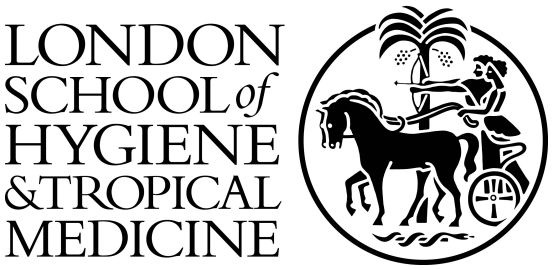

Development and validation of serological assays to evaluate malaria transmission

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Thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy

University of London

March 2023

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No funding received

Declaration

I, Tate Ewart Oulton, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

In 2007, the aim to eradicate malaria was revived and reinvigorated, taking into account the experiences and lessons of the earlier efforts initiated in the mid 1950's. In particular, it was recognised that improvements to diagnosis and surveillance would be essential for directing malaria control and elimination strategies across a highly heterogeneous disease landscape. In response, novel approaches to detecting, measuring and modelling malaria have been developed and implemented leading to elimination successes and significant reductions in disease burden. However, it remains clear that further improvements in this area are needed to tackle malaria where existing efforts have so far failed to break transmission. The work presented in this thesis demonstrates a 'pipeline'of technical and methodological development, aiming to identify, characterise and apply the use of serological biomarkers that may improve our understanding of malaria transmission within endemic settings. The specific studies and work included here describe a linear narrative, from conception to the implementation of serological biomarker approaches, through: 1) the development and optimisation of protein microarray technology to enhance the investigation of malaria parasite antigens as potential serological biomarkers of interest; 2) the evaluation and comparison of two parasite protein production methods in the context of a protein antigen microarray; 3) the utilisation of recently developed assay methods and analysis to investigate sexual stage specific humoral responses as potentially informative biomarkers in the context of malaria transmission; and 4) the application of established methods and known biomarkers to answer specific questions relating to the application of malaria diagnostics in field settings. Key findings include: 1) that the capture of protein antigen specific antibodies using microarray technology can be highly variable depending on specific assay materials and approaches utilised; 2) different protein antigen production methods can impact the capture of specific antibody, even between matched epitopes; 3) antibody responses to known sexual stage parasite antigens were induced in previously malaria naive individuals, developing later than anti-asexual responses; and 4) residual gametocytes after standard artemisinin-based combination therapy do not contribute to circulating levels of the parasite antigen HRP-2, and thus do not impact time to rapid diagnostic test negativity. Together these results demonstrate the complexity of establishing serological assay approaches for use in malaria research and surveillance, but also the potential for the further development and application of such methods to address important knowledge gaps.

Acknowledgements

First and foremost:

To *Chris Drakeley* — for taking me on as a fresh faced MSc student, sticking with me for so many years and for providing me with the opportunity and a huge amount of support to get this done. It is extremely appreciated.

To *Will Stone* — for the guidance and encouragement, even in the face of a number of meltdowns. But for your time, effort and friendship, this thesis would not have been finished! I hope this first experience hasn't put you off PhD supervision for life...

To *Kevin Tetteh* — for your mentorship and for leading me through the daunting process of becoming a research scientist. Even at the times when it seemed everything had gone wrong, you found a way for us to laugh about it.

To *Tom Hall* — for all your patience, instruction and kindness while showing me the ropes in 234, and for bringing me so warmly into the fold.

To *Jackie Cook* — for your support, advice and, crucially, perspective on all things during my time at LSHTM.

To *all the 'Drakeleys'* — for being such excellent colleagues and an all around good bunch of people. It's a pleasure to know and work with you all.

To *the study participants* included in this thesis, and all the other work I have conducted over the years — whose contribution to the advancement of public health cannot be overstated.

To family and friends:

To *my family*, Oultons and Burtons — for your continuous support and interest in my work, and for all the fun and laughs when things have been great, or things have been difficult. You have all made getting this done easier in so many ways.

To all my *friends at LSHTM* — you know who you are. Thank you for making working at the School so enjoyable, and for all the opportunities to let off steam. I owe you all a beer (or two).

Finally:

To *Alice* — for your endless patience, love, encouragement, advice and belief in me. For showing me how it's done. For putting up with me. For the kind words and the kicks up the arse, as and when they were needed. I couldn't have done this without you.

and *Flynn* — who arrived so shortly after completing my corrections. You are the perfect ending to all of this.

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Acronyms and initialisms

ACT Artemisinin-based combination therapy

AMA1 Apical Membrane Antigen 1

CSP Circumsporozoite protein

DBS Dried blood spots

DMFA Direct membrane feeding assay

EBA Erythrocyte binding antigen

ELISA Enzyme-linked immunosorbent assay

ETRAMP-5 Early Transcribed Membrane Protein 5

GLURP Glutamate rich protein

GMEP Global Malaria Eradication Programme

HRP-2 Histidine-rich protein 2

IFAT Immunofluorescence antibody test

iRBC Infected red blood cell

ITN Insecticide treated net

IVTT *In vitro* transcription/translation

malERA Malaria Eradication Research Agenda

MFI Median fluorescence intensity

MSP1 Merozoite Surface Protein 1

PCR Polymerase chain reaction

PfEMP1 *Plasmodium falciparum* erythrocyte membrane protein 1

pLDH *Plasmodium* lactate dehydrogenase

RBC Red blood cell

RDT Rapid diagnostic test

Rh Reticulocyte binding protein-like homolog

RIFIN Repetitive interspersed families of polypeptides

RPPA Reverse phase protein microarray

SBA Suspension bead array

SMFA Standard membrane feeding assay

STEVOR Subtelomeric variable open reading frame

TRI Transmission reducing immunity

uRDT Ultra-sensitive rapid diagnostic test

VSA Variant surface antigen

WHO World Health Organisation

Chapter 1

Introduction

The *Plasmodium* genus may be traced back at least 15-20 million years [1], with the earliest estimated hominin infections — transmitted from apes and gorillas — likely occurring within the last 400,000 years [2–4]. Through the course of this continuing co-evolutionary period, *Plasmodium* species have become highly efficient human parasites. Accordingly, malarial disease is deeply ingrained in human history, primarily as a result of its extraordinary past prevalence and mortality. Modern advances in our understanding of parasite and vector biology, treatment, epidemiology and control methods have led to drastic reductions in the prevalence and geographical range of disease, yet malaria remains one of the most serious global threats to human health [5]. Global trends in malaria incidence and mortality showed a steady decline from 2000 to 2015, this decline largely attributed to widespread distribution of mosquito nets and the increasing use of artemisinin-based combination therapy (ACT) [6]. However this downward trajectory appears to have slowed since 2015, and alarmingly appears to be reversing; the estimated number of global cases increased from 230 million in 2015 to 232 million in 2019, via an intermediate peak of 237 million cases in 2017 [7]. What's more, the impact of the recent SARS-CoV-2 pandemic appears to have had a significant impact of the incidence of disease, such that in 2021 there were an estimated 247 million cases, exceeding the case estimate for 2000 [7] However, this is likely an underestimate due to reduced testing (48.5 million fewer tests between 2019 and 2020 in the WHO Africa and South-East Asia regions) for malaria throughout 2020 and 2021 [7]. Thankfully, despite the challenges faced by healthcare systems, deaths were almost 0.3 million fewer than 20 years prior [7], though it is also probable that a proportion of malaria deaths have been mis- or unreported during the height of the global pandemic.

The outstanding threat posed by malaria is in part due to the biologically complex nature of malarial disease in humans, compounded by the often under-resourced and less developed nature of presently endemic areas, and further by recent stresses inflicted on global healthcare provision and infrastructure during the SARS-CoV-2 pandemic. Limited public health services, informal or basic housing and other socioeconomic factors all increase the risk of exposure and poor clinical outcomes [8].

In addition, considerable heterogeneity in the intensity of transmission, both within and between endemic regions, can make the implementation of intervention methods more difficult. Levels of malaria transmission naturally vary, ranging from perpetual holoendemicity, to disparate and low level patterns of infection, or a highly seasonal periodicity in relation to *e.g.* weather patterns that permit vector breeding [9]. Furthermore, there are distinct disparities in transmission between urban and rural environments, often linked to reduction of vector breeding sites as urbanicity increases [10], as well as relationships between exposure risk and occupation — particularly in the case of *P. knowlesi* [11]. Overall, widespread and substantial variation in transmission intensity, and thus levels of immunity and clinical presentation, leads to a highly diverse and nuanced picture of malarial disease at the global level.

If eradication is to be achieved, a refined and strategic approach to better targeting of treat-

ment and prevention at all levels of endemicity will be needed, bolstered by the development of new tools and methods to aid in such efforts. This work has been carried out in consideration of this need.

1.1 Human malaria parasite lifecycle and disease

1.1.1 Human *Plasmodium* lifecycle

Five species of *Plasmodium* are transmissible in humans — *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (including two subspecies: *curtisi* and *wallikeri*), and the zoonotic *P. knowlesi*. Human infection occurs during the blood feeding of female anopheline mosquitoes. While many species are implicated in malaria transmission, endemic areas are typically dominated by one or two species; *Anopheles funestus* and the highly efficient vectors of the *An. gambiae* complex drive the vast majority of malaria in sub-Saharan Africa [12]. Motile sporozoites pass from the mosquito salivary glands into the dermal tissue before migrating to the bloodstream. It is at this point that the human stage of the *Plasmodium* life cycle begins (**Figure 1.1**). In the case of *P. falciparum*, sporozoites are passively transported through the blood vessels to the liver, whereupon they initiate active invasion of hepatocytes. Once within the hepatic intracellular space, the parasites undergo schizogony, replicating into many thousands of asexual merozoites, which are released into the blood upon rupture of the host cell. Once back in circulation, merozoites rapidly bind and invade red blood cells (RBCs), whereupon further rounds of replication and subsequent release into the blood leads to the exponential increase in parasitaemia.

However, transmission of the parasite from human host to mosquitoes is dependent on sexual stage gametocytes. During the repeated rounds of blood stage replication, a proportion of parasites commit to a developmental pathway leading to the formation of distinctly male and female individuals termed gametocytogenesis. For mosquito infection to establish, both male and female gametocytes must be ingested during blood feeding, at which time male gametocytes exflagellate, releasing gametes able to fertilise the female oocyte. Post-fertilisation and situated within the mosquito mid-gut, the zygotic oocyte developmentally transitions through ookinete to oocyst stages, within which sporozoites develop thus completing the life cycle. Upon emergence from the oocyst, sporozoites migrate to the mosquito salivary glands whereupon they are injected along with saliva into the human host during blood feeding.

1.1.2 Human pathology

Malaria pathologies are principally induced by the asexual blood stage parasites. In the case of a primary infection, the number of circulating merozoites increases exponentially with each

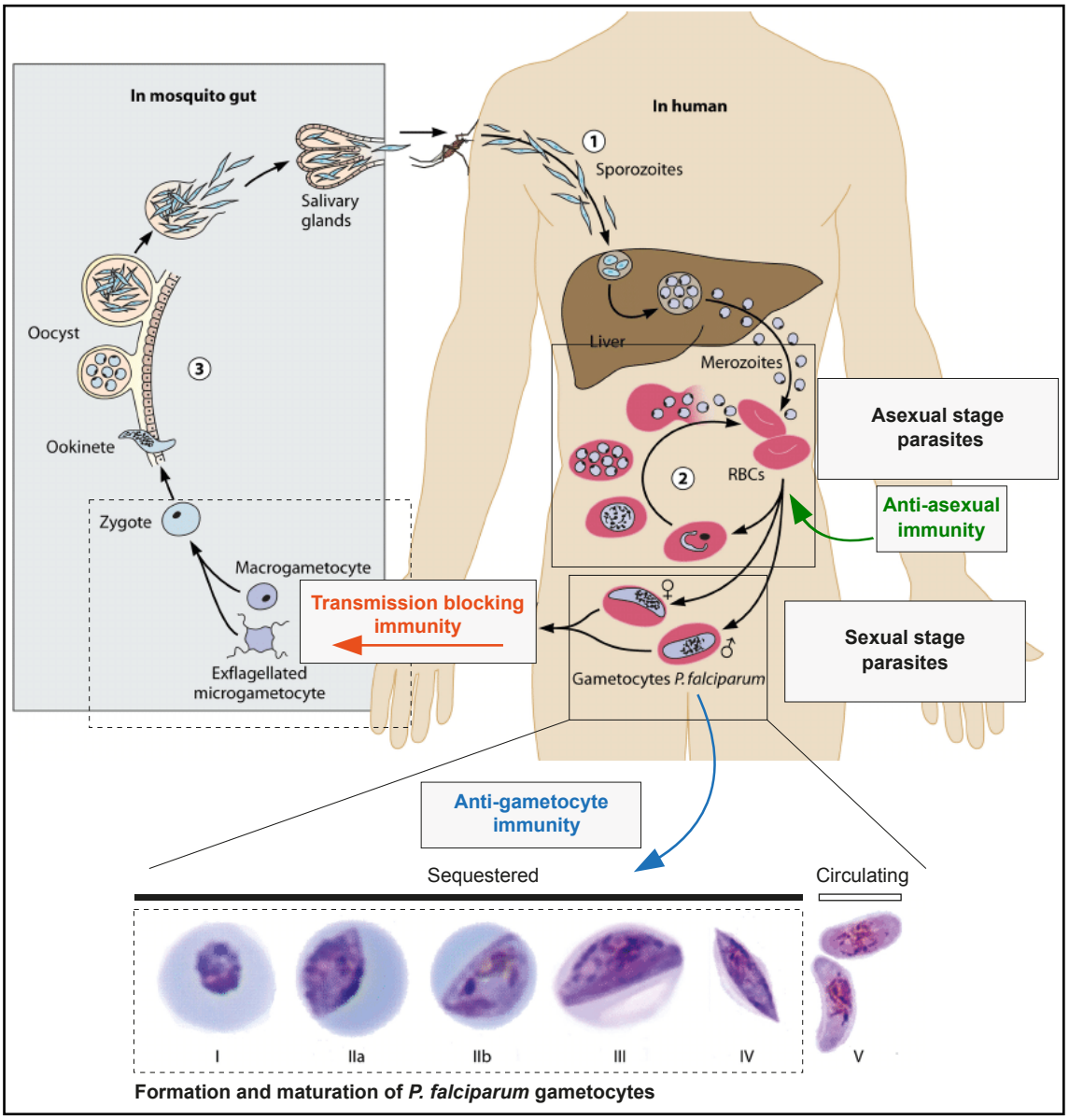

Figure 1.1. The lifecycle of *Plasmodium falciparum*, highlighting gametocyte development and key forms of anti-parasite immunity. Asexual and sexual stage parasites induce density reducing immune responses. Sexual stage antibody responses specific to gametes are capable of inducing transmission blocking effects that occur within the mosquito mid-gut. Sporozoites and liver stage parasites can induce immune responses, though these are outside the scope of this thesis. Adapted from Bousema and Drakeley, 2011 [13]

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round of schizogony. As newly released merozoites infect RBCs the number of infected red blood cells (iRBCs) increases correspondingly, leading to high levels of parasite burden in some cases; in severe cases $\geq$4% of total RBCs may become infected, defined as hyperparasitaemia [14]. After each round of asexual replication is complete, schizonts burst, destroying the RBC, ultimately leading to haemolytic anaemia and other associated pathologies.

Other features of severe disease result from the cytoadherence of iRBCs to the endothelial

surfaces of the blood vessels. Parasites within the iRBC upregulate expression of proteins such as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) — a family of variant proteins encoded by the *var* gene group [15], and repetitive interspersed families of polypeptides (RIFINs) [16]. Once exported to the membrane of the erythrocyte, PfEMP1 is involved in binding to a number of endothelial ligands such as CD36, ICAM-1 and TSP [17], removing the iRBC from circulation and preventing splenic clearance. Similarly, RIFINs facilitate the binding of iRBCs to other RBCs leading to clumping or 'rosetting' of erythrocytes. The build up of adherent iRBCs and RBCs within blood vessels leads to occlusion, reducing blood flow and thus inducing hypoxia, disruption to metabolism and ultimately organ failure.

A particular hallmark of severe disease is a dysregulation of immune responses to infection, or immunopathogenesis — a result of self-perpetuating proinflammatory cascades leading to a sepsis-like condition. Furthermore, immune mediated degradation of the microvasculature and the blood-brain barrier often results in significant, and generally fatal, brain damage [18]. However, the immune response to malaria infection, when regulated, is also capable of providing protection and significantly improving clinical outcomes.

1.1.3 Human infectiousness and potential to transmit

Although the infectious potential of an individual is influenced by a multitude of factors (*e.g.* specific vector and parasite characteristics) control of overall parasite density through the action of clinical immunity appears to be an important determinant of infectiousness. Fundamentally, individuals with a higher asexual burden will produce greater numbers of sexual stage parasites [13] and at a base level, evidence suggests that the transmission potential of an infected individual or population is broadly proportional to gametocyte density; individuals with more gametocytes will infect greater numbers of mosquitoes, and vice versa [19–21].

Other evidence suggests that a direct relationship between gametocyte load and infectiousness is imperfect [13, 22–24]. Indeed, the implementation of recently developed molecular techniques able to distinguish male from female gametocytes suggest that it is the sex ratio of sexual stage parasites that is particularly critical to successful mosquito infection and thus onward transmission [23, 25]. Complicating the picture further are the effects of clinical immunity and transmission reducing immunity (TRI), that may help to reduce transmission potential by influencing gametocyte sex ratio [26], density [27], or any other as yet unidentified characteristic.

Within a population living in the same endemic area, particular immune responses to malaria parasites may lead to disparities in infectiousness, with certain individuals being more or less likely to transmit. A person with greater infectious potential may have particular influence on localised transmission patterns, but at the population scale in low transmission settings, low-density, asymptomatic infections may constitute the greatest proportion of cases, and thus drive the majority of transmission events [28]. Equally, in areas of high parasite prevalence, levels of

natural immunity are likely to be much greater [29], which may have a considerable impact on the likelihood of transmission. In sum, the exact nature of transmission potential is unresolved and this lack of clarity has impeded efforts to combat malaria from the earliest modern initiatives through to the present day.

1.2 Malaria immunity

Although sterilising immunity does not appear to occur naturally, the adaptive immune system is able to provide increasing levels of protection with repeated infection, such that older children and adults in endemic areas are often able to live asymptomatically with active infection [30]. From the initiation of infection, *Plasmodium* parasites induce a range of immunological responses. In the case of *P. falciparum*, CD8+ T cells are known to provide functional protection [31–33], while other cell mediated responses target blood stage parasites; secretion of interferon gamma by Natural Killer T-cells, Natural Killer cells and CD4+ T-cells activates phagocytic cells leading to the phagocytosis of iRBCs [34, 35]. Alongside such cellular immune processes are humoral responses that are also implicated in protective immunity.

Antibodies are a well-documented component of protection against malarial disease. The importance of immunoglobulin mediated immunity to malaria was first demonstrated in the 1960s, through experiments in which transfer of IgG from immune adults to children suffering with severe clinical disease was shown to ease symptoms and reduce parasite density [36, 37]. Since this time, the evidence for the importance of adaptive humoral responses as a component of malarial immunity has grown considerably, and the characterisation of specific antibody responses to malaria parasites is a key focus of clinical and elimination research (reviewed in [38] and [39]). More specifically, antibodies have been demonstrated to play a number of definitive roles in immunity to malaria, including: opsonisation of sporozoites [40, 41], merozoites [42] and iRBCs [43]; the blockage of RBC invasion through receptor binding [44]; and the inhibition of cytoadhesion by iRBCs [45]. Evidence suggests that vast numbers of parasite proteins are capable of inducing measurable antibody responses after natural or artificial exposure — antibodies to over 2000 proteins out of a panel covering 91% of the *P. falciparum* proteome were detected in previously exposed Tanzanian adults after inoculation with sporozoites [46]. In particular, antibodies with affinity to certain parasite proteins are explicitly associated with immune protection (**Figure 1.2**), which will be discussed below.

1.2.1 Pre-erythrocytic humoral immunity

Pre-erythrocytic immunity (*i.e.* against sporozoites) is highly desirable, as an effective response at this stage of infection can prevent both clinical disease and transmission. Circumsporozoite protein (CSP), has been implicated in protective immunity for some time [47]. Specific to the

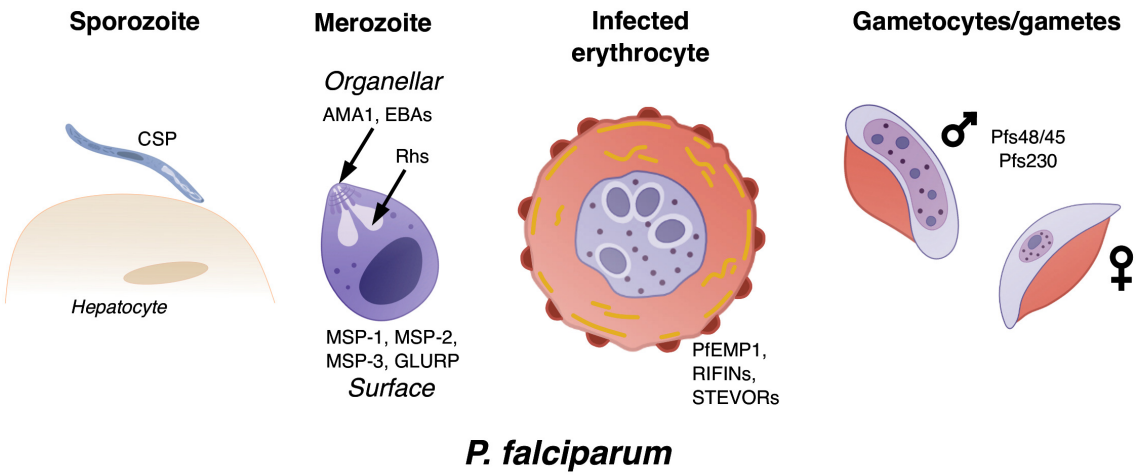

Figure 1.2. A selection of known, key P. falciparum antigen targets of humoral immune responses. Adapted from an unpublished figure by Tetteh, K.K.A., 2017. Created with BioRender.com.

sporozoite stage, investigation of responses to this highly abundant protein has led to the development of the only malaria vaccine currently in use (Mosquirix™[RTS,S]), and one other promising candidate undergoing clinical trials (R21/Matrix-M) [48], though questions remain around the efficacy and protection afforded against species other than *P. falciparum* [49, 50].

Although antibody responses to CSP are clearly implicated in protection, further evidence demonstrates that antibodies targeting a number of other sporozoite proteins are able to reduce or inhibit hepatocyte invasion [51, 52].

1.2.2 Blood stage humoral immunity

Induction of humoral responses to asexual parasites is broad and robust [53], primarily as a result of the sheer biomass of blood stage parasites and their exposure to the immune system. Such antibody responses target either parasite antigens presented on the membrane of infected erythrocytes or antigens present on the surface of free merozoites.

A characteristic of the first group of iRBC antigens is the extremely high levels of sequence variation seen both within and between parasite strains. These variant surface antigens (VSAs) comprise a number of multi-gene families, including PfEMP1 and RIFIN (already mentioned in 1.1.2 Human pathology), and subtelomeric variable open reading frame (STEVOR). As previously discussed, antibody responses to these proteins are implicated in the inhibition of cytoadherence, rosetting and, in the case of STEVORs, potentially erythrocyte invasion [38]. Though humoral responses to VSAs are induced during infection, and have been implicated in protective immunity [54–56] (reviewed in [57]), the highly polymorphic nature of these proteins and the parasite's ability to switch expression *in vivo* raises questions around strain specific immunity [58], immune evasion [59] and pathogenesis [60].

With regard to antigens localised on the merozoite surface or apical organelles, a number of well-studied proteins produced by *P. falciparum*, including Apical Membrane Antigen 1 (AMA1) and three merozoite surface proteins (Merozoite Surface Protein 1 (MSP1), -2 and -3), all induce antibodies that are strongly implicated in the prevention of severe clinical disease [61–66]. Despite these associations, developmental vaccines based on these targets have thus far failed to elicit functional protection against disease [67]. Other targets of current interest, known to induce antibody responses implicated in protective immunity to *P. falciparum*, include the erythrocyte binding antigens (EBAs) (orthologs of the Duffy-binding protein in *P. vivax*) [68, 69], reticulocyte binding protein-like homologs (Rhs) [70, 71] and glutamate rich protein (GLURP) [72], for example.

Importantly, many of the antibody responses to such proteins, whether protective or not, can be used to determine exposure to parasites, which will be discussed in 1.4.2.2 Serology as an epidemiological tool.

1.2.3 Anti-gametocyte and transmission reducing immunity

In addition to immune responses able to suppress clinical symptoms and clear asexual parasites, antibody responses able to reduce gametocyte density and impair gamete fertilisation within the mosquito mid-gut, and thus onward human infection, have more recently been identified.

The development of antibody responses to sexual stage parasites is strongly influenced by gametocyte physiology. As described earlier, during each cycle of asexual blood-stage replication, a fraction of merozoites become committed to a developmental pathway leading to the formation of male and female gametocytes [73–76], with these sexually committed parasites reaching maturity over five distinct stages (**Figure 1.1**). Of particular significance during this process is the upregulation and expression of a number of proteins in stage I-IV gametocytes that are believed to facilitate adhesion to bone marrow epithelial and stromal cells [13], in a similar fashion to the asexual parasites in the blood vessels. As a result, these developing parasites are inherently able to avoid such processes as splenic clearance and are somewhat shielded from circulating antibodies and other immune effector molecules.

With little evidence to suggest that mature, stage V gametocytes can be actively cleared from circulation [13], this period of sequestration appears to be a highly effective gametocyte survival strategy. Nevertheless, it is clear that antibody responses to early-stage gametocytes can be raised during the course of natural infection and are effective against their target; early-stage gametocyte specific antibodies have been implicated in antibody-mediated phagocytosis leading to reductions in gametocyte densities [27], and gamete specific antibodies are known to block transmission within the mosquito mid-gut when ingested during blood feeding [77].

The development of the majority of TRI effector antibodies is likely initiated through ex-

posure to developing gametocytes and the mature gametocytes that will remain in circulation after mosquito blood feeding; dead sexual stage parasites will be degraded over time, exposing numerous, possibly protective epitopes. Many such targets have been identified [78–80], though two proteins, Pfs48/45 and Pfs230 [81], continue to be of particular interest. Both proteins are expressed on sexual stage parasites, from the later half of gametocyte development until fertilisation, and are present on male and female gametes [82]. Pfs230 has been shown to be involved in the binding of male microgametes to RBCs and both are recognised to be involved in fertilisation [83, 84].

1.3 Malaria control, elimination and eradication

Historically, the objective of malaria intervention strategy was one of elimination (and ultimately eradication), primarily focused on vector control and drug treatment. The WHOs 1955 Global Malaria Eradication Programme (GMEP) initially led to great inroads in reducing disease burden, almost exclusively through the use of indoor residual insecticides (*e.g.* DDT) and the drug chloroquine for treatment and prevention of infection [85]. However, such a simplistic approach failed to account for the complex nature of malaria transmission, leading to a stalling of progress. The lack of impact was most notable in sub-Saharan Africa, where highly efficient vectors such as *Anopheles gambiae* drive transmission, and a lack of access to drugs needed for successful mass treatment regimens, undercut the efficacy of the GMEP [86]. What's more, the rise of insecticide resistance in mosquitoes and chloroquine resistance in *Plasmodium spp.* soon overwhelmed the 'one size fits all' approach of the GMEP, leading to significant rebounds in malaria incidence and endemicity [87]. As a result, the strategy for tackling malaria in following decades shifted to one of control of clinical disease by treatment through primary health care services, and prevention through the use of insecticides and insecticide treated bednets [85, 88, 89].

By the 1990s however, incidence of malaria had once again reached severe epidemic levels, sparking serious public health crises. A lack of financial support for control programmes and health care, mass migration, changes to land use, widespread civil unrest and the continued expansion of anti-malarial drug resistance led to an estimated 300-500 million malaria cases and 1.5-2.7 million deaths per year in the first half of the decade, with 90% of cases located in sub-Saharan Africa [88]. Despite the increasingly dire disease landscape, in a turn of great fortune and timing, new and highly efficacious drug treatments based on artemisinin reached the market. The roll-out of artemisinin therapies, alongside early iterations of rapid diagnostic tests (RDTs) which aided in the identification an targeting of infections [90], over the late 1990's and early 2000's led to significant improvements in the control of clinical disease and mortality, such that by 2006 the WHO formally recommended artemisinin as the front-line treatment for malaria and advocated for the administration of combined drug preparations (*i.e.* artemisinin

plus another, non-artemisinin based drug) termed ACT, to prevent the development of drug resistance. AsACTs lent hope to the possibility turning the tide on malaria, in 2007 at the Gates Malaria Forum, the prospect of aiming for eradication was seriously reconsidered [91, 92] — though by this time it was understood that a far more nuanced and scenario-specific approach would be necessary to achieve such a goal.

It is now recognised that for malaria control and elimination programmes to be successful, there must be strong integration of surveillance and the resultant data into health systems and engagement of local communities, rather than the more stand-alone interventions of the past [93]. The conventional methodologies of vector control (making use of novel insecticides), and provision of treatment with newer, more efficacious drugs continue to be crucial components of malaria eradication strategy, though efforts must be made to maximise both coverage and access to these measures. Of particular significance in this context are treatments able to block transmission through the targeting of sexual stage parasites, such as primaquine, which may aid in the reduction of asymptomatic infectious reservoir during mass drug administration initiatives [94]. However, an ability to evaluate progress and precisely target interventions is clearly a necessity — interventions such as those described above are effective at reducing transmission, but the timescale over which they are effective and the associated financial burden make these methods unsustainable in isolation. In 2011, the Malaria Eradication Research Agenda (malERA) project set out to identify the specific knowledge gaps that were impeding elimination efforts across a number of thematic areas through a series of consultations. The Consultative Group on Monitoring, Evaluation and Surveillance stressed the importance of shifting from passive measurement of morbidity and mortality to actively detecting infections, both symptomatic and asymptomatic, and measuring transmission [95]. Since that time, epidemiological analysis and feedback, underpinned by the development of new diagnostic tools, monitoring, modelling, and mapping of disease, has had a significant impact on the downward trajectory of malarial burden across the globe.

However, and as already mentioned, cases and deaths over recent years appear to once again be rebounding. Though this upward trend is generally applicable worldwide (though perhaps less so in the Americas), it is most acutely observable within the WHO African Region, which accounted for 95% and 96% of cases and deaths in 2021 respectively. In particular, between 2019 and 2020, case incidence increased from 225.5 to 233.6 per 1000 population at risk in the region as a result of disruption to malaria prevention activities due to SARS-CoV-2 [7]. Thankfully, estimates of a doubling of malaria mortality in 2020 under pessimistic circumstances [96] did not come to pass, with some countries surpassing historical levels of insecticide treated net (ITN) distribution, testing by RDT and seasonal malaria chemoprevention [7] thanks to the incredible dedication and commitment of healthcare workers and national health services in endemic countries. Such efforts led to far more modest increases in cases and deaths between 2020 and 2021. Nevertheless, both the general slowdown of incidence and mortality since 2015, and the

stark impact of the SARS-CoV-2 pandemic highlight the need for further innovation in our approach to malaria elimination, particularly in developing ways to identify and respond to local variations in malaria transmission, as laid out in updates to the malERA agenda in 2017 [97].

1.4 Malaria surveillance

Surveillance of infectious diseases has become a key component of modern public health. Collection of relevant data on disease — either actively, passively, or both — and epidemiological analysis of them, allows assessment of the current burden of disease, the monitoring of disease trends and transmission patterns, and the identification of outbreaks [98]. Such analysis is critical in demonstrating the need to for disease control measures, such as mass drug treatments or vaccination, but also for measuring the success and impact (or failure) of such interventions, particularly where disease elimination is a target. Furthermore, effective disease surveillance will allow the early detection of outbreaks, presenting opportunities to mitigate health impacts.

Appropriately, surveillance methodologies have been widely adopted by public health systems in malaria endemic regions, underpinning the epidemiological component of malaria elimination strategy. Such surveillance is principally based on the measurement of prevalence and incidence through the diagnosis and detection of infection.

1.4.1 Clinical diagnosis, or passive case detection

The use of light microscopy to identify and characterise infection is well proven and remains the 'gold standard' for parasitological diagnosis; Giemsa-stained blood films prepared on microscope slides are visually examined for the presence of parasites [14]. Although the direct financial costs of microscopy are low once infrastructure and expertise are in place, the training required to produce and maintain capable microscopists, and the time needed to accurately screen bloodfilms is considerable.

RDTs, based on the detection of parasite antigens in the blood in a lateral flow format, have gone a considerable way to reducing pressure on microscopic diagnostic services and have in many ways revolutionised point-of-care diagnostic testing in malaria endemic areas. In particular, RDTs have greatly aided in the implementation of the WHOs 'test-and-treat' policy, whereby suspected malaria cases are diagnostically confirmed before treatment, rather than presumptively treating any individuals presenting as febrile [14].

The ease of use, low cost and quick result has led to the highly routine use of RDT as a clinical diagnostic [5]. According to the WHO World Malaria Report 2021, 3.1 billion RDTs were sold by manufacturers between 2010 and 2020 (81% of these sales were in sub-Saharan Africa), with 2.2 billion of these distributed by national malaria programmes [5]. However, difficulties

with specificity for species other than *P. falciparum* and limited sensitivity, particularly in the context of low density infections, remain an issue for RDTs [90, 99, 100].

1.4.2 Surveillance and research diagnostics

Outside of individual diagnoses for clinical purposes, population level, or surveillance diagnostics, provide the basis for epidemiological research of malarial disease. Although clinical diagnostic data are also considered within surveillance analysis, other methods for measuring metrics of disease can provide greater sensitivity and specificity, as well as potentially providing information on exposure history.

Molecular detection of infection

More recently, molecular-based methods have drastically increased testing throughput and have simultaneously improved sensitivity and specificity by a significant margin compared to microscopy. Real-time polymerase chain reaction (PCR) techniques allow highly sensitive parasite quantification and speciation at sub-microscopic densities [101, 102], with similar approaches allowing for the detection of specific parasite developmental stages [103] or the determination of gametocyte sex-ratios [104], for example. Although PCR methods have become a mainstay of both qualitative and quantitative malaria metrics in a research context, material cost and expertise can be highly prohibitive to use in a point of care setting, and it is unclear that molecular techniques will ever become a standard surveillance diagnostic. As such, molecular diagnostics are generally limited to specific case uses; PCR techniques may be used to definitively speciate infection (*e.g. P. knowlesi* can be misdiagnosed due to morphological similarities to *P. malariae* [105, 106], and are often incorporated into epidemiological surveys and studies, but are not supported for front-line case detection [14].

Whilst diagnosis and speciation of a current malaria infection by the methods described above provides useful data from a clinical and epidemiological perspective, it is perhaps still painting a fairly broad picture of what may be a more complex scenario. Unless large cross-sectional studies have been undertaken repeatedly over time for comparison, measures of current prevalence and incidence may not be indicative of changing trends in exposure and transmission. However, the detection of parasite specific antibodies by serological methods has opened up the field of malaria surveillance to much greater depths of questioning.

Serology as an epidemiological tool

The use of serological methods to determine exposure history and transmission in malaria has been well established over the last 15 years [36, 107–111]. Measuring antigen specific antibody pre-

valence, or acquisition, allows high-resolution analysis of sampled populations, and can demonstrate highly reproducible trends when stratifying data by measures such as age [108, 111, 112], geography [113–115] and immunity [29, 116–118]. As anti-malarial antibodies accumulate within an individual, an immune 'signature ' is compiled, providing some insight into the infection history of that person. These signatures, when collated with epidemiological data on the individual such as infection status, age, or gender *etc.*, can be used to illustrate and predict aspects of disease. The most simple and demonstrable of these is historical exposure. An individual who has been exposed to *Plasmodium* species will (under normal conditions) produce antibodies specific to any number of parasite antigens (as already discussed in 1.2 Malaria immunity), which may then be detected by serological laboratory methods. As such, the detection of any parasite antigen-specific antibody is indicative of that person having ever been exposed to *Plasmodium*. On this basis, it is then possible to use measured antibodies to extrapolate whether another individual has experienced infection irrespective of their current infection status.

A development of this idea is to detect the presence of antibodies specific to particular antigens that provide a more detailed and informative picture of disease. A key example of antigens that fulfil this criteria are AMA1 and MSP1, both of which have been demonstrated as useful biomarkers for measuring long-term malaria transmission trends in both hyper- and hypoendemic areas [113, 119]. The age-stratified acquisition of antibodies specific to these two antigens, and the related half-life decay of those same circulating antibodies, can be used to monitor changes in transmission over time, where declines in seropositivity are indicative of reduced exposure [111, 112].

Recently, interest had grown in identifying antigen biomarkers that can produce higher resolution data as a result of differences in the kinetics of humoral responses to the parasite. Antibodies to parasite proteins such as Early Transcribed Membrane Protein 5 (ETRAMP-5) are understood to act as markers of short-term, or more recent exposure [120, 121]. In contrast to the long-term markers described above, short-term biomarkers would be used to detect antibody levels that may peak and decay in the weeks to months after infection. In this way, it would be possible to serologically ascertain whether an individual has acquired a new infection within a defined time period, for example when assessing the short term impacts of intervention studies [122].

Measuring humoral responses to sexual stage specific antigens could also provide relevant insight for surveillance. Although relatively little is understood about sexual stage immunity at this time, it is plausible that relationships between antibody responses to gametocyte antigens and infectiousness may exist. There is evidence to suggest that anti-gametocyte antibody responses rise and fall rapidly in line with gametocyte exposure [123], acting as potential indicators of current gametocyte carriage and thus infectiousness (see 1.2.3 Anti-gametocyte and transmission reducing immunity). As such, changes in the population prevalence, or levels of antibody response to certain sexual stage antigens, may be indicative of a corresponding in-

crease or decrease in infectiousness in line with gametocytaemia. In contrast, it is possible that decreases in gametocyte specific antibodies may indicate reduced anti-gametocyte immunity or TRI, leading to increased likelihood of transmission.

This level of data stratification is extremely useful in low endemicity settings or areas targeting malaria elimination, particularly if it is achieved using low-cost, field applicable methods such as an enzyme-linked immunosorbent assay (ELISA). However, there is growing evidence that more precise models of disease can be achieved through the analysis of antibody responses to multiple parasite antigens [121, 124]. As analytical approaches to malaria surveillance have developed, so too have the laboratory methods and assays that underpin the data generation.

1.5 Serological methodologies

1.5.1 Antibody detection

The earliest methods for detecting *Plasmodium* specific antibodies were relatively crude, relying on fixation of complement by antigen-bound immunoglobulins, or haemagglutination resulting from the cross-linking that occurs between antibody-antigen complexes. Both methods were dependent on visual confirmation of results, and required soluble preparations of antigen that were difficult to manufacture reproducibly at scale at the time. As such, these methods were soon surpassed by the immunofluorescence antibody test (IFAT), which is able to visibly demonstrate localisation of antibody bound to target antigens. The assay is based on the fixation of antigen or whole parasites to a solid support — in this case a microscope slide — followed by incubation with sera. Slides are subsequently probed with a fluorescently labelled secondary antibody specific for human immunoglobulins, and are then viewed under a fluorescent microscope. Despite the advantages of the IFAT compared to complement fixation and haemagglutination assays, it is still assessed by eye and requires great amounts of time and skill to perform successfully at scale. These shortcomings were likely one of the driving forces for the development of newer approaches, though the methodological principle formed the foundation for the next generations of serological immunoassays in malaria research (**Figure 1.3**).

The colourmetric quantification afforded by the ELISA is standardisable and objective (assuming that antigen production is similarly standardised; see 1.5.3 Producing antigenic targets below). Using microwell plates in place of microscope slides and an enzyme-substrate reporter in place of fluorescent labels, the colour change as substrate is catalysed can be measured by spectrophotometer giving a numerical result (**Figure 1.3**). The technique is simple to perform and materials are relatively cheap. As a result the ELISA has been ubiquitous in malaria serology over recent decades, and it has enabled the characterisation of key epidemiological trends across numerous studies [77, 108, 109, 111–115, 125, 126].

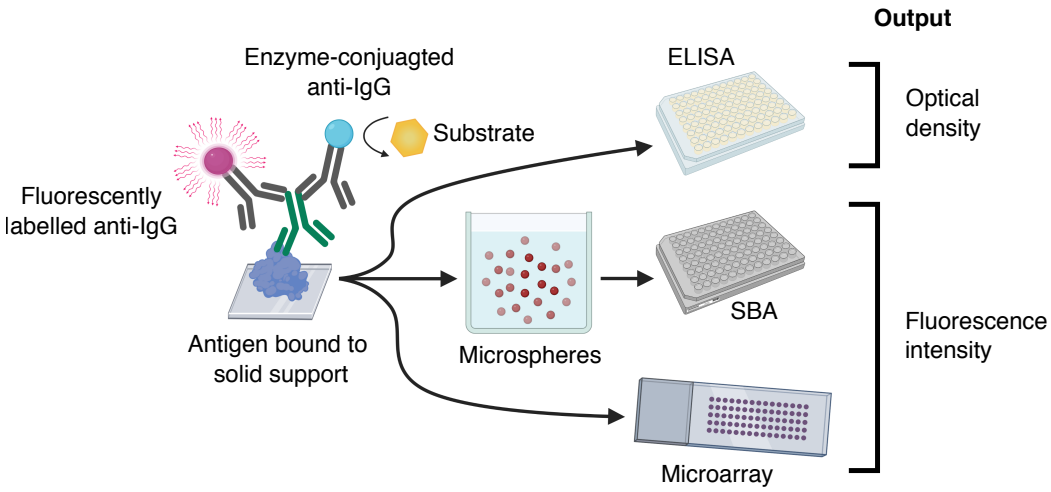

Figure 1.3. Methodology for the capture and detection of antigen specific antibody. Antigen is covalently bound to a solid support, *e.g.* 96-well microplate; plastic microspheres; specialised microarray slides. When probed with sample, specific antibody (green) binds to the target protein, which is then detected using a secondary antibody (grey) conjugated to a reporter, *e.g.* an enzyme-substrate system or a fluorescent dye molecule. Created with BioRender.com.

1.5.2 Producing antigenic targets

Another important change in the field of malaria serology was the advent of recombinant protein production. Until that time, whole or lysed parasites were the source of antigen for immunological assays, with non-human infectious, or zoonotic, *Plasmodium* species commonly used owing to the difficulty of obtaining or culturing human parasites [127, 128]. Crucially, this meant that all antibodies specific to any antigen present were captured and, in the case of zoonotic species, there was a reliance on cross-reactivity of antibodies between *Plasmodium* species [129]. Accordingly, such antigen preparations were only able to generally indicate infection or exposure, to varying degrees of sensitivity and specificity [130]. To improve upon these techniques, a reproducible source of known, purified antigen was needed.

The establishment of recombinant protein and peptide production techniques (**Table 1.1**) meant that individual, species-specific antigen targets could be produced and interrogated serologically. In this way it was finally possible to identify and characterise important immunogenic antigens, helping to elucidate protective immune responses and paving the way for vaccine development. Furthermore, such investigation enabled the interrogation of antigens outside the context of protective immunity; a number of potential vaccine targets were later found to be useful seroepidemiological targets, such as AMA1 and MSP1 (discussed in 1.2.2 Blood stage humoral immunity). These proteins could be reliably manufactured and used in an ELISA to conduct large scale surveys, giving standardised and comparable results.

Table 1.1. Common recombinant protein expression techniques for *Plasmodium* antigens.

Format	Expression system	Key points	References
Cellular	Bacterial (*e.g. E. coli*)	Low cost, simple culture conditions; Potential issues with protein solubility	[131, 132]
	Yeast (*e.g. P. pastoris*)	Eukaryotic processing; Growth conditions may require optimisation	[133, 134]
	Mammalian (*e.g.* Human embryonic kidney cells)	Very high level protein processing; Demanding culture conditions	[135]
	Insect-baculovirus	High levels of protein processing; Production of baculovirus vectors is time consuming	[136]
Cell free (*in vitro* transcription/translation)	Bacterial lysates	Fast, simple expression; Higher yields may not be possible	[59, 77, 120]
	Wheat germ		[137–139]

The cost and effort required to express proteins and peptides by some of these methods is now so low that it is now feasible to produce large numbers of potentially interesting protein antigens based purely on *in silico* predictions of immunogenicity. The *P. falciparum* genome contains over 5000 protein encoding genes [140], many of which may be capable of eliciting antibody responses. Although far fewer of these are likely to be of any clinical or epidemiological relevance, there are still potentially many hundreds or thousands of antigens to be investigated. Furthermore, measuring antibodies responses to single targets is not representative of the true breadth of immunity induced by natural infection, and is thus liable to miss variation in population immune responses or genetic polymorphism in parasite strains. With these caveats in mind, the newest generation of serological assays have been developed to allow the detection of distinct antigen specific antibody responses to multiple antigens simultaneously.

1.6 Multiplexing antibody detection assays

The ability to serologically screen samples against multiplexed biomarker targets has led to significant increases in throughput, whilst reducing workloads and time-associated costs. New assay platforms have enabled the characterisation of putative antigen biomarkers on a scale of tens to hundreds at a time (**Figure 1.4**), and similarly allow the incorporation of multiple antigen response variables into epidemiological models [77, 120, 121, 141–145].

The suspension bead array (SBA) is one such technology that allows multiplexed screening of antigen targets. In this approach, a protein is covalently bound to fluorescently coded microspheres or 'beads' that can be held in liquid suspension (**Figure 1.3**). The fluorescent code of each bead 'set' is unique, such that when the beads are interrogated by a cytometric instrument, each bead may identified as belonging to its set. By assigning one antigen per bead set it is possible to distinguish antigen bound beads even when mixed [146]. Much like an ELISA, with each bead set acting as a well of an antigen coated microplate, beads are incubated with sample before probing with a fluorescently labelled detection antibody. The flexibility of SBAs has led

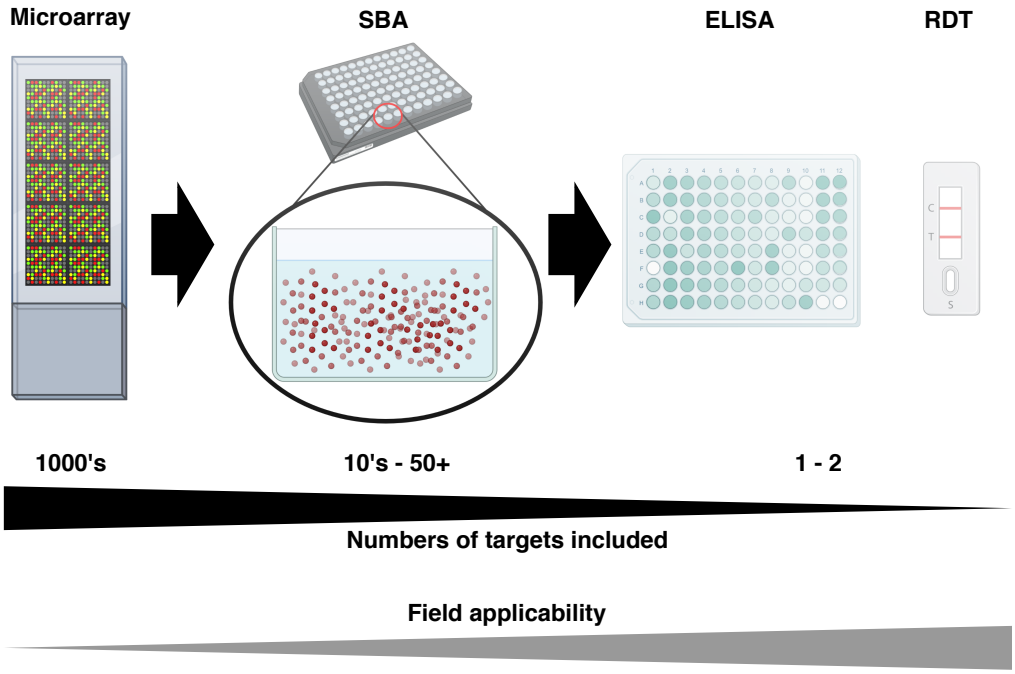

Figure 1.4. Representative illustration of a research pipeline for the discovery of biomarkers through to application. The assay platforms utilised at each stage may be selected as appropriate according to the research questions being asked, or end-use application. Assay platforms are ordered according to the typical number of antigen targets that may be included for screening. Created with BioRender.com.

to their widespread adoption within the field of malaria serology, with optimised approaches for tens of antigens at a time demonstrating high levels of performance and insight [143–145, 147].

