and 1:600 for RH5 ELISAs. These were then serially diluted 9-fold and added to wells in duplicate. The positive control was sera from hyperimmune individuals, diluted 1:3600 for MSP1 and GST ELISAs, 1:8000 for AMA1 ELISAs and 1:24,000 for RH5

ELISAs. The same positive controls were run in duplicate on each plate to allow for standardization of plate-to-plate variation. PBS/T was the negative control.

Plates were incubated for 2 hours at RT, after which they were washed 6x with PBS/T. Plates were incubated with secondary antibody (50µl/well of goat anti-human total IgG (γ-chain)(Sigma A3187) diluted 1:1000 in Caesin blocker) for 1 hour at RT. Plates were washed 6x with PBS/T and 100µl of DB added to each well.

Optical density was read at 405nm (OD405). Plates were read after 5 minutes incubation in the dark at RT or until positive control wells gave an expected OD405. Antibody units (dilution of sample required to give OD = 1.0) were multiplied by a conversion factor if the internal reference was > ±10% if the expected value.

2.2.4.2.2 Anti-schizont and Anti-MSP2 ELISAs

Individual wells of Dynex Immunolon 4HBX ELISA plates (Dynex Technologies Inc) were coated with 50ng of recombinant MSP2 (Dd2 allele)[349] per 100µL of carbonate coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.3). *P. falciparum* schizont extract (A4 and 3D7 parasite strains) was coated onto wells in PBS.[350] Plates were incubated overnight at 4°C, before washing four times in PBS/T, and blocking for 5 hours at RT with 1% skimmed milk in PBS/T (blocking buffer). Wells were washed again and incubated overnight at 4°C with 100µL of test sera (1:1000 dilution in blocking buffer). Plates were then washed 4x and incubated

for 3 hours at RT with 100μL of HRP-conjugated rabbit anti-human IgG, (Dako Ltd.) at 1:5000 dilution in blocking buffer before final washing and detection with H_2O_2 and O-phenylenediamine (Sigma). The reaction was stopped with 25μL of 2M H_2SO_4 per well and absorbance read at 492nm. The same positive controls (hyper-immune sera) were run in duplicate on each plate to allow for standardization of plate-to-plate variation. As high background reactivity was seen with non-malaria exposed sera (or UK sera) in results using 3D7 lysate, final results were obtained using A4 lysates (ELISA ODs to A4 and 3D7 lysates were highly correlated, data not shown).

2.2.4.2.3 IFN-γ ELISA

IFN-γ ELISA was performed using 'Human IFN gamma ELISA Ready-SET-Go!' (eBioscience, UK) as per the manufacturer's instructions. The standard curve range of the kit was 4-500 pg/mL, with baseline responses in healthy human volunteers predicted to be ≈0 pg/mL. At all time-points, neat serum or a 1:5 dilution was tested.

2.2.4.3 Functional assays of immunity to *P. falciparum*

2.2.4.3.1 *In vitro* growth inhibition activity assay

The ability of antibodies to inhibit growth of *P. falciparum* 3D7 strain parasites *in vitro* was assessed by a standardised GIA assay using purified IgG as previously described[235]. This assay was performed at the GIA Reference Center (Laboratory of Malaria and Vector Research, NIH). Serum from participants in VAC049 and KCS were tested against 3D7 parasites at 10mg/ml. IgG was purified from serum using protein G columns and purified polyclonal IgG samples were dialysed and

concentrated. Concentrated IgG was pre-absorbed with uninfected human O+ RBCs for 1 hour. *P. falciparum* late trophozoites and schizonts were mixed with test or control samples and culture medium in 96-well culture plates. The final concentration of the culture was 0.3 ± 0.1% parasitaemia, 1% haematocrit in growth medium. Each sample was tested in triplicate. Cultures were maintained for a single growth cycle (40-42 hours) and relative parasitaemia quantified by biochemical determination of parasite lactate dehydrogrenase.

2.2.4.3.2 Antibody dependent respiratory burst assay

ADRB activity assay was undertaken according to Jenner SOP: J196.

Merozoite Lysate (Mz) Preparation: 3 sets of *P. falciparum* Mz lysate were produced by D. Llewellyn at the Jenner Institute, University of Oxford using the following method: *P. falciparum* 3D7 clone parasites were maintained routinely in culture, as described previously[351]. 3 x 30mL cultures of *P. falciparum* at 10% hematocrit and 10% parasitaemia were grown. Parasitophorous vacuolar membrane (PVM)-enclosed merozoite structures (PEMS) were harvested and re-suspended at a concentration of 18.5 x 10^5 schizonts/mL in PBS.

Preparation of human polymorphonuclear neutrophils (PMNs): These were prepared fresh for each assay and used within 3 hours of collection. PMNs were isolated from whole blood collected in EDTA vacutainers (BD Bioscience). 4 mL blood was layered upon 5mL polymorphprep (Axis-Shield Diagnostics) and centrifuged at 450 *x g* for 40 minutes. The band containing PMNs was isolated and washed in PMN buffer (HBSS, 1% glucose, 0.1% BSA). Contaminating RBCs were lysed with ice cold 0.2% NaCl added for 20 seconds before the restoration

of isotonic conditions. Cell viability was confirmed by Trypan blue (Sigma) and purity determined by Giemsa stained slide before suspension in neutrophil buffer at 1 x 10^7 PMNs/mL. Giemsa stained slides were examined under a 100x oil immersion objective on a Leica DM2000 microscope and PMN purity assessed in at least 5 fields of view. If PMN purity was <95%, final dilution in neutrophil buffer was adjusted to ensure a final suspension of 1 x 10^7 PMNs/mL. RBC counting solution (RBCCS) was used to assess RBC contamination. If PMN purity was below 75% due to red blood cell contamination, the RBC lysis step was repeated.

Assessment of ADRB activity: 100 µL *P. falciparum* PEMS at 18.5 x 10^5 schizonts/mL was adsorbed onto Nunc opaque Maxi-sorp 96-well plates at RT overnight. Plates were then washed 3x with PBS and blocked for 1 hour with Casein block solution (Pierce) before a second set of 3x washes. 100 µL serum diluted 1:50 in PBS was then added and incubated for 1 hour at 37°C. Within 2 minutes of a final wash of the assay plate in PBS, 50µL isoluminol (0.04 mg/mL) (Sigma) and 50µL of isolated human PMNs at 1 x 10^7 PMNs/mL were added to each well and luminescence was read every 2 minutes for 1 hour.

Every sample was tested with a 3 different neutrophil donors. Maximum relative light units (RLU) were recorded and indexed against the hyperimmune positive control ('reference') maximum RLU as below:

$$\text{Indexed RLU} = \frac{Absolute\ maximum\ RLU\ of\ test\ sample}{Absolute\ maximum\ RLU\ of\ reference}$$

After indexing, the mean of the three replicates for each sample was reported.

2.2.4.4 Haemoglobinopathies

Genotyping for HbS and the common African 3.7-kb α-globin α^+-thalassaemia deletion were conducted by PCR as described in detail previously[352-354]. Participants with one α-globin deletion (-α/$\alpha\alpha$) were defined as heterozygous α-thalassemia, and those with two α-globin deletions (-α/-α) were defined as homozygous α-thalassemia. In KCS volunteers were excluded if they were heterozygous or homozygous for α-thalassemia or sickle haemoglobin.

2.2.4.5 Parasite genotyping

Parasite genotyping was performed in KCS only.

Sample Preparation: A 5ml blood sample collected in sodium heparin tubes (BD bioscience) was centrifuged at 440 *x g* for 5 minutes. Plasma was removed using a pipette and stored. The remaining cells and buffy coat were re-suspended with 5mL yellow RPMI. This suspension was then layered onto 3ml of Lymphoprep (Axis-shield 1114545) in a 15ml centrifuge tube and spun for 20 minutes at 440 *x g*. The layers above the RBC pellet were then discarded and 10mls of RPMI added to the RBC pellet. This was then centrifuged at 1200 *rpm* for 5 minutes and the supernatant aspirated and removed down to the RBC interface.

Removal of Granulocytes from RBC pellet: Granulocytes were separated from the remaining RBC pellet by making a 40% cell suspension with warm yellow RPMI (1.5 x pellet volume). An equal volume (2.5 x pellet volume) of warm plasmion (Bellon France) was then added and left to stand for 10 minutes in a 37°C incubator. After 10 minutes the supernatant was aspirated and discarded. The pellet was re-suspended with 10mls RPMI, centrifuged at 2000 *x g* for 5 minutes

after which the supernatant was aspirated and discarded. The pellet was re-suspended up to a final volume of 5mL using yellow RPMI and then centrifuged at 1200 *rpm* for 5 minutes.

Preparation of parasite RNA for later Analyses: 1000µl of warmed trizol (Invitrogen) was added to 100µl aliquots packed cells (100 p/µl). After thorough mixing these samples were stored at -80°C.

Preparation of parasite DNA for later analyses: CF11 columns in 10ml syringes were made as described in section 2.2.3.2, with the exception that approximately 11ml of CF11 powder was added so each syringe was filled to approximately 5.7mL. PBS was added to remaining packed cells so that the total sample volume equalled twice the original whole blood volume. The PBS volume was adjusted according to what had already been used for the Trizol step (i.e. if a 5ml sample was collected and 300µl of sample was removed for the trizol step, PBS was added to the sample at this point in order to make a total volume of 9.4mls).

The CF11 columns were pre-wet with 6ml of PBS followed by the blood-PBS mixture, both passing by gravity. 6ml of PBS was then added to the column as a wash and the plunger used to gently push the remaining sample through the column. The filtrate was then centrifuged at 1000 *x g* at RT for 10 minutes following which the supernatant was discarded and the pellet stored at -20°C.

Parasite Genotyping: MSP2 genotypes of the parasite DNA samples were assessed using the Agilent Bioanalyzer to examine the qPCR products following amplification of the MSP2 gene[355].

2.2.5 Transcriptomics

2.2.5.1 Sample collection & immediate processing

In KCS and VAC049, whole blood (PAXgene tubes) and PBMC samples were taken for gene expression studies at various, differing time points (Figure 2-4). PAXgene Tubes (Quiagen) contain a proprietary reagent composition based on a patented RNA stabilization technology. This reagent composition protects RNA molecules from degradation by RNases and minimizes induction of gene expression.

Immediately after sampling, PAXgene tubes were stored upright at room temperature (18°C to 25°C) for a minimum of 2 hours and a maximum of 72 hours before transferring to -20°C and subsequently to -80°C. Immediately after sampling, PBMCs were separated from whole blood using Lymphoprep and Leucosep tubes as described in section 2.2.4.1. One million PBCMs (un-stimulated) were then added to 350µl of RLT-βME buffer, stored at -20°C and transferred to -80°C within one week.

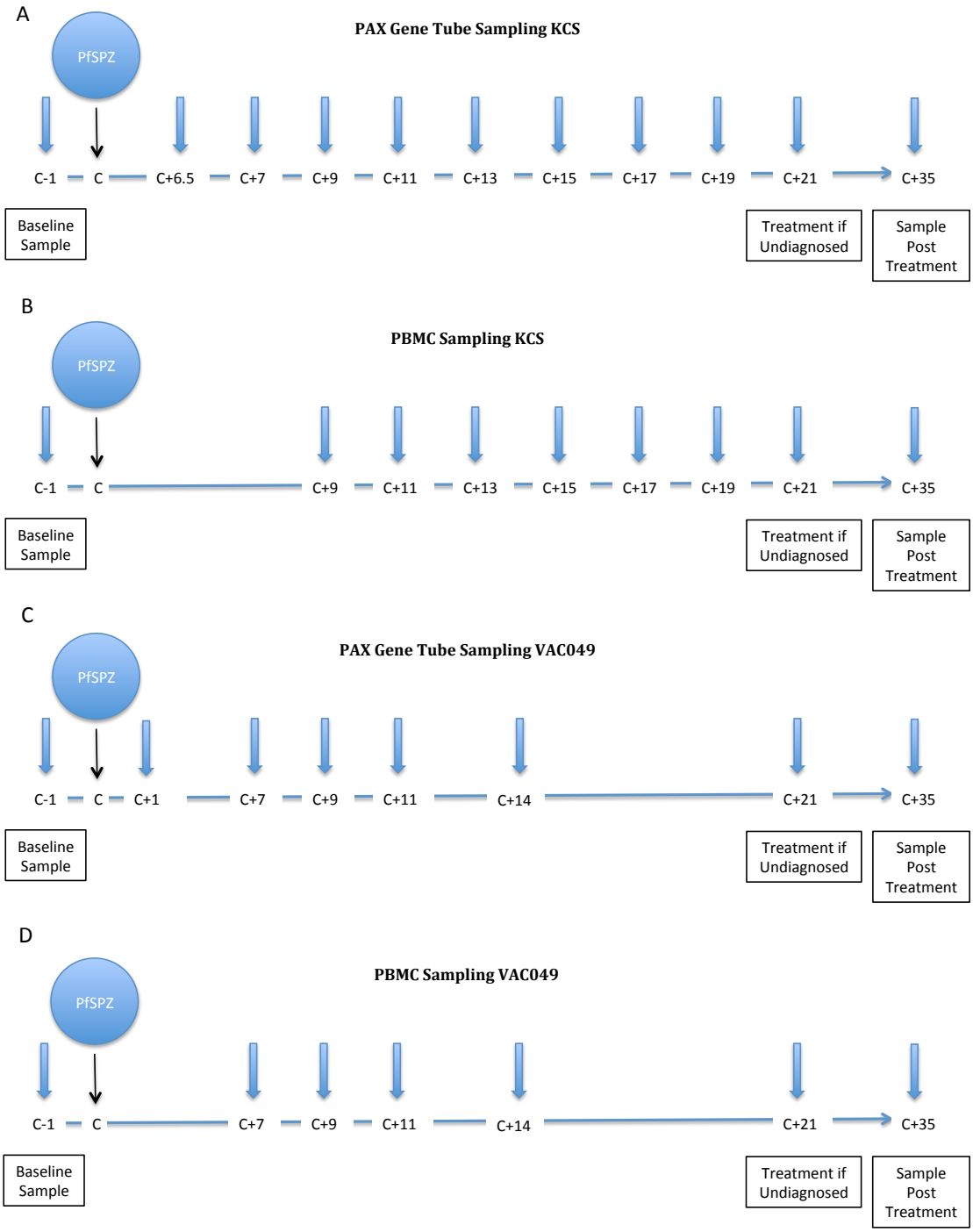


Figure 2-4: Schedule of collection of PAXgene and PBMC samples for KCS and VAC049

Blue arrows represent sample collection. C = challenge (administration of PfSPZ Challenge. (A) KCS PAXgene Samples (n=201): A sample was also taken at diagnosis if a PAXgene tube had not been drawn in the preceding 24 hours. (B) KCS

PBMC Samples (n=123). (C) VAC049 PAXgene Samples (n=117): A sample was also taken at diagnosis. (D) VAC049 PBMC Samples: A sample was drawn within 24 hours of diagnosis (but not if diagnosed on C+7, C+7.5, C+11 or C+11.5).

2.2.5.2 RNA extraction & amplification

RNA was manually extracted and purified from PAXgene samples using PAXgene-Blood-RNA-Kit (Quiagen). Briefly, nucleic acids pelleted following centrifugation were washed and re-suspended in optimised buffers. RNA was selectively bound to a silica membrane as contaminants passed through and DNase added to remove trace amounts of bound DNA. After the wash steps RNA was eluted in elution buffer and heat-denatured. During this process, microRNA was extracted and stored using RNeasy spin columns (Quiagen).

In order to improve the sensitivity of expression array data, Globin (α and β) mRNA (up to 70% of total mRNA population) was removed using the GLOBINclear™ Human Kit (Ambion). Briefly, globin mRNA was hybridised with biotinylated oligonucleotides and then mixed with streptavidin magnetic beads. Streptavidin bound to the biotinylated oligonucleotides, captures the globin mRNA on magnetic beads, which are pulled to the side of the tube with a magnet. RNA depleted of globin mRNA was then transferred to a fresh tube.

RNA was manually extracted from PBMC samples and purified using RNeasy® Mini Kit (Quiagen). Following lysis, the sample was added to an RNeasy Mini spin column where RNA molecules <200 nucleotides bind to the silica-based membrane and contaminants are washed away. This procedure allows

enrichment of mRNA.

Extracted RNA was quantified using spectrophotometry (Nanodrop 2000, Thermo-Scientific). The quality of extracted RNA for a selection of samples was assessed using on-chip gel electrophoresis (Bioanalyzer 2000, Agilent). GLOBINclear™ was repeated on RNA from 3 KCS PAXgene samples with initial poor quality control (QC) measures (205 C+35, 216 C-1 and 105 C+35) and the samples relabelled (205 C+35R, 216 C-1R and 105 C+35R respectively).

Further processing of RNA was then performed by the High-throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, University of Oxford according to Illumina® protocols. This included a first and second-strand reverse transcription step followed by a single *in vitro* transcription amplification that incorporated biotin labelled nucleotides.

2.2.5.3 Microarrays

Hybridisation to the Illumina BeadArray microarray platform was performed at the High-throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, University of Oxford according to manufacturer's protocols. 11µl of samples at a concentration of 50ng/µl were randomly allocated for hybridisation across Illumina HT-12 v4 BeadChips (47,231 probes) to avoid geographic biases in expression estimates. The same operator performed all the microarrays in four batches.

The BeadArray technology consists of 3µm silicon beads, which are randomly assembled into microscopic wells on a silicon chip. Each bead has hundreds of thousands of copies of a single oligonucleotide (50 base pair sequence) that acts

as the capture sequence (probe) for a specific target RNA sequence. There is redundancy in detection with multiple beads sharing the same oligonucleotide sequence: on average 30 replicate beads in the Human HT12-v4 array. In addition, more than one probe is usually designed for each gene. Probes usually target the 3' end of the gene.

Quantitation was performed using the Illumina iScan system and raw probe level intensities were outputted using Illumina's GenomeStudio version 1.6.0.

2.2.5.4 Quality control of microarray data

Raw data were imported into the R statistical software[356] for further processing and analysis using BioConductor packages[357]. Raw signal intensities were background corrected (using array-specific measures of background intensity based on negative control probes), prior to being transformed and normalised using the Variance Stabilization and Normalization (vsn) package.[3]

A range of QC checks were undertaken including assessment of technical control and sample metrics, comparison of boxplots of normalised expression values between samples, hierarchical clustering of all samples and principal component analysis (PCA) to identify potential outlier samples. Microarrays were repeated for two PAXgene samples from KCS with poor QC metrics (151 C-1 and 216 C-1R) and the best quality results used for subsequent analysis.

2.2.5.5 Differential gene expression analysis using limma

Differentially expressed genes were identified using the widely accepted statistical R package; limma[358] (Linear Models for Microarray Analysis) and arrays weighted by their quality score. Data were also adjusted for microarray

hybridisation scan dates. Age and gender were matched between subjects with a history of exposure to malaria (DefExp) and those without (MinExp). Nominal p-values were corrected for multiple testing using the false discovery rate (FDR) controlling procedure of Benjamini and Hochberg[5].

Limma is designed to analyse complex experiments involving comparisons between many RNA targets simultaneously. The package fits a linear model to the expression data for each gene followed by an empirical Bayes method that borrows information across genes to shrink the variance to a common value. This means the variance estimates for a particular probe are more reliable even when there are a small number of samples.

The differential expression method was used to identify:

1. Differences across the whole time course (*whole series analysis*).
2. Differences between two time points:
    a. Differences between baseline (C-1) and a subsequent time point (e.g. C6.5 v C-1) (*baseline comparisons*).
    b. Differences between two consecutive time points (e.g. C+7 versus C+6.5) (*consecutive comparisons*).
3. Differences between MinExp and DefExp groups.
4. Differences between patients who demonstrated a delay to diagnosis (DTD) and those who did not (NDTD).

In KCS, volunteers were classified as demonstrating a DTD if treatment was started > 2 times the standard deviation in days after the mean time to diagnosis

of the MinExp group (i.e. those diagnosed >13.2 days post administration of PfSPZ Challenge). Four volunteers, all in the DefExp group, demonstrated a DTD (Volunteers 110, 150, 151 and 220).

For the KCS PAXgene dataset, we restricted analyses to time points where at least 21 samples were available. As result, the series for analysis included the following time points:

- C-1: n=27 – sample 216 outlier excluded
- C+6.5: n=28
- C+7: n=28
- C+9: n=28
- C+11: n=24 – samples 178, 189, 127, 209 missing
- DIAG: n=22 - samples 105, 110, 167, 197, 216, 220 missing.
- C+35: n=27 – sample 220 missing

2.2.5.6 Functional principal components analysis

Changes in gene expression can be modelled as a function of time. One of the main limitations of linear models is that they cannot account for the nonlinear nature of a transcriptional time series dataset. This is illustrated in Figure 2-5 where both Genes A and B have the same fold change estimate over time and would be considered to be equally differentially expressed with a linear model.

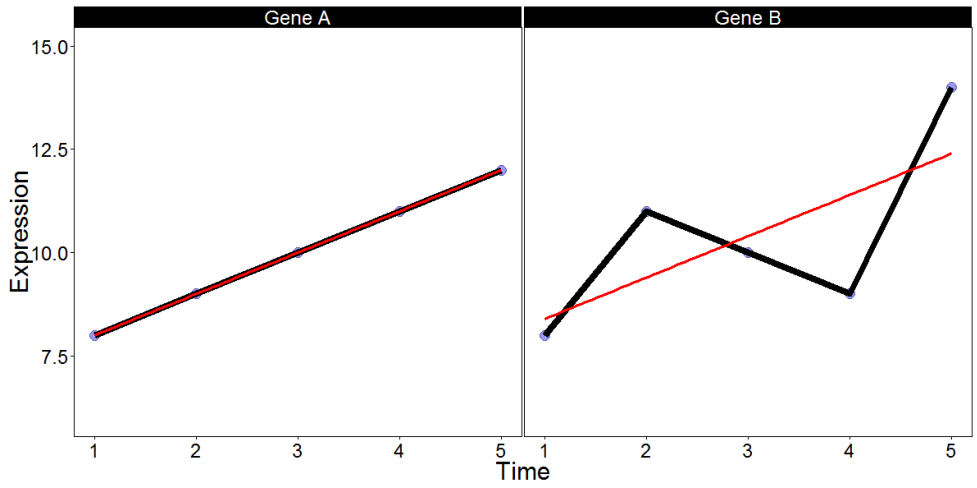


Figure 2-5: Hypothetical expression of Gene A and B overtime post-CHMI

Y axis = probe expression.

Functional principal components analysis (fPCA) is an alternative time course analysis method which tests for significant deviation from mean probe expression over the entire time course[359,360]. This approach predicts each probe's expression trajectory, respectively, under the null and the alternative hypotheses. An *F*-type statistic is then constructed, and the empirical p-value distribution obtained for each probe. By estimating each individual subject's expression profile and combining this data, fPCA is a robust method to select clusters of functionally related genes based on similarities in their expression pattern over time. We performed this analysis with 10,000 permutations of sample labels according to published algorithms[359,360]. Time points were treated as uniform intervals. All data from subject 216 were excluded from the fPCA analysis as the baseline sample did not pass QC.

2.2.5.7 Enrichment analysis of differentially significant probes

Probes were annotated with gene information using the relevant BioConductor annotation package and Ingenuity Pathway Analysis.

Biological pathway enrichment was generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com).

Modular mapping was used to present up or down regulation of pathways, where each gene set was assigned to a module using online pathway databases and published literature[361,362].

2.2.6 Statistical Analyses

Statistical analyses for transcriptomics data are outlined in section 2.2.5. Other statistical analyses were carried out using Prism v.5.03 (Graphpad, USA). Individual, geometric mean (GM) or median responses for measurements within each group were described. Parasite densities and certain other measurements were log transformed to remove skewness, with 1 added to each value to allow transformation of zero values.

Comparisons between two groups were conducted using Mann-Whitney tests, or Wilcoxon matched-pairs signed rank test when data were paired. Comparisons between three or more groups were assessed by means of a Kruskal-Wallis test (independent groups) or a Friedman test (paired data/repeated measures of samples). Correlations were assessed using Spearman's rank correlation coefficient. Time to treatment was presented using Kaplan-Meier survival curves

and between groups comparisons made using the log-rank test. All p values in this thesis are two sided and p values <0.05 were considered significant.

CHAPTER 3: ASSESSMENT OF THE SAFETY AND INFECTIVITY OF

PfSPZ CHALLENGE ADMINSTERED IM IN UK MALARIA-NAÏVE ADULTS (VAC049)

3.1 Authorship statement

The work presented in this Chapter is part of a collaborative effort. Unless otherwise stated, all work was undertaken by myself.

The conduct of the trial was greatly assisted by the Jenner Institute Clinical Trials team (including Alison Lawrie, Rachel Roberts, Ian Poulton, Mary Smith, Raquel Lopez Ramon and Nicholas Anagnostou) and staff at Sanaria Inc (including Stephen Hoffman, Peter Billingsley, Eric James and Kim Lee Sim).

Nick Edwards and Domtila Kimani performed the qPCR, Alex Spencer and Rhea Longley processed and stored samples for later immunological and transcriptomic studies and Alexander Douglas assisted with the parasite modelling.

Blood samples for FBC, biochemistry and serology (for HIV, Hepatitis B and C) were processed at the Oxford University Hospitals' Trust laboratories, Oxford.

3.2 Introduction

The development of cryopreserved, aseptic *P. falciparum* sporozoites (SPZ) for injection (PfSPZ Challenge) is a practically feasible potential solution to aid the conduct of CHMI studies in Africa[222]. It also provides the opportunity to reduce variation in infectious dose between parallel clinical trials at multiple sites or sequential clinical trials at the same site[310]. However, in the first clinical trial where PfSPZ Challenge was administered ID) which enrolled malaria-naïve Dutch subjects, in all three dose groups (2,500 SPZ, 10,000 SPZ and 25,000 SPZ)

only 5/6 subjects (83%) were successfully infected, with no differences between groups in time to blood-film or qPCR positivity or parasite kinetics[311]. The failure to identify a dosing regimen that successfully infected 100% of volunteers limits the future application of PfSPZ Challenge[311]. Given that murine studies demonstrate that IM injection is associated with greater infectivity than ID[312], the next logical step was to assess the safety and infectivity of PfSPZ Challenge administered IM in humans in order to identify a regimen that reliably infected all subjects.

3.2.1. Null hypothesis

There is no significant difference in the infectivity of PfSPZ Challenge administered by needle and syringe by ID or IM routes.

3.2.2. Study objectives

- *Primary Objective:* To determine the infectivity rates of PfSPZ Challenge administered in various ID and IM regimens.
- *Secondary Objective:* To assess the safety of PfSPZ Challenge administered in various ID and IM regimens.
- *Tertiary Objectives:* To determine the dynamics of *P. falciparum* parasite growth following PfSPZ Challenge administered in various ID and IM regimens.

3.2.3. Study design

The study was an open label, non-randomised pilot study with blinded laboratory outcome assessment (Table 3-1). The study was conducted at the

CCVTM, University of Oxford and was allocated the study code 'VAC049.' Healthy, malaria-naïve males and non-pregnant females aged 18-45 years were invited to participate. An identical group to that in the Dutch study was included in the trial design (Group 1; 2,500 SPZ ID)[311] to allow comparison of results across the two centres and an assessment of batch effect on PfSPZ Challenge infectivity.

Group	No. of subjects	Total Dose of PfSPZ	Total Volume Administered	Route of Administration	No. of injection sites
1	6	2,500 SPZ	50µl x 2*	ID	Two*
2	6	2,500 SPZ	50µl x 2*	IM	Two*
3	6	25,000 SPZ	50µl x 2*	IM	Two*

Table 3-1: Overview of trial groups VAC049

** 1 injection per deltoid area. Each injection was dosed in a separate syringe. SPZ = P. falciparum sporozoites (PfSPZ Challenge); ID = intradermal; IM = intramuscular.*

3.2.4. Study outcomes

Volunteers were treated when pre-defined criteria were met. These included: blood film, qPCR and clinical data (Chapter 2). Time to diagnosis (TTD), LBI and PMR were calculated (methods detailed in Chapter 2).

3.3 Results

3.3.1 Study participants

Recruitment took place between September and November 2011. Eighteen healthy malaria-naïve adult participants (8 female and 10 male) underwent CHMI in November 2011 (Figure 3-1). The mean age of participants was 24.2 years (range 18 – 37) (Table 3-2).

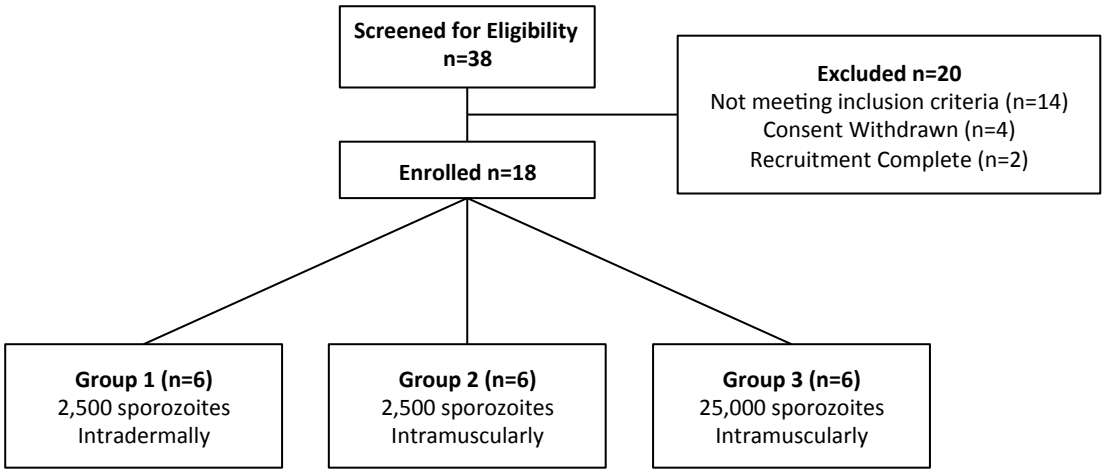


Figure 3-1: Flow chart of volunteer recruitment for VAC049

20 participants were excluded following screening for the following reasons: significant psychiatric history (n=6); consent withdrawn (n=4); recruitment complete (n=2); lack of response from General Practitioner to medical screening letter (n=2); unexplained systolic murmur, elevated alanine transaminase, unexplained tachycardia and significant prior malaria exposure (n=1 for each complaint).

	2,500 ID	**2,500 IM**	**25,000 IM**
	n = 6	**n = 6**	**n = 6**
Sex			

Male	3	3	4
Female	3	3	2
Age at screening (years)			
Mean ± SD	23.8 +/- 5.0	26.2 +/- 7.6	22.5 +/- 3.0
Median	22.00	23.50	21.00
Min, Max	20, 33	18, 37	20, 28

Table 3-2: Demographics of enrolled volunteers in VAC049

ID = intradermal; IM = intramuscular.

All participants received injection of PfSPZ Challenge as scheduled with the exception of one volunteer in Group 1 who received approximately 10% less than the scheduled dose due to some of the inoculum leaking from the administration site post-injection. All participants completed the study as scheduled. The time between thawing and administration of PfSPZ Challenge was < 30 minutes for all subjects.

3.3.2 Infectivity of PfSPZ Challenge

Five out of six participants receiving 2,500 SPZ ID in Group 1, 3/6 participants receiving 2,500 SPZ IM in Group 2 and 6/6 participants receiving 25,000 SPZ IM in Group 3 were successfully infected with malaria (Figure 3-2 and Table 3-3). Of note, the participant in Group 1 who received a lower dose of PfSPZ Challenge than planned was not infected with malaria. The median TTD was 13.2, 17.8 and 12.7 days for 2,500 SPZ ID, 2,500 SPZ IM and 25,000 SPZ IM respectively (*Kaplan Meier analysis; p=0.024 log rank test*). No significant difference in TTD was seen

between volunteers receiving 2,500 SPZ ID and 25,000 IM (*Kaplan Meier analysis; p=0.592; log rank test*).

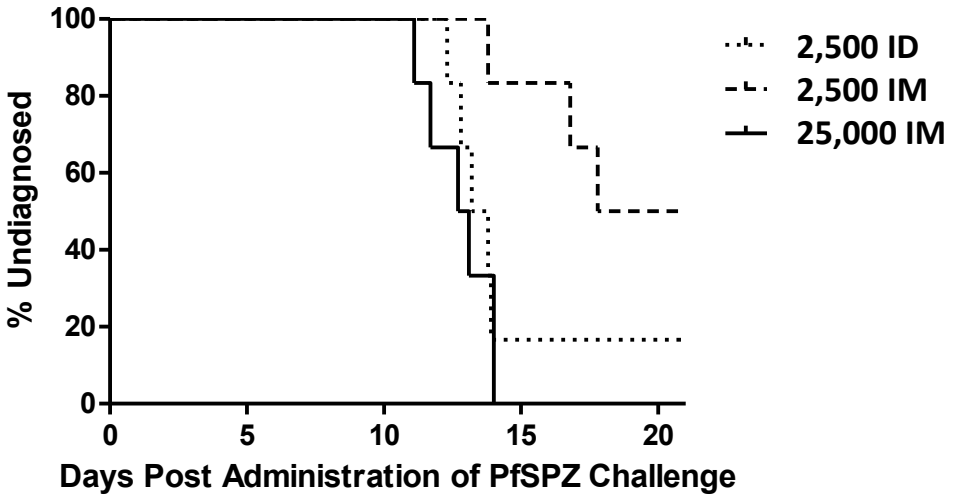


Figure 3-2: Time to patent parasitaemia for VAC049

Days post-administration of PFSPZ Challenge = day between injection and diagnosis. p=0.024 log rank test. Median pre-patent period = 13.19 days for 2,500 SPZ ID, 17.8 days for 2,500 SPZ IM, 12.72 days for 25,000 SPZ IM. ID = intradermal, IM = intramuscular.

	2,500 ID n=6	**2,500 IM n=6**	**25,000 IM n=6**
Symptomatic with positive BF	1 (17%)	2 (33%)	2 (33%)
Symptomatic with negative BF	0	0	0
Asymptomatic with positive BF & PCR > 500 parasites/ml	4 (66%)	1 (17%)	4 (66%)
Reached Day 21 undiagnosed (asymptomatic, BF & PCR negative)	1 (17%)	3 (50%)	0

Withdrew Consent or Excluded From Study Prior to Primary Endpoint	0	0	0

Table 3-3: Criteria for treatment of subjects in VAC049

BF = blood film; ID = intradermal; IM = intramuscular.

3.3.3 Modeling of parasitaemia measured by qPCR

Figure 3-3 plots the qPCR results for each individual in the trial. No positive results were obtained from any of the 82 blood samples from the four individuals who were not diagnosed with malaria (246 individual replicate qPCR reactions; Table 3-4). A significant difference in LBI was seen between groups in agreement with the pre-patent periods (*p=0.016 Kruskal-Wallis test*; Figure 3-4). However, no significant difference in LBI was seen when this analysis was restricted to successfully infected subjects *(p=0.144 Kruskal-Wallis test*). No significant difference in LBI was seen between subjects receiving 2,500 SPZ ID and 25,000 SPZ IM (*p=0.238 Mann Whitney test*). Of note, all PfSPZ dosing regimens assessed led to markedly lower LBIs compared to that reported in mosquito-bite CHMI studies in European centres (240,000 – 2,835,000 parasites)[305]. As expected, there was no difference in PMRs between groups (*p=0.518 Kruskal-Wallis test;* Figure 3-4), however an inverse correlation was seen between LBI and PMR, which was most likely an artefact of the modelling method (Figure 3-4).

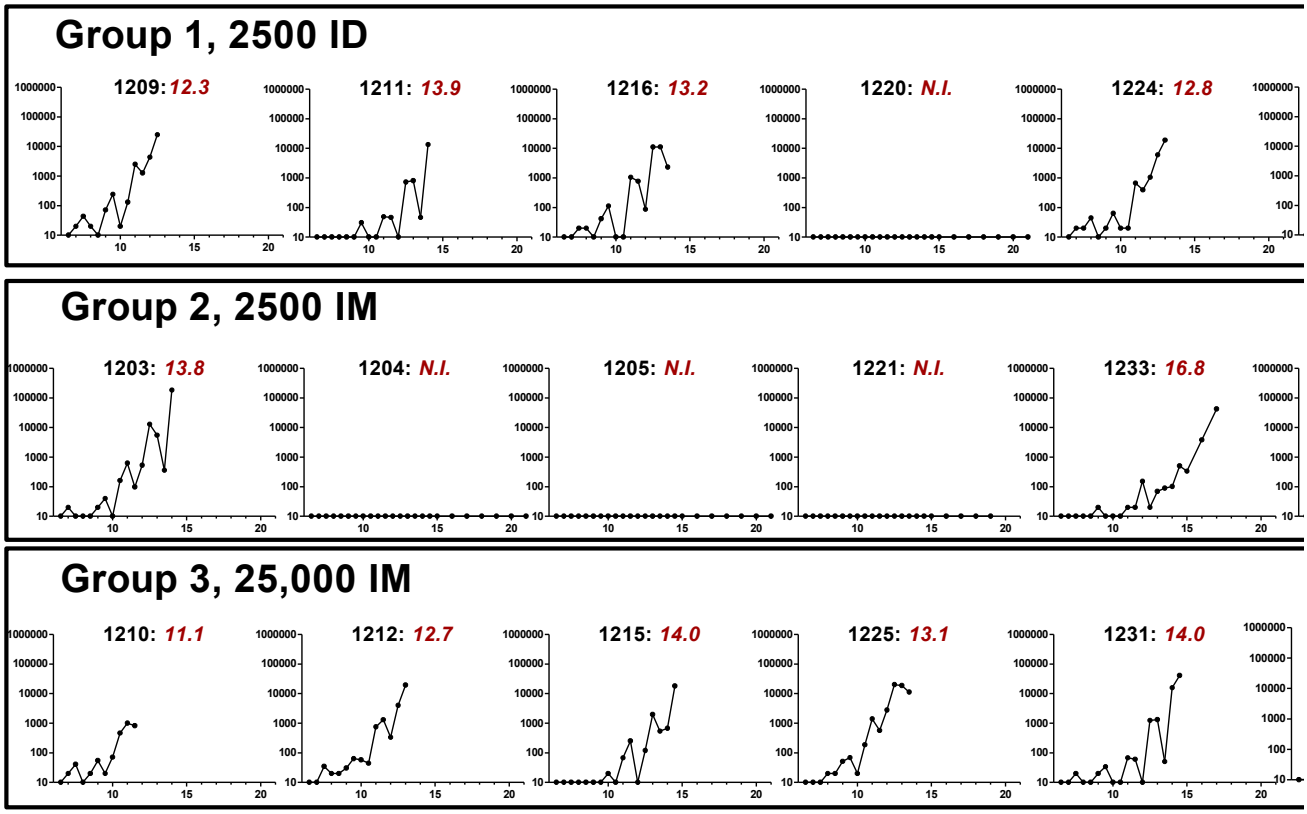


Figure 3-3: qPCR data for VAC049

Y axis = qPCR (p/mL). X axis = days post-injection of PfSPZ Challenge. Black subtitles indicate subject identification numbers; red subtitles indicate time to diagnosis by thick film microscopy in days. NI = not infected.

	vol no.	C+6.5	C+7	C+7.5	C+8	C+8.5	C+9	C+9.5	C+10	C+10.5	C+11	C+11.5	C+12	C+12.5	C+13	C+13.5	C+14	C+14.5	C+
2,500 ID	1209	5	7	23	12	5	62	232	10	122	2491	1269	4348	25033					
	1211	0	0	0	0	0	5	14	0	5	39	24	5	720	807	36	13523		
	1216	0	0	5	5	5	32	103	0	5	1047	766	78	11209	11234	2327			
	1220	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	1224	0	5	5	23	5	13	54	6	14	660	386	1044	6031	19075				
	1228	0	5	5	5	5	6	7	5	19	178	21	413	4316	1565	68	59262		
2,500 IM	1203	5	5	0	0	5	18	20	5	153	617	89	526	12995	5538	352	185818		
	1204	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	1205	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	1221	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	1233	0	0	0	0	5	5	0	0	5	11	5	143	19	60	80	92	497	3
	1236	0	0	0	0	5	6	0	0	0	5	5	0	5	12	26	26	56	3
25,000 IM	1210	5	5	21	5	13	30	5	62	456	1000	820							
	1212	0	5	17	14	5	21	54	48	35	745	1302	326	4026	19715				
	1215	0	0	0	0	0	0	5	5	5	58	246	5	111	1974	528	666	18139	
	1225	0	0	5	14	11	42	59	18	178	1396	558	2798	20309	18951	11380			
	1231	0	5	5	0	5	7	16	0	5	58	51	5	1227	1317	41	15954	41087	
	1235	0	5	9	24	14	85	204	5	98	3289	3695	2214						

Table 3-4: qPCR data for VAC049

Date = parasites/ml measured by qPCR. Top row represents day of follow-up visit post administration of PfSPZ Challenge. Neg = qPCR negative including time points where at least 2/3 replicates were negative; Red = time-point where treatment started.

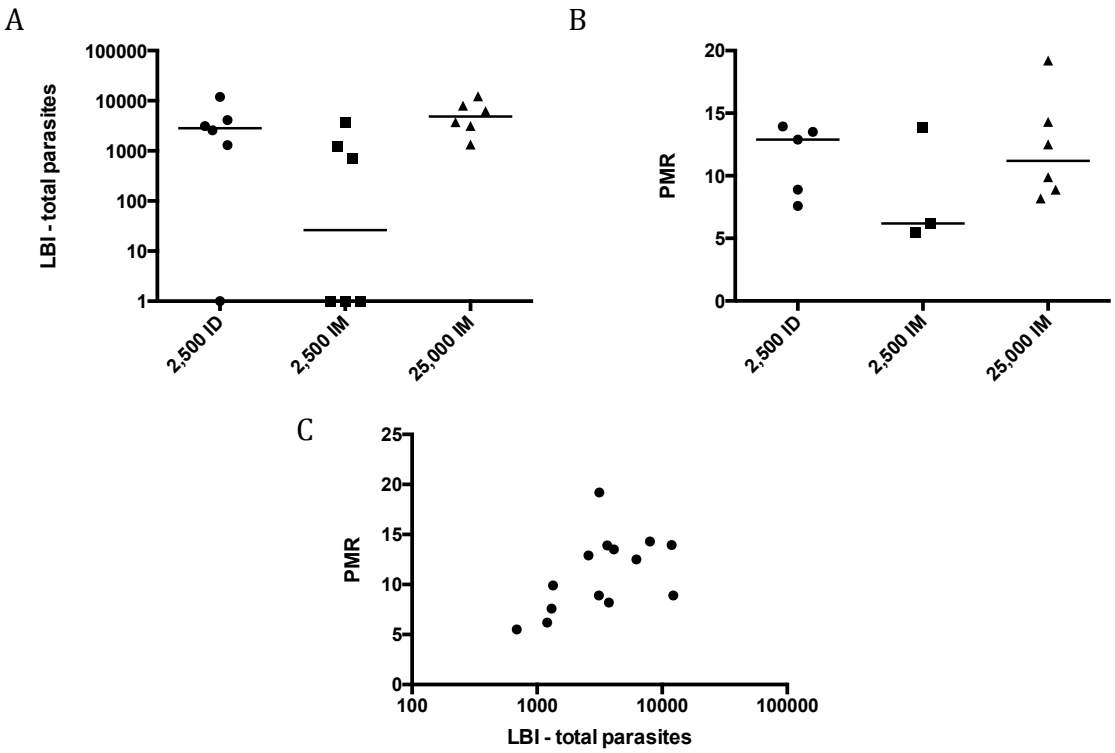


Figure 3-4: Parasite modelling data for VAC049

(A) Estimation of liver to blood inoculum (LBI) according to group, median values indicated (2,500 ID = 2844 parasites, 2,500 IM = 345 parasites, 25,000 IM = 4985 parasites). p=0.016 for all volunteers (n=18), p=0.144 for infected volunteers only (n=14) Kruskal Wallis test. (B) Estimation of parasite multiplication rate (PMR) median values indicated (p=0.423 Kruskal Wallis test). (C) Correlation between LBI and PMR.

3.3.4. Safety Data

3.3.4.1 PfSPZ Challenge Reactogenicity

All AEs following injection of PfSPZ Challenge were mild and short lived (Figure 3-5). No serious AEs occurred. One participant who received 25,000 SPZ IM

described gum bleeding on teeth-brushing, starting the evening following injection of PfSPZ Challenge. FBC and clotting assays were normal and there was no visible pathology on examination. This AE resolved within 2 days and did not recur. No laboratory AEs related to PfSPZ Challenge injection were noted.

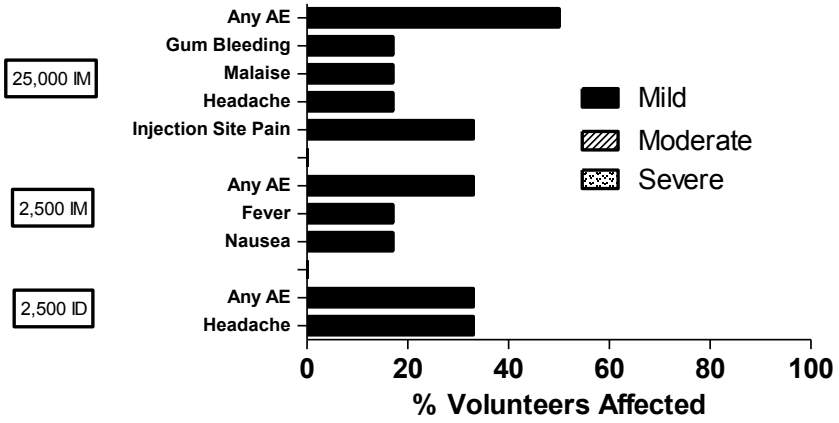


Figure 3-5: AEs related to PfSPZ Challenge for VAC049

AEs deemed definitely, probably or possibly related to PfSPZ Challenge infection (excluding symptoms related to resultant P. falciparum infection) in VAC049. Data are combined for all AEs for all volunteers receiving the same dose of PfSPZ. There were no serious AEs.

3.3.4.2 PfSPZ induced clinical *Plasmodium falciparum* infection

One of the 14 participants diagnosed with *P. falciparum* malaria experienced no symptoms or signs of malaria infection. The total duration of symptoms in participants with symptomatic malaria infection ranged from 1-12 days (mean 6.0 +/- 3.12 days), similar to that seen at a recent mosquito-bite CHMI trial at the CCVTM[309], and there appeared no difference in duration of symptoms of malaria

between the groups (Figure 3-6). Two volunteers diagnosed with malaria (14.3%) had a fever prior to diagnosis (38.2°C & 38.3°C) and one volunteer developed a fever post-treatment (37.6°C). Nine out of 14 participants diagnosed with *P. falciparum* (64.3%) experienced at least one AE post-challenge that was severe in intensity (Figure 3-7). There were no serious AEs. No participants were admitted for in-patient management of malaria infection. Safety bloods taken at C+9, C+35, C+90 and within 24 hours of diagnosis demonstrated transient laboratory abnormalities at frequencies and severities expected following *P. falciparum* infection (Figure 3-8)[284].

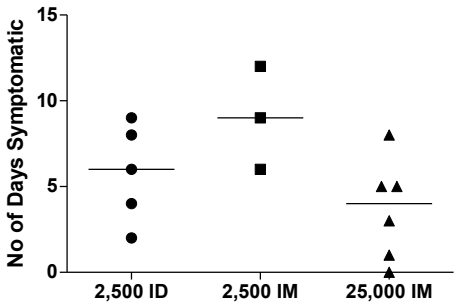


Figure 3-6: Duration of symptoms and signs associated related to malaria infection in VAC049

Duration of symptoms and signs related to malaria infection in individuals diagnosed with malaria in VAC049 (n=14). 2,500 SPZ ID: mean 5.8 days, median 6.0 days. 2,500 SPZ IM: mean 9.0 days, median 9.0 days. 25,000 SPZ IM: mean 3.7 days, median 4.0 days. Median values for each group are indicated.

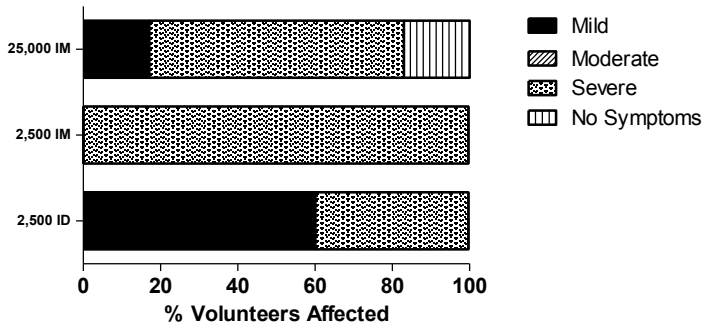


Figure 3-7: Maximum severity of any AE related to malaria infection in VAC049

Maximum severity of any AE deemed possible, probably or definitely related to malaria infection in individuals diagnosed with malaria in VAC049 (n=14). Laboratory AEs excluded.