Microarray technology has also seen a resurgence within a serological context. Although initially developed for nucleic acids research, microscale printing techniques have been utilised to produce reverse phase protein microarrays (RPPAs), whereby spots of protein are deposited at high density on specialised microscope slides before screening with sample serum (**Figure 1.3**). Such approaches have been shown to be effective in profiling antibody responses to pathogen for over a decade [148, 149], and RPPA methods are increasingly utilised in malarial studies [77, 120, 121, 150]. A particular strength of microarray platforms is the ability to incorporate analysis of antibody responses to very large numbers of antigenic targets at a time, with high density peptide arrays even facilitating 'immunomic' level screening of thousands of potential epitopes [151, 152]. The opportunity to cast the net so widely at relatively little additional expense enables investigation of numerous antigens that may otherwise have been discounted if other methods were to be used.

A further advantage of such technologies is an improvement in sensitivity (*i.e.* the lower limit of detection, or true positivity rate), dynamic range (*i.e.* the scale, or ratio, from minimum to maximum signal) and granularity (*i.e.* the level of detail, or minimum signal increment) of

data afforded by the automated fluorescence-based reporting typically employed by SBAs or RPPAs, rather than colourmetric measures of an ELISA for example. While these more technically advanced approaches may be less well suited to front-line, routine usage, the increased analytical power and flexibility they provide had led to their widespread adoption within a research context.

The large, high-dimensional data sets generated by these assay techniques are highly in keeping with the development and application of new modelling approaches and machine learning techniques that are being applied to malaria research and epidemiology [120, 124, 153, 154]. For example, in one recent study antibody responses to 23 different *P. vivax* antigens were shown to be associated to different demographic variables known to relate to risk of malaria, and responses to a subset of 8 antigens incorporated into a classification algorithm to predict recent exposure in a low transmission setting [137]. With an ability to deal with large numbers of variables and types of data, these analytical methods can be best exploited by incorporating additional assay measurements.

1.6.1 Extending assay capabilities

Malaria serology to date has primarily focused on detection of IgG antibodies, which constitute by far the greatest proportion of antibody in serum [155]. However, many of the serological methods described may be easily adapted to detect other antibody classes [156] or subclasses [157]. With the distinctive kinetics and effector functions of each class and sub-class orchestrating different immunological outcomes, detection of highly particular antibody responses may lead to the identification of specific mechanisms of immunity or useful epidemiological trends. Although not yet commonplace, developments to enable multiplexing of analytes (*e.g.* IgG-1, -2, -3 and -4; IgM and IgG) alongside multiplexed antigen targets could further transform the approach to malaria serology (see 3.7 Additional work and figures).

Other avenues of investigation could include evaluation of antibody function. While infection can induce a range of detectable specific antibody responses, not all of these will be effectively protective and differences may be indicative of important epidemiological characteristics. Complement fixing antibodies have been shown to inhibit blood stage replication, and assays capable of detecting the binding of C1q to immune complexes (antigen bound to antibody) have been developed to assess immune protective associations between antibodies that do or do not fix complement effectively [139, 158]. Similarly, antibodies with a high avidity to specific antigen indicate a maturation of humoral immune response to infection. Measurements and comparison of antibody avidity, through the use of chaotropic agents on antibody-antigen interactions, could prove to be useful disease indicators [159, 160].

1.7 Developing a research pipeline

In addressing the need to develop new tools and analytical methods for malaria surveillance and diagnosis, it is clear that new or refined laboratory techniques can play a major role. The ability to screen, identity and characterise serological biomarkers at such scale and power implies that it is simply a matter of time until new informative targets are identified. However, without a strategy in place to make best use of the available technology, there is a risk that the wait may be longer than is necessary.

The work in this thesis broadly describes a 'pipeline' research strategy; from the development and evaluation of assays for the discovery of new biomarkers, through characterisation of candidate markers and finally to application. It similarly lays the groundwork for 'down-selection' processes that can be implemented to make the most effective use of the available technologies: highly multiplexed platforms such as the microarray can be utilised to conduct broad screening of candidate antigens as recombinant proteins or peptides; bioinformatic approaches can then be used to design SBAs that can then be used to drill down into the characteristics of a smaller panel of antigenic or biomarker targets in a more versatile and scalable format; and finally specific biomarker assays can be optimised and put to use to answer defined research questions or developed into simple tools for use in clinical or field settings (**Figure 1.4**). Elements of this sort of approach are already being used in studies, for example determining a minimum panel of antigen specific antibody responses to detect exposure in low density infections [121], or looking to identify new antibody responses associated with transmission reducing immunity, alongside already established markers [77].

As such pipeline strategies are refined, the pathway for discovering and developing the tools called for to assist with malaria elimination will become more efficient. Accordingly, innovative methodologies may help to address the current knowledge gaps in malaria surveillance, thus accelerating elimination strategy. The following chapters broadly describe such a research pipeline, with an endpoint focus on research questions surrounding sexual stage immunity and gametocyte detection. Ultimately these data can feed into the development of strategies aiming to improve surveillance of malaria transmission and transmission blocking interventions.

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Chapter 2

Rationale, aim and objectives

As the reinvigorated ambition to eradicate malaria has grown in recent decades, it has become clear that new strategies and tools are required to achieve elimination across a range of highly diverse endemic settings. Particularly in areas where malaria transmission has fallen or has historically been low — leading to greater heterogeneity of prevalence and incidence — enhanced levels of resolution in disease surveillance and clinical diagnosis are needed to help build clearer pictures of who is transmitting and becoming infected with malaria parasites, and where and when it happens. This will be achieved through the development and application of new laboratory and analytical techniques, and the characterisation of potential biomarkers. Improvements in these areas will be of great benefit in the context of determining what intervention measures may be most effective within a specific setting, how to approach their implementation and measuring efficacy and outcomes.

2.1 Aim

The aim of the research presented in this thesis is to demonstrate the development and application of serological, biomarker-based methodologies within the context of better understanding malaria infection and transmission in endemic settings.

2.2 Objectives

The studies included here cover a 'pipeline' approach to serological biomarker discovery, characterisation and application. The specific objective of each is to:

1. Develop the use of microarray technology and techniques to facilitate the discovery of biomarkers that could contribute to the elimination and eradication of malaria;

    Hypothesis: Compare and optimise materials, reagents and methodology forStandardisation of microarray approaches to *Plasmodium* antigen screening will help to reduce inconsistencies and artefacts in data generated by protein microarray, thus strengthening and improving methods to identify biomarkers that may be useful in efforts to control and eliminate malaria.

    Approach: Compare and optimise materials, reagents and methodology for the production and processing of a reverse phase protein microarray for the serological assessment of antibody reactivity to *Plasmodium* antigens.

2. Compare two protein expression methods in the context of a microarray, to determine whether differences in attributes of an antigen, in relation to the protein expression method utilised, affects antibody reactivity and subsequent analytical interpretation of biomarker performance;

   Hypothesis: The antibody reactivity to protein antigens used in serological assays varies by protein expression system. The use of specific protein expression methodologies is dependent on study context and application, with some expression systems more suited for high-throughput overview screening of antigenic targets, and others more sensitive characterisation of responses to targets.

   Approach: Compare antibody responses to matched *Plasmodium* protein antigens produced using two different recombinant protein expression systems on a reverse phase protein microarray platform.

3. Investigate potential biomarkers of gametocyte exposure and the relationship between gametocyte specific antibodies and gametocyte density, and length of gametocyte carriage;

   Hypothesis: Antibody responses to gametocyte specific antigens are induced proportionally to gametocyte exposure, and the responses induced after single exposure are known to be associated with transmission reducing immunity.

   Hypothesis: Evaluate the induction and kinetics of antibody responses to sexual stage *Plasmodium* parasites in a naive cohort during a controlled human malaria infection.

4. Apply serological, biomarker based techniques to determine whether residual gametocytes after standard treatment can confound RDT diagnostics.

   Hypothesis: Residual gametocytes after standard anti-asexual stage drug treatments may contribute to circulating levels of HRP-2, thus confounding ultra-sensitive diagnostics based on the detection of HRP-2 in the blood.

   Approach: Quantify, using a multiplex ELISA array, levels of the parasite protein HRP-2 over time in cohorts with and without residual gametocytes after treatment, and compare levels of HRP-2 to time to test negativity by standard and ultra-sensitive rapid diagnostic tests.

Chapter 3

An evaluation of microarray slide substrates and materials: optimising a serological screen of a panel of purified recombinant *Plasmodium falciparum* antigens by protein microarray

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Student ID Number	1404292/RITD	**Title**	Mr
First Name(s)	Tate		
Surname/Family Name	Oulton		
Thesis Title	Development and validation of serological assays to evaluate malaria transmission		
Primary Supervisor	Chris Drakeley		

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Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

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SECTION E

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Title:

An evaluation of microarray slide substrates and materials: optimising a serological screen of a panel of purified recombinant *Plasmodium falciparum* antigens by protein microarray

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Abstract

To achieve the targeted elimination of malaria, new biomarkers of disease are required, to direct and evaluate intervention measures. High-throughput, multiplex technologies such as the protein microarray have significantly improved the ability to identify and characterise informative antibody responses to parasite antigens. Here we have developed and optimised a bespoke microarray for use in the serological screening of purified *Plasmodium falciparum* protein antigens. A range of materials, including slide substrates and buffers, were tested for their ability to capture antibody responses to a select panel of 5 antigenic targets. Of the 480 unique experimental conditions tested, 8 specific combinations were found to be represented within the top 10% of ranked conditions for 4 out of the 5 antigens. Between these 8 conditions, 3 were found to produce highly correlated data (r=0.91–0.98) and all displayed high levels of inter-assay reproducibility. These findings allow us to recommend a number of experimental conditions in the context of malaria serology by protein microarray, and also highlight the considerable impact material choices and methodological approach can have on the generation of protein microarray data.

3.2 Introduction

The use of serological methods in the surveillance of malaria endemic settings is widely recognised. Typically, antibody reactivity to a small number of well characterised parasite proteins is used to monitor trends of exposure and immunity to *e.g.* determine changes in malaria transmission at the population level over time [1–4], or support targeted approaches in areas where transmission is very low and normal metrics of disease are more difficult to measure [5–8]. Such methodologies have, and may, play a part in reducing both incidence and prevalence of malaria, and thus many endemic areas are now realistically targeting disease elimination.

However, as population exposure declines, surveillance systems are likely to face challenges: asymptomatic, low density infections are likely to come to represent the greatest proportion of cases [9], and specific population groups (*e.g.* adults and men, in relation to occupational and behavioural factors) may become disproportionally at risk [10]. In these instances, existing methodologies used for surveillance may struggle to fully characterise disease in a way that may help to understand how to completely disrupt transmission. Measures such as the entomological inoculation rate, used to ascertain the intensity of parasite transmission by mosquitoes, can be used to determine whether elimination can be achieved after intervention [11], though such an approach is painstaking and highly labour intensive. Established serological methods, largely based on asexual stage vaccine candidate antigens with uncertain linkage to protection, are relatively logistically simple and allow dissection of long-term immunological trends and infection history, but an ability to describe patterns of disease in a more refined and focussed way may prove to be valuable. Examples include: identifying groups or individuals at increased or reduced risk of reinfection [12]; measuring the time since infection in the context of months rather than years based on short lived antibody responses [13, 14]; identifying risk factors for infection [5]; or making spatial predictions on areas susceptible to outbreaks of disease [6]. Serological tools with such capacity can help to improve the sensitivity and granularity of surveillance methods, but to progress further in this area, novel immunogenic antigen biomarkers must be identified, characterised and assessed for suitability for inclusion in bioassays.

Interrogation of serological responses to any meaningful proportion of *Plasmodium* antigens can be laborious due to the sheer number of candidates. As such, technologies have shifted towards multiplex approaches in an attempt to overcome the restrictions of traditionally monoplex assays. While platforms such as multiplex bead arrays continue to gain traction in this area [15–17], microarray technologies provide an opportunity to significantly improve sample, target and analyte throughput [18, 19]. The versatility and high-throughput nature of the protein microarray platform makes it an appealing approach for biomarker discovery and characterisation; the ability to examine hundreds of protein targets simultaneously is highly effective in reducing cost, time and sample volume requirements when compared to techniques such as an ELISA [20–22].

Microarray based approaches have already been widely utilised in the context of malaria serology. Microarrays of *Plasmodium* protein antigens have been successfully applied toward general characterisation of population level transmission and immunity [23–25], strain specific immunity [26], aspects of clinical immunity to disease [27–30], transmission reducing immunity [31, 32], vaccine target evaluation [33] and in the determination of infection history [13, 14]. Crucially, the ability to measure antibody responses to much larger panels of putative markers, coupled with the power of multivariate modelling and machine learning approaches, allows for the potential development of far more sensitive and granular serological tools. For example, unique combinations of antibody responses may act as 'fingerprints' for specific infection characteristics, such as estimates of recent exposure to parasites [13, 14].

Often, microarrays for such studies are produced as bespoke products, consisting of discrete panels of targets defined by specific criteria such as expression in sexual stage parasites [31] or polymorphic variants [30], but it is currently unclear what bearing any differences in methodology may have on data output. The ability to not only examine specific panels of proteins, but to do so under optimal and standardised conditions, allows experimental data to be compared and examined with much greater confidence. The success of protein-based antibody capture assays is dependent on a number of factors such as epitope availability and orientation, abundance of non-specific molecules in samples, ease of processing, and protein lability — all of which may be affected or determined by the methods, reagents and materials used.

Microarrays are typically manufactured using a 'printing' process, whereby picolitre-volumes of material are spotted onto a substrate coated microscope slide — nitrocellulose film or one of a number of chemically reactive coatings (*e.g.* aldehyde functional groups). Printing methods include contact-based (*i.e.* grids of pins, loaded with target material are physically pressed to the slide substrate) or non-contact ink jet derived systems. Material to be printed is mixed with a print buffer to achieve a viscosity that facilitates a consistent application and morphology of spots.

Taking into consideration the potential impact these variables may have on the output of a microarray assay, we have evaluated a number of key components and procedures in the manufacture and processing of a *Plasmodium falciparum* derived, purified recombinant protein microarray for use in serological screens using a small panel of representative *P. falciparum* protein antigens. These components are slide composition and substrate, printing buffer and blocking reagents.

3.3 Results

Array condition evaluation

We first sought to evaluate a range of slide substrates, print buffers and blocking buffers. A small panel of purified recombinant antigens, of varying known reactivity in a hyperendemic malaria control (CP3; a pool of Tanzanian adult, hyperendemic sera), were printed in three different print buffers across 10 slide types (**Figure 1**; **Supplementary table 1**). All were assayed utilising four blocking buffers at four dilutions, to give 480 unique array conditions. All arrays were otherwise treated uniformly to allow direct comparisons between conditions.

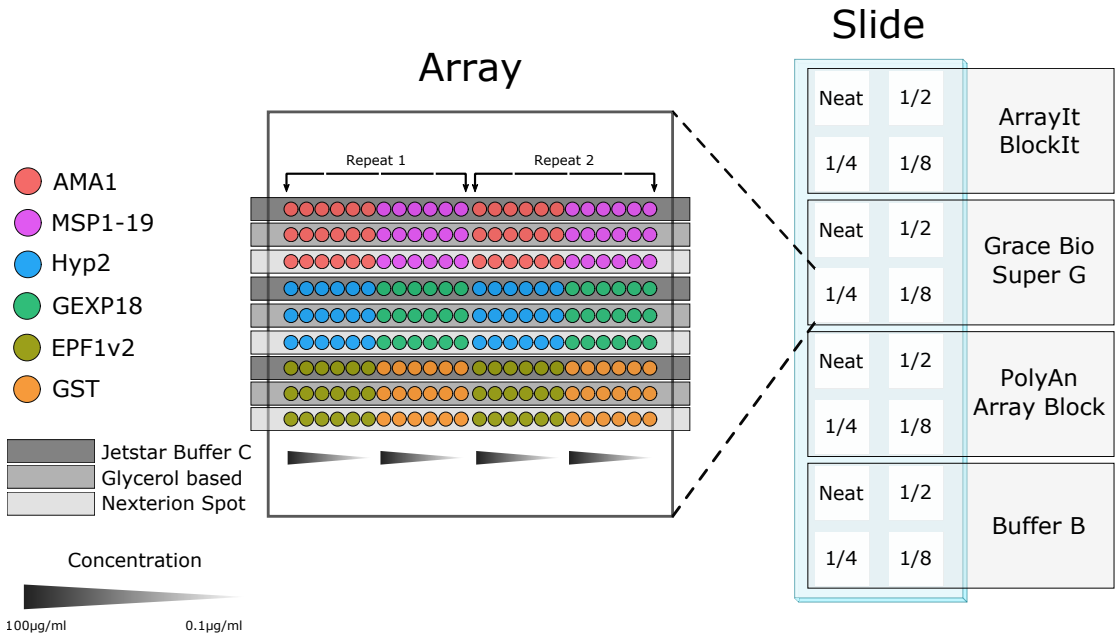

Figure 1. Optimisation array design. A panel of six antigens (coloured circles), at six concentrations (100–0.1 µg/ml), were printed in each of three print buffers (dark to light grey boxes), in duplicate, within the 16 arrays per slide. Each slide (including the sample later applied) was treated using each of the four block buffers, at four dilutions.

The hyperendemic sera and malaria naive negative controls (pooled European sera from non-exposed individuals, N=10) were screened under every array condition. Raw MFI data was processed as described below and values were converted to a ratio of reactivity, where positive control values were divided by the corresponding signal in the negative control.

First, printing concentrations for each antigen, across all conditions, were compared to determine where optimal reactivity occurred (**Figure 2A**). Both AMA1 and MSP1-19 show clear concentration-dependent responses, while EPF1 and Hyp2 show sudden increases in signal at 25 µg/ml and 100 µg/ml respectively. Individual responses to GEXP18 showed some variation, but median reactivity was negligible and no general trend across all conditions was observed.

To this end, further analysis only considered antigens printed at 100 μg/ml to capture the best representative data.

To assess the performance of each array condition, individual normalised $\log_2$(positive/negative) MFI ratios for each antigen were ranked by magnitude. The top 10% of ranked conditions for each antigen were then compared to identify commonalities. When considering all five antigens, no conditions were found to be equally represented, however eight conditions were found to be common between AMA1, MSP1-19, Hyp2 and EPF1 (**Figure 2B**). Very few conditions captured any level of reactivity to GEXP18, which was expected based on the very limited reactivity observed shown in **Figure 3A**. As such, GEXP18 was excluded from further analysis.

When comparing the eight common conditions between antigens, no one antigen achieved a consistent level of response, with some conditions showing closer groupings of antigens (*e.g.* condition number 432, **Figure 2B**), while others displayed a more dispersed distribution (*e.g.* condition number 478, **Figure 2B**). Furthermore, where differences in MFI signal between antigens were more variable, there was a tendency for one of the four antigens to demonstrate a markedly greater or lesser signal; MSP1-19 and Hyp2 are considerably distinguished as the highest and lowest responding antigens respectively, compared to AMA1 and EPF1, in condition numbers 142 and 478 (**Figure 2B**).

Interestingly, six of eight of the conditions with high signal for all four antigens included nitrocellulose slides (four with Avid and two with Nova), and only one chemical slide type was featured in this group (the SciChip Aldehyde). Five of the six nitrocellulose slide conditions were also paired with the Jetstar protein print buffer C (AJ). All four blocking buffers appeared in the eight common conditions, and the PolyAn (PA) buffer occurred in five conditions.

Printing consistency and intra-assay reproducibility

To examine any effect of condition on consistency of the printing process and intra-assay reproducibility, we evaluated duplicate antigen spots in each array. Pearson's correlation coefficients between the 72 duplicate spots in all 480 tested conditions were generally high, though correlation was moderate to poor for a number of conditions, and some slide types were clearly more variable (*e.g.* 3D_epoxy_COP) than others (*e.g.* Nova) (**Figure 3A**). The eight common top performing conditions show a very high level of correlation between duplicate spots within arrays compared to all conditions (labelled black points in **Figure 3A**).

Overall antigen responses were then compared between the top eight conditions to quantify variability in reactivity. Only 21% (6/28) of all top condition comparisons achieved greater than 80% correlation (Pearson's correlation coefficient, r=0.83-0.98, **Figure 3B**), indicating some considerable inter-antigen variability. Despite this, three conditions, 98, 102 and 112, showed excellent signal correlation (r=0.91–0.98; **Figure 3B**), each of which shared both a common

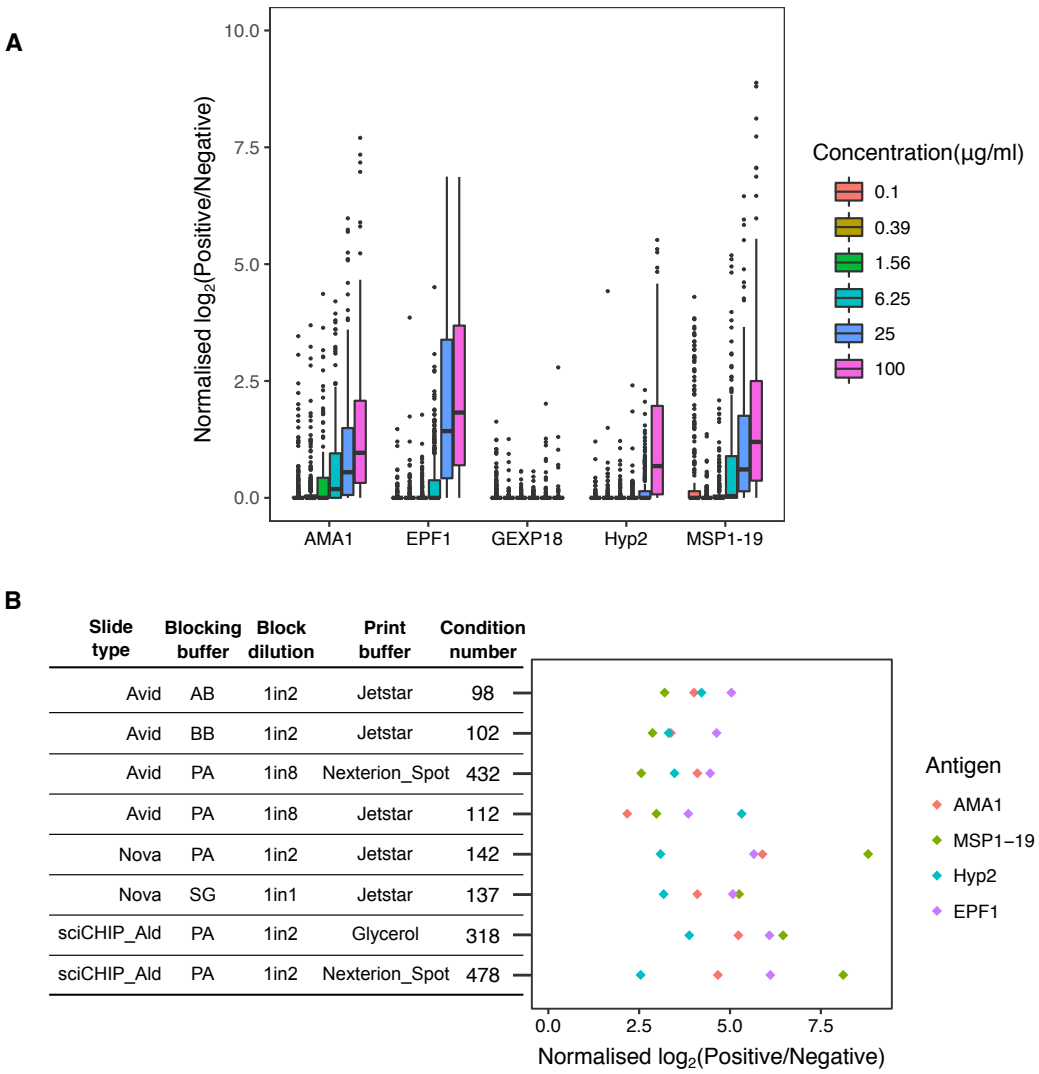

Figure 2. Antigen specific responses by experimental conditions. A The normalised log_2(Positive/Negative) values for all 480 conditions, for all antigens, printed in a six-point dilution curve. **B** Normalised log_2(Positive/Negative) values for the eight conditions which have a response in the top 10% for four antigens: AMA1, MSP1-19, Hyp2, and EPF1. AB – ArrayIt BlockIt; BB – Buffer B; PA – PolyAn Blocking Buffer; SG – Super G.

slide type and print buffer (Avid slides and Jetstar protein print buffer C; **Figure 2B**).

Inter-assay reproducibility

The eight top performing array conditions were reprinted and screened accordingly against the hyperimmune positive control and WHO Pf positive control standard (10/198; WHO Pf reference reagent) and UK naive pool in four replicate arrays. MFI signal detected in dilution curves of MSP1-19 and AMA1 were plotted showing highly reproducible curves of reactivity within each condition in both the CP3 hyperimmune pool (**Figure 4A**) and WHO_Pf+ (**Figure 4B**).

3.4 Discussion

Protein microarray platforms are increasingly utilised in malarial antigen biomarker identification and characterisation [13, 14, 23, 24, 26, 29–34], though little data on the standardisation and optimisation of such methods is published. A lack of understanding as to how contrasting technical approaches may influence microarray data and analysis makes comprehension of, and comparisons between, studies difficult. As such, we have evaluated the performance of a protein microarray, under a wide range of material, reagent and sample conditions, to identify optimised assay conditions for an representative panel of purified recombinant *P. falciparum* protein antigens, and have demonstrated the impact choice and implementation of materials may have on reported data.

Through the assessment of serum responses to a panel of antigenic targets, across 480 unique array conditions, we have found eight top performing combinations of materials and reagents common to four of five representative malarial antigens. Responses to the one excluded antigen (GEXP18) were not found to be well captured in any of these eight conditions. GEXP18 has been identified as a target of transient immunity during the early stages of infection and thus has been proposed as a marker of recent exposure [13]. As such, antibody responses to this antigen are likely poorly represented in the chronically and highly exposed individuals making up the hyperendemic positive pool, as shown by the low signal across all 480 conditions.

Overall, printing consistency (*i.e.* correlation of duplicated spots) was very good. Within the eight common conditions identified, three were found to share the same combination of slide type and print buffer (Avid nitrocellulose slide and ArrayJet buffer). This finding strongly suggests that the choice of slide substrate and reagents has an impact on detectable signal, likely by influencing factors such as background reactivity, protein stability and topographical epitope availability. Furthermore, the variability and magnitude of response to each antigen under the top eight conditions suggests that particular substrates and reagents are especially suited to some antigens; the size or polarity, for example, of specific amino acid residues in sequence may facilitate greater or lesser binding of antigen to substrate. The Nexterion Spot buffer — a printing buffer designed for DNA arrays — was unexpectedly identified within two of the eight common top conditions which suggests potential physical and chemical properties working in conjunction or synergy to best immobilise and present protein antigens in the context of an antibody capture assay.

In contrast to this study, most experimental microarrays will contain hundreds of protein antigen targets, of which the considerable majority will not have been individually evaluated under different array conditions. This leaves an assumption that all tested proteins will behave equally well on the chosen slide type and buffers; unlike nucleic acid based arrays, where targets to be spotted are homogenous mixtures of chemically and structurally similar molecules, protein arrays will consist of highly variable and complex molecular units. While it is largely

unfeasible to assess every target to be printed in multiple array conditions, our finding of conditions that produce highly replicable results for a small panel of diverse antigens is encouraging. Additionally, our methodology was restricted by a lack of specific positive controls for each antigenic target, although such limitations are generally unavoidable at the early stages of serological characterisation of antigens, and any experiment able to address this would be extremely large and unwieldy. However, the hyperimmune pool used adequately captured responses to four of the five targets screened and we consider our approach in this study to be broadly appropriate to our specific application aims and transferable to future iterations of a full antigen panel array.

Considering these shortcomings, the flexible nature and customisability of the platform provides the opportunity to develop our methodology as required in future. Groups or families of antigens with particular characteristics (*e.g.* genetic variants of protein families, or proteins containing a particular domain *etc.*) may demonstrate improved performance under alternative conditions, which may advocate for grouped sub-arrays depending on the context of the experimental questions to be addressed. As throughput is the driving force behind microarray technology, it is most likely that any such specialised sub-panels of proteins would be down-selected from a larger panel as targets of interest, and optimised specifically. In addition, we envisage the microarray system as the top level of a triage pipeline for biomarker discovery, where array data informs the transfer of targets to comparatively moderate throughput, but more sensitive multiplex (*e.g.* multiplex bead array) or monoplex field applicable (*e.g.* ELISA or lateral flow assay) systems.

The optimisation of a bespoke protein microarray for the serological interrogation of human IgG responses to *Plasmodium* antigens provides an excellent foundation for biomarker discovery, with considerable potential to expand into additional areas. At present, we have focussed on *P. falciparum* proteins, but the principles developed here are translatable to other *Plasmodium* species, and to non-malarial pathogens — either as independent or combined array panels. Similarly, our methodology is applicable to the investigation of additional antibody classes (*e.g.* IgM, IgA) and sub-classes (*e.g.* IgG1, IgG3), with the potential to multiplex these analytes where technically feasible. Such an integrated approach is highly desirable in terms of its ability to generate highly comprehensive data sets, while utilising significantly less materials, reagents and sample compared to alternative techniques.

Previous studies have considered the effects of microarray design on data quality [35–38], and we have similarly established that particular combinations of array conditions have considerable impact on the performance of the platform. This study has considered multiple aspects of array manufacture and processing using purified recombinant protein targets, allowing us to establish an application-specific platform, in-house. The dissection and fine-tuning of microarray methodologies allows greater confidence in data output compared to more generalised approaches. In summary, our results demonstrate the clear need to consider materials, reagents and procedures when utilising this assay platform, and highlight the potential to develop highly

specialised and specific protein microarrays for a range of future applications. Further, we have identified a number of reagents and materials that we recommend for use in the case of a protein microarray of recombinant *P. falciparum* antigens.

3.5 Methods

Recombinant antigens

The methods used in the production and purification of the recombinant antigens for this study are as previously described [39, 40]. Based on published literature the antigens were selected to represent historical markers of exposure — AMA1 [40] and MSP1-19 [39]; markers predicted to be associated with recent exposure — Hyp2 and GEXP18 [13]; and a member of the Maurer's cleft proteins implicated in merozoite release, previously characterised as a target of naturally acquired immunity — EPF1 [41]. Further details of all target antigens can be found in **Supplementary table 2** Following purification each protein was dialysed against 1xPBS, assayed for protein content and stored in aliquots at -20°C until needed.

Serum samples

Three serum samples were used to examine the effect of material and methodological differences in the manufacture and processing of the microarrays. The WHO *P. falciparum standard* (10/198; NIBSC, UK) and an adult hyperimmune pool (CP3; Tanzanian adult pooled hyperendemic sera) were used as malaria positive samples, and a pool of adult malaria-naive serum from UK residents (N=10) was used as a negative control.

Microarray fabrication

Arrays were printed using a non-contact (*i.e.* ink-jet based) microarray printer (Marathon Classic, ArrayJet, UK) in a 16-array format (2 x 8 layout) (**Figure 1**). Prior to printing, each protein was diluted to twice the desired final concentration in PBS, before mixing 1:1 with a microarray print buffer. Proteins were printed in duplicate within each array, across a 6-point dilution series with an assigned volume of 100 pL/spot. Following printing, slides were subjected to specific treatment conditions, defined by the manufacturer, to maximise protein immobilisation. Briefly, following printing nitrocellulose coated slides were left to dry inside the printer in climate controlled conditions of approximately 60% humidity and 18°C overnight. Chemically treated glass and copolymer slides were dried under vacuum in the presence of desiccant for 24 hours, followed by rehydration at 75% relative humidity for 48 hours in a sealed chamber. After immobilisation, all slides were stored under vacuum with desiccant at 4°C until required.

Further detail concerning materials used in the proceeding sections are summarised in **supplementary table 1**.

Microarray slide substrates

Ten microarray slide substrates were selected for assessment based on compatibility with protein printing applications: Nova and Avid nitrocellulose coated slides (GraceBioLabs); aldehyde (PolyAn and Scienion) treated slides; 2D epoxysilane (Scienion and PolyAn), 3D epoxysilane and 3D epoxysilane (cyclic olefin polymer) (both PolyAn) treated slides; 3D N-hydroxysuccinomide and 3D N-hydroxysuccinomide (cyclic olefin polymer) treated slides.

Print buffers

The operating parameters of the microarray printer required an optimal level of viscosity to be maintained in the final protein-print buffer mix. This was essential to achieve reproducible spot morphology and printing consistency between slides and print runs. To this end, we evaluated three print buffers: the proprietary Jetstar Optimum Microarray Printing Buffer (ArrayJet, UK), a custom prepared glycerol-based buffer [42], and, although specified for the printing of of oligonucleotides, we included the proprietary Nexterion Spot (Schott, Germany) to compare its performance with the protein specified print buffers.

Blocking buffers

Four blocking buffers were assessed for their ability to reduce non-specific protein binding, background fluorescence and for their compatibility with each slide substrate tested; BlockIt Blocking Buffer Plus (ArrayIt Corporation, CA, USA), SuperG Blocking Buffer (Grace BioLabs, OR, USA) and PolyAn Blocking Buffer (PolyAn, Germany). In addition, we also included blocking buffer B (1xPBS, 1% BSA, 0.05% Tween 20, 0.05% sodium azide, 0.5% polyvinyl alcohol, 0.8% polyvinyl pyrrolidone) [43], developed for use with the MagPix suspension bead array platform (Luminex Corp., TX, USA). Each blocking buffer was tested at the following dilutions: neat, 1/2, 1/4 and 1/8. Where appropriate, blocking buffers were diluted with 1xPBS.

Screening for immunoglobulin G reactivity

Slides were loaded into 4 x 16 well hybridisation cassette (ArrayIt Corporation, CA, USA) to allow samples to be processed in parallel. 200 µl of blocking buffer was applied to each array, the wells sealed to prevent evaporation (repeated at each incubation step), and incubated for one hour, on an orbital shaker set to 100 rpm, at room temperature (RT). The arrays were washed by adding 200 µl of PBS/Tween (0.01%) (Sigma) to each well and left shaking (100 rpm) for two

minutes/wash for a total of three washes. Care was taken not to allow arrays to dry out between wash steps.

The pooled serum samples were diluted to 1/400 in blocking buffer and 100 µl added to each array and left to incubate for one hour at RT, at 100 rpm. Arrays were washed as before and 100 µl of goat anti-human IgG Alexa Fluor 633 conjugate antibody (Invitrogen) diluted to 1/1000 in blocking buffer, was applied to each slide and incubated for one hour at RT, shaking at 100 rpm and protected from light. Arrays were washed three times in PBS/T, followed by a single wash in ultrapure water (Milli-Q). The slides were then centrifuged at 1800 xg to dry and stored with desiccant, under vacuum, at 4°C until use.

Imaging of processed slides

Array images were acquired using a GenePix 4300A Microarray Scanner (Molecular Devices, CA, USA) set to a resolution of 5 µm. Due to the contrasting treatment of each array, the photomultiplier tube (PMT) adjustments (*i.e.* the amplification of detected signal) were made per array condition to maximise signal, whilst minimising signal saturation within antigen spots. Images were analysed using GenePix Pro Microarray Analysis Software v7.0 (Molecular Devices), utilising an array specific GenePix Array List (GAL) file to extract raw median fluorescence intensity (MFI) values for antigen spots (foreground) and local spot backgrounds (background).

Analysis

Data extracted from images were analysed using R (Foundation for Statistical Computing, Austria, https://www.R-project.org/). Raw data were processed based on previously described methodology [32, 44]. Briefly, raw background values were first corrected using the backgroundCorrect (normexp) function of the *limma* package, and subtracted from raw foreground MFI values. Corrected background-subtracted MFI values were then Log2-transformed, and finally normalised against the mean value of buffer control spots. Spots were printed in duplicate and MFI values were averaged, on the condition that the MFI of each duplicate was within twice that of the other. Where this rule was violated, results for that pair of spots was excluded from further analysis. Sample reactivity to purified GST was subtracted from the signal for all GST-tagged recombinant proteins on a per sample basis to account for any background reactivity to the fusion tag. Where appropriate, negative normalised $\log_2$(MFI) values were adjusted to zero.

3.6 Supplementary material

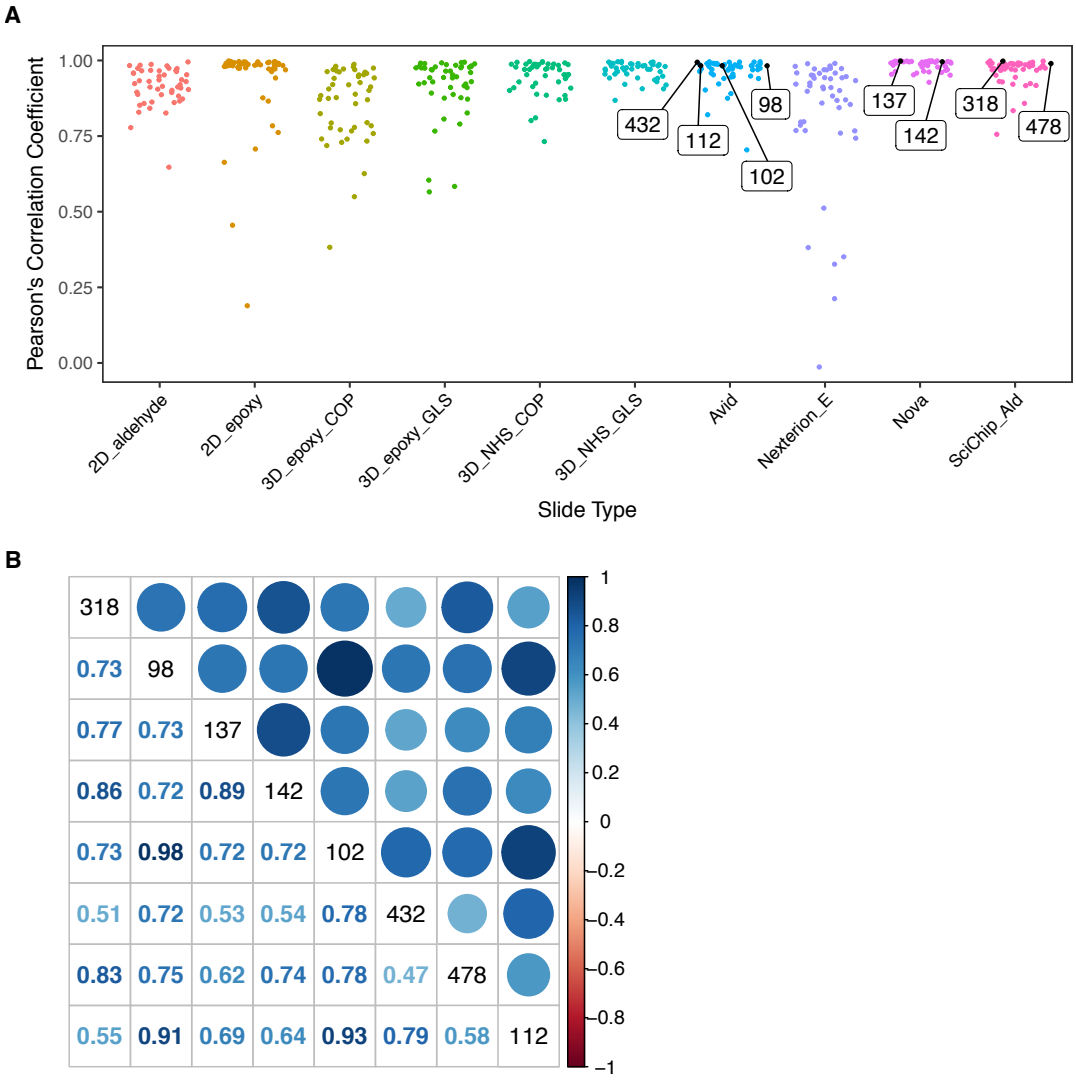

Figure 3. Correlation of responses between experimental conditions. A Correlation between all duplicates for each of 480 conditions. Points in black indicate the Pearson's correlation coefficient for the 8 selected conditions, indicated with labels. **B** Overall correlation between all $\log_2$(Positive/Negative) data points for each of the 8 selected conditions. Values shown in the left of the matrix are absolute Pearson's correlation coefficients, while colour scale and circle size represent the strength of relationship. For both plots normalised $\log_2$(MFI) values were used without adjusting negative values to zero.

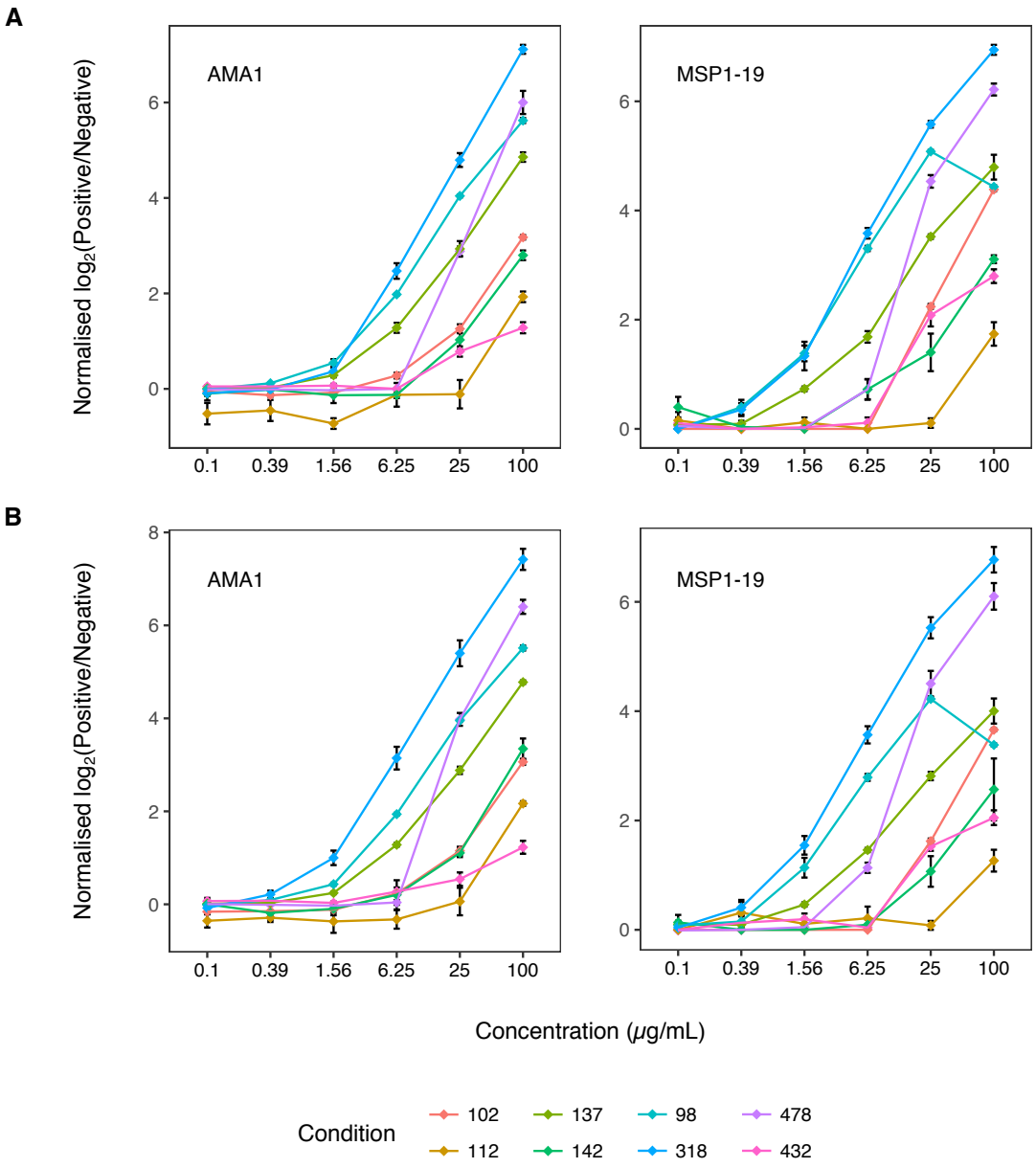

Figure 4. Reproducibility of captured responses in the top eight conditions. Responses in the positive control samples **A** CP3 and **B** NIBSC 10/198 (WHO reference reagent). Data are the geometric mean ± SEM (black bars) of the normalised $\log_2$(Positive/Negative) from 4 replicate arrays of each of the 8 selected conditions.

Supplementary table 1. Microarray reagents and materials.

Experimental condition	Reagent	Abbreviation	Manufacturer
Slide type	ONCYTE AVID; Nitrocellulose	AVID	GraceBio Labs
	ONCYTE NOVA; Nitrocellulose	NOVA	GraceBio Labs
	3D Epoxysilane; Chemical	3D_epoxy_GLS	PolyAn
	3D Epoxysilane (COP[a] slide); Chemical	3D_epoxy_COP	PolyAn
	2D Epoxysilane; Chemical	2D_epoxy	PolyAn
	3D N-Hydroxysuccinimide; Chemical	3D_NHS_GLS	PolyAn
	3D N-Hydroxysuccinimide (COP[a] slide); Chemical	3D_NHS_COP	PolyAn
	2D Aldehyde; Chemical	2D_aldehyde	PolyAn
	sciCHIP Aldehyde; Chemical	sciCHIP_ald	Scienion
	Nexterion Epoxysilane; Chemical	Nexterion_E	Scienion
Print buffer	JETSTAR protein printing buffer C	Jetstar	ArrayJet
	Nexterion Spot	Nexterion_spot	Schott
	Glycerol print buffer	Glycerol	-
Blocking buffer	BlockIt Plus	AB	Arrayit Corp
	Super G	SG	GraceBio Labs
	PolyAn Blocking Buffer	PA	PolyAn
	Buffer B	BB	-

[a] COP - Cyclic olefin polymer; plastic-based slide

Supplementary table 2. Target antigen details.

Gene ID	Name	Description	Allele
PF3D7_1133400	AMA1	Apical membrane antigen 1	FV0
PF3D7_0930300	MSP1	Merozoite surface protein 1	3D7
PF3D7_1002000	Hyp2	Plamodium exported protein	3D7
PF3D7_0402400	GEXP18	Plasmodium exported protein	3D7
PF3D7_1101800	EPF1	Exported protein family 1	3D7

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3.7 Additional work and figures

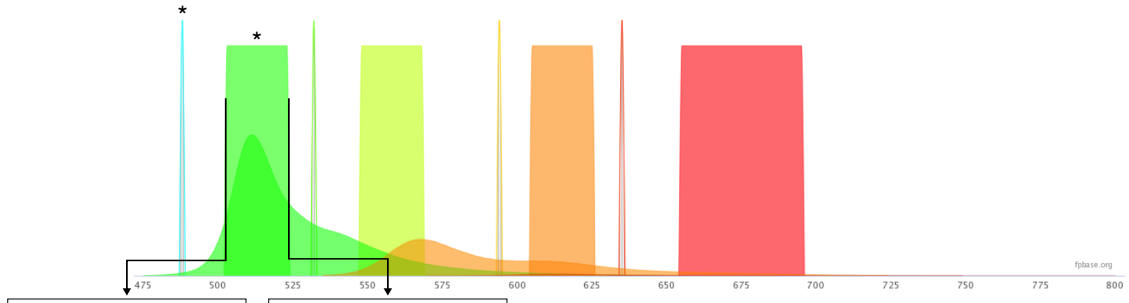
Multiplex microarray system upgrade

The experiments described within this chapter are based on the detection of human IgG by a fluorescently labelled α-huIgG antibody. Briefly, a laser within the microarray scanner energetically excites the fluorescent label (fluorophore) at a specific wavelength, leading to the emission of photons at a lower energy state. These photons are filtered by wavelength to increase specificity, detected and converted to median fluorescence intensity (MFI). As different fluorophores are excited, and emit photons, at different wavelengths it is possible to differentiate between labels even when mixed. The GenePix 4300A Microarray Scanner used in the study above is fitted with four lasers — 635nM red, 594nM yellow, 532nm green and 488nM blue — which facilitates the ability to multiplex at least four different fluorophores within a single experiment. The ability to simultaneously detect multiple analytes within a sample is highly desirable from a logistical perspective, and significantly increases the ability to characterise potential biomarkers. To this end, the microarray platform was developed and optimised to allow the multiplex detection of the four human IgG sub-classes: IgG1, IgG2, IgG3 and IgG4.

To achieve this objective, four fluorophores and compatible fluorescence bandpass filters were identified to allow distinct separation of emission spectra (**Additional figure 1**). These custom filters were purchased and fitted to the microarray scanner, and bespoke α-huIgG1-4 fluorophore conjugated reagents were procured.