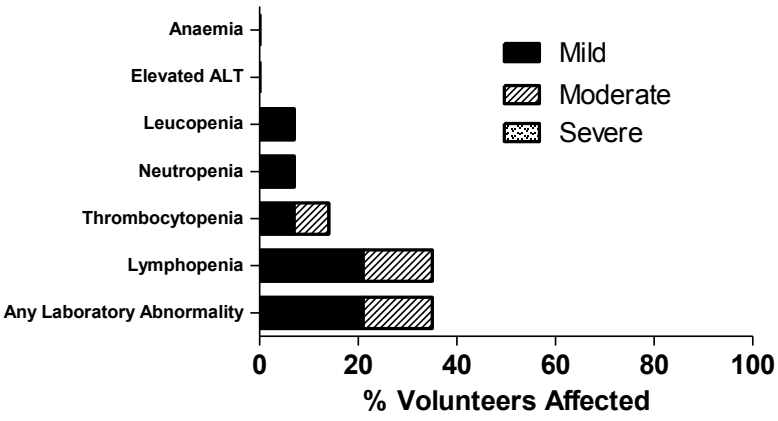


Figure 3-8: Laboratory AEs related to malaria infection in VAC049

Laboratory AEs deemed possibly, probably or definitely related to P. falciparum infection. For 'any laboratory abnormality' only the highest intensity laboratory AE per subject is counted. ALT = Alanine transaminase.

3.4 Discussion

This study increases the clinical evidence that PfSPZ Challenge is a potent and safe product capable of inducing *P. falciparum* infection in malaria-naïve individuals. In those participants who developed symptomatic *P. falciparum* infection, the severity and duration of symptoms were reassuringly similar to those seen following CHMI administered by mosquito-bite at the same site[309], and no concerning AEs were noted following injection of PfSPZ Challenge.

This was the second study to assess the infectivity of 2,500 SPZ of PfSPZ Challenge administered ID. The result for this dosing schedule (5/6 participants successfully infected, pre-patent period 13.2 days) is near identical to that reported by *Roestenberg et al* (5/6 participants successfully infected, pre-patent period 13.0 days)[311]. Given that different batches of PfSPZ Challenge, manufactured two years apart, were used for each of these trials, this result demonstrates the reproducibility of PfSPZ Challenge as a challenge agent.

For the first time, this study has identified an administration regimen for PfSPZ Challenge (25,000 SPZ IM) that successfully infects 100% of participants. However, given there was no difference in pre-patent period or LBI between 25,000 SPZ IM and 2,500 SPZ ID it is likely that the increased proportion of individuals successfully infected with 25,000 SPZ IM is consistent with chance.

In contrast to murine data, administration of 2,500 SPZ ID was associated with higher infectivity (in terms of proportion of participants infected, duration of pre-patent period and LBI) than 2,500 SPZ administered IM, suggesting ID administration is a more effective route for delivering SPZ to the liver in humans.

The reason for the discrepancy between murine and human models is not clear, however, this finding might be intuitive given that ID injection by needle and syringe most closely resembles injection via a mosquito's proboscis. Alternatively, other factors such as administration volume could impact on infectivity.

In VAC049, a dose response was seen when PfSPZ Challenge was administered IM, with 25,000 SPZ appearing more infectious than 2,500 SPZ. This is in contrast to the study by *Roestenberg et al*[311], where increasing the dose of SPZ ID failed to translate into an increase in the proportion of participants successfully infected. The reason for this difference is unclear but supports the evaluation of higher doses of PfSPZ Challenge IM, which may, in contrast to ID administration, lead to identification of a dose that reliably infects 100% of participants.

The pre-patent periods of infected participants in this trial were longer than those seen in participants undergoing CHMI by mosquito-bite at the same site[309]. This and parasite modelling data, support the conclusion that PfSPZ Challenge (administered by needle and syringe in the dosing regimens evaluated in VAC049) is not as effective at delivering SPZ to the liver as five mosquito-bites.

Having identified an IM administration regimen for PfSPZ Challenge that was both safe and infectious, the next step in this work was to conduct the first modern sporozoite CHMI study in Kenya, using PfSPZ Challenge (Chapter 4).

CHAPTER 4: ASSESSMENT OF THE SAFETY AND INFECTIVITY OF PfSPZ CHALLENGE ADMINSTERED IM

IN KENYAN ADULTS (KCS)

4.1 Authorship statement

The work presented in this Chapter is part of a collaborative effort. Unless otherwise stated, all work was undertaken by myself.

The conduct of the trial was greatly assisted by the KEMRI Centre for Clinical Trials team (including Elizabeth Juma, Bernhards Ogutu, Amina Salim, Daniel Njenga, Ken Awuondo, Brett Lowe, Charles Magiri and Alfred Muia) and staff at Sanaria Inc (including Stephen Hoffman and Peter Billingsley).

Sean Elias, Charles Magiri, Alfred Muia and Michelle Muthui processed and stored samples for later assays, Domtila Kimani performed the qPCR, Michelle Muthi performed the parasite genotyping and Philip Bejon assisted with the parasite modelling. Screening of samples for haemoglobinpathies was performed by Alex Macharia.

Blood samples for FBC, biochemistry and serology (for HIV, hepatitis B and C) were processed at the Lancet Laboratories, Nairobi.

4.2 Introduction

The conduct of modern CHMI trials in Africa has been greatly limited by the lack of appropriate entomology staff and facilities necessary to conduct mosquito-bite CHMI studies[287]. Promising safety and infectivity data from early trials of PfSPZ Challenge, including VAC049 (see Chapter 3)[311,316] made sporozoite CHMI studies in Africa a practical reality for the first time. As well as allowing earlier assessment of the efficacy of candidate malaria vaccines and drugs in the target population, CHMI studies in Africa would provide a novel method of assessing the dynamics and mechanisms of NAI in individuals where the timing and infectious load is predetermined[222]. Detailed, regular sampling in such studies would allow, for the first time, detailed analysis of both changes in parasitaemia and immunological responses, particularly in the liver and early blood-stages of infection, over time.

Currently, there is no validated assay that can predict NAI[222]. If such an assay were identified, this would greatly aid research by allowing stratification of individuals according to NAI and targeted, detailed analysis of their immunological responses. It is assumed that individuals with NAI can control parasite growth *in-vivo*. Samples from malaria exposed individuals that can reduce PMR in a CHMI setting could therefore be used to validate candidate assays of NAI, such as those measuring GIA or ADRB activity, or as the basis of more exploratory, hypothesis-generating studies to identify novel mechanisms of NAI.

This work set out to perform the first sporozoite CHMI study in Kenya. Primarily, the objective was to assess the safety and infectivity of PfSPZ Challenge administered IM in Kenyan adults. However, the study design included adults with varying degrees of prior exposure to malaria in order to allow an evaluation, for the first time, of the effect of NAI on parasite growth dynamics post-CHMI.

At the time of the study design, no modern CHMI study had been performed in Africa. The trial was therefore a pilot study, with an empirical design, the results of which were hoped to inform and rationalise future CHMI studies in Africa.

4.2.1. Null hypothesis

There is no difference in the parasite growth rates between subjects with minimal and definite prior exposure to malaria.

4.2.2. Study objectives

- *Primary Objective:* To establish and assess the CHMI model using IM administered PfSPZ Challenge in individuals with varying degrees of prior exposure to *P. falciparum* infection in an African setting.
- *Secondary Objective:* To examine the relationship between NAI to *P. falciparum* malaria and laboratory assays of anti-malarial responses that could reflect NAI.

4.2.3. Study design

The study was an open label, non-randomised pilot study with blinded laboratory outcome assessment (Figure 4-1). The study was conducted at the

KEMRI Centre for Clinical Research, Nairobi and was allocated the study code 'KCS.' Healthy, males and non-pregnant females aged 18-40 years were invited to participate.

A dose of 125,000 SPZ IM was chosen for the main study cohort (n=20), 10-fold higher than the largest dose administered in VAC049. It was hoped that given the clear dose-response relationship in infectivity seen with IM PfSPZ Challenge in VAC049 (Chapter 3)[316], this dose would increase the number of infected hepatocytes, and ensure successful infection of all participants. It was hypothesised that individuals with NAI could clear low-level parasitaemia prior to detection by blood film or even qPCR. For this additional reason, a higher number of SPZ was chosen in order to ensure detectable parasitaemia post-CHMI. Since the maximum dose of PfSPZ Challenge administered IM to humans at the time of study design was 25,000 IM[316], two additional, small lead-in cohorts receiving lower doses of 25,000 (n=4) and 75,000 (n=4) SPZ IM were included in the study design to allow assessment of safety prior to administration of 125,000 SPZ IM.

Given the lack of a known assay to reliably assess NAI, both serological (anti-schizont and anti-MSP2 ELISA responses) and historical geographical data were used as criteria for grading prior exposure to *P. falciparum,* as a surrogate measure of NAI (see Chapter 2).

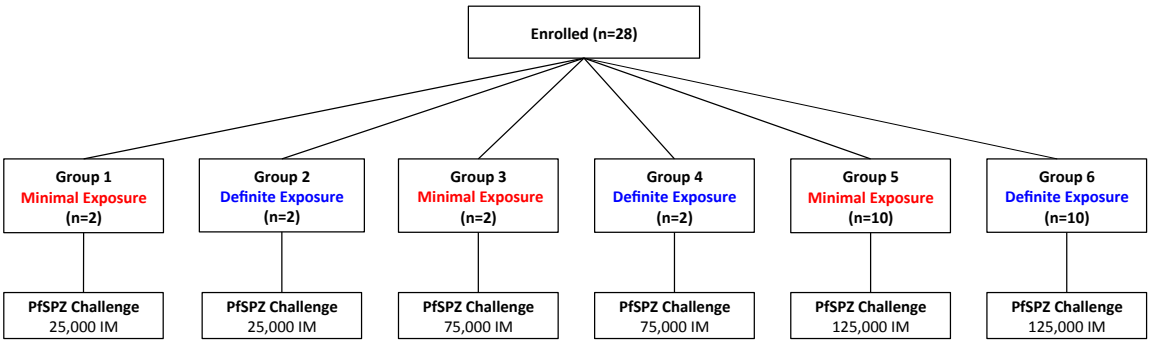


Figure 4-1: Overview of trial groups in KCS

Total dose was split between two 50µl injections, which were administered IM, one in each deltoid area.

4.2.4. Study outcomes

The criteria for treatment were different to VAC049 as it was not possible to perform qPCR in real time. Volunteers were treated when blood-film positive and/or displaying severe signs or symptoms consistent with malaria (see Chapter 2). qPCR was performed retrospectively and LBI and PMR calculated using the same methods as VAC049 (see Chapter 2).

4.3 Results

4.3.1 Study participants

Recruitment took place between March and April 2013. One hundred and forty six subjects were screened and 28 healthy adults (11 female and 17 male) underwent CHMI in May 2013 (Figure 4-2). The mean age of participants was 25 years (range 19-31 years) (Table 4-1).

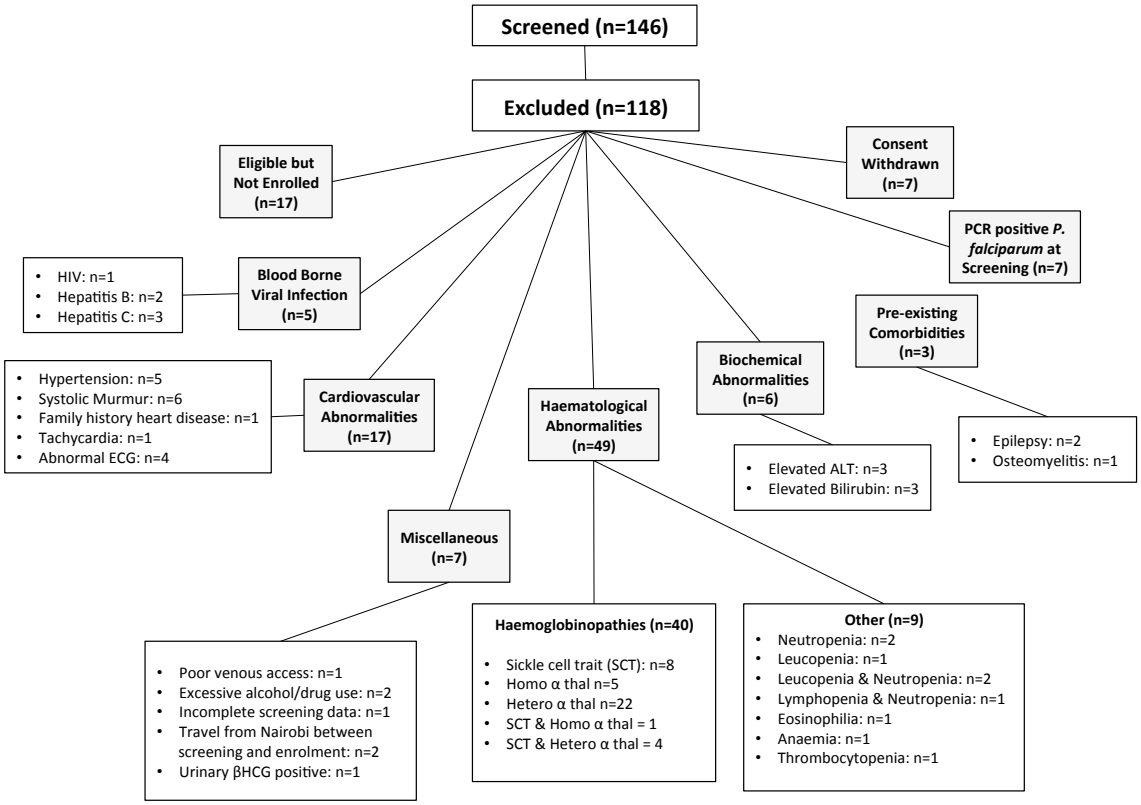


Figure 4-2: Reasons for exclusion of subjects from KCS

ALT = alanine transaminase; βHCG = 'β human chorionic gonadotropin; thal = thalassemia.

	25,000	**75,000**	**125,000**
	n = 4	**n = 4**	**n = 20**
Sex			
Male	2 (50%)	4 (100%)	11 (55%)

Female	2 (50%)	0	9 (45%)
Age at screening (years)			
Mean (SD)	24 (1.6)	23 (2.5)	26 (2.9)
Median	24	23	26
Min, Max	22, 26	19,25	22,31

Table 4-1: Demographics of enrolled volunteers in KCS

The distribution of anti-schizont and anti-MSP2 antibody OD readings at screening of enrolled volunteers with definite prior exposure (DefExp) and minimal prior exposure (MinExp) to malaria is shown in Figure 4-3. A significant correlation was seen between results from these two assays (*p=<0.0001, R=0.58*). All participants received injection of PfSPZ Challenge and completed the study as scheduled. The time between thawing and administration of PfSPZ Challenge was < 30 minutes for all subjects.

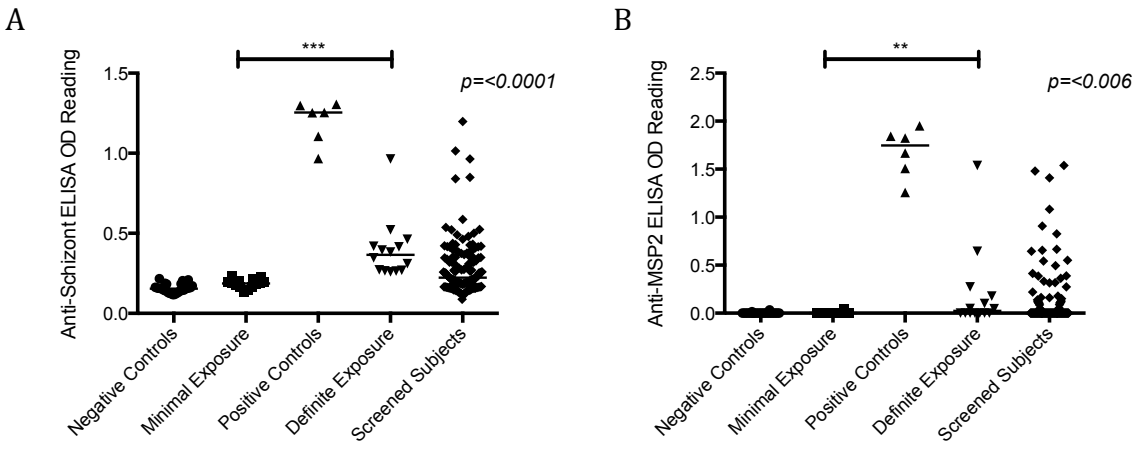


Figure 4-3: Antibody ELISA absolute OD Readings measured at screening for KCS

(A) Anti-schizont. (B) Anti-MSP2. Serum diluted 1:1000. Negative controls = OD readings from UK malaria naïve adults (n=30). Positive controls = OD readings from hyperimmune Kenyan adults living in malaria-endemic regions (n=6). Minimal exposure = subjects enrolled in groups 1, 3 and 5 (n=14). Definite exposure = subjects enrolled in groups 2, 4 and 6 (n=14). Screened subjects = all volunteers that had blood drawn at screening (n=145). A significant difference was seen between minimally and definitely exposed volunteers for both antigens (Anti-schizont = p=<0.0001; Anti-MSP2 = p=0.006; Mann-Whitney test). Median values represented by lines through each dataset.

4.3.2 Time to diagnosis

All volunteers were diagnosed with malaria with the exception of one volunteer in Group 2 (Volunteer 110), who was asymptomatic and blood film negative at all time-points, despite blood-stage infection confirmed by qPCR (Figure 4-4 and Table 4-2). This volunteer had the highest anti-schizont and MSP2 antibody OD readings at screening of all enrolled participants (Figure 4-3). There was no apparent relationship between dose of PfSPZ Challenge and TTD in MinExp volunteers (Median 12.6, 11.8 and 12.0 days for Groups 1, 3 and 5 respectively; *p=0.571* log rank test; Figure 4-4) or DefExp volunteers (Median 16.4, 13.3 and 11.9 days for Groups 2, 4 and 6 respectively; *p=0.332* log rank test; Figure 4-4); no significant difference in TTD between the four Kenyan volunteers and the 6 malaria-naïve UK volunteers in VAC049 who received 25,000 SPZ IM (median TTD 12.5 versus 13.0 days. *p=0.487* log rank test; Figure 4-4)[316] and no

significant difference in TTD between MinExp and DefExp volunteers (median 12.2 and 12.1 days respectively; *p=0.171* log rank test; Figure 4-4).

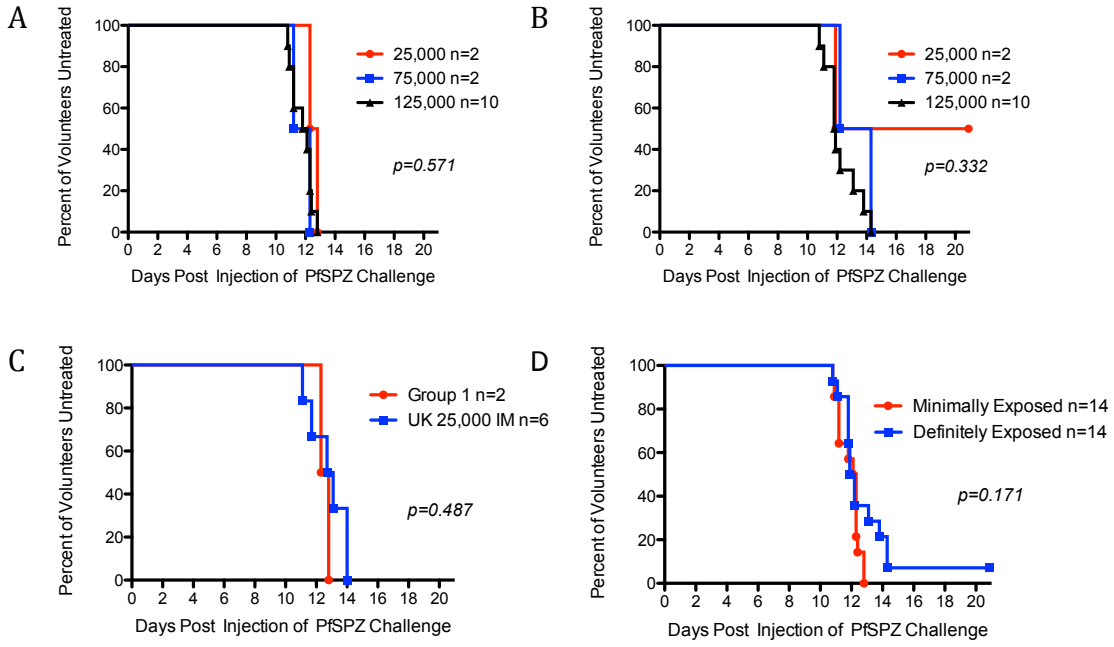


Figure 4-4: Time to patent parasitaemia for KCS

Time to patent parasitaemia in days between injection of PfSPZ Challenge and diagnosis. (A) Effect of dose of PfSPZ Challenge on TTD in MinExp. Median pre-patent period = 12.6 days for Group 1, 11.8 days for Group 3 and 12.0 days for Group 5 (Log-rank test; p=0.571). (B) Effect of dose of PfSPZ Challenge on TTD in DeExp. Median pre-patent period = 16.4 days for Group 2, 13.3 days for Group 4 and 11.9 days for Group 6. (C) Comparison of pre-patent period between VAC049 volunteers receiving 25,000 SPZ IM (n=6) and MinExp (Group 1; n=2) receiving PfSPZ Challenge 25,000 IM. Median pre-patent period = 12.9 days and 12.6 days respectively (Log-rank test; p=0.487). (D) Effect of prior exposure to P. falciparum

on TTD. Minimally exposed = Groups 1, 3 and 5. Definitely exposed = Groups 2, 4 and 6. Median pre-patient period = 12.2 days for Minimally exposed and 12.1 days for definitely exposed (Log-rank test; p=0.171).

	25,000 n=4	**75,000 n=4**	**125,000 n=20**
Symptomatic with positive BF	0	0	5 (25%)
Symptomatic with negative BF	0	0	0
Asymptomatic with positive BF	3 (75%)	4 (100%)	15 (75%)
Reached Day 21 undiagnosed (asymptomatic, BF negative)	1 (25%)	0	0
Withdrew Consent or Excluded From Study Prior to Primary Endpoint	0	0	0

Table 4-2: Criteria for treatment of subjects in KCS

BF = blood film.

4.3.3 Modeling of parasitaemia measured by qPCR

Figure 4-5, Figure 4-6 and Table 4-3 show the qPCR results over time for each individual in the trial. There was no significant difference in PMR or LBI between groups (*p=0.251* and *p=0.557* respectively; *Kruskall-Wallis test* Figure 4-7) or between MinExp and DefExp participants (*p=0.206* and *p=0.597* respectively; *Mann Whitney test* Figure 4-7). A significant correlation was seen between anti-schizont antibody OD at screening and PMR (*Spearman rank =-0.384, p=0.044*) but not LBI (*Spearman rank =-0.124, p=0.529*) *(*Figure 4-7). No significant correlation was seen between anti-schizont OD and PMR when volunteer 110 was excluded from the analysis (*p=0.112, R=0.313*).

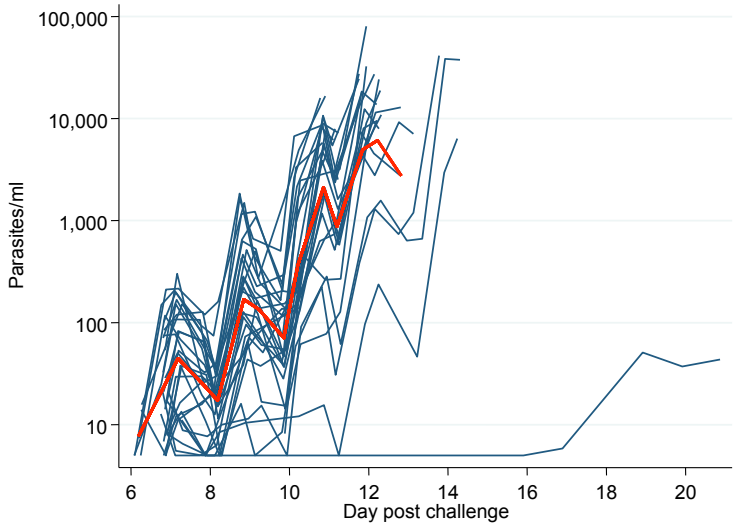


Figure 4-5: qPCR-measured parasite density overtime for KCS

Y-axis = parasites/mL measured by qPCR. X-axis = days post infection of PfSPZ Challenge. Red line = mean parasite density over time for all subjects.

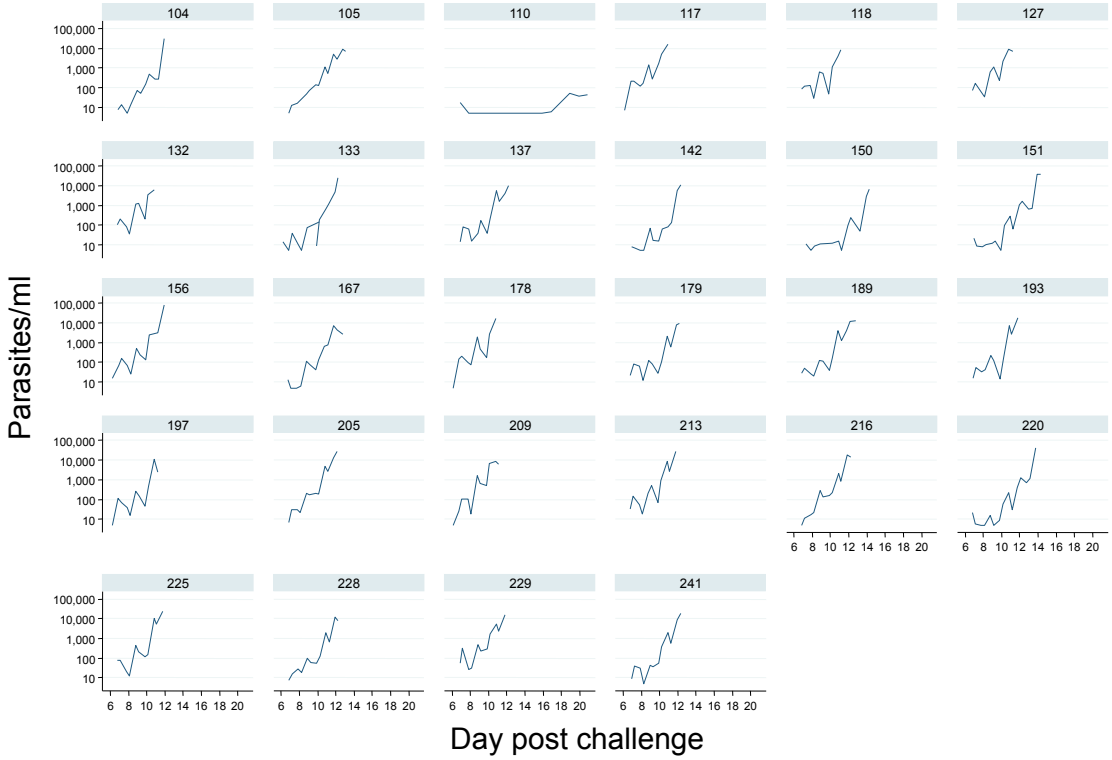


Figure 4-6: qPCR-measured parasite density over time for each subject in KCS

Y-axis = parasites/mL measured by qPCR. X-axis = days post-injection of PfSPZ Challenge. Black subtitles indicate participant identification numbers.

Vol ID	Group No.	D6.5	D7	D7.5	D8	D8.5	D9	D9.5	D10	D10.5	D11	D11.5	D12	D12.5	D13	D13.5	D14	D14.5	D15
167	1	N	13	5	5	6	114	74	40	131	633	736	7434	4544	2767				
228	1	N	8	16	28	19	96	61	54	131	1889	655	12373	7997					
104	2	N	8	13	5	14	71	51	149	485	264	270	32528						
110	2	N	18	N	5	N	N	N	N	N	N	N	5	N	N	N	N	N	N
133	3	14	5	38	11	5	73	140	8	189	658	1102	4868	24390					
197	3	5	117	69	38	16	271	152	47	459	10614	2541							
150	4	N	N	11	5	8	10	N	N	12	16	5	97	238	47	2957	6410		
179	4	N	21	81	65	12	127	79	28	106	2086	587	8077	9538					
117	5	7	211	216	120	161	1497	271	1705	4924	16549								
118	5	N	84	120	127	30	628	530	48	1160	3788	7979							
127	5	N	71	168	N	35	651	1088	239	2151	9012	7220							
132	5	N	101	199	80	35	1157	1223	206	3335	5825								
142	5	N	8	7	5	5	65	17	15	62	78	128	5763	10863					
189	5	N	29	51	26	19	124	114	37	163	3911	1298	3997	11529	12868				
205	5	N	7	29	30	22	204	172	206	193	4743	2730	12939	27366					
216	5	N	5	11	17	21	283	138	154	232	2088	871	18527	13700	9435				
225	5	5	75	77	20	13	428	201	117	151	10691	5448	24513						
241	5	N	9	38	31	5	44	37	55	366	1986	582	8983	18812					
105	6	N	5	12	16	5	46	73	137	129	1172	515	4909	2787	9203	7123			
137	6	N	14	83	64	15	38	164	37	202	5670	1626	4038	9627					
151	6	N	21	9	8	10	11	15	5	91	285	62	1084	1583	640	667	38535	37755	
156	6	16	67	153	67	25	519	243	134	2489	N	3179	80426						
178	6	5	150	208	95	74	1850	465	165	2654	15945								
193	6	N	16	53	33	41	220	123	14	177	7491	2559	17997						
209	6	5	25	108	106	19	1648	663	507	6705	8468	6216							
213	6	N	33	153	56	19	211	503	73	950	8685	2596	27438						
220	6	N	21	6	5	5	16	5	8	58	225	31	383	1306	735	1201	41407		
229	6	N	54	301	26	30	466	228	295	1694	5291	2375	16175						

Table 4-3: qPCR data for KCS

Data = parasites/mL measured by qPCR. Top row represents day of follow-up visit post-administration of PfSPZ Challenge. N = PCR negative including time-points where at least 2/3 replicates were negative. Red = time-point treatment started.

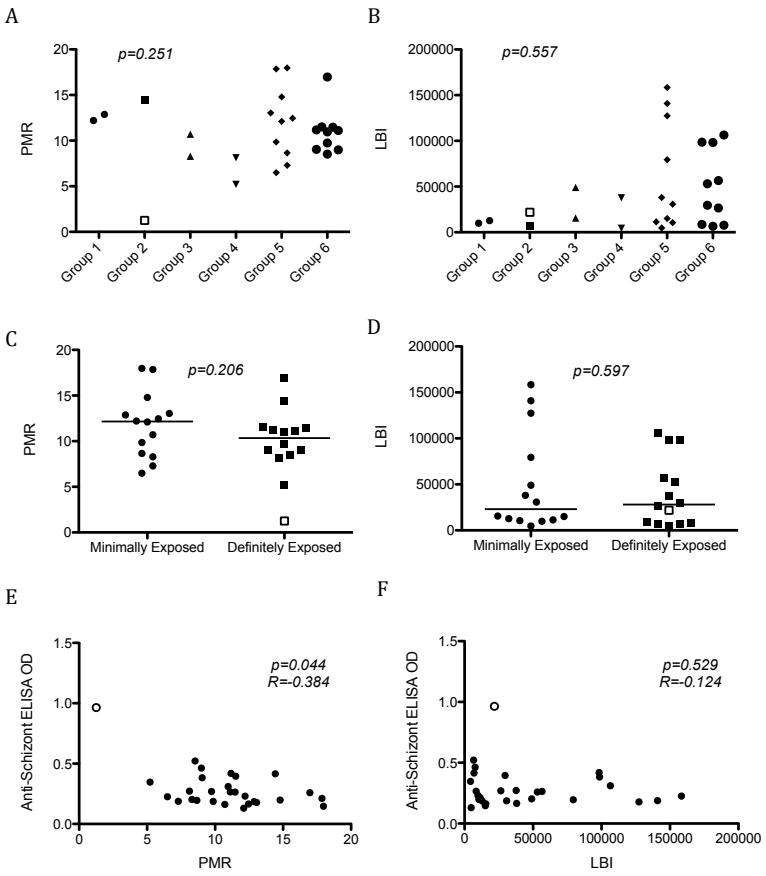


Figure 4-7: Parasite modelling data for KCS

(A) Dot plot of PMR according to Group (p=0.251; Kruskal-Wallis test). (B) Dot plot of LBI according to Group (p=0.547; Kruskal-Wallis test). (C) PMR according to prior exposure to malaria (p=0.206; Mann-Whitney test). (D) LBI according to prior exposure to malaria (p=0.700; Mann Whitney test). (E) Relationship between PMR and anti-schizont ELISA OD measured at screening (Spearman rank =-0.384; p=0.044). (F) Relationship between LBI and anti-schizont ELISA OD measured at screening (Spearman rank =-0.123; p=0.534). In all graphs Volunteer 110's data points are represented as open points. Median values are represented by a straight line in relevant plots.

The qPCR data for Volunteer 110 are shown in Figure 4-8. Volunteer 110 had a notably reduced PMR (1.3) in comparison to the other 27 volunteers (median 11.1). Importantly, Volunteer 110 was qPCR positive on C+7 (mean 18 p/mL; triplicate readings; 14, 38 & 0 p/mL, positivity at this time-point confirmed using a second PCR method[330]) and then subsequently qPCR negative until C+19, suggesting control of blood-stage parasite growth rather than an extremely low or undetectable LBI. Moreover, the LBI in this volunteer was mid-range, in comparison to all other subjects (Figure 4-7).

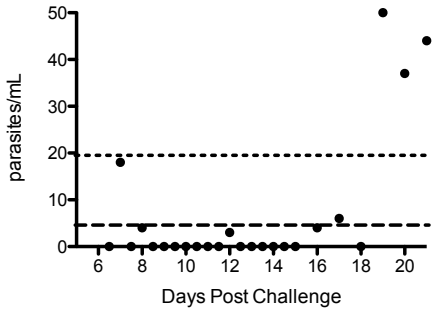


Figure 4-8: qPCR results post-CHMI for Volunteer 110 in KCS

Volunteer 110 received 75,000 SPZ IM in group 2. Long dashed line = LLD (i.e. a probability of >50% of ≥1 positive result among three replicate PCR reactions) for qPCR assay (5 parasites/mL). Short dashed line = LLQ (defined as %CV<20%) for qPCR assay (20 parasites/mL).

MSP2 genotypes of the blood-stage infections were compared using the Agilent Bioanalyzer to examine the qPCR products following amplification of the MSP2 gene[355]. The fragment sizes were similar to within 10 base pairs (within the error of the method) and therefore all infections were clonal (NF54) and

assumed to result from injection with PfSPZ Challenge rather than being naturally acquired.

4.3.4. Safety data

4.3.4.1 PfSPZ Challenge reactogenicity

PfSPZ Challenge was well tolerated with only one AE possibly, probably or definitely related to injection of sporozoites noted: mild injection site pain in a participant in Group 5 lasting for the day of injection only.

4.3.4.2 PfSPZ induced clinical *Plasmodium falciparum* infection

No unexpected or serious AEs occurred. Twenty-two of the 27 participants diagnosed with malaria were asymptomatic at the time of diagnosis (81%; MinExp n=10, DefExp n=12), however all developed at least one symptom or sign consistent with malaria infection before treatment was completed (Figure 4-9). The total duration of symptoms in participants with symptomatic malaria infection ranged from 1-26 days (median 4 days) (Figure 4-9). Particularly long-lasting AEs (>20 days) occurred in 8 participants (MinExp n=6, DefExp n=2) and included headache (n=5), anorexia (n=4), low back pain (n=2), fatigue (n=2) and arthralgia (n=1). There was no difference in the number, duration or severity of symptoms of malaria between MinExp and DefExp participants (Figure 4-9 and Figure 4-10).

Seventeen out of 27 participants diagnosed with *P. falciparum* (63%) experienced at least one AE post-CHMI that was severe in intensity (Figure 4-10). Three volunteers diagnosed with malaria (11%) had a fever (>37.5°C) prior

to diagnosis (MinExp n=2, DefExp n=1) and 17 participants (63%) developed a fever following treatment (MinExp n=8, DefExp n=9). Safety bloods taken at C+9, C+35, C+90 and within 24 hours of diagnosis demonstrated transient laboratory abnormalities at frequencies and severities expected following *P. falciparum* infection in CHMI studies (Figure 4-11)[284,309].

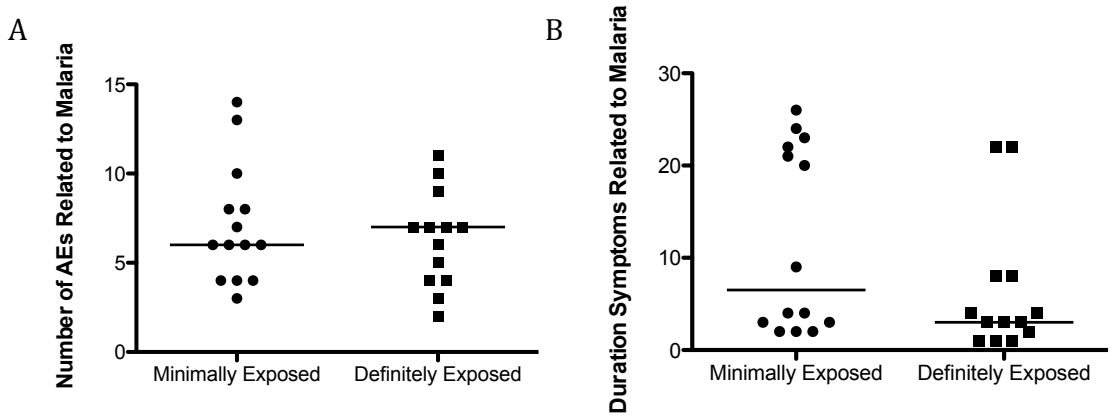


Figure 4-9: Number and Duration of AEs associated with clinical malaria in KCS

Data from volunteers diagnosed with malaria (n=27). Only AEs possibly, probably or definitely related to clinical malaria infection were included in analysis. Laboratory AEs were excluded. (A) Total number of AEs in MinExp (mean = 7.1, median = 6.0) and DefExp volunteers (mean = 6.3, median = 7.0) (Mann-Whitney test; p=0.750). (B) Duration of AEs in MinExp (mean = 11.8, median = 6.5) and DefExp volunteers (mean = 6.3, median = 3.0) (Mann-Whitney test; p=0.142). The median value is represented by a straight line through each plot.

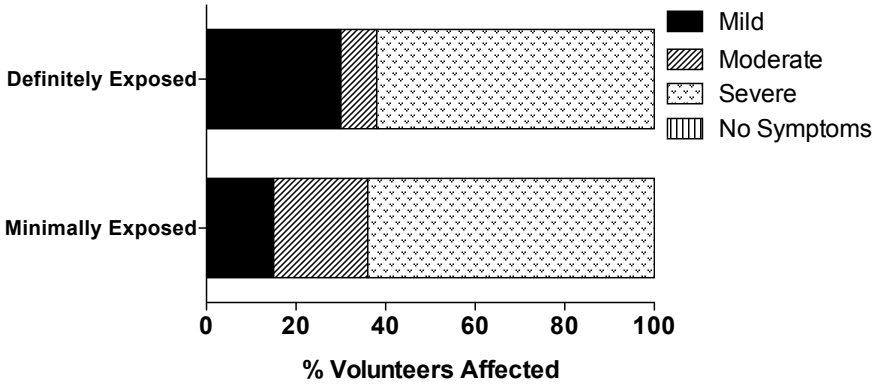


Figure 4-10: Maximum severity of AEs related to malaria infection in KCS

Only AEs deemed possibly, probably or definitely related to P. falciparum infection from individuals diagnosed with malaria (n=27) were included. Laboratory AEs were excluded.

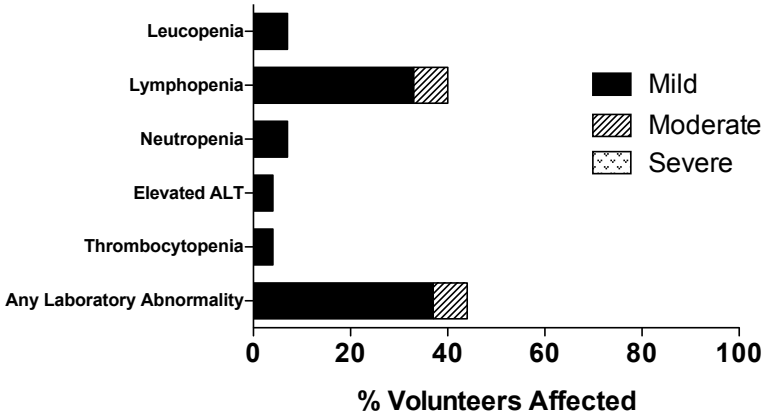


Figure 4-11: Laboratory AEs related to malaria infection in KCS

Only AEs deemed possibly, probably or definitely related to P. falciparum infection were included. For 'any laboratory abnormality' only the highest intensity laboratory AE per subject is counted. ALT = Alanine transaminase.

4.4 Discussion

The primary aim of this pilot study was to establish the parameters for safely and practically performing CHMI studies in Kenya. Given the unknown effects of prior exposure to malaria on CHMI, individuals with both MinExp and DefExp to *P. falciparum* and therefore, presumed varying levels of NAI were included. This study has shown that the CHMI model using PfSPZ Challenge is safe in African adults with no significant differences in AEs seen between DefExp and MinExp volunteers. Importantly, a similar safety profile was seen to that reported in malaria-naïve subjects who underwent CHMI, with the exception that Kenyan participants experienced AEs of a notably longer duration than malaria-naïve volunteers, the reason for which is unclear[305,309,316].

This study importantly confirms the infectivity of PfSPZ Challenge in malaria-exposed individuals, supporting the future use of PfSPZ Challenge in malaria endemic regions to test vaccines and anti-malarial drugs. Acknowledging the necessarily small group sizes in both trials, in contrast to VAC049 (Chapter 3)[316], no significant increase in infectivity (measured by LBI, TTD or proportion of participants infected) was seen in KCS with increasing dose of PfSPZ Challenge IM. This suggests 25,000 SPZ may be a sufficient dose to reliably ensure infection when administered IM, with the caveat that this regimen leads to lower LBI than traditional 5 mosquito-bite CHMI studies[305].

However, since the conduct of VAC049 and KCS, other trials using PfSPZ Challenge administered by direct venous inoculation (DVI) have suggested this route of administration can reliably ensure successful infection in CHMI trials with only 3,200 SPZ[313]. Since DVI administration can reduce the variation in LBI between volunteers and allow an infectious dose more closely approximating that seen in 5 bite mosquito-bite CHMI studies[313], this dose and route of

administration of PfSPZ Challenge is being used in the future to assess the efficacy of certain candidate malaria vaccines (*Hoffman SL, personal communication*). It is however, currently unclear whether 3,200 SPZ administered by DVI in the context of NAI can consistently lead to infection and a detectable parasitaemia.

Of my screened cohort, only five individuals had anti-schizont OD readings of the same order as 'hyperimmune' controls (Figure 4-3). Due to a high degree of previously undiagnosed co-morbidities in these five individuals, only one of these volunteers was subsequently eligible to participate in the study (Volunteer 110). In practice therefore, our study included subjects with varying but probably low to moderate prior exposure to *P. falciparum*, and this may help explain why only one out of 14 DefExp individuals was able to control parasite growth *in vivo*. Nevertheless, there was no overlap between the anti-schizont reactivities of those with definite versus minimal exposure.

Despite the lack of 'hyperimmune' individuals in the study, there was still preliminary evidence of the potential of the CHMI model to detect differences in individuals' NAI to *P. falciparum*. This is demonstrated first by the relationship between the anti-schizont OD reading at screening and PMR post-CHMI (Figure 4-7), and second by the case of the individual with the highest anti-schizont ELISA OD at screening (Volunteer 110) who remained asymptomatic and blood-film negative despite qPCR confirmed *P. falciparum* infection. Comparison of PMRs from this study with the only other published measurement of PMR in the context of NAI[339] showed that this volunteer had a similar PMR to those measured in Gambian adults participating as unvaccinated controls in a malaria

vaccine field efficacy study[339,363]. However, it should be noted these latter adult volunteers had presumably been infected multiple times before with local circulating strains against which they had developed effective immunity, in contrast to this study which used the historical laboratory reference strain NF54, which, although of African origin, may not completely reflect the strains against which the volunteers were naturally exposed in the past. Other volunteers in KCS appeared to have PMRs broadly similar to malaria-naïve controls post-CHMI administered by mosquito bite[305,309,335]. Nevertheless, key methodological differences in qPCR analysis and study design (including 'drug clearance' before qPCR sampling) between these studies and limited other trials reporting vaccine-induced reduction in parasite growth post-CHMI[238,364], make comparison of PMRs across these various settings largely difficult to interpret[222].

Examination of Volunteer 110's qPCR data over time show a positive qPCR result on C+7.0 followed by a run of mostly negative results, until C+19.0 when all subsequent qPCR results became positive. Whilst it is not possible to draw definite conclusions regarding the mechanism(s) by which this volunteer controlled parasite growth, this pattern of qPCR positivity could suggest the first wave of merozoites released from the liver invaded RBCs and thus were detectable at C+7.0 post-CHMI and not completely controlled by anti-merozoite immunity. The subsequent run of negative qPCR results would be consistent with control of parasite growth mediated by existing anti-PfEMP1 or anti-iRBC antibodies, followed by loss of control of parasite growth. This could occur following a *var* gene switch, leading to a change in variant surface antigen to one that is not recognised by existing antibodies[365,366]. Alternatively, there is a

possibility that the late increase in qPCR seen in this volunteer represented gametocytaemia. Given that no asexual parasites were seen at any time-point on blood-film this unlikely, however future trials should consider performing gametocyte-specific qPCR in such scenarios to test for this possibility[367].

Having completed the KCS, the next step in this work was to reflect on and discuss lessons learnt conducting the first modern CHMI study in Kenya (Chapter 5).

CHAPTER 5: LESSONS LEARNT FROM CONDUCTING THE FIRST MODERN CONTROLLED HUMAN MALARIA INFECTION STUDY IN KENYA

5.1 Introduction

Whilst well established in northern centres, only three CHMI trials, all using PfSPZ Challenge, have been performed in Africa to date: the first in Tanzania[368], the second in Kenya as outlined in this thesis[317], and the third in Gabon *(Lell et al, unpublished).* In order to increase the international capacity to assess the efficacy of novel malaria vaccines without the need for costly field studies, it is anticipated that the number of African trial sites conducting CHMI studies will notably increase in the near future (*SL Hoffman, personal communication*).

Performing CHMI studies in the developing world entails logistical and ethical challenges different to those well understood and anticipated in northern centres, for example: ensuring subjects are not infected with naturally transmitted parasites whilst under follow-up, guaranteeing ease of access to intensive care facilities and undertaking thoughtful engagement with the local community.

As KCS was the first modern CHMI study performed in Kenya, a large amount of consultation and iterative design was undertaken to ensure the safety of volunteers and integrity of the study data. Given these modifications and lessons learnt from conduct of the trial itself are likely to be highly relevant to other African CHMI centres, this Chapter seeks to reflect in detail on these experiences and summarise them as learning points that may be relevant for other CHMI sites.

5.2 Learning points

5.2.1. Consultation

A detailed plan for community sensitisation for the study was drawn up in advance of the study (Table 5-1). Four years before the start of the trial, consultations began with key stakeholders in Kenya (Box 5-1). At these multi-disciplinary meetings, ethical, logistical, funding and scientific issues were discussed. All parties supported the establishment of a CHMI centre in Kenya as a key objective in line with national and regional research priorities. A decision was made for Nairobi to be the preferred site for the first CHMI study given the ease of access to tertiary medical centres, the relative lack of natural malaria transmission and large concentration of educated individuals best placed to provide informed consent. Initial discussions focused on developing an insectary capable of supporting mosquitoes imported from abroad for mosquito-bite CHMI studies[287]. However when PfSPZ Challenge became available, this option was chosen due to its practical advantages[310].

Conclusion: Detailed discussion with key stakeholders years ahead of the planned study was an important two-way process, increasing understanding and acceptance of the CHMI model by key national stakeholders and allowing important feedback to guide the study design and increase speed of ethical and regulatory approvals at a later date.

	Universities	ERC	Public	Participants
Consultation / Gate Keeping	Seek advice from senior managers and request permission to discuss study with students	Discussion regarding suitability of study in Kenya and ethical concerns. Advised on compensation levels. Approved study.	Nil	Nil planned
Sensitisation	General meetings outlining role of KEMRI & study	Nil planned	Nil	General meetings outlining role of KEMRI & study and invitation to attend a more detailed information meeting
Feedback	Meeting with University management to feedback results	Written report of study findings, challenges and lessons learnt	Nil	Presentation of study findings in 'grand round' setting

Table 5-1: Community sensitisation plan for KCS

ERC = KEMRI Ethics Review Committee. KEMRI = Kenyan Medical Research Institute.

- Ministry of Health
- Pharmacy and Poisons Board (National Regulatory Agency)
- Kenya Medical Research Institute (KEMRI) Ethics Research Committee
- National Commission for Science, Technology and Innovation
- Kenya Medical Research Institute
- Consortium for National Health Research Kenya (NGO)
- University of Nairobi Institute of Tropical & Infectious Diseases
- University of Nairobi School of Medicine
- Ministry of Health Division of Vaccines and Immunization

Box 5-1: Kenyan stakeholders consulted in advance of submission of study for ethical and regulatory approval for KCS

5.2.2. Ethical and regulatory approvals

Kenya has a multi-tiered system of human subjects research review where proposals go through three or more rounds of review prior to approval. Following review by the KEMRI Kilifi Centre Scientific Committee (CSC), the study was reviewed by the national KEMRI Scientific Steering Committee (SSC) and then later approved by the KEMRI Ethics Review Committee (ERC). The ERC requested additional information regarding access to intensive care facilities, the rationale for the proposed amount of monetary compensation (see section 5.2.4) and a clear description of how the target population of educated adults would be approached. Final ethical approval to conduct the study was received 6 months after initial submission to the CSC.

The University of Oxford requires OXTREC approval for all clinical trials it sponsors overseas. Prior to approval, OXTREC requested justification for the proposed amount of monetary compensation, clarification on consent processes for long-term storage of samples and subsequent assays on these samples and

assurances regarding the sensitivity of PfSPZ Challenge to the proposed anti-malarial therapy. Ethics approval from OXTREC took 2 months from initial submission.

Prior to regulatory approval, the Kenyan Pharmacy and Poisons Board requested assurances as to how PfSPZ Challenge would be transported and stored at -140°C. Regulatory approval took 3 weeks from initial submission. An import permit was granted for PfSPZ Challenge and the two required diluents for injection (25% HAS and PBS), which were imported from the USA. A test shipment labeled as PfSPZ Challenge and diluents passed through customs with no delays. PfSPZ Challenge is subject to an Investigational New Drug Application filed with the US Food and Drug Administration (FDA) and sponsored by its manufacturer, Sanaria Inc. In this capacity the FDA reviewed and approved the study.

Just prior to the start of screening, the international CHMI community was made aware of a second cardiac serious AE occurring in a mosquito-bite CHMI study assessing a malaria vaccine at RUMC Nijmegen, The Netherlands.[328] The KCS study protocol and consent form were updated to reflect this new information and add an ECG to the investigations performed on all individuals undergoing screening. Approval was sought for these amendments from the relevant ethical and regulatory authorities. All approvals were obtained within 37 days of the applications, allowing the study to go ahead according to the original timelines.

Conclusion: All bodies were supportive of the study and recognised its importance. The initial multi-stage approval process did take a considerable time but ensured the study was rigorously reviewed. Given that the dates of the CHMI

aspect of study were required to be set well in advance (see section 5.2.6), the swift approval of the substantial amendment was extremely important as it allowed the study to proceed on schedule with appropriate updated information for volunteers.

5.2.3. Community sensitisation

All community sensitisation occurred following approval of the study and at a local, rather than national level. Given the novel and complex study nature of the study[369], community sensitisation focused on local medical schools as it was felt this population was most likely to have some existing understanding of the concept of research. Letters were sent to the heads of the three main medical schools in Nairobi, requesting permission to present the study to staff and students. Three meetings occurred approximately 6 weeks before the start of screening, and took the form of a formal presentation by the Principal Investigator on the background and purpose of CHMI studies, followed by details of the planned study. Approximately 200 individuals in total attended these meetings. A list of questions raised at the meetings is given in Box 5-2 and the reasons attendees gave to participate or not in the study are shown in Box 5-3. About half of individuals at the meetings said they would consider participating in the study. Posters advertising the trial were placed at the medical schools and interested individuals were asked to contact the study team to attend an individual information meeting and receive the VIS. Two hundred and forty three individuals subsequently received the VIS and 146 attended for screening visits at a later date.

Conclusion: Meetings at the medical schools provided an excellent forum to explain the study to a target, educated audience and discuss and explain all aspects of the study. For future studies, the VIS should be updated to address those questions raised at community sensitisation meetings that were not addressed in the VIS.

Questions Addressed in the VIS:

- Does one have to stay in hospital throughout the study?
- What is the compensation that will be given?
- What are the risks of participating?
- If it happens that I do not react as expected, what will happen?
- The method of injection is new - is it safe?
- How do you compare mosquito and injection?
- What will make you choose me to participate in the study?
- Will I be subjected to pricking (blood sampling)?
- What if I have commitments at the time of the study?
- In brief, what message can I give my friends about this study?
- How many people will participate?
- What are your expectations?
- I know my parents will want to know – are there other people involved in the study other than students say for example a doctor or just other people?
- What if I react on day 1?
- With the dates of the study procedures you have given it seems this might interfere with our studies and we all know our education should come first. What do you have to say about this?
- What are the benefits of participation in this study?
- Is there risk of infection?
- Now that we are going to take part in the study who will do the research, KEMRI or the students?
- How much of our time do you need for the study?

Questions not Addressed in the VIS:

- Where can I get more information about this product?
- Will you be involving only people from Nairobi or also outside Nairobi?
- Do you just sit down the whole day doing nothing?
- Is visiting allowed?
- Will there be a document to say I have participated in this study, like a certificate or a letter saying I was involved?

Box 5-2: Questions raised at community sensitisation meetings for KCS

Reasons Given to Participate
- I am patriotic and I would love to take part in this study to help my country. - One day I will have my own family and by my participation in this particular study I will be helping to generate very important medical knowledge that will one day help my own children and other families in Kenya. - For curiosity. - I would have been happy to volunteer in the malaria vaccine study (RTS,S) if given the opportunity as I have seen it has generated very useful data.
Reasons Given Not to Participate
- I am scared of my parents' reaction. - I will be having job interviews during the time. - I am scared of needles. - Staying away for that long and being unable to do my things. - It is a new thing – we do not know what to expect.

Box 5-3: Reasons given at community sensitisation meetings to participate or decline to participate in KCS

5.2.4. Consent

5.2.4.1 Informed consent

At screening, at least 24 hours after individuals received the VIS, volunteers discussed the study in detail in a one-on-one setting with Kenyan study investigators who were also physicians. All volunteers who presented for screening were happy to consent to participate in the study. Prior to obtaining written consent, all volunteers were required to undertake a written

questionnaire to assess their understanding of the study. Volunteers were required to answer all questions correctly before being allowed to consent to the study and were only allowed to attempt the questionnaire a maximum of three times. After each attempt, the participant was allowed to ask for clarification of any points from the study investigator. Of the 146 screened volunteers, 100% required at least two attempts and 55% required three attempts to correctly answer all questions on the questionnaire. Volunteers completed two, written consent forms; one to participate in the study and one to allow the long-term storage of samples taken during the course of the study.

To meet inclusion criteria, all volunteers were required to provide evidence of completion of secondary education (typically completed at age 16-18 years). It was anticipated that participants would have some existing understanding of the concept of research and might raise different questions and concerns to participants in studies conducted at field sites. In particular, it was emphasised that in contrast to many studies, KCS would involve making healthy individuals unwell (for a short while). Key concerns I anticipated volunteers might raise were similar to those discussed in screening appointments, namely concern that the trial was 'high risk,' the volume of blood taken and the need for an extended inpatient stay (Table 5-2).

Question Category	Number of questions asked n = 167	Percentage (%)
Effects of Malaria Infection and Treatment: Short and Long-term	27	16%
Details of Inpatient Stay and Travel out of Nairobi	20	12%
Availability of Test Results	19	11%
Blood Sampling	13	8%
Further explanation of study logistics	13	8%
Parasite strain used in CHMI	13	8%
Prior studies of PfSPZ Challenge	10	6%
Rationale for Allocation to Groups	9	5%
Malaria Treatment	7	4%
Study Start Date	7	4%
Compensation	6	4%
Post-CHMI Follow-up	5	3%
Purpose of the Research	4	2%
Possibility of Failure of Infection	3	2%
Criteria for Malaria Treatment	3	2%
Failure to Cure Malaria Infection	2	1%
Injection Sites of PfSPZ Challenge	2	1%
Proof of participation in the Study	2	1%
Resistance of PfSPZ Challenge to Anti-Malarial Drugs	2	1%

Table 5-2: Questions raised by volunteers at screening appointments for KCS

5.2.4.2 Compensation for study participation

Volunteers were offered financial compensation for transport expenses and time away from potential income generation or actual work in order to participate in the study. Based on local wages and comparison with the Tanzanian CHMI study initiated in March 2012[368], this was $50 (Kshs. 4,000) for each overnight stay, $12 (Kshs. 1,000) for each scheduled clinic visit, and $6 (Kshs. 500) for each return journey to clinic (maximum approximately $1200). The ERC and OXTREC agreed that these amounts would neither unduly coerce potential participants nor set a difficult precedent for other research conducted within the programme.

The availability of financial compensation for enrolled volunteers but not the amount was disclosed at the community sensitisation meetings. Only volunteers receiving the VIS at later information meetings were aware of the amount paid as compensation for participation in the study.

Conclusion: The informed consent questionnaire was a valuable tool to identify areas where volunteers' understanding was lacking and facilitate focused discussion with the study investigators. However, the fact all volunteers had to take the questionnaire at least two times suggests volunteers had difficulty understanding the complex study. To rectify this in the future, the length of the information meetings could be extended to allow even more detailed discussion of the study details. The wide range and sophistication of questions asked both at screening and at the community sensitisation meetings was a reassuring indication that individuals were engaging with the study. The financial compensation offered to participants did not appear to unduly influence volunteers' decisions to participate in the study as supported by the fact of the 243 individuals receiving the VIS only 146 attended for screening.

5.2.5. Recruitment and target population

The occupations of screened volunteers are shown in Table 5-3. Despite only promoting the study in medical schools, only 54% of screened volunteers were students, suggesting dissemination of news of the study went beyond our advertising targets.

Occupation Category	Frequency (n)	Percentage (%)
Student	78	54%
Unemployed	25	17%
Business	6	4%
Casual Labourer	4	3%
None/ Not Specified	4	3%
Researcher	4	3%
Social Worker	3	2%
Acrobat	1	1%
Broker	1	1%
Community Health Worker	1	1%
Contractor	1	1%
Counsellor	1	1%
Driver	1	1%
Engineer	1	1%
Footballer	1	1%
Housekeeper	1	1%
Journalist	1	1%
Nurse	1	1%
Online Writer	1	1%
Plumber	1	1%
Sales and Marketing	1	1%
Sales and Services	1	1%
Secretary	1	1%
Self Employed	1	1%
Stylist & Beautician	1	1%
Teacher	1	1%
TV Producer	1	1%
Waitress	1	1%

Table 5-3: Occupations of screened volunteers in KCS

(Data collected for n=145)

As the first CHMI study undertaken in Kenya, the exclusion criteria were extensive (Chapter 2) and only 45/146 (31%) of the screened volunteers were eligible to participate in the study (Figure 4-2). Key factors were the high degree of previously undiagnosed co-morbidities and haemoglobinopathies.

5.2.5.1 Haemoglobinopathies

Given the known, significant protective effect of sickle cell trait and both heterozygous and homozygous α-thalassemia against severe malaria[49,52], volunteers with these conditions were excluded from the study. This did, however, markedly reduce the proportion of eligible volunteers. As these haemoglobinopathies only confer mild or no protection against mild malaria and asymptomatic parasitaemia[52], future CHMI studies could consider including such volunteers. Indeed, in the recent Tanzanian CHMI study using PfSPZ Challenge, a considerable number of enrolled volunteers were heterozygous for α thalassemia and no evidence of an effect on parasite growth dynamics (PGD) was seen post CHMI[368]. An added advantage of including volunteers with these haemoglobinopathies in future CHMI studies is the ability to assess the efficacy of novel vaccines and drugs in participants more representative of the target population at large.

5.2.5.2 Classifying prior exposure to *P. falciparum*

One of the aims of the study was to assess PGD in volunteers with varying degrees of prior exposure, and therefore presumed NAI to *P. falciparum*[20]. Although there is little or no natural transmission of malaria in Nairobi[370], there was confidence in the ability to recruit volunteers with varying degrees of prior exposure to *P. falciparum* given the highly migratory nature of the population of Nairobi.

Initially, volunteers were to be divided into two main cohorts: individuals with no evidence of prior exposure to *P. falciparum* (Group 1, 3 and 5) and individuals

with evidence of marked prior exposure (i.e. semi-immune; Groups 2, 4 and 6). Given the lack of a known assay to reliably assess NAI,[20] both serological and historical geographical data were to be used as criteria for grading prior exposure to *P. falciparum,* as a surrogate measure of NAI (Table 5-4). Data were collected at screening regarding volunteers' place of birth, location of schooling and higher education and time spent in Nairobi directly prior to screening. Serologically, two key antigens were chosen; total schizont antigens and MSP2 on the basis of their published, positive association with prior exposure to *P. falciparum*[371-375].

Groups	Malaria Exposure	Anti-Pf serology*	History of Malaria Infection	Time Spent in Endemic Region
1, 3, 5	None	All Negative	None	Lived whole life in Nairobi or similar area where *Pf* is not endemic.
2, 4, 6	Maximal	High Positive	+/-	Vast majority of life spent in Western Kenya in area of high Pf transmission. Immediately prior to CHMI, should not have spent more than 6 months out of endemic region.

Table 5-4: Initial criteria for group allocation in KCS according to prior exposure to P. falciparum

**Anti-Pf serology = Anti-schizont and anti-MSP2 antibodies.*

Following collection of data from screening, it became clear these criteria for classifying volunteers were unworkable. Firstly, in the target, educated population, most had attended boarding schools and higher education institutions at multiple locations, considerable distances from their place of birth, making it extremely difficult to quantify time spent in malaria endemic regions (Table 5-5 and Table 5-6). Secondly, using CDC data on malaria endemicity ("Pf*...present in all areas (including game parks) (of Kenya) at altitudes below 2,500 m (8,202ft) with none in the highly urbanized, central part of the city of Nairobi*")[370], nearly all volunteers had spent significant time in a malaria endemic region (Table 5-5 and Table 5-6). Although information was collected regarding volunteers' reported history of episodes of blood-smear confirmed malaria infections, these were unable to be validated since many individuals had taken anti-malarial medications for presumed malaria infection unconfirmed by blood-smear and it was difficult to access medical records retrospectively.

Subject	Group	Place of Birth (POB)	Years in POB	Location Childhood	Location Primary School	Location High School	Location Higher Education	Location Most Time Spent
167	1	EMBU	20	EMBU	EMBU	EMBU	NAIROBI	EMBU
228	1	NAIROBI	24	NAIROBI	NAIROBI	NAIROBI	NAIROBI	NAIROBI
133	3	NAIROBI	19	NAIROBI	NAIROBI	NAIROBI	N/A	NAIROBI
197	3	ELDAMA RAVINE	15	ELDAMA RAVINE	ELDAMA RAVINE	MASENO	NAIROBI	ELDAMA RAVINE
117	5	NAIROBI	31	NAIROBI	NAIROBI	MURANG'A	NAIROBI	NAIROBI
118	5	KAKAMEGA	15	KAKAMEGA	KAKAMEGA	NAIROBI	NAIROBI	KAKAMEGA
127	5	NAIROBI	24	NAIROBI	NAIROBI	NAIROBI	NAIROBI	NAIROBI
132	5	MURANG'A	17	MURANG'A	MURANG'A	MURANG'A	NAIROBI	MURANG'A
142	5	MURANG'A	6	MURANG'A	NAIROBI	KIAMBU	NAIROBI	NAIROBI
189	5	NAIROBI	24	NAIROBI	NAIROBI	NAIROBI	NAIROBI	NAIROBI
205	5	NAKURU	17	NAKURU	KINARI	NGORIKA	NAIROBI	NAKURU
216	5	NAIROBI	20	NAIROBI	NAIROBI	KIAMBU	NAIROBI	NAIROBI
225	5	NAKURU	6	NAKURU	NAIROBI	KIAMBU	NAIROBI	NAIROBI
241	5	NAIROBI	23	NAIROBI	NAIROBI	NAIROBI	NAIROBI	NAIROBI

Table 5-5: Historical geographical data for MinExp volunteers in KCS

Subject	Group	Place of Birth (POB)	Years in POB	Location Childhood	Location Primary School	Location High School	Location Higher Education	Location Most Time Spent
104	2	MACHAKOS	15	MACHAKOS	MACHAKOS	MACHAKOS	NAIROBI	MACHAKOS
110	2	KAKAMEGA	22	KAKAMEGA	KAKAMEGA	KAKAMEGA	KAKAMEGA	KAKAMEGA
150	4	NAIROBI	6	NAIROBI	NYANZA	NYANZA	N/A	NYANZA
179	4	KITALE	14	KITALE	KITALE	TESO, WESTERN	NAIROBI	KITALE
105	6	MWINGI	17	MWINGI	MWINGI	MWINGI	NAIROBI	MWINGI
137	6	NAKURU	CAN'T RECALL	ELDORET	ELDORET	ELDORET	ELDORET	ELDORET
151	6	KENDU BAY	16	KENDU BAY	KENDU BAY	NAIROBI & KENDU BAY	NAIROBI	KENDU BAY
156	6	ISIBANIA	18	ISIBANIA	MIGORI	MBITA	NAIROBI	ISIBANIA
178	6	MURANG'A	25	MURANG'A	MURANG'A	MURANG'A	NAIROBI	MURANG'A
193	6	SIAYA	3	SIAYA	NAIROBI	NAIROBI	NAIROBI	NAIROBI

209	6	KITALE	14	KITALE	KITALE	NAKURU	NAIROBI	KITALE	
213	6	TAITA	18	TAITA	TAITA, MOMBASA	TAITA	NAIROBI	TAITA	
220	6	MERU	12	MERU	MERU	MERU	NAIROBI	MERU	
229	6	NAIROBI	26	NAIROBI	NAIROBI	MURANG'A	NAIROBI	NAIROBI	

Table 5-6: Historical geographical data for DefExp in KCS

On review, it was felt serological data were the most objective measure to assess prior exposure to *P. falciparum*. Given that anti-schizont ELISA results reflect reactogenicity to multiple antigens in parallel, it was decided to use this assay as the primary serological endpoint. (Indeed, subsequently published work has shown antibody responses to *P. falciparum* lysate can predict individuals with lower parasite density at first detection by qPCR following CHMI[176]).

Individuals were re-classified into those with evidence of minimal prior exposure (MinExp; Groups 1, 3 and 5) and those with definite evidence of prior exposure (DefExp; Groups 2, 4 and 6) on the basis of serological data alone (Table 5-7). In the final enrolled volunteers, it was ensured that the geographical history was consistent with the serological data (for example, that volunteers reporting lifelong residence in Nairobi were not in the DefExp group).

Groups	**Malaria Exposure**	**Primary Criterion: Anti-Pf serology***	**Secondary Criterion: Time Spent in Endemic Region**
1, 3, 5	Minimal	All Negative	Lived majority of life in Nairobi or area where Pf is not endemic.
2, 4, 6	Definite	Positive Anti-Schizont +/- Positive Anti-MSP2	Considerable time spent in areas of *Pf* transmission.

Table 5-7: Final criteria for group allocation according to prior exposure to P. falciparum for KCS

**Anti-Pf serology = Anti-schizont and anti-MSP2 antibodies.*

5.2.5.3 Asymptomatic parasitaemia at screening

Whilst malaria transmission is minimal in Nairobi,[370] it was important to ensure volunteers were not parasitaemic with naturally-acquired *P. falciparum* prior to administration of PfSPZ Challenge. Administration of a course of curative anti-malarials to all enrolled volunteers prior to CHMI was considered. However, there were concerns that even a medication with a reported short half-life could impact on PfSPZ Challenge infectivity or PGD[170]. Instead it was decided to exclude all volunteers qPCR positive for *P. falciparum* at screening. Seven volunteers (5%) were excluded using this criterion (Figure 4-2). All were asymptomatic: five with a 'low positive' unquantifiable result (below LLQ i.e. < 20 p/mL) and two with quantifiable results of 55 and 669 p/mL. These seven volunteers were treated with a course of anti-malarial therapy according to national guidelines and excluded from the study. Whilst it's not known when these individuals were infected with malaria, they may have been semi-immune volunteers capable of controlling parasitaemia; indeed 4/7 (57%) met the criteria for DefExp. Given the difficulty recruiting such individuals in Nairobi, it was an unfortunate effect of the study design that it was necessary to exclude such volunteers.

Conclusion: Given only 54% of screened volunteers were students, there was no clear advantage to exclusively targeting medical students and future studies should appeal to students of all disciplines.

Exclusion of volunteers heterozygous for α thalassemia markedly limited recruitment and such volunteers could be considered for inclusion in future CHMI studies. Grouping volunteers according to prior exposure to malaria, and

therefore NAI was extremely difficult given the lack of a validated assay and recognised methods to grade malaria exposure on the basis of geographical data. This was compounded by the highly migratory nature of the population and high degree of undiagnosed comorbidities. If future Kenyan CHMI studies seek to enrol 'hyperimmune' adults, it will be important to screen a considerably larger sample of individuals, do more targeted screening or move the study site to an endemic region. Whilst pre-screening of volunteers to create a database of appropriate individuals in advance of a CHMI study could be useful logistically, given the unknown dynamics of the loss of NAI, such volunteers would require rescreening ahead of enrolment to ensure accurate, timely data immediately prior to CHMI.

5.2.6. Study design

5.2.6.1 Schedule of enrolment

In order to minimise vials of PfSPZ Challenge used and ensure standardisation with other PfSPZ Challenge studies, staff from Sanaria Inc USA prepared and dispensed syringes of PfSPZ Challenge, which were required to be administered within 30 minutes of thawing of PfSPZ Challenge. For logistical reasons it was decided all volunteers would undergo CHMI in the same week. Given a safety review by the SMC was required prior to each dose escalation of PfSPZ Challenge, safety data had to be compiled in real-time and SMC members briefed in advance to allow a strict schedule of reviews prior to approval of each dose escalation to be met (Table 5-8). The fact all volunteers were enrolled in one week meant that the phase of inpatient follow-up lasted just 28 days and so intensive nursing and laboratory support was only required for a month.

Milestone	Documents to Be Reviewed	Date/Time of Email of Documents to be Reviewed	Timeline
Preparatory Meeting – Background Information	Review of initial clinical trial documentation	1 month before meeting	February 2013
Preparatory Meeting 2	Schedule of safety review during challenge week	1 week before meeting	Week beginning 29th April 2013
Enrolment of Cohort 1 (Groups 1 & 2)	Safety Data	Wednesday 8th May 2013 0500 GMT	48 hours post Enrolment of Cohort 1
Enrolment of Cohort 2 (Groups 3 & 4)	Safety Data	Friday 10th May 2013 0800 GMT	48 hours post enrolment of Cohort 2
Clinical Trial End	Safety & Infectivity Data	1 month before meeting	November 2013

Table 5-8: Safety monitoring committee schedule of meetings for KCS

5.2.6.2 In-patient setting

Depending on the setting, management of subjects undergoing CHMI is conducted on in-patient or out-patient basis[376]. Given the need to prioritise volunteer safety in this pilot study, the fact that considerable traffic congestion in Nairobi could seriously impede volunteers' ability to attend the medical centre in a timely fashion and the lack of ease of access to public health care, it was decided that volunteers would be managed as in-patients from the day before injection of PfSPZ Challenge until completion of anti-malarial therapy. The need for in-patient care was emphasised to participants at screening and again prior to enrolment. Clear protocols were established and explained to both staff and participants regarding the actions to be taken if a participant were to go missing[309]. Participants were encouraged to voice any concerns they had about

their living conditions, treatment or change in personal circumstances early so that issues could be addressed quickly and anti-malarial treatment initiated in a timely fashion if a volunteer wished to withdraw from the study and leave the in-patient clinic.

5.2.6.3 Duration of follow-up post-CHMI

As the first pilot study in Kenya, a follow-up schedule similar to European centres was adopted, with all undiagnosed participants treated at C+21[376]. One participant (110) who had an anti-schizont ELISA OD at screening similar to hyperimmune individuals was qPCR positive but blood-film negative (and therefore undiagnosed) on C+21. It would be extremely interesting to examine the PGD of this, and other hyperimmune individuals if a longer window of follow-up prior to treatment of undiagnosed participants were adopted. One benefit could be the ability to track the development of gametocytaemia. In KCS, no qPCR specific for gametocytes was performed, and so it is possible that the rise in qPCR seen in participant 110 from C+19 onwards reflected gametocytaemia rather than loss of control of the blood-stage of infection. In future studies, if qPCR specific for gametocytes were performed and a phenotype of individuals identified that were able to control blood-stage infection, remain asymptomatic but develop gametocytes, this could allow for the development of a much-needed clinical model to assess the efficacy of novel transmission-blocking vaccines[377].

In KCS, the risk of spread of the challenge strain of *P. falciparum* (NF54) into the surrounding area was thought to be minimal due to treatment of participants prior to the typical development of gametocytes and the lack of an appropriate mosquito vector in Nairobi. However, if the follow-up period prior to treatment

were extended in future studies, particularly in sites with local transmission of *P. falciparum*, careful thought should be given to the potential for natural transmission of the challenge parasite to local areas.

Conclusion: In the KCS study, the need to enrol all volunteers within one week and confirm this date in advance in order to coordinate with staff from Sanaria Inc. did limit the time available for screening. If local staff were trained to thaw and dilute PfSPZ Challenge and load syringes, then enrolment could be spread over a longer period, which would be especially useful if there is a difficulty recruiting appropriate participants. This should be possible as more of these studies are done in centres in malaria endemic countries.

Future CHMI studies aiming to examine the dynamics of NAI, the efficacy of blood-stage or transmission blocking vaccines should consider treating undiagnosed volunteers later than C+21. Performing qPCR in real-time to inform treatment decisions and measuring gametocytaemia using specific qPCR could also increase the applications of the CHMI model in Africa[378].

5.2.7. Clinical and laboratory resources

Given the complex and unique nature of CHMI studies, in particular the need for results to be available in real-time to guide clinical decision making, it was critical that both clinical and laboratory staff received detailed training in advance of the study. This involved the creation of 22 clinical and 29 laboratory study-specific standard operating procedures (SOPs), weeks of dedicated training sessions and 'dry runs' of the preparation and administration of PfSPZ Challenge. In order to adhere to internationally agreed standards[376] and achieve

consistency with northern CHMI centres, significant clinical and laboratory training and support were provided by both national and international collaborators. Since blood-film positivity was the primary criterion for the start of treatment, microscopy was a critical area of training. All microscopists passed a 2-week refresher-training course at the KEMRI-affiliated Malaria Diagnostics Centre of Excellence, Kisumu, Kenya approximately 3 months prior to working on study samples. Blood smears were prepared and interpreted according to a modified version of the "*Consensus SOP for Malaria Microscopy in the Context of Clinical Challenge Trials*"[317,376]. It was particularly important in the study to emphasise to the microscopists, who were used to working in a clinical, field setting, the importance of avoiding false positive results.

Conclusion: Support from established CHMI centres and detailed advanced training was essential for the first Kenyan CHMI study to take place safely and efficiently.

5.2.8. Feedback

On discharge, all participants were asked to complete an exit questionnaire providing feedback on their experience participating in the study. 19/28 (68%) of participants returned the anonymised questionnaire. Results were positive (Table 5-9) with participants reporting good understanding of the study and discharge procedures.

A written final report will be submitted to the ethical and regulatory bodies that approved the study and the results of the study presented to university

managers, local students and interested study participants when all analyses of study related data is completed.

Conclusion: Future CHMI studies in Kenya will be informed by feedback from volunteers, ethics and regulatory bodies and collaborators. It is crucial to feedback findings of the study to the key stakeholders in order to help maintain a working relationship for future CHMI studies.

Did you understand the study?	What was your state of health on discharge?	What was the attitude of the nursing staff?	Were procedures appropriately explained to you?	Was the discharge process clearly explained?	What was rating of p in the 1-3 / 4
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Not yet improved	Good	Sometimes	Clearly	7
Fully	Improved	Good	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Good	Always	Clearly	7
Fully	Good	Good	Always	Clearly	7
Fully	Good	Good	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Good	Sometimes	Clearly	7
Fully	Improved	Excellent	Always	Somehow clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Good	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7
Fully	Good	Excellent	Always	Clearly	7

Table 5-9: Feedback from volunteers enrolled in KCS

5.3 Conclusions

Performing CHMI studies in an African setting present unique but surmountable challenges. As the first pilot study performed in Kenya, there were many learning points, most unique to the African setting and it is hoped that the detailed discussion outlined above with inform future trials, both in Kenya and at other new CHMI sites in Africa.

CHAPTER 6: OPTIMISING QUANTITATIVE NUCLEIC ACID TESTING FOR CONTROLLED HUMAN MALARIA INFECTION STUDIES

6.1 Authorship statement

The work presented in this Chapter is part of a collaborative effort. Unless otherwise stated all work was performed by myself.

Nick Edwards at the Jenner Institute, University of Oxford, performed the qPCR for UK CHMI trials. Charles Magiri and Alfred Muia at KEMRI Centre for Clinical Research, Nairobi, performed WBC removal and extraction of parasite DNA for KCS. qPCR for KCS was performed Domtila Kimani at the KEMRI-Wellcome Trust Research Programme, Kilifi. KCS samples for qRT-PCR were prepared at site by Sean Elias. Sean Murphy's group at the University of Washington performed the qRT-PCR. Analysis of the qRT-PCR data was undertaken by myself in close collaboration with Sean Murphy.

6.2 Introduction

Various nucleic acid tests (NATs) have been used to detect and quantify pre-microscopy patent blood-stage parasitaemia. The most common nucleic acid target is the 18S rRNA-coding genes (rDNA) or the asexual-type 18S rRNAs themselves, which can be detected by nested polymerase chain reaction (PCR), quantitative PCR (qPCR), nucleic acid sequence-based amplification (NASBA) and quantitative reverse transcription PCR (qRT-PCR), as recently reviewed[379]. When applied in the post-CHMI setting, blood-stage infections can often be detected by NAT tests 2-5 days earlier than by blood smears, with most sensitive

NAT assays becoming positive around C+7 when the standard CHMI dose of five infectious mosquito bites is used[305,311,316,330,331].

As well as allowing calculation of the PMR of blood-stage parasites, modelling of NAT data allows estimation of the number of parasites released from the liver into the blood (LBI)[335]. LBI is an especially useful measurement for assessing the efficacy of drugs and vaccines designed to reduce or eliminate the sporozoite and/or liver-stage of development of the pre-erythrocytic malaria parasite. Parasite release from the liver after CHMI with 3D7 or NF54 sporozoites is thought to mainly start on C+6 but not be complete until C+7.5[336,380]; one report indicates detection as early as C+5 post-CHMI[331]. Given that many samples are NAT negative at these time points and direct measurement of LBI is therefore not possible, modelling methods are usually employed to estimate LBI[335]. If it were possible to improve NAT sensitivity at these early time-points to allow direct measurement of LBI, the accuracy and confidence of LBI measures would be markedly increased. A further application would be the ability to identify and study subclinical parasitaemias, particularly those that are detected unreliably by microscopy in field studies of natural immunity or vaccine efficacy.

There is much variation between centres in NAT methodology, making comparison of results difficult. However, most centres use qPCR to quantify DNA for the *P. falciparum* 18S ribosome (3 copies per parasite); have a lower limit of quantification (LLQ) of approximately 20 parasites/mL (p/mL); and rely on a three-stage process:

(i) Removal of human leukocytes (human DNA reduces the sensitivity of the assay) +/- lysis of RBCs (to release parasites).

(ii) Extraction of DNA.

(iii) Quantification of parasitic DNA using qPCR.

The qPCR protocol currently used by the University of Oxford involves filtration of fresh, whole blood to remove leukocytes using a multi-well plate (Whatman™ filter (WF)), followed by DNA extraction using a commercial kit (QiaAmp, Qiagen) and a Taqman-probe based qPCR reaction. The whole process is designed to be performed contemporaneously during an on-going trial[381]. At RUNMC, fresh blood samples are snap frozen with Zap-Oglobin, DNA is extracted using phenol-chloroform, and parasites quantified using the same Taqman-based qPCR assay, but the assay is not performed contemporaneously[341]. However both methods are time-consuming and costly, limiting to date their transfer to trial centres in the developing world.

This work set out to investigate potential modifications and alternatives to the Oxford qPCR protocol for quantifying *P. falciparum* with an aim to improving sensitivity at the LLQ, reducing costs and minimising the time demands of parasite quantification. The methods examined were;

1. CF11 filtration for leukocyte depletion;
2. Zap-Oglobin treatment of fresh whole blood samples;
3. Loop mediated isothermal amplification (LAMP);
4. qRT-PCR.

6.2.1 CF11 cellulose filtration for leukocyte depletion

Depletion of leukocytes from whole blood samples is an important requisite for multiple assays. Differential density centrifugation methods using sucrose solutions such as Lymphoprep™ are useful when viable leukocytes are required for subsequent immunological investigations. However when parasites need to be isolated for transcriptomic analysis or *in vitro* anti-malarial drug sensitivity surveillance[382], column filtration and removal of leukocytes is an alternative method to reduce genomic contamination from the host[383]. In contrast to certain commercially available filters (such as Plasmodipur or WFs), easy to produce filtration columns, using the cellulose powder CF11, are cheap, low tech and easy to use, filtering out a higher proportion of leukocytes using size exclusion and/or interaction between cellulose hydroxyl groups and leukocyte surface molecules[384].

Comparison of leukocyte depletion assessed using an automated haematology analyser showed CF11 columns to be superior to Plasmodipur filters, reducing the mean initial leukocyte count from 9.4 x 10^3 per µl (95% CI 5.2-13.5) to 0.01 x 10^3 (95% CI 0.01-0.03) and 0.7 x 10^3 per µl (95% CI 0.4-1.8) respectively[385]. Quantification of 30 *P. vivax* infected field samples by microscopy before and after CF11 filtration showed only a minor loss in parasitaemia (≤ 7.1% of initial counts) with no stage-specific retention of iRBCs seen[385]. In another study comparing CF11 column filtration of *P. falciparum* infected samples to a Lymphoprep™/Plasmodipur (LP) method prior to microarray sequencing, CF11 was more effective at reducing human DNA contamination (mean 15.9% vs 2.4% respectively; *p=0.03*) with comparable total DNA recovered with each method

(mean 4.64µg for CF11 vs 2.86µg for LP; *p=0.46*)[384]. These data have led to CF11 columns being widely used in the field for professing of samples for genomic studies of *P. falciparum*[386], however to date CF11 filtration has not been used in the processing of samples for qPCR in CHMI studies.

Given the complexity and considerable cost of the use of WFs in the Oxford CHMI qPCR protocol, this work sought to evaluate CF11 filtration as a cheaper and less time consuming alternative method with the potential to remove a greater proportion of leukocytes than WFs and improve qPCR sensitivity.

6.2.2 Zap-Oglobin treatment of fresh samples

Zap-Oglobin is a reagent that causes lysis of RBCs and is frequently used in flow cytometry experiments. For CHMI studies performed at RUNMC, fresh blood samples for qPCR are snap frozen with Zap-Oglobin. DNA is extracted using phenol-chloroform at a later date, and parasites quantified using the same Taqman-based qPCR assay as that used in Oxford[341]. Despite the lack of removal of leukocytes (and their DNA), this method gives similar sensitivity to the Oxford qPCR protocol (*N. Edwards personal communication*). Given this protocol removes the need for filtration of leukocytes and the time and expense this involves, assessment of whether snap freezing samples in Zap-Oglobin followed by DNA extraction using Qiagen QiAmp columns could give similar or improved sensitivity compared to the existing Oxford qPCR protocol was a key question.

6.2.3 Loop mediated isothermal amplification

LAMP is an alternative, highly sensitive and specific method of detecting nucleic material[343,387]. This isothermic method employs a DNA polymerase and a set of 4-6 highly specific primers that recognise 4-6 distinct sequences on the target DNA segment. The final products are stem-loops of DNA, which can be measured by a variety of well-recognised methods including turbidity and immunofluorescence[387]. LAMP has been widely deployed because of its simplicity, rapidity, high efficiency and specificity. Expensive equipment is not necessary and there are notably fewer preparation steps compared to conventional real-time PCR assays[387]. These features make LAMP an attractive alternative to real-time PCR for the detection of *P. falciparum* in resource poor settings.

LAMP has been used as an experimental method of detecting *P. falciparum* in clinical samples in a range of settings[388-392]. The assay does not cross react with DNA from other *Plasmodium* species and was sensitive, specific and cheap. However, all studies to date have designed the assay to provide binary results only (samples were positive or negative), limiting the application for studies requiring quantification for modelling of PMRs or LBI.

If it were possible to develop and validate a LAMP assay that was able to reliably quantify *P. falciparum* this would provide a cheaper and simpler alternative to the Oxford qPCR protocol, which would be more easily applied in field studies.

6.2.4 qRT-PCR

Each ring-stage parasite genome contains relatively few copies of the rDNA sequence targeted by DNA-based quantitative PCR (qPCR; 2-4 copies depending on the precise 18S rDNA sequence specificity). At the published 5 p/mL LLD for the Oxford qPCR method[316], 15 copies of the Oxford assay's target sequence are present in 1 mL of whole blood (3 copies per parasite genome). The assay involves DNA extraction from 0.5 mL of blood, and the use of 10% of this DNA in each of three replicate qPCR reactions. The LLD roughly corresponds to the parasite density at which it becomes probable that one or more qPCR reactions will contain at least one target sequence, resulting in just-detectable DNA amplification with a cycle threshold (C_T) of ~41 cycles. Because of the low number of target sequences per parasite, this LLD is somewhat higher than the parasite density at which it becomes probable that at least one parasite will be present in the blood from which DNA is extracted. The LLQ is higher still.

In contrast, in the University of Washington (UW) qRT-PCR method[346], ~3500 copies of the target 18S rRNA sequences are present in each ring-stage parasite. At the 20 p/mL published LLD for the UW qRT-PCR assay, 1 parasite is present in 0.05 mL of whole blood, which gives rise to 3500 copies of the qRT-PCR 18S rRNA target detected at a C_T of ~33.5 cycles at the LLD. Thus, in qRT-PCR, sampling of a parasite-containing volume of blood (or not) dictates the LLD, since each parasite contains a readily detectable 'quantum' of target sequences. Thus, because the rRNA is present at a concentration approximately three logs higher than that of the parent rDNA genes, qRT-PCR can be performed on

relatively small volumes of blood and achieves high sensitivity without the need for replicates since the amount of rRNA per parasite is so high.

To increase the sensitivity of Oxford qPCR to detect infections at <5 p/mL, larger volumes of blood could be processed, or a larger proportion of the DNA extracted from a 0.5 mL blood sample could be used in each PCR reaction. In either case, enhanced sensitivity would require maintenance of a reasonable ratio of parasite DNA to blood-derived potential PCR inhibitors. Sufficient removal of human DNA and other potential PCR inhibitors such as Hb from large samples is challenging (*Douglas AD, unpublished observations*). Because of the low number of target copies per parasite, this problem cannot readily be overcome by diluting the extracted DNA. In addition, it is relatively inconvenient to collect, store and process 5-10 mL volumes of whole blood for PCR-based diagnostics.

To increase the sensitivity of qRT-PCR to detect infections at <20 p/mL, a 10-fold larger sample (0.5 mL vs. 0.05 mL) could be collected and preserved in 10-fold more lysis buffer (20 mL vs. 2 mL). With this approach, the LLD could be reduced to 2 p/mL in a manner that could not be achieved with a DNA-based assay. This approach was tested using duplicate 0.5 mL qRT-PCR samples (hereafter referred to as 'large volume' qRT-PCR) alongside other approaches including the standard Oxford qPCR protocol, the standard 0.05 mL qRT-PCR method from UW and a qRT-PCR approach for 0.05 mL blood on dried blood spot (DBS) cards also from UW. Samples from both KCS and VAC052 (n=32), a UK mosquito-bite CHMI study assessing the efficacy of malaria vaccines ChAd63-MVA expressing AMA1, ME-TRAP and CS (*Rampling et al. Manuscript in preparation*) were used.

6.3 Results

6.3.1 CF11 cellulose filtration for leukocyte depletion

The efficiency of leukocyte depletion was compared between CF11 columns and the WFs routinely used in the Oxford qPCR protocol. Two whole blood samples spiked with 100,000 *P. falciparum* parasites were used to make two, 10-fold dilution series: A and B (see Chapter 2). Aliquots from both dilution series were then filtered either with WFs or CF11 filters and the concentration of DNA in the end samples compared. These results showed no significant difference in total DNA present in samples filtered using either method (Figure 6-1) (*p=0.12).* When data were corrected to calculate total DNA post-filtration accounting for differences in the filtered sample volume from which DNA was eluted (0.5ml for WF samples and 2ml for CF11 samples) and elution volume for each method (50µl for WF samples and 100µl for CF11 samples), there was still no significant difference between WFs and CF11 columns at removing white cells(Figure 8-1) (*p=<0.10).*

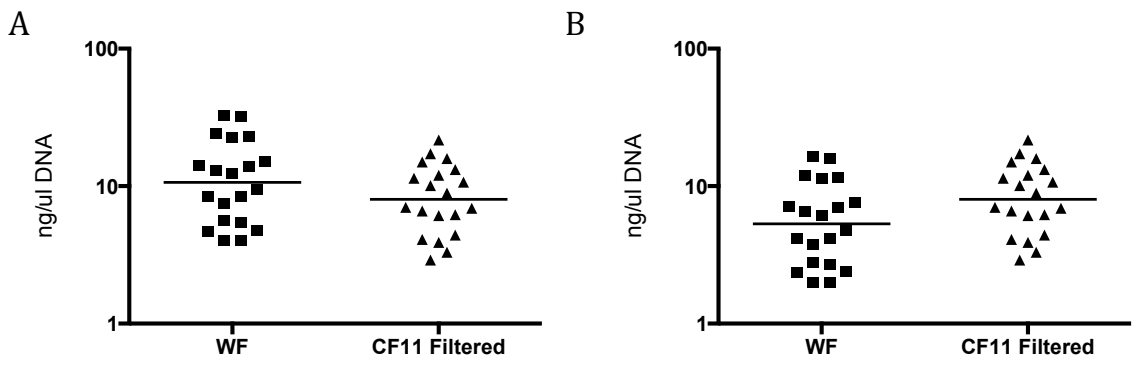


Figure 6-1: Comparison of total DNA persisting post Whatman or CF11 filtration of spiked whole blood samples

Total DNA measured by nanodrop for samples from two dilution series (A & B) filtered using Whatman™ filters (WF) or CF11 columns. (A) Results according to protocol methods; WF v CF p=0.12. (B) Results corrected for ng/µl in starting 2mL sample; WF v CF p=<0.10. Paired t tests.

qPCR performed at a later date on these samples using the Oxford qPCR protocol (with appropriate modifications to account for the increased sample size of the CF11 samples) showed that WF of samples gave the most accurate qPCR results, particularly at lower parasite concentrations (Figure 6-2). However, CF11 filtration of samples led to increased qPCR sensitivity, with all *P. falciparum* spiked CF11 samples giving a positive result, even those below the qPCR LLQ (Table 6-1).

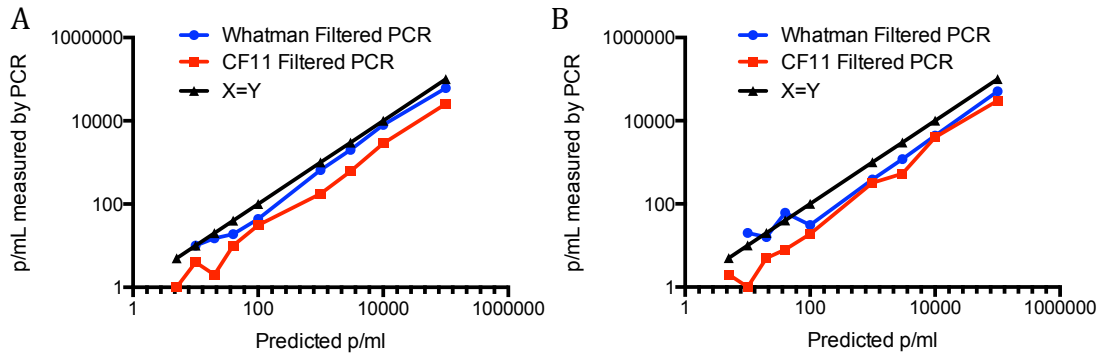


Figure 6-2: Comparison of qPCR results from spiked whole blood samples filtered using Whatman and CF11 filters

qPCR results for samples spiked with known concentrations of P. falciparum parasites filtered using Whatman filters or CF11 columns. A) Dilution series A. B) Dilution series B (see Chapter 2).

Predicted p/mL	WF Dilution Series A	CF 11 Dilution Series A	WF Dilution Series B	CF 11 Dilution Series B
0	0	0	0	0
5	0	1	0	2
10	10	4	20	1
20	15	2	16	5
40	19	10	61	8
100	44	31	31	19
1,000	660	177	387	323
3,000	2013	611	1197	536
10,000	8012	2868	4389	4018
100,000	61423	25687	51172	30438
Sensitivity	*89%*	*100%*	*89%*	*100%*

Table 6-1: qPCR data for spiked whole blood samples filtered using Whatman or CF11 filters

Sensitivity = number of true positives/(number of true positives + number of false negatives).

Comparison of the sensitivity of qPCR at low parasitaemias for WF and CF11 filtered samples was undertaken using samples from VAC049. Samples processed using WF gave more accurate results than those filtered using CF11, with higher qPCR values in WF samples (Table 6-2). The CF11 protocol did not appear more sensitive than the WF protocol; there were three occasions when CF11 samples were positive earlier than the equivalent WF sample and two occasions when WF samples were positive earlier than the equivalent CF11 sample.

	C+7		C+9	
Vol ID	**CF11**	**WF**	**CF11**	**WF**
1203	3	8	5	18
1204	N	N	N	N
1205	N	N	N	N
1209	N	7	14	62
1210	0.4	8	1	45
1211	N	N	N	N
1212	N	N	0.4	21
1215	N	N	N	N
1216	N	N	2	32
1220	N	N	N	N
1221	N	N	N	N
1224	0.2	12	N	13
1225	0.04	N	4	42
1228	N	10	2	9
1231	N	N	N	20
1233	0.2	N	N	11
1235	1.8	N	21	128
1236	N	N	1	19

Table 6-2: qPCR data for VAC049 samples filtered using Whatman filters or CF11

WF= Whatman filters. qPCR was performed six times on each sample and the mean result shown. N=qPCR negative. Blue shaded box = qPCR positive but value < qPCR LLQ. Pink shaded box = qPCR positive with value >LLQ. Green shaded boxes highlight the earliest time-points for each volunteer where one filtration method was superior to another at detecting a positive result, even if <LLQ.

6.3.2 Zap-Oglobin treatment of fresh samples

qPCR results from C+7 or C+7.5 samples collected in two UK mosquito-bite CHMI vaccine studies (VAC045[340] and VAC052 (*Rampling et al., in preparation*)) and snap frozen with or without Zap-Oglobin, were compared to results generated using the Oxford qPCR protocol (Table 6-3). Generally, whole blood samples snap frozen with Zap-Oglobin were more sensitive at detecting *P. falciparum* than samples snap frozen without Zap-Oglobin. However, samples processed according to the Oxford qPCR protocol appeared more sensitive for detecting *P. falciparum*, with generally higher qPCR values. On the basis of this preliminary evidence, there was little to support further investigation of the use of whole blood samples with or without Zap-Oglobin as a possible method of improving the Oxford qPCR method.