Additional figure 1. Fluorophore emission profiles by laser and filter combinations. Asterisks denote the active laser (thin peak), filter (solid bar) and the associated fluorophore (text in tables). Black brackets with arrows indicate the lower and upper limit of the filter bandpass between which fluorescence is detected, and tables below show the percentage detected emission of each fluorophore at that limit. Adapted from FPbase Spectra viewer (https://www.fpbase.org/spectra/).

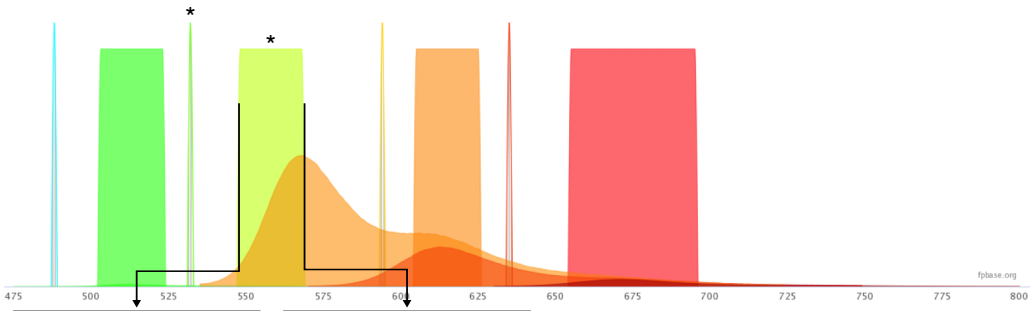
488nM laser; 513/20 filter; BODIPY FL



Fluorophore	% emission
BODIPY FL*	0.24
Texas Red	0.00
AF555	0.00
AF647	0.00

Fluorophore	% emission
BODIPY FL*	0.24
Texas Red	0.00
AF555	0.00
AF647	0.00

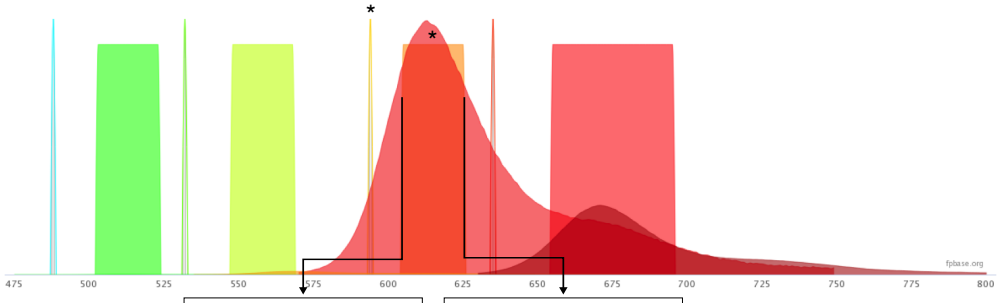
532nM laser; 558/20 filter; AF555



Fluorophore	% emission
BODIPY FL	0.00
Texas Red	0.00
AF555*	0.08
AF647	0.00

Fluorophore	% emission
BODIPY FL	0.00
Texas Red	0.00
AF555*	0.50
AF647	0.00

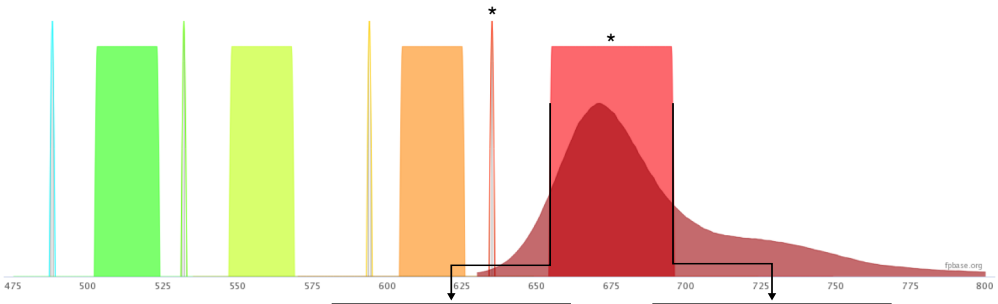
594nM laser; 615/20 filter; Texas Red



Fluorophore	% emission
BODIPY FL	0.00
Texas Red*	0.82
AF555	0.01
AF647	0.00

Fluorophore	% emission
BODIPY FL	0.00
Texas Red*	0.75
AF555	0.00
AF647	0.00

635nM laser; 675/40 filter; AF647

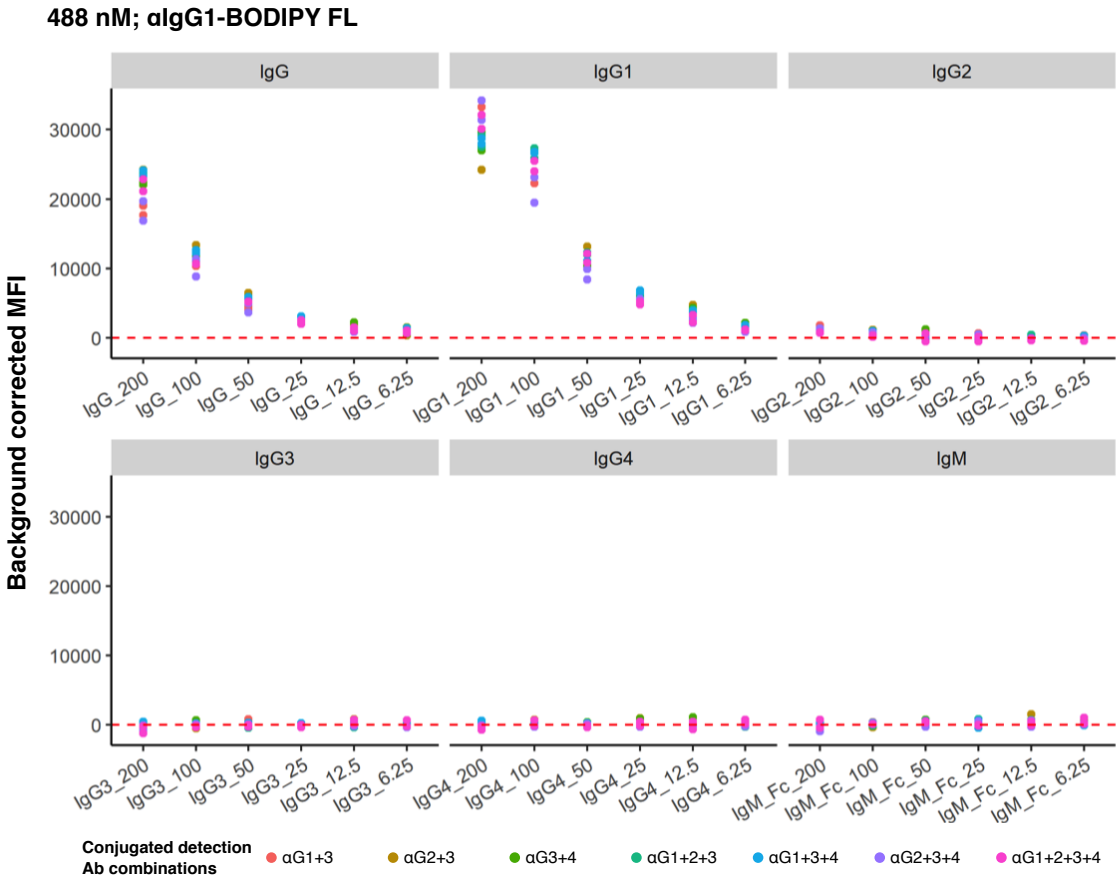


Fluorophore	% emission
BODIPY FL	0.00
Texas Red	0.00
AF555	0.00
AF647*	0.35

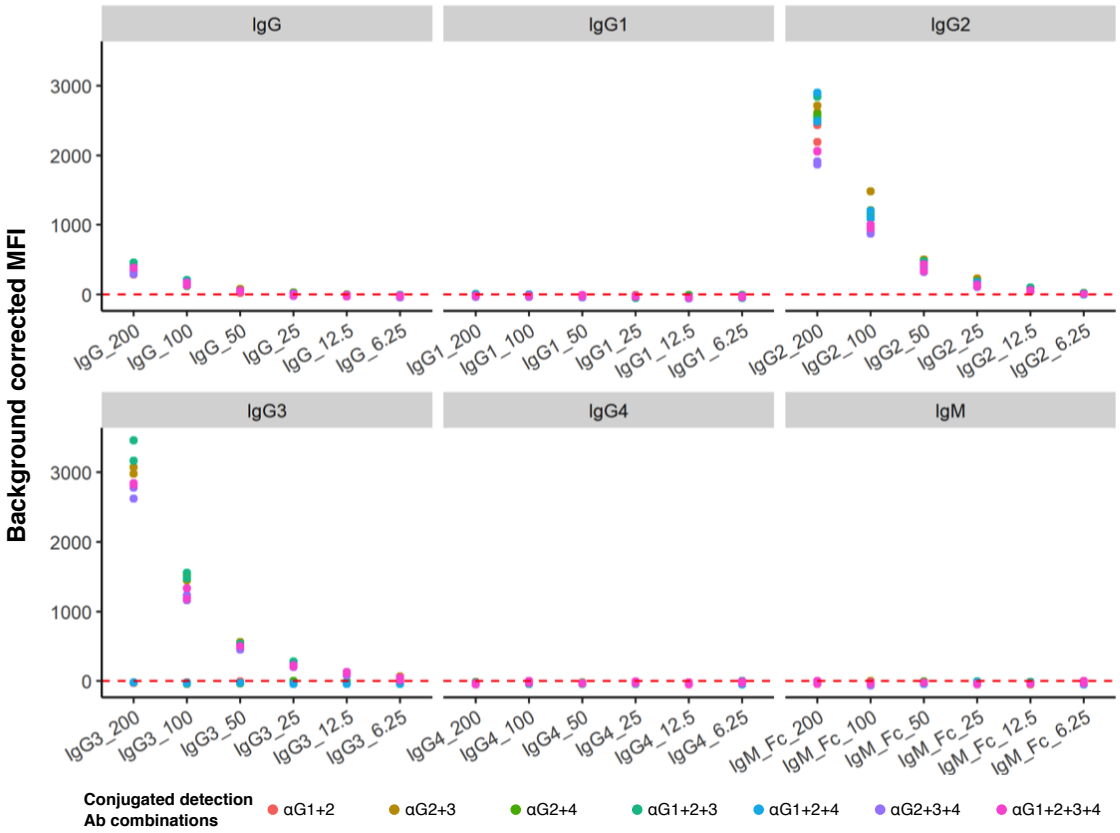
Fluorophore	% emission
BODIPY FL	0.00
Texas Red	0.00
AF555	0.00
AF647*	0.29

Initial tests of specificity in multiplex were conducted by screening detection antibody combinations at 1/1000 dilutions against standard curves of purified total IgG, IgG1, IgG2, IgG3, IgG4 and IgM (**Additional figure 2**). All detection antibodies showed good specificity for their target, though some unexpected signal was observed in the 594 nM (αIgG2-Texas Red) channel where αIgG3 was included in the detection antibody mixture, suggesting a degree of fluorescence spill-over. To address this issue, a titration of αIgG3 was carried out.

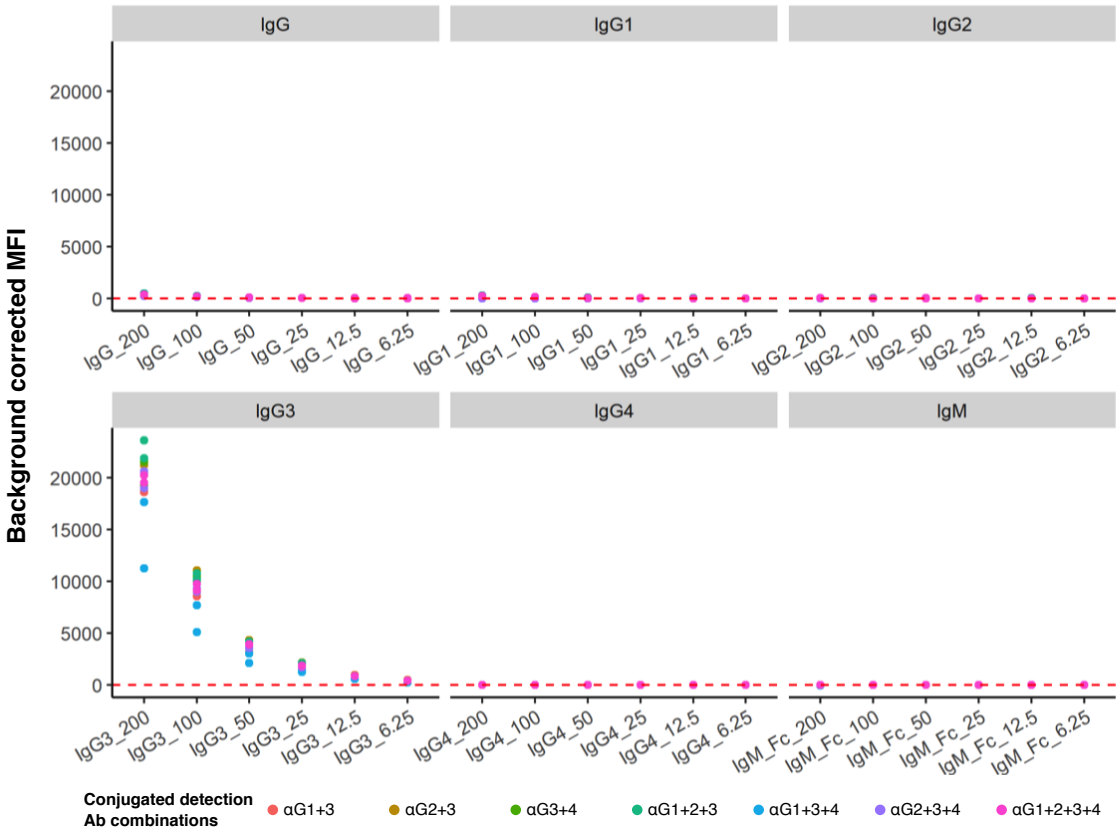
Additional figure 2. Conjugated detection antibody specificities in multiplex. Combinations of fluorescently conjugated detection antibodies were screened against duplicate purified standard curves of total IgG, IgG1-4 and IgM. Point colours indicate the detection antibody combination used. Plot titles indicate the active laser and the conjugated detection antibody expected to show signal against its target standard curve.

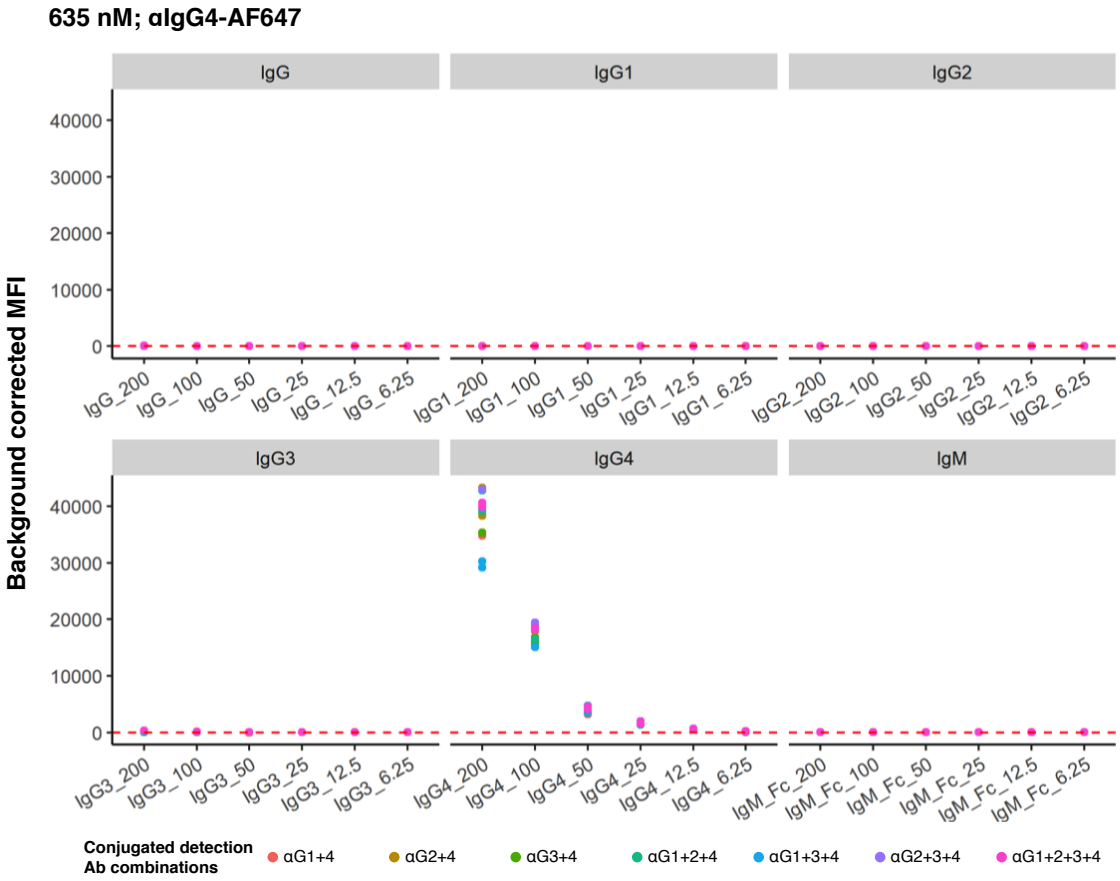


594 nM; αIgG2-Texas red

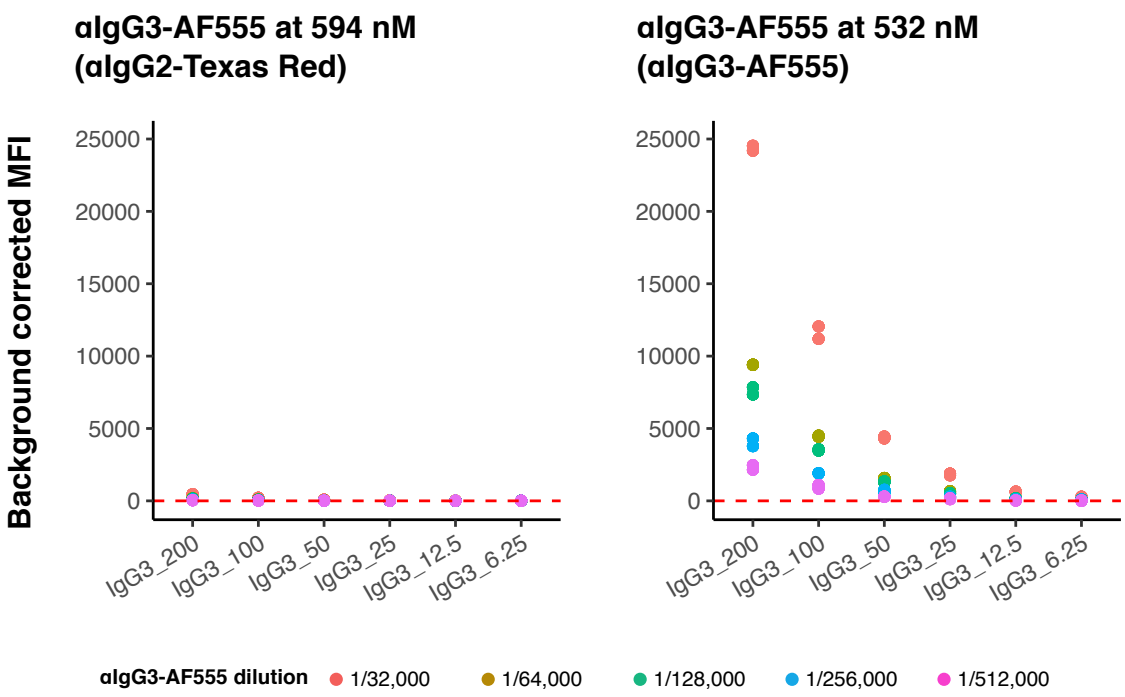


532 nM; αIgG3-AF555





A dilution curve of αIgG3 from 1/32,000 to 1/512,000 was screened against the same purified standard curves described above. Over all dilutions tested, signal in the 594 nM (αIgG2-Texas Red) channel were greatly reduced, whilst signal in the 532 nM (αIgG3-AF555) channel was maintained (**Additional figure 3**).



Additional figure 3. Titration of αIgG3-AF555. Signal in the 594 nM (αIgG2-Texas Red) channel (left) and the 532 nM (αIgG3-AF555) channel (right) for a titration of αIgG3-AF555 against the purified IgG3 standard curve.

Chapter 4

Plasmodium falciparum serology: A comparison of two protein production methods for analysis of antibody responses by protein microarray

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Student ID Number	1404292/RITD	**Title**	Mr
First Name(s)	Tate		
Surname/Family Name	Oulton		
Thesis Title	Development and validation of serological assays to evaluate malaria transmission		
Primary Supervisor	Chris Drakeley		

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SECTION E

Student Signature	[redacted]
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Title:

Plasmodium falciparum serology: A comparison of two protein production methods for analysis of antibody responses by protein microarray

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Abbreviations:

IVTT, in vitro Transcription/Translation

Keywords:

Malaria, Protein array, Protein microarray, Reverse phase protein array, Serology

Abstract

The evaluation of protein antigens as putative serologic biomarkers of infection has increasingly shifted to high-throughput, multiplex approaches such as the protein microarray. In vitro

transcription/translation (IVTT) systems — a similarly high-throughput protein expression method — are already widely utilised in the production of protein microarrays, though purified recombinant proteins derived from more traditional whole cell based expression systems also play an important role in biomarker characterisation. Here we have performed a side-by-side comparison of antigen-matched protein targets from an IVTT and purified recombinant system, on the same protein microarray. The magnitude and range of antibody responses to purified recombinants was found to be greater than that of IVTT proteins, and responses between targets from different expression systems did not clearly correlate. However, responses between amino acid sequence-matched targets from each expression system were more closely correlated. Despite the lack of a clear correlation between antigen-matched targets produced in each expression system, our data indicate that protein microarrays produced using either method can be used confidently, in a context dependent manner, though care should be taken when comparing data derived from contrasting approaches.

4.2 Introduction

To date, the majority of malaria serologic studies have focussed on antibody responses to a small number of well-characterised, highly immunogenic *Plasmodium falciparum* antigens that have proven to be reliable markers of exposure to infection [1–8]. However, *P. falciparum* expresses more than 5000 proteins, each a potential antibody target [9, 10]. Advances in technology have led to the development of new assay platforms that allow proteome scale investigation of antibody responses, such as the protein microarray [11, 12] — boasting significantly greater experimental throughput than more classical monoplex methods (*e.g.* ELISA) [13, 14]. The ability to simultaneously interrogate large numbers of putative targets, using low volumes of sample, significantly increases the rate at which an individual's antibody responses to antigens can be characterised. As such, protein microarray based approaches to biomarker identification and humoral response profiling in malaria, and other infectious diseases, have been increasingly adopted [15–24].

One widely utilised form of the protein microarray is based on an in vitro transcription/translation (IVTT) system [25] — where protein products are produced through a PCR, in vivo recombination cloning and an in vitro expression pipeline, before being printed onto arrays [18]. In principle, whole organism proteome microarrays can be fabricated simply and quickly, enabling analysis of all potential protein driven immune responses to a pathogen. Cell-free synthesis (CFS) is a technique first established over 50 years ago as a means to dissect the molecular mechanisms around protein expression. More recently, the technique has been used as a high throughput expression platform to explore a number of diverse biological processes [26, 27]. At its simplest, the approach utilises the crude extract containing the transcription and translation machinery from the cell, performing the process of protein expression without the constraints of the cell. This allows a wide variety of proteins to be expressed including those that would be deemed toxic if expression was attempted within the confines of the cell membrane [28]. CFS systems based on *Escherichia coli* (*E. coli*) are among the most widely used of the IVTT systems [27] and have helped to transform the narrative around a number of areas including biomarker discovery for infectious diseases [18, 29, 30]. Despite the widespread uptake of the approach there remain some issues around the technique. This includes significant heterogeneity of expression, leading some research groups to describe the mechanisms of the process as a "black box". Therefore, the inherent heterogeneity between products is not assessed for every target making it difficult to normalise for reactivity between protein spots, which represent an impure mix of *E. coli* and target protein. In addition to the *E. coli* cell-free expression platform, other approaches have been employed in the characterisation of protein targets for immunological assessment. The wheat germ cell-free expression system in particular has also proven to be an important platform in the advancement of biomarker discovery and malaria vaccine research [31–34]. This is not the focus of the current study.

In contrast to the IVTT array methodology, the printing of purified proteins is cheaper and typically more quantifiable. Uniform amounts of product can therefore be incorporated into arrays, increasing confidence when comparing quantitative antibody responses between antigenic targets [35] and assessing relative immunogenicity. The process can be modified to support the scale up of recombinant proteins, and furthermore, affinity purification of protein targets reduces the risk of undesired background reactivity due to expression system components, and in part truncated proteins. However, the time required to produce panels of purified proteins is far in excess of the IVTT system, particularly for large numbers of targets, unless supported by an automated production platform [36–38]. For both the IVTT and purified protein *E. coli* systems, although the production of complex conformational proteins is possible it can sometimes be a challenge [39, 40]. These challenges are in part due to the expression of proteins foreign to the bacteria, the speed at which bacteria express proteins, only partially mitigated with a reduction in expression temperature; and the lack of essential molecular chaperones to aid correct folding/refolding of proteins [41–43].

Here we present a comparison between IVTT based and purified proteins on a single microarray. For clarity proteins produced using the IVTT system will simply be referred to as IVTT proteins, and those produced by conventional *E.coli* expression will be referred to as purified proteins. Matched malarial protein targets from each methodology were assessed for comparative reactivity in serum from Ugandan participant samples (n = 899) [44] to determine the suitability of each approach in the context of high-throughput profiling of serological responses to protein antigens.

4.3 Methods

Ethics statement

All serum samples were collected after written informed consent from the participant or their parent/guardian. The protocol for sample collection was reviewed and approved by the Makerere University School of Medicine Research and Ethics Committee (#2011–149 and #2011–167), the London School of Hygiene and Tropical Medicine Ethics Committee (#5943 and #5944), the Durham University School of Biological and Biomedical Sciences Ethics Committee, the University of California, San Francisco, Committee on Human Research (#11–05539 and #11–05995) and the Uganda National Council for Science and Technology (#HS-978 and #HS-1019).

Serum samples

Sera were originally collected as part of a comprehensive longitudinal surveillance study conducted in three sub-counties in Uganda (Walukuba, Jinja District; Kihihi, Kanungu District, and Nagongera, Tororo). The study design and methods have been previously reported and are described in detail elsewhere [44]. A sub-selection of samples (n = 899) was made from individuals across a breadth of recorded clinical episodes of malaria to ensure a range of sero-reactivity.

Protein targets

Purified protein expression

Recombinant proteins were generated and expressed in *Escherichia coli* as glutathione S-transferase (GST)-tagged fusion proteins using previously described methods: PfMSP1-19 [45]; MSP1 block 2 [46]; ACS5, ETRAMP4 & HSP40 [19]; ETRAMP5 [19, 47]; EBA181[48]; MSP4 [49]; MSP5 [50]; MSP7 [51]; and GAMA [52]. The exception to this was PfAMA1, which was expressed as a histidine tagged protein in *Pichia pastoris* [53]. Purification of the expressed proteins was performed using affinity chromatography (Glutathione Sepharose 4B (GE Healthcare Life Sciences) or HisPur Ni-NTA (Invitrogen) resins for GST and His tagged proteins, respectively). Protein concentration was assessed using the Bradford protein assay, with quality, and purity assessed by resolution on a 4-20% gradient SDS-PAGE.

IVTT protein expression

An IVTT system was used to express proteins of interest as previously described [18]. Briefly, *P. falciparum* DNA (3D7 isolate) coding sequences were PCR-amplified and cloned into T7 expression vectors via homologous recombination. Target sequences were expressed at 21°C for 16h in *E. coli*-based, cell-free transcription/translation reactions, and products were printed onto arrays as unpurified, whole reaction mixtures.

Overview of compared IVTT and purified protein antigens

We assessed antibody responses to protein targets mapping to eleven antigens (*i.e.* distinct gene products), each represented on the array by at least one IVTT and one purified protein target. Full details are in **table 1** and **supplementary table 1**. The number of purified protein targets varied according to availability, while the number of IVTT targets was dependent on the exon composition of each the gene sequence; multiple exon sequences were expressed as multiple protein targets based on exon delineation. Similarly, single exon gene sequences were generally expressed as a single protein. As a result, of the 11 antigens investigated, 8 were repres-

ented by >1 IVTT or purified protein target; 5 had >1 IVTT protein target (EBA181, HSP40, MSP1, MSP4 and MSP5) and 5 had >1 purified protein target (ACS5, ETRAMP4, ETRAMP5, HSP40 and MSP1). Near identical IVTT proteins (1 terminal amino acid difference in length) were produced independently and printed in parallel for two antigens: MSP4 and MSP5 as expression controls. Sequence information used in the design and expression of the purified *E. coli* proteins were generally smaller than the equivalent proteins expressed in the IVTT cell-free systems. This was done to limit the sequence length to below 1kb as expression of proteins larger than 1kb in *E. coli* can contribute to poor or failed expression yields [42, 43]. Truncation of target sequences was based on *in silico* mapping of each protein sequence to focus on regions of predicted immunogenicity based on the in silico analysis. Empty GST vectors were expressed and the purified GST used in background correction for proteins with this tag. His-tag vector was not expressed as it has proven impossible to express and purify the 6xhistidine tag in isolation.

Table 1. Description of antigens and their corresponding IVTT and purified protein targets.

Protein	Description	Full length (amino acids)	Protein target/expression system	Size (Start amino acid - End amino acid)
ACS5	Acyl CoA synthase	811	IVTT_1	811 (1–811)
			Pure_1	117 (294–410)
			Pure_2	160 (414–573)
			Pure_3	150 (578–727)
AMA1	Apical membrane antigen 1	622	IVTT_1	622 (1–622)
			Pure_1	450 (97–546)
EBA181	Erythrocyte binding antigen 181	1567	IVTT_1	754 (1–754)
			IVTT_2	752 (737–1488)
			Pure_1	585 (755–1339)
ETRAMP4	Early transcribed membrane antigen 4	136	IVTT_1	136 (1–136)
			Pure_1	25 (28–52)
			Pure_2	61 (76–136)
ETRAMP5	Early transcribed membrane antigen 5	181	IVTT_1	181 (1–181)
			Pure_1	86 (26–111)
			Pure_2	47 (135–181)
GAMA	GPI-anchored membrane antigen	738	IVTT_1	738 (1–738)
			Pure_1	99 (68–166)
HSP40	Heat shock protein 40 type II	402	IVTT_1	134 (80-213)
			IVTT_2	171 (213-401)
			Pure_1	83 (71-153)
			Pure_2	189 (214-402)
MSP1	Merozoite surface protein 1	1720	IVTT_1	870 (1-870)
			IVTT_2	868 (853-1720)
			Pure_1	45 (64-108)
			Pure_2	35 (54-63;109-133)
			Pure_3	116 (1605-1720)
MSP4	Merozite surface protein 4	272	IVTT_1	162 (1-162)
			IVTT_2	161 (1-161)
			IVTT_3	110 (163-272)
			Pure_1	65 (43-107)
MSP5	Merozoite surface protein 5	272	IVTT_1	172 (1-172)
			IVTT_2	171 (1-171)
			Pure_1	61 (147-207)
MSP7	Merozoite surface protein 7	351	IVTT_1	351 (1-351)
			Pure_1	175 (177-351)

Protein microarray

Prior to printing, Tween 20 was added to purified proteins to yield a final concentration of 0.001% Tween 20. Arrays were printed onto nitrocellulose-coated slides (AVID, Grace Bio-Labs, Inc., Bend, OR, USA) using an Omni Grid Accent microarray printer (Digilabs, Inc., Marlborough, MA, USA). Alongside proteins of interest, buffer (PBS) and no-DNA (empty T7 vector reactions) were included as controls to allow for background normalisation of purified and IVTT proteins respectively.

Sample probing

For analysis of antibody reactivity on the protein microarray, serum samples were diluted 1:200 in a 3 mg mL^{-1} *E. coli* lysate solution in protein arraying buffer (Maine Manufacturing, Sanford, ME, USA) and incubated at room temperature for 30 min. Arrays were rehydrated in blocking buffer for 30 min. Blocking buffer was removed, and arrays were probed with pre-incubated serum samples using sealed, fitted slide chambers to ensure no cross-contamination of sample between pads. Slides were incubated overnight at 4°C with agitation. Arrays were washed five times with TBS-0.05% Tween 20, followed by incubation with biotin-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:200 in blocking buffer at room temperature. Arrays were washed three times with TBS-0.05% Tween 20, followed by incubation with streptavidin-conjugated SureLight P-3 (Columbia Biosciences, Frederick, MD, USA) at room temperature protected from light. Arrays were washed three times with TBS-0.05% Tween 20, three times with TBS, and once with water. Arrays were air dried by centrifugation at 500 xg for 5 min and scanned on a GenePix 4300A High-Resolution Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA). Target and background intensities were measured using an annotated grid file (.GAL).

Data normalisation

Microarray spot foreground and local background fluorescence data were imported into R (Foundation for Statistical Computing, Vienna, Austria) for correction, normalisation and analysis. Local background intensities were subtracted from foreground using the backgroundCorrect function of the *limma* package [54]. The backgroundCorrect function was then further applied to GST-tagged purified proteins, whereby background-corrected GST fluorescence was subtracted from background-corrected target fluorescence to account for any GST-specific reactivity in samples. All data were then $\log_2$ transformed and the mean signal intensity of buffer and no-DNA control spots were subtracted from purified and IVTT proteins respectively to give a relative measure of reactivity to targets over background (**Supplementary figure 1**) [22].

4.4 Results

Table 1 summarises the purified and IVTT protein targets for each antigen, with further detail in **supplementary table 1**. In brief, we assessed IgG antibody responses to 35 antigenic targets, derived from 11 well-characterised *P. falciparum* protein antigens (distinct gene products). Each antigen was represented by at least one IVTT and one purified protein target.

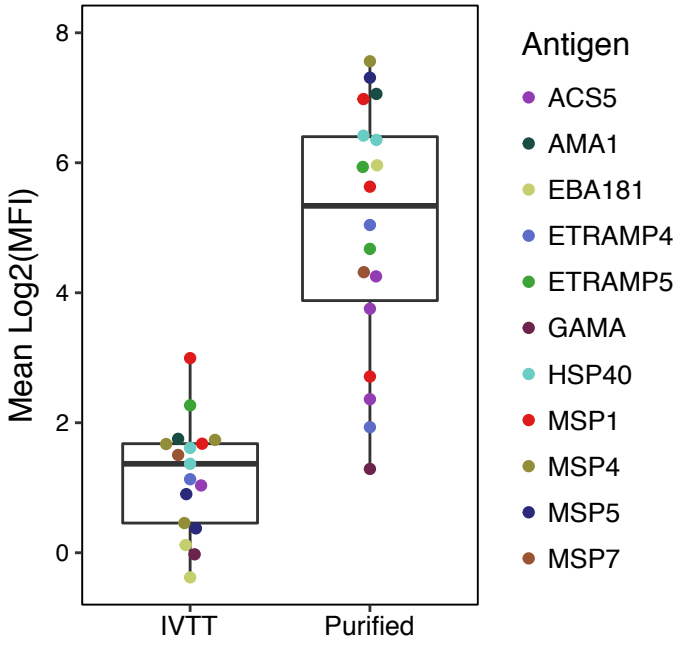


Figure 1. Mean magnitude of antibody responses to targets. The mean magnitude of response of each protein target stratified by expression system, presented with median and interquartile range of all mean responses.

Magnitude of responses between expression systems

The magnitude of response to all protein targets was compared by antigen to evaluate differences in seroreactivity between IVTT derived and purified protein targets. As expected, responses varied significantly between antigens and between the protein targets mapping to each antigen.

Mean responses to all targets were compared by expression system (**Figure 1**) revealing a greater range of response to purified proteins (IQR Log2MFI = 3.88–6.40) than IVTT proteins (IQR Log2MFI 0.46–1.68), and a greater magnitude of response to purified than IVTT targets (p = <0.001). Similarly, the range and median intensity of individual antibody responses was found to be greater for purified proteins than their IVTT counterparts (*e.g.* AMA1 — IVTT_1, median [IQR] Log2MFI = 1.66 [0.80–2.53]; Pure_1, median [IQR] Log2MFI = 7.92

[6.16–8.52]) for all targets (p = <0.001) except MSP1 Pure_2, which more closely reflected the level of reactivity to the two MSP1 IVTT targets (**Figure 2**).

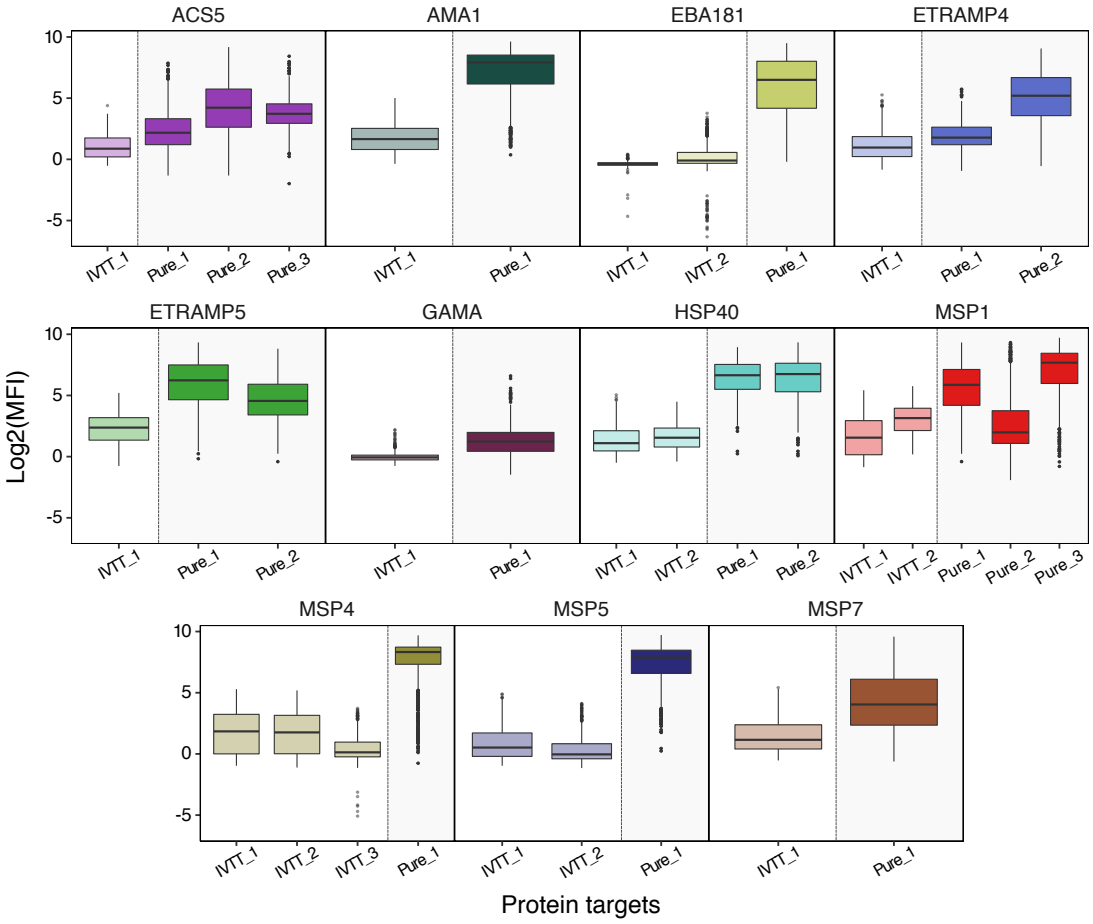


Figure 2. Magnitude and range of response to IVTT and purified proteins. All sample responses (n = 899) to all protein targets grouped by antigen, presented with median and interquartile range.

Correlation of responses between antigen matched targets

Considering all at least partially sequence matched IVTT and purified protein targets (*i.e.* excluding pairwise comparisons where purified protein sequence were completely non-overlapping with IVTT sequence for the same antigen) there was no evidence for a general correlation in mean response between expression platforms (Spearman's rho (r_s) = 0.279, p = 0.23). Antibody responses to all protein targets for each antigen were therefore compared individually (representative example in **figure 3** and all antigens in **supplementary figure 2**). This allowed for comparison between sequence matching IVTT and purified protein targets (*e.g.* HSP40 IVTT 2 vs. HSP40 Pure 2), non-matching IVTT and purified protein targets (*e.g.* HSP40 IVTT 2 vs. HSP40 Pure 1), and matching or non-matching targets produced in the same system (*e.g.* HSP40 IVTT 1 vs. HSP40 IVTT 2). Correlations were highly variable (r_s = 1.00 to -0.045)

though all but one (GAMA; r_s = -0.045, p = 0.17) demonstrated a degree of positive, if not always statistically significant, association.

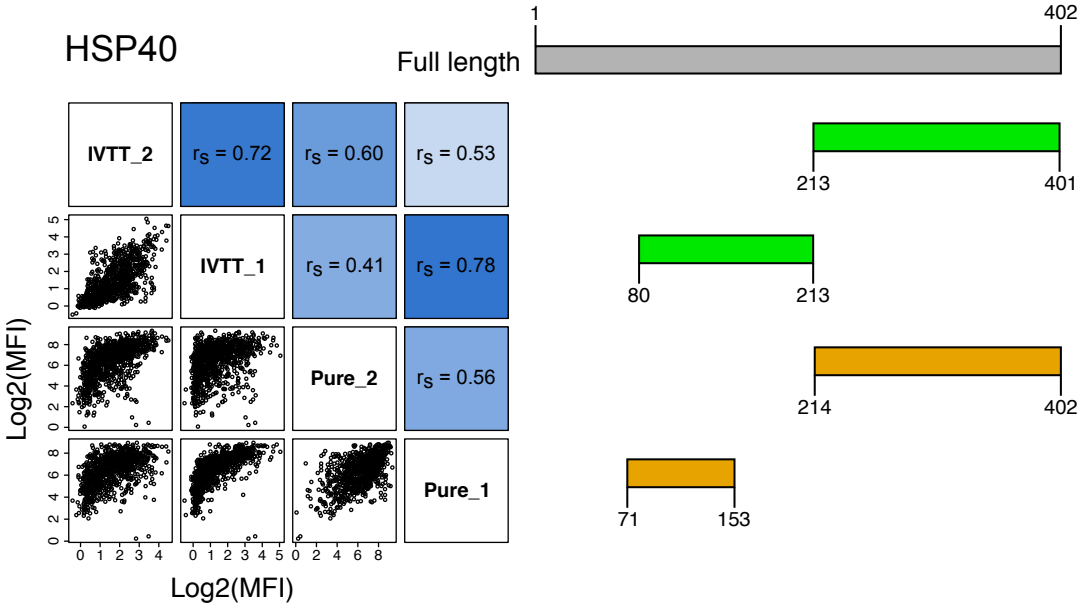


Figure 3. Correlation of antibody responses and sequence mapping. A representative example correlogram of multiple antigen-matched targets (left). Spearman's rank correlation reported (r_s) and increasing blue colour scale indicates relative strength of correlation based on calculated correlations for all proteins included in this analysis. Protein schematic (right) represents amino-acid aligned representation of IVTT (green) and purified (orange) proteins to the full-length native protein (grey). Proteins in the correlogram and schematic are correspondingly aligned. Corresponding axes are adjacently below or to the left of each protein.

Multiple IVTT targets were produced for EBA181, HSP40, MSP1, MSP4 and MSP5. For all other than MSP5, non-sequence matching IVTTs were produced; correlation co-efficients for these targets were between 0.37 and 0.73 (**Supplementary figure 2**). For EBA181 and MSP1, IVTT targets overlap by 17 amino acids — equivalent to a small peptide in terminal regions unlikely to cover immunogenic epitopes. As such, these targets were considered non-overlapping. For MSP4 and MSP5, duplicate IVTT protein products were generated for each gene, with each duplicate protein identical to the other except for the omission of one N- or C-terminal amino acid. These respective targets resulted in near perfect correlation of antibody responses (MSP4 r_s = 1.00, p = <0.001; MSP5 r_s = 0.94, p = <0.001). Multiple purified protein targets were produced for ACS5, ETRAMP4, ETRAMP5, HSP40 and MSP1 — none of which overlap. Correlation between these purified protein targets in each antigen varied between 0.31 and 0.59 (**Supplementary figure 2**).

For the 8 antigens with >1 IVTT or purified protein target, the greatest level of correlation was found between an IVTT and purified target in 4/8 instances; between two IVTT targets (IVTT-IVTT) in 3/8 instances; and between two purified targets (purified-purified) in 1/8 in-

stances (**Supplementary table 2**). Comparing correlations between antigen-matched IVTT and purified proteins only, overlapping targets correlate more highly than non-overlapping targets. Sample sizes were too low to test the significance of this trend within antigens (**Figure 4**).

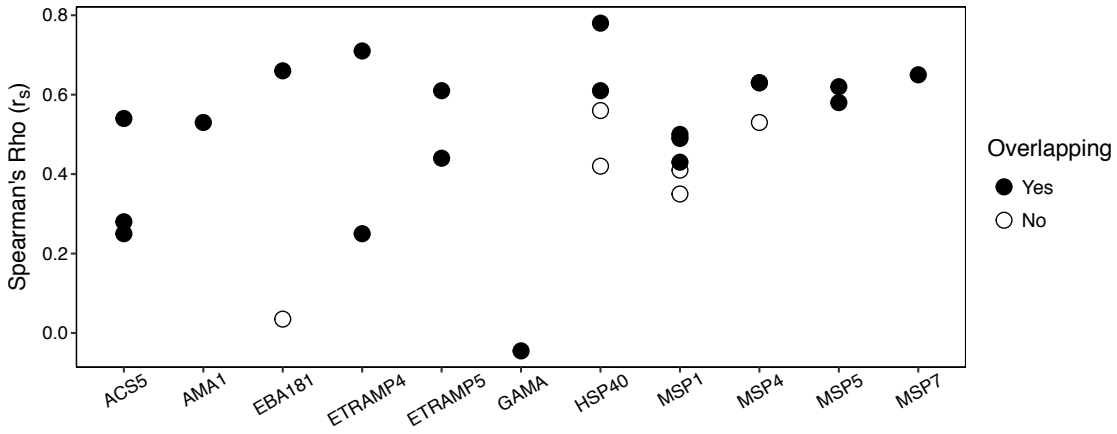


Figure 4. Spearman's rho correlation coefficients between antigen-matched IVTT and purified proteins. Targets with overlapping amino acid sequences are indicated by closed circles, compared to non-overlapping sequences indicated by open circles.

4.5 Discussion

Protein microarrays are a practical approach to the serological screening of large numbers of putative malaria antigen biomarkers. The throughput and flexibility of the microarray platform presents an opportunity to interrogate malarial antibody responses at a scale far exceeding traditional mono- or multiplex approaches, agnostic of predicted immunological targets. Here we have evaluated matched antigenic targets produced using two *E. coli*-based expression techniques — in vitro transcription/translation (IVTT), and purified, whole-cell recombinants — in the context of a protein microarray. We found that the magnitude of antibody responses to purified protein targets was generally higher than for their IVTT counterparts, and that correlation between protein target pairs at the individual serum sample level was variable and related to degree of sequence homogeneity between targets. Our findings warn against direct comparisons of microarray data from proteins produced in different expression platforms without careful cross-validation of sequences and allelic types. However, our data do provide support for the use of both IVTT and purified protein microarray platforms in the context of early-stage antigen biomarker identification to feed into experimental pipelines where candidate proteins may be interrogated by methods providing higher resolution analysis.

In building this study, we predicted that the magnitude of responses to IVTT products — which tended to be longer, often representing single exon sequences and therefore potentially containing more epitopes — would be greater than purified targets truncated based on species-

specificity or domain boundaries which potentially represented fewer epitopes. Contrary to this prediction, we found that purified proteins captured a greater range and magnitude of responses (Purified, IQR Log2MFI = 3.88–6.40; IVTT, IQR Log2MFI 0.46–1.68; p = <0.001). The greater level of reactivity to purified targets may relate to differences in the amount of protein deposited on the array, where consistent and defined amounts of purified protein are spotted in contrast to the unquantified, and likely variable IVTT products. These findings recommend a degree of caution in interpretation of array data from two different platforms, for example: MSP5 showed the second highest mean MFI for any purified protein, but showed among the lowest mean MFI of any IVTT protein.