Vol ID	Oxford Protocol	WB Snap Freeze + ZAP	WB Snap Free - Zap
VAC045 1402	53	26	26
VAC045 1404	14	4	6
VAC045 1405	5	1	0
VAC045 1406	ND	ND	ND
VAC045 1408	ND	10	0
VAC045 1416	61	13	5
VAC045 1419	103	100	41
VAC045 1420	15	9	6
VAC045 1429	26	ND	2
VAC045 1431	316	176	127
VAC045 1436	207	79	49
VAC045 1444	104	65	35
VAC052 1302	64	130	114
VAC052 1303	11	ND	18
VAC052 1305	ND	ND	ND
VAC052 1308	ND	ND	ND
VAC052 1309	ND	ND	ND
VAC052 1310	40	32	15
VAC052 1312	47	24	3
VAC052 1335	ND	ND	ND
VAC052 1337	329	129	68
VAC052 1338	ND	ND	ND
VAC052 1339	70	ND	12
VAC052 1330	12	16	5

Table 6-3: qPCR data for samples collected post-CHMI following Zap-Oglobin treatment

qPCR data (parasites/mL) for samples taken from malaria-naïve volunteers on C+7 (VAC045) or C+7.5 (VAC052) post-mosquito-bite CHMI. WB = whole blood. ZAP = Zap-Oglobin. ND=Parasites not detected on qPCR. Yellow shaded box = sample identified as positive when Zap-Oglobin treated only. Green shaded box = whole blood sample giving higher qPCR value than Oxford protocol. Pink shaded box = sample identified as qPCR positive when run using Oxford qPCR protocol but not Zap-Oglobin treated whole blood sample.

6.3.3. Loop mediated isothermal amplification

LAMP was performed using whole blood samples spiked with known concentrations of *P. falciparum*. Sybr green was added to the reactions to assess if changes in fluorescence overtime could allow quantification of starting nucleic material. Samples with parasitaemia ≥ 71 p/mL all had rapid, indistinguishable increases in fluorescence over time (Figure 6-3). Samples with parasitaemia < 71 p/mL did not have any meaningful increase in fluorescence. Gel electrophoresis of the LAMP products confirmed failure of amplification of parasite DNA in samples with concentrations < 71 p/mL (Figure 6-3). In addition, for the samples where LAMP had successfully amplified the starter DNA, no difference in the amount of LAMP product was seen with differing parasitaemias. Given the lack of LAMP at the low concentrations of parasitaemia typically seen early in CHMI studies, no further investigation of this method was undertaken.

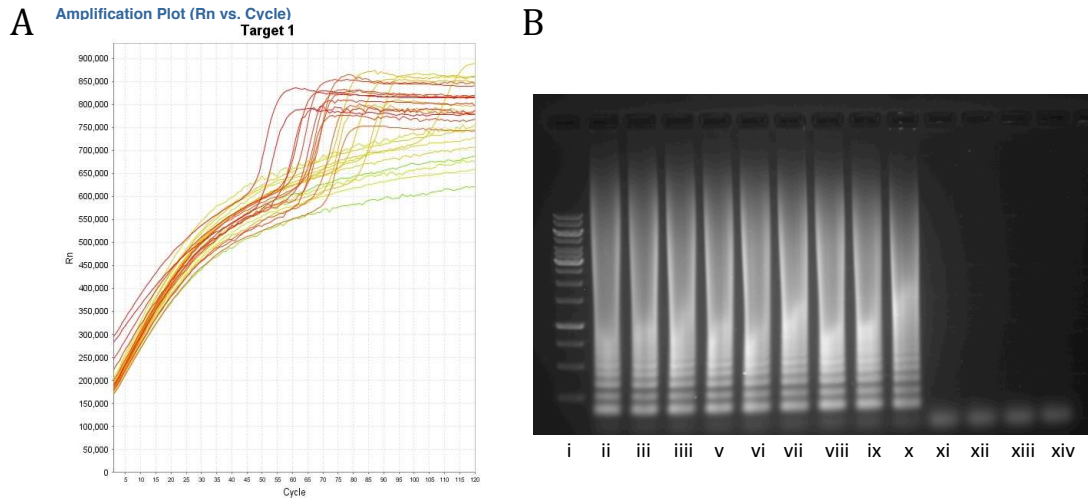


Figure 6-3: LAMP results for whole blood spiked P. falciparum parasites

(A) Fluorescence plot overtime. Rn (normalized reporter): fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. (B) Agrose gel of LAMP products. Samples in wells left to right; (i) Ladder, (ii) Sample 1 (S1) (872,071 p/mL), (iii) S1b, (iv) S2 (80819 p/mL), (v) S2b, (vi) S3 (6706 p/mL), (vii) S3b, (viii) S4 (590 p/mL), (ix) S4b, (x) S5 (71 p/mL), (xi) S5b, (xii) S6 (7 p/mL), (xiii) S6b, (xiv) No template control. 'b' samples are 1 in 2 dilution of corresponding sample tested by LAMP in by the same method; e.g. S1b = 1 in 2 dilution of S1 i.e. approximately 436,036 p/mL

6.3.4 qRT-PCR

Starting with samples from C+7.5 in VAC052 *(Rampling et al., in preparation)*, Oxford qPCR and UW-based standard (0.05 mL RNA/DNA) and large volume (0.5 mL RNA/DNA) qRT-PCR assays were performed to see if increasing the volume of blood tested by qRT-PCR enhanced sensitivity. This sample set included 32 subjects, 27 of who developed patent parasitaemia by C+21, (6 of whom showed

a significant delay to diagnosis) and 5 who did not develop malaria infection by C+21 (*Rampling et al., in preparation*). Of the samples collected on C+7.5 from the 27 subjects who were diagnosed by C+21, 19/27 were positive (≥LLD) by Oxford qPCR, 20/27 by UW standard volume qRT-PCR and 24/27 by UW large volume qRT-PCR. The only subjects negative by all assays were those who either did not develop malaria infection and 3/6 who showed a significant delay in TTD.

Overall 'clinical sensitivity' at C+7.5 (true positives/(true positives + false negatives) using study-protocol-defined definitions of malaria was 70.4% for Oxford qPCR, 74.1% for UW-based standard volume qRT-PCR and 88.9% for large volume qRT-PCR assay. 'Clinical specificity' (true negatives/(true negatives + false positive) using study-protocol-defined definitions of malaria was 100% for all assays.

Since the larger volume RT-PCR assay showed increased sensitivity at C+7.5 in VAC052, samples collected during the first cycle of expected parasitaemia in KCS trial (C+6.5-C+7.5) from the 27 subjects diagnosed with malaria were assessed. 100% of these subjects were diagnosed with malaria and so specificity of the assays could not be compared for these samples. Large volume qRT-PCR assay was most sensitive in this dataset, followed by Oxford qPCR, standard volume qRT-PCR and DBS qRT-PCR (Table 6-4). On C+6.5, the large volume qRT-PCR assay detected the highest number of positive specimens (10/27), followed by Oxford qPCR (7/27) and UW standard volume qRT-PCR assay (1/27). Amongst samples positive on C+6.5, all large volume qRT-PCR (n=10) and standard volume qRT-PCR assay samples (n=1) were quantifiable within the limits of the assay (i.e. ≥LLQ), whereas all samples positive by Oxford qPCR (n=7) were in the

'low positive' range (i.e. ≥LLD). By C+7.0, most samples were qualitatively positive by all assays (27/27 by large volume qRT-PCR, 26/27 by Oxford qPCR, 19/27 by standard volume qRT-PCR and 14/26 by DBS qRT-PCR), which increased further by C+7.5.

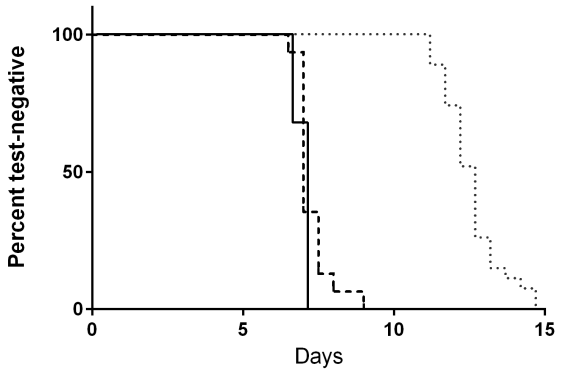


Figure 6-4: Time to positivity (>LLD) for microscopy, Oxford qPCR and large volume qRT-PCR in KCS

n=27. Kaplan-Meier survival curve representing the rate of conversion from negative to positive NAT results by test method. Solid line, UW large volume qRT-PCR; heavy dashed line, Oxford qPCR; light dotted line, microscopy diagnosis.

Diagnosis by method (blood volume)*	**C+6.5**	**C+7.0**	**C+7.5**
UW large volume qRT-PCR (0.5 mL)	37.0%	100.0%	100.0%
Oxford qPCR (0.5 mL)	25.9%	96.3%	100.0%

UW standard qRT-PCR (0.05 mL)	3.7%	70.4%	88.9%
UW DBS qRT-PCR (0.05 mL)	0.0%	50.0%	84.6%
	n=27 (n=26 DBS)		

Table 6-4: 'Clinical sensitivity' by NAT assay for values ≥LLD for KCS

**Clinical trial diagnosis of malaria by C+21 as the gold standard comparator. n=27.*

A large proportion of samples tested by Oxford qPCR were identified as 'low positive' results (i.e. LLQ > result ≥ LLD) (27/60 positive qPCRs on C+6.5–C+7.5) whereas a low proportion of positive qRT-PCR samples were in the low positive range (0/61 large volume qRT-PCR, 3/43 standard volume qRT-PCR and 5/35 DBS qRT-PCR). On average, the large volume qRT-PCR and the Oxford qPCR assays became positive (>=LLD) on C+7.0 compared to C+12.5 by microscopy (Figure 6-4), giving a 5.5 day average lead time over microscopy.

Next, inter-assay comparison of quantitative results was performed using all tests that generated results >LLQ. With the exception of the pairing of Oxford qPCR results with DBS qRT-PCR results, all datasets showed statistically significant concentration-dependent correlation with one another (Figure 6-5). The strongest correlation was observed between the large and standard volume qRT-PCR assays performed at UW. While Oxford and UW assays showed strong correlation, when comparisons were made using Bland-Altman to look for quantitative agreement and bias, a consistent quantitative shift was observed between Oxford qPCR compared to liquid sample-based UW qRT-PCR assays

(large and standard volume) (Figure 6-6), consistent with previous reports[379]. DBS qRT-PCR results also diverged from UW liquid results, and this shift resulted in apparent alignment between Oxford qPCR and UW DBS qRT-PCR results.

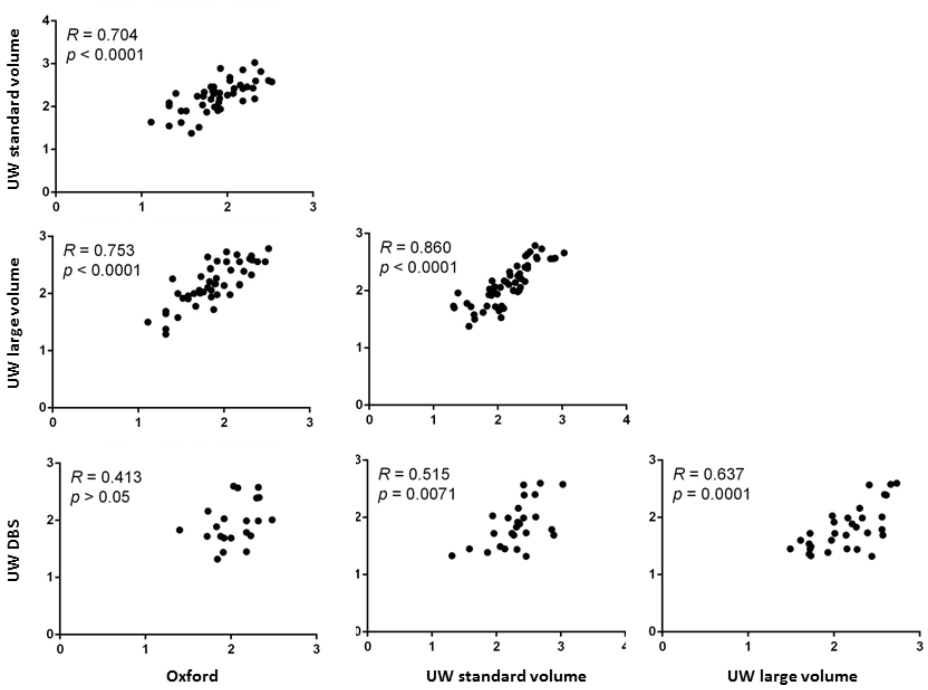


Figure 6-5: Correlation analyses of data from varying NAT methods

Data were compiled from all samples in KCS and where two methods produced results ≥LLQ. Spearman rank correlation. Units for x and y axes are log_{10} p/mL whole blood.

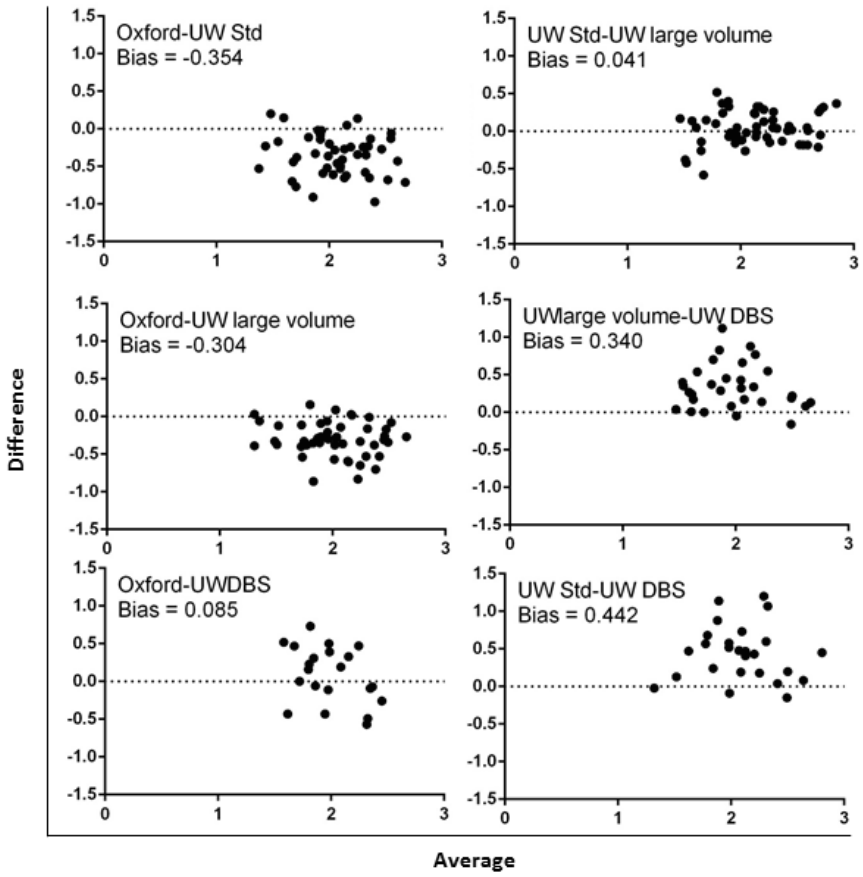


Figure 6-6: Agreement analyses of data from varying NAT methods.

Data were compiled from all samples in KCS and where two methods produced results ≥LLQ and plotted using a Bland-Altman chart showing the average result (x-axis) and the difference between results (y-axis) for each sample. The labels indicate the mathematical order used to calculate difference. Units for x- and y-axes are log_{10} p/mL whole blood as determined for paired samples. The average difference from a value of 0.0 log_{10} p/mL equals the bias.

A comparison was undertaken of LBI estimated from the maximum qPCR value obtained with Oxford qPCR between C+6.5 and C+7.5 (4 data-points: C+6.5, C+7.1, C+7.3, C+7.5; the first cycle peak method[335]) and the UW large volume

qRT-PCR result at C+7.5 for subjects in VAC052. Sterilely-protected volunteers were NAT negative by all assays and were excluded from the analysis. Comparison of the ability to calculate LBI using different datasets is shown in Table 6-5. Although LBI could be estimated for 81% of participants (n=21) using Oxford qPCR data ≥LLD, LBI could only be quantified using Oxford qPCR data ≥LLQ for 67% of participants (n=18). Use of qRT-PCR data ≥LLQ allowed LBI to be calculated for 85% of participants (n=23).

LBI		**Oxford qPCR: n (%)**			
		Able to estimate using data ≥LLQ	Able to estimate using data ≥LLD	Not Able to estimate using data ≥LLQ	Not Able to estimate using data ≥LLD
UW large volume qRT-PCR: n (%)	Able to estimate using data ≥LLQ	18 (67%)	22 (81%)	5 (19%)	1 (4%)
	Not able to estimate using data ≥LLQ	0 (0%)	1 (4%)	4 (15%)	3 (11%)

Table 6-5: Comparison of ability to estimate LBI using peak method for subjects: qRT-PCR large volume vs. Oxford qPCR method

Data from VAC052. Sterilely protected volunteers were NAT negative by all assays and were excluded from the analysis. n=27.

In KCS, LBI for the study publication was calculated with simple linear regression using Oxford qPCR values[317]. LBI could not be calculated with this method using

the qRT-PCR data given that samples were only taken at 3 time points during the first cycle of parasitaemia. For the purposes of comparison, LBI was therefore estimated for the KCS study with the first cycle peak method using Oxford qPCR and large volume RT-PCR data ≥LLQ and ≥ (Table 6-6). Use of qRT-PCR increased the proportion of volunteers for whom LBI could be quantified more accurately using data ≥LLQ (Table 6-7).

Liver-to-Blood Inoculum	**Peak Method***			
	≥ LLQ		≥ LLD	
	Min	Max	Min	Max
Oxford qPCR	97,608**	1,396,941**	33,768	1,396,941
UW large volume qRT-PCR	37,800	1,855,630	37,800	1,855,630

Table 6-6: Estimations of LBI for KCS using data generated using different NAT methods

**Units are total number of parasites estimated to be released from the liver. **n=20 (8 volunteers were qPCR negative up to C+7.5). All other analyses n=28.*

LBI		**Oxford qPCR: n (%)**			
		Able to estimate using data ≥LLQ	Able to estimate using data ≥LLD	Not Able to estimate using data ≥LLQ	Not Able to estimate using data ≥LLD
UW large volume qRT-PCR: n (%)	Able to estimate using data ≥LLQ	20 (71%)	28 (100%)	8 (29%)	0
	Not Able to estimate using data	0	0	0	0

	≥LLQ				

Table 6-7: Comparison of ability to estimate LBI using peak method for subjects in KCS: qRT-PCR vs. Oxford qPCR methods

n=28.

6.4 Discussion

6.4.1 CF11 Cellulose filtration for leukocyte depletion

Published data have shown an advantage of CF11 filtration over Plasmodipur filters when processing *Plasmodium* infected blood samples[384,385]. However, this work is the first time CF11 filtration has been compared to WFs. This data using samples from a CHMI study have shown WFs to be superior both in terms of leukocyte depletion and parasite retention. It is important to note key differences in the WF and CF11 protocols used. For WF samples, DNA was eluted in 50µl of Tris from 0.5mL of WF blood. In contrast for CF11 filtered samples, DNA was eluted in 100µl of Tris from 2mL of CF11 blood. Whilst this difference was applied with an aim to increase the sensitivity of samples filtered using CF11, this may have instead increased parasite retention in the CF11 column.

Of note, samples used had low parasitaemias (0-100,000 p/mL), typical of those seen in early samples taken post-CHMI[287]. *Venkatesan et al* noted that leukocyte contamination increased with decreasing parasitaemia and recommended an ideal minimum parasite density for CF11 filtration of 10,000,000 p/mL[384,386].

The fact that samples tested in this work had considerably lower parasitaemias than this may have contributed to the poor result seen with CF11 samples. Other differences to the protocol established by *Venkatesan et al* (smaller sample volume; 2mL v 3mL, pushing the sample through the column rather than allowing passage by gravity) may also have contributed to the difference in results.

The lack of clear evidence of superiority of CF11 over WF for removing leukocytes in CHMI study samples, and the fact CF11 filtration was time-consuming to perform, mean the CF11 protocol assessed is not a viable alternative to WF. Future work could assess a novel prototype non-woven fabric filter, which has been shown to be superior to CF11 at removing leukocytes and retaining infected RBCs, as a cheaper alternative to WF[383].

6.4.2 Zap-Oglobin treatment of fresh samples

The addition of Zap-Oglobin to samples prior to extraction of parasite DNA allows RUNMC to avoid removal of leukocytes from samples. However, these data show that when using the Oxford PCR protocol, Zap-Oglobin treated whole blood had reduced sensitivity compared to samples filtered with WFs. Given that the RUNMC qPCR protocol has similar sensitivity to the Oxford qPCR protocol, it may be that Zap-Oglobin treated samples require subsequent DNA extraction to be performed using phenol-chloroform rather than the QiaAmp kit in order to have maximal sensitivity. Given the potential hazards associated with exposure to phenol-chloroform and the fact the RUNMC qPCR protocol is no more sensitive than the Oxford qPCR protocol, no further work was undertaken to investigate the use of Zap-Oglobin.

6.4.3 LAMP

The simplicity of method, isothermic nature of the reaction and clear dichotomy of results in terms of positive versus negative are why LAMP is such an attractive method to assess for parasitaemia in the field. However, these data showed limited sensitivity of LAMP at low parasite concentrations, despite published evidence of successful amplification from samples as low as 8p/mL[393]. However, the speed of the isothermic nature of the reaction meant that it was impossible to use change in fluorescence as a measure of starting DNA. Whilst some centres have used turbidity as a gross measure of parasitaemia, in the context of CHMI trials where the aim is to increase sensitivity and accuracy at concentrations <20 p/mL, LAMP is not a viable method of to quantify parasites.

6.4.4 qRT-PCR

The UW large volume qRT-PCR assay accelerated early malaria diagnosis compared to other assays. If the large volume qRT-PCR was applied at all pre-diagnosis time points post-CHMI, it is likely that the increased sensitivity could speed diagnosis if used in real time as a criterion to initiate treatment in CHMI studies assessing pre-erythrocytic drugs and vaccines. Data generated from such an approach could improve the accuracy of LBI estimation in those trials where early time points (up to C+7.5) were frequently negative by standard assays, such as those using PfSPZ Challenge[311,316]. In studies where early time points are qualitatively positive by Oxford qPCR or standard UW qRT-PCR, the large volume qRT-PCR could improve the accuracy of absolute LBI. However, given variability in the challenge inocula used in mosquito-bite CHMI studies, analyses are usually

restricted to intra- not inter-study data, so the absolute qPCR or qRT-PCR (and calculated LBI) values are not as critical for comparisons between studies. If there is a move toward more CHMI trials using highly standardised inocula such as PfSPZ Challenge, then it may be possible to perform inter-trial comparisons where use of the more sensitive large volume qRT-PCR method at multiple sites could be especially useful. Ultrasensitive diagnosis using this type of approach could also be useful in studies of Duffy- negative subjects challenged with large numbers of *Plasmodium vivax*-infected mosquitoes since these subjects do not develop patent parasitaemia but can have detectable parasite nucleic acids in their blood following successful liver-stage development and abortive erythrocyte infection (*Herrera, personal communication.).*

When the LBI was calculated using all data (including data inferred on samples below the LLQ but ≥LLD), there were minimal differences between the proportions of individuals with positive LBI calculated using the large volume qRT-PCR and the Oxford qPCR approaches. While preferable to use most accurate data (i.e., ≥LLQ), given the considerable greater cost of large volume qRT-PCR, it may be preferable to use standard Oxford qPCR or qRT-PCR methods and include values <LLQ but ≥LLD when calculating LBI endpoints.

Comparisons of NAT methods showed a high degree of concentration-dependent correlation. The liquid-based assays at UW had the highest degree of quantitative agreement by Bland-Altman analysis, while a consistent quantitative shift was seen between Oxford qPCR and both liquid sample-based UW qRT-PCR assays. This shift was likely due to loss of parasites during Oxford filtering of whole blood and to differences in the matrix used for plasmid DNA calibrators at

Oxford, as has been previously reported[329]. The UW DBS qRT-PCR also diverged from the UW liquid-based samples, likely due to incomplete recovery of the target from the DBS surface[346]. The apparent alignment between Oxford qPCR and UW DBS qRT-PCR results is likely due to losses in both assays.

Whilst qRT-PCR performed on whole blood preserved on DBS cards had the lowest overall sensitivity of the methods compared, the DBS samples were still positive days before microscopic diagnosis. These samples were collected and initially stored in a field setting with the use of desiccant and humidity indicators before being shipped to the UW testing facility. Therefore, DBS qRT-PCR could offer an attractive option for field studies where early (C+6.5-C+7.5) diagnosis is not critical.

6.5 Conclusions

Of the methods explored in order to improve on the sensitivity of the Oxford qPCR protocol, large volume qRT-PCR was the most promising. However, the considerable cost of the increased volume of lysis buffer required to process the increased sample volumes necessary for this method are currently prohibitive. Given that analyses showed that LBI calculated using Oxford qPCR data ≥LLD considerably improved the accuracy of LBI at no extra cost, this is the most appropriate current strategy and indeed this approach was employed in the analysis of parasite data from VAC049 and KCS.

CHAPTER 7: UNDERSTANDING THE IMMUNE RESPONSE TO CONTROLLED HUMAN MALARIA INFECTION USING *IN VITRO* ASSAYS

7.1 Authorship statement

The work presented in this Chapter is part of a collaborative effort. Unless otherwise stated all work was performed by myself.

For KCS, Sean Elias, Charles Magiri and Alfred Muia processed and stored samples for later assays. Alex Spencer and Rhea Longley did the same for VAC049.

David Llewellyn provided *P. falciparum* merozoite lysate for the ADRB activity assay and assisted with processing of these samples. Carole Long's group at the international GIA assay reference laboratory, NIH-NIAID, performed the GIA assays. Sarah Silk and Kathryn Milne assisted with ELISAs against AMA1, $MSP1_{19}$ and RH5. Andrew Armitage and Katherine Wray assisted with the hepcidin assay.

Screening of sample C-1 from volunteer 1221 in VAC049 was performed by Laurent Decosterd at the University Hospital of Lausanne, Switzerland. Anti-schizont antibodies were assessed by Faith Osier's group at the KEMRI-Wellcome Trust Research Programme, Kilifi. Assays of serum CRP, iron, ferritin, transferrin and transferrin saturation were performed by Selvaraj Nambiar at the Oxford University Hospitals' Trust, Oxford.

Nicola Williams (The Centre for Statistical Medicine, University of Oxford), and Eleni Giannoulatou (Victor Chang Cardiac Research Institute, Sydney) assisted with statistical analysis.

7.2 Introduction

In vitro assays have been used extensively to improve understanding of the immune responses to malaria. Given the large number of blood-stage proteins identified (Figure 7-1), there is a huge selection of antigens against which immunological responses could be assessed using *in vitro* assays. That, in combination with the complexity of the immune response to malaria and limited sample volumes collected in KCS and VAC049, meant the choice of assays performed using samples needed to be rationalised.

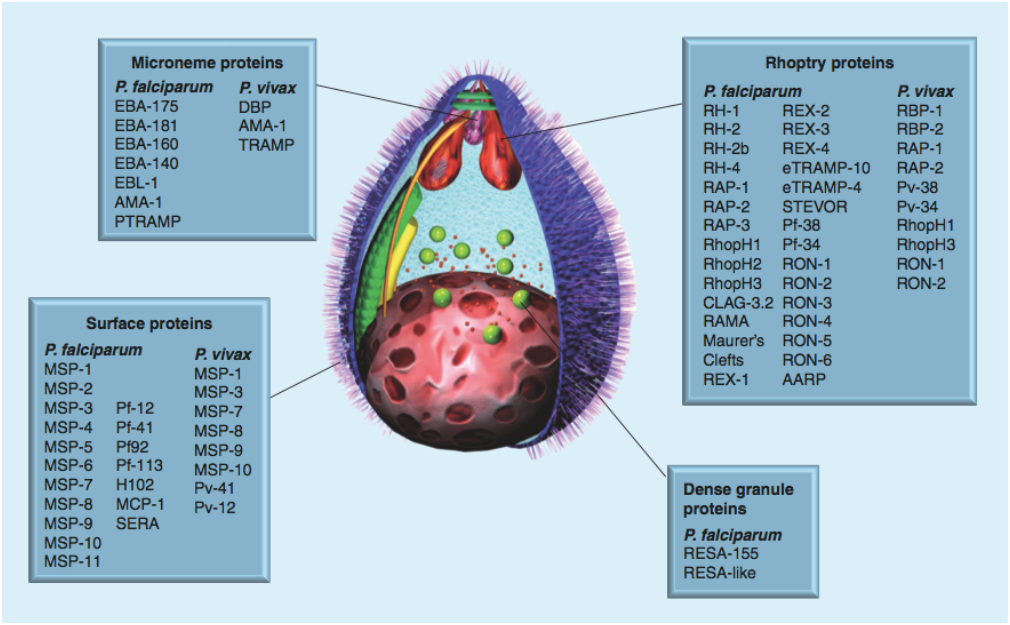


Figure 7-1: Proteins identified in the merozoite stage of P. falciparum

Adapted from Patarroyo et al [394].

7.2.1 Quantification of antibody and cellular responses to key antigens

Firstly, antibody and T cell responses to the blood-stage antigens; MSP1, AMA1 and reticulocyte binding protein homolog 5 (RH5) were assessed given their importance as potential vaccine candidates and existing data available for comparison from various settings. Antibody responses to schizont extract were assessed for KCS volunteers at C+35 to allow comparison with results from screening.

MSP1 is the most abundant and well-studied protein on the merozoite parasite and is found as part of a complex with the proteins MSP6 and 7 in the maturing schizont. Primary processing during merozoite release from schizont cleaves MSP1 into four fragments of 30, 38, 42 and 83 kDa. During erythrocyte invasion, the 42 kDa fragment is cleaved into two fragments of 33 and 19 kDa. The 33 kDa region along with the protein complex is shed, whilst the 19 kDa fragment is maintained on the parasite as it enters the erythrocyte[395,396]. This, along with the knowledge that MSP_{19} is more conserved than $MSP1_{33}$ made it a target for early vaccine studies[397]. Conflicting data from clinical studies has made the association between antibody responses to MSP1 and NAI difficult to interpret[398-400]. These data and polymorphisms in MSP1 have led to the suggestion that MSP1 may be an important antigen in immune evasion[401]. Comparison of antibody responses to $MSP1_{19}$ and AMA1 between subjects following ChAd63-MVA immunisation, CHMI and natural exposure suggest that complex differences in qualitative aspects of antibody responses to these antigens depend on the means of their induction[402].

AMA1 is located within the micronemes of the merozoite and sporozoite and is involved in cellular invasion of erythrocytes and hepatocytes. Antibodies to AMA1 that disrupt this process have been shown to prevent invasion, and as such AMA1 has become a leading antigen in vaccine development[403]. However, AMA1 is relatively polymorphic within malaria species[404]. Although individuals have been shown to produce antibodies recognising both conserved and polymorphic regions of AMA1[405], a single amino acid change can abolish cross-strain neutralisation[406]. As a result, a major aim of AMA1 vaccines is to maximise allelic coverage or target conserved regions of AMA1[407-409]. Multiple field studies have associated antibodies to AMA1 with exposure and clinical immunity[405,410-412]. However, AMA1 vaccines to date have shown disappointing levels of efficacy in Phase IIa/b clinical trials[241,413,414]. Whilst one AMA1 formulation reportedly showed strain-specific efficacy in a field trial in Malian children[415], assessment in a subsequent blood-stage CHMI study in the UK showed no efficacy (*Payne et al. submitted*).

Rhoptry antigens are important during erythrocyte invasion, binding to receptors on the RBC surface[416]. RH5 is one such antigen and, given its highly conserved nature, has become a leading vaccine candidate antigen[223]. Individuals exposed to *P. falciparum* develop limited anti-PfRH5 antibodies, but when detected, these have been associated with protection against symptomatic malaria and high-density parasitaemia[417]. It is thought that the essential function of RH5 has limited the development of polymorphisms in spite of evidence that anti-RH5 antibodies may be clinically protective[223,418]. Given the limited volume of serum samples available for VAC049, RH5 ELISAs were performed for KCS

only.

7.2.2 Assessment of candidate functional assays

A functional assay that could reliably predict blood-stage immunity would have extremely important applications, for example helping identification of individuals with NAI and understanding of the mechanism(s) of their immunity, or de-selection of blood-stage vaccines that aim to, but fail to, successfully mimic NAI[222]. Functional assays of natural or vaccine-induced immunity can be broadly grouped into those assessing either anti-iRBC or anti-merozoite antibody responses. Whilst it is largely accepted that responses to parasite antigens expressed on the surface of the iRBC are particularly important for NAI, it is also felt that responses against merozoites play a role[225].

It was decided to assess anti-merozoite antibody responses in VAC049 and KCS using two leading functional assays: the GIA assay and ADRB activity assay. As discussed in Chapter 1, both these assays have been suggested as potential measures of NAI, but in the case of each assay, no data have definitively confirmed or refuted such roles to date

7.2.3 Characterisation of IFN-γ responses

Interferon signalling, particularly pathways involving IFN-γ are known to play a crucial role in the immune response to, and immunopathology of malaria (Chapter 1). However, changes in IFN signalling over the time course of infection and the role of IFNs in mediating immunity to malaria remain to be definitively clarified, particularly in African subjects. It was decided therefore to assess changes in IFN-γ overtime in both KCS and VAC049 to better define the changes

seen in early pre-patent blood-stage infection and the effect of prior exposure on these.

7.3 Results

7.3.1 ELISAs

Antibody responses in UK adults in VAC049 to $MSP1_{19}$, and AMA1 were significantly induced following a single infection (Figure 7-2 and Table 7-1). $MSP1_{19}$ and AMA1 responses at C+35 in VAC049 were comparable to those reported in UK controls following mosquito-bite CHMI[402]. Comparison of baseline ELISA results between UK subjects and MinExp volunteers in KCS showed no significant difference in responses (Figure 7-2). A significant difference in responses to $MSP1_{19}$ was seen between VAC049 volunteers and MinExp subjects in KCS at C+35 but not to AMA1 (Figure 7-2).

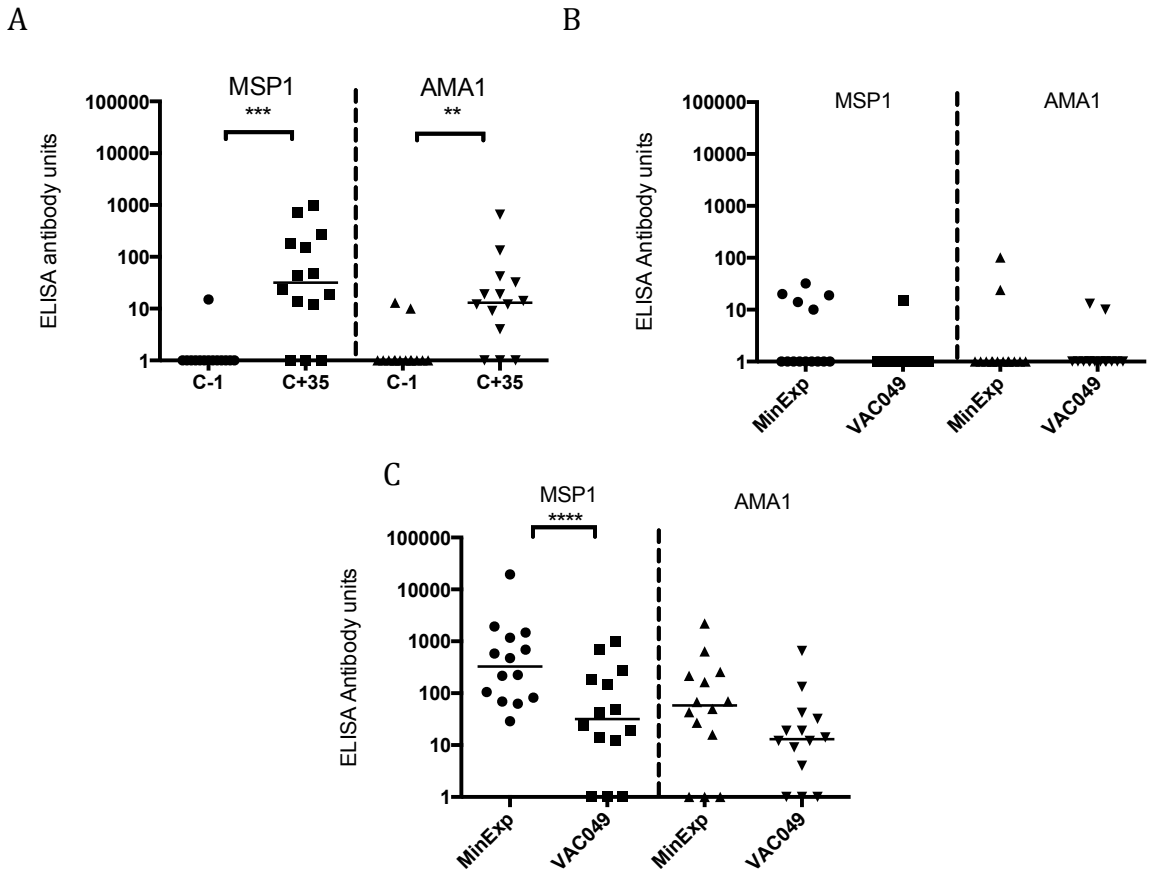


Figure 7-2: IgG Antibody responses pre and post-CHMI for VAC049 and KCS

(A) VAC049 (n=14). (B) Antibody responses at C-1 for VAC049 and MinExp volunteers from KCS. (C) Antibody responses at C+35 for VAC049 and MinExp volunteers from KCS. Median values are indicated. Comparisons were performed using Wilcoxon or Mann Whitney tests as appropriate.

Comparison	MSP1 IgG Titre		AMA1 IgG Titre		GIA		ADRB	
	p	***r***	***p***	***r***	***p***	***r***	***p***	***r***
VAC049 C-1 v C+35	**0.001**		**0.004**		**0.0002**		0.855	
Parasitaemia at diagnosis v C-1	**<0.0001**	-0.034	**<0.0001**	-0.408	0.935	0.0274	0.454	-0.2176
Parastaemia at diagnosis v C+35	0.669	0.126	0.245	0.332	0.069	0.503	0.594	-0.156
VAC049 C-1 v KCS MinExp C-1	0.09		0.73		**<0.0001**		**0.016**	
VAC049 C+35 v KCS MinExp C+35	**0.006**		0.077		0.124		0.282	

Table 7-1: Analysis of antibody, GIA and ADRB activity for VAC049

Only subjects infected in VAC049 were included (n=14). GIA was not available for VAC049 volunteer 1224 at C-1. Correlations were performed using Spearman rank test. Comparisons were performed using Wilcoxon or Mann Whitney tests as appropriate.

A significant difference in antibody responses at C-1 and C+35 was seen between DefExp and MinExp volunteers in KCS for all antigens except RH5 (Figure 7-3 and Table 7-2). Antibody responses to all antigens except RH5 were significantly boosted following CHMI for both DefExp and MinExp volunteers in KCS (Figure 7-3 and Table 7-3). Of note, the highest antibody responses to $MSP1_{19}$ and AMA1 in KCS were still lower than those seen following vaccination of UK adults with viral vectored vaccines encoding these antigens, but were similar to those recently reported for a cohort of naturally-immune Kenyan adults[309,402].

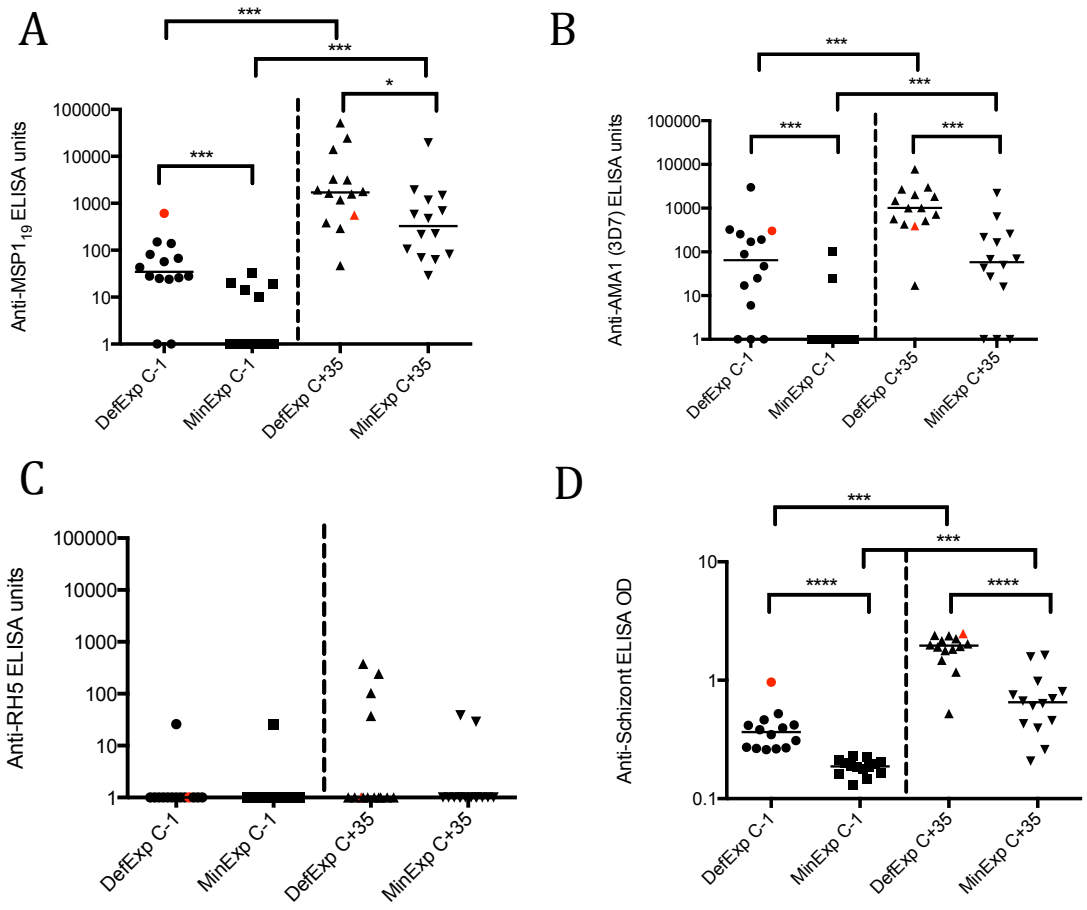


Figure 7-3. IgG antibody responses for MinExp and DefExp subjects in KCS

(A) MSP1$_{19}$. (B) AMA1. (C) RH5. (D) Schizont. Median values are indicated. Mann Whitney tests and Wilcoxon tests as appropriate. Data from volunteer 110 highlighted in red.

MinExp vs DefExp	MSP1 IgG Titre	AMA1 IgG Titre	RH5 IgG Titre	Schizont ELISA OD	GIA	ADRB
C-1	**0.0002**	**0.0005**	0.222	**<0.0001**	0.222	**0.0005**
C+35	**0.019**	**0.0003**	0.229	**<0.0001**	0.449	**0.0001**

Table 7-2: Comparison of antibody responses in KCS between MinExp and DefExp volunteers

Mann Whitney tests.

Comparison	MSP119 IgG Titre		AMA1 IgG Titre		RH5 IgG Titre		Schizont OD		
	p	*r*	*p*	*r*	*p*	*r*	*p*	*r*	
C-1 v C+35	**<0.0001**		**<0.0001**		**0.045**		**<0.0001**		**<**
Parasitaemia at diagnosis v C-1	**0.042**	0.395	0.066	0.359	0.193	0.259	0.202	0.253	
Parastaemia at diagnosis v C+35	**0.036**	0.405	0.208	0.208	0.960	0.011	**0.037**	0.404	
PMR v C-1	0.886	-0.029	**0.018**	-0.443	0.491	-0.136	**0.044**	-0.384	
PMR v C+35	0.168	0.268	0.197	-0.251	0.147	-0.600	0.330	-0.191	
GIA C-1 v C-1	0.960	0.010	**0.007**	0.501	**0.007**	0.500	0.427	0.157	
GIA C-1 v C+35	0.165	-0.270	**0.015**	0.454	0.188	0.256	0.314	0.197	
GIA C+35 v C-1	0.483	0.138	0.340	0.187	0.471	0.142	0.333	0.190	
GIA C+35 V C+35	**0.005**	0.517	0.064	0.354	0.754	0.062	0.058	0.362	
ADRB C-1 v C-1	**0.042**	0.386	**0.0003**	0.630	0.421	0.158	**0.0002**	0.649	
ADRB C-1 v C+35	0.191	0.255	**0.002**	0.554	**0.032**	0.407	**0.001**	0.589	
ADRB C+35 v C-1	**0.031**	0.409	**0.011**	0.471	0.842	0.040	**0.001**	0.578	
ADRB C+35 v C+35	**0.0002**	0.649	**0.0001**	0.658	0.111	0.308	**<0.0001**	0.886	

Table 7-3: Multiple analyses of antibody, GIA and ADRB activity for KCS

n=28. Correlations were performed using Spearman rank test. Comparisons were performed using Wilcoxon or Mann Whitney tests as appropriate. Anti-schizont antibody responses at screening are listed as C-1. GIA results <0% were assigned 0%. These analyses were hypothesis driven and key findings require replication in future studies.

For KCS analyses including all volunteers, only antibody responses at C-1 to schizont and AMA1 showed a significant correlation to PMR (*p=0.044* and *p=0.018* respectively) (Table 7-3), although these relationships were lost when volunteer 110 was excluded from analyses (*p=0.529* and *p=0.049* respectively). However, a trend for decreasing antibody responses with increasing PMR was seen for all antigens. No correlation was seen between antibody responses at C+35 and PMR (Table 7-3). Only IgG responses to $MSP1_{19}$ and schizont at C-1 and to $MSP1_{19}$ at C+35 correlated with number of parasites at diagnosis when all volunteers were included in the analyses (Table 7-3). For VAC049, no antibody responses were shown to correlate with the number of parasites at diagnosis (Table 7-1).

7.3.2 Cellular assays

PBMC samples from three subjects enrolled in KCS (137, 142, 216) at three time points (C-1, diagnosis and C+35) were thawed and mBC and T cell ELIspots to AMA1 and MSP1 performed as previously described[419]. Positive control wells failed for both ELIspots for all three C-1 samples and one diagnosis sample, and the same results were seen when these assays were repeated using other aliquots of the same samples.

These assays were then performed on PBMC samples from the same time-points from a further three subjects in KCS (104, 105, 132) and a similar pattern of results was seen; positive control wells failed for both ELIspots for all C-1 samples and two diagnosis samples. Flow cytometry performed prior to

knowledge of the ELIspot results showed that the samples which failed ELIspot QC contained dead cells.

The most likely cause of the loss of cells is a freezer failure some time between the diagnosis of some of the subjects and before the C+35 visit. Given the lack of live cells in C-1 samples and the resultant lack of baseline data, no further work on cellular assays was undertaken.

7.3.3 Functional assays

7.3.3.1. GIA

GIA was assessed using purified IgG from serum of VAC049 volunteers before and after CHMI. No result was obtained for volunteer 1224 at C-1 due to a technical failure. A number of unexpected results were seen. Firstly, one subject (1221) was shown to have GIA of 99.9% at C-1 (expected to be < 20%) (Figure 7-4). This volunteer received 2,500 SPZ IM and was not successfully infected with *P. falciparum*. They had no detectable antibodies against $MSP1_{19}$ or AMA1 suggesting no prior exposure to malaria. The sample was screened for the presence of 14 anti-malarial medications (Piperaquine, Amodiaquine, Desethyl-amodiaquine, Chloroquine, Pyronaridine, Quinine, Sulfadoxine, Pyrimethamine, Mefloquine, Artesunate, Artemether, Dihydroartemisinin, Lumefantrine and Desbutyl-lumefantrine) with an adaption of the previously published liquid chromatography-tandem mass spectrometry assay using stable isotopically labelled internal standards for quantification[420].

The volunteer was shown to have a plasma concentration of 11ng/mL of quinine at C-1 (LLQ of the liquid chromatography 2.5ng/mL). The presence of quinine

was confirmed by full scan mass spectrum analysis and comparison with a quinine standard. This result was confirmed by repeat testing and controls were negative. Screening of plasma samples from the same volunteer at C+7, C+9, C+11 and C+14 did not detect the presence of any anti-malarial drugs. The assay of GIA was repeated using C+7 sample and showed 0% inhibition.

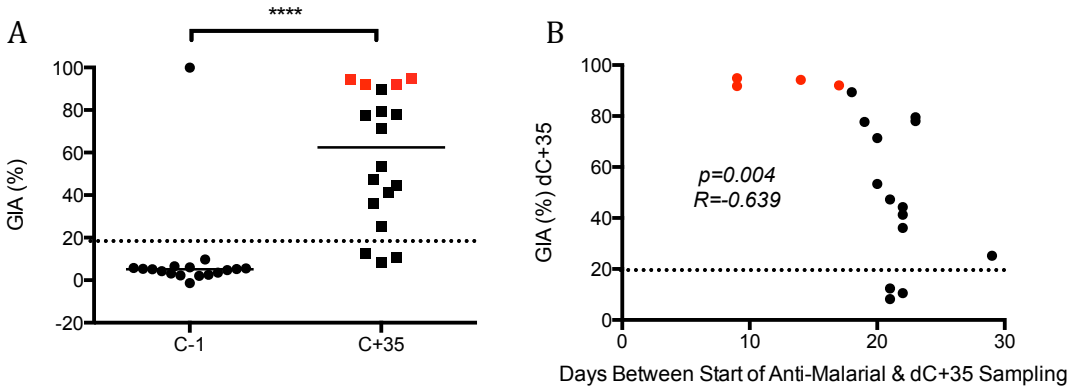


Figure 7-4: VAC049 GIA

(A) Pre and post–CHMI. Wilcoxon test. (B) Correlation between GIA at C+35 and days between start of anti-malaria therapy and sampling at C+35 visit. Spearman rank test. Volunteers not successfully infected are highlighted in red. GIA expected to be <20% in malaria-naïve individuals (dotted line).

The second interesting finding was that GIA increased post-CHMI across the cohort, but most markedly in the four subjects that were not successfully infected in VAC049 (i.e. qPCR negative from C+6.5 until C+21) (Figure 7-4). This finding was in contrast to other studies where no GIA was induced in malaria-naïve adults following a single CHMI[241,402]. A correlation was seen between GIA at C+35 and days between start of anti-malarial therapy and sampling at C+35.

Given that uninfected subjects in VAC049 were treated at C+21, at least 3 days later than the other subjects, they had a shorter interval between completion of their drug therapy and sampling at C+35. It is likely therefore that the increase in GIA seen post-CHMI is secondary to a residual effect from Malarone, persisting despite purification of IgG, rather than mediated by antibody induced following a single malaria infection.

Analysis of GIA pre and post-CHMI in KCS showed a significant increase post-infection (*p=<0.0001*) (Figure 7-5 and Table 7-3). No significant difference in GIA was seen between MinExp and DefExp subjects at C-1 or C+35 (Figure 7-5 and Table 7-2). Volunteer 110, who had a markedly reduced PMR *in vivo* (1.3; median 11.1), demonstrated no GIA prior to CHMI (-0.4%) and only minimal GIA post-CHMI (27%). The subject with the highest GIA at C-1 (volunteer 150; 46%) had DefExp to malaria and a reduced PMR (5.2). When all volunteers were included in the analysis, no correlation was seen between GIA and PMR or days between start of anti-malarial therapy and C+35 sampling (Figure 7-5). However, the same anti-malarial therapy was used in both KCS and VAC049, and it is likely that GIA measured at C+35 in KCS was influenced to some degree by Atovaquone/Proguanil and so these results must be treated with caution. GIA at C-1 was only shown to correlate with AMA1 IgG titres at C-1 and C+35 (*p=0.007* and *p=0.015* respectively) (Table 7-3). GIA at C+35 was only shown to correlate with $MSP1_{19}$ IgG titres at C+35 (*p=0.005*) (Table 7-3).

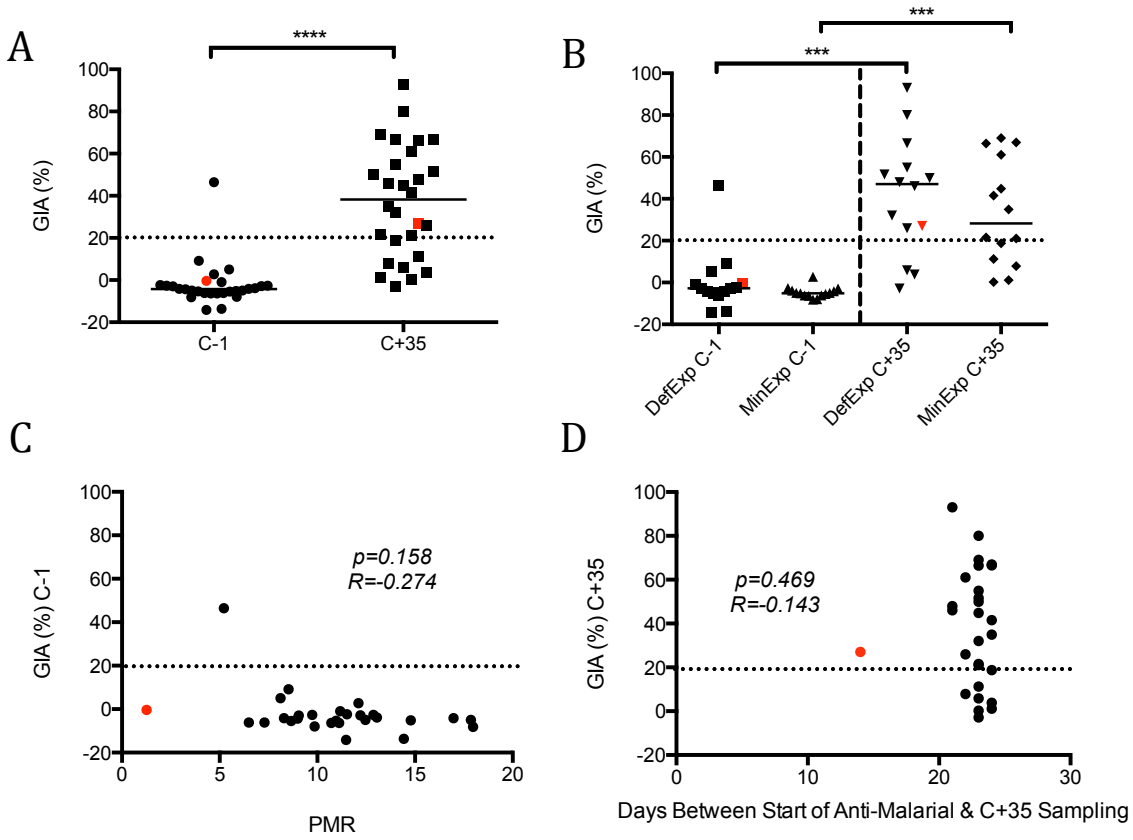


Figure 7-5: KCS GIA

(A) Pre and post-CHMI. (B) MinExp and DefExp volunteers. (C) Correlation between GIA at C-1 and PMR. (D) Correlation between GIA at C+35 and days between start of anti-malaria therapy and sampling at C+35 visit. Data for volunteer 110 is highlighted in red. Median values are indicated. Wilcoxon or Mann Whitney tests as appropriate. Spearman rank test for correlations.

6.3.3.2 ADRB activity assay

In VAC049, no significant increase in ADRB activity was seen post-CHMI in infected volunteers (Figure 7-6 and Table 7-1). Analysis of ADRB activity pre and

post-CHMI in KCS showed a significant increase post-infection (*p=<0.0001*) (Figure 7-7 and Table 7-3).

A significant difference in ADRB activity was seen between MinExp and DefExp subjects at both C-1 and C+35 (Figure 7-7 and Table 7-2). Volunteer 110, had the highest ADRB at C-1, however at 0.44 this was considerably lower than the hyper-immune serum used as the positive control in the assay. No correlation was seen between PMR and ADRB activity (Table 7-3). ADRB activity at C-1 was only shown to correlate with $MSP1_{19}$, AMA1 and schizont IgG titres at C-1 (*p=0.042*, *p=0.0003*, *p=0.0002* respectively) and AMA1 and schizont antibody responses at C+35 (*p=0.002* and *p=0.001* respectively) (Table 7-3). ADRB activity at C+35 correlated with antibody responses to all antigens at C-1 and C+35 except RH5 (Table 7-3).

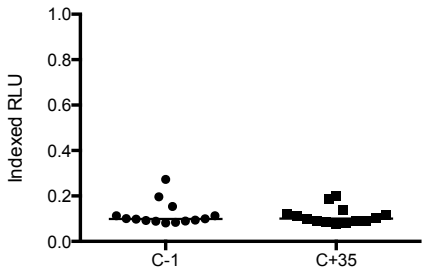


Figure 7-6: VAC049 ADRB activity

ADRB activity pre and post-CHMI for subjects infected in VAC049 (n=14). p=0.670. Wilcoxon test. RLU = relative light units.

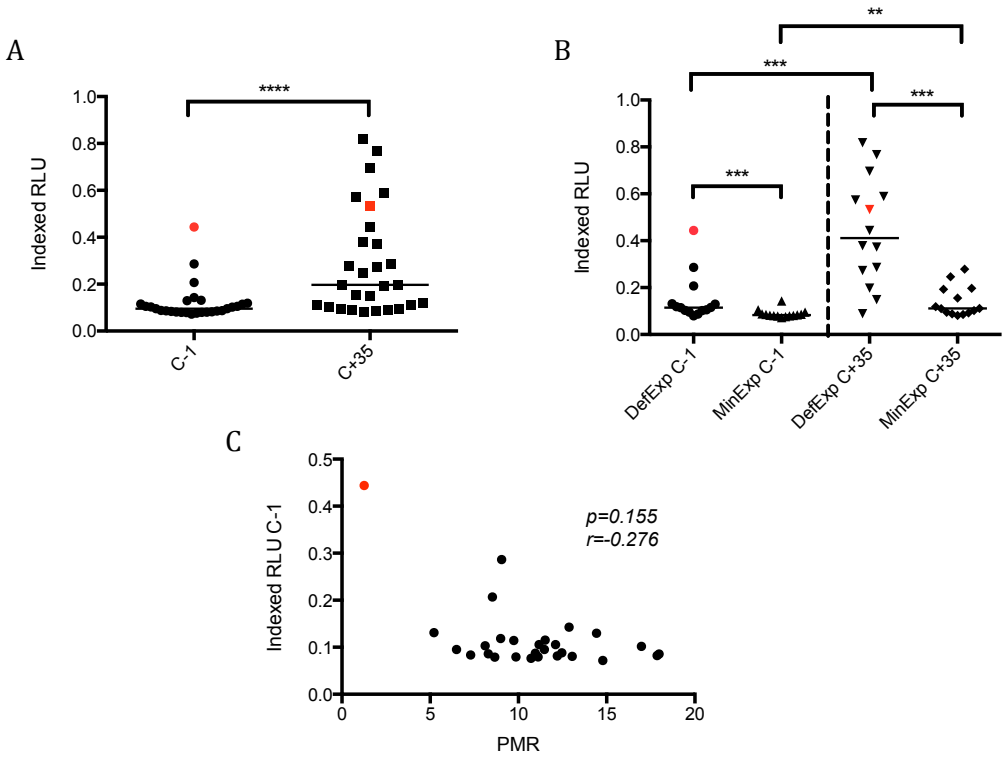


Figure 7-7: KCS ADRB activity

(A) All volunteers pre and post-CHMI. (B) MinExp and DefExp volunteers. (C) Correlation between ADRB activity at C-1 and PMR. Data for volunteer 110 is highlighted in red. Median values are indicated. RLU = relative light units. Wilcoxon or Mann Whitney tests as appropriate. Spearman rank test for correlation.

7.3.4 IFN-γ

Due to limited sample volumes, only serum samples were available for IFN-γ testing for KCS and plasma samples for VAC049. To assess the comparability of IFN-γ results from these two samples types, IFN-γ concentrations in paired serum and plasma samples from unvaccinated control volunteers participating in a mosquito-bite CHMI study[309] were assessed. No significant difference in IFN-

γ levels was seen in these samples at baseline or diagnosis and a close correlation was seen between serum and plasma samples (Figure 7-8). Comparison of IFN-γ results from VAC049 and KCS was therefore performed, however of note, samples were collected at different time points post-CHMI.

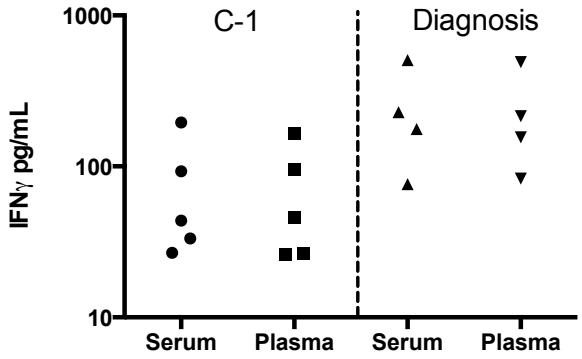


Figure 7-8: Comparison of IFN-γ in paired serum and plasma samples

Unvaccinated control volunteers undergoing CHMI by mosquito-bite in vaccine trial[309] (n=6). Wilcoxon test; C-1: p=0.635, Diagnosis: p=0.250. Correlation between serum and plasma results; p=<0.0001, r=1. Spearman rank.

No notable change in IFN-γ concentration was seen over time in uninfected volunteers in VAC049, where all comparisons of time-points post-CHMI with baseline (C-1) showed a fold change of <2 (Figure 7-9). Differences in dose of PfSPZ Challenge and LBI and small sample numbers limited direct comparison of IFN-γ over time between groups in VAC049, however, IFN-γ was seen to increase with increasing parasitaemia in each group (Figure 7-9).

Analysis of changes in IFN-γ in KCS overtime showed that concentrations of IFN-γ significantly increased between C-1 and C+9/C+11 and remained significantly

elevated from baseline at C+35 (Figure 7-10). No increase in IFN-γ was seen in volunteer 110 post-CHMI (Figure 7-10). No correlation was seen between IFN-γ and temperature or CRP in either study (data not shown).

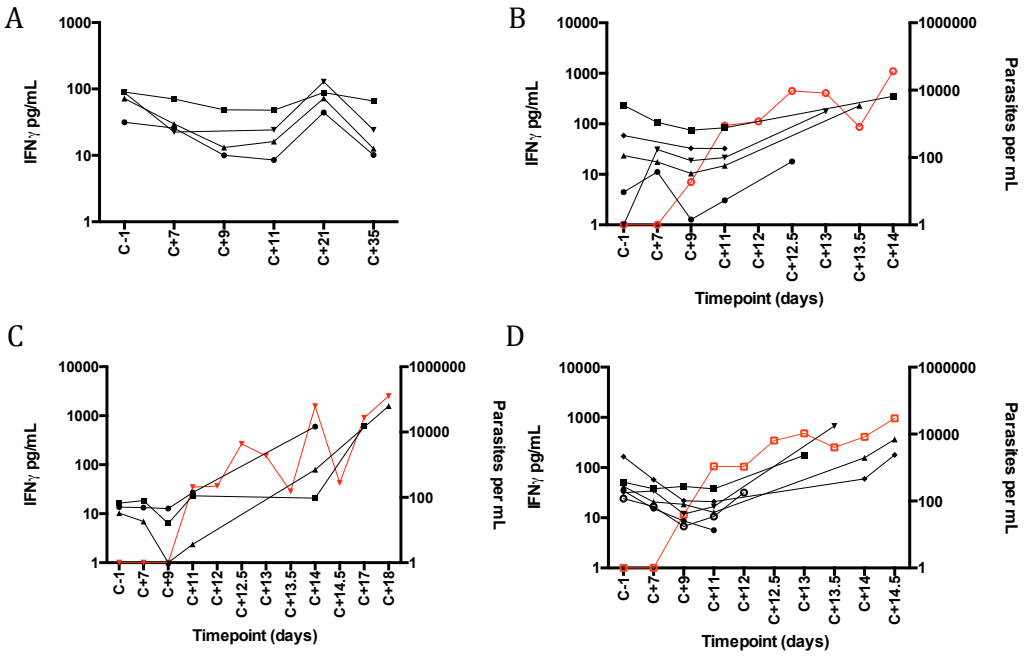


Figure 7-9: VAC049 changes in IFN-γ over time post-CHMI

(A) Uninfected volunteers (n=4). (B) Infected volunteers in Group 1: PfSPZ 2,500 ID (n=5). (C) Infected volunteers in Group 2: PfSPZ 2,500 IM (n=3). (D) Infected volunteers in Group 3: PfSPZ Challenge 25,000 IM (n=6). Red line = mean parasitaemia for group.

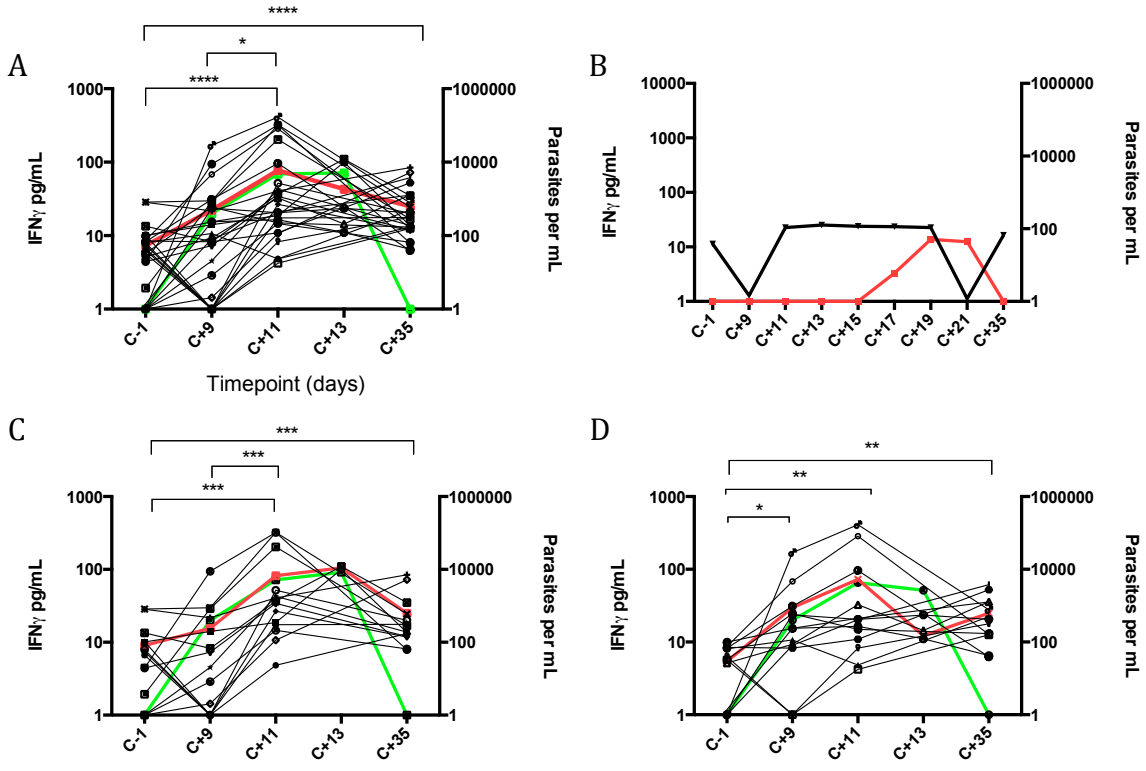


Figure 7-10: KCS Changes in IFN-γ over time post-CHMI

(A) All volunteers excluding 110 (n=27). (B) Volunteer 110. (C) MinExp volunteers (n=14). (D) DefExp volunteers excluding volunteer 110. (n=13). Statistical analysis used Wilcoxon test and was not performed with C+13 data as limited data-points available at this time-point. Mean parasitaemia measured by qPCR shown by green line (p/mL). Mean IFN-γ concentration shown by red line.

In both studies a significant increase in IFN-γ was seen between baseline and diagnosis (Figure 7-11). A significant correlation was seen in KCS between parasitaemia and IFN-γ (*p=<0.0001*), where for each unit increase in log IFN-γ there was a 0.57 increase in log parasitaemia (95 CI 0.26-0.89) (Figure 7-12). The same analysis for VAC049 showed a similar result (*p=<0.0001*) where for each unit increase in log IFN-γ there was a 1.4 increase in log parasitaemia (Figure 7-12).

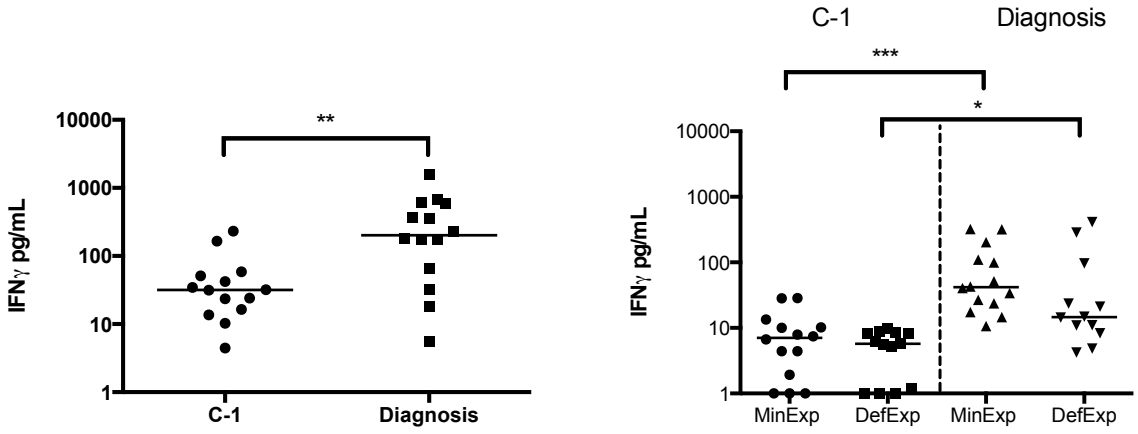


Figure 7-11: Comparison of IFN-γ at C-1 and diagnosis for VAC049 and KCS

(A) VAC049 (n=14) (B) KCS (n=27). For KCS, sample taken closest to diagnosis was used as 'diagnosis.' Wilcoxon test.

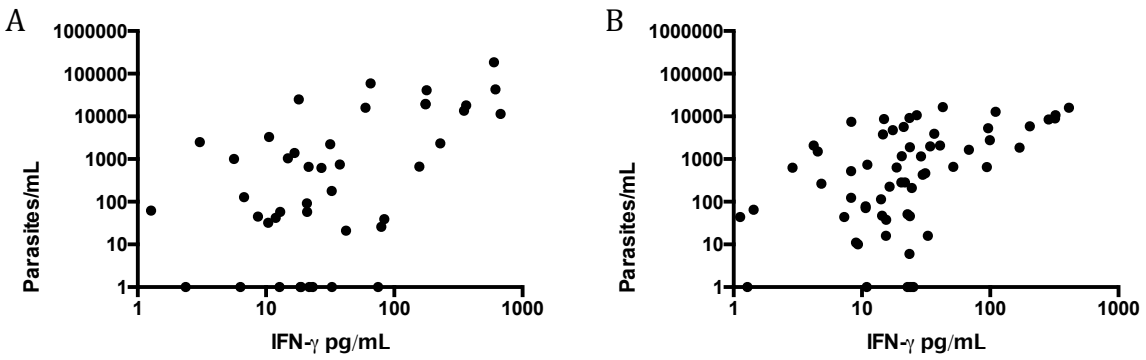


Figure 7-12: Correlation between IFN-γ and parasitaemia

(A) KCS: For each unit increase in log IFN-γ a 0.57 increase in log parasitaemia was seen (p=<0.0001. 95%CI 0.26-0.890) (B) VAC049: For each unit increase in log IFN-γ there was a 1.4 increase in log parasitaemia (p=<0.0001. 95 CI 0.87-1.94). qPCR data from C+9 until diagnosis was included. (Including time -points with zero values for parasitaemia in analysis highly skewed the data making a random effects model invalid). Random effects model.

Differences in TTD meant that volunteers differed considerably in parasitaemia at set time-points post-CHMI. In order to help address this problem and aid comparison of data across volunteers and studies, the time-points of sampling were synchronised according to diagnosis (Figure 7-13). Using these data, a significant difference in the time course of changes in IFN-γ between VAC049 and KCS was seen (*p=<0.0001; random effects regression analysis using random intercept model).* No difference in the time course of parasitaemia post-CHMI was seen between volunteers diagnosed with *P. falciparum* in VAC049 and KCS (*p=0.38; random effects regression analysis using random intercept model*), suggesting the difference in time course of IFN-γ between the studies was not

explained by a differences in patterns of parasitaemia. No significant difference was seen in the time-course of IFN-γ for MinExp and DefExp volunteers diagnosed with malaria in KCS when data corrected for timing of diagnosis were used (*p=0.077; random effects regression analysis using random intercept model).*

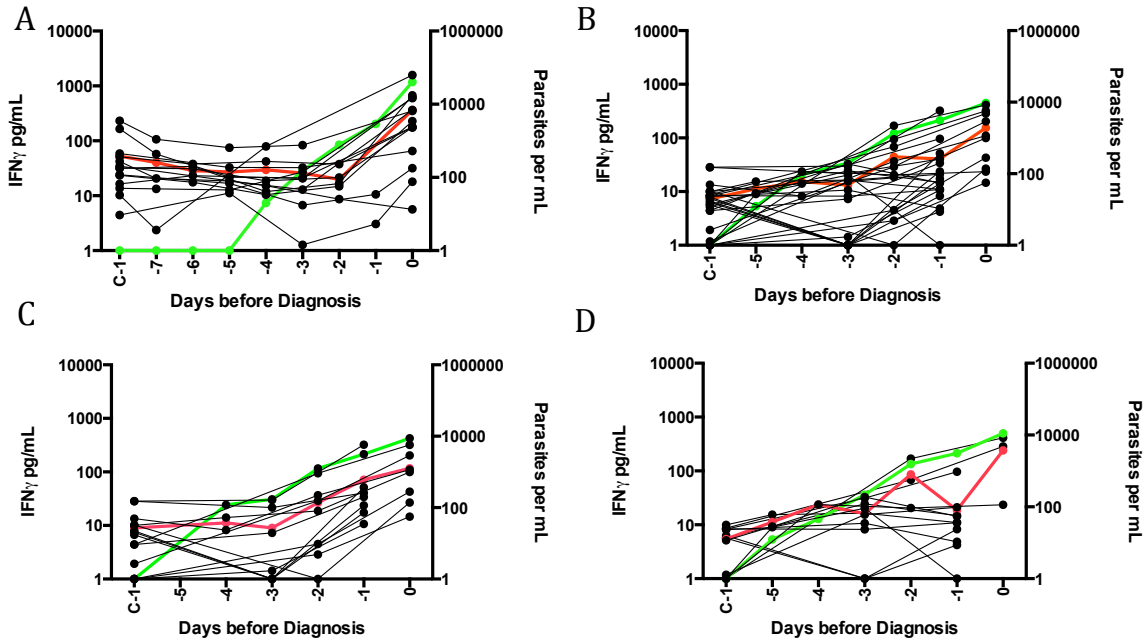


Figure 7-13:VAC049 changes in IFN-γ post-CHMI synchronised according to diagnosis

(A) VAC049 infected volunteers (n=14) (B) All diagnosed volunteers in KCS (n=27) (C) MinExp volunteers in KCS (n=14) (D) DefExp volunteers in KCS diagnosed with malaria (n=13). 0 = day of diagnosis, -1 = 1 day before diagnosis). Time-points on the same day (e.g. -5.5 and -5) were combined as a single time-point (-5). Red line = mean IFN-γ concentration. Green line = mean parasitaemia (p/mL).

There was a significant difference between IFN-γ measured at baseline and diagnosis between participants in VAC049 and KCS, however the difference at diagnosis was lost when the analysis adjusted for baseline IFN-γ (Figure 7-14). A significant difference in IFN-γ concentration was seen in KCS between MinExp and DefExp volunteers at diagnosis, however this effect was lost when the analysis was adjusted for baseline (Figure 7-14).

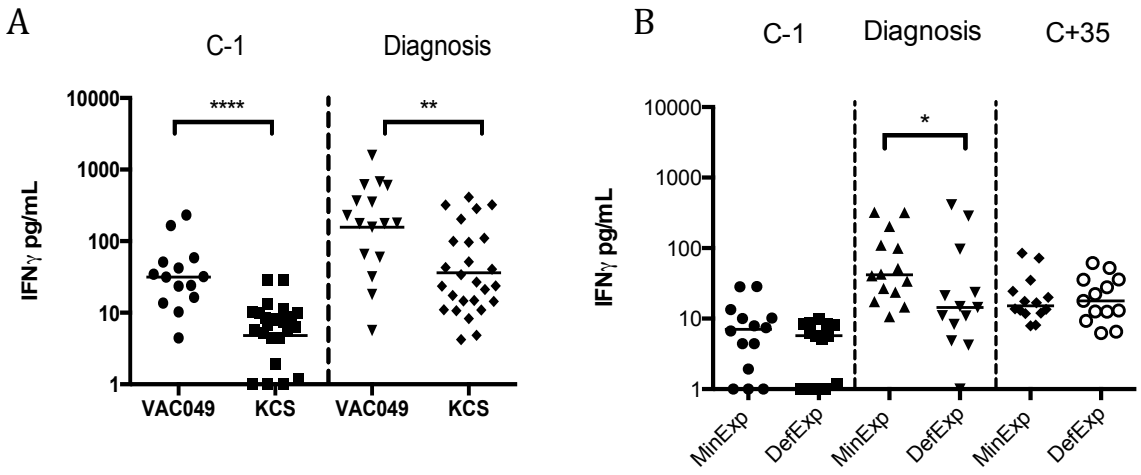


Figure 7-14: Comparison of IFN-γ between VAC049 and KCS

(A) Volunteers diagnosed with malaria in VAC049 and KCS. NB: No significant difference in IFN-γ was seen between VAC049 and KCS at diagnosis when the analysis was adjusted for baseline values (p=0.1; linear regression). (B) MinExp and DefExp volunteers diagnosed with malaria in KCS. NB: No significant difference in IFN-γ was seen between MinExp and DefExp volunteers at diagnosis when the analysis was adjusted for baseline values (p=0.09; linear regression). Mann Whitney tests.

Changes in IFN-γ overtime post-CHMI have been described in two publications. *Elias et al* reported changes in IFN-γ in 6 unvaccinated, UK malaria naive control volunteers post-CHMI by mosquito-bite. IFN-γ followed a similar time course to that seen in VAC049 and KCS with peak levels at diagnosis of a similar magnitude[172].

Walther et al reported changes in IFN-γ and other cytokines in 18 UK malaria-naive volunteers from three mosquito-bite CHMI studies[82]. For their analysis, all time points were reassigned values according to the time of the first measurement post-CHMI >50 parasites/ml by qPCR (time-point 0). They also restricted analysis to those individuals with at least one time-point post-CHMI with a fold change >2 from their individual baseline. Analysis of IFN-γ data from VAC049 and KCS using these criteria showed that, in agreement with *Walther et al*, the majority of volunteers in both studies demonstrated a > two-fold increase in IFN-γ at least 2 days after parasitaemia had increased > 50 p/mL (Figure 7-15). In VAC049 and KCS, the maximum mean fold change in IFN-γ post CHMI was 31 and 54 respectively, in comparison to approximately 15-fold reported by *Walther et al*[82].

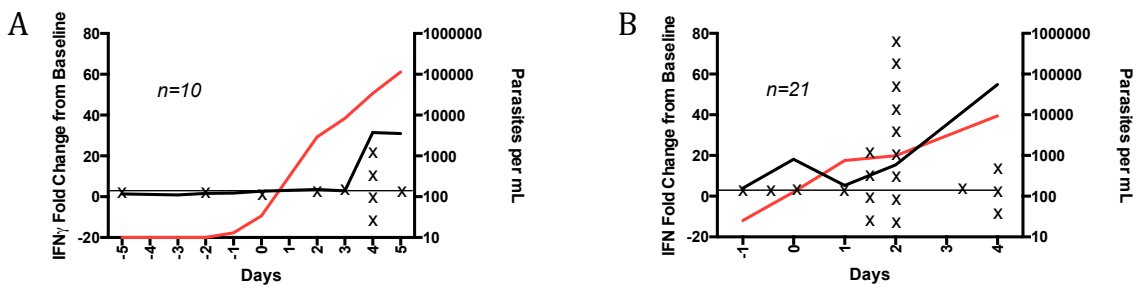


Figure 7-15: KCS change in IFN-γ post-CHMI with samples synchronised to first detection of > 50 p/mL

(A) VAC049 (B) KCS. The black line represents the magnitude of cytokine up-regulation (expressed as mean fold increase over baseline) for subjects showing at least a 2-fold increase in IFN concentration over their own individual baseline at least one time-point (excluding C+35). Time-points with data from < 2 volunteers were excluded. The red line indicates mean parasitaemia (p/mL measured by qPCR) over the study period for subjects included in analysis. The horizontal line indicates the cut-off for up-regulation of IFN-γ (2-fold) and crosses (X) on this line indicate the time the first sample giving a value above the cut-off was collected for each donor.

As previously reported, not all volunteers diagnosed with malaria developed a >2 fold increase in IFN-γ (IFN-γ^-) (VAC049: n=4, KCS n=6). 5/6 of the volunteers diagnosed with malaria in KCS who were IFN-γ^- were in DefExp groups. There was no significant difference in the PMRs of these six volunteers compared to other subjects, although they did have significantly higher anti-schizont antibody responses at baseline (*p=0.005, Mann Whitney test*). There appeared to be nothing remarkable about the four volunteers in VAC049 who were diagnosed

with malaria but IFN-γ⁻. Of note, volunteers that did not generate a > two-fold increase in IFN-γ post-infection did not have significantly higher baseline measures of IFN-γ when compared to other volunteers diagnosed with malaria in the same study (*VAC049 p=0.767, KCS p=0.11).*

7.4 Discussion

7.4.1. Antibody responses

A single malaria infection following administration of PfSPZ Challenge was capable of inducing a significant increase in antibody responses against $MSP1_{19}$, and AMA1 in malaria-naïve adults in VAC049, with titres similar to those reported following mosquito-bite CHMI.[402] Of interest there was a significant difference in C+35 antibody responses to $MSP1_{19}$ between VAC049 volunteers and MinExp subjects in KCS. This could be explained by differences in dose of PSPZ Challenge administered between the trials or suggest MinExp subjects in KCS had some degree of prior exposure to malaria that lead to boosting of mBC responses following CHMI. For DefExp volunteers in KCS, only antibody titres at C+35 to $MSP1_{19}$ and AMA1 were of a similar order to that reported for hyperimmune individuals[402], supporting the suggestion that DefExp subjects in KCS likely had only mild to moderate prior exposure to malaria.

In contrast to data from other UK malaria-naïve subjects undergoing mosquito-bite CHMI, no correlation was seen between antibody responses to $MSP1_{19}$ at

C+35 and p/mL at diagnosis for either study[402], suggesting antigenic burden alone may not explain antibody concentrations.

In KCS, at C-1, volunteer 110 had the highest antibody titre to $MSP1_{19}$ but not to AMA1. However, in the KCS cohort, only antibody responses at C-1 to AMA1 correlated with PMR. These findings suggest that antibody responses to $MSP1_{19}$ and AMA1 are associated with exposure but not necessarily predictive of NAI.

7.4.2. GIA assay

One key limitation of functional assays is that they commonly assess responses to a single laboratory strain of *P. falciparum.* NAI is likely to be 'personalised,' determined by an individuals' own unique history of exposure to various locally circulating strains of parasite. Whether an individual is shown to have activity against a single laboratory strain *in vitro* that they may not have encountered before is therefore not a reliable assessment of an individuals' ability to control parasite growth in the field. By the same reasoning, current CHMI studies provide limited insight into an individuals' NAI, as volunteers are exposed to a single parasite strain, pre-determined from a limited number, that may or may not resemble those volunteers have encountered previously in the field.

The GIA assay measures the ability of purified IgG to inhibit the growth of 3D7 blood-stage parasites *in vitro* over one life-cycle. Volunteer 110 had undetectable GIA prior to CHMI and yet was able to control parasite growth *in vivo*. In contrast, subject 150 who had 40% GIA at C-1, was unable to control parasite growth to the same degree and was diagnosed with malaria following CHMI. This apparent inconsistency could be explained if the parasite used in the GIA assay

differed from the challenge strain, for example in var gene expression. Alternatively, volunteer 110 could be able to control parasite growth by a mechanism that does not inhibit parasite invasion and growth directly and so would not be detected in the GIA assay.

The finding of quinine in a C-1 sample for a volunteer in VAC049 is significant and difficult to explain. Given that a single, oral dose of 500mg quinine sulphate in healthy adults leads to a peak plasma concentration of 3.3mg/L three hours after administration, and the half-life of quinine is approximately 12 hours[421], the concentration of quinine seen would be consistent with the volunteer taking a single 500mg tablet of quinine approximately 4 days before C-1. However, on questioning, the volunteer denied taking any medications before or during the study, other than those prescribed by the study team (of note, anti-malarial medications are widely and easily available for purchase in the UK). Quinine is present in certain soft drinks, and the volunteer did recall possibly drinking 'gin and tonic' around the time of CHMI.

Tonic water contains approximately 58mg/L of quinine[422]. Intake of 200mL (11.6mg) of tonic water would thus lead to a peak plasma concentration of 0.077mg/L[422], which would decay to 11ng/mL in approximately 1 day. The therapeutic range of quinine is 0.2mg/L – 2mg/L[422], suggesting the levels measured would be non-therapeutic *in vivo*, but were sufficient to impact on parasite growth and show 100% GIA *in vitro*. Indeed, the EC50 (estimated drug dose required to inhibit parasite growth by 50%) for quinine has been shown to be 4 x 10^8 M for *P. falciparum* 3D7[423]. Given that quinine is known to exert its anti-malarial effect on the blood-stage of infection[423,424], the lack of quinine

detected in our volunteer when expected to be parasitaemic (dC+6.5 onwards) makes it is extremely unlikely that the drug impacted on blood-stage parasite growth *in vivo*. Whilst quinine is not known to have an effect on sporozoites, it should be noted that this volunteer was not successfully infected with PfSPZ Challenge[316]. Considering that 50% of this group (n=6) were not infected, it is very unlikely that the finding of quinine in this volunteer's serum impacted on the study's outcome. However, the finding still has a number of important implications for CHMI studies:

Firstly, whilst volunteers may be unlikely to, it is possible that they could imbibe large enough volumes of tonic water to reach therapeutic plasma concentrations of quinine that could impact on PMR. These results show that even low concentrations of quinine, 4-fold below the therapeutic plasma level, can significantly impact on GIA *in vitro* assay findings and may impact on other parasitological assays not tested here. It would therefore seem sensible to ask participants to abstain from drinking tonic during a CHMI study.

Secondly, the finding of quinine in this volunteer's serum and the presumed effect of residual Atovaquone/Proguanil on GIA in VAC049 and KCS importantly suggest that anti-malarials, previously assumed to be removed by IgG purification with a Protein G column and subsequent dialysis, can give falsely high GIA results. Further work is needed to repeat this finding and investigate methods to ensure removal of anti-malarial medications from serum prior to the GIA assay. In the interim, caution should be taken interpreting GIA results from samples taken following anti-malarial therapy or where the use of anti-malarial drugs is unknown (e.g. field studies).

Finally, CHMI studies are used to screen large numbers of novel malaria drugs and vaccines in order to ensure that only the most promising candidates progress to more costly field studies. Given the necessary small numbers of participants in each CHMI study it is important that investigators are confident any observed efficacy (either sterilizing protection, or substantial delay in TTD) is due to the drug or vaccine in question and not to an antimalarial substance that a participant may have surreptitiously or inadvertently self-administered. Mass spectrometry offers a relatively cheap (approximately $165 per sample), simple and sensitive method to simultaneously screen for a number of anti-malarial drugs, and should be routinely performed on individuals demonstrating drug or vaccine efficacy in order to increase investigator confidence in study conclusions.

7.4.3. ADRB activity assay

Whilst infected malaria-naïve volunteers in VAC049 failed to develop ADRB activity following a single malaria infection, both MinExp and DefExp subjects in KCS had a significant increase following CHMI supporting the conclusion that MinExp volunteers had some degree of prior exposure to malaria. The increase in ADRB activity following CHMI was more marked in DefExp volunteers, possibly due to improved B cell memory or increased titres of antibody capable of giving ADRB activity in this group. Significant correlations were seen between ADRB activity at baseline and C+35 and antibody responses to $MSP1_{19}$, AMA1 and schizont at these time points, further supporting the suggestion that ADRB activity correlates with exposure.

No correlation was seen between ADRB activity at C-1 or C+35 and parasitaemia at diagnosis or PMR. However volunteer 110 who demonstrated an ability to control parasitaemia post-CHMI did have the highest ADRB activity at baseline, suggesting the relationship between ADRB activity and NAI warrants further investigation.

The ADRB activity assay assesses the ability of donor neutrophils to produce ROS in the presence of test serum and parasites. The assay is performed in triplicate, with neutrophils from a new donor used with each replicate. One key limitation of the study is therefore that the assay relies on neutrophils that are not from the subjects in question, meaning results need to be interpreted with caution.

7.4.4. IFN-γ

Comparing IFN-γ responses between volunteers, trials and over time was extremely difficult given variations in infectious dose and resultant parasitaemia and TTD between subjects. In addition, sampling schedules differed between trials: for example in VAC049 blood was drawn at the point of diagnosis but the comparator samples in KCS were those collected closest to diagnosis, up to 1.5 days before treatment was started. However, synchronisation of data according to diagnosis or when parasitaemia crossed a specified threshold did help compensate for some of these limitations.

IFN-γ was seen to increase with rising parasitaemia, with a greater increase in parasites seen per log-fold increase in IFN-γ in VAC049 than KCS. The reason for this is unclear, but might reflect the effect of prior malaria exposure and primed

immune responses in volunteers enrolled in KCS. Significant differences in absolute IFN-γ were seen at diagnosis between volunteers in VAC049 and KCS and between MinExp and DefExp volunteers. However, these comparisons were no longer significantly difference when analyses were corrected for differences in baseline values, highlighting the importance of adjusting for this variable in analyses. This finding and the considerable spread of data in all groups at all time-points suggest that IFN-γ is not affected by prior mild-moderate exposure to malaria, at least in these studies.

Malaria-naïve UK volunteers had higher baseline IFN-γ concentrations than the Kenyan volunteers, which was of interest given one might predict a higher prevalence of minor co-infections that could stimulate an inflammatory response in the Kenyan cohort. As has previously been reported[82], in both studies a number of individuals diagnosed with malaria failed to generate a > two-fold increase in IFN-γ. Whilst the KCS data suggest IFN-γ$^-$ volunteers were those with greater prior exposure malaria, the fact that a number of VAC049 volunteers also failed to demonstrate a notable increase in IFN-γ suggests prior exposure does not explain the magnitude of IFN-γ response following infection. Indeed, analysis from another UK mosquito-bite CHMI suggests this finding may simply reflect differences between individuals in innate profiles to early blood-stage malaria infection[82].

7.5 Conclusions

Antibody responses to schizont and the ADRB activity assay appeared to be the most promising measures of prior exposure to malaria and warrant further investigation regarding their relationship to NAI.

CHAPTER 8: USE OF MICROARRAYS TO UNDERSTAND THE IMMUNE RESPONSE TO CONTROLLED HUMAN MALARIA INFECTIONS

8.1 Authorship statement

The work presented in this Chapter is part of a collaborative effort. Unless otherwise stated all work in this chapter was performed by myself.

Separation of peripheral blood mononuclear cell (PBMCs) and storage at known concentrations in RLT-buffer was performed by Sean Elias for KCS and Rhea Longley for VAC049.

RNA amplification and microarrays were performed by Karene Argoud of the High-throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, University of Oxford.

Quality control and initial analysis of the data was performed by myself and Helen Lockstone of the High-throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, University of Oxford. These findings were verified by Adaikalavan Ramasamy and Julius Muller of the Jenner Institute Transcriptomics Core Group, University of Oxford. Julius Muller performed the functional principal component (fPCA) and modular analyses with myself. All pathway analyses were performed by myself.

8.2 Introduction

Whilst gene expression studies have been performed using samples from CHMI trials (see Chapter 1), none of these have included data from African subjects. The samples taken in VAC049 and KCS provide a novel opportunity to describe

and contrast the gene expression changes seen in early liver and blood-stage malaria infection in UK, African, malaria-naïve and malaria-exposed individuals. The large number of whole blood and PBMC samples taken at multiple time points in both VAC049 and KCS studies allow for unprecedented, detailed analysis over time of these changes. There is also an opportunity to examine for correlations between gene expression and key immunological and parasitological outcomes such as ADRB activity and PMR. Whilst only one volunteer in KCS was able to control parasite growth *in vivo*, examination of this volunteer's gene expression profile over time could provide hypothesis generating insights into novel mechanisms of NAI.

8.3 Objectives

(a) To compare gene expression over time between UK and Kenyan malaria naïve volunteers following infection in the VAC049 and KCS CHMI studies.

(b) To compare gene expression over time between Kenyan adults with MinExp and DefExp prior malaria exposure in KCS.

(c) To compare gene expression over time between Kenyan adults in KCS demonstrating a delay in TTD and those that did not.

(d) To compare the gene expression over time of volunteer 110 in KCS who was infected but undiagnosed with malaria with other subjects in the study.

(e) To identify genes correlating with key clinical and parasitological outcome measures.

8.4 Results

8.4.1 Interrogation of extracted RNA quality and sample selection prior to amplification of mRNA

PAXgene and PBMC samples were available for multiple time-points post CHMI for KCS and VAC049. Given limited resources, it was only possible to perform microarrays on a selection of samples available. In order to prioritise samples, a small selection of PAXgene and PBMC samples from each study were evaluated for sample quality (Table 8-1).

	RNA ng/µl*	**260/280***	**260/230***	**RIN****	**28s/18s****
KCS PAXgene (n=6)	61.54 *(14.02)*	2.00 *(0.06)*	0.54 *(0.32)*	7.10 *(1.20)*	1.42 *(0.44)*
VAC049 PAXgene (n=6)	87.73 *(17.27)*	2.04 *(0.03)*	0.57 *(0.31)*	7.60 *(0.30)*	1.58 *(0.28)*
KCS PBMC (n=8)	135.7 *(77.45)*	2.09 *(0.13)*	1.01 *(0.50)*	9.59 *(0.68)*	2.39 *(0.28)*
VAC049 PBMC (n=10)	120.5 *(124.5)*	2.092 *(0.06)*	1.237 *(0.65)*	6.93 *(0.66)*	1.2 *(0.34)*

Table 8-1: RNA quality data for PAXgene and PBMC samples

*Mean data for selection of PAXgene and PBMC samples from KCS and VAC049. All samples were eluted in 30µl. Numbers in brackets = standard deviation. *As measured by spectrophotometer. **As measured by gel electrophoresis. RIN = RNA integrity number. 28s/18s = measure of RNA quality – expected ratio = 2:1.*

The PBMC samples yielded higher concentrations of RNA than the PAXgene samples. PBMC samples from the KCS study had a higher RNA integrity number (RIN) than those from VAC049, possibly because the VAC049 samples had degraded as a result of being stored considerably longer prior to RNA extraction than those from KCS (2.5 years versus 1 year). All samples were however suitable for microarray analysis. Given the KCS dataset was the most novel, it was decided to perform microarrays on both PAXgene and PBMC samples from this study in order to allow validation of any interesting findings across two sample sets.

To measure the effect of GLOBINclear™ on RNA quality and quantity, RNA was assessed before and after removal of Globin RNA in a small selection of PAXgene samples (Table 8-2). GLOBINclear™ resulted in a mean loss of 22% of extracted RNA, consistent with the manufacturer's advice and likely to represent loss of globin RNA only. Whilst no reduction in RNA quality was seen (as assessed by the ratio of absorbance at 260nm and 280nm (260/280), the process of removing globin RNA may have introduced a contaminant (a marked reduction in 260/230 ratio was seen post GLOBINclear™) and caused some degradation of RNA. This however was felt to be insignificant and it was decided to remove Globin from all KCS PAXgene samples prior to amplification of RNA.

	PRE Globin Clear					POST Globin Clear					
	µg*	260/ 280*	260/ 230*	RIN **	28s/ 18s**	µg*	260/ 280*	260/ 230*	RIN **	28s/ 18s**	% RNA Retained
VAC049 PAXgene (n=6)	3.00	2.08	1.33	-	-	2.64	2.04	0.57	7.60	1.58	88
KCS PAXgene (n=6)	2.12	2.01	1.54	-	-	1.84	2.00	0.54	7.10	1.42	89

Table 8-2: Data pre and post removal of Globin mRNA for PAXgene samples from KCS and VAC049

*Mean data pre and post 'Globin Clear' for a selection of PAXgene samples from KCS and VAC049. *As measured by spectrophotometer. **As measured by gel electrophoresis. RIN = RNA integrity number. 28s/18s = measure of RNA quality – expected ratio = 2:1.*

RNA was extracted from 201 KCS PAXgene samples and 123 KCS PBMC samples. The quality of RNA prior to amplification is summarised in Table 8-3. In contrast to earlier findings, the whole blood samples collected in PAXgene tubes yielded significantly higher concentrations of RNA than PBMC samples. However, this RNA was of a lower quality than that extracted from PBMC samples. No samples were excluded on the basis of these QC measures.