In addition to differences in the magnitude of mean responses to targets stratified by expression system, we observed a greater range of individual sample responses, stratified by antigen, to purified proteins than in sequence matched IVTT-expressed targets (*e.g.* AMA1 - IVTT_1, median [IQR] Log2MFI = 1.66 [0.80–2.53]; Pure_1 (*P. pastoris* produced), median [IQR] Log2MFI = 7.92 [6.16–8.52]; p = <0.001). The *P. pastoris* AMA1 was included as a control for the evaluation of the production of a conformational protein. AMA1 is a complex structure comprised of three domains defined by three disulphide bonds. Production of AMA1 in *P. pastoris* has been fully characterised in terms of correct folding of the purified protein [53, 55] and this observation is likely a reflection of antibody reactivity to correctly folded (*P. pastoris*) and incorrectly folded AMA1 (IVTT). We acknowledge that a lack of correct folding in other purified and IVTT products may impact on epitope recognition by antibodies raised to native protein during infection. However, human antibody responses are composed of a polyclonal response to each antigen, which will include both confirmation and linear epitopes. Whilst questions remain about the appropriateness of using unfolded protein fragments in serological screens, such reagents remain the most widely utilised and efficient approach in this context at present.

Considering all antigenic targets together, we found no evidence of correlation in mean reactivity to sequence matched targets between expression systems (r_s = 0.28, p = 0.23). In the context of this study, this was not unexpected taking into account the differences observed in magnitude of response between IVTT and purified proteins, and that the length of native protein sequence coverage between IVTT and purified targets was highly variable. More broadly, it is perhaps less reassuring that matched targets derived from different expression systems lack more obvious relationships in antibody response than have been demonstrated in other studies [17, 56], though Kobayashi et al. report relatively similar results for a smaller number of targets expressed in *E. coli* (purified proteins) and IVTT systems specifically [29]. It is likely that protein concentration disparities between the two approaches are one of the drivers of this heterogeneity. However, without attempting to quantify the exact amount of protein generated in the small volume of IVTT reactions we are unable to address this here. Although in this current study targets grouped by antigen displayed highly variable correlations of response, it is encour-

aging that sequence matched proteins did generally display stronger correlations of response than non-sequence matched targets. Further, this may indicate the importance of capturing specific epitopes within expression sequences when producing antigens by either expression method.

Despite the lack of a clearly defined relationship between antigen-matched targets from the evaluated expression systems, we remain confident that microarrays utilising IVTT or purified recombinant proteins are able to produce compelling and biologically relevant data. Indeed, our data show age-dependent trends in antibody responses (typical of highly endemic populations) [3, 4, 7] irrespective of expression system (**Supplementary figure 3**), lending weight to the applicability of either methodology in serological assays [57–70].

The IVTT system lends itself to microarray applications, as vast numbers of proteins, or even entire proteomes, may be produced at scale relatively quickly. However, for application to serology there is concern that expressed proteins are not quantified before printing, and that expression levels of product may vary considerably; product yield in bacterial-based IVTT systems is generally considered to be lower (typically 1 mg mL^{-1} or less) though higher protein yields have been reported [71, 72]. This has been shown to be due to an inherent heterogeneity with IVTT components, although this weakness is an area of active research [26, 73]. Similarly, it is important to acknowledge that the unpurified nature of printed reaction mixtures may mask, or otherwise adversely affect, the detection of antibody reactivity in a sample; Davies et al. report IVTT reaction compositions of 99% *E. coli* lysate to 1% target protein [18], though this will vary considerably, at scale, in practice.

In contrast to IVTT-based microarrays, printing purified protein allows a highly quantifiable approach to be taken. Affinity purification and dialysis of expression products substantially reduces the risk of background reactivity to bacterial components, and the simple determination of target protein concentrations allows defined quantities of product to be spotted, providing much greater confidence when comparing reactivity between targets. However, these advantages come at a substantial cost; the need for *in silico* analysis to design vectors, transfection procedures, expression and purification drastically slows the rate at which putative targets can be produced and screened. Shorter, epitope specific sequences may in theory be transposed from IVTT systems with a view to generating more granular serological screens, though we accept that truncated protein targets will in some cases favour linear B cell epitopes, while missing conformational epitopes. However, for measuring exposure to infection there is less importance on the targeting of confirmation epitopes than would be required for protective epitopes [58].

The primary benefit of the microarray platform is the ability to screen orders of magnitude more targets simultaneously than more standard serological assays. Our analysis shows that both IVTT and purified proteins can be successfully used to capture malarial protein antigen-specific antibody responses on a protein microarray. Although correlations of response between

expression systems are not as strong as may have been expected, a number of acknowledged technical differences in the methods of protein production may account for this finding. In addition to the *E. coli in vivo* and IVTT systems utilised here, high-throughput wheat germ cell free systems have been successfully used to conduct large scale serological screens of putative antigen biomarkers [74, 75], alongside chemically synthesised peptide arrays [58, 59]. High-throughput mammalian and baculovirus expression systems have also been pioneered for the production of recombinant proteins [37, 76]. Differences in expression efficiency and the homology to native epitopes achieved by the assortment of available approaches likely have considerable impact on the capture of antibody from sample. This variability should be accounted for both in terms of choosing an experimental approach and comparative analysis between different methods. We suggest that further investigation of differences in seroreactivity to sequence-matched proteins derived from contrasting expression systems is needed to shed light on the parity between such data that is already widely published. It should also be noted that it is unlikely that any single expression platform will satisfy the demands of all recombinant expression projects due to varying importance such as protein folding, proteins activity (*e.g.* enzymes) and glycosylation. In addition, *E. coli* expression has the advantage of low cost, flexibility and easy scale-up.

Considering the data presented here more broadly, observed trends lend support to the utilisation of both IVTT and purified arrays depending on the objectives and context of hypotheses to be investigated. The strengths and weaknesses of each expression system should dictate the chosen approach on a case-by-case basis. For example, very high-density proteome level screening to identify shortlists of candidate markers based on binary categorisation of seropositivity may be best achieved using IVTT systems. In contrast, smaller numbers of shortlisted targets expressed as purified proteins may allow for more nuanced characterisation of antibody responses on a more continuous scale. As already described, the key limitation in the production of purified recombinants in our current expression pipeline is throughput. The adaption of our methods to increase the capacity of protein production would improve our ability to more widely mine the biomarker information derived from the IVTT platform. As such, we are currently exploring a number of existing approaches to address this methodological bottleneck [38, 77].

In summary, the IVTT protein microarray approach has proven to be a powerful, high-throughput, biomarker discovery platform with applicability across a range of infectious diseases. When combined with a cheap, scalable and flexible protein expression platform such as the *E. coli in vivo* expression platform we have the ability to mine potential diagnostic and vaccine related targets.

Acknowledgements

We thank all the study participants who participated in the original PRISM study (Program for Resistance, Immunology, Surveillance, and Modelling of Malaria in Uganda; East Africa ICEMR) from which the test samples were taken. The authors give thanks to James Beeson for the provision of purified recombinant protein EBA140 RIII-V, Ross Coppel for the provision of MSP4 and MSP5, and Tony Holder for the provision of GAMA and MSP7. KKAT was supported by a Bloomsbury SET Award (Innovation Fellowship to KKAT; BSA14) under the UKRI Connecting Capabilities Fund (CCF). WS is supported by Sir Henry Wellcome fellowship (number 218676/Z/19/Z) from the Wellcome Trust (UK).

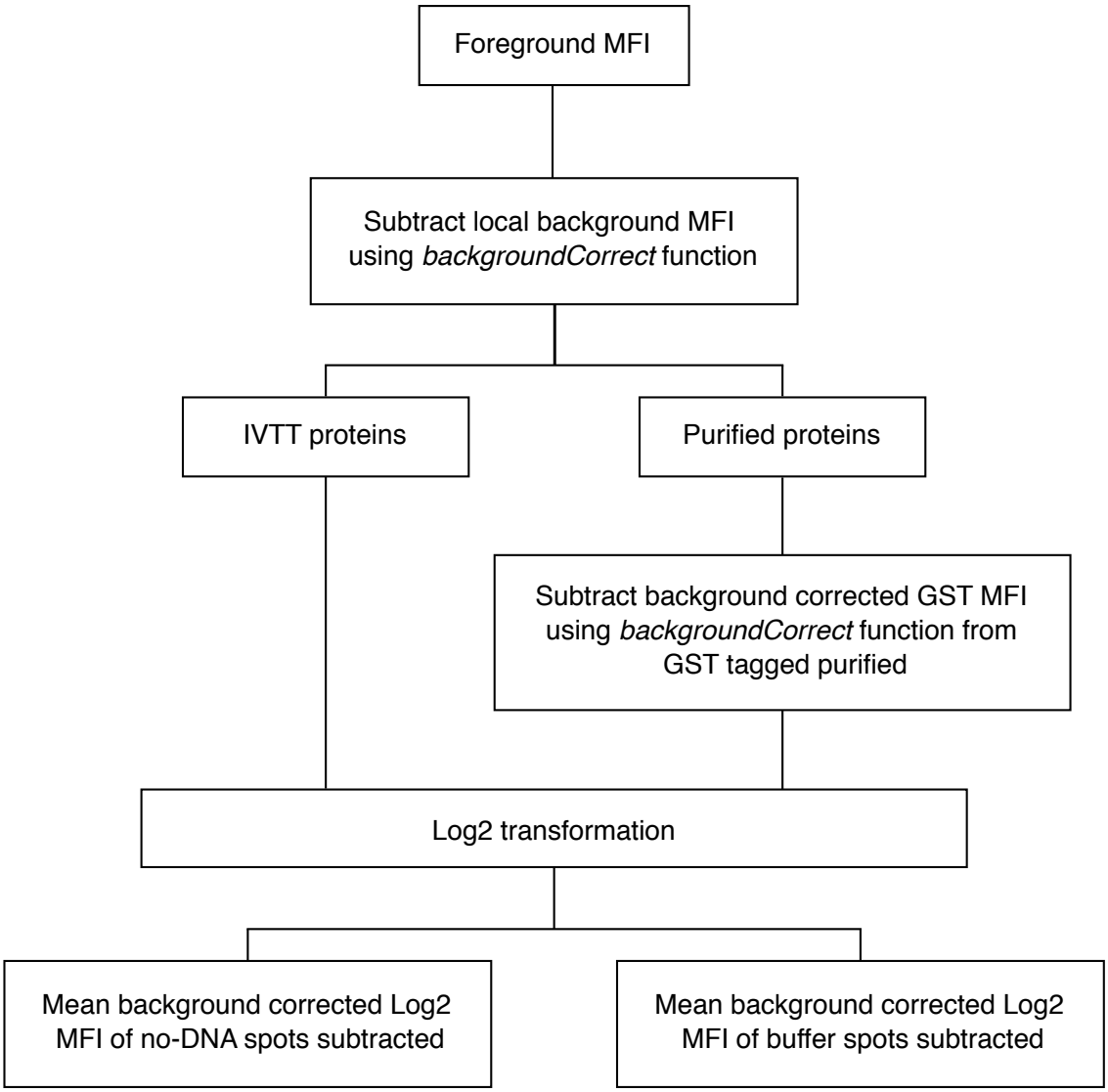
Conflict of Interest Statement

The authors have declared no conflict of interest.

4.6 Supplementary material

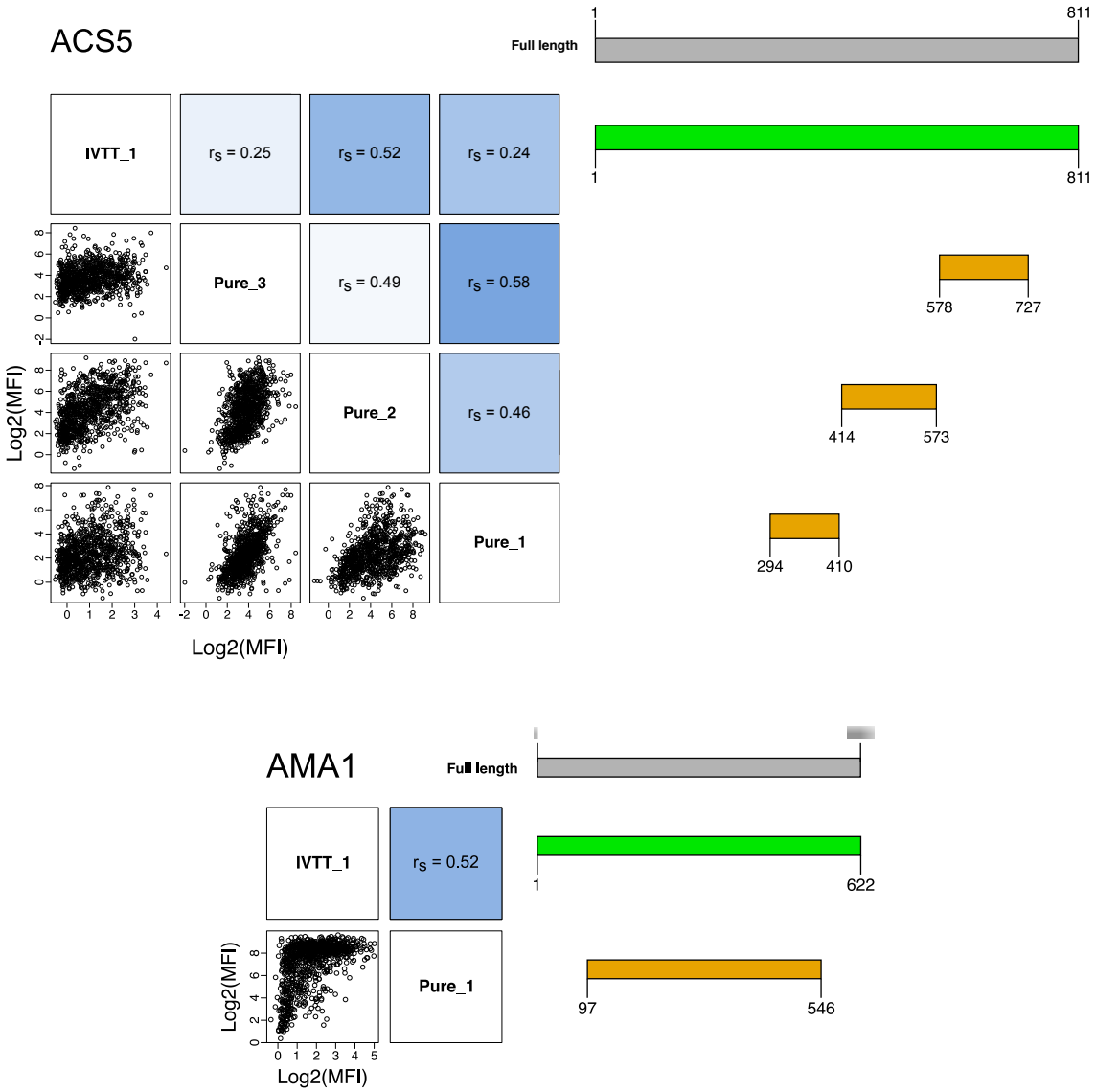
Supplementary table 1. Detail of expressed protein targets. A key to the simplified nomenclature used for specific proteins in text is provided.

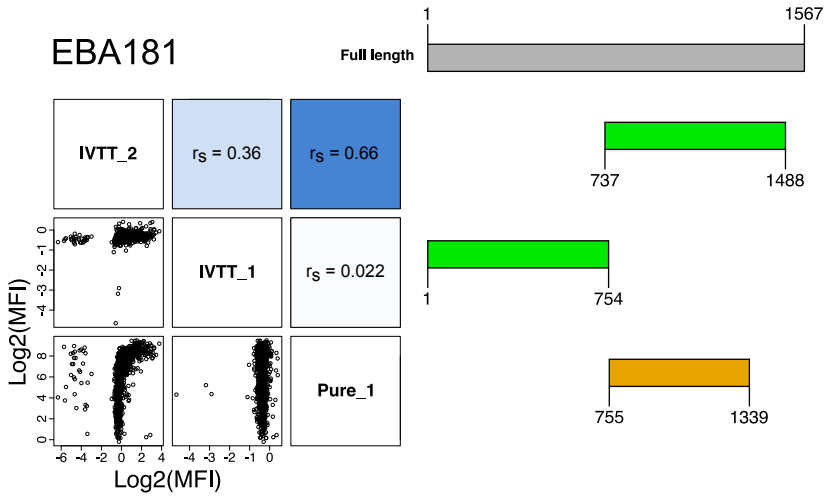
Gene ID	Name	Description	Full length (Amino Acids)	Allele	Protein target (simplified name)	Expression System	Size (Start Amino Acid - End Amino Acid)	Reference
PF3D7_0731600	ACS5	Acyl CoA synthase	811		IVTT_PF07_0129e1s1 (IVTT_1)	IVTT	811 (1–811)	
				3D7	ACS5 Ag 2 (Pure_1)	PR[a]	117 (294–410)	Helb et al. (2015)[19]
				3D7	ACS5 Ag 3 (Pure_2)	PR	160 (414–573)	Helb et al. (2015)[19]
				3D7	ACS5 Ag 4 (Pure_3)	PR	150 (578–727)	Helb et al. (2015)[19]
PF3D7_1133400	AMA1	Apical membrane antigen 1	622		IVTT_PF11_0344 (IVTT_1)	IVTT	622 (1–622)	
				FV0	AMA1 (Pure_1)	PR	450 (97–546)	Collins et al. (2007)[53]
PF3D7_0102500	EBA181	Erythrocyte binding antigen 181	1567		IVTT_PFA0125ce1s1 (IVTT_1)	IVTT	754 (1–754)	
					IVTT_PFA0125ce1s2 (IVTT_2)	IVTT	752 (737–1488)	
				3D7	EBA181 RIII-V (Pure_1)	PR	585 (755–1339)	Richards et al. (2010)[48]
PF3D7_0423700	ETRAMP4	Early transcribed membrane antigen 4	136		IVTT_PFD1120c.1o1 (IVTT_1)	IVTT	136 (1–136)	
				3D7	Etramp 4 Ag 1 (Pure_1)	PR	25 (28–52)	Helb et al. (2015)[19]
				3D7	Etramp 4 Ag 2 (Pure_2)	PR	61 (76–136)	Helb et al. (2015)[19]
PF3D7_0532100	ETRAMP5	Early transcribed membrane antigen 5	181		IVTT_PFE1590w (IVTT_1)	IVTT	181 (1–181)	
				3D7	Etramp 5 Ag 1 (Pure_1)	PR	86 (26–111)	Helb et al. (2015)[19]
				3D7	Etramp 5 Ag 2 (Pure_2)	PR	47 (135–181)	Helb et al. (2015)[19]
PF3D7_0828800	GAMA	GPI-anchored membrane antigen	738		IVTT_PF08_0008(neg clone) (IVTT_1)	IVTT	738 (1–738)	
				3D7	GAMA N-terminal (Pure_1)	PR	99 (68–166)	Hinds et al. (2009)[52]
PF3D7_0501100.1	HSP40	Heat shock protein 40 type II	402		IVTT_PFE0055ce3s1 (IVTT_1)	IVTT	134 (80-213)	
					IVTT_PFE0055ce4s1 (IVTT_2)	IVTT	171 (213-401)	
				3D7	HSP40 Ag 1 (Pure_1)	PR	83 (71-153)	Helb et al. (2015)[19]
				3D7	HSP40 Ag 3 (Pure_2)	PR	189 (214-402)	Helb et al. (2015)[19]
PF3D7_0930300	MSP1	Merozoite surface protein 1	1720		IVTT_PFI1475w-s1 (IVTT_1)	IVTT	870 (1-870)	
					IVTT_PFI1475w-s2 (IVTT_2)	IVTT	868 (853-1720)	
				3D7 (K1 type); repeat only	MSP1 bk2 3D7 Rep (Pure_1)	PR	45 (64-108)	Polley et al. (2003)[46]
				Allelle specific flanking region (based on K1-type allele)	MSP1 bk 2 K1 flank (Pure_2)	PR	35 (54-63;109-133)	Polley et al. (2003)[46]
					PfMSP1_19 (Pure_3)	PR	116 (1605-1720)	Burghaus et al. (1994)[45]
PF3D7_0207000	MSP4	Merozite surface protein 4	272		IVTT_PFB0310c.1o2 (IVTT_1)	IVTT	162 (1-162)	
					IVTT_PFB0310c-e1 (IVTT_2)	IVTT	161 (1-161)	
					IVTT_PFB0310c.2o2 (IVTT_3)	IVTT	110 (163-272)	
				D10	MSP 4 (Pure_1)	PR	65 (43-107)	Wang et al. (2000)[49]
PF3D7_0206900	MSP5	Merozoite surface protein 5	272		IVTT_PFB0305c.1o2 (IVTT_1)	IVTT	172 (1-172)	
					IVTT_PFB0305c-e1 (IVTT_2)	IVTT	171 (1-171)	
				D10	MSP 5 (Pure_1)	PR	61 (147-207)	Black et al. (2002)[50]
PF3D7_1335100	MSP7	Merozoite surface protein 7	351		IVTT_PF13_0197 (IVTT_1)	IVTT	351 (1-351)	
				3D7	MSP7 (Pure_1)	PR	175 (177-351)	Pachebat et al. (2001)[51]

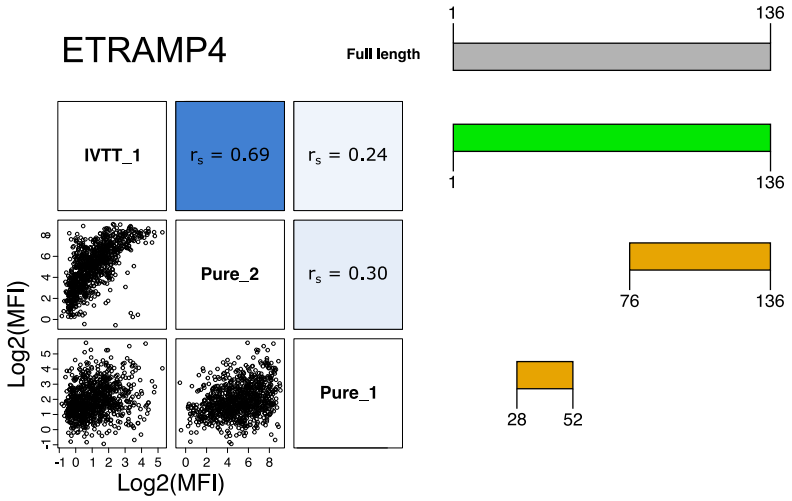


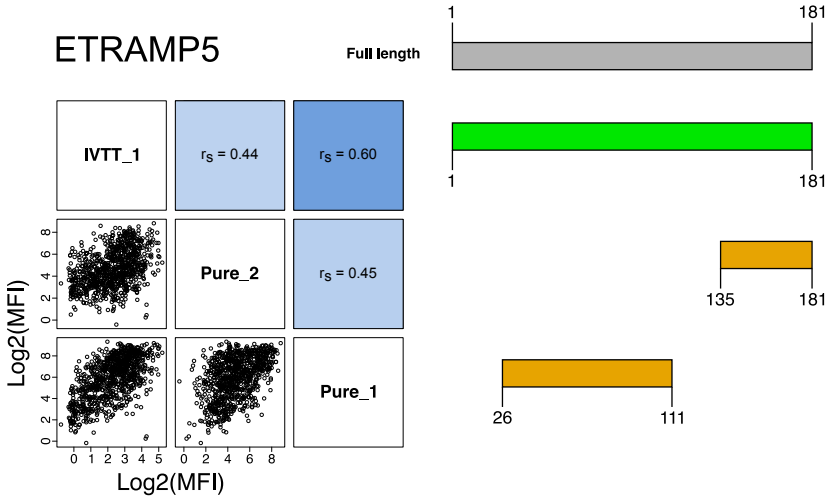
Supplementary figure 1. Data normalisation processes for IVTT and purified protein spots. After local background correction using the backgroundCorrect function from the *limma* package, purified protein spots were additionally corrected for possible GST reactivity by subtracting GST reactivity using the same function. After Log2 transformation, IVTT and purified proteins were normalised to background control spots of empty T7 vector and PBS buffer control spots respectively.

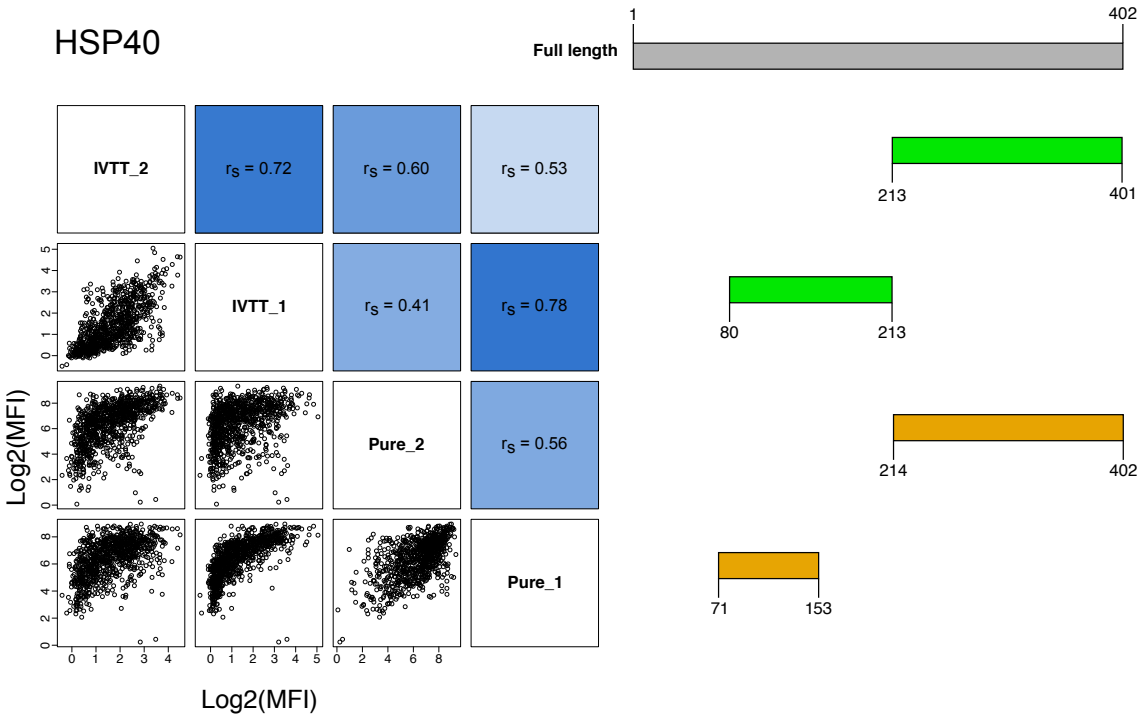
Supplementary figure 2. Correlation of antigen-matched targets. Correlogram of multiple antigen-matched targets (left). Spearman's rank correlation reported (r_s) and increasing blue colour scale indicates relative strength of correlation based on calculated correlations for all proteins included in this analysis. Protein schematic (right) represents amino-acid aligned representation of IVTT (green) and purified (orange) proteins to the full-length native protein (grey). Proteins in the correlogram and schematic are correspondingly aligned.

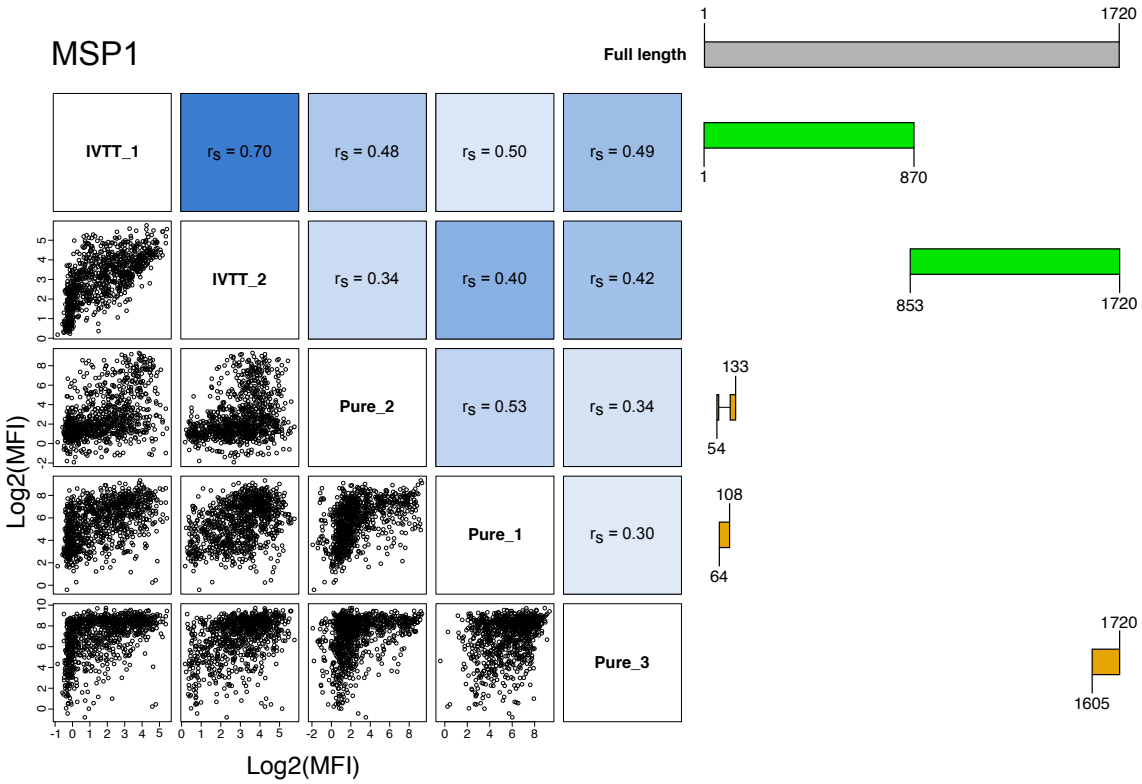


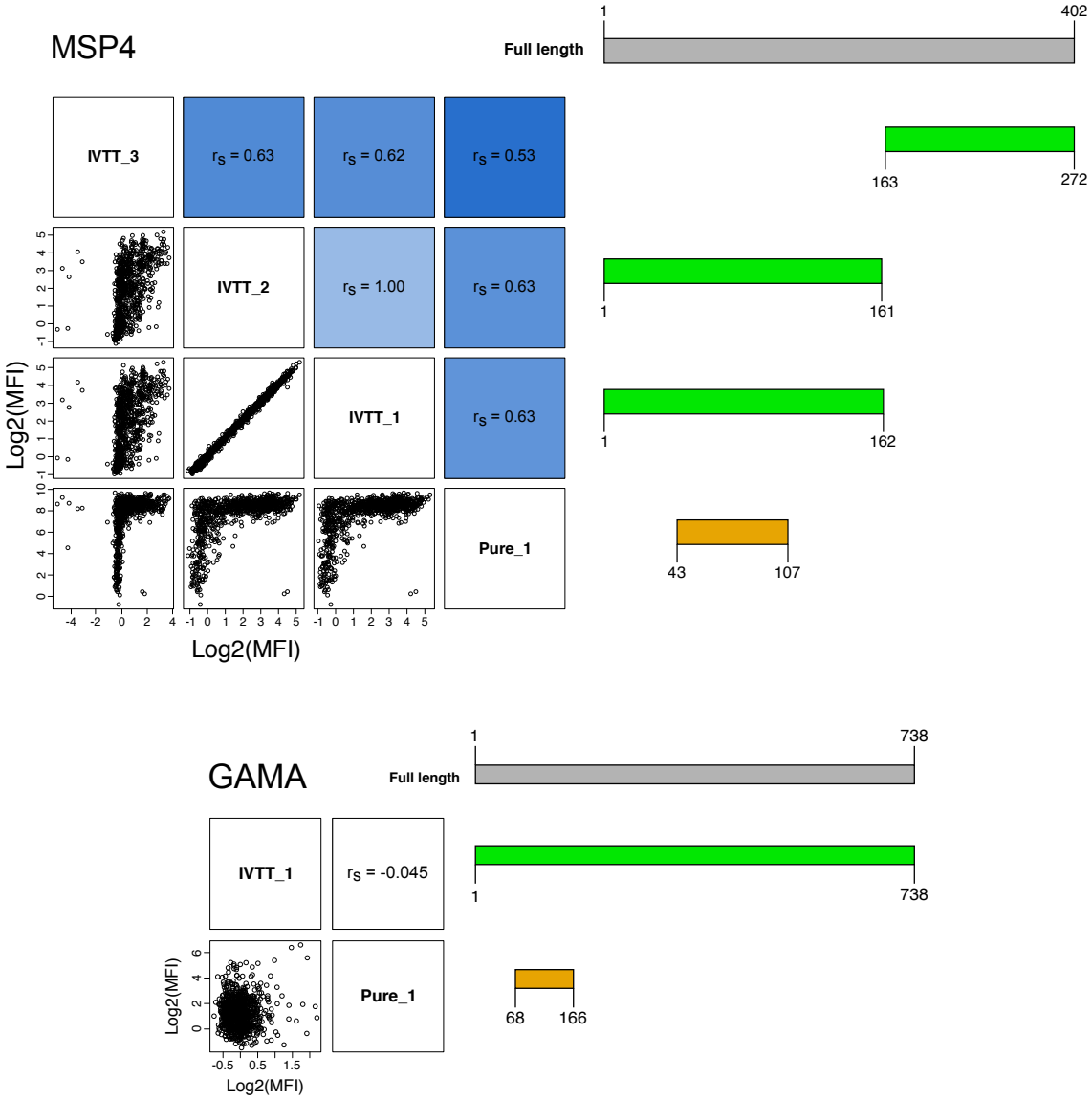

EBA181
Full length
1
1567
IVTT_2
rS = 0.36
rS = 0.66
737
1488
IVTT_1
rS = 0.022
1
754
Pure_1
755
1339
Log2(MFI)
Log2(MFI)


ETRAMP4
Full length
1
136
IVTT_1
rs = 0.69
rs = 0.24
1
136
Pure_2
rs = 0.30
76
136
Pure_1
28
52
Log2(MFI)
Log2(MFI)


ETRAMP5
Full length
1
181
IVTT_1
rS = 0.44
rS = 0.60
1
181
Pure_2
rS = 0.45
135
181
Pure_1
26
111
Log2(MFI)
Log2(MFI)


HSP40
Full length
1
402
IVTT_2
IVTT_1
Pure_2
Pure_1
rS = 0.72
rS = 0.60
rS = 0.53
rS = 0.41
rS = 0.78
rS = 0.56
213
401
80
213
214
402
71
153
Log2(MFI)
Log2(MFI)


MSP1
Full length
1
1720
IVTT_1
IVTT_2
Pure_2
Pure_1
Pure_3
rS = 0.70
rS = 0.48
rS = 0.50
rS = 0.49
rS = 0.34
rS = 0.40
rS = 0.42
rS = 0.53
rS = 0.34
rS = 0.30
1
870
853
1720
133
54
108
64
1720
1605
Log2(MFI)
Log2(MFI)

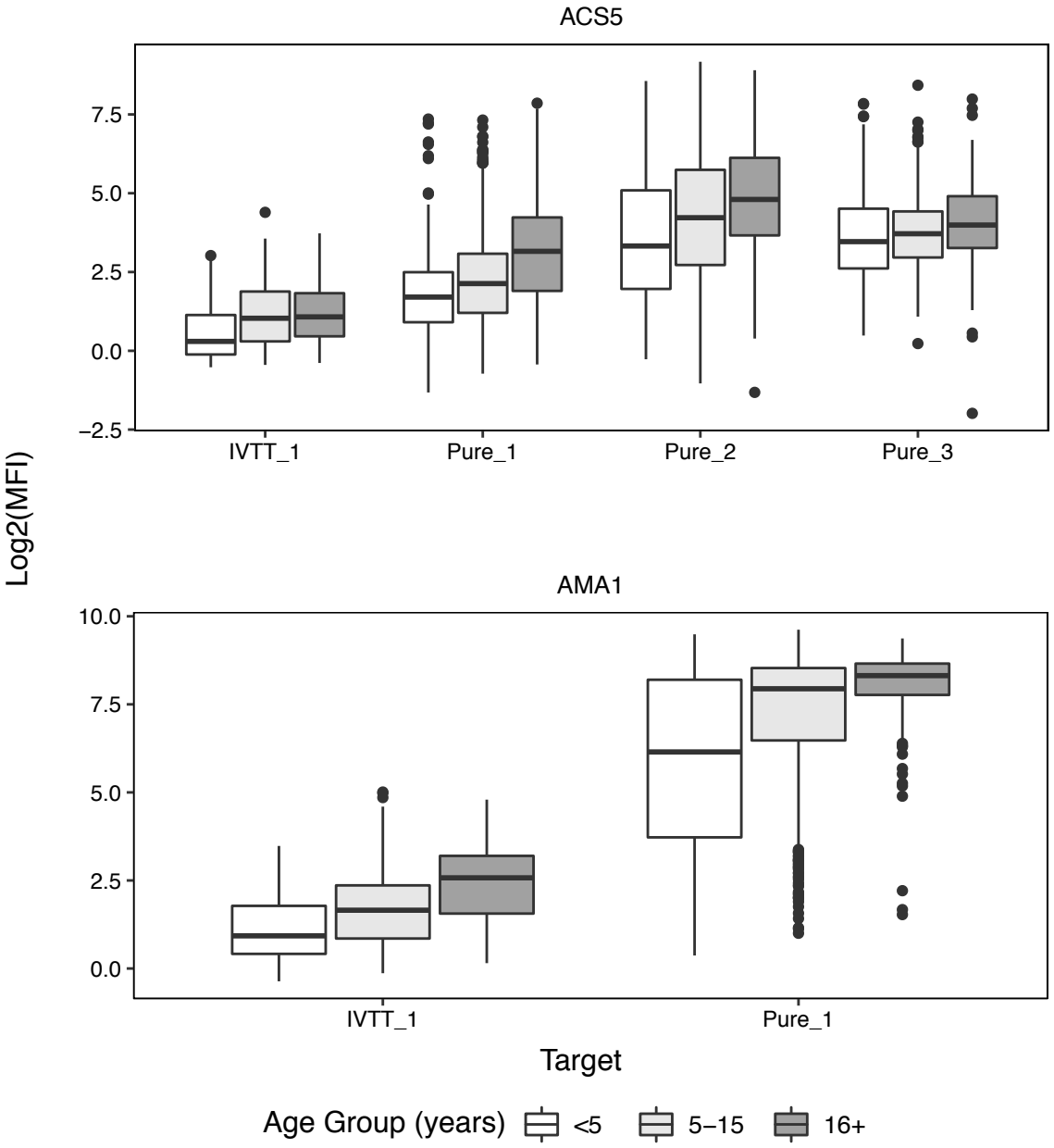

MSP4
Full length
1
402
IVTT_3
r_S = 0.63
r_S = 0.62
r_S = 0.53
163
272
IVTT_2
r_S = 1.00
r_S = 0.63
1
161
IVTT_1
r_S = 0.63
1
162
Pure_1
43
107
Log2(MFI)
Log2(MFI)
GAMA
Full length
1
738
IVTT_1
r_S = -0.045
1
738
Pure_1
68
166
Log2(MFI)
Log2(MFI)

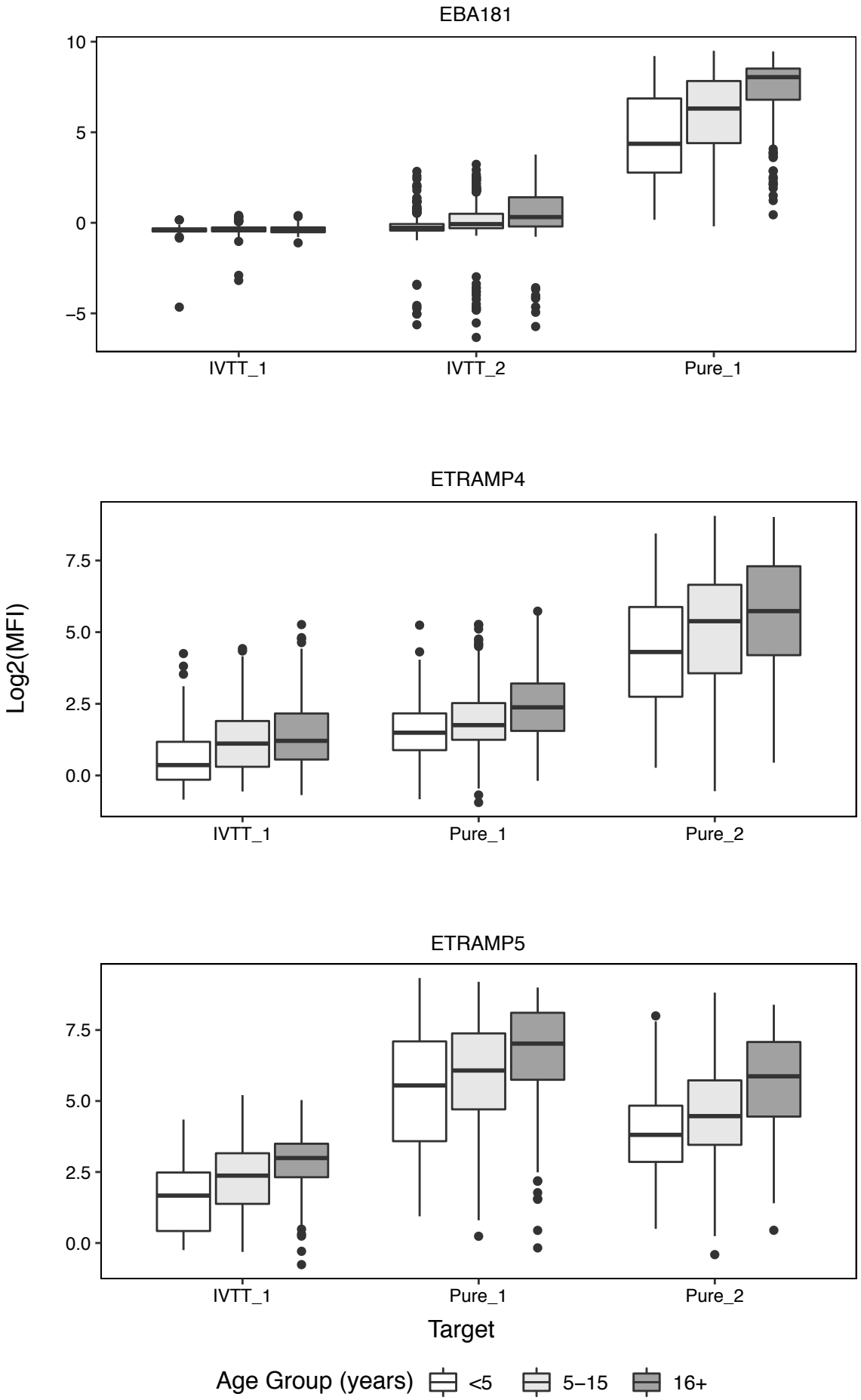
Supplementary table 2. Correlation coefficient results for all protein pairs. Protein targets are grouped by antigen, and all possible combinations within each antigen group are shown.

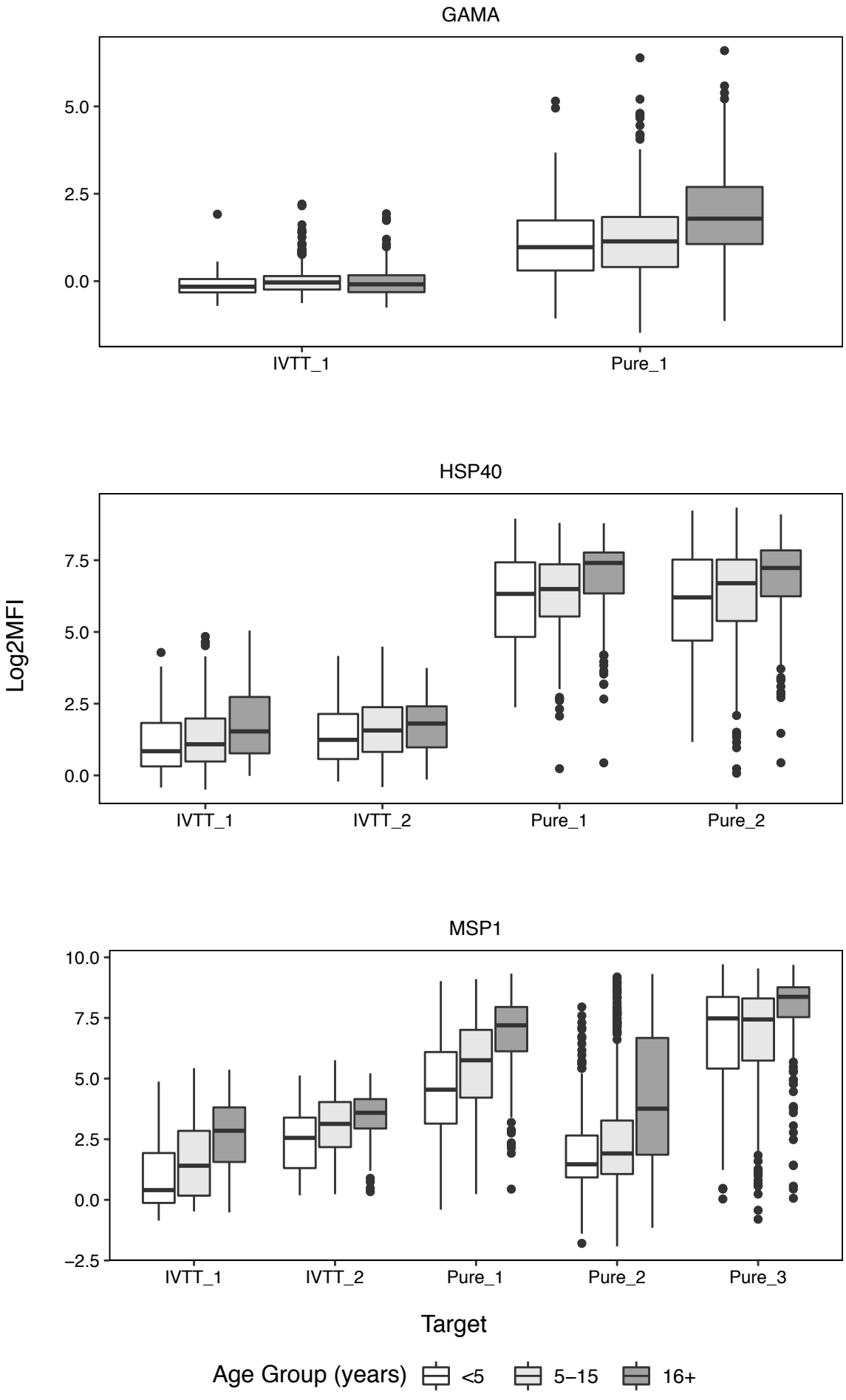
Antigen	Combination member 1	Combination member 2	Correlation coefficient (r_s)	Overlapping	Length 1 (Amino Acids)	Length 2 (Amino Acids)
ACS5	IVTT_1	Pure_3	0.28	Yes	811	150
	IVTT_1	Pure_2	0.54	Yes	811	160
	IVTT_1	Pure_1	0.25	Yes	811	117
	Pure_2	Pure_3	0.50	No	160	150
	Pure_1	Pure_3	0.59	No	117	150
	Pure_1	Pure_2	0.47	No	117	160
AMA1	IVTT_1	Pure_1	0.53	Yes	622	450
EBA181	IVTT_1	IVTT_2	0.37	No	754	752
	IVTT_2	Pure_1	0.66	Yes	752	585
	IVTT_1	Pure_1	0.035	No	754	585
ETRAMP4	IVTT_1	Pure_2	0.71	Yes	136	61
	IVTT_1	Pure_1	0.25	Yes	136	25
	Pure_1	Pure_2	0.32	No	25	61
ETRAMP5	IVTT_1	Pure_2	0.44	Yes	181	47
	IVTT_1	Pure_1	0.61	Yes	181	86
	Pure_1	Pure_2	0.45	No	86	47
GAMA	IVTT_1	Pure_1	-0.045	Yes	738	99
HSP40	IVTT_2	IVTT_1	0.73	No	171	134
	IVTT_2	Pure_2	0.61	Yes	171	189
	IVTT_2	Pure_1	0.56	No	171	83
	IVTT_1	Pure_2	0.42	No	134	189
	IVTT_1	Pure_1	0.78	Yes	134	83
	Pure_1	Pure_2	0.57	No	83	189
MSP1	IVTT_1	IVTT_2	0.71	No	870	868
	IVTT_1	Pure_2	0.49	Yes	870	35 (10;25)*
	IVTT_1	Pure_1	0.50	Yes	870	45
	IVTT_1	Pure_3	0.49	No	870	116
	IVTT_2	Pure_2	0.35	No	868	35 (10;25)*
	IVTT_2	Pure_1	0.41	No	868	45
	IVTT_2	Pure_3	0.43	Yes	868	116
	Pure_2	Pure_1	0.54	No	35 (10;25)*	45
	Pure_2	Pure_3	0.35	No	35 (10;25)*	116
	Pure_1	Pure_3	0.31	No	45	116
MSP4	IVTT_3	IVTT_2	0.63	No	110	161
	IVTT_3	IVTT_1	0.62	No	110	162
	IVTT_3	Pure_1	0.53	No	110	65
	IVTT_2	IVTT_1	1.00	Yes	161	162
	IVTT_2	Pure_1	0.63	Yes	161	65
	IVTT_1	Pure_1	0.63	Yes	162	65
MSP5	IVTT_1	IVTT_2	0.94	Yes	172	171
	IVTT_1	Pure_1	0.61	Yes	172	61
	IVTT_2	Pure_1	0.58	Yes	171	61
MSP7	IVTT_1	Pure_1	0.65	Yes	351	175

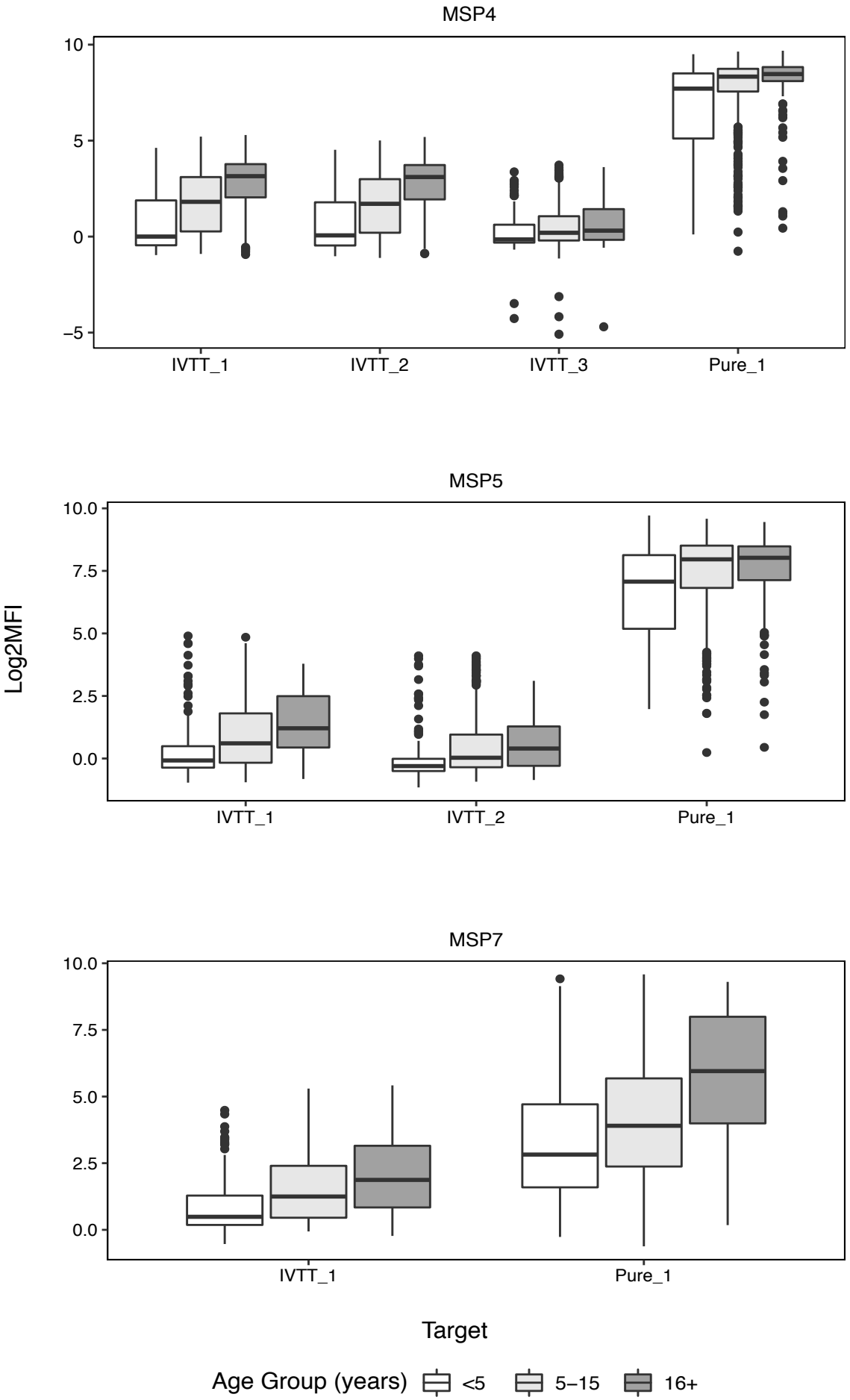
* Figures in brackets indicate length of linked fragments

Supplementary figure 3. Magnitude and range of response to IVTT and purified proteins, stratified by age. Magnitude and range of response to IVTT and purified proteins, stratified by age. All sample responses (n = 899) to all protein targets grouped by antigen, presented with median and interquartile range.




EBA181
10
5
0
−5
IVTT_1
IVTT_2
Pure_1
ETRAMP4
Log2(MFI)
7.5
5.0
2.5
0.0
IVTT_1
Pure_1
Pure_2
ETRAMP5
7.5
5.0
2.5
0.0
IVTT_1
Pure_1
Pure_2
Target
Age Group (years)
<5
5–15
16+


GAMA
5.0
2.5
0.0
IVTT_1
Pure_1
HSP40
Log2MFI
7.5
5.0
2.5
0.0
IVTT_1
IVTT_2
Pure_1
Pure_2
MSP1
10.0
7.5
5.0
2.5
0.0
−2.5
IVTT_1
IVTT_2
Pure_1
Pure_2
Pure_3
Target
Age Group (years)
<5
5–15
16+


MSP4
10
5
0
−5
IVTT_1
IVTT_2
IVTT_3
Pure_1
MSP5
Log2MFI
10.0
7.5
5.0
2.5
0.0
IVTT_1
IVTT_2
Pure_1
MSP7
10.0
7.5
5.0
2.5
0.0
IVTT_1
Pure_1
Target
Age Group (years)
<5
5–15
16+

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4.7 Addendum of corrections

The following text addresses corrections made at the request of examiners, but that is not included in the original published article:

Edit to 'Correlation of responses between antigen matched targets'

Considering all at least partially sequence matched IVTT and purified protein targets (*i.e.* excluding pairwise comparisons where purified protein sequence were completely non-overlapping with IVTT sequence for the same antigen) there was no evidence for a general correlation in mean response between expression platforms (Spearman's rho (r_s) = 0.279, p = 0.23). Antibody responses to all protein targets for each antigen were therefore compared individually (representative example in **figure 3** and all antigens in **supplementary figure 2**), considering that successful expression of different epitopes within each native protein may be variable by expression system, and that responses to different epitopes may still correlate in terms of overall reactivity to the whole antigen. It is important to understand the relationships between differentially expressed protein fragments from the same gene as serological studies often describe antibody responses to an antigen as a whole, rather than explicit epitopes or defined reactive fragments. Furthermore, protein antigen microarrays will most likely contain multiple gene-matched antigen fragments, within the use case of the high throughput triaging of potential biomarkers, to make best use of the technology. Antigen fragments produced using different expression platforms will expectedly display variation in reactivity, some of which will be explainable by the degree of sequence overlap, though not necessarily exclusively.

Edit to 'Discussion'

…we predicted that the magnitude of antibody responses to IVTT products — which tended to be longer, often representing single exon sequences and therefore potentially containing more epitopes — would be greater than purified targets truncated based on species specificity or domain boundaries which potentially represented fewer epitopes. However, this hypothesis did not influence any expectations around the suitability of either protein expression system for producing reactive targets, and each expression methodology was considered in the context of it's applicability for the application — in this case, high-throughput screening of putative biomarkers.

Chapter 5

The acquisition of humoral immune responses targeting *Plasmodium falciparum* sexual stages in controlled human malaria infections

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SECTION A – Student Details

Student ID Number	1404292/RITD	**Title**	Mr
First Name(s)	Tate		
Surname/Family Name	Oulton		
Thesis Title	Development and validation of serological assays to evaluate malaria transmission		
Primary Supervisor	Chris Drakeley		

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Where was the work published?	Frontiers in Immunology		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I contributed to the general study and analysis design. I carried out the Luminex based antibody quantification component of the laboratory work included in this study and completed the analysis of the data generated from the Luminex experiments above. I facilitated data gathering, processing, QC and analysis for the microarray experiments. I wrote the first draft with the other authors.

SECTION E

Student Signature	[redacted]
Date	. .

Supervisor Signature	[redacted]
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Title:

The acquisition of humoral immune responses targeting *Plasmodium falciparum* sexual stages in controlled human malaria infections

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Keywords:

malaria, *Plasmodium falciparum*, sexual stage, gametocyte antigens, antibody responses, controlled human malaria infection (CHMI)

Abstract

Individuals infected with *P. falciparum* develop antibody responses to intra-erythrocytic gametocyte proteins and exported gametocyte proteins present on the surface of infected erythrocytes. However, there is currently limited knowledge on the immunogenicity of gametocyte antigens and the specificity of gametocyte-induced antibody responses. In this study, we assessed antibody responses in participants of two controlled human malaria infection (CHMI) studies by ELISA, multiplexed bead-based antibody assays and protein microarray. By comparing antibody responses in participants with and without gametocyte exposure, we aimed to disentangle the antibody response induced by asexual and sexual stage parasites. We showed that after a single malaria infection, a significant anti-sexual stage humoral response is induced in malaria-naïve individuals, even after exposure to relatively low gametocyte densities (up to ~1,600 gametocytes/mL). In contrast to antibody responses to well-characterised asexual blood stage antigens that were detectable by day 21 after infection, responses to sexual stage antigens (including transmission blocking vaccine candidates Pfs48/45 and Pfs230) were only apparent at 51 days after infection. We found antigens previously associated with early gametocyte or anti-gamete immunity were highly represented among responses linked with gametocyte exposure. Our data provide detailed insights on the induction and kinetics of antibody responses to gametocytes and identify novel antigens that elicit antibody responses exclusively in individuals with gametocyte exposure. Our findings provide target identification for serological assays for surveillance of the malaria infectious reservoir, and support vaccine development by describing the antibody response to leading vaccine antigens after primary infection.

5.2 Introduction

Gametocytes are the only life stage of *Plasmodium falciparum* that can initiate successful infection in anopheline mosquitoes. The human infectious reservoir in malaria endemic areas is therefore defined by the presence of mature male and female gametocytes in the blood. Interventions to reduce this reservoir or prevent transmission by direct interference with sexual stage development inside mosquitoes could facilitate efforts to achieve malaria elimination [1, 2].

From the point of erythrocyte invasion by a sexually committed merozoite it takes 10 to 12 days for *P. falciparum* gametocytes to fully mature; during this time, they pass through five distinct developmental forms (stages I-V). Immature gametocytes sequester primarily in the bone marrow and spleen outside the peripheral circulation [3–5]. They are released back into the circulation to fully mature, after which they can be transmitted to mosquitoes during a blood meal. In the mosquito midgut, gametocytes egress from the host cell, and differentiate into male and female gametes that rapidly undergo fertilisation. In humans, intact immature gametocytes produce proteins that are exported to the erythrocyte surface and elicit an immune response [6]. Early reports suggest that naturally acquired antibodies can directly affect gametocyte morphology and maturation, and as a result these antibodies may be able to affect gametocyte numbers and time in circulation [7, 8]. Recent evidence indicates that antibodies specific to putative immature gametocyte erythrocyte surface antigens may promote phagocytosis [6], but to what extent gametocytes are specifically targeted and killed in circulation remains unclear. In contrast, there is abundant evidence that immune responses to intra-erythrocytic gametocyte proteins can inhibit gamete fertilisation in the mosquito midgut, when gametes are exposed to the blood meal content after egress from the red cell [9]. These target antigens form the basis of advanced transmission blocking vaccines [10].

At present, little is known about antibodies specific for gametocyte proteins, besides the well-characterised gamete fertility proteins (Pfs48/45 and Pfs230). Studies indicate that these proteins may not be the sole contributors to natural transmission blocking immunity [11], so there is an imperative to investigate immune responses to a wider sexual stage protein catalogue. The use of gametocyte specific antibodies as biomarkers of gametocyte carriage and infectiousness may also help identify the infectious reservoir in population-wide surveillance. Prior studies that identified gametocyte-enriched or specific proteins used proteomic data, without reference to immunogenicity [11, 12]. Studies assessing anti-gametocyte antibody responses have focused on naturally exposed populations and are thus complicated by lack of effective controls and inherent variance in prior parasite exposure. Controlled human malaria infection (CHMI) models [13] in which malaria naïve volunteers are deliberately infected with *P. falciparum* parasites, provide a powerful tool to study immune responses during a well-characterized primary infection. Classical CHMI does not allow for evaluation of interventions affecting transmission, as gametocytes arise approximately 10 days [14] after asexual parasitaemia peaks, by which

time participants have received full curative treatment that does not allow for gametocyte development. Recently, the CHMI model has been adapted to allow safe induction of gametocytes in study participants [12, 15, 16]. In these models, volunteers were infected with *P. falciparum* 3D7 parasites and sub-curative treatment of asexual parasites allowed the development of viable mature male and female gametocytes. Infection by injection of infected red blood cells appeared to induce higher gametocyte densities and a higher likelihood of infecting mosquitoes, compared to infection through mosquito bites [16] and resulted in lower inflammation [17].

Here, we assessed antibody responses to sexual stage antigens among participants of a CHMI transmission study [16] after a single induced infection. We examined the immunogenicity of gametocyte proteins, the acquisition of gametocyte-specific antibodies and their association with preceding gametocyte exposure. Bead-based antibody assays allowed us to assess antibody responses to sub-units of the transmission blocking vaccine antigens Pfs48/45 and Pfs230, with comparison to well characterised antibody biomarkers of blood stage infection. Using a protein microarray, we set out to identify novel antigens that are targeted by antibodies uniquely induced after gametocyte exposure, which is of value in the context of serological assay development for gametocyte surveillance.

5.3 Materials and methods

Clinical trial samples

Samples were collected in a CHMI transmission trial conducted between May and November 2018 [16]. Individuals were infected either by the bites of 5 *P. falciparum* 3D7 infected mosquitoes (SPZ Gct, n=12), or by intravenous injection with ~2,800 *P. falciparum* 3D7 infected human erythrocytes (BS Gct, n=12). Parasitaemia was monitored by 18s quantitative polymerase chain reaction (qPCR); after parasitaemia reached a prespecified treatment threshold, participants received a gametocyte permissive sub-curative dose of piperaquine (480 mg) [16]. Serum and citrate plasma samples were collected on prespecified time points prior to and after challenge infection. Plasma samples were selected for analyses from blood samples taken prior to challenge (C-1), and at days C+7 (BS Gct), C+9 (SPZ Gct), C+21 and C+36 in BD Cell Preparation Tubes with sodium citrate. One serum sample was selected from day C+51, which was collected in BD SST™ II Advance tubes.

As a control for gametocyte exposure in our antibody assays, additional plasma samples were analysed from control participants in a CHMI study where no gametocyte exposure was anticipated. Although gametocyte exposure was deemed highly unlikely due to early curative treatment, the absence of gametocytaemia was not formally demonstrated. This study was conducted between April 2011 and March 2012 [18]; volunteers were infected by mosquito bite (SPZ

Control: 5 *P. falciparum* 3D7 infected mosquitoes, participants n=5) or an intravenous blood stage injection (BS Control: 1,962 *P. falciparum* 3D7 infected erythrocytes, participants n=5) and treated with a standard curative regimen of atovaquone/proguanil upon thick smear positivity. Here, we analysed antibody responses in 5 volunteers infected by mosquito bite (SPZ Control, acting as a control for SPZ Gct) and 5 infected with blood stages (BS Control, acting as a control for BS Gct). Plasma samples were collected prior to challenge (C-1), and at days C+7 (BS Control only), C+9 (SPZ Control only), C+21 and C+36 in BD Cell Preparation Tubes with sodium citrate. Plasma samples were collected in BD Cell Preparation Tubes with sodium citrate.

Asexual parasite densities were determined by 18S qPCR on prespecified timepoints as described previously [16, 18, 19]. Gametocyte densities were determined using quantitative reverse-transcriptase polymerase chain reaction (qRTPCR) for *ccp4* (female) and *pfmget* (male) messenger RNA, with a limit of detection of 0.1 male or female gametocyte/µL [20].

Both trials were performed at the Radboud university medical centre (Nijmegen, the Netherlands) following approval by the central committee on research involving human subjects (CCMO) under NL34273.091.10 and NL63552.000.17. All study participants provided written informed consent and both trials were registered at clinicaltrials.gov under NCT01236612 and NCT03454048. Antibody responses were specified as an exploratory outcome measure in the CHMI transmission study; the current analyses are thereby ancillary to the main study evaluation that focused on safety, gametocyte density and infectivity.

Gametocyte and asexual ELISA

Gametocyte and asexual extracts were prepared as described previously [21]. Nunc MaxiSorp™ 96-wells plates (ThermoFisher) were coated overnight at 4°C with 100 µl, equivalent to 75,000 gametocytes or 40,000 asexual parasites, per well. Plates were blocked with 5% skimmed milk in PBS and subsequently incubated with an 1:50 dilution of citrate plasma. Detection was done with 1:40,000 dilution Goat anti-Human IgG HRP (Invitrogen, Cat. No. 31412). ELISAs were developed by adding 100 µL tetramethylbenzidine and stopped with 50 µL 0.2M H_2SO_4. Absorbances were read at 450nm on an iMark™ microplate absorbance reader (Bio-Rad).

ELISA analyses were performed using Auditable Data Analysis and Management System for ELISA (ADAMSEL FPL v1.1). We included serial diluted control serum from a Dutch missionary that experienced many malaria episodes as a standard curve. The standard curve was plotted on a logarithmic scale and fitted to a power trend line ($R2 > 0.99$), optical density (OD) measurements for each test sample (average of duplicates that were no more than 25% different) were converted to arbitrary units (AU) relative to the control serum, where undiluted control serum was defined to contain 100 AU of IgG.

Bead-based antibody quantification

IgG antibodies against 21 antigens, one targeting pre-erythrocytic stages (Circumsporozoite protein [CSP] [22]), 15 targeting the asexual blood stage (Erythrocyte binding antigen [EBA140, EBA175 and EBA181] [23]; Early transcribed membrane protein 5 [Etramp-5] [24]; Glutamate rich protein 2 [GLURP-R2] [25]; Heat shock protein 40 [HSP40] [24]; Merozoite surface protein 1-19 [MSP1-19] [26], Merozoite surface protein 2 [MSP2-ch150/9 (3D7 family allele) [27], and MSP2-DD2 (FC27 family allele) [28]; Schizont egress antigen 1 [SEA-1] [29]; Skeleton binding protein 1 [SBP-1] [30]; Apical membrane antigen 1 [AMA1] [31]; and Reticulocyte binding protein homologue [RH2.2 [32], RH4.2 [33], RH5.1 [34]] and five (belonging to 2 proteins) targeting sexual stages [four fragments of Pfs48/45; Pfs230-CMB [35]] were quantified for all samples for each participant using a Luminex MAGPIX© suspension bead array, as described previously [36]. The proper conformation of Pfs48/45 recombinant proteins was validated using conformational dependent rat monoclonal antibodies [37]. A complete list of antigens is provided in **supplemental table 1**. Briefly, plasma/serum samples were assayed at a dilution of 1:200. Secondary antibody was an R-phycoerythrin conjugated goat anti-human IgG (Jackson Immuno Research, PA, USA; 109-116-098) diluted to 1:200. Data are presented as background adjusted median fluorescence intensities (MFI), or the same measure as a log2 ratio of each individual's adjusted MFI at baseline.

Protein microarray

De novo protein microarrays were designed and printed at the University of California, Irvine, to assess antibody responses to a panel of gametocyte enriched *P. falciparum* proteins. The backbone for protein selection was an analysis of specificity for the gametocyte stage, as scored by determining frequency of detection across 11 proteomic analyses. This analysis is described in detail elsewhere [12]. In summary: Proteins were binned from low to high abundance and weighted according to the retrieval rates of proteins in two curated lists of 'gold standard' gametocyte and asexual genes, consisting of genes that are known to be specific for either asexual stages (n=45) or gametocytes (n=41). High expression of gametocyte gold standard proteins with concurrent absence of non-gametocyte gold standard proteins resulted in a high gametocyte score, calculated from the fraction of retrieved gametocyte genes over retrieved non-gametocyte genes. All scores were log-transformed and summed over all data sets.

Full criteria for inclusion on the array are presented in **supplemental table 2**. In addition to gametocyte specificity, proteins were prioritized according to their likelihood of gamete surface expression (based on gene ontological terms, domain prediction, or empirical evidence), or association with gametocyte exposure [38], transmission blocking immunity [39] or antibody recognition of immature gametocyte infected red blood cell (giRBC) surfaces [6]. In total, a selection

of 600 unique *P. falciparum* genes were selected for cloning. Sequences encoding the proteins were obtained from a 3D7 strain reference genome, with sequences longer than 1000 amino acids split into multiple fragments (overlaps of at least 17 amino acids). PCR amplification and cloning were successful for 568 unique genes (making up 943 distinct sequences) all of which were expressed in an in vitro transcription and translation (IVTT) system (5 Prime, Gaithersburg, MD, USA) according to manufacturer instructions, and as described previously [39, 40]. Arrays were printed onto 8-pad nitrocellulose-coated glass AVID slides (Grace Bio-Labs, Inc., Bend, OR, USA) using an Omni Grid Accent robotic microarray printer (Digilabs, Inc., Marlborough, MA, USA). Each array and subarray contained IgG positive controls, for quality control, and negative controls containing the products of the IVTT reaction without PCR vector, for data normalisation. Samples were processed as described previously, with small deviations. Samples were probed at a final dilution on of 1:200, with secondary antibody (Southern Biotech, Goat Anti-Human IgG-TXRD) at a concentration of 0.5 µg/mL [39]. Arrays were scanned on a GenePix 4300A High-Resolution Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA). Local background was assessed for each protein target automatically, with foreground MFI determined using irregular threshold pixel density mapping. Correction for background was done for each spot using the 'backgroundCorrect' function of the *limma* package [41]. Background corrected values were transformed using the base 2 logarithm and normalised to systematic effects by subtracting the median signal intensity of the negative IVTT controls (internally within four subarrays per sample). The final normalised data are a log2 MFI ratio relative to the background reactivity of each sample/sub-array: a value of 0 represents equality with the background, and a value of 1 indicates a signal twice as high.

Data analysis

Data analysis was performed using R (R foundation for statistical computing, Vienna, Austria; version 4.1.2) [42], STATA (StataCorp. 2021. College Station, TX: StataCorp LLC; Release 17), or Graphpad PRISM (Graphpad software, San Diego, CA, USA; version 8). Total parasite and gametocyte area under the curve (AUC) was computed using GraphPad prism software with the formula AUC = $(\Delta X)^*(Y_1 + Y_2)/2$, where X is the time in days and Y the parasite density at a given timepoint. Correlation between parasite and serological data were analysed by Spearman's rank order correlation. For the analysis of breadth in the purified antigen antibody assays, antigens were considered 'recognised' for a given timepoint and participant if an antibody response (log transformed background adjusted MFI) exceeded the mean plus 2*SD of all individuals (n=34) against the same antigen pre-challenge. For the analysis of breadth in the protein microarray antibody assays, antigen recognition was defined as any log2 MFI ratio value greater than 1 (double the signal with respect to the internal array control). Serological data were log transformed and compared between timepoints within cohorts by paired t-tests, and between cohorts by students t-tests. For recombinant protein assays, significance thresholds were adjus-

ted by Bonferroni correction for comparisons of multiple antigens. Comparison between array responses between timepoints and cohorts were conducted with bayes moderated t-tests with adjustment for false discovery [43].

5.4 Results

In the CHMI transmission trial, 12 participants were infected by mosquito bite (SPZ Gct) and 12 participants were infected by intravenous injection of asexual parasites (BS Gct) [16]. All participants developed parasitaemia with median onset of qPCR detectable parasitaemia on day 7 (Interquartile range (IQR) day 7–9) in SPZ Gct and on day 5 (IQR day 5–5) in BS Gct (**supplemental figure 1**). To clear asexual parasitaemia but permit gametocyte maturation, participants received treatment with low dose piperaquine (480mg) on day 12.25 (Median, Interquartile range (IQR) day 10.5 – 12.5) in SPZ Gct, and all BS Gct participants were treated on day 8. This resulted in qRT-PCR detectable gametocytes post treatment in 11/12 participants in SPZ Gct and 12/12 participants in BS Gct [16].

In the control CHMI cohorts, 5 participants were infected by mosquito bite (SPZ Control) and 5 participants were infected by intravenous injection of asexual parasites (BS Control) with the same *P. falciparum* 3D7 parasite clone as the CHMI transmission trial. In contrast to the CHMI Gct trial, participants from control cohorts received a full curative treatment with atovaquone/proguanil that does not permit gametocyte maturation, initiated on day 12.3 (Median, IQR day 9.8–12.3) in SPZ Control and on day 8 (IQR day 8-8) in BS Control. As such, these participants were included in our analyses as negative controls without gametocyte exposure (**table 1**), although there was no formal demonstration of gametocyte negativity.

Neither peak total parasite density nor AUC were significantly different between SPZ Gct and SPZ Control (peak density, p=0.091; AUC, p=0.058), and between BS Gct and BS Control (peak density, p=0.673; AUC, p=0.206) or between SPZ Gct and BS Gct (peak density, p=0.478; AUC, p=0.977). However, significantly higher peak gametocyte densities were observed in BS Gct compared to SPZ Gct (p<0.001, Mann-Witney U; **table 1**) [16]. Total parasite AUC and gametocyte specific AUC were positively associated for both SPZ Gct and BS Gct (**supplemental figure 2A**); a tighter correlation was observed for BS Gct, reflecting the controlled timing of blood stage infection and subsequent schizogony in this cohort.

Table 1. Parasitaemia and gametocytaemia for different cohorts.

		Mosquito bite (sporozoite) infection		Asexual stage infection	
		SPZ Control	SPZ Gct	BS Control	BS Gct
Total parasites	Median peak density in parasites/µL (IQR)	7,413 (2,336 – 28,371)	32,807 (7,137 – 50,831)	44,668 (27,184 – 74,505)	27,700 (9,818 – 81,091)
	Median AUC in parasites/µL/day (IQR)	8,682 (2,782 – 29,890)	37,654 (15,430 – 71,484)	62,470 (39,606 – 103,950)	38,735 (11,366 – 75,145)
Gametocytes	Median peak density in gametocytes/µL (IQR)	ND	14 (10 – 64)	ND	1,304 (308 – 1,607)
	Median AUC in gametocytes/µL/day (IQR)	ND	1,574(596 – 3,018)	ND	11,043 (2,715 – 14,866)

AUC, Area under the curve; IQR, Interquartile range; ND, Not determined.

Antibody response to crude gametocyte extract does not reflect preceding gametocyte exposure

Extracts from mixed asexual stage and mature gametocytes from laboratory cultured *P. falciparum* NF54 were prepared to assess antibody responses in the CHMI participants to native parasite proteins (**figure 1**). At C+35/36 after infection, a statistically significant increase in anti-asexual stage antibodies was found compared to baseline in both SPZ Gct and BS Gct ($p<0.0001$) and corresponding SPZ Control ($p<0.001$) and BS Control ($p=0.002$). Anti-asexual stage IgG antibody levels post infection were not statistically different between infection routes SPZ Gct and BS Gct ($p=0.22$), and borderline significant between SPZ Control and BS Control ($p=0.050$).

IgG levels against gametocyte extract were also significantly higher post CHMI compared to baseline in the SPZ Gct and BS Gct groups ($p<0.0001$). There was no statistically significant difference in antibody responses to gametocyte extract between SPZ Gct and BS Gct cohorts ($p=0.31$). Although participants in control cohorts were not exposed to sexual stage parasites during their CHMI, some subjects infected by mosquito bites (SPZ Control) showed an increase in anti-sexual stage IgGs in response to infection. On average, antibody responses to gametocyte extract were higher after infection for both control cohorts (SPZ Control, $p=0.034$, BS Control, $p=0.0017$), though for SPZ Control this increase was disproportionally driven by a single volunteer (after exclusion, $p=0.075$).

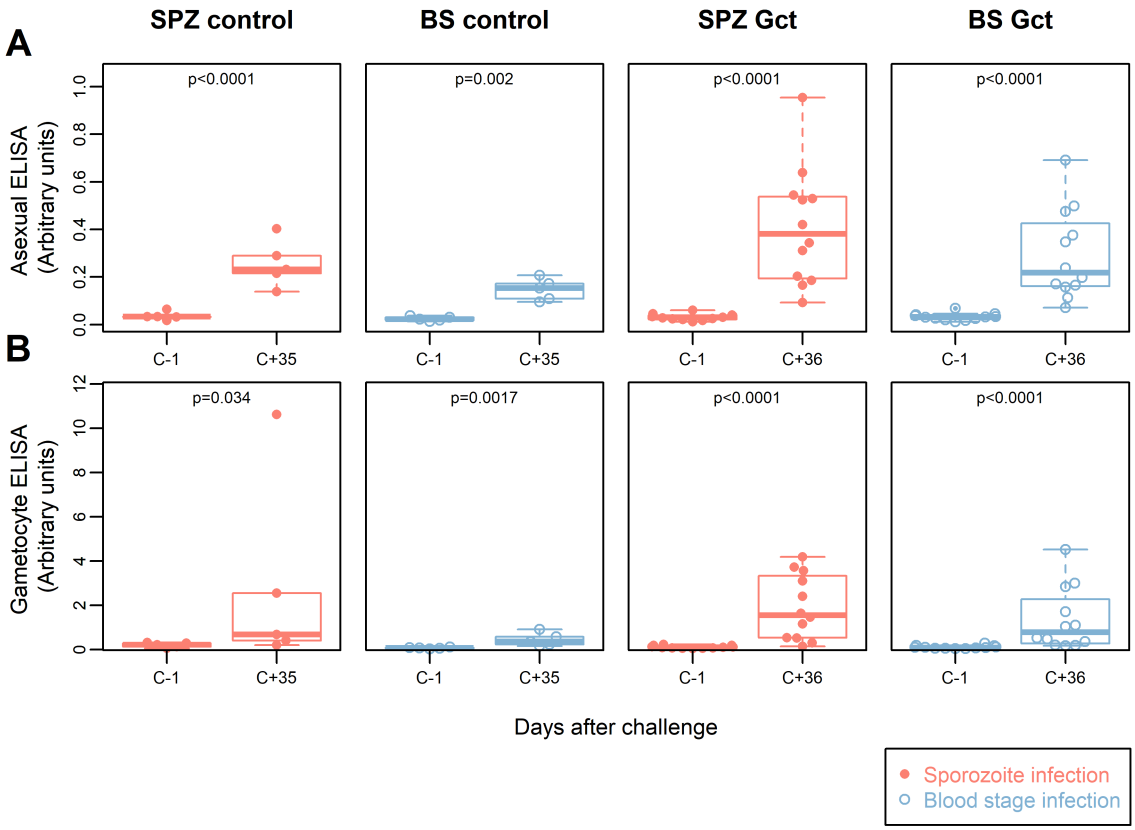

Figure 1. Participant parasite exposure and antibody response to crude parasite extracts. IgG antibody responses to crude blood stage and gametocyte extracts. In all plots, red (top) denote SPZ Gct (sporozoite infection) and blue (bottom) denotes BS Gct (Asexual infection). **A.** Anti-asexual antibody response pre- and post- (day 36) infection. **B.** Anti-gametocyte antibody response pre- and post- (day 36) infection (right). p=p-values from paired t-tests on log-transformed data. ns = Not Significant at p>0.05.

There were no statistically significant correlations between total parasite AUC or gametocyte AUC and either asexual or gametocyte antibody responses by ELISA (**supplemental figure 2B and 2C**).

Sexual stage-specific antibodies are induced after limited gametocyte exposure

We observed that antibody responses to crude gametocyte extracts cannot be used to discriminate between responses to asexual and sexual parasite stimuli. This indicates, perhaps unsurprisingly, an abundance of proteins of unknown stage-specificity in the gametocyte extract that formed the basis of this assay. We therefore analysed antibody responses to 21 well-characterized asexual stage, sporozoite, and sexual stage recombinant proteins, including TBV candidates Pfs48/45 and Pfs230, in a multiplexed bead-based antibody assay.

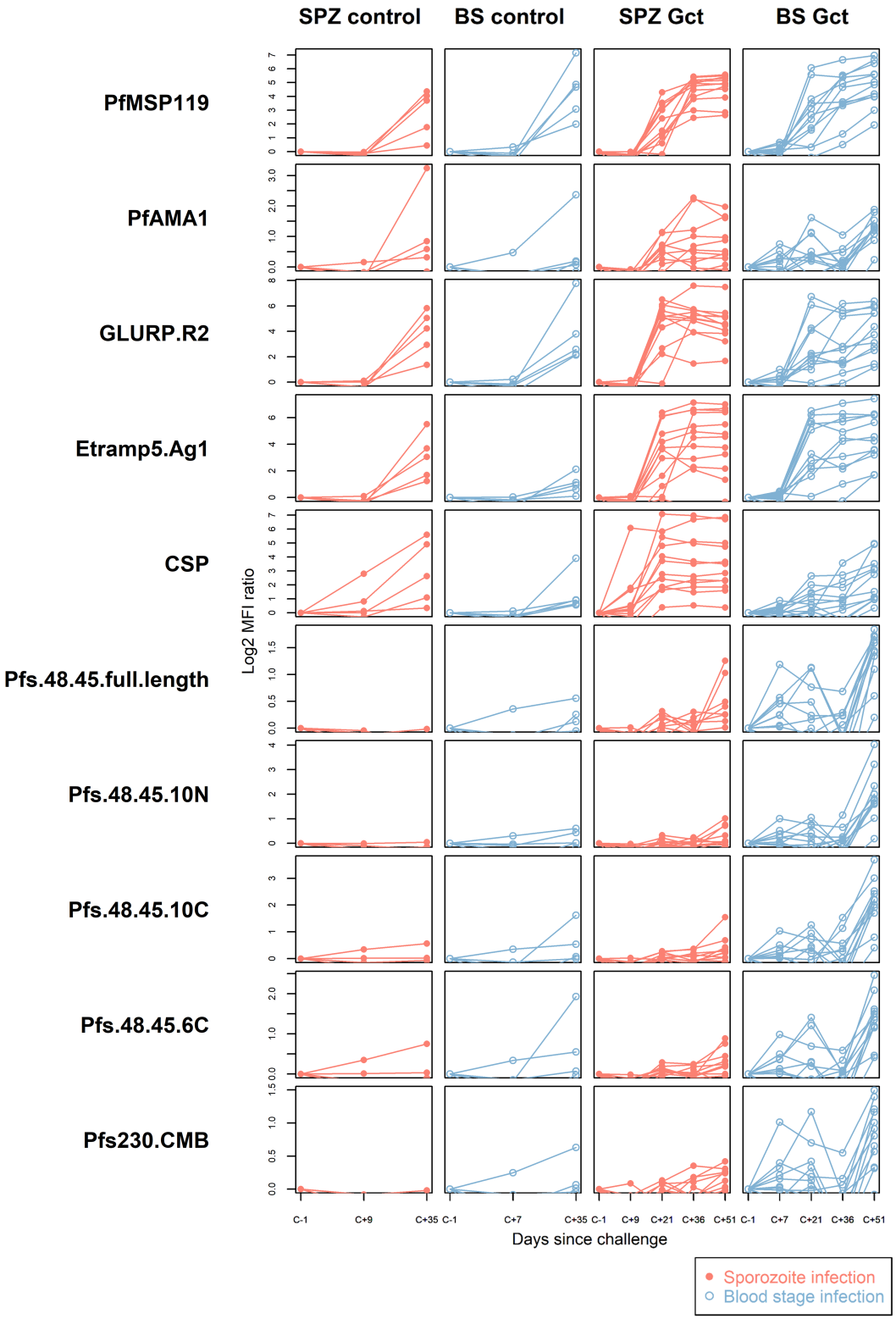

Figure 2. Antibody responses to purified recombinant proteins. IgG antibody responses to a selection of purified sporozoite, asexual and sexual stage antigens. Statistical analysis of response over time compared to baseline for all antigens is in **supplemental table 3**. In all plots, red solid circles denote mosquito bite (sporozoite) 10 infection cohorts (SPZ Control, SPZ Gct) and blue hollow circles denote asexual parasite infection (BS Control, BS Gct). Data are presented as log2 median fluorescence intensity (MFI) ratios of response over baseline, each line representing a single individual.

At baseline, the median number of antigens recognised per individual in SPZ Gct and BS Gct were 0.5 and 0, respectively (**supplemental figure 3**). By C+36, all individuals were seropositive to at least one antigen, with a statistically significant increase in antibody breadth in SPZ Gct (p=0.025), but not BS Gct (p=0.12). At C+51, 21/21 antigens were recognised by at least one individual in both cohorts. Between C+36 and C+51, there was no significant increase in breadth of response in SPZ Gct (p=0.23), but a significant increase in BS Gct (p=0.015). Breadth of antigen recognition in the control cohorts was similar to the CHMI groups at relative time points; the increase in breadth scores from baseline to C+35 was statistically significant in both SPZ Control (p=0.041) and BS Control (p=0.0086) and the change in median score was equal between control groups (SPZ Control, change in median = +2; BS Control, change in median = +2). In contrast, the increase in the median number of antigens recognized in the CHMI groups over the same time period was nearly twice as great in SPZ Gct (change in median = +3.5) compared to BS Gct (change in median +2).

As observed in the cell extract assays, quantitative antibody responses to specific recombinant antigens increased significantly over the period of observation (**figure 2**). In the CHMI Gct cohorts, the earliest independently significant increases in antibody response were observed at C+21 for a small number of non-sexual stage antigens (**supplemental table 3**). However, after adjustment for multiple comparisons the only significant response observed at day 21 was against CSP in the SPZ Gct cohort (p=0.001). At C+35/36 a greater number of antigens showed increased responses, with those to PfMSP1-19 in SPZ Gct and BS Gct, and GLURP-R2 in SPZ Gct remaining significant after adjustment for false discovery. At C+51, several asexual antigens remained significantly elevated including ETRAMP5 and GLURP-R2 in SPZ Gct, EBA175, MSP2-DD2 and PfAMA-1 in BS Gct, and PfMSP1-19 in both cohorts. After adjustment for false discovery, antibody responses to the sexual stage antigens Pfs230, Pfs48/45 full length, and the 10C fragment of Pfs48/45 were all significantly higher at C+51 compared to baseline in BS Gct, but not SPZ Gct. No statistically significant antibody responses to gametocyte antigens were observed in SPZ Gct at any point, or in either cohort before C+51.

In the control cohorts, independently significant responses were observed for HSP40 in SPZ Control, and EBA140 and PfMSP1-19 in BS Control; these increases were not statistically significant after adjustment for false discovery. It should be noted that samples were only available until C+36 for these cohorts.

Responses to gametocyte antigens occur after responses to asexual antigens

To determine possible shifts in antibody responses during follow-up, supportive of responses specifically induced by gametocytes, we next compared antibody responses and their relative rankings between days C+35/36 and C+51. For this, antibody responses to all antigens were ranked by median magnitude of response in each cohort (**supplemental figure 4**) and compared between C+35/36 and C+51 timepoints.

Within the CHMI Gct cohorts, median magnitude-ranked responses to sexual stage antigens were most increased between C+36 and C+51 in BS Gct, with Pfs48/45-10C, Pfs48/45-10N and Pfs48/45 full length moving up by 5, 4 and 5 positions, respectively (**supplemental table 4**). In SPZ Gct, Pfs48/45-10C and Pfs48/45-10N moved up by 1 and 2 positions, though Pfs48/45 full length fell by 1 position. Little to no change was observed for Pfs48/45-6C or Pfs230-CMB in either cohort between timepoints. Sexual stage antigen rankings at C+35/36 between SPZ Gct and SPZ Control were similar, while median responses tended to rank higher in BS Control than in BS Gct at the equivalent timepoints.

Antibody response to gametocyte infected erythrocyte surface antigens are among those 6 correlated with cumulative gametocyte exposure

The correlation between each recombinant protein biomarker and asexual or gametocyte exposure was assessed by analysing antigen-specific antibody data from the final timepoint of observation (C+51) in the CHMI Gct cohorts (**supplemental figure 5**, **supplemental table 5**) in relation to prior parasite biomass (*i.e.* area under the curve of density over time). In SPZ Gct, responses to Pfs230 and Pfs48/45-10N were independently correlated with asexual and gametocyte AUC, but these correlations were not statistically significant after adjustment for multiple comparisons. For BS Gct, several asexual and sexual stage responses were independently correlated with asexual and gametocyte AUC; after adjustment only PfMSP1-19 showed a statistically significant positive correlation with asexual AUC (R^2=0.64, p=0.0017), and only Pfs48/45-10C showed a statistically significant positive correlation with gametocyte AUC (R^2=0.64, p=0.0019).

To identify novel antibody biomarkers of gametocyte exposure, 943 protein targets (mapping to 568 gene IDs) were printed on microarrays, following selection for their enrichment in gametocyte stages based on transcriptomic and proteomic evidence (**supplemental table 2**), or inclusion as known *Plasmodium* biomarkers. Antibody breadth increased significantly after infection (C+35/36) in all cohorts except for SPZ Control **figure 3A**. Mean magnitude of response to all antigens for each participant increased significantly in SPZ Gct and BS Gct, but

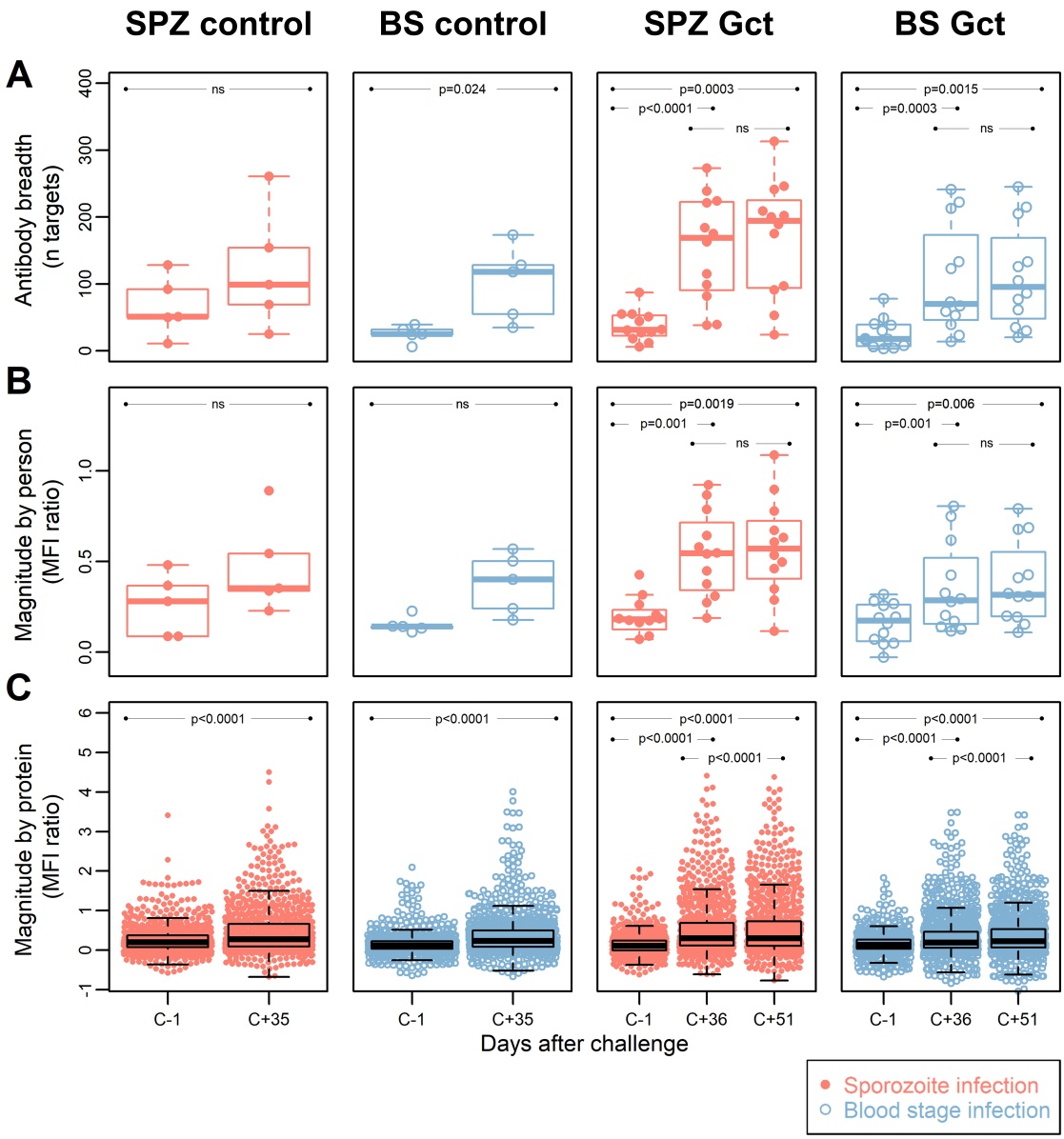

Figure 3. Antibody breadth and magnitude. Data points for each individual within each cohort group are represented with dots in a beeswarm pattern. Overlayed boxplots represent the median (thick line), interquartile range (box limits) and the $25^{th}/75^{th}$ percentiles plus 1.5*IQR (whiskers). **A.** Antibody breadth is the number of protein targets (out of a total of 943) with responses above background, for each individual. For SPZ Gct, the mean MFI ratio of antibody responses to all proteins for one individual was -0.47 at C-1; this data point was not included in the plot, but the parameters of the relevant box plot were calculated from all data points. **B.** Antibody magnitude is shown as the mean magnitude of response by each individual in a cohort group/timepoint to all protein targets. Magnitude of response is shown as a log2 MFI ratio, where 0 represent no change relative to background, and 1 represents doubling with respect to background. **C.** Antibody magnitude is shown as the mean magnitude of response to each protein target by all individuals in a cohort group/timepoint, with units as in **B**. P-values are from paired two-sided t-tests for difference between C-1 and C+35/36, C-1 and C+51, or C+36 and C+51, as indicated. MFI: median fluorescence intensity; IQR: Interquartile range. p = p-value from paired t-tests. Ns = Not significant at p=0.05.

not in the controls, while a significant increase in mean magnitude of response to each target protein was observed for all cohorts (**figure 3B and 3C**). Correlation in mean response to each target between the two included post-infection timepoints (C+36 compared to C+51) was near perfect for SPZ Gct (R^2=0.99, p<0.0001) and BS Gct (R^2=0.98, p<0.0001).

At the level of individual targets, 216 of the 943 IVTT protein targets on the array displayed a significant increase in antibody response from pre-infection to either C+36 or C+51 in SPZ Gct, and 91 showed a significant increase in BS Gct. Four of the 91 targets with statistically significant responses in BS Gct were uniquely responsive in this cohort: PF3D7_0905300 (dynein heavy chain, putative; 1.65 fold increase compared to baseline), PF3D7_1302000 (EMP1-trafficking protein; 1.31 fold increase), and PF3D7_0721100 (conserved protein, unknown function; 1.32 fold increase), and PF3D7_1306500 (MORN repeat protein, putative, 1.15 fold increase) (**supplemental table 6**). In the control cohorts, the only statistically significant increase in antibody response (C-1 to C+35) after adjustment for multiple comparisons was PF3D7_0206800 (MSP2) in SPZ Control.