	RNA ng/µl*	260/280*	260/230*	RIN**	28s/18s**
KCS PAXgene	145.80 *(97.93)*	2.02 *(0.07)*	0.76 *(0.38)*	7.66 *(0.63)*	1.16 *(0.31)*
KCS PBMC	75.39 *(52.79)*	2.08 *(0.11)*	0.98 *(0.43)*	9.62 *(0.41)*	2.20 *(0.46)*
	p=<0.0001	*p=<0.0001*	*p=<0.0001*	*p=<0.0001*	*p=<0.0001*

Table 8-3: Quality of RNA extracted from KCS PAXgene and PBMC samples

*Mean data for RNA extracted from 201 PAXgene and 123 PBMC samples from KCS, prior to amplification. All samples were eluted in 30µl. *Measured by spectrophotometer for all samples. **RIN and 29s/18s measured by gel electrophoresis for 28 PAXgene tube samples (14%) and 31 PBMC samples (25%). Numbers in brackets = standard deviation; RIN = RNA integrity number. Statistical comparison by Mann Whitney test. 28s/18s = measure of RNA quality – expected ratio = 2:1.*

8.4.2 Quality control data for KCS PAXgene and KCS PBMC samples

QC and normalisation was initially performed on all 324 samples (PAXgene and PBMC) combined (Appendices 7-1 and 7-2). PCA and hierarchical clustering showed that sample type (PAXgene versus PBMC) had a clear and dramatic effect on quality metrics. It was therefore decided to process and analyse PAXgene and PBMC samples as two separate data sets. QC and normalisation were repeated for each dataset and outlier samples removed following consideration of QC metrics, PCA plots and probe signal intensities. Five samples were removed from the PAXgene dataset (Appendix 7-3) and nil from the PBMC dataset.

Given the greater number of samples and time points in the PAXgene data set, and concerns regarding the quality of PBMC samples (see Chapter 6), it was decided to continue analysis using only the PAXgene dataset and to keep the PBMC dataset to potentially verify any findings of interest. QC following removal of the five outlier samples from the PAXgene dataset is shown in Appendices 7-4 and 7-5.

The PAXgene dataset was then filtered to remove probes with low detection values; only probes with a detection p value ≤0.01 in at least one sample were retained for analysis. This reduced the number of probes for subsequent analysis from 47,231 to 30,985.

8.4.3 Time course analysis of KCS PAXgene dataset - all volunteers

The null hypothesis was that there is no significant change in gene expression over the time course. This was tested by investigating for any significant change in gene expression between one or more pairs of time points (whole series analysis). This analysis showed 9371 probes differentially expressed at 5% FDR over the time course. This was reduced to 1020 probes by requiring the fold change (FC) to be > +/- 1.5. Pathway analysis of this list showed significant enrichment of seven canonical pathways all relating to the innate immune system (Table 8-4).

Canonical Pathway	p value
Interferon Signalling	2.42E-11
Activation of IRF by Cytosolic Pattern Recognition Receptors	1.27E-06
Crosstalk between Dendritic Cells and Natural Killer Cells	1.93E-06
TREM Signalling	7.39E-06
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	7.94E-06
PI3K Signalling in B Lymphocytes	1.30E-05
Death Receptor Signalling	4.79E-05

Table 8-4: Canonical Pathways differentially expressed in the KCS PAxgene dataset whole series analysis

p value < E10-4, FDR 5%, FC >+/- 1.5. TREM = triggering receptor expressed on myeloid cells.

In order to understand in greater detail the patterns of gene expression over time, the following analyses were performed;

(a) *Baseline Comparisons:* Expression at each time point was compared with baseline (C-1) (e.g. C+6.5 v baseline).

(b) *Consecutive Comparisons:* Expression at each time point was compared to the previous one to assess progression of change (e.g. C+7 v C+6.5).

A matrix of baseline and consecutive comparisons is shown in Appendix 8-6. The number of differentially expressed probes in each of the baseline and consecutive analyses is shown in Table 8-5. Given that pathway analysis is not informative for very large probe lists, various fold change thresholds were explored (Table 8-6). A fold change of 1.25 gave a suitable number of genes for each analysis for further investigation.

	Baseline Comparisons						Consecutive Comparisons				
	C+6.5 v BL	C+7 v BL	C+9 v BL	C+11 v BL	DIAG v BL	C+35 v BL	C+7 v C+6.5	C+9 v C+7	C+11 v C+9	DIAG v C+11	C+35 v DIAG
Down-regulated	846	1451	1454	3016	3752	895	1027	418	512	2268	3221
No Change	28771	27495	27161	25176	23780	28797	29464	29898	30298	26360	23439
Up-regulated	1368	2039	2370	2793	3453	1293	494	669	175	2357	4325

Table 8-5: Numbers of differentially expressed probes in the baseline and consecutive comparisons for KCS PAXgene dataset

FDR 5%. BL = Baseline (C-1).

Comparison	No FC	1.25 FC	1.5 FC
C+6.5 v BL	1776	313	28
C+7 v C+6.5	1239	167	5
C+9 v C+7	853	185	32
C+11 v C+9	587	69	3
DIAG v C+11	3659	1720	276
C+35 v DIAG	5608	3048	766
C+7 v BL	2647	786	140
C+9 v BL	2963	978	322
C+11 v BL	4524	2002	486
DIAG v BL	5376	2859	840
C+35 v BL	1699	372	17

Table 8-6: Number of analysis-ready molecules differentially expressed for various comparisons for KCS PAXgene dataset with different fold change filters.

FDR <5%. BL = Baseline (C-1). FC = fold change.

The greatest number of probes differentially expressed compared with baseline was seen at diagnosis, and the greatest change in probe expression between consecutive time points was seen between diagnosis and C+35 (Table 8-6). Both baseline and consecutive analyses were significantly enriched for many canonical pathways, particularly those related to the innate immune system and pro-inflammatory responses (Tables 8-7 and 8-8). Between C+7 and C+9, there was enrichment of interferon signalling (*p=6.43E-15*) and pathways related to activation of the innate immune system including: activation of IRF by cytosolic PRRs (*p=1.21E-06*) and the role of PRRs in recognition of bacteria and viruses (*p=1.37E-05*)) and antigen presentation (*p=2.07E-05*). Between C+11 and diagnosis (DIAG) there was further enrichment of these pathways and enrichment of numerous, additional pathways known to amplify the inflammatory immune response, including TREM (*p=2.86E-10*) and natural killer cell signalling (*p=4.75E-08*).

Canonical Pathway	C+6.5 v BL	C+7 v BL	C+9 v BL	C+11
Mitochondrial Dysfunction	-	**1.05E-08**	2.50E-02	2.50
Renal Cell Carcinoma Signalling	2.53E-02	**4.37E-05**	5.05E-03	8.14
Heme Biosynthesis II	8.66E-03	2.04E-04	**2.51E-05**	**2.52**
Interferon Signalling	-	-	1.47E-04	**1.44**
IL-1 Signalling	-	1.98E-02	-	**1.51**
Role of NFAT in Regulation of the Immune Response	-	-	-	**2.35**
UVA Induced MAPK signalling	-	-	-	**7.26**
CD40 Signalling	-	-	-	**2.67**
Glucocorticoid Receptor Signalling	1.43E-04	2.58E-02	-	**7.77**
ERK5 Signalling	-	8.65E-03	6.96E-04	**7.89**
IL-8 Signalling	7.97E-04	1.34E-03	2.86E-03	**7.97**
Heme Biosynthesis from Uroporphyrinogen-III I	-	7.86E-03	1.26E-02	**9.06**
TREM1 Signalling	5.11E-03	-	-	
Antigen Presentation Pathway	-	-	-	
Natural Killer Cell Signalling	-	-	-	3.71
Crosstalk between Dendritic Cells and Natural Killer Cells	-	-	-	
Pathogenesis of Multiple Sclerosis	-	-	7.15E-03	4.98
PI3K Signalling in B Lymphocytes	1.48E-02	-	3.14E-02	1.71
PCK0 Signalling in T Lymphocytes	-	-	-	
Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	8.21E-04	-	-	1.36
IL-10 Signalling	4.80E-03	4.02E-02	-	5.39
Dendritic Cell Maturation	-	-	-	
Toll-like Receptor Signalling	2.90E-02	-	-	
NF-KB Signalling	-	-	-	1.32
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	8.21E-04	-	-	1.36
OX40 Signalling Pathway	-	-	-	
Type 1 Diabetes Mellitus Signalling	-	-	-	2.77
CTLA4 Signalling in Cytotoxic T Lymphocytes	-	4.28E-02	-	4.07
iCOS-iCOSL Signalling in T Helper Cells	-	-	-	4.14

Table 8-7: Canonical Pathways differentially expressed in at least one baseline comparison for KCS PAXgene dataset.

P value < E10-4. FDR <5%, FC > 1.25. BL = baseline (C-1); DIAG = diagnosis.

Canonical Pathway	C+6.5 v BL	C+7 v C+6.5	C+9 v C+7	C+
Interferon Signalling	-	-	**6.43E-15**	
Activation of IRF by Cytosolic Pattern Recognition Receptors	-	-	**1.21E-06**	
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	4.37E-02	-	**1.37E-05**	
Antigen Presentation Pathway	-	-	**2.07E-05**	
Crosstalk between Dendritic Cells and Natural Killer Cells	-	3.29E-02	**-**	
TREM1 Signalling	5.11E-03	-	-	
Pathogenesis of Multiple Sclerosis	-	-	2.87E-03	
Death Receptor Signalling	-	-	9.74E-03	
Communication between Innate and Adaptive Immune Cells	4.36E-02	2.67E-02	-	
Natural Killer Cell Signalling	-	-	-	4
Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	8.21E-04	-	-	
Role of NFAT in Regulation of the Immune Response	-	-	-	
PCK0 Signalling in T Lymphocytes	-	-	-	
Dendritic Cell Maturation	-	4.43E-02	-	
Calcium-induced T Lymphocyte Apoptosis	-	-	-	
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	8.21E-04	2.58E-02	-	
Type 1 Diabetes Mellitus Signalling	-	-	1.63E-02	
Toll like Receptor Signalling	2.90E-02	2.48E-03	-	
OX40 Signalling Pathway	-	-	-	
IL-10 Signalling	4.80E-03	2.01E-03	-	
Tec Kinase Signalling	-	-	-	
iCOS-iCOSL Signalling in T Helper Cells	-	-	-	
PI3K Signalling in B Lymphocytes	1.48E-02	-	-	
NF-KB Signalling	-	-	-	
Allograft Rejection Signalling	-	-	-	
CD28 Signalling in T Helper Cells	-	-	-	

Table 8-8: Canonical Pathways differentially expressed in at least one consecutive comparison for KCS PAXgene dataset.

p value < E10-4 FDR < 5%, FC > 1.25. BL = baseline (C-1). DIAG = diagnosis.

Modular analysis of the baseline comparisons confirmed up regulation of genes in interferon modules (M1.2, M3.4 and M5.12) from approximately C+9 until diagnosis and up regulation of genes in modules relating to neutrophils (M5.15) and erythrocytes (M6.18, M3.1 and M2.3) throughout infection, with some changes in erythrocyte modules persisting post treatment (Appendix 8-7 and Figure 8-1). Variable up and down regulation of modules relating to inflammation was seen at each time point. There was also down regulation of genes in the modules relating to B (M4.10), T (4.15) and NK (M3.6) cells towards diagnosis. Hierarchical clustering of modular results showed grouping of samples collected close to and at diagnosis, but no clustering of subjects according to prior exposure to malaria or time to diagnosis (data not shown).

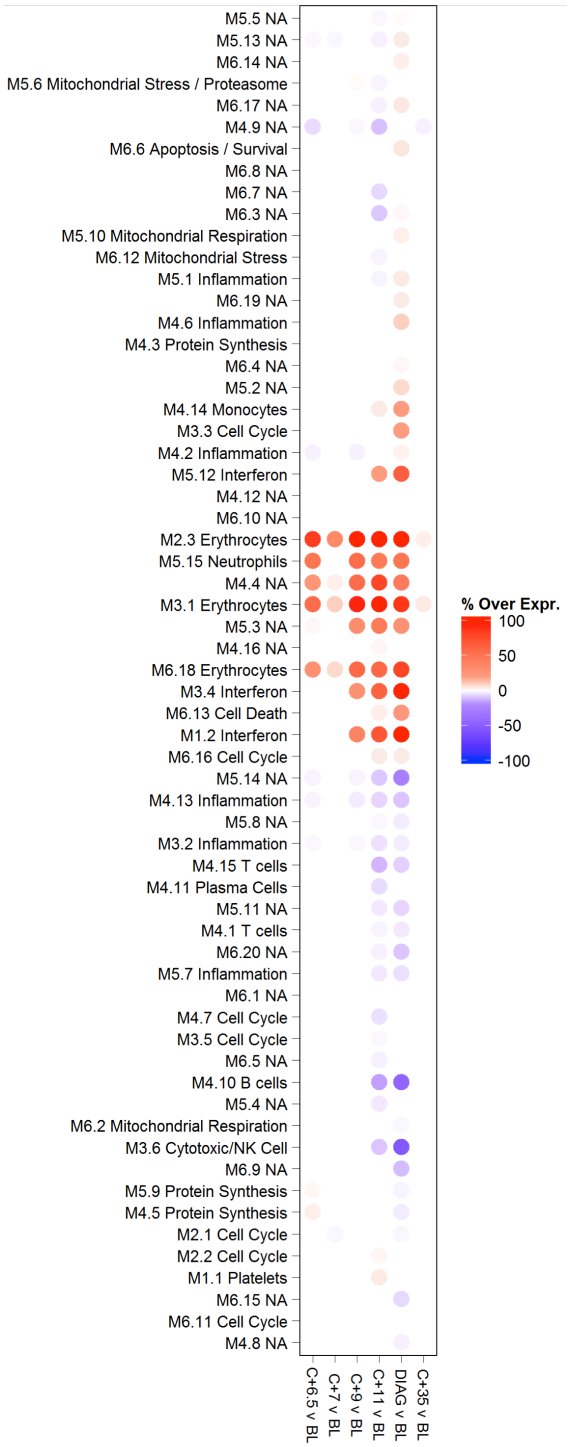


Figure 8-1: Mean modular changes in gene expression for KCS PAXgene dataset summarised according to baseline comparisons

n=28, except baseline (BL) v diagnosis (n=27). FDR < 5%, FC>1.5. Y axis = previously defined modules of pre-determined co-dependant genes[362].

The multiple comparisons above provided a detailed, overview of the changes in gene expression during the time course. Given the large amount of data to potentially explore, it was decided to narrow subsequent analysis to three key areas;

(i) More detailed characterisation of changes in the ***interferon-signalling pathway*** given its known importance in NAI and prominence in the initial results.

(ii) More detailed analysis of genes differentially expressed in the ***diagnosis versus baseline*** analysis given the greatest number of genes were differentially expressed in this analysis and this comparison was the most likely to reflect immunological changes at the peak of blood-stage parasitaemia.

(iii) More detailed characterisation of the differentially expressed genes in the ***C+6.5 versus baseline*** analysis given this comparison provides important insights into the expression changes associated with the liver stage of infection, a stage of the life-cycle nearly impossible to study in naturally occurring infections.

(i) Interferon signalling pathway

An overview of the interferon signalling canonical pathway is shown in Figure 8-2. This pathway became increasingly significantly enriched in baseline analyses from C+9 until diagnosis, returning to baseline values post treatment (Table 8-7 and Figure 8-3). The greatest change in pathway enrichment occurred between C+7 and C+9 (*p=6.43E-15*), with a smaller change between C+11 and diagnosis

(*p=2.36E-09*) (Table 8-8 and Figure 8-4). Both IFN-γ and Type I/III interferon pathways appeared to be up-regulated (Figure 8-3 and Figure 8-4). The gene expression changes in the IFN-γ pathway in the baseline versus diagnosis comparison were broadly similar to those seen in PBMC samples taken during a mosquito-bite CHMI study of malaria-naïve American volunteers (Figure 8-5)[84]. Change in expression over time of the 17/34 genes in the interferon signalling pathway differentially expressed in the diagnosis versus baseline analysis are shown in Appendix 7-8.

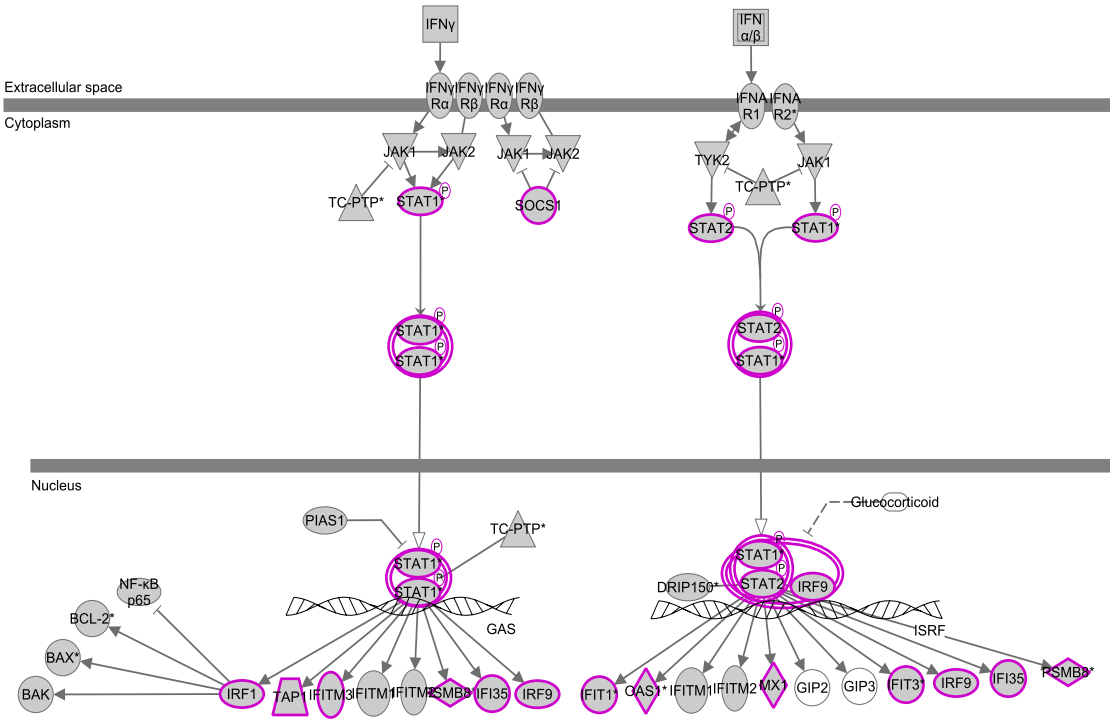


Figure 8-2: Interferon (IFN) signalling pathway (source Ingenuity).

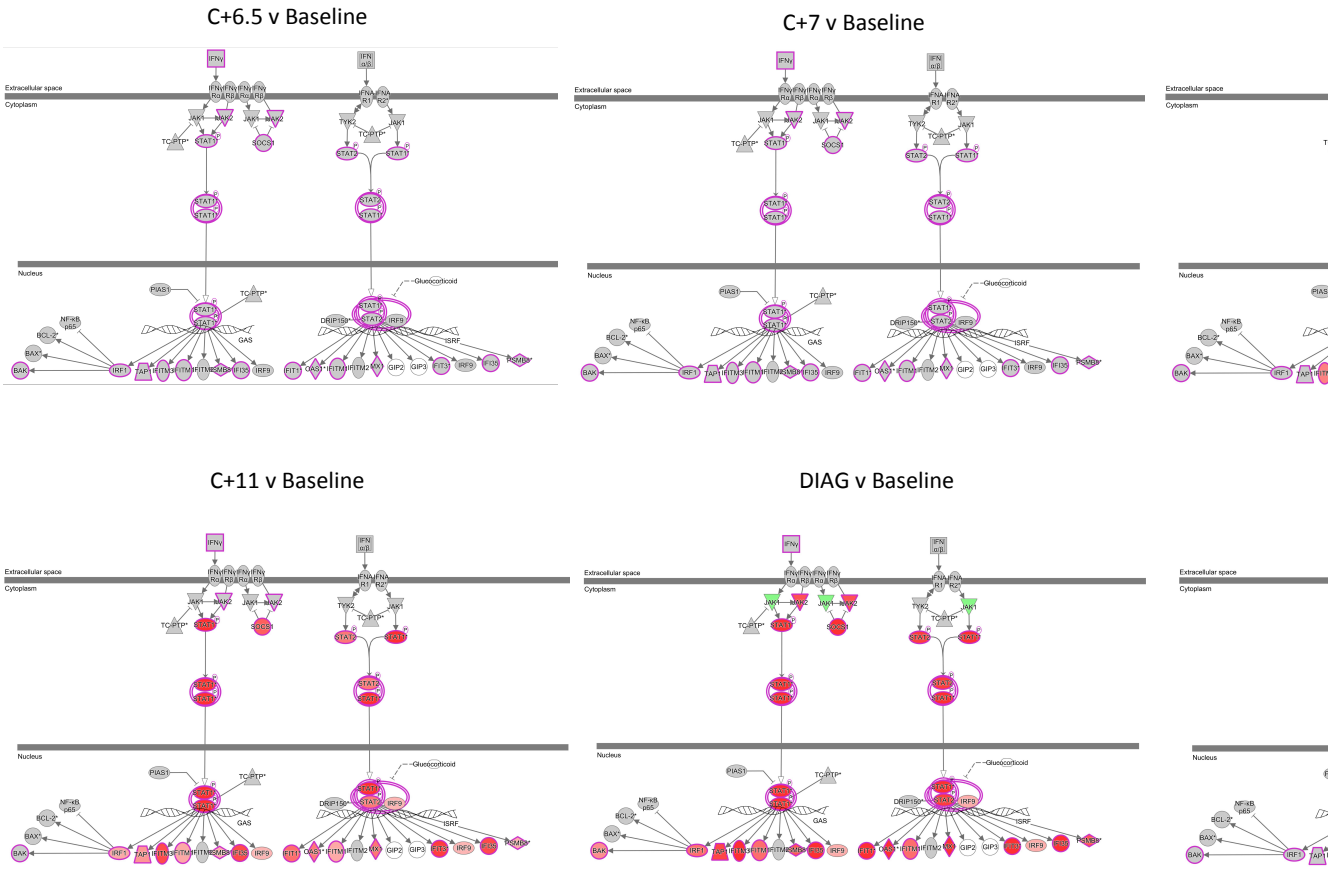


Figure 8-3: Changes in gene expression in the IFN-signalling pathway: baseline comparison analysis for KCS PAXgene dataset

FDR < 5%, FC > 1.25. Red=up regulation. Green = down regulation.

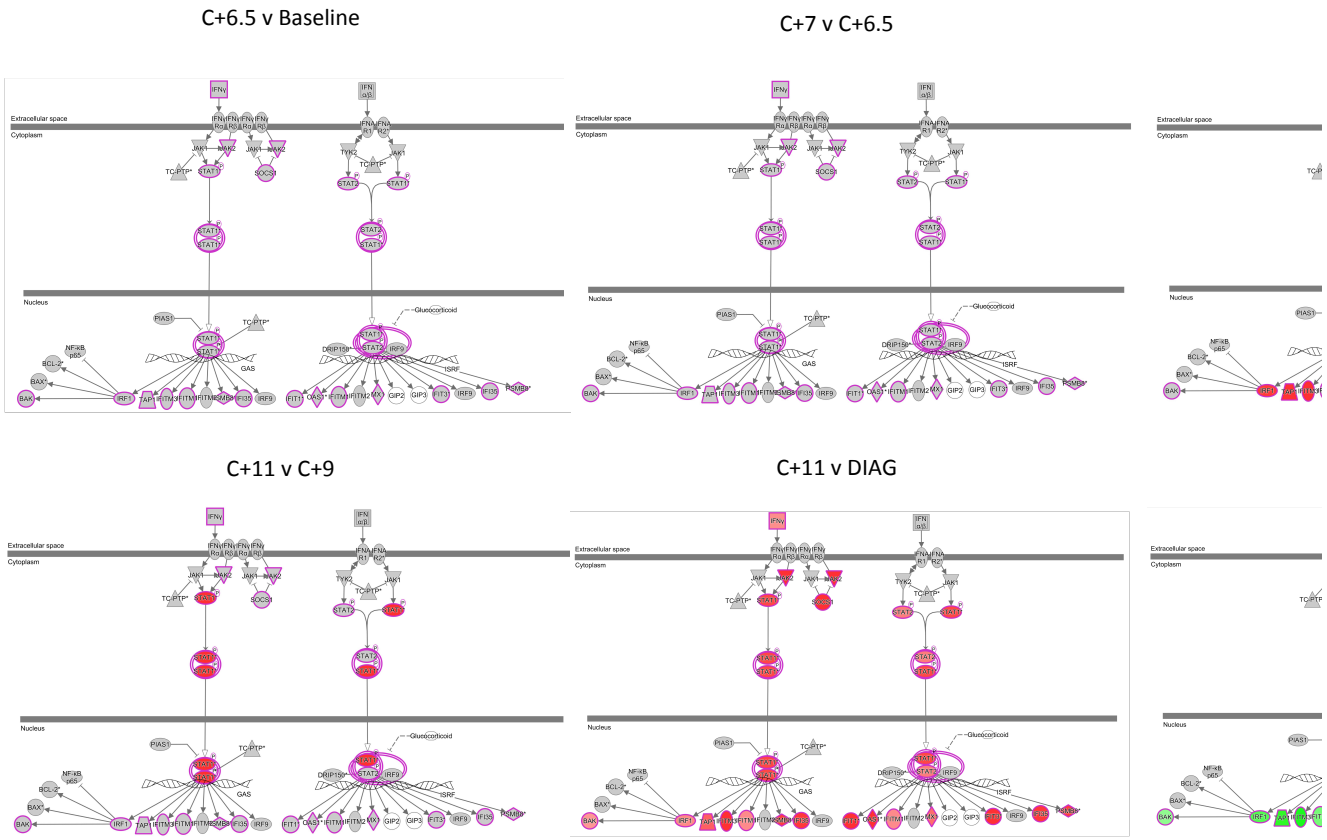


Figure 8-4: Changes in gene expression in the IFN-signalling pathway: consecutive comparison analysis for KCS PAXgene dataset.

FDR < 5%, FC > 1.25. Red=up regulation. Green = down regulation.

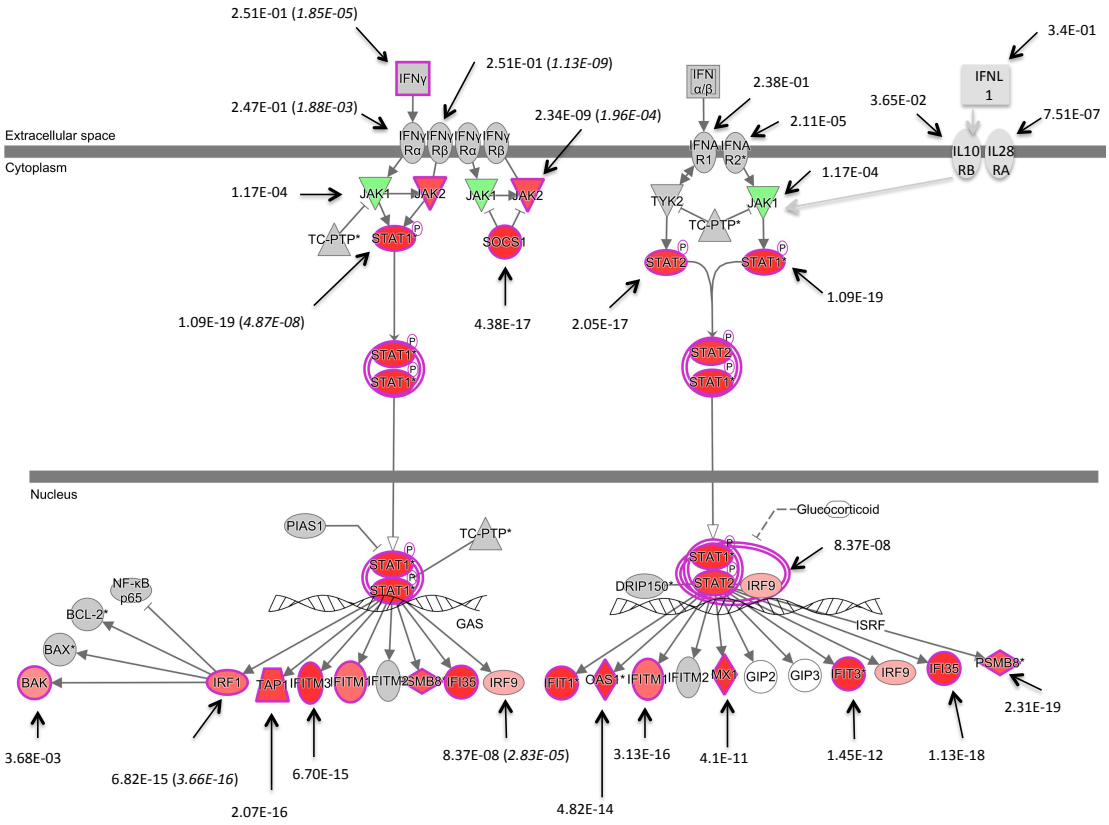


Figure 8-5: FDR for change in gene expression in IFN-signalling pathway in diagnosis v baseline analysis for KCS PAXgene dataset.

FC > 1.25. FDR in brackets are data from PBMC samples from CHMI study of US volunteers[84]. Red=up regulation. Green=down regulation.

(ii) Diagnosis versus baseline analysis

The top differentially expressed genes in this analysis included the inducible chemokines *CXCL9* and *CXCL10* (Table 8-9 and Figure 8-6). Both *CXCL9* and *CXCL10* bind to the receptor *CXCR3* (which is expressed primarily on activated T cells and NK cells) and facilitate chemo attraction of T cells, helping recruit activated T cells to sites of inflammation. *CXCL9*, also known as monokine induced by gamma interferon (MIG) is induced by IFN-γ, whilst *CXCL10* is induced by a variety of innate stimuli that include IFN-α/β and IFN-γ[425]. Both chemokines have been studied in relation to malaria[426]: *CXCL9* levels have been shown to correlate with parasite density and be increased in the plasma of patients with severe malaria[427], whilst *CXCL10* is increased in the serum and plasma of children with cerebral malaria and correlates with mortality[428-430]. Indeed, in samples taken at diagnosis in a UK CHMI study, elevated *CXCL9* levels were seen coincident with a reduction in peripheral blood B-cell subsets expressing *CXCR3*, suggesting migration of the cells away from the periphery[172]. By contrast, *ANKRD22* (Ankyrin Repeat Domain 22), a protein-coding gene markedly up-regulated in the analysis, is not known to have any association with inflammation or infection.

In a study of Kenyan children presenting acutely unwell, *Griffiths et al* identified a number of genes as markers of parasitaemia as opposed to other forms of infection (Table 8-10)[264]. Changes in the expression of these genes was also seen in another study of naturally acquired malaria infection in African children[265]. Analysis of the differential expression at diagnosis versus baseline of some of these genes in our dataset showed markedly lower fold changes, presumably

because subjects in our CHMI study were diagnosed earlier, at lower parasitaemias than those presenting with acute malaria in the field. However genes *C1QB* and *C3AR1* relating to the complement system, had extremely low FDR values in our analysis, and could show promise as biomarkers of early blood-stage malaria infection.

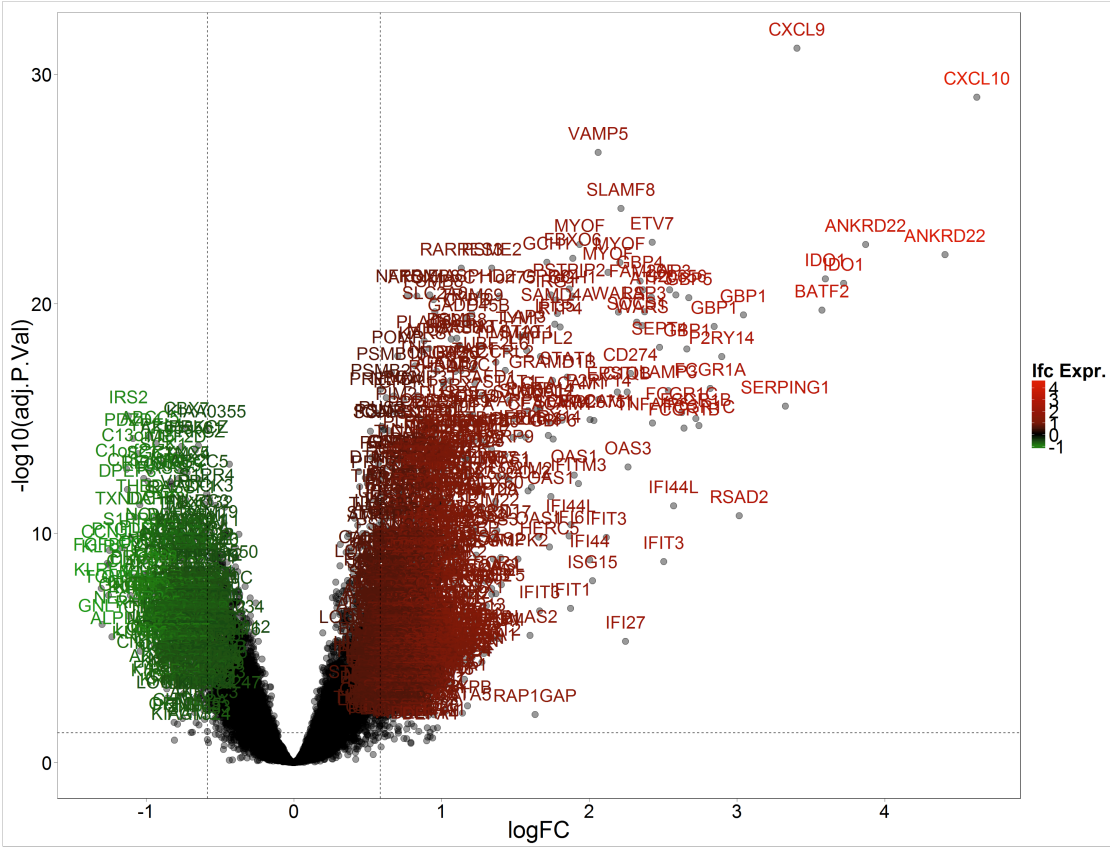


Figure 8-6: Volcano plot of KCS PAXgene dataset: diagnosis versus baseline analysis.

FDR < 5%, FC > 1.25. Red = up-regulated. Green = down-regulated.

	Molecule	Log Ratio	FDR (q value)
Up Regulated	CXCL10	4.365	5.75E-28
	ANKRD22	4.190	2.86E-23
	IDO1	3.481	1.43E-20
	BATF2	3.353	4.58E-19
	CXCL9	3.224	5.75E-28
	SERPING1	3.143	4.79E-16
	RSAD2	2.936	3.84E-15
	P2RY14	2.686	1.30E-17
	GBP1	2.673	6.99E-20
	FCGR1B	2.651	2.73E-17
Down Regulated	CCNJL	-1.179	1.86E-09
	GNLY	-1.167	7.40E-11
	ALPLL	-1.111	9.89E-03
	KLRF1	-1.105	2.23E-08
	TGM3	-1.101	3.47E-10
	IRS2	-1.088	9.08E-18
	DPEP3	-1.062	2.86E-16
	PTGDR	-1.040	6.68E-11
	TXNDC12	-1.022	1.83E-12
	THBD	-1.016	1.25E-16

Table 8-9: Up and down-regulated genes for KCS PAXgene dataset: Diagnosis versus baseline analysis

Top 10 genes differentially up or down-regulated ranked according to Log ratio. FDR <5%, FC >1.25.

	Idaghdour et al[265]		KCS	
Loci	Log Ratio	FDR (q value)	Log Ratio	FDR (q value)
C1QB	11.15	8.72E-19	2.079	8.17E-16
MMP9	11	1.12E-12	0.701	9.87E-01
C3AR1	1.33	5.80E-07	0.77	1.02E-12
ILI8R	2.83	7.96E-07	0.07	7.33E-01
HMOX1	2.08	1.10E-08	0.145	4.39E-01

Table 8-10: Changes in gene expression of genes identified previously as potential biomarkers of parasitaemia

Log fold changes and FDR values for some genes identified by Griffiths et al[264] as potential biomarkers of parasitaemia. KCS values =Results from KCS PAXgene dataset: diagnosis versus baseline analysis.

The predicted upstream regulators of the differentially expressed genes in the diagnosis versus baseline analysis showed small log fold changes that were highly significant (Table 8-11). As expected, the pro-inflammatory molecules IFN-γ (*p=2.28E-34*) and TNF (*p=1.21E-19*) were up-regulated, consistent with their known role in the immune response against blood-stage malaria[21]. Of interest was the down regulation of IL1RN (interleukin 1 Receptor Antagonist) (*p=5.06E-23*), presumably resulting in potentiation of the pro-inflammatory response mediated by IL-1[431]. However, interestingly the top upstream regulator IFNL1 (IFN-λ1) (*p=7.40E-35*) has no known role in the immune response to blood-stage malaria.

Upstream Regulator		Log Ratio	Molecule Type	Predicted Activ
IFNL1	Interferon Lamda 1	-0.163	cytokine	Activat
IFNG	Interferon gamma	0.259	cytokine	Activat
MAPK1	Mitogen-activated protein kinase 1	-0.265	kinase	Inhibite
IFNA2	Interferon alpha 2		cytokine	Activat
IL1RN	Interleukin 1 receptor antagonist	0.893	cytokine	Inhibite
TGM2	Transglutaminase 2	1.307	enzyme	Activat
TNF	Tumour necrosis factor	0.781	cytokine	Activat
IFN α	Interferon alpha		group	Activat
CNOT7	CCR4-NOT transcription complex, subunit 7	-0.127	transcription regulator	Inhibite
TLR3	Toll-like receptor 3	-0.098	transmembrane receptor	Activat
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	1.022	kinase	Activat
STAT1	Signal transducer and activator of transcription 1	1.736	transcription regulator	Activat
IRF1	Interferon regulatory factor 1	0.886	transcription regulator	Activat
PAF1	Paf1, RNA polymerase II associated factor 1	0.037	other	Activat
Immunoglobulin			complex	Activat

Table 8-11: Predicted upstream regulators for KCS PAXgene dataset: diagnosis versus baseline analysis

Upstream regulators with p value of overlap <E-09 and activation z score >2. FDR < 5%, FC > 1.25.

Analysis of the downstream effects of the differentially expressed genes showed significant changes relating to immune cell trafficking, cellular movement, haematological system development and function and inflammatory response (Table 8-12). The most significant downstream effect however was up-regulation of an antiviral inflammatory response. Interestingly, significant down regulation of proliferation of immune cells and activation of lymphocytes was seen. Network analysis confirmed a central role for TNF, IFNα, IFNL1 and the pro-inflammatory transcription factor NF-κB complex (Appendices 8-9 and 8-10).

Downstream Effect	p-value	z score	Number of Molecules
Antiviral response	7.00E-16	2.89	46
Antimicrobial response	3.63E-14	2.49	61
Cell movement of mononuclear leukocytes	2.29E-09	2.14	68
Homing of mononuclear leukocytes	2.46E-08	2.15	44
Chemo taxis of mononuclear leukocytes	4.63E-08	2.04	43
Cell movement of leukocytes	6.38E-08	2.10	84

Table 8-12: Predicted downstream effects predicted from KCS PAXgene dataset: diagnosis v baseline analysis

P=<E-07, activation z score >+/- 2. FDR < 5%, FC > 1.25.

(iii) C+6.5 versus baseline analysis

In contrast to published data reporting a central role of Type I and II interferons in the liver-stage of infection[57,82], analysis highlighted differential expression of the interleukin signalling pathways for IL-6, IL-8 and IL-9 (Table 8-13). IL-8 (or CXCL8) can trigger inflammation in cells such as neutrophils, leading to

chemotaxis, respiratory burst, granule release, and increased cell adhesion. IL-9 is a Th2-type cytokine, a potent T-cell and mast cell growth factor and enhances the production of IgE from B-cells. IL-6 is a regulator of acute-phase responses and a lymphocyte stimulatory factor. Little published data to date has supported a role for these interleukins in immune response against the liver-stage of infection[122,432-435].

Canonical Pathway	P value
Glucocorticoid Receptor Signalling	7.97E-05
IL-6 Signalling	4.08E-04
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	4.91E-04
IL-8 Signalling	5.21E-04
IL-9 Signalling	1.72E-03

Table 8-13: Canonical pathways differentially expression in KCS PAXgene dataset: C+6.5 versus baseline analysis

Top 5 canonical pathways differentially expressed. FDR < 5%, FC > 1.25.

The top differentially expressed genes in this analysis included up-regulation of *MGST1* (*p=3.10E-18*; a glutathione S-transferase involved in cellular defence against active electrophilic compounds), *FADS1* (*p=1.37E-14*; Fatty acid desaturase 1 which regulates unsaturation of fatty acids) and *ELANE* (*p=2.61E-07*; a neutrophil expressed elastase) and down regulation of DDIT4 (*p=2.07E-12*; DNA-damage-inducible transcript 4), *PRDX6* (*p=6.80E-10*; a gene with a role in phospholipid turnover and protection against oxidative injury) and WAPAL (*p=4.50E-07;* a regulator of sister chromatid cohesion in mitosis), broadly

suggesting a cellular response against stress (Figure 8-7 and Table 8-14).

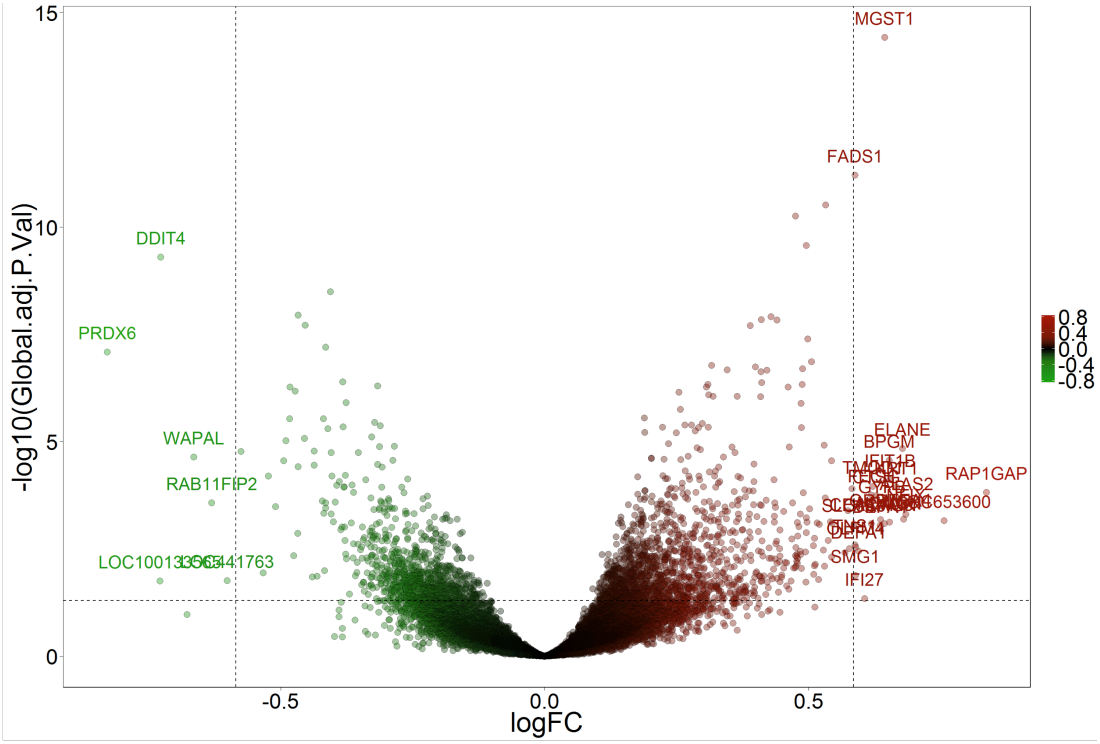


Figure 8-7: Volcano plot of KCS PAXgene dataset C+6.5 v baseline analysis.

Red = up-regulated. Green = down-regulated. FDR < 5%, FC > 1.25.

	Molecule	Log Ratio	FDR (q value)
Up-regulated	*RAP1GAP*	0.837	4.18E-06
	DEFA1 (includes others)	0.756	2.64E-05
	ALAS2	0.686	8.37E-06
	NFIX	0.684	1.76E-05
	DEFA4	0.680	2.37E-05
	ELANE	0.678	2.61E-07
	KRT1	0.667	3.00E-06
	HEMGN	0.653	2.92E-05
	BPGM	0.652	5.46E-07
	MGST1	0.644	3.10E-18
Down-regulated	*PRDX6*	-0.828	6.80E-10
	DDIT4	-0.727	2.07E-12
	WAPAL	-0.664	4.50E-07
	RAB11FIP2	-0.631	8.24E-06
	TSC22D3	-0.575	1.69E-11
	CXCL8	-0.533	8.70E-04
	TAF15	-0.523	1.49E-06
	CRISPLD2	-0.494	5.60E-07
	DERL2	-0.483	4.02E-08
	CLEC4E	-0.482	5.83E-09

Table 8-14: Up and down-regulated genes for KCS PAxgene dataset: C+6.5 versus baseline analysis.

Top 10 up and down-regulated genes ranked according to Log ratio. FDR < 5%, FC > 1.25.

IL-13 was the top predicted upstream regulator in this analysis (Table 8-15; *p=3.51E-14*). An immunoregulatory cytokine produced by activated Th2 cells, IL-13 is involved in several stages of B cell maturation and differentiation. It also down regulates macrophage activity thereby inhibiting production of pro-inflammatory cytokines and chemokines. Whilst no role for IL-13 in the liver-stage of malaria has been described, this interleukin is known to modulate

resistance to intracellular pathogens such as *Leishmania major*, *Leishmania mexicana* and *Listeria monocytogenes*[436].

Upstream Regulator	Log Ratio	Molecule Type	Activation Score	p value of Overlap
IL-13	-	Cytokine	0.857	1.10E-13
GATA1	0.369	Transcription Regulator	-	1.98E-07
GFI1	-0.078	Transcription Regulator	-	2.85E-06
CEBPE	0.113	Transcription Regulator	-	5.23E-06
IgG	-	Complex	-0.412	8.27E-06
BTK	0.036	Kinase	-	1.09E-05
TGM2	0.364	Enzyme	1.603	2.91E-05
CEBPA	0.475	Transcription Regulator	0.79	3.87E-05
EGFR	-0.108	Kinase	-1.071	4.69E-06
IL4	-0.096	Cytokine	0.079	6.77E-05
INS	-0.015	Other	0.805	7.82E-05
SLC4A1	0.485	Transporter		9.22E-05
ELANE	0.678	Peptidase		9.22E-05

Table 8-15: Upstream regulators for KCS PAXgene dataset: C+6.5 v baseline analysis

p value of overlap <E-04. FDR < 5%, FC > 1.25.

Analysis of the downstream effects of the differentially expressed genes was very similar to that predicted for tuberculosis (*p=4.99E-17*) and bacterial infection (*p=1.98E-13*). Of note, similarity to the predicted gene changes seen in parasitaemia (*p=1.02E-03*) and malaria (*p=5.87E-03*) were notably lower. Network analysis confirmed a central role for TNF in concordance with published literature (Appendix 8-11)[437].

8.4.4 Time course analysis of KCS PAXgene dataset according to exposure

The time course analysis was repeated comparing gene expression at each time point between subjects with DefExp and MinExp prior exposure to malaria. In addition, the whole series was analysed to identify genes differentially expressed between DefExp and MinExp volunteers at one or more time points.

At a FDR of 25%, 205 molecules were differentially expressed over the whole series between the two groups (Table 8-16) with differential expression of canonical pathways relating to interferon signalling and antigen presentation (Table 8-17). The most differentially expressed gene between the two groups was RPS6KA2, which was up-regulated in DefExP volunteers compared to MinExp and is known to be important in cell growth and differentiation (Table 8-18). Upstream regulators IFN-γ, IFNL1, IL1RN and TNF were all predicted to be down-regulated in DefExp compared to MinExp subjects, whilst MAPK1 was up-regulated (Table 8-18). Unfortunately too few genes were differentially expressed between groups at individual time points to allow more detailed description of changes in gene expression between the groups over time, although it appeared that the majority of these changes were seen C+11 (Table 8-16).