To further distinguish antibody responses elicited by the principally asexual stimulus of the control CHMI studies from responses to the asexual and gametocyte stimulus of the Gct studies, the mean magnitude of response to each target post-infection was compared between control and test cohorts according to infection methodology. A threshold for negligible change in response between time points in the control cohorts was set arbitrarily as any percent difference in MFI of 7.5% or less, while a positive response cut-off for the Gct cohorts was set at 25%. 348 targets showed increases in magnitude (>25% absolute fold change) from pre- to post- (C+36 or C+51) infection in the Gct cohorts (**figure 4**). Of these, 67 were responsive only in the SPZ Gct and/or BS Gct CHMI and not in their respective control cohorts; 53 were unique to SPZ Gct (median maximum percent change between cohorts at C+36 or C+51: 37.5% [IQR 30.7-47.4]), 8 were unique to BS Gct (35.0% [30.3-39.0]), and 6 were shared in both (SPZ Gct: 37.1% [32.9-41.6], BS Gct: 38.6 [29.0-44.1]) (**supplemental table 6**). Nineteen targets were uniquely identified in the day 36 analysis, 6 in the day 51 analysis, and 42 were identified as responsive in both. A sub-selection of putative biomarkers of gametocyte exposure was made and is shown in (**table 2**). This includes the four targets described above with significantly higher antibody responses (day C+36 and/or 51) compared to baseline in BS Gct but not SPZ Gct, six with responses in both SPZ Gct and BS Gct but not in their methodological control cohorts, and six with response in either SPZ Gct or BS Gct where a response was observed at C+51 but not at C+36 (two targets met more than one of these criteria). There was no significant correlation between antibody responses to these targets and total parasite AUC in either SPZ or BS Gct (**supplemental figure 6A**), and no correlation between response and gametocyte AUC in SPZ Gct (**supplemental figure 6B**). For BS Gct, 4 targets showed independently significant or borderline significant associations with gametocyte AUC: PF3D7_0721100 (R^2=0.33, p=0.051; conserved *Plasmodium* protein, unknown function), PF3D7_1302000 (R^2=0.47, p=0.014; EMP1-trafficking

protein [PTP6]), PF3D7_0726400 (R^2=0.37, p=0.037; conserved *Plasmodium* membrane protein, unknown function), and PF3D7_1016300 (R^2=0.34, p=0.046; glycophorin binding protein [GBP]). None of these remained significant after adjustment for false discovery.

The 69 array targets meeting any of our criteria for further investigation (**supplemental table 6**) mapped to 64 unique gene IDs; three IDs were represented by two peptides (PF3D7_1250100; osmiophilic body protein (G377), PF3D7_0212400; conserved *Plasmodium* membrane protein, unknown function, PF3D7_1328000; conserved *Plasmodium* protein, unknown function) and one was represented by three peptides (PF3D7_1038400; gametocyte specific protein Pf11-1). Association with cell membranes was highly represented (n/N=27/65) among predicted gene ontological terms (**supplemental table 6**). Antibody responses to several well-characterised blood stage antigens were associated with gametocyte exposure in our analyses, including PF3D7_1228600 (merozoite surface protein 9, MSP9), PF3D7_0711700 (erythrocyte membrane protein 1, PfEMP1 [VAR]), and PF3D7_0102200 (ring-infected erythrocyte surface antigen, RESA). Well-characterised sexual stage antigens included PF3D7_1302100 (gamete antigen 27/25 [G27/25]), PF3D7_1102500 (parasite/early gametocyte exported protein PHISTB/GEXP02]), and PF3D7_1038400 (gametocyte-specific protein [Pf11-1]). Overall, 4/64 targets were specific to asexual stages, 42 were specific or enriched in gametocytes, 17 showed more evenly shared stage expression, and 1 was unclassifiable. Antibody responses to eleven of the 64 gene products identified here were noted for their association with naturally acquired transmission blocking immunity (TBI) in a previous analysis of individuals from Burkina Faso, Cameroon and Gambia [39]. Nine of the 65 genes (three also linked with TBI) were identified as putative early gametocyte erythrocyte surface antigens in a previous analysis of rodent infections and sera from Malawi [6].

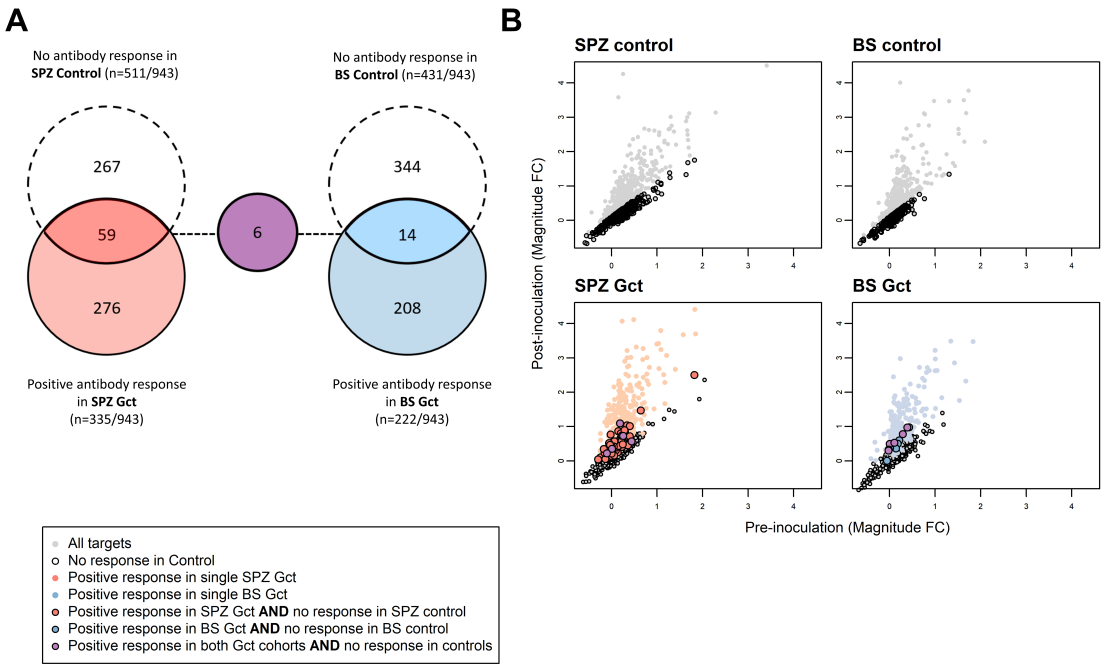

Figure 4. Differences in antibody response to microarray targets associated with gametocyte exposure. A. Array targets demonstrating minimal response (<7.5% increase in the mean magnitude of response between pre- [day 0] and post- [day 35] inoculation timepoints) in the control CHMI cohorts are shown in orange for SPZ Control (sporozoite inoculum) and blue for BS Control (asexual inoculum). Array targets demonstrating a positive response (>7.5% increase in the mean magnitude of response between pre- [day 0] and post- [day 36] inoculation timepoints) in the control CHMI cohorts are shown in dashed circles for each cohort, with overlap representing those targets with minimal response in control and positive response in Gct cohorts. **B.** Axes show pre- and post-inoculation mean magnitude of response (log2 MFI ratio) to each target on the array. Mean magnitude for each target is represented by a single marker. Grey markers are those with increased responses after inoculation in the Control cohorts. Orange and blue markers are as in **A**. Targets with responses only in the CHMI Gct cohorts are outlined in black.

Table 2. Antibody targets putatively linked with higher gametocyte exposure as identified by protein microarray. Summary table listing targets with significantly higher antibody responses (day C+36 and/or 51) compared to baseline in BS Gct but not SPZ Gct (*n=4), in both SPZ Gct and BS Gct compared to their methodological control cohorts (**n=6), and in either SPZ Gct or BS Gct where a response was observed at C+51 but not at C+36 (***n=6 [overlapping]). BS = Antibody response in blood stage infection cohort, SPZ = Antibody response in sporozoite infection cohort, 36 = Antibody response at C+36 compared to methodology matched control, 51 = Antibody response at C+51 compared to methodology matched control. Full details of these targets and all targets described in the text are given in **supplemental table 6**.

Gene ID	Name	Description	Criteria for selection	Note
PF3D7_0905300		dynein heavy chain, putative	*	
PF3D7_1306500		MORN repeat protein, putative	*	Naturally acquired transmission blocking immunity (mosquito stage) (39)
PF3D7_1351000		phosphatidylinositol transfer protein, putative	BS/51***	
PF3D7_1125200		ubiquitin-like domain-containing protein, putative	BS/51***	
PF3D7_0721100		conserved Plasmodium protein, unknown function	BS/51*/***	Early giRBC membrane antigen, putative (6)
PF3D7_1409400		conserved Plasmodium membrane protein, unknown function	SPZ/51***	
PF3D7_1135600	CAPD3	condensin-2 complex subunit D3, putative	SPZ/51***	
PF3D7_1016300	GBP130	glycophorin binding protein (GBP)	SPZ/51***	Early giRBC membrane antigen, putative (6)
PF3D7_1328000		conserved Plasmodium protein, unknown function	SPZ and BS/36 and 51**	
PF3D7_0307900		conserved Plasmodium protein, unknown function	SPZ and BS/36 and 51**	
PF3D7_0726400		conserved Plasmodium membrane protein, unknown function	SPZ and BS/36 and 51**	Early giRBC membrane antigen, putative (6) AND Naturally acquired transmission blocking immunity (mosquito stage) (39)
PF3D7_0201900	EMP3	erythrocyte membrane protein 3 (EMP3)	SPZ and BS/36 and 51**	Early giRBC membrane antigen, putative (6)
PF3D7_1320800		dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	SPZ and BS/36 and 51**	
PF3D7_1302000	PTP6	EMP1-trafficking protein (PTP6)	SPZ and BS/36 and 51*/**	Early giRBC membrane antigen, putative (39)

Summary table listing targets with significantly higher antibody responses (day C+36 and/or 51) compared to baseline in BS Gct but not SPZ Gct (*n=4), in both SPZ Gct and BS Gct compared to their methodological control cohorts (**n=6), and in either SPZ Gct or BS Gct where a response was observed at C+51 but not at C+36 (***n=6 [overlapping]). **BS** = Antibody response in blood stage infection cohort, SPZ = Antibody response in sporozoite infection cohort, 36 = Antibody response at C+36 compared to methodology matched control, 51 = Antibody response at C+51 compared to methodology matched control. Full details of these targets and all targets described in the text are given in Supplemental Table 6.

5.5 Discussion

To improve our understanding of naturally acquired immunity after gametocyte exposure, we assessed antibody responses to antigens in parasite and gametocyte extracts, selected recombinant *P. falciparum* proteins and a large panel of gametocyte-enriched proteins in volunteers from CHMI cohorts with different exposures to gametocytes. We showed that antibody responses to sexual stages are induced after a single exposure to relatively low gametocyte densities (peak densities up to ~1,600 gametocytes/mL). The antibody response to sexual stage-specific proteins was higher in participants exposed to higher gametocyte densities and was observed later than the response to well-characterised asexual antibody responses. Furthermore, we identified a list of known and new antigens that elicit antibodies that are associated with gametocyte exposure.

A handful of studies aimed to identify antibody responses to gametocyte-specific antigens in naturally exposed individuals. Several seroepidemiological studies, limited to the well-known gametocyte antigens Pfs48/45 and Pfs230 [44, 45], demonstrated rapid and short-lived gametocyte-specific antibody responses [46, 47], which do not necessarily increase with age as responses to some asexual antigens do [44, 48]. Skinner et al. analysed antibody responses in the sera of Malian children to a large panel of putatively gametocyte-specific antigens based on the first published *P. falciparum* proteome [38]. Comparing responses before and after the malaria season, they identified high seroprevalence of antibodies to the proteins Pfs16, PF3D7_1346400, PF3D7_1024800 and PfMDV1, indicating that these may be important biomarkers of gametocyte exposure. More recently, Muthui et al. set out to analyse naturally acquired antibody responses to seven antigens selected based on potential gametocyte surface expression, including Pfs230, PfMDV1 and five previously uncharacterized gametocyte antigens [49]. They demonstrated antibody responses to all seven antigens, and suggested that PF3D7_0303900, PF3D7_1314500, and PF3D7_0208800 may have potential as markers of high gametocytaemia. Though undoubtably useful, these studies were not designed to assess the immune response to incident infection or accurately validate serological biomarkers as indicators of prior gametocyte exposure. In field settings, such assessments are challenging and require longitudinal observations before and after infection with sensitive quantification of parasite and gametocyte exposure. Complementary to the studies in natural gametocyte exposure populations, CHMI provides a unique opportunity in which the absence of prior exposure is guaranteed, and parasitaemia and gametocytaemia are monitored with high precision to provide metrics for cumulative exposure to different parasite life stages. In our current study, all CHMI cohorts showed an overall increase in antibody responses after infection to antigens in asexual as well as gametocyte extracts. CHMI control cohorts were exposed to asexual parasites but probably not to mature gametocytes: while gametocyte commitment may have occurred [50] and, potentially, early-stage gametocytes may have developed, early treatment of volunteers with a curative regimen of atovaquone/proguanil upon thick smear positivity makes it very unlikely that gametocytes

completed maturation. Nevertheless, responsiveness in our gametocyte ELISA in CHMI control cohorts is not unexpected since the majority of antigens are shared between parasite stages [12], and responses to crude gametocyte extract were previously shown to be a poor predictor of gametocyte carriage [51].

Importantly, responses against sexual stage specific antigens were highest in our cohort that was exposed to the highest gametocyte burden, with antibodies to Pfs48/45 and Pfs230 showing a statistically significant increase during follow-up in the BS Gct group. In this cohort, anti-Pfs48/45 antibody responses were also strongly associated with the preceding gametocyte biomass; this response was observed only at day 51 after CHMI, indicating a slight lag from peak gametocytaemia (c. day 20) to its commensurate response (not observed at day 36) compared to the peak in parasite density (day 8) and the observation of a response (from day 21). Given the relative sizes of the asexual and gametocyte biomass and their anticipated antigenic insults, these findings are broadly in line with expectations. It is noteworthy though that despite the relative scarcity of gametocytes, antibody responses to some sexual stage antigens (Pfs48/45-10C) were ranked higher than the majority of asexual antigens, indicating that the native Pfs48/45 protein is highly immunogenic. One unexpected finding was the antibody response to CSP after infection with blood stage parasites; we hypothesize that the abundance of low complexity, highly immunogenic repeat regions shared by CSP and some blood stage antigens may have resulted in a degree of cross-reactivity [52], Unexpected blood stage antigen reactivity has been observed previously after RTS'S vaccination [53].

Pfs48/45 and Pfs230 represent two well-described gametocyte antigens, but as there are hundreds of proteins predicted to be enriched or specifically expressed in gametocytes [12, 54], these two represent a tiny fraction of antigens that are likely to induce antibodies during gametocyte exposure. The ability to detect gametocyte-specific antibodies in the BS Gct cohort encouraged us to try to identify other gametocyte antigens that induced specific responses. We thus compared antibody responses to a large panel of gametocyte-enriched proteins on a protein microarray between the SPZ Gct and BS Gct cohorts. A long list of gene products (n=64) was identified for further analysis if they: 1) Showed significant antibody response to infection in the high (BS Gct) but not in the low (SPZ Gct) gametocyte cohort; or 2) Showed antibody responses in either the low or high Gct cohorts while having a negligible response in control CHMI cohorts. Encouragingly, we identified multiple antigens that are known to be gametocyte-specific, including Pfs16 [55], Pfg27 [55], Pf11-1 [56] and Pfg377 [57]. Furthermore, there was considerable overlap with gametocyte proteins on the surface of infected red blood cells identified by Dantzler et al. [6], and with antigens that were identified as being associated with functional transmission reducing activity [39]. Given that antibody responses in our CHMI cohort participants were low, as compared to individuals with rare functional anti-gametocyte immunity [39, 58], and as expected, based on a single relatively low infection burden, we did not expect or assess the functionality of responses but focused on the kinetics of antibody acquisition and

the potential utility of responses as biomarkers of gametocyte exposure.

Our findings are based on detailed assessments of parasite exposure and antibody responses following a first encounter with *Plasmodium* parasites in a small number of volunteers. A limitation of this study is the relatively low gametocyte exposure with median peak densities of 1,304 (IQR 308–1,607) gametocytes/mL combined with a relatively short duration of exposure. Whilst these densities are similar to that observed in many asymptomatic infections in endemic settings, much higher densities and, in particular, much longer exposure to gametocytes can be observed in naturally infected individuals [59]. As such, it is conceivable that our analysis did not identify all markers of epidemiologically relevant gametocyte carriage. A second limitation, as described above, is that we did not formally demonstrate absence of gametocyte exposure in control cohorts. If gametocytes had developed in control subjects, our comparisons between cohorts may have resulted in a conservative interpretation of unique gametocyte responses. A further limitation is the limited number of timepoints for immunological assessments. Control cohorts did not have a late timepoint (C+51) of sampling and we were unable to examine antibody longevity beyond day 51. Assessing the duration of detectable antibody titers, isotype and avidity beyond this timeframe, and whether re-infection will boost and/or change these responses will be valuable in the characterization of antibodies as biomarkers of infectivity and could have implications for the development of transmission blocking vaccines (TBV). Natural boosting of antibody responses has been noted as an advantage for TBVs that target pre-fertilisation gametocyte antigens. We found that all BS Gct participants showed an increase in antibodies to TBV candidate antigens Pfs48/45 and Pfs230, including the Pfs48/45-6C and Pfs230CMB fragments that are similar to TBV targets currently in human trials (Ref. [60] and clinicaltrials.gov: NCT04862416). Our findings suggest relevant natural boosting of Pfs48/45 and Pfs230 antibody responses; we further report on the first distinct analysis of responses against full length Pfs48/45, as well as three Pfs48/45 fragments: 10C, 10N and 6C.

In conclusion, we found increased humoral responses to *P. falciparum* sexual stages after exposure to a single CHMI, irrespective of gametocyte densities. The cohort with highest gametocyte exposure showed more sexual-stage specific responses compared to the cohort exposed to low gametocytes, while overall parasite responses were higher in SPZ Gct. Using a protein microarray we identified potential gametocyte specific antigens.

Acknowledgements

This work was supported by a Sir Henry Wellcome fellowship (number 218676/Z/19/Z) from the Wellcome Trust (UK) awarded to WS. TB, RMdJ and MA are supported by a fellowship from the European Research Council (ERC-CoG 864180; QUANTUM). CD and TO are supported by a project grant from the Bill and Melinda Gates foundation (INDIE OPP1173572),

and KKAT is supported by the Intellectual Ventures Global Good Fund and a UKRI Connecting Capabilities fund/Bloomsbury SET (CCF-17-7779). MM and PN are supported by a Programme grant from the UKRI Medical Research Council (MR/T016272/1) and Wellcome Center award 104111. We would like to thank James Beeson for provision of recombinant protein, and Marga van de Vegte-Bolmer, Geert-Jan van Gemert, and Kjerstin Lanke for their assistance with laboratory assays, and Jackie Cook and David Macleod for their data analytical assistance.

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5.6 Supplemental material

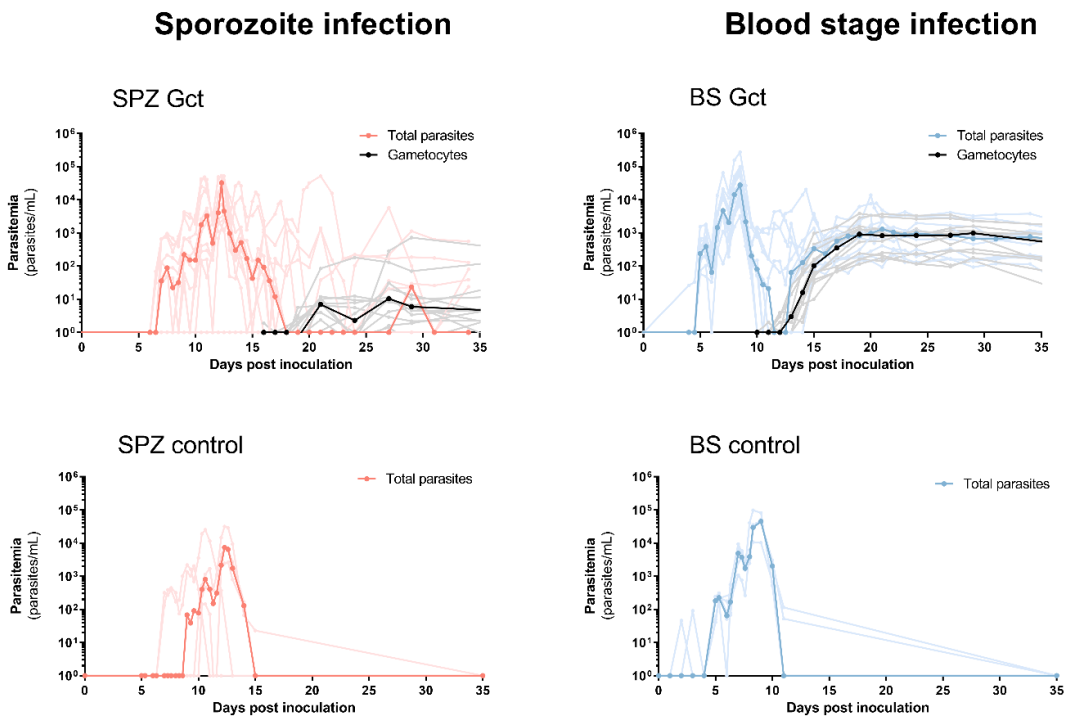
Supplemental table 1. Recombinant antigens used in the luminex bead-based antibody quantification assay. Refs. [1–11]

Gene ID	Acronym	Description	Location	Expression tag	Strain	Reference
PF3D7_0304600	CSP	Sporozoite surface. Component of RTS,S vaccine	Sporozoite	n/a	3D7	[1]
PF3D7_1301600	EBA140 RIII-V	Erythrocyte binding antigen 140; erythrocyte invasion	Apical organelles, micronemes	GST	3D7	[2]
PF3D7_0731500	EBA175RII_F2	Erythrocyte binding antigen 175; RBC binding region via glycophorin A	Apical tip	GST	3D7	[2]
PF3D7_0102500	EBA181 RIII-V	Erythrocyte binding antigen 181; involved in erythrocyte invasion	Apical tip	GST	3D7	[2]
PF3D7_0532100	Etramp 5 Ag 1	Early transcribed membrane protein. Integral PVM protein	iRBC, PVM	GST	3D7	LSHTM
PF3D7_1035300	GLURP R2	Glutamate rich protein R2	Merozoite Surface	n/a	F32	[3]
PF3D7_0501100.1	HSP40 Ag 1	Heat shock protein 40	iRBC	GST	3D7	LSHTM
PF3D7_0206800	MSP2 CH150/9	CH150/9 allele of MSP2. Full-length.	Merozoite surface	GST	CH150/9	[4]
PF3D7_0930300	MSP1-19	19kDa fragment of MSP1 molecule.	Merozoite surface	GST	Wellcome	[5]
PF3D7_0206800	MSP2 Dd2	Dd2 allele of MSP2. Full-length.	Merozoite surface	GST	DD2	[6]
PF3D7_1021800	PfSEA-1	Schizont egress antigen	iRBC	GST	3D7	LSHTM
PF3D7_0501300	SBP1	Skeleton-binding protein; translocation of PfEMP1 to RBC surface via Maurer's cleft	iRBC	GST	3D7	LSHTM
PF3D7_1133400	AMA1	Apical membrane antigen 1	Micronemes	His	FVO	[7]
PF3D7_1335400	Rh2_2030	Reticulocyte-binding protein homolog 2; involved in erythrocyte invasion	Merozoites; Rhoptries	GST	3D7	[8]
PF3D7_0424200	Rh4.2	Reticulocyte-binding protein homolog 4; involved in erythrocyte invasion	Merozoites; Rhoptries	GST	3D7	[9]
PF3D7_0424100	Rh5.1	Reticulocyte-binding protein homolog 5; involved in erythrocyte invasion	Merozoites; Rhoptries	His	3D7	[10]
PF3D7_1346700	Pfs48/45-10C	Gametocyte/gamete surface protein, 10C fragment	Mature gametocytes/gametes	His	3D7	Radboud
PF3D7_1346700	Pfs48/45-10N	Gametocyte/gamete surface protein, 10N fragment	Mature gametocytes/gametes	His	3D7	Radboud
PF3D7_1346700	Pfs48/45-6C	Gametocyte/gamete surface protein, 6C fragment	Mature gametocytes/gametes	His	3D7	Radboud
PF3D7_1346700	Pfs48/45-Full length	Gametocyte/gamete surface protein, Full Length	Mature gametocytes/gametes	His	3D7	Radboud
PF3D7_0209000	Pfs230-CMB	Gametocyte/gamete surface protein, CMB fragment	Mature gametocytes/gametes	His	3D7	[11]

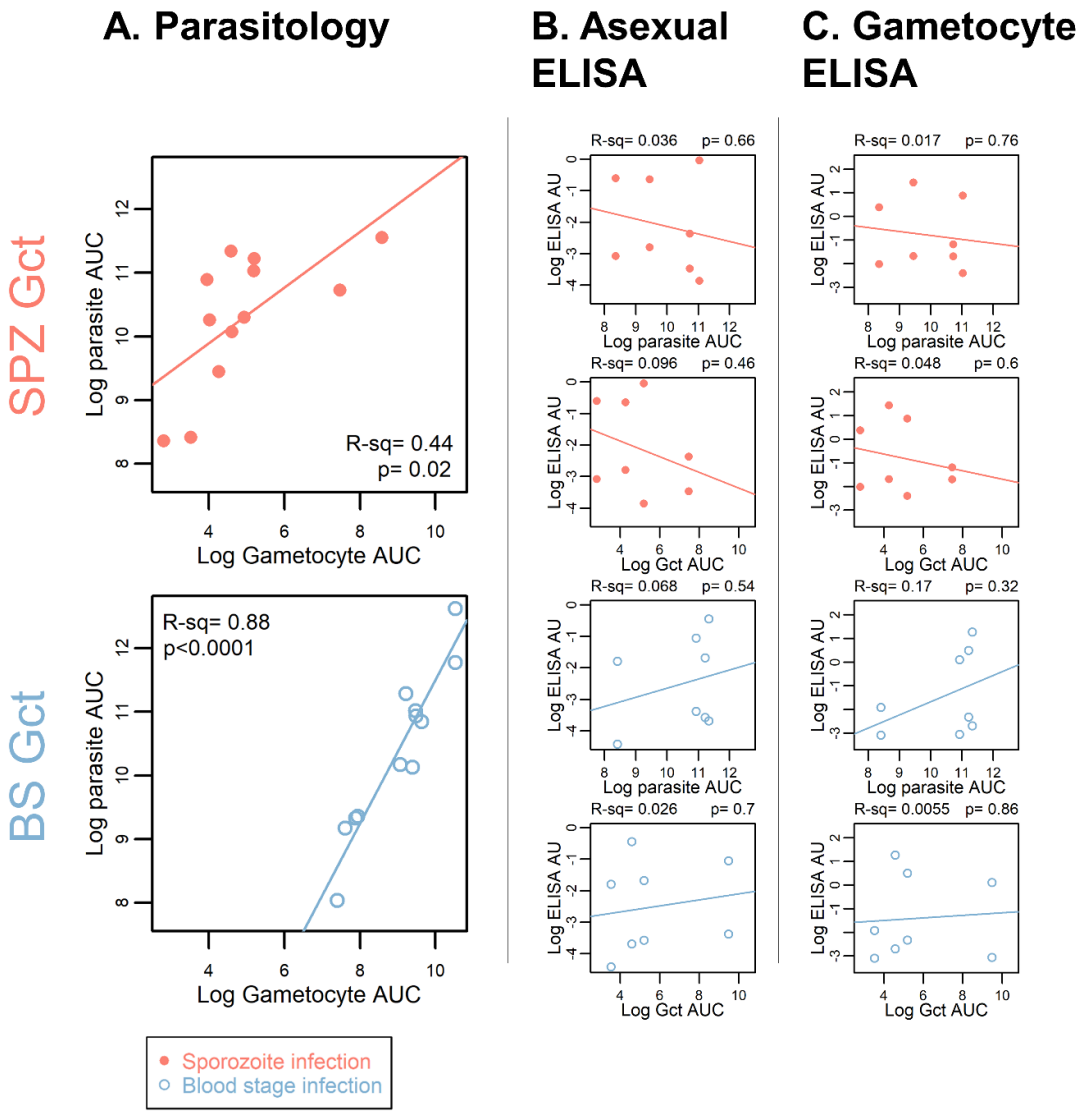
Radboud: Produced in house at Radboudumc; LSHTM: Produced in house at LSHTM

Supplemental table 2. Selection criteria for genes expressed as proteins on the gametocyte protein microarray. Selection was made as follows, initially prioritizing proteins with characteristics necessary for surface level expression and less conservative gametocyte specificity (selection for involvement in TRI), before including proteins with greater gametocyte specificity regardless of their function (selection of markers of exposure): **1.** Proteins with evidence for presence or enrichment in gametocytes (score >0) PLUS presence of transmembrane domains (tm), signal peptides (sp), or GO term indicating membrane or surface expression (excluding mitochondrial proteins) [n=372] **2.** Proteins without unique peptides in any proteomic analysis, or evidence of translational repression in literature [12] PLUS high gametocyte transcript score (>9.63), PLUS presence of TM/SP/GO term indicating membrane or surface expression (excluding mitochondrial proteins) [n=36, cumulative total = 407] **3.** Gametocyte protein score >-10 (some evidence of gametocyte expression) PLUS presence of GPI anchor [13] or predicted export protein (PlasmoDB) [n=60, cum. total = 436] Proteins putatively exported by and specific to early or late gametocytes [14] n=30, cum. total = 443] **5.** Gametocyte protein score of >9.69 (Gametocyte enriched), regardless of protein structure/function, and an average peptide score of 9 in each of the gametocyte databases [n=107, cum. total = 518) **6.** Gametocyte specific proteins from early literature 14,19[15, 16]. PLUS seropositive in field sera in >50% of samples after transmission season or with >20% sero-prevalence increase after transmission season [17] (n=43, cum. total 451) **7.** Gold standard gametocyte proteins in list used to generate transcript and protein score [n=41, cum. total = 538) **8.** A priori protein selection. Markers of sexual stage exposure, transmission blocking vaccine candidates, 6-cys proteins, proteins implicated in gamete fertility, asexual markers etc. (n=37, cum. total = 561) **9.** Inclusion based on previous gametocyte array analysis [18] of differently reactive proteins in naturally occurring transmission blockers [n=45, cum. total = 580), **10.** Inclusion based on association with the giRBC surface of immature gametocytes [19] [n=47, cumulative total and total number of IVTT targets on array = 600]. Of the 600 proteins selected for inclusion, 568 were successfully expressed and printed on the array (943 IVTT protein targets).

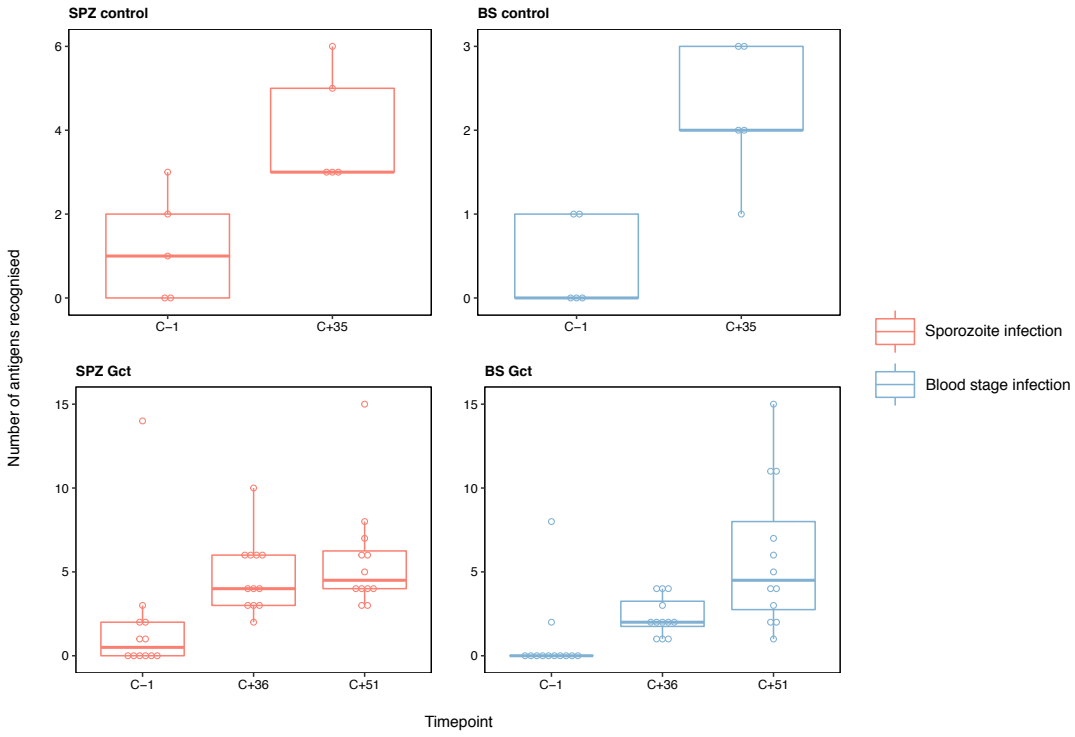
Criteria ID	Genes matching criteria	*Genes added*	Gene cumulative total	Criteria for inclusion	Simplified criteria
1	372	*372*	372	Gametocyte protein score >0 PLUS (presence of TM/SP/GO term indicating membrane OR surface expression (excluding mitochondrial proteins))	Protein moderately upregulated in gametocytes, and indication of membrane expression (excluding mitochondrial)
2	36	*35*	407	Zero protein evidence in any proteomic analysis, or evidence of translational repression in literature PLUS high gametocyte transcript score (>9.63) PLUS (presence of tm/sp/go term indicating membrane OR surface expression (excluding mitochondrial proteins))	Zero protein scores but gametocyte specific transcript (evidence of failure to detect protein, and/or mRNA storage/translational repression)
3	60	*29*	436	Gametocyte protein score >-10 (some evidence) PLUS (presence of GPI anchor [12] OR predicated export protein (Public domain).	Presence of protein in gametocytes, plus GPI anchor, or export association
4	30	*7*	443	Putatively exported by and specific to early or late gametocytes [13]	Additional exported proteins specific to early gametocytogenesis
5	107	*75*	518	Gametocyte protein score of >9.69, regardless of protein structure/function, and an average peptide score of 9 in each gametocyte proteome database	Highly upregulated in gametocytes regardless of structure/function
6	43	*13*	531	Gametocyte specific proteins [14] seropositive in field sera in >50% of samples after transmission season OR with >20% seroprevalence increase after transmission season [15]	Gametocyte specific in the first gametocyte proteomic analysis [14] with evidence of immune recognition in Skinner et al [15] (>50% samples after transmission season or >20% increase)
7	41	*7*	538	Gold standard gametocyte protein in list used to generate transcript and protein score	Gold standard gametocyte proteins
8	37	*23*	561	*A prioiri* selection. Markers of sexual stage exposure, TBV candidates, 6-cys proteins, proteins implicated in gamete fertility, and markers of asexual exposure	*A priori* selection: markers of asexual and sexual stage, vaccine candidates
9	45	*19*	580	Inclusion based on correlation with transmission blocking immunity [16]	Inclusion based on association with transmission blocking immunity in our earlier work [16]
10	47	*20*	**600**	Inclusion based on recognition by mice/immune sera and presence on giRBC surface [17]	Inclusion based on presence and recognition on giRBC surface (early gametocytes) [17].



Supplemental figure 1. Parasite densities over time in the CHMI cohorts. Total parasite densities were determined by 18S quantitative polymerase chain reaction. Shaded lines represent data from individual participants, bold lines indicate the median values. Gametocyte densities are the sum of ccp4 and pfmget quantitative reverse-transcription polymerase chain reaction (qRT-PCR) data. Gray lines represent data from individual participants and the black line the median gametocyte density.



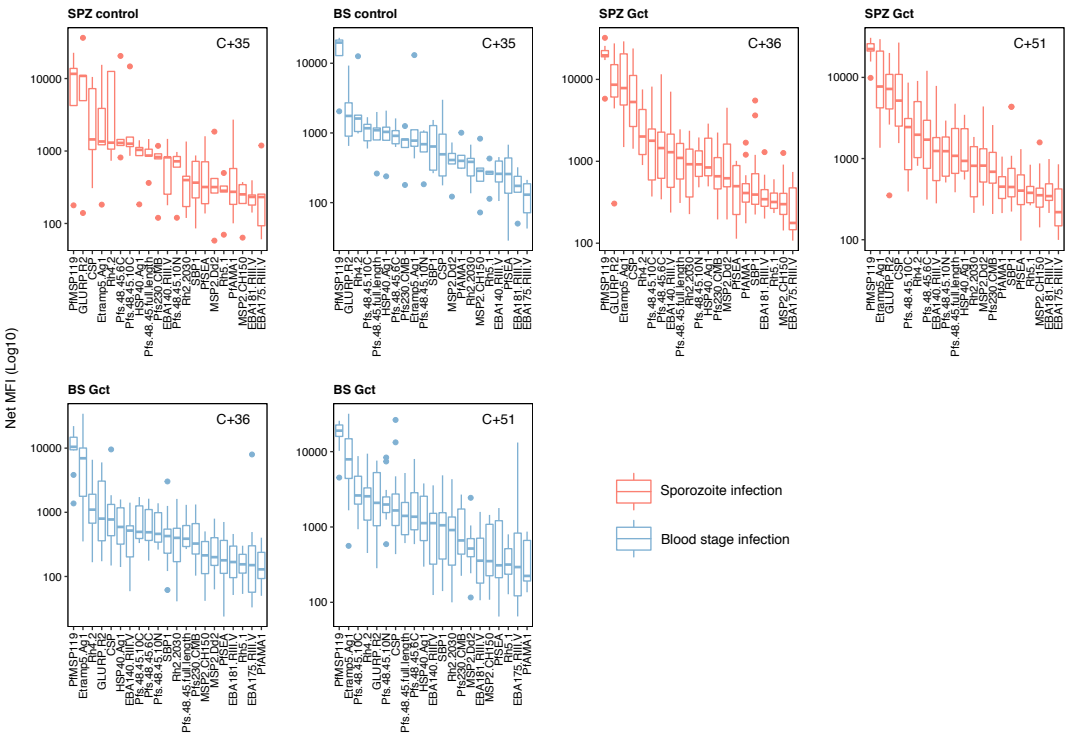
Supplemental figure 2. Participant parasite exposure and antibody response to crude parasite extracts. In all plots, red (top) denote Gct A (sporozoite infection) and blue (bottom) denotes Gct B (Asexual infection). **A.** The association of total parasite and gametocyte density areas under the curve (AUC) **B.** The association of total parasite AUC (top) and gametocyte AUC (bottom) with antibody response to asexual parasite extract. **C.** The association of total parasite AUC (top) and gametocyte AUC (bottom) with antibody response to gametocyte extract. r = Spearman's Rho, p = p-value from Spearman's rank test.



Supplemental figure 3. Cumulative breadth of positive antibody responses to recombinant antigens. Participants were considered to recognise an antigen (*i.e.* be seropositive) if background corrected MFI values were greater than the mean background corrected MFI plus 2 standard deviations of all individuals at baseline. In all plots red boxes indicate mosquito bite (sporozoite) infection cohorts (SPZ Gct, SPZ control) and blue boxes denote asexual parasite infection (BS Gct, BS control).

Supplemental table 3. Differences in net MFI for antibody responses assessed in bead based assay between baseline (C-1) and subsequent timepoints. Differences were tested with paired t-tests to account for repeated measures and adjust for variable baseline antibody reactivities. Values are p-values unadjusted for multiple comparisons (<0.05=red). Values below the Bonferroni adjusted threshold for significance are highlighted in green. All differences are positive (higher response after baseline). Responses at day C+7/9 were not significantly increased over baseline for any antigen.

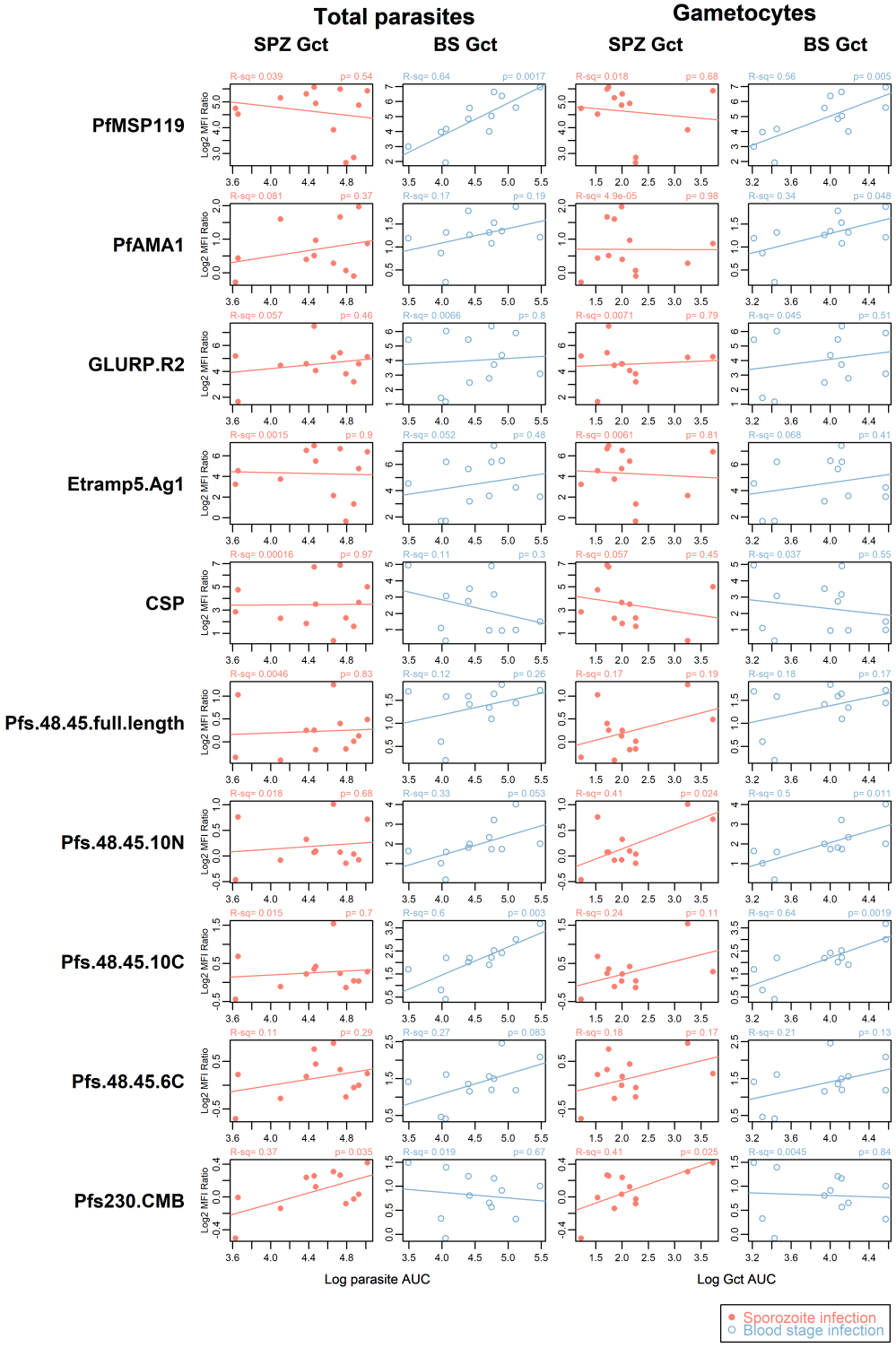
This table is too large to include at an appropriate scale. The file is available to view and download at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.930956/full#supplementary-material or `https://tinyurl.com/54etz3dh` listed as 'Table 1.xlsx'



Supplemental figure 4. Magnitude of antibody response to all recombinant antigens. Background corrected MFI values for all antigens, presented on a log10 scale on the y-axis, ordered by median response (strong bars) on the x-axis. In all plots red boxes indicate mosquito bite (sporozoite) infection cohorts (SPZ Gct, SPZ control) and blue boxes denote asexual parasite infection (BS Gct, BS control).

Supplemental table 4. Position of sexual stage antigens in ranked median magnitude of response amongst all antigens. Colour scale indicates the number of positions moved up (green), down (red), or if there is no change (white), between C+36 and C+51 for the CHMI cohorts.

	SPZ Gct		BS Gct		SPZ Control	BS Control
	C+36	C+51	C+36	C+51	C+35	C+35
Pfs48/45-10C	6	5	8	3	7	4
Pfs48/45-10N	11	9	10	6	12	10
Pfs48/45-6C	7	7	9	9	6	7
Pfs48/45 full length	9	10	13	8	9	5
Pfs230-CMB	13	14	14	14	10	8



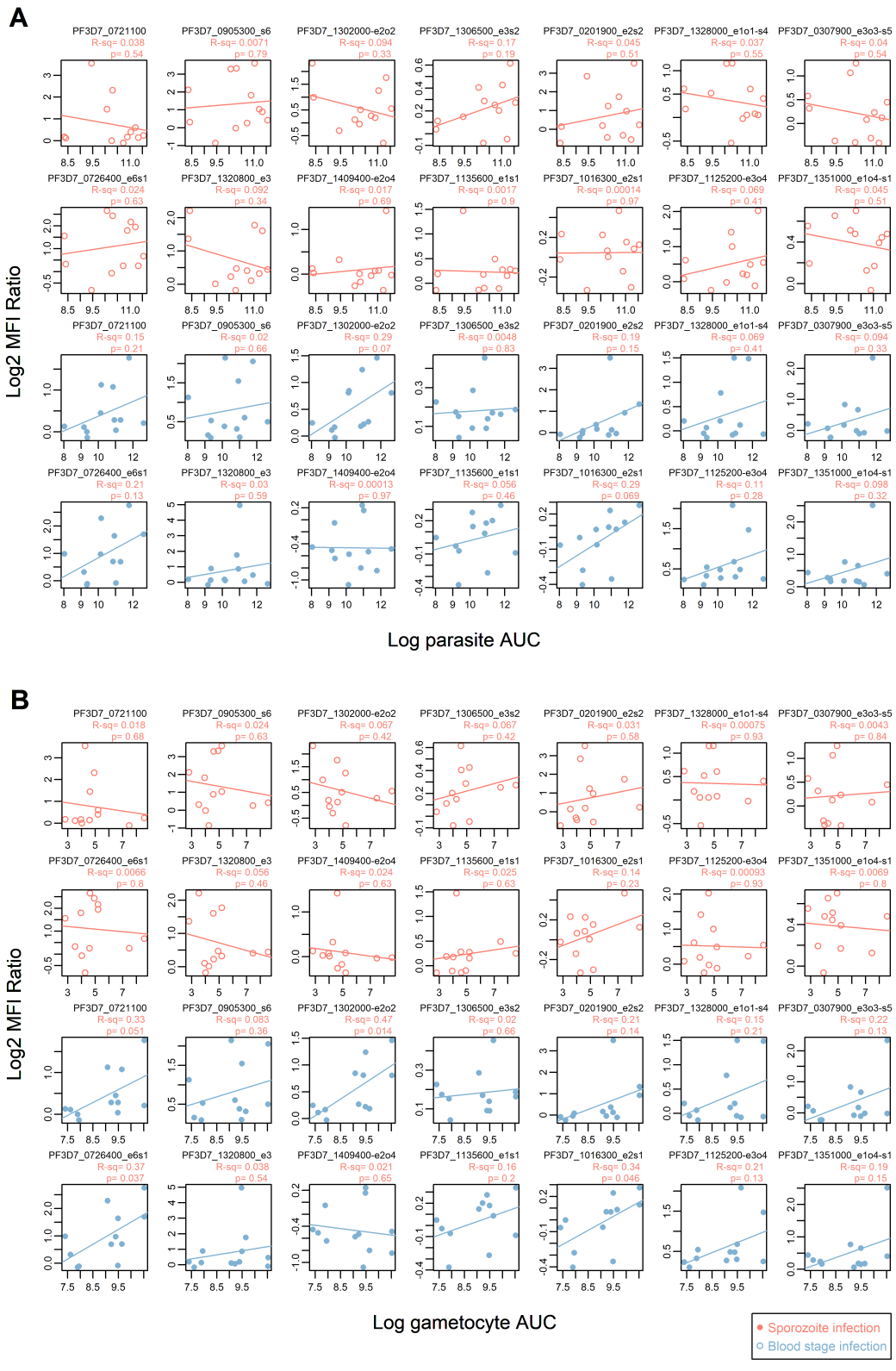
Supplemental figure 5. Correlation between total parasite and gametocyte AUC and antibody response to selected antigens. R^2 and p-value are from Spearman's rank order correlation, with trend line from simple linear regression. In all plots, red solid circles denote mosquito bite (sporozoite) infection cohorts (SPZ Gct, SPZ control) and blue hollow circles denote asexual parasite infection (BS Gct, BS control). Antibody assay data are presented as log2 MFI ratios of response over each individual's baseline.

Supplemental table 5. Correlation between total parasite and gametocyte AUC and antibody response to all recombinant antigens. R^2 and p-value are from Spearman's rank order correlation. Antibody assay data used were log2 MFI ratios of response over each individual's baseline. Parasite AUC was log transformed for analysis. Values are p-values unadjusted for multiple comparisons (<0.05=red). Values below the Bonferroni adjusted threshold for significance are highlighted in green. Serology data are all from the latest timepoint observed in the trials (C+51). AUC: Area under the curve.