Analysis	FDR 5%	FDR 10%	FDR 25%
Whole Time Course	48	80	205
Baseline	0	0	9
C+6.5	0	0	0
C+7	0	0	0
C+9	0	0	0
C+11	0	10	37
DIAG	0	0	0
C+35	0	0	1

Table 8-16: Numbers of analysis-ready molecules differentially expressed for various comparisons between MinExp and DefExp volunteers using KCS PAXgene dataset

Canonical Pathway	p value
Interferon Signalling	3.01E-04
NRF2-mediated Oxidative Stress Response	1.61E-03
Glutathione-mediated Detoxification	1.70E-03
Antigen Presentation Pathway	5.29E-03
UVA-Inducted MAPK Signalling	9.78E-03

Table 8-17: Canonical Pathways differentially expressed between MinExp and DefExp subjects in whole series analysis using KCS PAXgene dataset

FDR 25%, no FC filter.

DefExp Group in Relation to MinExp					
Differentially Expressed Genes			**Predicted Upstream Regulators**		
Molecule	*FDR (q value)*	*Median Log FC*	*Molecule*	*Median Log FC*	*p value of overlap*
RPS6KA2	6.19E-10	0.425	*MAPK1*	0.887	3.15E-08
NQO2	4.74E-08	0.561	*IFNL1*	-0.094	1.20E-06
PRUNE2	2.83E-06	-0.828	*IFNG*	-0.106	4.27E-06
HRK	5.14E-06	0.707	*IL1RN*	-0.19	3.90E-04
PARP3	6.12E-06	-0.334	*IFNA2*	-	9.74E-04
POMC	2.98E-05	-0.613	*ERK1/2*	-	3.37E-03
KIAA0040	1.63E-04	-0.617	*TNF*	-0.037	1.91E-02

Table 8-18: Differentially expressed genes and upstream regulators in whole series analysis comparing MinExp and DefExp subjects in KCS PAXgene dataset

Top differentially expressed genes and upstream regulators (activation z score >+/- 2). Median Log FC = median fold change over time course. Shaded molecules = predicted status down-regulated. FDR 25%, no FC filter.

An interaction analysis was also performed to test whether the DefExp and MinExp subjects showed a different response between DIAG and baseline or C+35 and baseline. However, no probes passed even a lenient threshold (FDR>50%).

8.4.5 Time course analysis of KCS PAXgene dataset according to time to diagnosis

Four volunteers (110, 150, 151 and 220) demonstrated a delay in TTD (DTTD). All were in the DefExp group. Volunteers 150, 151 and 220 were diagnosed at C+14.3, C+14.3 and C+13.8 days post-administration of PfSPZ Challenge

respectively, while volunteer 110 was undiagnosed at C+21. The time course analysis was repeated comparing gene expression at each time point between the four subjects with a DTTD and those without (NDTTD). In addition, the whole series was analysed to identify genes differentially expressed between DTTD and NDTD volunteers at one or more time points.

At an FDR of 25%, 91 molecules were differentially expressed over the whole time course between the two groups (Table 8-19). Enrichment analysis showed differential expression of IL-17A and IL-17F pathways between the groups (Table 8-20). Both IL-17A and IL-17F can induce the expression of diverse pro-inflammatory cytokines and chemokines from a large variety of cells, and as such can mobilise, recruit and activate neutrophils[438]. Whilst there is only limited evidence to support the role of IL-17 in the immune response against malaria[439,440], the IL-17 cytokine group is known to be important in the pro-inflammatory, anti-microbial response[441-444].

Down regulation of pro-inflammatory molecules such as RELA (NF-κB), CXCR5 (also important in neutrophil chemotaxis)[445], CD40 and TLR4 was seen in subjects demonstrating a DTTD compared to those not (Table 8-21). However, it is impossible to ascertain whether these changes were the cause for the DTTD or a result of it.

Analysis	FDR 5%	FDR 10%	FDR 25%
Whole Time Course	29	49	91
Baseline	0	0	1
C+6.5	0	0	0
C+7	0	0	0
C+9	0	1	1
C+11	0	0	2
DIAG	0	0	0
C+35	0	0	0

Table 8-19: Numbers of analysis-ready molecules differentially expressed for various comparisons between subjects with a delay to diagnosis and those without for KCS PAXgene dataset

Canonical Pathway	p value
Role of IL-17F in Allergic Inflammatory Airways Disease	6.37E-04
Role of IL-17A in Arthritis	1.43E-03
Gαi Signalling	1.51E-03
Leukotriene Biosynthesis	1.69E-03
γ-glutamyl Cycle	1.69E-03
IL-10 Signalling	2.77E-03

Table 8-20: Canonical Pathways differentially expressed between subjects with a delay to diagnosis and those without in whole series analysis using KCS PAXgene dataset

FDR 25%; no FC filter; p<E-04.

DTTD Group in Relation to NDTTD					
Differentially Expressed Genes			**Predicted Upstream Regulators**		
Molecule	*FDR (q value)*	*Median Log FC*	*Molecule*	*Median Log FC*	*p value of overlap*
H6PD	2.89E-06	-0.534	*RELA*	-0.113	2.68E-03
XRN2	2.89E-06	0.975	*ASB9*	-	4.12E-03
KCNH3	3.11E-06	-0.807	*CD40*	-0.276	6.30E-03
*CD01**	7.44E-06	1.166	*Igm*	-	7.20E-03
SNHG17	9.86E-06	-0.561	*RPP38*	-0.062	1.23E-02
CCS	3.10E-05	0.580	*PTPN22*	0.249	1.23E-02
RPS6KA2	7.08E-05	0.931	*IL17A*	-0.208	1.61E-02
L1TD1	1.73E-04	0.645	*STK40*	-0.048	1.64E-02
*PALM**	1.73E-04	0.678	*NEAT1*	0.080	1.64E-02
*CXCL5**	6.75E-04	-0.964	*TLR4*	-0.014	1.87E-02

Table 8-21: Differentially expressed genes and upstream regulators in whole series analysis comparing subjects with a delay to diagnosis and those without in KCS PAXgene dataset

Top differentially expressed genes and upstream regulators (all activation z scores <+/- 2). FDR 25%. Median Log FC = median fold change over time course.

8.4.6 Time course analysis of KCS PAXgene dataset using functional principal component analysis

fPCA analysis of data from all volunteers did not yield any significant results. However, when the analysis was repeated after removal of volunteers demonstrating a DTTD and volunteer 110, two prominent eigenfunctions describing the trajectory of change in expression profile over time of a group of genes were identified (Figure 8-8). The first eigenfunction (EF1) explained 95.2% of the variation in data and represented a monotonic change with time until day of diagnosis before returning towards baseline. The second

eigenfunction explained an additional 4.6% of the variation and had maximal expression change around C+7, then plateaued until C+10.5 before returning towards baseline. EF2 also appeared to further change post treatment.

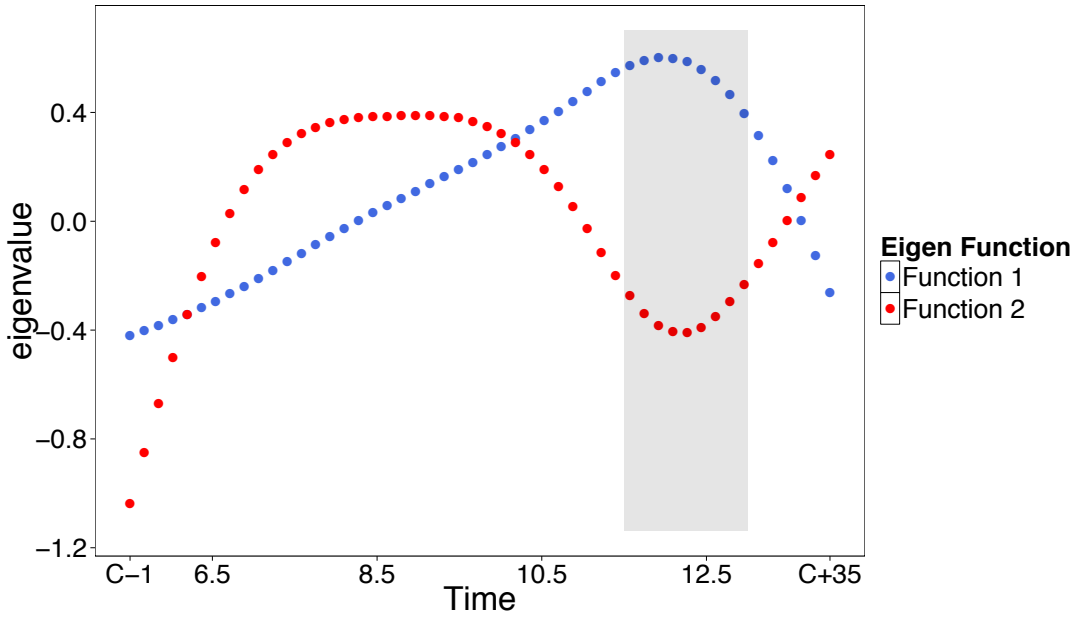


Figure 8-8: First two eigenfunctions from fPCA analysis of KCS PAXgene dataset

Four volunteers demonstrating a DTTD and volunteer 110 excluded (n=23). Grey shading = time window of diagnosis. FDR < 5%, FC > 1.25.

1,066 probes were detected with FDR < 5% for differential expression over time compared to mean expression levels. In order to identify groups of genes with functionally similar expression trajectories, K-means clustering was applied to the eigenscores of these two eigenfunctions. This clearly identified two major clusters of genes (Figure 8-9). Broadly speaking, in terms of EF1, probes in cluster 1 (red) were up-regulated and probes in cluster 2 (blue) were down-regulated at diagnosis compared to baseline (Figure 8-10). Individual expression

trajectories for probes with the largest positive (Appendix 8-12) and negative (Appendix 8-13) eigenscores for EF1 showed that the peak change in expression was increasingly delayed as TTD increased. This peak was not seen in the four individuals demonstrating a DTTD.

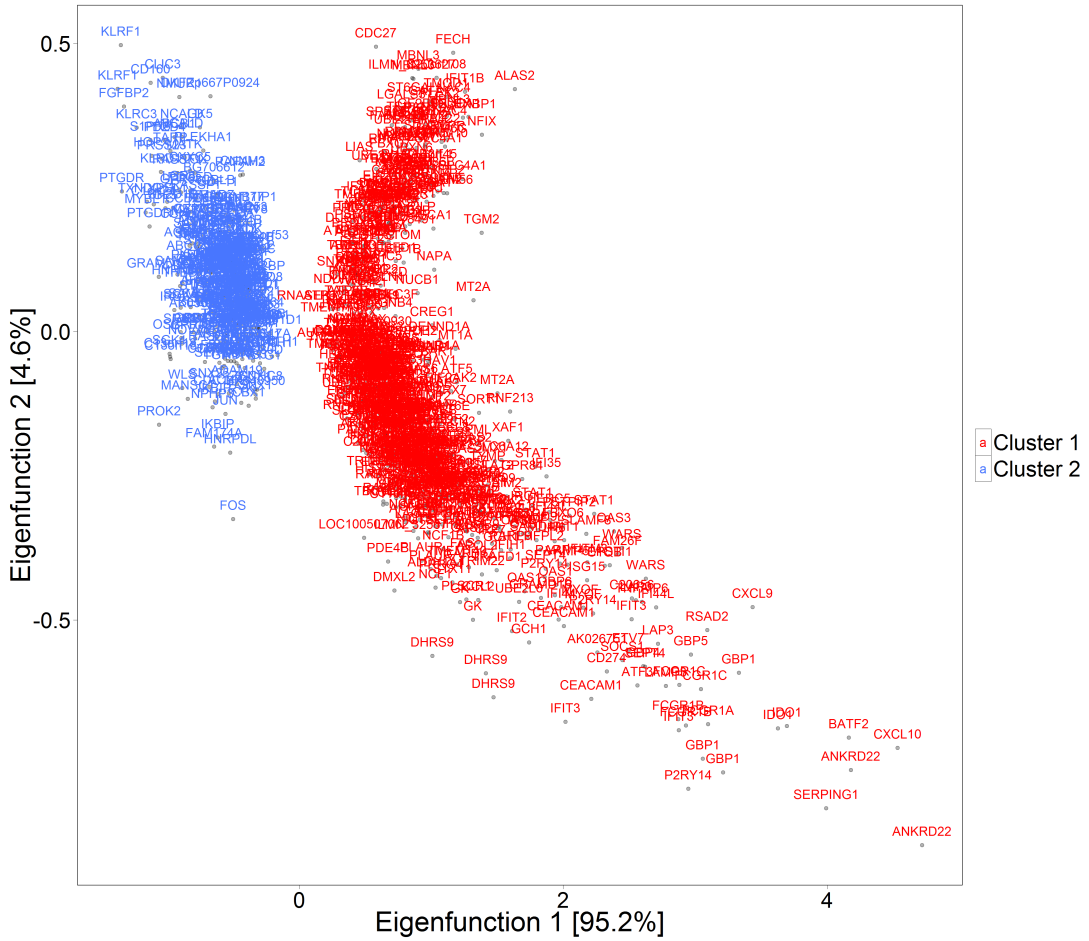


Figure 8-9: Eigenscores for 1,066 probes from fPCA analysis of KCS PAXgene dataset

Four volunteers demonstrating a delay to diagnosis and volunteer 110 excluded (n=23). F1=eigenfunction 1. F2 = eigenfunction 2. Red = cluster 1. Turquoise = cluster 2. FDR < 5%, FC > 1.25.

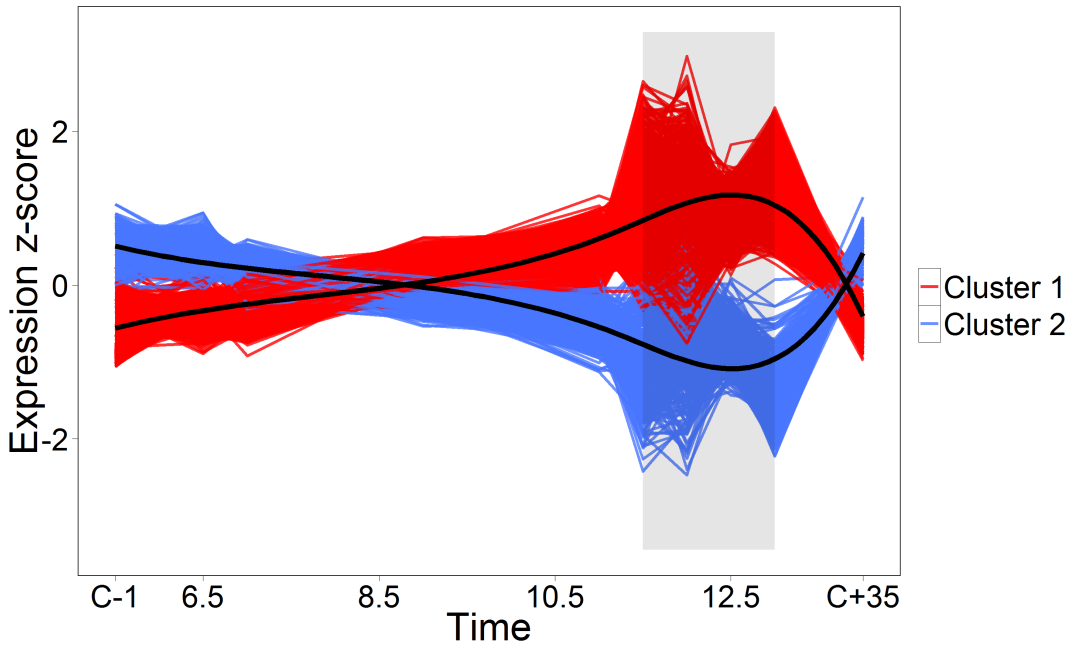


Figure 8-10: Individual changes in probe expression overtime from fPCA analysis of KCS PAXgene dataset

Changes in probe expression overtime (mean, scaled). *Four volunteers demonstrating a DTD and volunteer 110 excluded, coloured according to cluster (n=23). FDR < 5%, FC > 1.25.*

Cluster 1 (red) was significantly enriched for many canonical pathways related to the innate immune system, pro-inflammatory responses and antigen presentation, with only one pathway related to immune regulation (IL-10 signaling) (Table 8-22). Examples of genes in this cluster included *CXCL10, CXCL9, ANKRD22, RSAD2, STAT1, IFIT3* and *FCGR1A* (Figure 8-9 and Table 8-23).

Cluster 2 (blue) was only significantly enriched for three canonical pathways (ceramide signaling, CCR5 Signalling in Macrophages and UVB-Induced MAPK Signalling), which had notably lower *p* values than those pathways enriched in

cluster 1. Ceramide is important in cell signaling and can cause cell cycle arrest and apoptosis. It can also induce activation of NF-κB and a subsequent pro-inflammatory response. It is unexpected therefore to see this pathway down-regulated and could be as a result of regulation of the pro-inflammatory immune response. CCR5 regulates leukocyte chemotaxis in inflammation. Down regulation of this pathway was again unexpected and could be as a result of movement of leukocytes from the blood to the spleen as blood-stage infection progresses. Examples of genes in this cluster included *FOS* and *JUN*, which form the AP1 transcription factor that regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections (Figure 8-9 and Table 8-23)[446].

Cluster 1	Interferon Signalling	1.41E-15
	Activation of IRF (Interferon Regulatory Factor) by Cytosolic Pattern Recognition Receptors	1.15E-12
	Antigen Presentation Pathway	1.28E-12
	TREM1 (triggering receptor expressed on myeloid cells - 1) Signalling	1.34-08
	Death Receptor Signalling	7.74E-08
	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses*	9.40E-08
	Type 1 Diabetes Mellitus Signalling	1.02E-07
	Dendritic Cell Maturation	1.73E-07
	Toll-like Receptor Signalling	1.90E-07
	Role of PKR in Interferon Induction and Antiviral Response	2.01E-07
	Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	7.85E-07
	iNOS Signalling	4.11E-06
	Role of RIG1-like Receptors in Anti-viral Innate Immunity	2.45E-05
	Protein Ubiquitination Pathway	2.78E-05
	OX40 Signalling Pathway	2.90E-05
	IL-10 Signalling	3.25E-05
	Acute Phase Response Signalling	4.64E-05
Cluster 2	Ceramide Signalling	1.57E-05
	CCR5 Signalling in Macrophages	4.03E-05
	UVB-Induced Signalling	9.21E-05

Table 8-22: Canonical Pathways differentially expressed in Cluster 1 and Cluster 2 from eigenfunction 1 derived from fPCA analysis of KCS PAXgene dataset

N=23; Four volunteers demonstrating a delay to diagnosis and volunteer 110 were excluded. p value < E10-4. FDR < 5%, FC > 1.25.

Cluster 1		Cluster 2	
Molecule	*FDR (q value)*	*Molecule*	*FDR (q value)*
CXCL9	5.00E-04	GLS	3.27E-03
BATF2	1.38E-03	YPEL1	6.61E-03
CXCL10	1.38E-03	ABCC5	9.62E-03
STAT1	1.65E-03	RASSF1	9.62E-03
GBP1	1.65E-03	PPP1R8	1.04E-02
PSME2	1.65E-03	PTGDR	1.04E-02
PSTPIP2	1.65E-03	ERN1	1.06E-02
STAT2	1.65E-03	DSTYK	1.43E-02
TYMP	1.65E-03	TXNDC12	1.44E-02
UBE2L6	1.65E-03	CXXC5	1.47E-02

Table 8-23: Top differentially expressed in cluster 1 and 2 from eigenfunction 1, derived from fPCA analysis of KCS PAXgene dataset

N=23; Four volunteers demonstrating a delay to diagnosis and volunteer 110 were excluded. p value < E10-4. FDR < 5%, FC > 1.25.

Predicted upstream regulators in cluster 1 (i.e. up-regulated molecules post-infection) were very similar to those seen in the diagnosis versus baseline analysis and included the pro-inflammatory cytokines: IFNL1 (*p=6.12E-58*), IFN-γ (*p=4.45E-56*), TNF (*p=1.05E-22*) and Type I interferons ILNα2 (*p=1.90E-49*) and IFNβ1 (*p=4.13E-17*) (Table 8-24). Up regulation of TLR3 was seen (*p=4.56E-30*) which is interesting given the ligands for TLR3 are thought to be viral double stranded and single stranded RNA, and TLR3 is not known to have a role in the detection of parasite antigens[447]. Some molecules involved in immune modulation were also up-regulated including: CNOT7 (which can regulate interferon signalling by targeting STAT1)[448], SOCS1 (suppressor of cytokine

signalling 1)[449,450], and USP18 (which may down regulation interferon responses)[451,452]. Only two upstream regulators were predicted for cluster 1 with *p=<E-03*: ESR1 (*p=1.49E-04*) and SMARCA4 (*p=2.99E-04*). The top predicted downstream effects for cluster one included viral infection (*p=1.76E-17*) and antiviral response (*p=3.87E-14*). The top predicted downstream effects for cluster two included cell death and survival (*p=4.46E-06*) and cellular compromise (*p=4.46E-06*)

Upstream Regulator	Molecule Type	p-value of overlap
IFNL1	cytokine	6.12E-58
IFNG	cytokine	4.45E-56
IFNA2	cytokine	1.90E-49
MAPK1	kinase	7.54E-49
IL1RN	cytokine	2.01E-33
TLR3	transmembrane receptor	4.56E-30
TCR	complex	1.71E-27
CNOT7	transcription regulator	3.49E-24
IRF1	transcription regulator	5.76E-23
TGM2	enzyme	6.07E-23
TNF	cytokine	1.05E-22
STAT1	transcription regulator	1.67E-22
Interferon alpha	group	3.18E-22
EIF2AK2	kinase	1.42E-21
PAF1	other	4.21E-18
IFNB1	cytokine	4.13E-17
SOCS1	other	7.62E-16
STAT2	transcription regulator	8.61E-15
TAB1	enzyme	9.55E-14
RELA	transcription regulator	1.96E-13
USP18	peptidase	3.40E-13
GAPDH	enzyme	4.02E-13
CD40	transmembrane receptor	5.70E-13
NFkB (complex)	complex	7.79E-13
IL27	cytokine	8.22E-13
MAP3K7	kinase	1.11E-12
Immunoglobulin	complex	1.78E-12
IRF2	transcription regulator	1.86E-10
IL1A	cytokine	2.40E-10
IFN Beta	group	7.75E-10

Table 8-24: Upstream regulators for cluster 1 from eigenfunction 1 from fPCA analysis of KCS PAXgene data

N=23; Four volunteers demonstrating a delay to diagnosis and volunteer 110 were excluded. p value < E10-4. FDR < 5%, FC > 1.25.

8.4.7 Comparison of gene expression between volunteer 110 and KCS cohort using PAXgene dataset

Given only one individual (110) was capable of controlling parasite growth *in vivo* it is not possible to analyse gene expression patterns associated with NAI in my study. However, given the considerable data from this individual, across many time points, there is value in attempting to identify any key differences in gene expression in this individual compared to the cohort.

Comparison of z scores for all probes across the time course showed that gene expression for volunteer 110 did not appear to differ dramatically from the cohort at any time point (Appendix 8-14). For volunteer 110, 262 probes were identified with z scores > ± 3 at least one time point. Pathway analysis of these probes was enriched for processes related to malignancies such as melanoma, pancreatic and non-small lung cell cancer signalling (*p=4.17E-04, p=2.01E-03* and *p=2.17E-03* respectively). When this probe list was restricted to those with z scores > ± 3 at more than one time point, 14 probes were identified, 12 of which mapped to known genes (Figure 8-11). None of these genes are known to have a specific role in the immune response.

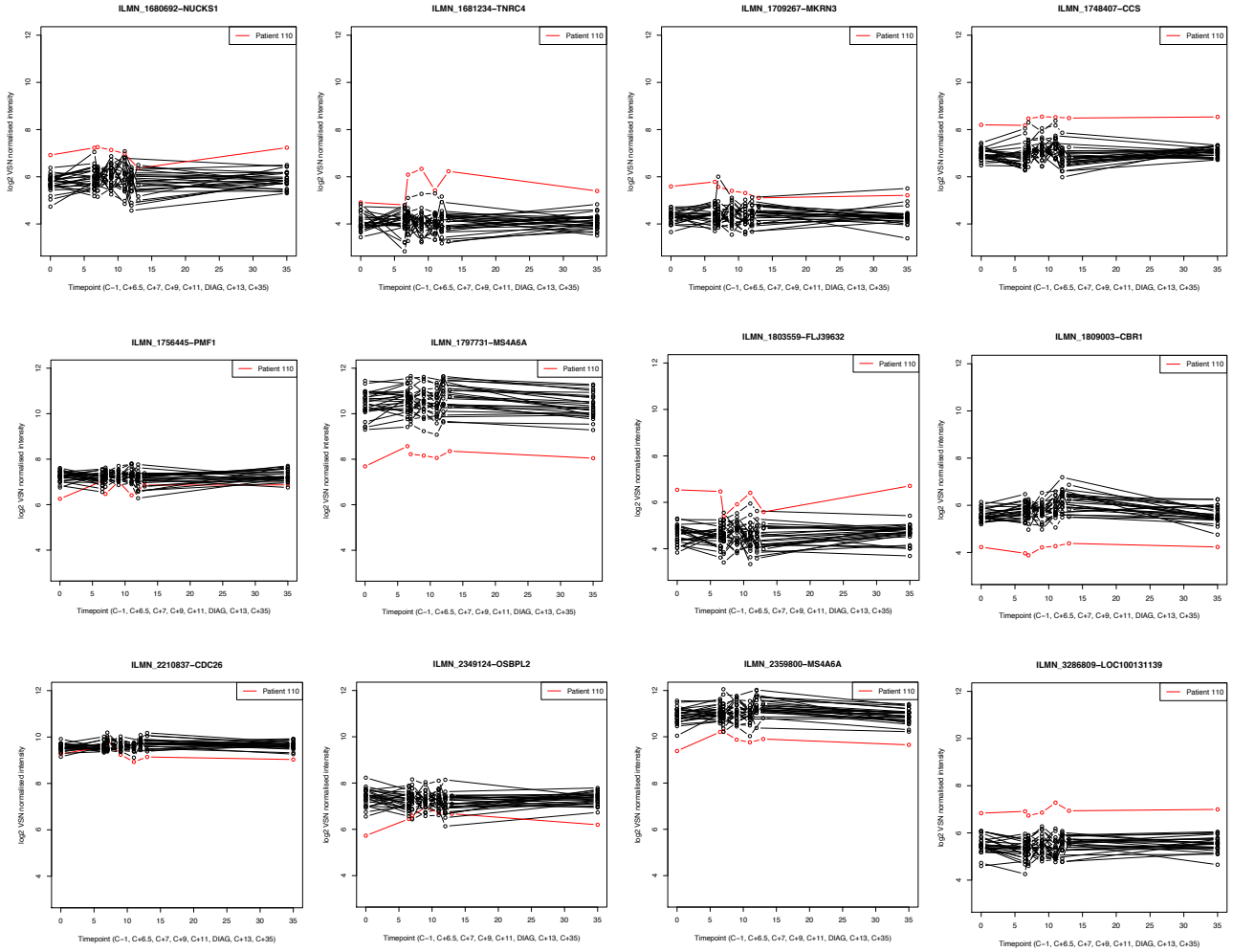


Figure 8-11: Probes mapping to known genes with z scores >± 3 at > one time-point for Volunteer 110 using KCS PAXGene dataset.

8.4.8 Correlation analyses of gene expression from KCS PAXgene dataset with key outcome measures

Gene expression data were interrogated to assess for correlation between gene expression profiles at particular time points and key measures of parasite growth and immunological function using the R function cor.test (Table 8-25). No probes were shown to significantly correlate (adjusted p value <0.05) with any of the measures assessed.

		Gene Expression						
		Baseline	C+6.5	C+7	C+9	C+11	DIAG	C+35
ADRB	Baseline	✓				✓	✓	
	C+35	✓				✓	✓	✓
GIA	Baseline						✓	
	C+35						✓	
PMR	-	✓				✓	✓	
LBI	-	✓	✓	✓				
p/ml at DIAG	-	✓					✓	

Table 8-25: Correlation analyses between gene expression profiles and key measures of parasite growth and immunological function for KCS PAXgene dataset.

Tick = analysis performed. Grey box = analysis not performed. ADRB = antibody-dependent respiratory burst assay; GIA = growth inhibition activity assay; PMR = parasite multiplication rate; LBI = liver to blood inoculum; p/ml = parasites per ml. DIAG = diagnosis. Baseline = day before CHMI (C-1).

8.4.9 Changes in leukocytes over time course in KCS

Microarray results are heavily influenced by sample cell-type composition. PAXgene samples contain RNA from whole blood and as a result, gene expression studies from such samples are dominated by signals from neutrophils. Microarrays do not distinguish between variations in gene expression resulting from physiological change and that resulting from a difference in cell-type frequency, and this distinction is crucial in order to fully interpret any findings[453].

In order to understand the impact of variation in cell type over the time course on these results microarray results, changes in leukocytes over the time course in KCS where these data were available (all time points except C+6.5) were analysed. These results showed that between baseline and diagnosis there was a trend for a reduction in lymphocytes and increase in neutrophils (Figure 8-12), however statistical analysis showed only a significant change in lymphocyte and monocyte absolute concentrations over the time course (*p=<0.0001 and p=0.001 respectively; Kruskall Wallis test*).

Given that white cell count data were not available for all samples, it was not possible to use these data as a covariant in the analysis, as has been done in another study[265]. However, of note there was no significant change in total white cell count over the time course in the KCS (*p=0.103; Kruskall Wallis test*).

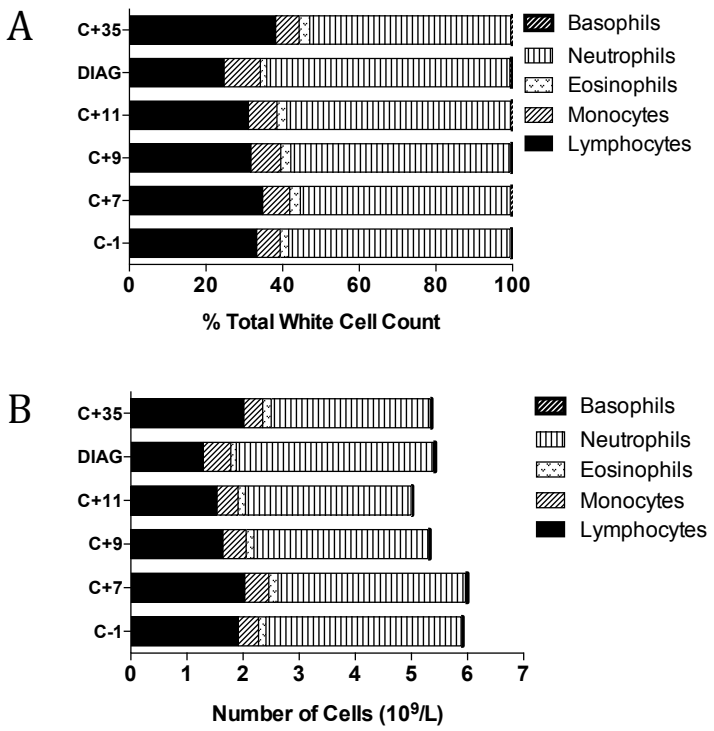


Figure 8-12: Changes in leukocytes over time course in KCS

(A) Change in contribution of various cell types to total leukocyte count over time course in KCS. (B) Absolute number of leukocytes at each time point for KCS. For (A) & (B) mean values for cohort are presented excluding data from volunteers at time points that were not included in the final KCS PAXgene dataset.

8.5 Discussion

Given the considerable translational potential of transcriptomics, there are surprisingly few gene expression studies published from clinical trials relating to malaria. Indeed, only one published study has described changes in gene expression over time post-CHMI and this was for *P. vivax* in a Columbian population[266]. Analysis of KCS data is therefore the first *P. falciparum* CHMI study to provide information on changes in gene expression at multiple time points post-*P. falciparum* infection, in African subjects, including volunteers with prior exposure to malaria.

In agreement with much published work (see Chapter 1), results confirmed a central role for interferons in the response to early blood-stage infection. As well as supporting the well-documented role of IFN-γ, the data confirm a role for Type I IFNs. Indeed, the changes in expression of genes related to IFN signalling seen in the diagnosis versus baseline analysis are very similar to those reported by *Ockenhouse et al* (Figure 8-5), despite differences in sample type (PAXgene versus PBMC) and method of inducing infection (PfSPZ Challenge versus mosquito-bite)[84].

Type III IFNs are not known to have a role in the immune response against any stage of malaria (Chapter 1). It was therefore of interest that IFN-λ1 was the most significant, predicted upstream regulator in both the diagnosis versus baseline and fPCA analyses. Type III IFNs were only identified in 2003[454,455] and there remains controversy as to the levels of IFNL receptors (IRNLRs) on immune cells[456]. However, knowledge of the role of Type III IFNs in anti-viral immunity has been increasing and the restricted receptor distribution of IFNLRs has lead to their investigation as a less noxious alternative to pegalated IFN-α in

the treatment of chronic viral hepatitis[456]. These data support further investigation, for example using animal models or population studies of polymorphisms, to elucidate the role of IFNL1 and other Type III IFNs in blood-stage malaria.

Counter intuitively, progressive down regulation in expression of genes in modules relating to B (M4.10), T (4.15) and NK (M3.6) cells was seen between baseline and diagnosis. However, given there was an increase in predicted cell movement, homing and chemotaxis of leukocytes at the peak of blood-stage infection, and a reduction in white cell count between baseline and diagnosis, this down-regulation could reflect redistribution of cells rather than reduced activation. Also seen was an up-regulation in genes relating to innate sensing and antigen presentation, including for example TLR expression, reflecting the innate hypersensitivity seen following malaria infection[37].

This work is the first to investigate the transcriptomic changes associated with the liver-stage of *P. falciparum* in humans. In contrast to published data reporting a central role of Type I and II interferons[57,82], this analysis highlighted differential expression of the interleukin signalling pathways for IL-6, IL-8 and IL-9 during the liver-stage of infection, with IL-13 predicted as the top upstream regulator. These data suggest the role of these interleukins warrants further investigation, particularly as there is currently little or no published evidence to support a role for these interleukins in the immune response against the liver-stage of malaria[122,432-435].

Predicted downstream effects of the differentially expressed genes at the peak blood-stage of infection (diagnosis versus baseline analysis) was most consistent

with an 'antiviral response' presumably due to the predominance of changes in gene expression relating to IFN signalling. Predicted downstream effects at the liver-stage of infection (C+6.5 versus baseline analysis) was similar to that predicted for tuberculosis (*p=4.99E-17*) and bacterial infection (*p=1.98E-13*). Similarity to the predicted gene changes consistent with 'parasitaemia' and 'malaria' were nil at the peak of blood-stage infection and extremely low for the liver-stage (*p=1.02E-03* and *p=5.87E-03* respectively*).* The downstream effects analysis in Ingenuity® is based on prior knowledge of expected causal effects between genes and biological functions stored in the 'Ingenuity® Knowledge Base,' "*a repository of curated biological and functional annotations created from millions of individually modeled relationships between proteins, genes, complexes, cells, tissues, drugs, and diseases*"[457]. On the basis of these data, this repository requires updating in regard to malaria infection.

A difference in gene expression was seen between subjects with MinExp and DefExp over the time course, predominantly driven by differences at C+11. These differences related to IFN signalling and antigen presentation and the upstream regulators IFN-γ, IFNL1, IL1RN and TNF, which were all relatively down-regulated in subjects with DefExp. Whilst acknowledging that this analysis was performed with an FDR of 25%, these data suggests prior exposure to malaria is associated with a relative reduction in pro-inflammatory response, at least in early blood-stage infection, as has been reported previously in a cohort of Malian children[96].

Given that only volunteer 110 demonstrated the ability to control parasite growth *in vivo,* the analyses were unable to identify patterns of gene expression

associated with NAI. No genes were shown to correlate with PMR and comparison of volunteer 110's gene expression profile over the time course with the cohort did not reveal differential expression of any biologically plausible genes. Comparison of gene expression over the time course between volunteers demonstrating a DTTD and those not, showed differential expression of IL-17A and IL-17F pathways between the groups. However, given this analysis was performed with an FDR of 25% and DTTD is a soft definition of NAI, these findings are of questionable significance.

Despite the high quality of RNA and large number of samples retained for analysis post-microarray, there were a number of limitations to the analysis. The use of PAXgene tube samples meant the extracted RNA came from whole blood and was dominated by neutrophils. Whilst providing insight into the transcriptional changes seen in this compartment, the samples do not provide information on changes in gene expression in other important organs such as the spleen, liver and bone marrow[132]. The analyses also do not correct for the effect of differences in cell type proportions on gene expression both between individuals and across time points. The data were grouped and analysed according to the set time points when sampling occurred (for example C+7, C+9). Whilst this approach was a practical necessity, differences in times of administration of PfSPZ Challenge between volunteers and variations in parasite concentrations between volunteers at each time point meant there was heterogeneity between the subjects that was difficult to control.

Whilst acknowledging these limitations, these results still provide important lessons for future transcriptomic studies in CHMI trials, particularly in Africa.

Comparison of RNA quality and quantity from PAXgene and PBMC samples showed that whilst PBMCs provided higher concentrations of RNA, the quality of RNA extracted from PBMCs reduced as the length of storage increased. Given the practical advantages of collection and storage of PAXgene tube samples, the high quality of RNA seen with the KCS PAXgene dataset and the known changes in gene expression associated with cryopreservation of PBMC samples[458], RNA stabilised whole-blood samples rather than PBMC samples are preferable for use in African CHMI studies.

Following detailed analysis over the time course of early malaria infection, key time points for changes in gene expression coincided with biologically important stages; i.e. C+6.5 (liver-stage) and diagnosis (peak blood-stage infection). Future CHMI studies where control of parasite growth is not anticipated could therefore restrict RNA sampling to these two time-points. However, given future CHMI studies in Africa are likely to include hyperimmune subjects where the dynamics of parasite growth and NAI are not known, it would be prudent to continue sampling at regular, multiple time-points. In such studies, a challenge of analyses will be distinguishing gene expression profiles of NAI from those simply related to parasite density i.e. distinguishing cause from effect.

Whilst it would be preferable to confirm the findings of these analyses using the KCS PBMC samples such work does not warrants the considerable financial cost this would entail. Instead, future work should prioritise microarray analysis of PAXgene samples collected in VAC049 to see they provide validation for findings identified in KCS. Work should also analyse stored microRNA (miRNA) from KCS

PAXgene samples given the increasingly important reported role of miRNA in the pathogenesis of infection and immunity to malaria[459].

CHAPTER 9: CONCLUDING REMARKS AND FUTURE DIRECTIONS

9.1 Summary

CHMI studies have become a vital tool to accelerate vaccine and drug development against *P. falciparum*[287,289,460], but to date, have had extremely limited use in the study of NAI, primarily due to the lack of access to appropriate facilities to perform mosquito-bite CHMI trials in malaria endemic countries[222]. This thesis describes the first assessment of PfSPZ Challenge administered IM in humans, and the first attempt to use the modern CHMI model to explore the dynamics and mechanisms of NAI. Using samples collected post-CHMI from both UK malaria-naïve volunteers and Kenyan subjects with varying prior exposure to *P. falciparum,* this work reports and compares the findings of key *in vitro* assays including GIA and ADRB activity in order to understand the effect of prior exposure to malaria on these measures. The necessary small sample sizes of these pilot studies limit the interpretation of these findings. Nonetheless, this work should greatly inform future CHMI studies in malaria endemic regions and encourage further use of the CHMI model, with appropriately powered samples sizes to increase understanding of NAI.

9.2 Conclusions and future work

9.2.1 PfSPZ Challenge

This work adds to the increasing evidence of the safety and infectivity (given appropriate dosing and administration) of PfSPZ Challenge, particularly in malaria-exposed individuals. As well as informing dosing choices for future

CHMI studies using PfSPZ Challenge, the infectivity data also have important implications for dosing of whole sporozoite vaccines currently in development[461]. PfSPZ Challenge was simple to prepare and administer, needing only a BSC and readily available laboratory equipment. These practical advantages and the ability to reduce variation in infectious dose in CHMI trials across multiple sites mean PfSPZ Challenge is an important development that could realistically accelerate the conduct of CHMI studies in malaria-endemic regions in the short term. One important limitation of the PfSPZ Challenge CHMI model is that, dependent on route of administration, the resulting *P. falciparum* infection either undergoes a modified skin-stage or circumvents the skin completely (as in the case of DVI). Given the importance of the skin-stage for priming of subsequent immunological responses[56], this limitation has important implications for groups using PfSPZ Challenge to assess vaccine or naturally-induced immunity to malaria.

9.2.2 Antimalarial use in CHMI studies

The finding of quinine in the serum of a volunteer participating in VAC049 was an unexpected and important result. Given that candidate malaria vaccines and drugs are selected for further development on the basis of CHMI trials involving necessarily small numbers of subjects, it is important that investigators are confident of the integrity of efficacy data. Screening for the presence of anti-malarials in selected samples is relatively cheap and simple to perform and should be undertaken routinely for individuals demonstrating efficacy in CHMI trials.

The effect of residual Atovaquone/Proguanil on C+35 GIA results is another important finding. Previously presumed to be removed during IgG purification, the persistence of this anti-malarial in serum post-processing has important implications not only for interpretation of results from GIA and other assays involving live parasites, but also for investigators planning sampling post-CHMI who may wrongly presume anti-malarials have been eliminated by C+35.

9.2.3 CHMI trials as a method of studying NAI

KCS was the first modern study to use the CHMI model to attempt to understand the dynamics and mechanisms of NAI. Given there was no precedent for the trial, many aspects of the study design, for example dose and route of PfSPZ Challenge and classification of individuals' prior exposure to malaria had to be based on best available evidence at the time. Whilst the results were disappointing in that only one individual demonstrated the ability to control parasite growth *in vivo*, the work has shown for the first time, proof of principal that NAI can impact on PMR post-CHMI in a detectable fashion. These findings suggest that the CHMI sporozoite model could allow identification of a heterogeneous group of participants with varying degrees of NAI, for whom the relationship between PMR and candidate assays of blood-stage immunity can be examined to try to gain greater understanding of the mechanisms of NAI to *P. falciparum* malaria.

However, a number of considerations limit the ability of the CHMI model to evaluate NAI reliably: As individuals are treated when blood-film positive, the model only allows assessment of the liver and early blood-stages of infection, and not the later stages of infection typically seen in the field when parasitaemia may be higher (including impact on gametocytaemia and transmission). In

addition, in contrast to the field-setting where it is likely individuals are exposed to low dose parasites of numerous, different strains over an extended period, CHMI studies involve exposing individuals to a single, strain of parasite, administered at a high dose, as a single bolus. Both of these factors could affect the nature or magnitude of the immune response generated, and mean that NAI that is strain or antigen-load dependent fails to be detected.

Given KCS was the first CHMI study to be undertaken in Kenya, participant safety was rightly the primary objective of the study. As a result, subjects were managed as in-patients in a large city, and treated at C+21, as is the case for CHMI trials in northern centres. However, 'northern' CHMI studies are typically designed with the objective of assessing vaccine efficacy against sporozoite, liver or early blood-stage infection. CHMI studies seeking to study NAI require a different approach that prioritises enrolment of hyperimmune individuals and extends follow-up prior to treatment - to allow detailed assessment of the interactions between the immune response and parasitaemia overtime. Given the first CHMI study in Kenya has now been performed safely, it is hoped that future studies can be performed in malaria endemic regions, without the need for in-patient monitoring or early treatment in the absence of symptoms.

9.2.4 Using in vitro assays to predict NAI

A key question of this work was whether an *in vitro* assay could be identified that could predict an individual's ability to control parasitaemia (a measure of NAI) *in vivo.* Although only one individual in KCS was qPCR positive but undiagnosed by C+21 (volunteer 110), analysis of PMRs showed a spread of values, allowing analysis of correlation of PMR with a number of *in vitro* assays.

Neither of the two candidate functional assays assessed (GIA and ADRB activity assay) were shown to correlate with PMR. However, volunteer 110 did have the highest ADRB activity at baseline, suggesting this assay warrants further investigation with regard to NAI. Baseline antibody responses to AMA1 and schizont extract were the only measures to correlate with PMR, however it is difficult to distinguish whether these measures simply reflect exposure rather than NAI. No gene expression profile was shown to correlate with PMR, and it likely that larger sample sizes with a greater proportion of individuals capable of controlling parasite growth *in vivo* will be necessary to identify associations between gene expression or other candidate measures and NAI. Indeed, as NAI is likely to be strain-specific and multi-mechanistic it is unlikely that a single assay will reliably predict NAI in all circumstances.

9.2.5 Effect of prior exposure to IFN-γ response post-CHMI

In agreement with published evidence, this work supports the finding that the magnitude of serum IFN-γ responses to malaria infection is determined by the degree of prior exposure: In Chapter 6, a greater increase in parasites per log-fold increase in IFN-γ was seen for malaria-naïve volunteers (VAC049) than African (KCS) subjects when concentrations of IFN-γ were directly measured. Indeed, DefExp volunteers in KCS had lower IFN-γ concentrations at diagnosis than UK subjects in VAC049. In addition, in Chapter 7, analysis of changes in gene expression profiles predicted that upstream regulators including IFN-γ were down regulated in DefExp compared to MinExp subjects. This paradoxical effect could be a protective mechanism to minimise the pathological effects of the pro-inflammatory immune response to malaria or a manipulation by the parasite to

evade the immune response. Until this response is fully understood, caution should be exercised with vaccines or treatments that aim to manipulate the pro-inflammatory immune response.

9.2.6 Gene expression studies

Studies of changes in gene expression in clinical malaria have been limited to date, despite the considerable potential application of such work. This work presents the first analysis of time course changes in gene expression of post-CHMI with *P. falciparum* and the first analysis of expression changes in African individuals post-CHMI. Whilst the findings confirmed the known central role for IFN signalling, the results included a number of novel findings, including the un-described role of Type III IFNs in blood-stage malaria and IL-17 in the immune response to the liver-stage of infection. Future work should seek to examine whether these novel findings are supported by directing measuring IL-17 and IFN-λ1 concentrations over the time course in KCS, and assessing whether similar results are seen in another population, for example by analysing PAXgene samples from VAC049. Mouse models could also be explored to address these hypotheses.

9.2.7 Limitations of Small Sample Sizes in CHMI Studies

In the great majority of CHMI studies performed to date, group sizes have been in single figures[287]. This reflects practical and ethical limitations on the numbers of individuals that can be recruited and challenged safety at one time. For this reason, CHMI studies, including those in this work, are usually designed to assess proof of concept rather than look for statistically significant associations. For the

purposes of this thesis, statistical analyses have been applied to aid interpretation of the data. However, the small sample sizes mean the conclusions reached must be considered tentative, and require replication in future, appropriately powered studies.

9.3 Final remarks

This work has provided proof of principal, both that sporozoite CHMI studies can be undertaken safely in malaria-endemic populations and that the model can be used to study NAI. There is now a need to build on this preliminary work by performing a CHMI study including a greater proportion of individuals capable of controlling parasite growth *in vivo*, so that any analyses are powered to more accurately determine the relationship between candidate assays of NAI and PMR *in vivo*.