	Total parasite AUC				Gametocyte AUC			
	SPZ Gct		BS Gct		SPZ Gct		BS Gct	
	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*
CSP	0.00016	0.97	0.11	0.3	0.057	0.45	0.037	0.55
EBA140.RIII.V	0.0084	0.78	0.077	0.38	0.014	0.71	0.15	0.22
EBA175.RIII.V	0.06	0.44	0.099	0.32	0.014	0.71	0.2	0.14
EBA181.RIII.V	0.16	0.2	0.0041	0.84	0.073	0.4	0.0034	0.86
Etramp5.Ag1	0.0015	0.9	0.052	0.48	0.0061	0.81	0.068	0.41
GLURP.R2	0.057	0.46	0.0066	0.8	0.0071	0.79	0.045	0.51
HSP40.Ag1	0.18	0.17	0.003	0.87	0.26	0.088	0.01	0.76
MSP2.CH150	0.09	0.34	0.0013	0.91	0.22	0.12	5.20E-06	0.99
MSP2.Dd2	0.27	0.081	0.062	0.44	0.15	0.21	0.11	0.29
PfAMA1	0.081	0.37	0.17	0.19	4.90E-05	0.98	0.34	0.048
PfMSP119	0.039	0.54	0.64	0.0017	0.018	0.68	0.56	0.005
Pfs230.CMB	0.37	0.035	0.019	0.67	0.41	0.025	0.0045	0.84
Pfs.48.45.10C	0.015	0.7	0.6	0.003	0.24	0.11	0.64	0.0019
Pfs.48.45.10N	0.018	0.68	0.33	0.053	0.41	0.024	0.5	0.011
Pfs.48.45.6C	0.11	0.29	0.27	0.083	0.18	0.17	0.21	0.13
Pfs.48.45.full.length	0.0046	0.83	0.12	0.26	0.17	0.19	0.18	0.17
PfSEA	0.16	0.2	0.082	0.37	0.093	0.34	0.049	0.49
Rh2.2030	0.29	0.073	0.02	0.66	0.14	0.23	0.063	0.43
Rh4.2	0.0047	0.83	0.053	0.47	0.027	0.61	0.027	0.61
Rh5.1	0.23	0.12	0.0021	0.89	0.18	0.17	0.0022	0.88
SBP1	0.11	0.29	0.21	0.13	0.15	0.21	0.26	0.092

Supplemental table 6. Antibody targets with differential response between cohorts or over the time course of infection. Proteins were classified based on protein expression profiles, using raw peptide counts obtained from proteomics data annotations on PlasmoDB (plasmodb.org version 56, 15 Feb 2022). Data originally published in Silvestrini, F., et al., Mol Cell Proteomics, 2010; Florens, L., et al., Mol Biochem Parasitol, 2004; Lasonder, E., et al., Nucleic Acids Res, 2016; Oehring, S.C., et al., Genome Biol, 2012.

This table is too large to include at an appropriate scale. The file is available to view and download at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.930956/full#supplementary-material or `https://tinyurl.com/54etz3dh` listed as 'Table 2.xlsx'



Supplemental figure 6. Correlation between asexual and gametocyte AUC and antibody responses to a selection of potential antibody biomarkers of gametocyte exposure. A. Correlation of antibody response to curated array targets and total parasite AUC. **B.** Correlation of antibody response to curated array targets and gametocyte AUC. R^2 and p-value are from Spearman's rank order correlation, with trend line from simple linear regression. In all plots, red solid circles denote SPZ Gct and blue hollow circles denote BS Gct. Array data are presented as log2 MFI ratios as described in the methods.

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5.7 Addendum of corrections

The following text addresses corrections made at the request of examiners, but that is not included in the original published article:

Edit to 'Discussion'

Assessing the duration of detectable antibody titers, isotype and avidity beyond this timeframe, and whether re-infection will boost and/or change these responses will be valuable in the characterization of antibodies as biomarkers of infectivity and could have implications for the development of transmission blocking vaccines (TBV).

In the context of biomarker identification, characterisation of gametocyte specific antibody responses may allow for the determination of gametocyte exposure history, whereby stronger responses may be indicative of frequent or extended periods of gametocytaemia and thus infectiousness. Similarly, an understanding of which antibodies (or combinations of antibodies) are able to completely block transmission could allow the identification of infectious and non-infectious individuals within a population. In theory, these kinds of data could be stratified (*e.g.* spatially) to help target the administration of transmission blocking drugs, or perhaps even correlated with responses to asexual or pre-erythrocytic antigens that are more typically included in serological surveys. However, in most instances it can be assumed that any individual within an endemic area will have been exposed to gametocytes at some stage, but that the vast majority of individuals will not naturally possess transmission blocking immunity. Accordingly, the application of gametocyte biomarkers is perhaps limited to large scale serological surveys where infections are not explicitly diagnosed. In contrast, there is a clear application for the discovery and characterisation of sexual stage antibody responses linked with transmission blockade at the gametocyte or gamete level. Whilst existing, or vaccine induced, asexual antibodies may provide clinical protection, the ability to also prevent onward transmission at a population level is highly desirable and would likely be of significant value in the context of elimination. Data has shown that effective transmission reducing activity does not solely rest on reactivity to targets such as Pfs48/45 or Pfs230 or complement fixing antibodies ([1]. This suggests that responses to other targets, including antigens that may be expressed on both sexual and asexual parasites, may play a significant role in transmission reducing immunity. It is crucial to understand the kinetics of all transmission reducing responses, in as much as that a TBV should ideally induce a rapid and long-lasting antibody response to avoid the need for multiple booster doses. The data presented in this study indicate that initiation of gametocyte specific responses are delayed in comparison to asexual specific responses, and thus longer study follow ups may be required to capture the peak and wane of sexual stage antibodies after exposure. Similarly, such considerations would be important in the context of the timing of vaccine administration in relation

to transmission peaks. Relatedly, it would be prudent to consider the effect of natural boosting of antibody responses upon reinfection, as this has been noted as an advantage for TBVs that target pre-fertilisation gametocyte antigens.

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Chapter 6

Persistence of *P. falciparum* HRP-2 antigenaemia after artemisinin combination therapy is not associated with gametocytes

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Student ID Number	1404292/RITD	**Title**	Mr
First Name(s)	Tate		
Surname/Family Name	Oulton		
Thesis Title	Development and validation of serological assays to evaluate malaria transmission		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Malaria Journal		
When was the work published?	06.12.2022		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I conducted the HRP-2 quantification laboratory work. I conducted analysis of the data I generated, and of existing data from work completed earlier in the study. I wrote and prepared the manuscript from draft to final submission, with comments from the additional authors.

SECTION E

Student Signature	[redacted]
Date	. .

Supervisor Signature	[redacted]
Date	. .

Title:

Persistence of *P. falciparum* HRP-2 antigenaemia after artemisinin combination therapy is not associated with gametocytes

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Abstract

Background

In some settings, sensitive field diagnostic tools may be needed to achieve elimination of falciparum malaria. To this end, rapid diagnostic tests (RDTs) based on the detection of the *Plasmodium falciparum* protein HRP-2 are being developed with increasingly lower limits of detection. However, it is currently unclear how parasite stages that are unaffected by standard drug treatments may contribute to HRP-2 detectability and potentially confound RDT results even after clearance of blood stage infection. In this study, we assessed the detectability of HRP-2 in periods of post-treatment residual gametocytaemia.

Methods

A cohort of 100 *Plasmodium falciparum* infected, gametocyte positive individuals were treated with or without the gametocytocidal drug primaquine (PQ) alongside standard artemisinin-based combination therapy (ACT) in the context of a randomised clinical trial in Ouelessebougou, Mali. We used a quantitative ELISA to measure levels of HRP-2, and compared time to test negativity using a standard and -sensitive RDT (uRDT) between residual gametocyte positive and negative groups.

Findings

Time to test negativity was longest by uRDT, followed by ELISA and then standard RDT. No significant difference in time to negativity was found between the treatment groups with and without residual gametocytes: uRDT (HR 0.79 [95% CI 0.52-1.21], p = 0.28), RDT (HR 0.77 [95% CI 0.51-1.15], p = 0.20) or ELISA (HR 0.88 [95% CI 0.59-1.32], p = 0.53). Similarly, no difference was observed when adjusting for baseline asexual parasite density. Quantified levels of HRP-2 over time were similar between groups, with differences attributable to asexual parasite densities. Furthermore, no difference in levels of HRP-2 was found between individuals who were or were not infectious to mosquitoes (OR 1.19 [95% CI 0.98-1.46], p = 0.077).

Interpretation

Surviving sexual stage parasites after standard ACT treatment do not contribute to the persistence of HRP-2 antigenaemia, and appear to have little impact on RDT results.

Funding

This work was supported by the Gates Foundation (INV-002098). WS is supported by a Sir Henry Wellcome fellowship (218676/Z/19/Z) from the Wellcome Trust (UK).

Keywords:

Malaria; rapid diagnostic tests; lateral flow; ultra-sensitive RDT; HRP-2; gametocytes; antigenaemia; infectiousness

Research in context

Evidence before this study

Widespread malaria elimination continues to be an elusive objective in many endemic areas. Evidence shows that advances in disease surveillance are needed to better target interventions and assess efficacy. Rapid diagnostic tests (RDTs), based on the detection of malaria parasite proteins such as HRP-2 in the blood, already account for the majority of global malaria diagnoses. Further evolution of RDT technology has led to increasingly lower limits of detection to more easily identify the low parasite density infections that are missed by standard RDTs and other field diagnostic methods. However, it is unclear if the persistence of HRP-2 after an infection is resolved (naturally or due to treatment) may lead to an increase in false positive results where new, ultra-sensitive RDTs are used. The sexual stage of malaria parasites, gametocytes, are responsible for the transmission of parasites to mosquitoes and can persist after asexual parasites are cleared. Existing evidence indicates that HRP-2 is produced by developing gametocytes and is internalised in the mature gametocyte. Two conflicting studies have examined the association between gametocytes and the performance of standard RDTs. As such, concern remains that gametocytes, which typically survive standard malaria treatment, may produce and/or release detectable HRP-2, further extending the window of RDT positivity after clearance of active asexual stage infection.

Added value of this study

In this study, we compared groups of individuals with and without gametocytes following treatment. Our results showed that time to test negativity for standard and ultra-sensitive RDTs did not differ significantly between groups. Using quantitative methods, we found that levels of HRP-2 did not vary according to the presence or absence of sexual stage parasites, but that any differences were attributable to the initial density of asexual stages.

Implications of all the available evidence

Our results indicate that surviving sexual stage parasites after treatment do not affect time to negativity by standard or ultra-sensitive RDT, or levels of circulating HRP-2. In addition, our findings support previous data showing that asexual parasites are the main source of HRP-2 during infection, and that initial asexual parasite density primarily determines how long an individual will test positive by RDT. Importantly, this study provides evidence to support the use of ultra-sensitive RDTs in diagnosis and surveillance in spite of persisting sexual stage parasites following standard malaria treatment.

6.2 Introduction

Historically, diagnosis of malaria infection has been dependent on a visual detection of blood stage parasites by blood film microscopy. However, the 2011 Malaria Eradication Research Agenda (malERA) process concluded that novel and improved tools are needed to detect and target transmission at its lowest levels for use in elimination contexts. This conclusion stemmed from the growing body of evidence that submicroscopic parasite densities were capable of maintaining a viable reservoir of infection in humans [1]. Although previous elimination successes have been achieved with little focus on low density or asymptomatic cases, persistence of malaria in areas even with high coverage of control measures and treatment strongly suggests that improved surveillance and targeting of the transmission reservoir is necessary to achieve elimination more widely [2].

Earlier innovations in the '00s led to the development of immuno-assay based tools, which facilitate parasite detection in blood quickly and cheaply. Such rapid diagnostic tests (RDTs) are primarily based on the capture of *Plasmodium* protein antigens in a lateral flow assay format. Current RDTs are based on the detection of a select combination of secreted parasite proteins that allow some species differentiation such as lactate dehydrogenase and aldolase; for *P. falciparum* the most commonly used is histidine rich protein-2 (HRP-2) [**Organization2021**, 3, 4].

HRP-2 is produced and secreted by all asexual parasites and developing sexual-stage gametocytes, and is known to be localised within mature gametocytes [5, 6]. Thus, HRP-2 is highly abundant in the blood during infection. Detection will be a function of parasite density and the duration of infection, but even relatively low-density infections (200 parasites/μL) produce levels of HRP-2 that are reliably detectable by RDT [7]; the lower limit of detection typically falling between 600 to 1000pg protein/mL [8]. In line with recommendations made by malERA, developments in the design of HRP-2 based malaria RDTs has led to greatly increased sensitivity; these ultra-sensitive RDTs (uRDT) may detect as little as ~80pg/mL [8, 9]. Such improvements in sensitivity are valuable in the context of enhanced detection of infection in endemic settings, particularly when lower density, asymptomatic infections are prevalent [1].

However, detectable HRP-2 will persist in circulation even after live parasite clearance following treatment. Attempts to derive parasite densities based on quantification of HRP-2 [9] are confounded by this stability of HRP-2 protein in the blood; in a study of individuals from Angola, Senegal and Tanzania, the half life of HRP-2 in the blood was demonstrated to be 3.0-4.7 days [10]. It is understood that time to HRP-2 RDT negativity is thus largely determined by factors including peak parasite density and genetic variation in HRP-2 expression. Differences in HRP-2 expression by parasite life cycle stage may also be involved, though it is currently unclear as to if, and how significantly, mature sexual stage parasites contribute to circulating levels of HRP-2.

The importance of this question is specific to *P. falciparum*, the only human parasite to express HRP-2. *P. falciparum* gametocytes can persist for weeks after resolution of asexual infection following treatment with standard artemisinin combination therapies (ACTs), which are incompletely effective against mature gametocytes [11, 12]. Though the exact mechanisms and efficacy of the gametocytocidal activity of ACTs are unclear at present, the sterilising gametocytocidal activities of the drug primaquine are well recognised [13, 14]; WHO policy recommends a single dose of primaquine (PQ) with ACT for *P. falciparum* malaria in low transmission areas to further reduce the risk of transmission [15]. As such, any significant contribution of HRP-2 by surviving sexual stages after ACT without PQ may affect estimates of RDT positivity particularly from uRDTs. Similarly, an understanding of gametocyte related HRP-2 levels may provide insights into post treatment infectivity, where gametocytes that survive continue to render an individual infectious to mosquitoes, even in the absence of asexual parasites [11, 16, 17].

In this study, we have quantified HRP-2 dynamics in a cohort of infected, gametocyte positive Malian individuals treated with an ACT (either pyronaridine-artesunate or dihydroartemisinin-piperaquine) with and without a single low dose of the gametocidal drug primaquine to rapidly clear gametocytes. This study allows us to evaluate the specific contribution of the post-treatment gametocytes to HRP-2 levels after asexual clearance. Additionally, we compare quantitation of HRP-2 to standard and ultra-sensitive RDT and, through the determination of individual infectivity to mosquitoes by membrane feeding assays, have examined the association between transmission potential and measurable HRP-2.

6.3 Methods

Study location and sample collection

The study was carried out in Ouelessebougou, Mali, in a trial conducted between September and January 2020. 100 asymptomatic individuals aged 5-50 years (inclusive), with a sexual stage density of $\geq$16 gametocytes/μL, were recruited and randomly assigned to one of four treatment arms: pyronaridine-artesunate (PA) (n = 25), PA plus single low dose primaquine (PA-PQ) (n = 25), dihydroartemisinin-piperaquine (DP) (n = 25), and DP plus single low dose primaquine (DP-PQ) (n = 25). All participants were followed for 49 days post initial treatment, receiving a full clinical and parasitological examination (including asexual and sexual stage parasite density measures by microscopy [asexual at baseline only] and qRT-PCR) on days 1, 2, 7, 14, 21, 28, 35, 42 and 49 (to day 28 only in PQ treated groups). A subsequent dose of DP at day 21 was given to ensure continued prophylaxis for the duration of the study.

Ethics

Ethical approval was granted by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry of the University of Science, Techniques, and Technologies of Bamako (Bamako, Mali), and the Research Ethics Committee of the London School of Hygiene and Tropical

Medicine (London, UK). The trial was registered on clinicaltrials.gov under identifier NCT04049916.

Parasite quantification

Individuals were recruited to the trial on the basis of blood smear examination and gametocyte quantification, but ring stages and gametocytes were subsequently quantified by highly sensitive qRT-PCR, as described [13]. Briefly, EDTA blood (EDTA VACUETTE tube, Greiner Bio-One, Kremsmünster, Austria) was aliquoted into RNA protect cell reagent (Qiagen, Hilden, Germany) and stored at -80°C until temperature tracked shipment on dry ice to Radboud university medical center (Nijmegen, Netherlands) for assay performance. Total nucleic acids were extracted using a MagNAPure LC automated extractor (Total Nucleic Acid Isolation Kit-High Performance; Roche Applied Science, Indianapolis, IN, USA). Male and female gametocytes were quantified in a multiplex reverse transcriptase quantitative PCR (RT-qPCR) assay as described [13]. Asexual stages were quantified by quantification of SBP1 transcripts, as described elsewhere [18]. Samples were classified as negative for a particular gametocyte sex if the qRT-PCR quantified density of gametocytes of that sex was less than 0·01 gametocytes per μL (*i.e.* one gametocyte per 100 μL of blood sample).

Rapid diagnostic testing

Two rapid diagnostic tests were utilised in this study analysis: the Standard Diagnostics, Inc. (SD) BIOLINE Malaria Ag P.f. RDT (SD/Alere, Yongin-si, Republic of Korea, Cat. 05EK50), the Alere Malaria Ag P.f RDT ULTRA SENSITIVE (SD/Alere, Yongin-si, Republic of Korea). Each are based on the detection of HRP-2 was used according to the manufacturers instruction. All Standard RDTs were performed at the same day where the blood was collected. The Alere Malaria Ag P.f RDT ULTRA SENSITIVE was performed at the same day as blood collection for all participants until day 21 visit. The other test were completed retrospectively using the frozen EDTA sample. For both RDTs the results were read and recorded separately by two lab technicians and in case of discrepancy the result was given by a third technician.

HRP-2 quantification

Levels of HRP-2 were determined using the Quansys Q-plex Human Malaria (5-plex) kit, Q-View Imager LS and Q-View software v3.13 (Quansys Biosciences, Logan, UT, USA). The assay was carried out according to the protocol and with reagents supplied. Briefly, neat serum samples were pre-diluted 2:5 in calibrator diluent, and subsequently diluted 1:4 in complete assay diluent for a final dilution of 1:10. The assay calibration curve was prepared as directed. 50 μL of prepared sample and duplicate wells of calibration curve material were added to the plate

and incubated on a shaker at 500 RPM for 2h at room temperature (RT). Plates were washed three times using an automated microplate washer (BioTek ELx405, Agilent, Santa Clara, CA, USA) before 50 µL of detection mix was added to each well and subsequently incubated for 1h at 500 RPM RT. Plates were then washed 3 times and 50 µL of provided Streptavidin-HRP was added, and incubated for 30min at 500RPM RT. Plates were then washed 6 times before 50 µL of prepared substrate was added. Plates were imaged within 5 minutes of the addition of substrate, using an exposure time of 270 seconds and standard image processing. Image processing and analysis was carried out within the Q-View software, with the quantified calibration curve generated using the inbuilt 5-parameter logistic regression model.

Mosquito feeding

Mosquito feeding was carried out as previously described. Approximately 75 locally reared *Anopheles gambiae* were allowed to feed for 15–20 min on venous blood samples (Lithium Heparin VACUETTE tube; Greiner Bio-One, Kremsmünster, Austria) through a prewarmed glass membrane feeder system (Coelen Glastechniek, Weldaad, Netherlands). All surviving mosquitoes were dissected on the seventh day after the feeding assay; midguts were stained in 1% mercurochrome and examined for the presence and density of oocysts by expert microscopists.

Data analysis

Data analysis was conducted using R (R foundation for statistical computing, Vienna, Austria; v3.6.3) [19]. Asexual parasite and gametocyte density data were compared between groups by Wilcoxon signed rank-sum test, and comparisons between gametocyte prevalence between group and time-point were conducted by Fisher's exact test. Quantified HRP-2 values that fell below the lower limit of quantification (LLOQ) were treated as LLOQ/2 For the analysis of HRP-2 prevalence, HRP-2 positivity was defined as any quantity of HRP-2 greater than the mean plus 3*SD of HRP-2 in individuals treated with ACT+PQ at day 49 post-treatment (n = 45). Multivariate survival analysis of time to RDT and HRP-2 negativity between groups was conducted by Cox proportional hazards regression. Differences in quantified HRP-2 levels between groups over time were compared by linear regression modelling and differences between mosquito infectious and non-infectious individuals were compared by logistic regression.

6.4 Results

Participants were categorised according to artemisinin treatment with (ACT+PQ) or without (ACT) the gametocytocidal drug primaquine to allow for the evaluation of the contribution of gametocytes to HRP-2 levels and RDT positivity. As such, at baseline each treatment group

consisted of 50 individuals. At enrolment, median asexual stage parasite density by qRT-PCR was greater in the ACT+PQ group (**Supplementary figure 1**), though not significantly so (Wilcoxon rank-sum, p = 0.26) and gametocyte densities were similar between groups **Table 1**.

Table 1. Baseline characteristics.

Treatment group	N	Female, n (%)	Age, median (range)	Baseline asexual density by qPCR, median (IQR)	Baseline gametocyte density by qPCR, median (IQR)
ACT	50	21 (42%)	11.5 (5-40)	289.3 (29.4-1724.5)	77.3 (37.1-124.0)
ACT + PQ	50	31 (62%)	10 (5-47)	692.4 (55.1-4247.6)	60.3 (24.8-175.9)

Asexual parasite density fell rapidly after initial treatment, with median asexual density reaching zero in both groups by day 7 (p = 0.57), remaining as such for the duration of the study (**Table 2 and supplementary figure 1**). Whilst gametocyte density declined over the full study period in both groups, gametocyte clearance occurred much more rapidly in individuals treated with primaquine. A statistically significant decrease in gametocyte density was observed by day 2 in the ACT+PQ group (Wilcoxon rank-sum, p = 0.05) whilst such a decrease was not observed in the ACT group until day 7 (p = 0.003). By day 7, gametocyte positivity had fallen to 65.2% in the ACT+PQ treatment group but remained at 100% in the ACT group (**Table 2 and figure 1**). Median gametocyte density fell to 0 by day 14 in the ACT+PQ group, but took until day 42 to drop to the same density in the ACT group. A far greater number of individuals remained gametocyte positive in the ACT group (90.7%) compared to the ACT+PQ group (11.4%) at day 28 (Fisher's exact test, p = <0.0001) (**Table 2 and figure 1**).

Survival analysis of test positivity by standard RDT, uRDT and HRP-2 quantification shows that the longest duration of test positivity was with uRDT then HRP-2 quantification and standard RDT respectively (**Figure 2**). Treatment with primaquine did not significantly influence the time to test negativity by uRDT (Cox proportional hazards ratio (HR) 0.79 [95% CI 0.52-1.21], p = 0.28), RDT (HR 0.77 [95% CI 0.51-1.15], p = 0.20) or HRP-2 (HR 0.88 [95% CI 0.59-1.32], p = 0.53), though the observed trend is suggestive of a slight delay in time to negativity in the ACT-PQ group relative to the ACT group – likely an effect of greater asexual parasite density at baseline leading to increased HRP-2 antigenaemia (**Supplementary figure 2**). However, no effect on time to test negativity was found when adjusting for baseline asexual density with any of the three tests: uRDT, HR 0.82 [95% CI 0.53-1.26], p = 0.36; HRP-2, HR 0.93 [95% CI 0.61-1.41], p = 0.727; and RDT, HR 0.83 [95% CI 0.55-1.26], p = 0.39.

Quantified levels of HRP-2 were similar between groups at all time-points, though a tendency towards an increased concentration was observed in the ACT+PQ group, in line with greater asexual parasite densities at baseline (**Figure 3**). Linear regressions revealed no statist-

Table 2. Parasite densities by study visit.

Study visit (days)	Treatment group	N	Asexual density by qPCR, median (IQR)	Gametocyte density by qPCR, median (IQR)
0	ACT	50	289.3 (29.4-1724.5)	77.3 (37.1-124.0)
	ACT + PQ	50	692.4 (55.1-4247.6)	60.3 (24.8-175.9)
2	ACT	50	0 (0-1.2)	62.6 (30.23-116.5)
	ACT + PQ	49	0.2 (0-1.5)	38.6 (10.3-114.2)
7	ACT	50	0 (0-0.1)	41.6 (17.0-68.2)
	ACT + PQ	48	0 (0-0.2)	0.1 (0-0.8)
14	ACT	49	0 (0)	19.2 (9.2-36.4)
	ACT + PQ	48	0 (0-0.1)	0 (0-0.1)
21	ACT	44	0 (0)	4.9 (2.1-12.7)
	ACT + PQ	46	0 (0)	0 (0)
28	ACT	45	0 (0)	0.8 (0.1-2.3)
	ACT + PQ	45	0 (0)	0 (0)
35	ACT	45	0 (0)	0.1 (0-0.7)
	ACT + PQ	45	NA	NA
42	ACT	44	0 (0)	0 (0-0.1)
	ACT + PQ	45	NA	NA
49	ACT	44	0 (0)	0 (0)
	ACT + PQ	45	NA	NA

ically significant difference between treatment groups, nor were any differences observed when adjusting for baseline asexual density (**Table 3**). Levels of HRP-2 declined rapidly over 14 days post-treatment, with HRP-2 positivity falling from 92% to 42.9% and 94% to 45.8% in the ACT and ACT+PQ groups respectively (**Table 3**). By day 21, variability in quantified HRP-2 levels between treatment groups was negligible and most individuals were approaching the lower limit of detection of the assay (**Table 3**). HRP-2 positivity continued to decrease over time until all individuals were found to be negative according to the assay cutoff by day 49.

Mosquito infectivity was analysed at day 0, 2 and 7 within the ACT group. HRP-2 levels were not significantly different between mosquito infectious and non-infectious individuals (Odds ratio (OR) 1.19 [95% CI 0.98-1.46], p = 0.077), nor was any significant difference observed when adjusting for baseline gametocyte density and day of study visit (OR 1.23 [95% CI 0.96-1.59], p = 0.12) (**Figure 4A**). Looking at only individuals who were infectious to mosquitoes, linear regression showed no significant relationship between the percentage of mosquitoes infected and levels of HRP-2 with (p = 0.31) or without adjustment for baseline gametocyte dens-

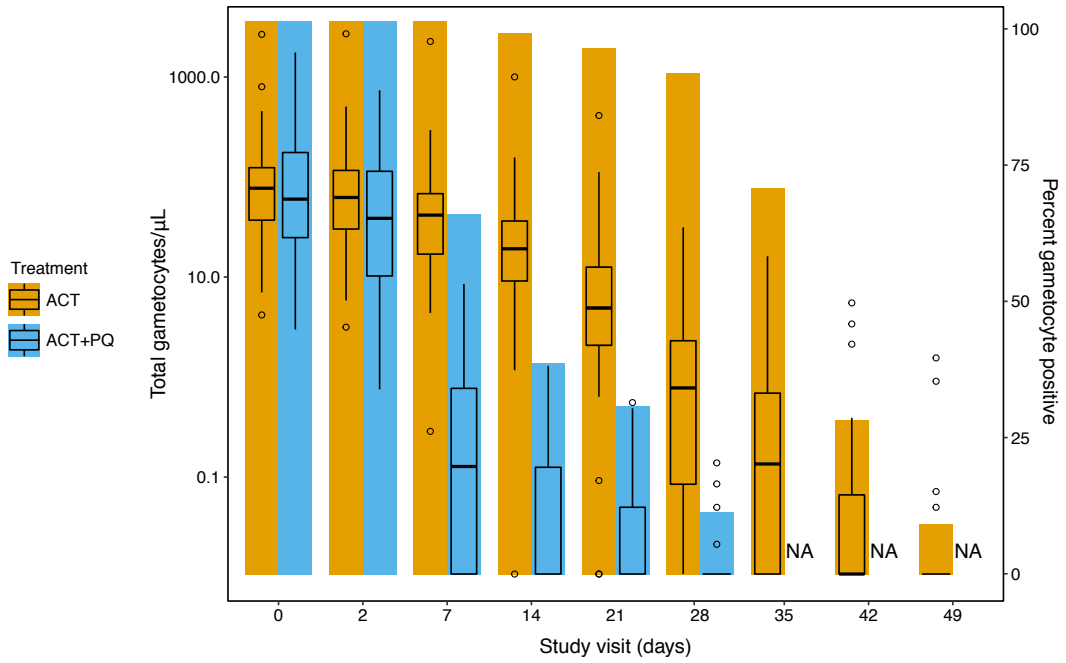


Figure 1. Gametocyte density and positivity at each study visit. Boxes represent gametocyte density (left axis) measured by RT-qPCR, presented as median and interquartile range. Bars represent percentage gametocyte positivity (right axis).

ity and study visit day (p = 0.83) (**Figure 4B**). Finally, when comparing time to test negativity, no significant difference was found between mosquito infectious and non-infectious individuals with and without adjustment for baseline gametocyte density (**Supplementary figure 3 and supplementary table 1**).

6.5 Discussion

Malaria diagnosis by HRP-2-based rapid diagnostic tests is widespread. Prior to this study, it was unknown if the presence of gametocytes contributes to the persistence of HRP-2 after ACT treatment. Understanding how gametocytes may contribute to HRP-2 levels and RDT positivity would improve interpretation of RDTs and uRDTs. If gametocytes are detectable after standard ACT via HRP-2-based diagnostics, it will also be important to establish the infectivity of such individuals. In this study we have evaluated the contribution of sexual stage parasites to RDT and uRDT positivity, and HRP-2 levels over time in malaria-infected, gametocyte positive individuals treated with or without the gametocytocidal drug primaquine. As expected, gametocytes were rapidly cleared after PQ treatment but we observed no significant differences in time to negativity by standard RDT, uRDT or quantified HRP-2. Correspondingly, levels of HRP-2 were found to be similar between the two treatment groups, with any differences likely attributable to baseline asexual parasite densities. These results address the concern that the

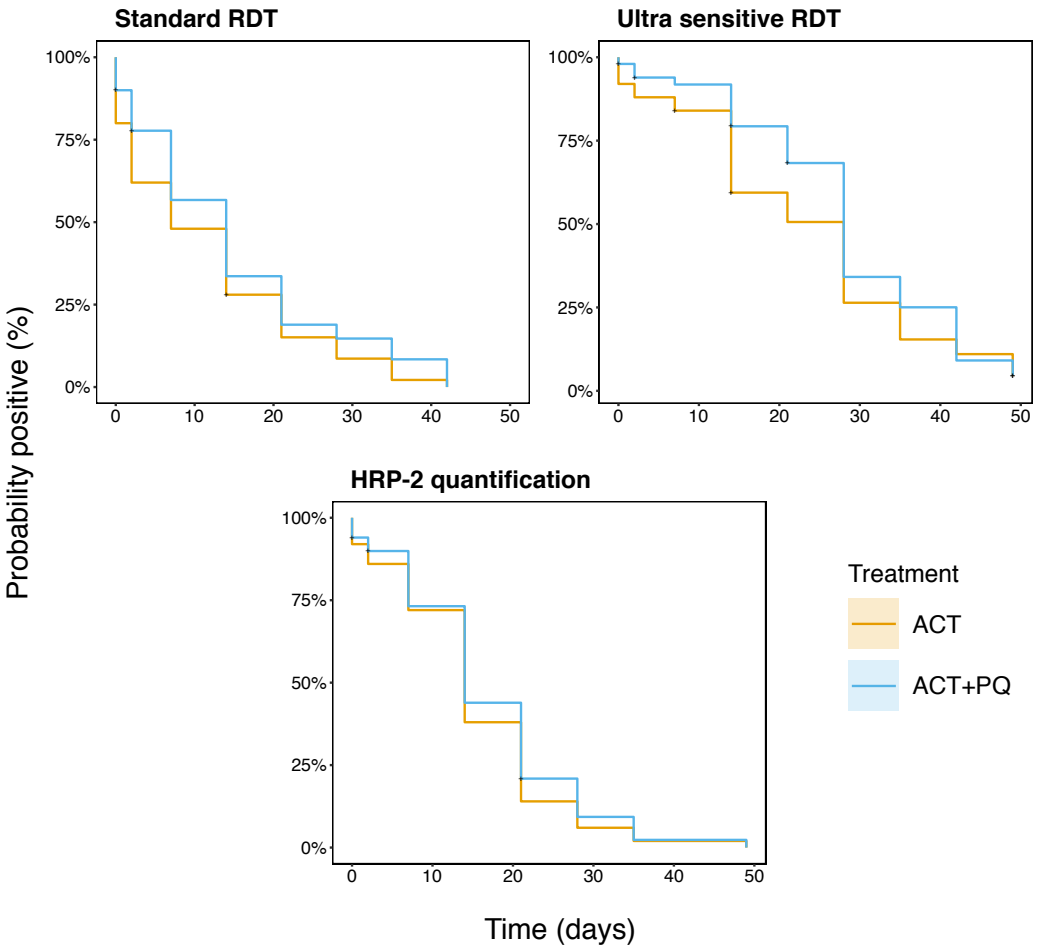


Figure 2. Kaplan-Meier plots showing time to negativity by standard RDT, ultra-sensitive RDT and quantified HRP-2 between the ACT group (orange) and the ACT+PQ group (blue).

lower limits of detection afforded by HRP-2-based uRDTs may lead to false-positive test results in individuals who do not harbour replicating blood-stage infection, but have not yet cleared gametocytes from circulation [20, 21]. Our data suggest that surviving gametocytes after ACT do not significantly contribute to HRP-2 load.

The lag between parasite and HRP-2 clearance after standard ACT treatment has led to concerns that RDT results may often be confounded [20, 22]. Evidence suggests that HRP-2 is actively produced and secreted by asexual and early sexual stage parasites, and is internalised by mature gametocytes – though it is unclear when transcription and translation ceases [5, 6]. HRP-2 has been proposed to play a role in the metabolism of haem, and also in the facilitation of cytoadherence [23]; a lack of active production by mature gametocytes is thus fitting for what are metabolically quiescent, circulating parasites, and this has led to a lack of concern that mature gametocytes are of significance in the context of persistent HRP-2 antigenaemia post-treatment.

Table 3. Test positivity and HRP-2 levels by study visit.

Study visit (days)	Treatment group	N	RDT positive (%)	Ultra sensitive RDT positive (%)	HRP-2 positive (%)	HRP-2 concentration (pg/mL), median (IQR)	p-value	Adjusted p-value
0	ACT	50	40 (80%)	46 (92%)	46 (92%)	>2800 (2704.3->2800)	0.597	0.689
	ACT + PQ	50	45 (90%)	49 (98%)	47 (94%)	>2800 (>2800->2800)		
2	ACT	50	31 (62%)	44 (88%)	41 (82%)	1469.6 (538.2->2800)	0.375	0.452
	ACT + PQ	49	39 (79.69%)	46 (93.9%)	44 (89.8%)	>2800 (582.2->2800)		
7	ACT	50	26 (52%)	42 (84%)	38 (76%)	197.3 (69.3-400.8)	0.210	0.401
	ACT + PQ	48	30 (62.5%)	44 (91.7%)	35 (72.9%)	393.3 (53.1-2056.2)		
14	ACT	49	15 (30.6%)	29 (59.2%)	21 (42.9%)	40.7 (26.4-1600.6)	0.552	0.940
	ACT + PQ	48	21 (43.8%)	38 (79.2%)	22 (45.8%)	43.05 (26.3-229.6)		
21	ACT	44	9 (20.5%)	22 (50%)	9 (20.5%)	32.6 (25.8-46.6)	0.871	0.479
	ACT + PQ	46	15 (32.69%)	32 (69.6%)	11 (23.9%)	28.05 (24.8-49.7)		
28	ACT	45	5 (11.1%)	12 (26.7%)	4 (8.9%)	26.6 (23.9-32.6)	0.503	0.674
	ACT + PQ	45	9 (20%)	15 (33.3%)	8 (17.8%)	26.4 (23.9-30.5)		
35	ACT	45	2 (4.4%)	7 (15.6%)	3 (6.7%)	23.9 (23.7-27.5)	0.217	0.149
	ACT + PQ	45	5 (11.1%)	12 (26.7%)	2 (4.4%)	25.3 (23.7-32.6)		
42	ACT	44	0 (0%)	5 (11.4%)	1 (2.3%)	23.9 (23.7-25.8)	0.419	0.507
	ACT + PQ	45	0 (0%)	5 (11.1%)	2 (4.4%)	23.9 (23.7-27.5)		
49	ACT	44	0 (0%)	2 (4.5%)	0 (0%)	23.9 (23.7-26.4)	0.253	0.337
	ACT + PQ	45	0 (0%)	5 (11.1%)	0 (0%)	23.9 (<6.8-26.4)		

However, the evidence for the relationship between gametocytes and HRP-2 antigenaemia is currently conflicting and sparse. Residual sexual stage parasites have been shown to be both associated [24] and unassociated [25] with RDT positivity, though such studies lack sufficiently sensitive experimental approaches to draw firm conclusions. In light of the dropping threshold for HRP-2 detection in the context of uRDTs, it is important to clarify unambiguously whether persistent gametocytes following treatment contribute to false test positivity.

Multivariate analysis found that the primary factor associated with initial HRP-2 levels and subsequent decay was asexual parasitaemia at enrolment in the study, as has been demonstrated previously)[26, 27]. Although most blood stages are known to secrete HRP-2, asexual parasites by far constitute the greatest proportion of total parasitaemia, thus it follows that infections with higher asexual densities produce higher levels of HRP-2. The persistence of HRP-2 after ACT has been shown to be caused by the return of previously uninfected erythrocytes (containing exported HRP-2) to circulation after the physical removal of dead asexual parasites [28]. That residual gametocytes do not significantly contribute to HRP-2 levels after treatment with ACT in this study will facilitate use and interpretation of more sensitive diagnostics based on detection of HRP-2, however it will be important to further examine whether this relationship is maintained across varying transmission settings and between genetic HRP-2 variant parasites, where gametocyte densities may be higher or variants may produce greater levels of HRP-2.

When comparing time to negativity between the standard RDT and uRDT, results were as anticipated, with individuals testing negative sooner by RDT than uRDT due to lower sensit-

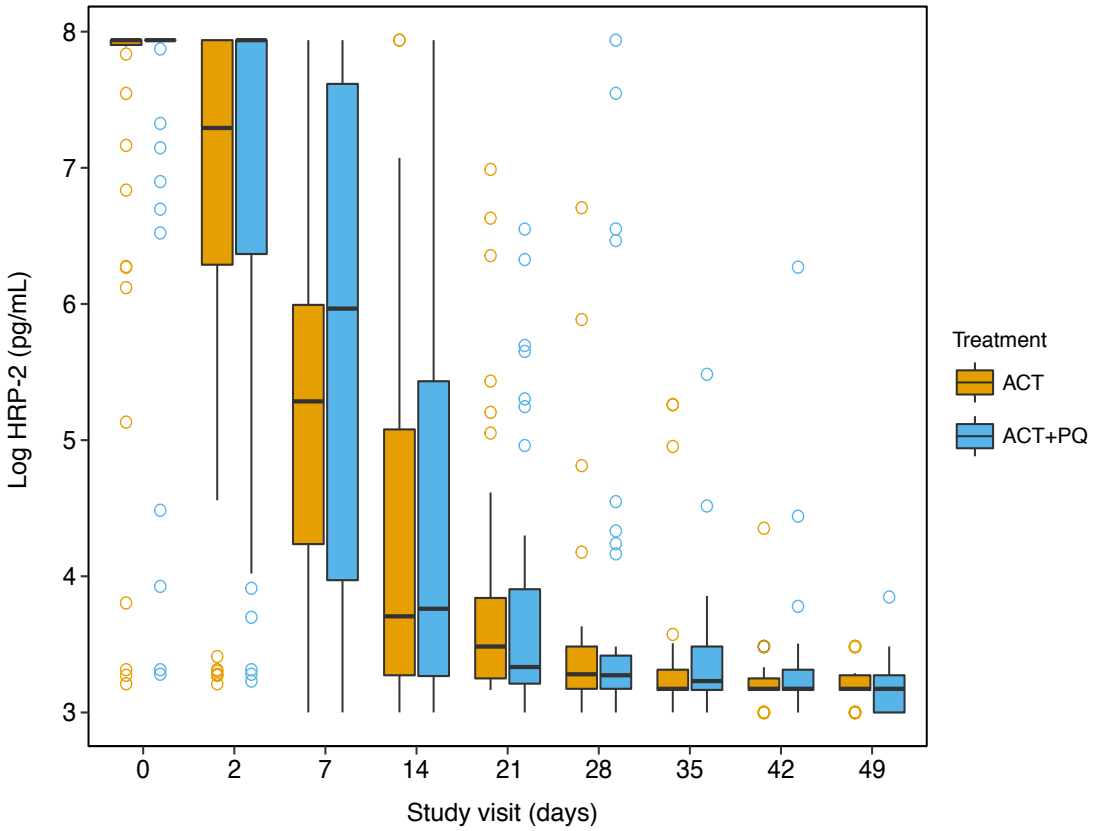


Figure 3. HRP-2 levels at each study visit by treatment group. Boxes are presented as median and interquartile range. For clarity of presentation in this figure only, individuals with HRP-2 levels lower than the lower limit of quantification (LLOQ) have been inflated to the LLOQ.

ivity. Unexpectedly, time to negativity based on quantified levels of HRP-2 was more similar to RDT than uRDT. This is likely due to the calculated cut-off of 54 pg/mL used to determine positivity by quantified HRP-2 versus the visual confirmation of the appearance of any line on the uRDT, which is sensitive down to 10-40 pg/mL [8]. The cut-off used in this study was calculated based on HRP-2 levels in individuals who were treated, and confirmed as continuously parasite negative by molecular methods over 49 days. As such, while the threshold for true HRP-2 negativity by this method may be less stringent, it is likely more representative of natural infections. Practically this finding supports a rationale for the more conservative use of RDTs post-treatment and highlights the importance of parasitological diagnosis when assessing potential treatment failure or reinfection. Within a research context, our findings support calls for the use of additional methods based on antigen quantification to differentiate active infection from residual antigenaemia; models based on the quantified decay and ratio of HRP-2 to p-LDH have shown an accuracy of 77.5% in distinguishing patients with and without current infection [29]. Future development in methods to quantify *Plasmodium* antigens in a lateral flow device format may also prove to be useful as a research tool. Such approaches are already in the early stages of application for diseases such as prostate cancer, whereby a portable reader

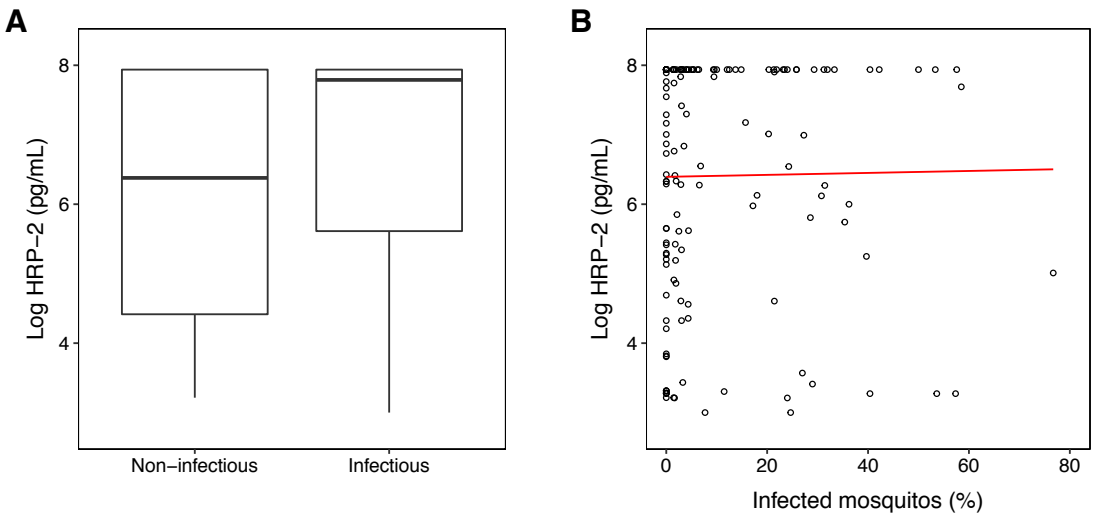


Figure 4. Levels of HRP-2 compared **A** between mosquito infectious and non-infectious individuals on days 0, 2 and 7 in the ACT group (boxes represent median and interquartile range), and **B** against the percentage of mosquitoes infected during feeding in mosquito infectious individuals (red line represents fitted linear regression model).

is able to measure the intensity of the test line, and thus quantity of prostate-specific antigen [30]. This type of innovation has the potential to be beneficial from a research perspective, quickly and easily providing epidemiological data to help direct RDT use — particularly in low transmission settings [31].

A difficulty in the context of such antigen quantification is accurately capturing the wide range of physiological concentrations observed during infection. In this study, HRP-2 levels were measured in samples diluted to 1:10, and yet signal exceeding the upper limit of quantification was observed in many samples — particularly at day 0 and 2. However, by increasing the dilution of samples to reduce saturation at the upper limit, the lower limit of quantification within a sample is correspondingly increased, leading to loss of data at the lower end of the quantifiable range. Whilst the issue of sample dilution will be highly relevant irrespective of the assay platform used, other approaches may provide greater levels of flexibility compared to commercial kits. Suspension bead array-based techniques have already been developed for the detection of HRP-2 and p-LDH [26, 27] and allow finer control of parameters such as the concentration of capture antibody used or the range of standard curve used to quantify the analyte.

In summary we have shown that the presence of gametocytes post antimalarial treatment appears to have little impact on time to negativity by HRP-2 based RDT and measurable levels of circulating HRP-2. Our data are consistent with other studies suggesting that asexual parasites are the primary source of HRP-2 which is perhaps unsurprising considering these parasites are multiplicative and present at markedly higher densities than gametocytes. These data provide clarity on the interpretation of RDT results and HRP-2 levels for future clinical and

epidemiological studies.

Contributors

TO, AM, CD, AD and WS designed the study. AM, KS, MD, AY, SMN, SS, SK, YS, AS, SFT and AD managed and conducted the trial. KL performed molecular parasite quantification assays. TO performed HRP-2 quantification assays. JB, TO and WS conducted analysis. CD, TO and WS prepared the manuscript.

Declaration of interests

The authors have declared no conflict of interest.

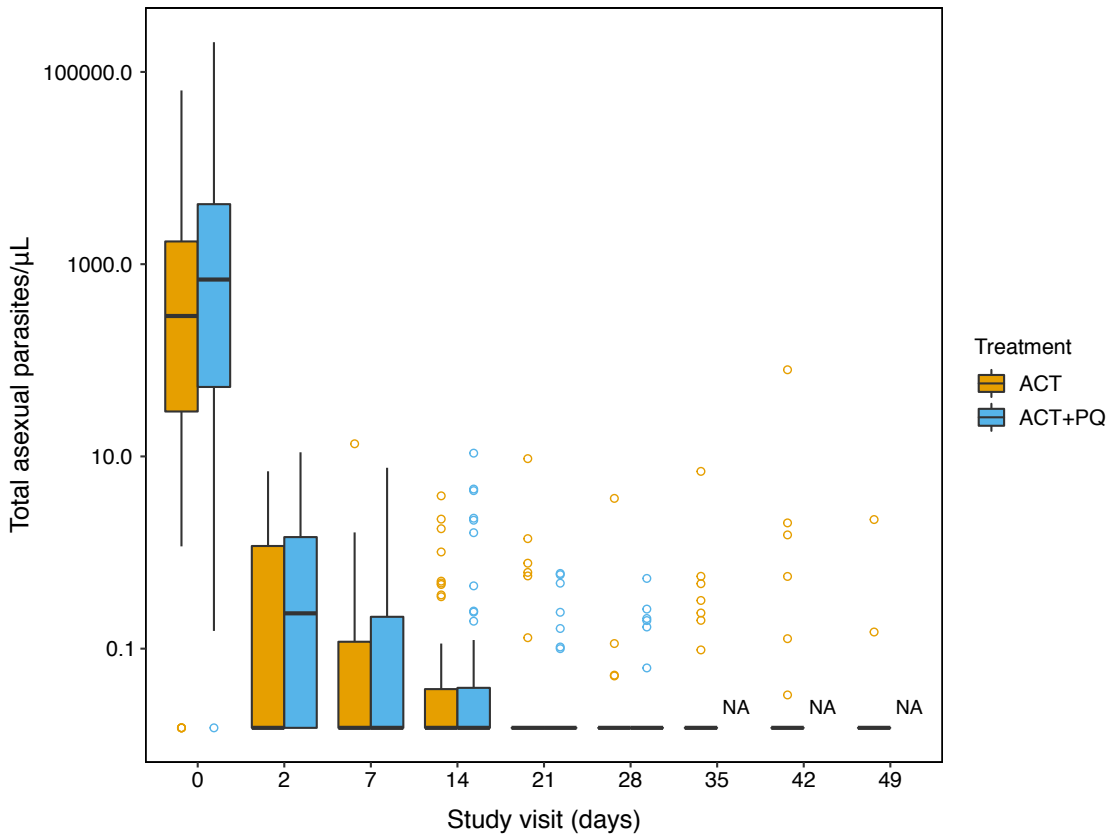
Acknowledgements

We thank the local safety monitor, members of the data safety and monitoring board, the study participants and the population of Ouélessébougou, Mali, for their cooperation.