APPENDICES

Appendix 2-1: Schedule of clinical reviews and blood sampling for VAC049

Timeline (days in relation to challenge)	Screening	C-1	C	C1	C6.5	C7	C7.5-C14	C15-C23	C35	C90
Attendance Number	1	2	3	4	5	6	7-20	21-29	30	31
Window (days)	N/A	-1	0	0	0	0	0	0	±5	±14
Medical History	X	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)
Clinical Assessment	X	(x)	(x)	(x)	X	(x)	(x)	(x)	(x)	(x)
Urinalysis	X									
Urinary β H-CG[£]	X	X			(x)	(x)	(x)	(x)		
Physical Observations	X	X	X	X	X	X	X	X	X	X
Informed Consent Quiz	X									
Medic Alert Card & Thermometer given to Volunteers			X							
Local & systemic events				X	X	X	X	X	X	X
Treatment for Malaria					(x)	(x)	(x)	X		
Thick Smear / PCR for malaria		3			3	3	3 x 14	3 x 9		
HBV, HCV, HIV (mL)	5									
Haematology (mL)	2	2					2^		2	2
Biochemistry (mL)*	3	3					3^		3	3
Anti- *P. Falciparum* serology (mL)[$]	2									
Exploratory Immunology	0	70		3		70**	90**	70**	70	70
Blood volume per visit (mL)	12	81	0	3	3	73	137	97	75	75
Cumulative blood volume (mL)	12	93	93	96	99	172	309	406	481	556

Table 5: *Schedule of clinical reviews*

C-1= Day Before Challenge, C6.5=6.5 days after challenge. (x)=If necessary. (Windows refer to time since last visit)

** Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, Liver Function Tests and Cholesterol. Cholesterol will only be tested at screening*

***70mls blood will be drawn for explorative immunology on C7, C11 and C21 if persistently undiagnosed with malaria. 10mls of blood will be drawn for cytokine analysis on C9 and C14 if persistently undiagnosed with malaria. If diagnosed with malaria, 70mls of blood will be drawn within 24 hours of diagnosis (but not if diagnosed on C7, C7.5, C11 or C11.5) and then no further blood drawn for explorative immunology until C35.*

^Blood will be drawn for haematology & biochemistry on C9 and within 24 hours of diagnosis. If a volunteer is slide positive before C9 then blood will be drawn for biochemistry and haematology within 24 hours of diagnosis and then not again until C35.

[£]Urinary β HCG will be performed in female volunteers prior to commencing anti-malaria treatment

[$]Anti-P.Falciparum serology may be tested at screening in certain individuals to help determine prior malaria exposure

Adapted from VAC049 protocol.

Appendix 2-2: Schedule of clinical reviews for KCS

Timeline (days in relation to challenge)	Screening	C-1	C	C+1 - C+23	C+35	C+90
Attendance Number	1	2	3	-	4	5
Window (days)	N/A	0	0	0	±5	±14
Medical History	X	(x)	(x)	(x)	(x)	(x)
Clinical Assessment	X	(x)	(x)	(x)	(x)	(x)
Urinalysis	X					
Urinary B-HCG	X	X		X		
Physical Observations	X	X	X	X	X	X
ECG	X					
Measurement of Height & Weight		X				
Informed Consent Quiz	X					
Administration of PfSPZ Challenge			X			
Medic Alert Card given to Volunteers			X			
Local & systemic events reviewed		X	X	X	X	X
Anti-Malarials Directly Observed				X		

Table 8: *Schedule of clinical procedures (excluding venepuncture)*

C-23= Challenge minus 23 days, C+6=6 days after challenge.
(x)=If necessary. (Windows refer to time since last visit). Shaded columns = time as in-patients

Adapted from KCS protocol.

Appendix 2-3: Schedule of venepuncture from screening until day of CHMI for KCS

	Screening	C-1	CHMI
NAT: WF, CF11 filter, and qRT-PCR (EDTA tube)	6	6	
FBC (EDTA tube)	2	2	
Clinical Biochemistry* (Plain tube)	3	3	
Serology: HIV, Hepatitis B & C (Plain tube)	5		
Cellular Studies (LiHep tube)		30	
Exploratory Serum (Plain tube)^	2	20	
Haemoglobinopathy screen (EDTA tube)		4	
PAXGene tube		3	
Vol (mls)	18	68	0
Cumulative Total (mls)	18	86	86

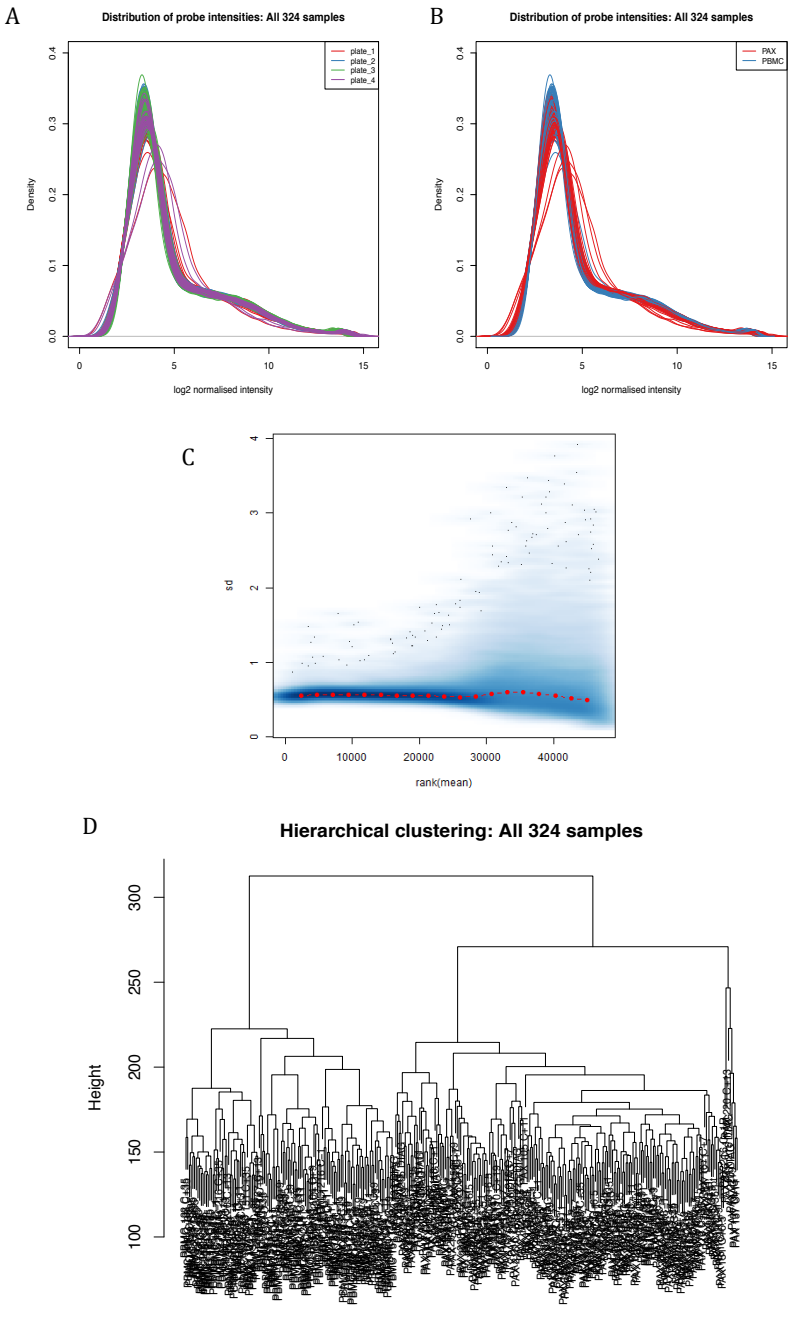
*C-1= day before administration of PfSPZ Challenge. FBC = full blood count. *Clinical biochemistry included Sodium, Potassium, Urea, Creatinine, Albumin, ALT and Bilirubin. ^Serum for anti-Pf schizont and anti PfMSP2 antibodies.*

Appendix 2-4: Schedule of venepuncture for KCS from C+6.5 until C+90

Days post CHMI	6	7	7.5	8	8.5	9	9.5	10	10.5	11	11.5	12	12.5	13	13.5	14	14.5	15	16	17	18	19	
NAT[$]	6	6	6	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Smear		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
FBC		2				2				2				2				2		2		2	
iochem*^						3																	
ellular tudies[%]						10				10				10				10		10		10	
erum[%]						10				10				10				10		10		10	
RNA analysis	3	3				3				3				3				3		3		3	
Parasite Typing																							
Vol (mls)	9	12	7	3	3	31	3	3	3	28	3	3	3	28	3	3	3	28	3	28	3	28	
um. Total (mls)	95	107	114	117	120	151	154	157	160	188	191	194	197	225	228	231	234	262	265	293	296	324	

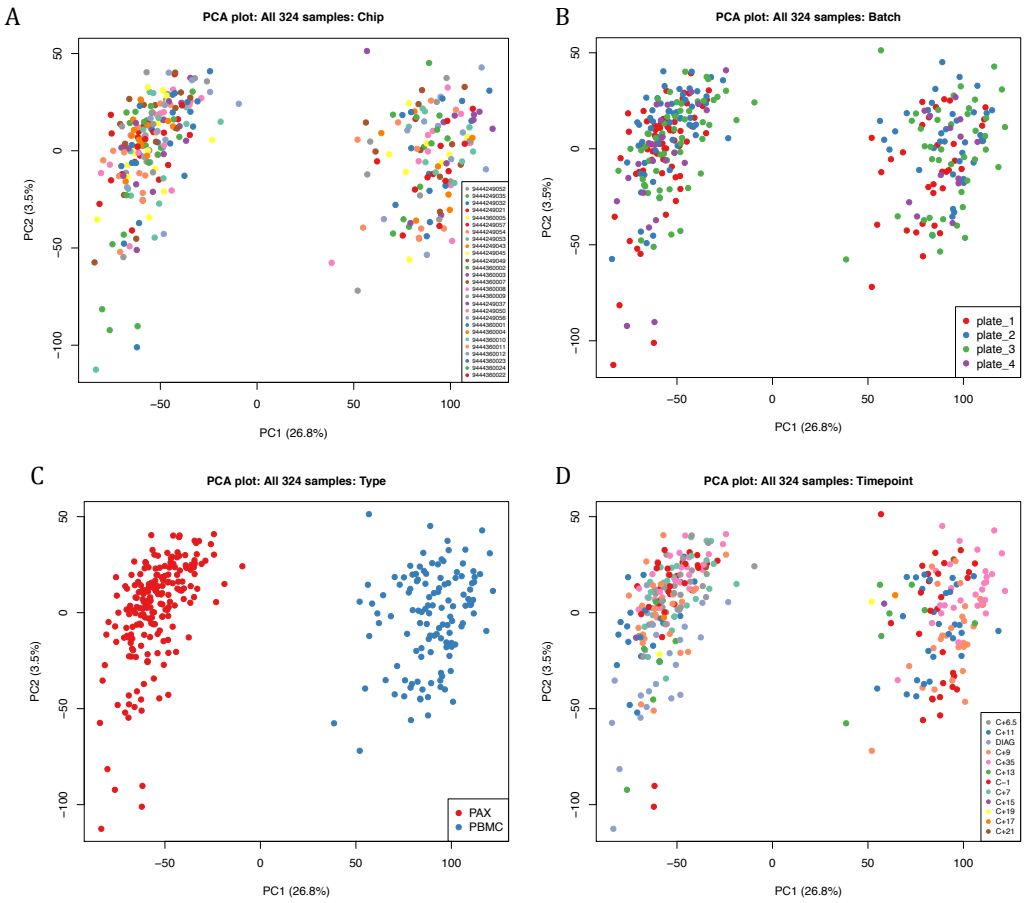
*No blood was drawn between day of Challenge and C+5. Samples for NAT, FBC and blood-smear were collected in EDTA vacutainers (BD Bioscience). *Biochemistry was collected in plain vacutainers (BD Bioscience) and tested for sodium, potassium, urea, creatinine, albumin, alanine transaminase (ALT) and bilirubin. Blood drawn for cellular studies was collected in lithium heparin vacutainers. Blood for parasite typing at diagnosis was collected in sodium heparin tubes (BD Bioscience). Blood for RNA analysis was collected in PAXgene tubes (Quiagen). %Serum drawn for exploratory immunology was collected in plain tubes. ^Blood was drawn for FBC & biochemistry on C+9 and within 24 hours of diagnosis. If a volunteer was slide positive before C+9 then blood was drawn for biochemistry and FBC within 24 hours of diagnosis and then not again until C+35. %Blood for exploratory immunology (serum and cellular studies) was drawn at C+ 9, every other day until diagnosis and then not again until C+35. &Blood for RNA analysis will not be taken at diagnosis if a sample has already been taken on the same day.*

Appendix 8-1: Microarray quality control data for KCS PAXgene and PBMC samples



n=324. (A) Distribution of probe intensities according to plate (batch) number. (B) Distribution of probe intensities according to sample type (PAXgene versus PBMC). (C) Mean variance plot. sd = standard deviation. (D) Hierarchical clustering.

Appendix 8-2: Principal component analysis of KCS PAXgene and PBMC samples

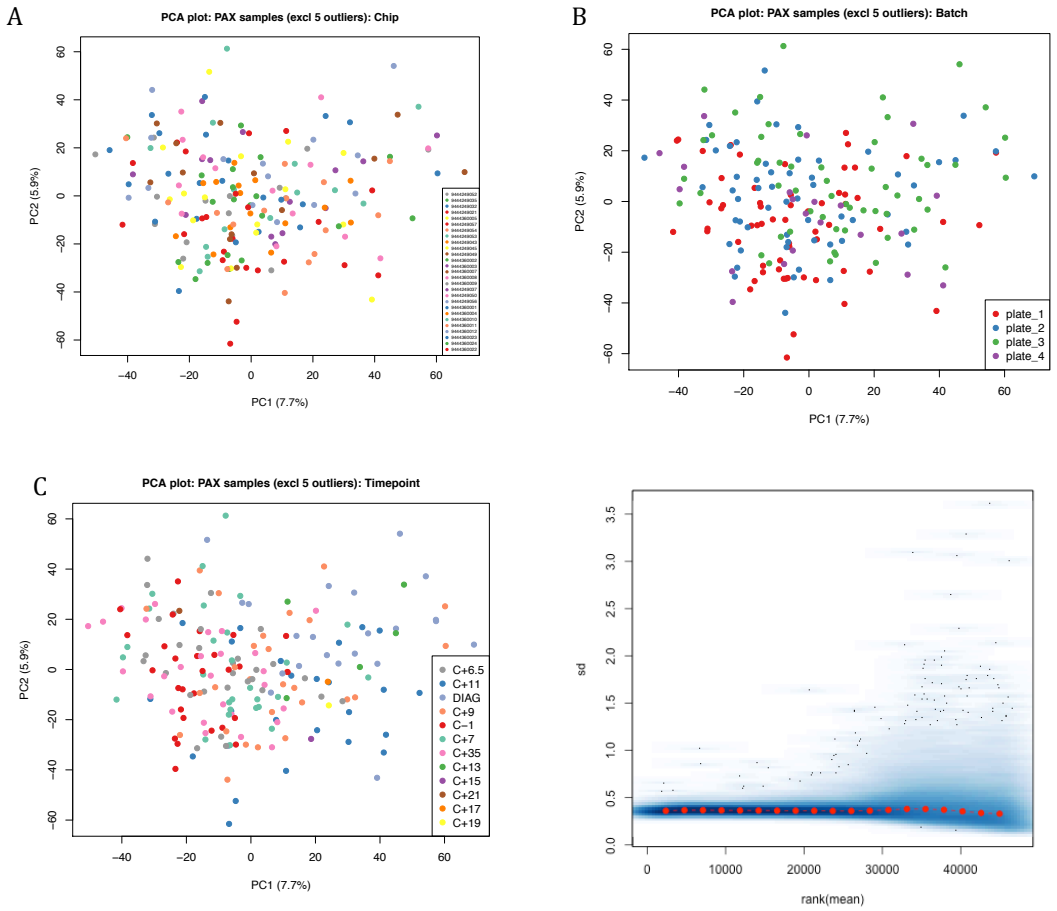


n=324. Individual points coloured according (A) Chip (B) Batch (C) Sample type (PAXgene versus PBMC) or (D) Time-point.

Appendix 8-3: Criteria for removal of five outlier samples from KCS PAXgene dataset.

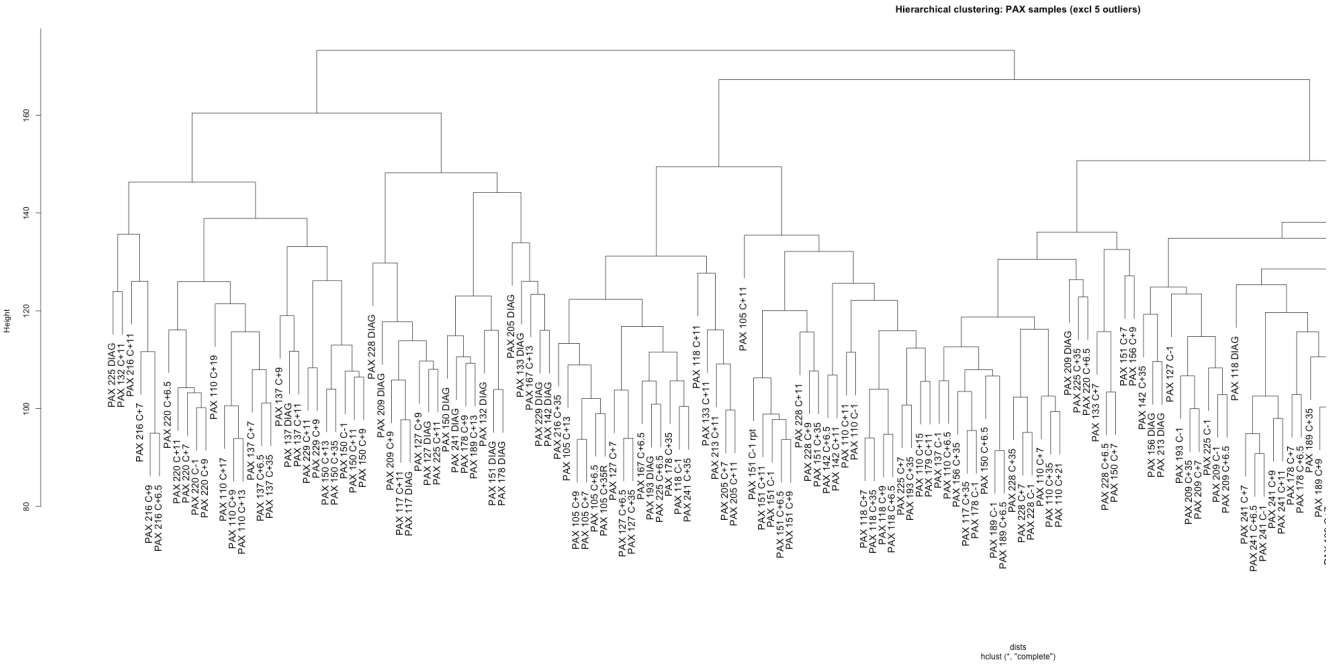
Sample	QC Metrics	PCA Plots	Probe Signal Intensity	Identified by Multiple Operators
PAX 220 C+13	✓	✓	✓	✓
PAX 216 C-1R rpt		✓	✓	✓
PAX 216 C-1 R		✓	✓	✓
PAX 216 DIAG		✓	✓	✓
PAX 220 DIAG		✓	✓	✓

Appendix 8-4: Quality control data from final KCS PAXgene dataset



n=196, excluding 5 outlier samples. Principal component analysis with points coloured according to (A) Chip (B) Batch or (C) Time-point. (D) Mean variance plot. sd = standard deviation.

Appendix 8-5: Hierarchical clustering of final KCS PAXgene dataset



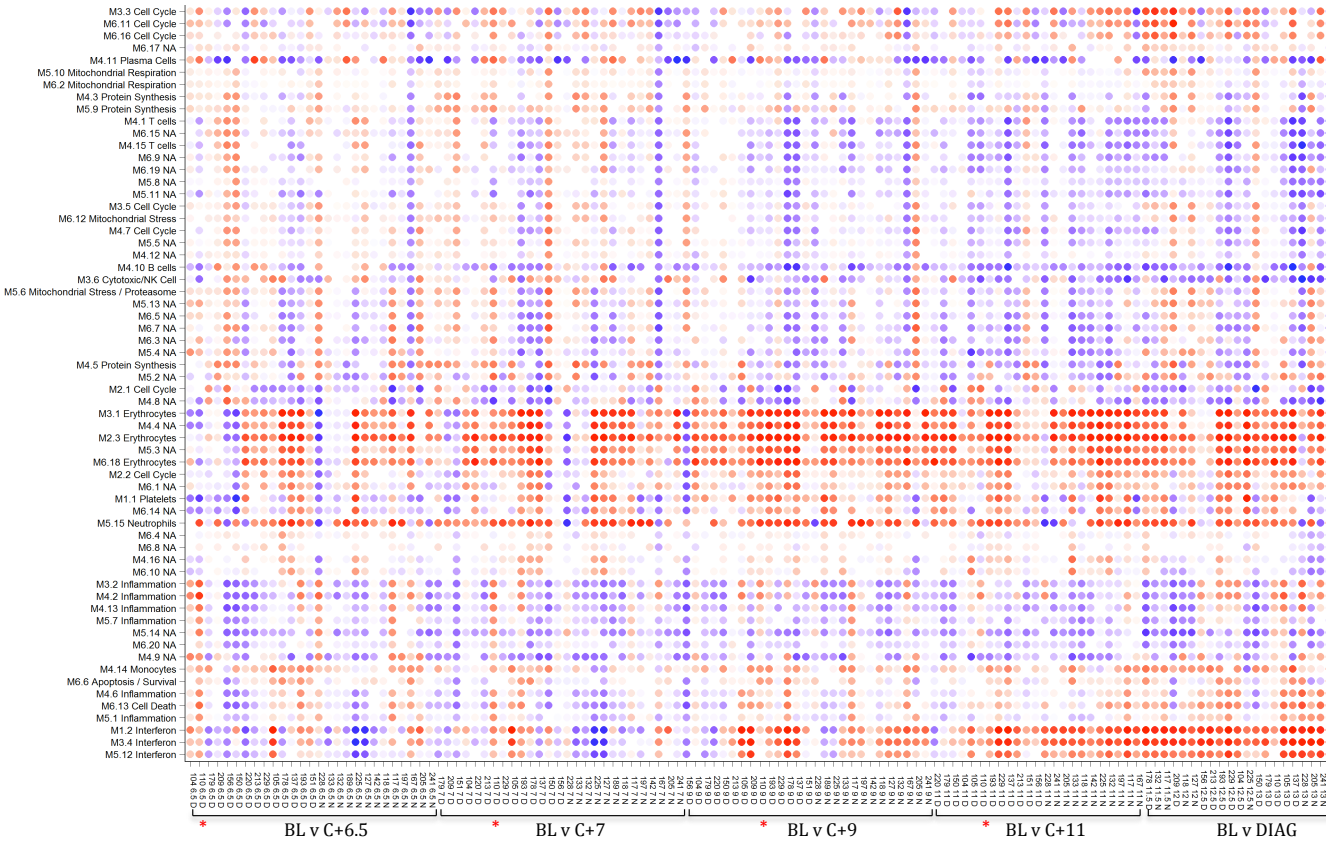
n=196 excluding 5 outlier samples.

Appendix 8-6: Matrix of comparisons used for time course analysis of KCS PAXgene dataset

Time points	C+6.5	C+7	C+9	C+11	DIAG	C+35
Baseline	BL	BL	BL	BL	BL	BL
C+6.5		CON				
C+7			CON			
C+9				CON		
C+11					CON	
DIAG						CON
C+35						

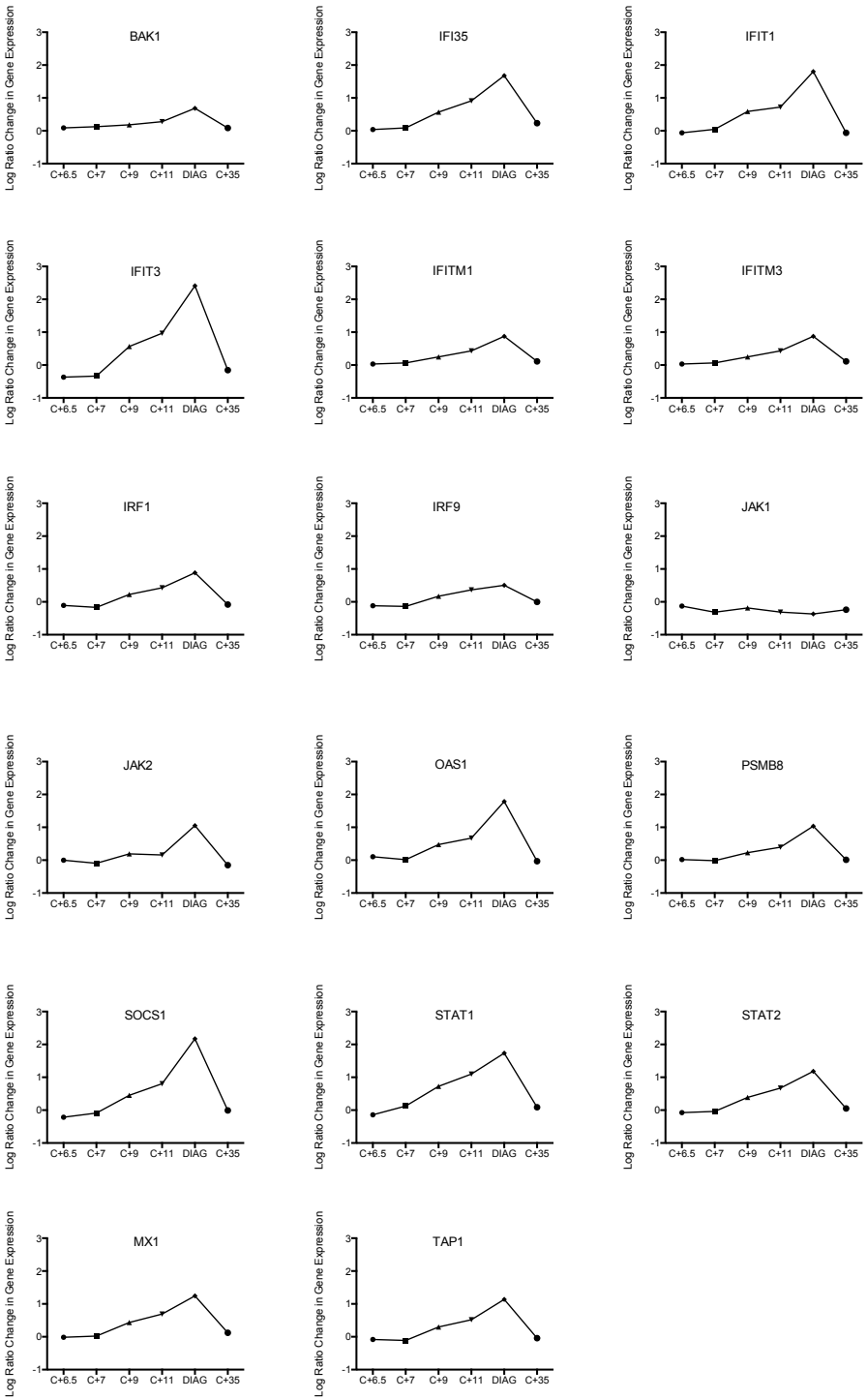
BL = comparisons for baseline analyses (C-1). CON = comparisons for consecutive analyses.

Appendix 8-7: Modular analysis of baseline comparison from KCS PAXgene dataset



BL = baseline. DIAG = diagnosis. D = DefExp. N = MinExp. FC>1.5. (No FDR cut off, as replicates for each subject at each time point were not available).=data from subject 110. Y axis = previously defined modules of pre-determined co-dependant genes[362].*

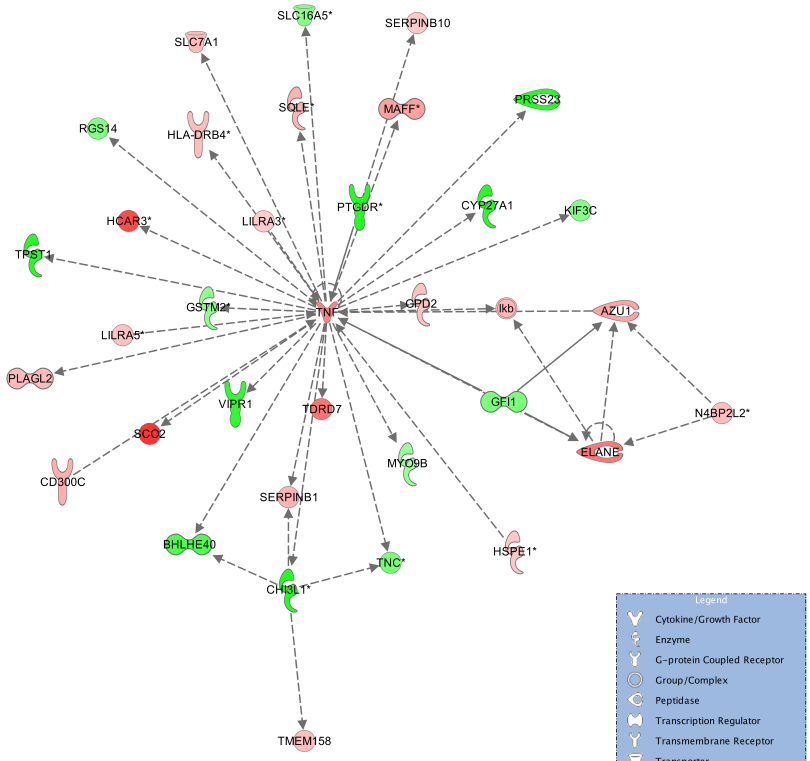
Appendix 8-8: Changes in expression for differentially expressed genes in the interferon-signaling pathway for diagnosis versus baseline analysis of KCS PAXgene dataset



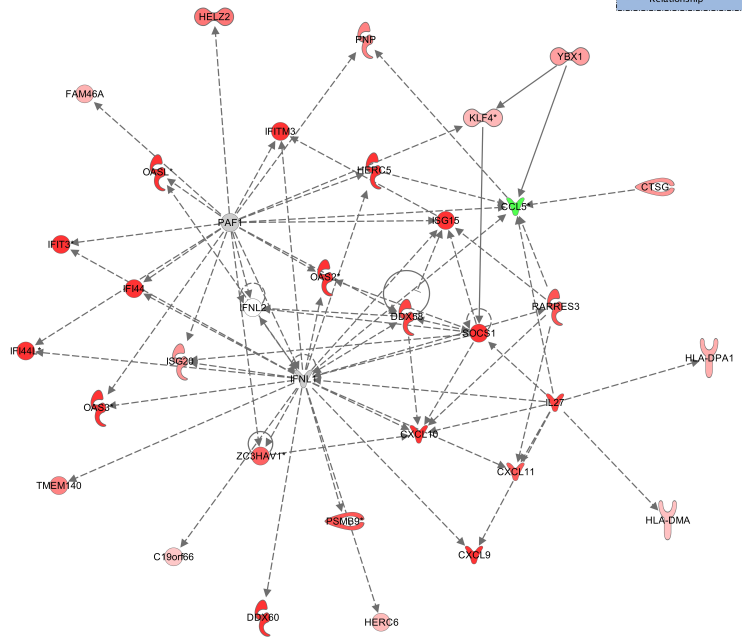
Log Ratio Changes. 17 out of a possible 34 genes. FDR < 5%, FC > 1.25.

Appendix 8-9: Selected predicted networks from KCS PAXgene dataset: diagnosis v baseline analysis (i)

A

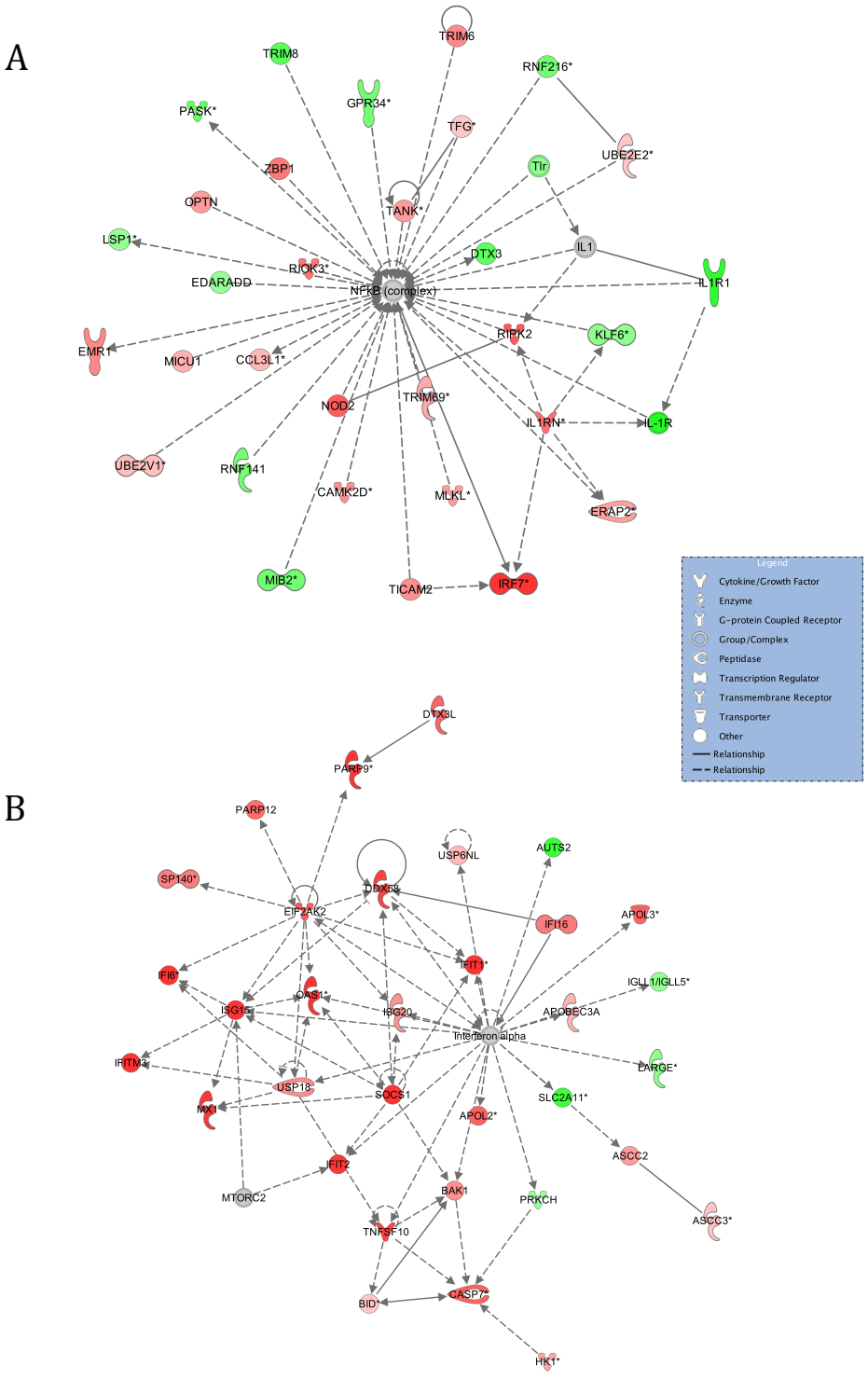


B



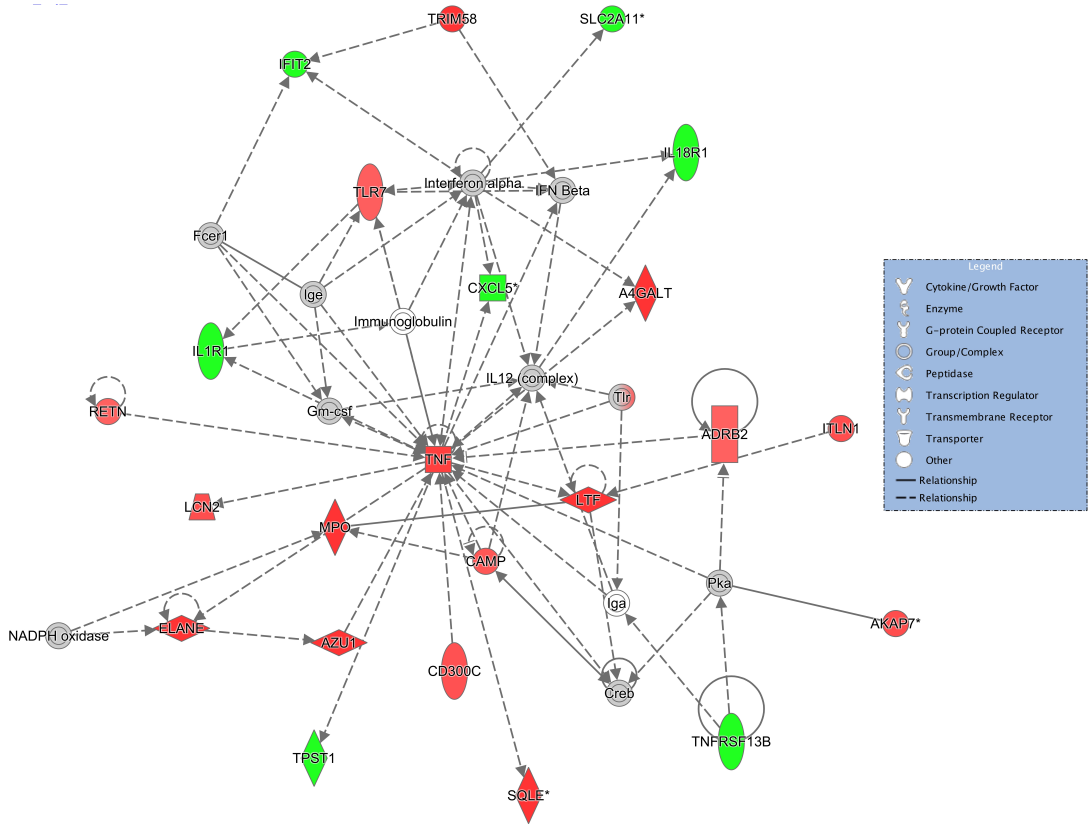
(A) TNF central: 34 molecules, p = 1x10E-26 (B) IFNL1 central: 32 molecules, p=1x10E-23 Red = up regulation, Green = down regulation. FDR < 5%, FC > 1.25.

Appendix 8-10: Selected predicted networks from KCS PAXgene dataset: diagnosis v baseline analysis (ii)



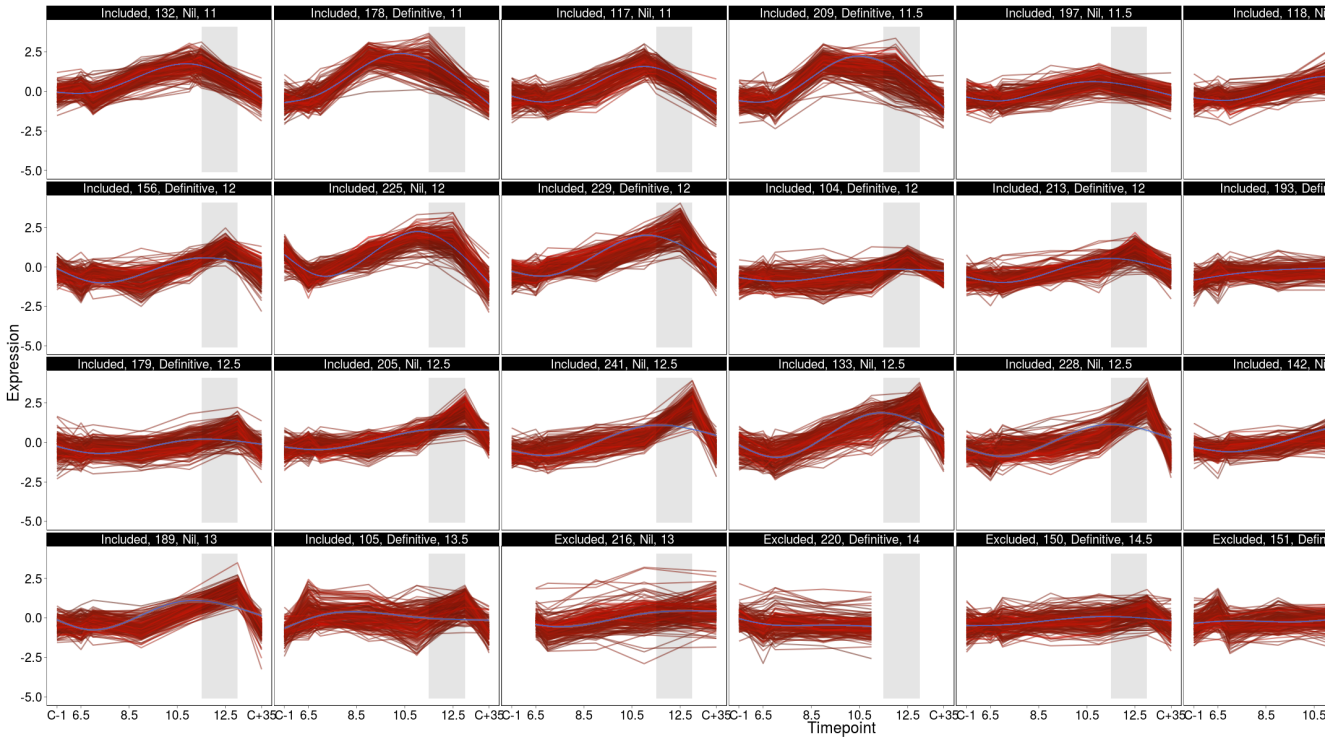
(A) NF-κB central: 31 molecules, p=1x10E-21. (B) IFNα central: 33 molecules, p=1x10E-24. Red = up regulation, Green = down regulation. FDR < 5%, FC > 1.25.

Appendix 8-11: Selected predicted network from KCS PAXgene dataset: C+6.5 v baseline analysis



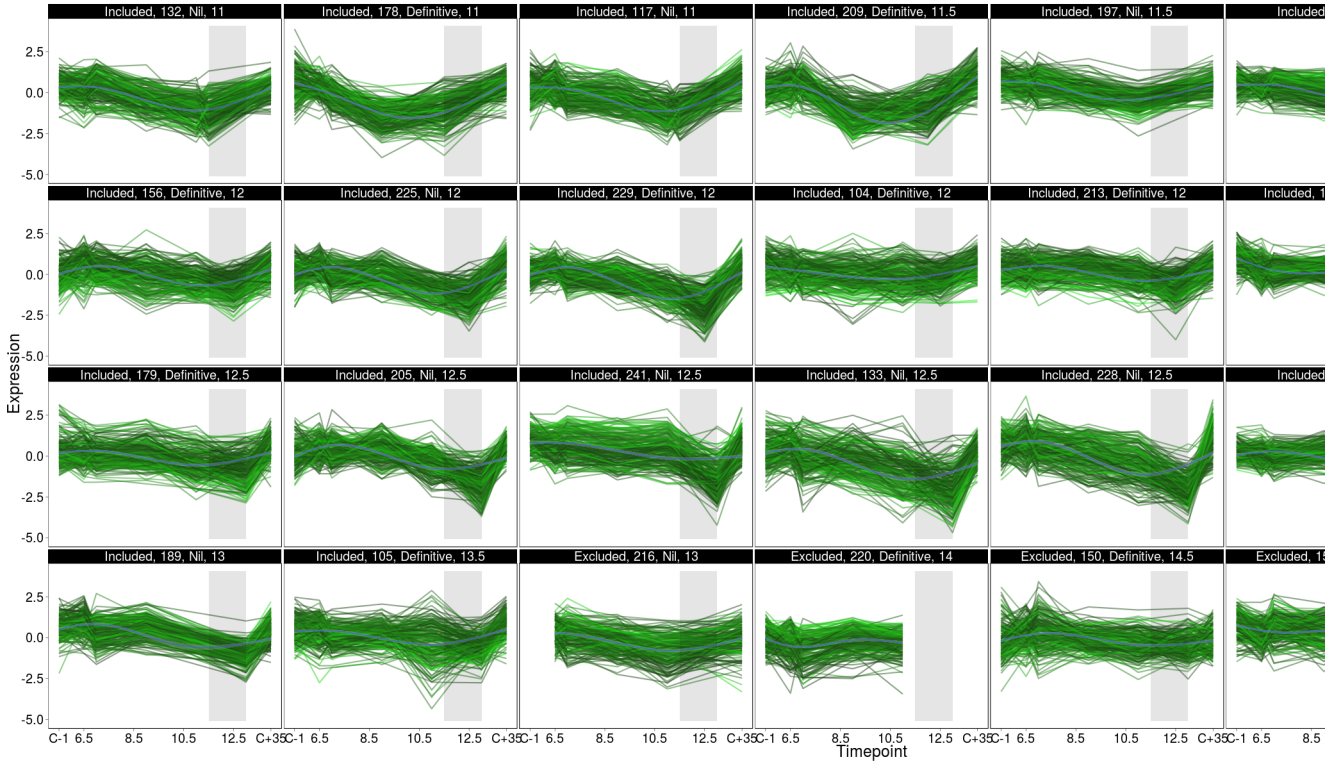
27 molecules, p=1x10E-40 FDR < 5%, FC > 1.25.

Appendix 8-12: Expression trajectory for probes with the largest positive eigenscore for eigenfunction 1 per subject for KCS PAXgene dataset.



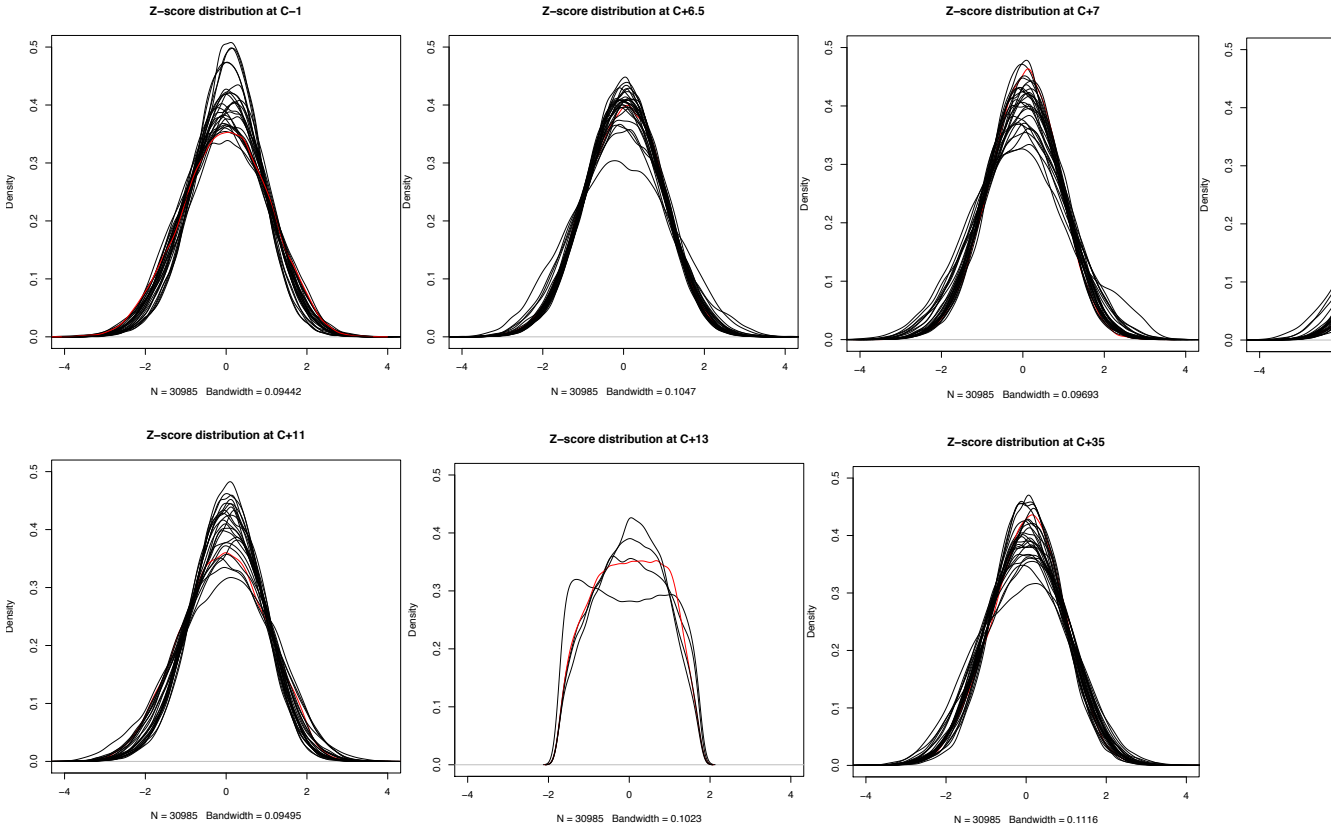
Expression trajectory (scaled) over time of top 100 probes Subjects ordered according to time to diagnosis. Included (n=23) = subjects included in fPCA. Excluded (n=5) = 4 KCS volunteers with DTD and volunteer 110. FDR < 5%, FC > 1.25.

Appendix 8-13: Expression trajectory for probes with the largest negative eigenscore for eigenfunction 1 per subject for KCS PAXgene dataset.



Expression trajectory (scaled) over time of top 100 probes Subjects ordered according to time to diagnosis. Included (n=23) = subjects included in fPCA. Excluded (n=5) = 4 KCS volunteers with DTD and volunteer 110. FDR < 5%, FC > 1.25.

Appendix 8-14: Histogram of z scores for all probes in KCS PAXgene dataset at various time points



Volunteer 110 in red.

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