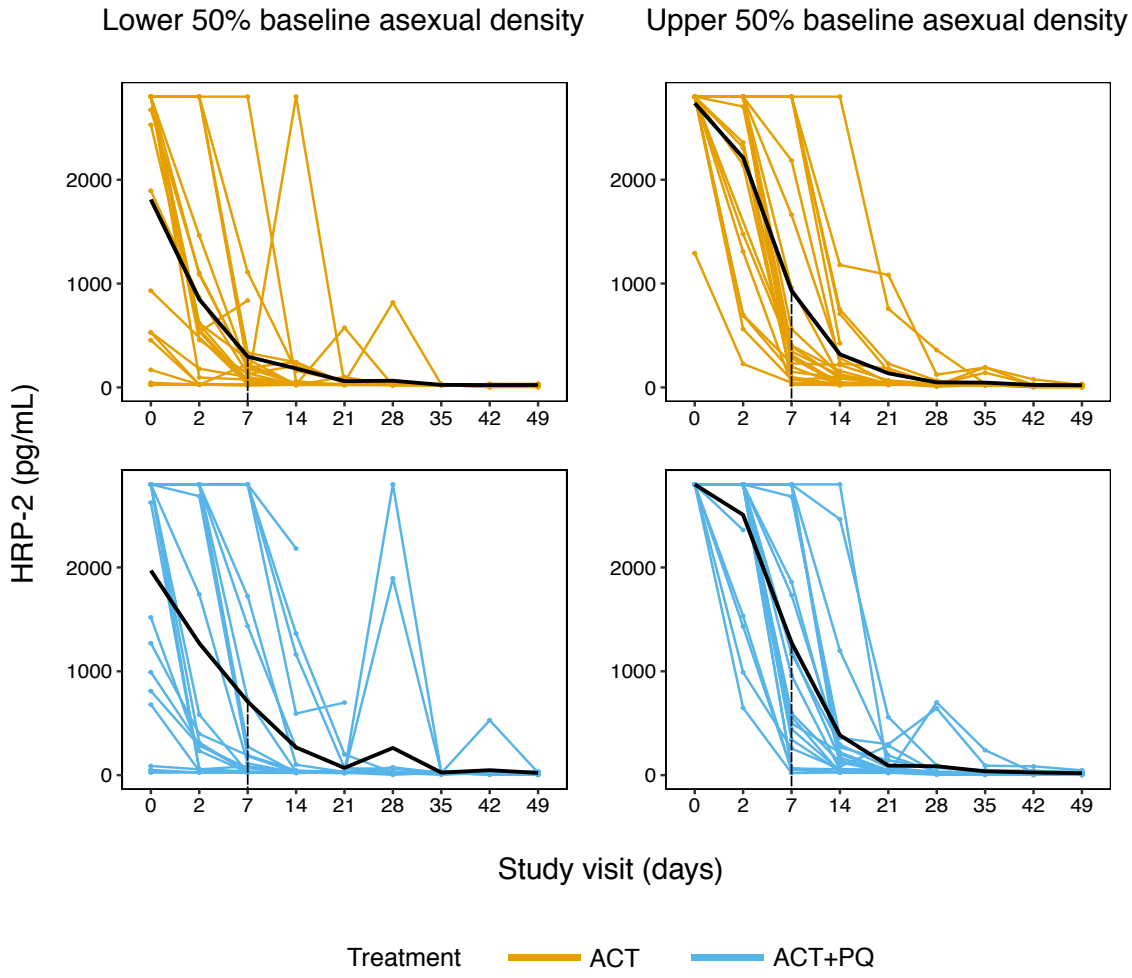
Data sharing statement

Data are available upon reasonable request to the corresponding author.

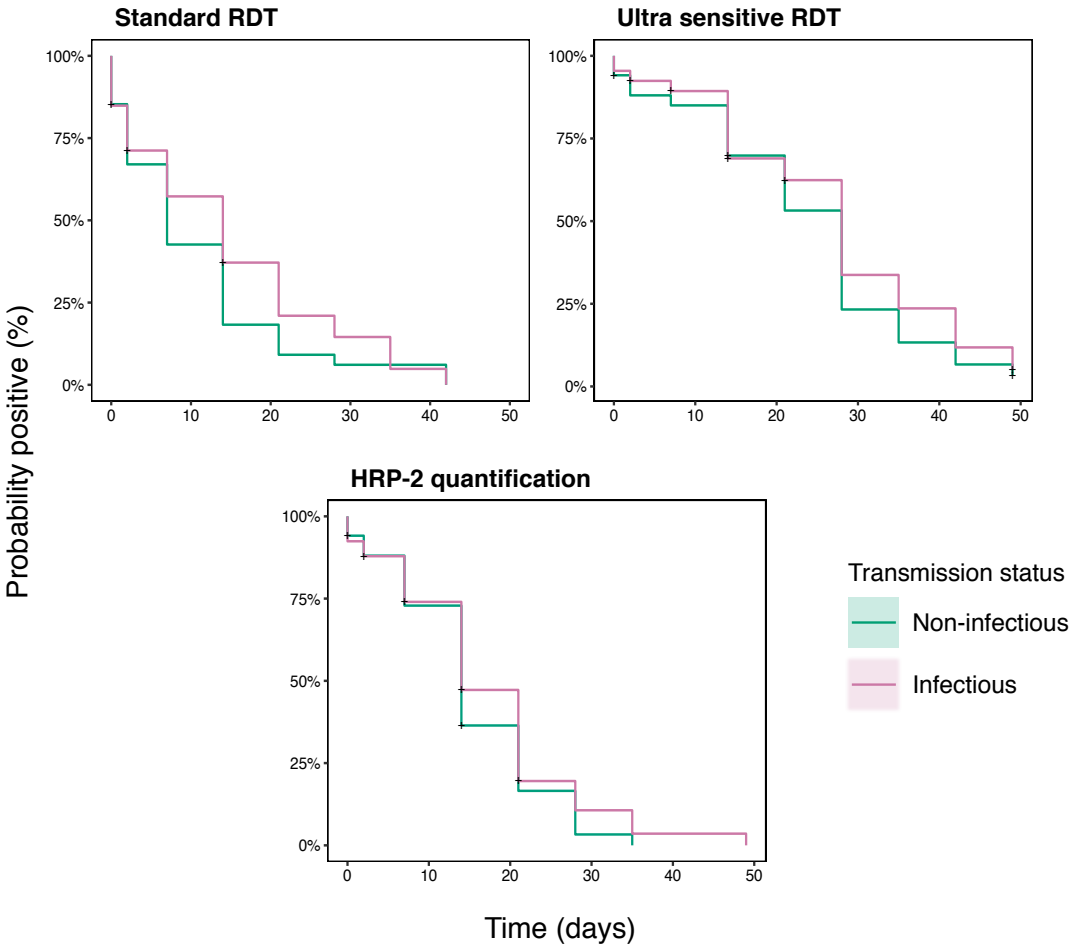
6.6 Supplementary material



Supplementary figure 1. The mean magnitude of response of each protein target stratified by expression system, presented with median and interquartile range of all mean responses.



Supplementary figure 2. Comparison of levels of HRP-2 between ACT and ACT+PQ group stratified by baseline asexual density. Solid black line represents mean levels of HRP-2. Dotted black lines at study visit 7 are to assist in identifying differences in mean levels of HRP-2 between groups and strata.



Supplementary figure 3. Kaplan-Meier plots showing time to negativity by standard RDT, ultra-sensitive RDT and quantified HRP-2 between the mosquito non-infectious (green) and mosquito infectious (red) individuals.

Supplementary table 1. Cox proportional hazards ratio by test, between infectious and non-infectious individuals. Reported with and without adjustment for baseline gametocyte density.

Test	Variate	Coefficients	Hazard ratio	95% CI	p
Standard RDT	Univariate	Infectious at day 0	0.76	0.49-1.16	0.197
	Multivariate	Infectious at day 0	0.76	0.49-1.18	0.229
		Baseline gametocyte density	1	1	0.763
Ultra sensitive RDT	Univariate	Infectious at day 0	0.79	0.51-1.24	0.309
	Multivariate	Infectious at day 0	0.80	0.50-1.27	0.344
		Baseline gametocyte density	1	1	0.607
Quantified HRP-2	Univariate	Infectious at day 0	0.79	0.51-1.22	0.286
	Multivariate	Infectious at day 0	0.79	0.50-1.24	0.301
		Baseline gametocyte density	1	1	0.677

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6.7 Additional work and figures

Implementation of a suspension bead array for the detection of HRP-2

Detection and quantification of HRP-2 in this chapter was carried out using an ELISA based methodology (Quansys Q-plex). However, initially an SBA approach was trialled, based on protocols shared by collaborators.

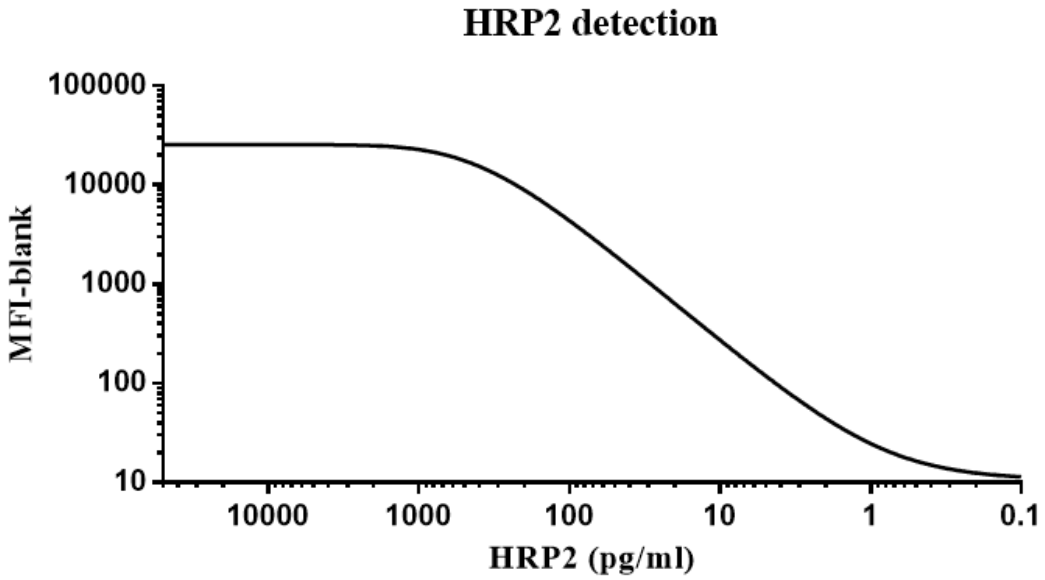
In principle, the assay is very similar to the antibody detection SBAs described elsewhere in this thesis, though with the following notable differences: a histidine-rich protein 2 (HRP-2) specific capture antibody is bound to the microspheres (or 'beads'), and a biotinylated HRP-2 specific antibody is used to facilitate detection, via phycoerythrin-conjugated streptavidin.

Assay preparation

Capture (MBS832975; MyBioSource, CA, USA) and detection (MBS832975; MyBioSource, CA, USA) antibodies were sourced (though were later discontinued and thus substituted accordingly: Capture, MPFM-55A; detection, MPFG-55A; both Immunology Consultants Laboratory Inc., OR,USA). Capture antibody was coupled to beads, while detection antibody was biotinylated (EZ-Link™Sulfo-NHS-LC-Biotinylation Kit; Thermo Fisher) each according to supplied protocols.

Initial testing and troubleshooting

Initial testing for successful bead coupling and biotinylation of detection antibody was assessed by screening a standard curve of recombinant HRP-2 protein type A (890015; MicroCoat, Germany) in a 22-point 2-fold serial dilution from 50 ng/mL. The expected result is a dose-response curve, saturating at 25,000 MFI, as in **additional figure 1**.



Additional figure 1. Expected titration curve of recombinant HRP-2 standard. Taken from *Bead suspension array for the quantification of malaria antigens PfHRP2 and pLDH Version 1, ISGlobal, Barcelona*.

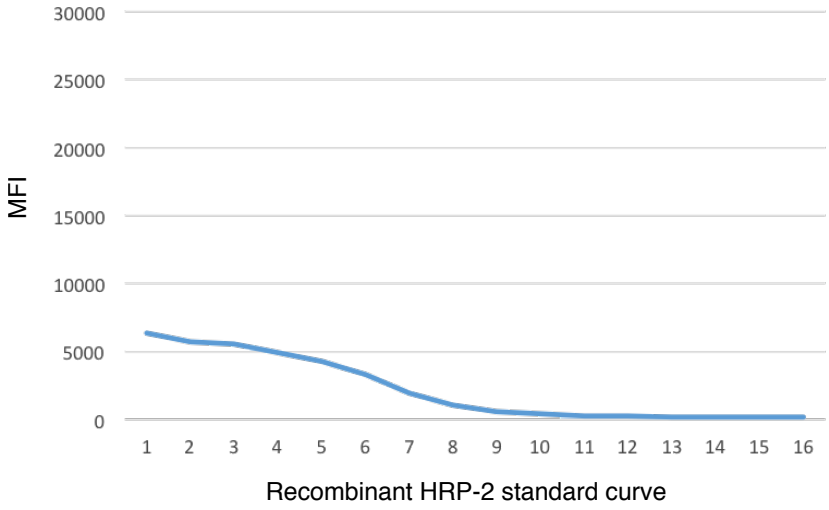
However, despite repeated attempts it was not possible to replicate such a curve, with maximum MFI typically falling between 4000-8000 (representative example in **additional figure 2**). Biotinylation of detection antibody was repeated, as suggested by the assay protocol, but this did not improve results. After repeated attempts to recover the assay at LSHTM, troubleshooting was next conducted on-site with collaborators at ISGlobal, Barcelona.

A 'chequerboard' approach to dissect the assay was conducted, using LSHTM and ISGlobal reagents (**Additional table 1**). Using this approach, it was possible to identify that the reagent at fault was the biotinylated detection antibody (Experiment 5). Accordingly, a new batch of detection antibody was biotinylated and tested on-site at the ISGlobal laboratory successfully.

Additional table 1. Chequerboard approach to reagent testing.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Beads (Capture antibody)	ISGlobal	LSHTM	LSHTM	ISGlobal	ISGlobal
Biotinylated detection antibody	ISGlobal	ISGlobal	ISGlobal	ISGlobal	LSHTM
Recombinant HRP-2	ISGlobal	LSHTM	ISGlobal	LSHTM	ISGlobal

Before large-scale testing could be conducted, the study was interrupted by SARS-CoV-2 lockdown measures. Unfortunately, when restrictions were finally eased, it was found that the assay no longer performed as expected and so new material and reagents were procured in an attempt to recover the assay. As already mentioned, the capture and detection antibodies



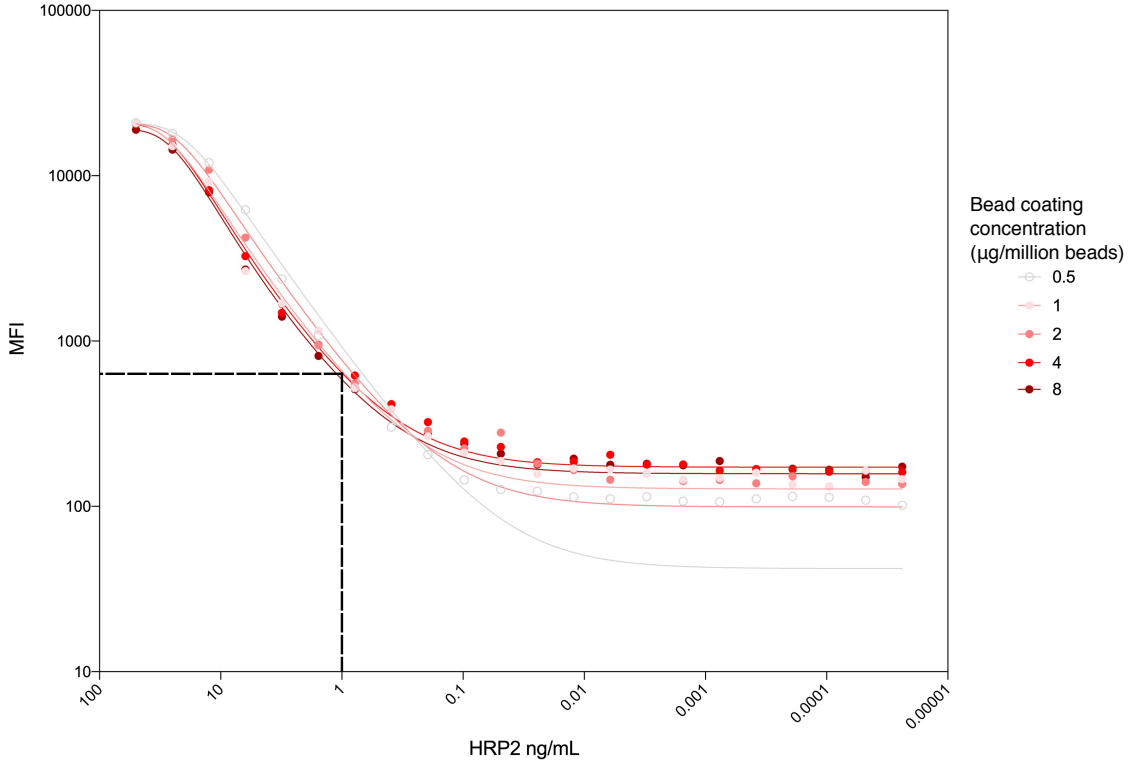
Additional figure 2. Representative actual titration curve of recombinant HRP-2 standard.

used previously had been discontinued. As such, a titration of each new antibody (bead coupling concentration, and biotinylated detection antibody concentration) was conducted to re-optimise the assay. Beads were coupled to capture antibody at a range of concentrations: 8 µg, 4 µg, 2 µg, 1 µg and 0.5 µg per million beads. Each bead set was then screened against a standard curve of recombinant HRP-2, and detection was carried out using the previous batch of biotinylated detection antibody.

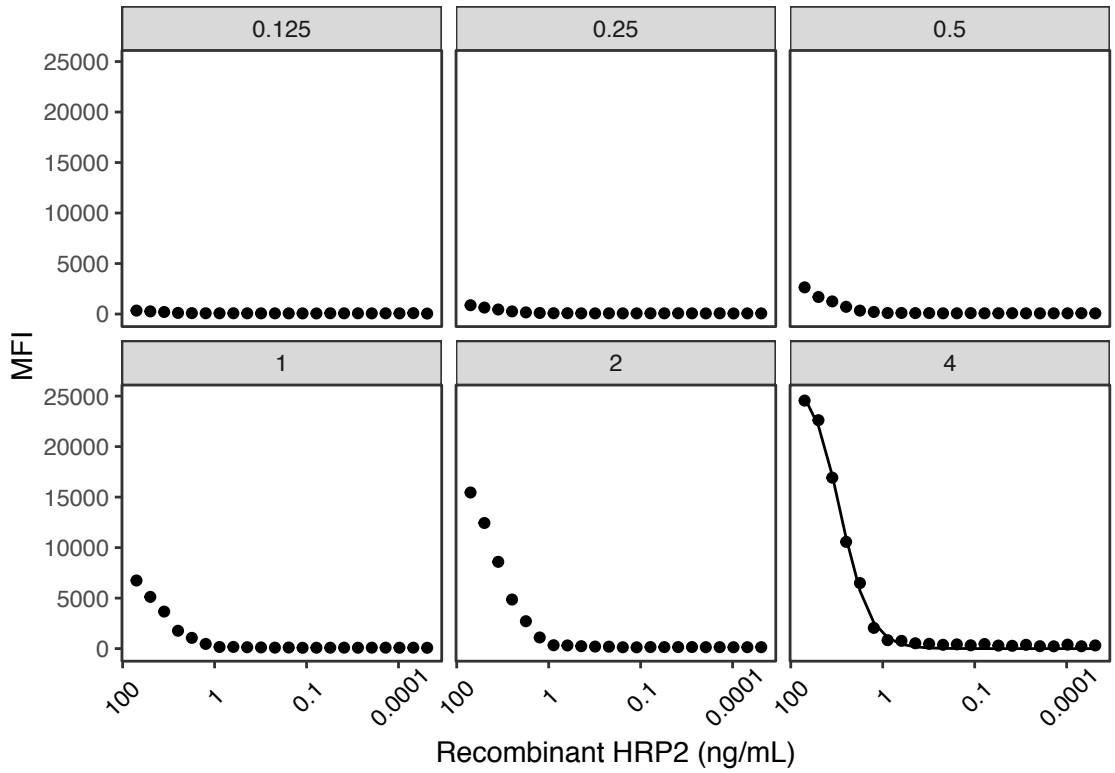
Performance was found to be similar between all bead coupling concentrations (**Additional figure 3**), although the range of detection was found to be limited — at 1 ng/mL of recombinant HRP-2, MFI was approximately 800, compared to a target of 25,000 according to the expected titration curve at the same point in the standard curve (**Additional figure 1**). However, as this experiment was conducted using the old biotinylated detection antibody, and the appropriate dose response curve was observed, it was thought that the limits of detection may be recovered when utilising the new biotinylated detection antibody. As such, a final coupling concentration of 0.5 µg/million beads was selected, to maximise the number of beads that could be coupled.

The new detection antibody was then biotinylated according to protocol and, using a new set of beads coupled at 0.5 µg/million beads, was then titrated out over 6 concentrations: 4 µg, 2 µg, 1 µg, 0.5 µg, 0.25 µg and 0.125 µg/mL (**Additional figure 4**). It was found that only a concentration of 4 µg/mL produced the expected dose response curve (notably 4 times more concentrated than is stipulated by the assay protocol using the original detection antibody clone) though, again, the expected sensitivity was far lower than anticipated.

Facing these difficulties, this SBA approach to HRP-2 was abandoned for the study included in this chapter with a view to continue to try to re-optimise the assay in future.



Additional figure 3. Titration of new capture antibody bead coupling. Black dashed line indicates approximate MFI at 1 ng/mL (1000 pg/mL) of recombinant HRP-2.



Additional figure 4. Titration of new biotinylated detection antibody. The concentration (µg/mL) of biotinylated detection antibody used is indicated over each plot.

Chapter 7

Discussion

Since the aim of malaria eradication was reinstated in 2007 at the Gates Malaria Forum, considerable progress towards reaching that target has been made, primarily through the widespread application of insecticide treated nets (ITNs) and artemisinin-based combination therapys (ACTs) [1–4]. However, success in reducing prevalence and incidence, through the targeted delivery of such interventions, have arguably been greatly enhanced by the development of new tools leading to improvements in diagnosis and surveillance. As a result, an increasing number of endemic areas have reduced transmission and are approaching elimination, though it is well recognised that further advances in detection of infection and measurement of transmission are needed to tackle the burden of residual or 'invisible' malaria. Serological methods have proved highly insightful in this area, providing the basis for rapid diagnostic tests (RDTs) and facilitating the measurement of key epidemiological trends through the detection of *Plasmodium* specific antibodies. For example, reductions in seropositivity to Apical Membrane Antigen 1 (AMA1) and Merozoite Surface Protein 1 (MSP1) have been used to describe decreases in exposure to *P. falciparum* and *P. vivax* in an area of low transmission in Indonesia, where conventional measures of transmission become less sensitive as parasite prevalence falls [5]. Similarly, serological endpoints for a selection of parasite antigens have been shown to improve the power and precision when measuring effect size after intervention in conjunction with molecular diagnostic methods [6]. As novel techniques and platforms become available, new serology-based approaches have the potential to unlock information crucial to addressing malaria elimination across endemic regions. This thesis aims to highlight the development, application, strengths and weaknesses of such innovations.

The topics covered here consider aspects of chapters 3 to 6 that were not, or only briefly, addressed in the paper-specific discussions.

7.1 Proteome-scale immunoassays

7.1.1 Microarray technology

Microarray technology was developed as a method for detecting and measuring large numbers of nucleic acid sequences through hybridization to complimentary immobilised target nucleic acid sequences on a solid slide support. Applications include characterisation of gene expression changes after *e.g.* developmental drug treatments in arthritis [7] or the influence of gene expression regulators (*e.g.* microRNA in cancer [8]).

This technique has since been extended to capture protein-protein interactions, thus facilitating the capture of antibodies, specific to immobilised antigen, from a serum sample. Such methodology has been adopted in the context of malaria vaccine target discovery, making use of an 'immunomics' (*i.e.* genome- or proteome-level) approach to screening afforded by such

techniques [9]. Early implementations of such methods have helped to establish the framework for developing multivalent malaria vaccine constructs [10], and proposed mechanisms of protection induced after vaccination with RTS,S [11]. In this particular study, comparisons were made between groups that received a full course of RTS,S or a comparator vaccine, revealing significantly lower breadth and magnitude of response to liver and asexual-stage parasites in the RTS,S group. This likely indicates a reduction in asexual parasite exposure through the blockage of sporozoite invasion of hepatocytes, leading to reduced asexual development. Interestingly, while the majority of antigens to which any reactivity was detected were shared between groups, significant differential reactivity was observed in the RTS,S group to circumsporozoite protein (CSP) (the antigen upon which RTS,S is based), but also to 3 additional proteins unrelated to the vaccine construct.

Such protein microarray approaches are similarly suited to early biomarker discovery thanks to the ability to incorporate so many putative antigenic targets into a single array. However, it is arguably this property that can make interpreting the data generated more difficult than other assays. As demonstrated in chapter 3, protein antigens behave differently dependent on materials and method. Whilst differences in reactivity to antigens between experimental conditions is to be somewhat expected irrespective of the assay platform used, it is objectively easier to optimise for a smaller number of targets. Enzyme-linked immunosorbent assays (ELISAs) and, to a great extent, suspension bead arrays (SBAs) can be refined to best capture antibodies to typically one to tens of antigens through titration of coating concentrations *etc.* However, this process may be limited by the availability of positive control samples (which is also true for microarrays).

By their nature, antigen reverse phase protein microarrays (RPPAs) for biomarker discovery are likely to contain numerous proteins about which very little is known, yet it would be incredibly logistically expensive to optimise and refine conditions for every target. What's more, with potentially such diverse proteins incorporated into the same microarray it is likely that optimal conditions for one antigen will be comparably poor for another. Despite this fact, the data presented in chapter 3 show that it is possible to develop approaches that are broadly appropriate for use with a range of protein antigens, though arguably RPPA methods are best suited to general identification of reactive targets for further investigation by ELISA or SBA rather than in-depth characterisation. With that said, it may also be feasible to establish a forward-to-reverse strategy — antigen targets of putative interest identified by microarray could be reassigned to an SBA format for further investigation, before being transferred back to a microarray for higher-throughput antigen screening.

At first glance, the use of RPPAs would appear to be limited to use strictly in a research context due to the cost and practicalities of the technique. However, the availability of plastic-based (*e.g.* cyclic olefin polymer) slides presents the possibility of developing arrays that are more suitable for use in a healthcare context. Although unlikely to be useful in a diagnostic capacity, the ability to routinely screen a patient for reactive antibodies to a panel of specific antigens along-

side a typical RDT could prove highly useful from an epidemiological perspective. For example, a recent study has shown that a panel of eight antigens are capable of identifying individuals that have been infected with *P. vivax* within the last nine months with 80% sensitivity and specificity, making them suitable candidates for anti-hypnozoite therapy [12]. This insight would be a useful tool for elimination, such that hypnozoite-derived relapses after standard blood-stage drug treatments can be greatly reduced. Assay procedures would need to be simplified and standardised to make such a test appropriate for use in a clinical setting, but if this were to be achieved it could transform the way serological data is collected and used. Examples of other potential metrics that could be measured if suitable antigen markers can be identified are recent exposure or transmission reducing immunity (TRI), as discussed in the introduction; either or both of these would greatly help to inform local surveillance initiatives and help to direct active intervention measures.

7.1.2 Peptide microarrays

Aside from the whole proteins or protein fragments used in the microarrays in chapter 3, 4 and 5, it is also possible to produce high density arrays of small, overlapping peptides. Peptides can be synthetically polymerised from individual amino acids using techniques such as photolithography [13], amino acid printing [14] or laser transfer [15]. Such approaches allow the production of arrays covering specific proteins of interest up to entire proteome level coverage, though any reactive epitopes will inevitably be linear in nature. However, using this type of array can be useful in the context of epitope mapping (*i.e.* isolating specific protein domains or sequences to which antibodies have affinity) and for identifying targets of immunity (*e.g.* vaccine candidates) or serological biomarkers (*e.g.* of exposure). For example, an array of 1912 individual peptides, covering 23 different malarial protein antigens, was used to identify and map B cell epitopes across the entire sequence of each protein that may be fruitful vaccine targets, showing good correlation of reactivity to known immunogenic targets, but also identifying novel epitopes (*e.g.* within glutamate rich protein (GLURP) — a component of a multivalent vaccine, GMZ2, currently in trials [16]) [17].

Smaller peptide arrays may be useful for comparing antibody reactivity to different pathogen strains; reactive epitopes within a highly immunogenic surface antigen, in common and unique between strains of *Borrelia burgdorferi*, have been elucidated using a small peptide microarray approach, in conjunction with ELISA and SBA methods [18]. In contrast, higher density designs are well suited to viral pathogens, as smaller proteomes are more easily covered. Entire viral proteomes may be compared for antibody reactivity, which has helped to identify a strongly IgG targeted epitope on the NS2B protein that acts as a biomarker for severe clinical phenotypes for Zika virus [19].

7.1.3 IVTT microarrays

The development of *in vitro* transcription/translation (IVTT) approaches to protein production — based on simple PCR-based methodologies to produce transcriptionally active PCR fragments (in contrast to plasmid transfected bacterial culture [20]) and subsequent cell-free, *in vitro* translation of amplified products [21] — led to the foundation of a new approach to protein microarray production. Since then, IVTT arrays have been readily adopted within a malaria serology context. Able to achieve similar target densities to the peptide arrays mentioned above, but with the benefit of also being able to produce longer protein sequences, IVTT arrays provide the potential to interrogate conformational epitopes, which are known to be important in a number of key *Plasmodium* biomarkers such as Pfs48/45 [22] and AMA1 [23]. The importance of conformation and stability in the capture of antigen specific antibody is considerable. In the case of Pfs48/45, one recent study has demonstrated an increase of 1-2 orders of magnitude in the level of functional immunity induced by a mutationally engineered, stabilised construct of Pfs48/45, versus wild-type, native protein [24]. It must be noted, however, that protein products from IVTT reactions are not typically purified and evaluated for characteristics such as folding or post-translational modification, so it cannot be assumed that correct protein folding has occurred. Indeed, the cysteine-rich nature of proteins such as Pfs48/45 lead to a large number of disulphide bonds in its native conformation, such that expression systems with high levels of post-translational processing, like an insect-baculovirus system, are much more suitable for the production of conformational targets [25]. What's more, conserved cysteine-rich domains are common in the *Plasmodium* proteome, with many located in erythrocyte invasion associated proteins (*e.g.* the erythrocyte binding antigens (EBAs), *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) and AMA1) [26] — all of which are antigens of particular interest from a serological perspective. This characteristic should therefore be considered in the context of protein expression systems with lower levels of protein modification.

The simple scalability of IVTT relative to other protein expression systems used for the production of protein microarrays enables far greater numbers of targets to be included in antigen panels. However, uncertainty around protein expression efficiency, both within and between rounds of production, coupled with the findings in chapter 3 around differences in reactivity to targets based on materials used to manufacture arrays, perhaps limits the usefulness of the system beyond high-level target identification. Given that microarray technology is well suited to such an application, this caveat is relatively inconsequential, although concerns around the consistency of protein production and reactivity are justified.

7.2 Comparing protein expression approaches across assay platforms

The differences in reactivity between proteins purified from *E. coli* expression systems and matched targets from IVTT systems, demonstrated in chapter 4, suggests that greater consideration should be given to the source of protein when interpreting results from serological screens against antigen panels. This is also likely to be true for other assay platforms and it would be prudent to further investigate potential variability between protein expression systems in this context, as this may have implications for the comparisons of data generated by different studies. Despite the variability in immunogenicity of sequence matched, but differently expressed, protein, there may be other properties inherent to products of a particular expression system that may be beneficial to specific applications. For example, the level of complete translation of transcripts, or post-translational modification, may have impacts on the stability of protein structure over time or under different environmental conditions (*e.g.* differences in the levels of protein phosphorylation carried out by a bacterial versus a eukaryotic baculovirus expression system have been shown to affect protein thermostability [27]). The final application of protein products should also be considered in this context — proteins that might be kept in frozen aliquots until required for use in an ELISA, compared to proteins coupled to microspheres for use in an SBA or printed onto a microarray, for later use.

The greater levels of reactivity observed against purified *E. coli* derived proteins in chapter 4 suggests that there may be a case for shifting production of putative targets of interest identified through IVTT arrays to other protein expression methods — in a similar fashion to the down-selecting of targets from microarrays to SBAs. The ability to refine expression using bacterial or wheat germ systems, for example, and quality check product before application (*e.g.* purity; quantity; stability; whether proteins remain in suspension *etc.*) allows for improved certainty and reproducibility when investigating antibody responses to antigens.

Overall, chapter 4 perhaps demonstrates a critical issue in biomarker discovery — which is that different methodologies can yield different results, and as such, there is a risk that targets of genuine interest may be missed (*e.g.* where targets may not express as expected, or at all in some cases) or overlooked (*e.g.* proteins expressed in a particular system may not be reactive compared to others). Whilst there is no obvious solution to this problem at this time, comparative studies and consideration of use case for particular technical approaches may help to rationalise interpretation of data.

In spite of these potential issues, using microarray techniques to screen protein antigens, irrespective of protein expression method, can be beneficial. For example, it could be argued that bacterially expressed proteins are better suited to methods such as SBA or ELISA, as production is unlikely to be of a scale capable of producing the 100+ targets that make microarray

approaches most useful. However, it is far more simple and cheap to include a new purified antigen for investigation on a microarray than it is to couple an antigen to microsphere beads and titrate it out for use in an SBA, which may also necessitate the purchase of additional bead regions *etc*. Relatedly, the ease with which antigens can be included or dropped from an exploratory panel make the approach highly flexible, allowing the quick production of study specific microarrays — antigens from multiple non-malarial pathogens can easily be incorporated, for example. Furthermore, the potential to simultaneously interrogate multiple analytes, such as different antibody classes or sub-classes (as discussed in 3.7 Additional work and figures) is a strong motivation for using the microarray platform even for smaller numbers of purified protein antigens.

7.3 Selecting and combining assay platforms, and methodological limitations

The three different methodological approaches used to investigate gametocyte specific antibody responses in chapter 5 are a demonstration of how the strengths of each platform can be combined to address research questions around biomarker discovery and characterisation. The use of ELISA and crude parasite extracts to describe broad immunological trends between study groups, followed by more thorough investigation of specific antibody responses by SBA and finally high-throughput screening of a gametocyte enriched, IVTT-based microarray to identify novel antigens of interest enables this study to investigate sexual stage immunity at multiple levels, helping to procure relevant data that may be missed by narrower approaches. In keeping with the research pipeline method, the targets identified by RPPA in chapter 5 could be transferred to an SBA format. The ability to swap antigens in and out of SBA panels allows for the refinement of standardised assays, as new antigens are identified and others are discounted; median magnitude of response to Pfs48/45-6C and Pfs230-CMB did not appear to increase over time in line with responses to other antigens, and so may be considered less informative. This would simply require the bead coating concentration of new antigens to be titrated out under the standardised assay conditions, although the inclusion of new target antigens is easier still on a microarray platform, as already discussed in this main discussion chapter (7.2 Comparing protein expression approaches across assay platforms).

An important point, already touched on in the study discussion in chapter 6, is the dilution at which samples are screened. Particularly in the case of the detection of antibodies, where highly immunogenic targets may be combined with antigens that elicit lower levels of response, but also in the direct antigen detection (*e.g.* HRP-2, see chapter 6), there may practical issues in achieving satisfactory results. Although antigens that induce lower levels of antibody can be conjugated to the solid support (*e.g.* SBA microbeads) at greater concentrations, this may not

always resolve difficulties in detecting reactive antibodies. In chapter 5, samples are screened at a 1/200 dilution to help capture antibodies specific to the sexual stage antigens included in the panel, despite the fact that the asexual targets included in the assay are typically screened at 1/400. This decision was made to address the fact that the biomass of gametocytes in an infection is much lower compared to asexual parasite, thus the amount of antigen exposed to the immune system is similarly low, leading to relatively reduced antibody responses. However, the increased concentration of sample risks losing data at the upper limit of detection for asexual antigens as signal saturates sooner. The solution to this problem is not obvious, short of running samples at two, or more, different dilutions — this would somewhat defeat the practical benefits of multiplex assays, though may be applicable in certain scenarios, such as additional avidity assays to measure the strength of binding between antibody and antigen [28]. At present, it is most likely that compromises may have to be made, with the inclusion of exclusion of targets, or sample dilution decisions, made on the basis of the research questions being asked.

Similarly, assay parameters around sample dilution may add a layer of complexity when comparing different serological studies. Fundamentally, raw output data from serological assays will vary according to the sample dilution used, and assays can be optimised accordingly [29]. As such, it can be difficult to directly compare results potentially leading to discrepancies between data sets where different sample dilutions are used. While this may not always be problematic in itself, strategies can be utilised to negate this issue, for example the conversion of adjusted median fluorescence intensity (MFI) to a ratio of signal against a known control (as in chapter 3) helps to account for contrasting assay conditions.

Measurement of parasite antigens by SBA or other formats, such as the ELISA-based Quansys system utilised in chapter 6, is likely best suited to answering epidemiological questions. Such assays are generally optimised for use with whole blood or serum, though the logistics of collecting such samples can be prohibitive in some cases. Dried blood spots (DBS) (whole blood absorbed into filter paper, and punched out to produce a disc of dried sample) are another standard form of sample collection for serological assays. The ease of storage and preservation make them preferable in many scenarios, and parasite antigens may be extracted via elution, though recovery appears to be reduced compared to serum or whole blood [30]. One limitation of the DBS approach is that the required final concentration of sample can make elution volumes very small, dependent on the volume of blood contained within each spot, which can make extraction difficult to impossible. If DBS are to be utilised for antigen detection assays, these material parameters should be considered prior to sample collection to enable simple execution.

7.4 Measuring gametocyte-specific immune responses and transmission reducing immunity

Shortcomings in the initial stage of the study approach in chapter 5 are highlighted in the detection of antibody responses to sexual stage parasite lysate by ELISA despite the lack of exposure to gametocytes in the control groups. This is most likely explained by the presence of antigens common to both sexual and asexual stage parasites in crude lysate, such as PfSRA [31], which are also likely to be present in the greatest abundance — the relative number of proteins exclusively expressed by each parasite stage will be far fewer than proteins that constitute the majority of the cellular structure at all stages of development. Cross reactivity between antigens may also be a factor in this context, suggested by the observation of reactivity to CSP in the cohort infected with blood stage parasites in the study presented in chapter 5. Antibody responses to non-sporozoite antigens such as MSP5 have been demonstrated after vaccination with RTS,S [32], with highly repetitive regions of low complexity a likely source of such cross reactive epitopes [33]. These issues highlight the difficulties in identifying stage specific antigens and corresponding antibody responses, and lend weight to the use of isolated protein targets rather than crude parasite preparations, as was conducted in the study. Even so, it would be difficult to draw firm conclusions from such serological screens. Identification of a true, stage specific antigen is dependent on the comparison of proteomic expression analysis of isolated parasite stages, whereas serological assays alone must be interpreted in light of potential cross-stage immunity — irrespective of the source of antigen.

The identification of responses to known and novel gametocyte specific antigens in gametocytaemic individuals in chapter 5 warrants further investigation. Although evidence surrounding anti-gametocyte immunity has grown more recently, there remain large gaps in our understanding of how such immune responses develop and the mechanisms by which they act [34]. At present, it is not clear what level or duration of gametocyte exposure is required to induce detectable natural responses, or how long such responses last. The original design of this particular study incorporated measurements of TRI through the use of standard membrane feeding assay (SMFA), and samples were collected accordingly, although only up to day 35/36. However, significant antibody responses to gametocyte antigens were only detected by SBA at day 51 (**Figure 5.2**), explaining the lack of any transmission blocking effect seen in the day 35/36 samples (unpublished observations). Such a finding sheds some light on the timescale of induction of anti-gametocyte immunity but is also an important consideration in study design for future investigations of this type. This is of particular relevance in the context of identifying antigens for vaccine development, where longer-lived antibody responses are more desirable than shorter-lived responses in terms of inducing lasting immunity. Measuring the length of decline of gametocyte-specific antibody response would likely require extended sampling periods beyond those of typical serological studies, as induction of such responses appears to be delayed

compared to anti-asexual responses. Furthermore, the effect of immune boosting as a result of exposure or re-inoculation in the months or years after initial vaccination may be important; antibody titres have been shown to significantly increase in non-malaria naïve individuals compared to malaria naïve individuals upon re-infection [35]. Such effects may have substantial implications for certain transmission blocking vaccine targets, such as Pfs25. While vaccine induced antibodies specific to Pfs25 have been shown to have a transmission reducing effect [36], the human immune system is not exposed to native antigen during natural infection as the antigen is expressed post-fertilisation whilst within the mosquito mid-gut, thus precluding the possibility of natural immune boosting.

As described above, assessment of patient infectiousness or TRI is carried out by membrane feeding assays. In the direct membrane feeding assay (DMFA) (using gametocyte positive whole blood from patients, with or without serum replacement) or standard membrane feeding assay SMFA (using laboratory cultured gametocytes with serum added), mosquitoes are fed through a membrane and later dissected to look for developing oocysts. Although these techniques are well recognised and utilised, the intricacies of maintaining stable mosquito colonies and the subsequent dissection process make membrane feeding assays difficult to conduct [37, 38]. In face of the challenges associated with both the DMFA and SMFA, development of alternative approaches to determining infectiousness are highly desirable. The discovery of a biomarker associated with TRI would be a great improvement on these current methods, potentially allowing identification of individuals with high levels of TRI (*e.g.* very high titre antibodies, or specific antibody repertoires) [39], and thus a low risk of transmitting. A potential example of this is is the 6C fragment of Pfs48/45, which is the target of a monoclonal antibody (TB31F [40]) currently under investigation as a transmission reducing treatment [41]. The detection of natural antibodies with affinity to this epitope may thus be indicative of functional levels of TRI. Alternatively, the presence of other gametocyte specific antibody responses, whether functionally protective or otherwise (*e.g.* antibody targets newly identified by microarray in the study in chapter 5), may be indicative of regular or historical gametocyte carriage. Measurements of such markers could potentially be used to identify specific target groups for transmission blocking drug treatments, or other interventions.

7.5 Application of immunoassays to address specific knowledge gaps

Chapter 6 demonstrates the application of a refined, parasite derived biomarker-based assay to answer specific questions of clinical and epidemiological importance. Confirmation that residual gametocytes after administration of standard ACT do not contribute to circulating histidine-rich protein 2 (HRP-2) and lengthen the time to RDT negativity, even when using

ultra-sensitive rapid diagnostic tests (uRDTs), has positive implications for the use of such diagnostic tools at point of care. The question answered by this study — whether more sensitive diagnostic tools may be confounded by unknown characteristics of infection — raises a critical issue. It is important to recognise that new or improved techniques to measure one feature of infection may obscure other facets of the disease, just as measuring antibody responses to single antigens may skew interpretations of malaria immunity (see 1.5.2 Producing antigenic targets).

The detection of additional antigens by RDTs, such as *Plasmodium* lactate dehydrogenase (pLDH), goes some way to countering the risk of taking too narrow an approach to parasite detection, in addition to improving sensitivity and specificity. Looking forward, work to identify novel antigenic markers for use in a standalone format, or to add to existing biomarker combinations would be desirable. Avenues of investigation should also consider 'host' markers, such as cytokines, chemokines or other immune-proteins, that may be indicative of aspects of disease. For example, a study measuring a panel of 25 inflammation associated proteins by SBA identified distinct profiles associated with sepsis, febrile malaria and febrile controls, with the chemokine CCL5 showing an AUC value of 0.96 ($p<0.0001$) differentiating between malaria and sepsis [42]. Another potentially advantageous development would be an ability to quantify levels of antigen within the lateral flow format. This is an approach already in the early stages of application for other diseases, such as a lateral flow device detecting prostate-specific antigen in prostate cancer in which the intensity of the test line (and so, by proxy, the amount of antigen present) can be digitally quantified by a portable reader [43].

An ability to rapidly quantify biomarkers at point of care could be beneficial from both a diagnostic, clinical outcome and epidemiological perspective; monitoring levels of HRP-2 after treatment (as in chapter 6) using a cheap and rapid test may help to identify instances of failure of treatment or reinfection. The potential impact of monitoring of biomarker levels in this way is strengthened by models able to predict the rate of clearance of antigens such as HRP-2, and the likely duration of RDT positivity after treatment, which can also provide data to help direct RDT use, particularly in low transmission settings [44]. As test sensitivities for specific antigens increases, it opens up the possibility of utilising antigen kinetics to inform on metrics such as time since infection or asexual parasite density. Developing this idea further, it may also be possible to measure antibody levels, allowing for the determination of seropositivity or negativity using a similar device format, thus facilitating a regular monitoring of immunity or exposure indicators at a population level without the need for large scale serological studies.

A significant issue facing current RDTs is the emergence of HRP-2 negative parasites across South America, Africa and Asia [45]. The increasingly widespread use of RDTs in malaria endemic areas is liable to act as a positive selection pressure on parasite strains that do not produce HRP-2 which will likely undermine the efficacy of front line RDT use [46]. Evidence suggests that other biomarkers such as pLDH may be able to bridge gaps in the detection of HRP-2 deleted parasites, but sensitivity falls at lower parasite densities [47] — a significant shortcoming

in that estimates suggest 20-50% of all human to mosquito transmission is caused by submicroscopic carriers when overall transmission falls to very low levels [48]. Accordingly, the more frequent monitoring of HRP-2 in patients with a confirmed parasitological diagnosis may assist in the identification and mapping of HRP-2 negative parasite populations, although this will of course need to take place in the context of active detection, as HRP-2 negative infections are inherently likely to be missed by RDT screening alone. Furthermore, the occurrence of such parasites warrants the identification of new biomarkers for use on RDTs — the ideal candidate being one that is essential for parasite survival to negate the risk of further deletion mutations. In principle, this is a good case for the type of research pipeline that this thesis describes.

7.6 Concluding remarks

As this thesis aims to demonstrate, serological methodologies have a range of use cases and roles to play in malaria surveillance, epidemiology and in elimination efforts. The identification of new biomarkers — whether parasite derived, antibody, or other host derived molecules — for use in these contexts will be important in developing new tools to address the short comings and knowledge gaps in current approaches.

Arguably, of particular importance is an ability to more closely measure exposure and transmission at a population level. Identifying markers able to elucidate time since last infection, or the transmission reservoir, for example, would provide the basis for improved targeting of intervention measures, especially where transmission has already declined and parasite prevalence may be low. Indeed, regular serological monitoring may be necessary to identify specific populations or geographical regions that drive remaining transmission (*e.g.* increased levels of immunity to markers suggestive of recent or frequent infection) or have become susceptible to reinfection (*e.g.* falling levels of protective immunity).

New techniques and platforms will likely play a role in the identification and application of new biomarkers. For example, biolayer inferometry — whereby antibody-antigen interactions are detected on the end of fibre-optic biosensors — provide rapid results (approximately 20 minutes) with reportedly high levels of sensitivity and an ability to multiplex analytes [49]. Evolutions in SBA platforms have also facilitated multiplex analyte measurements, such as the simultaneous detection of IgG and IgM to a panel of antigens coupled to microsphere beads [50].

Moving forward, research pipeline approaches in serology and biomarker discovery must be further refined to reflect the rapid change in our understanding of parasite-human interactions at a physiological and immunological level. An awareness of how different methodologies may be best suited to specific applications will help to clarify outstanding biological questions and contradictions in terms of measuring important metrics of disease (**Figure 7.1**).

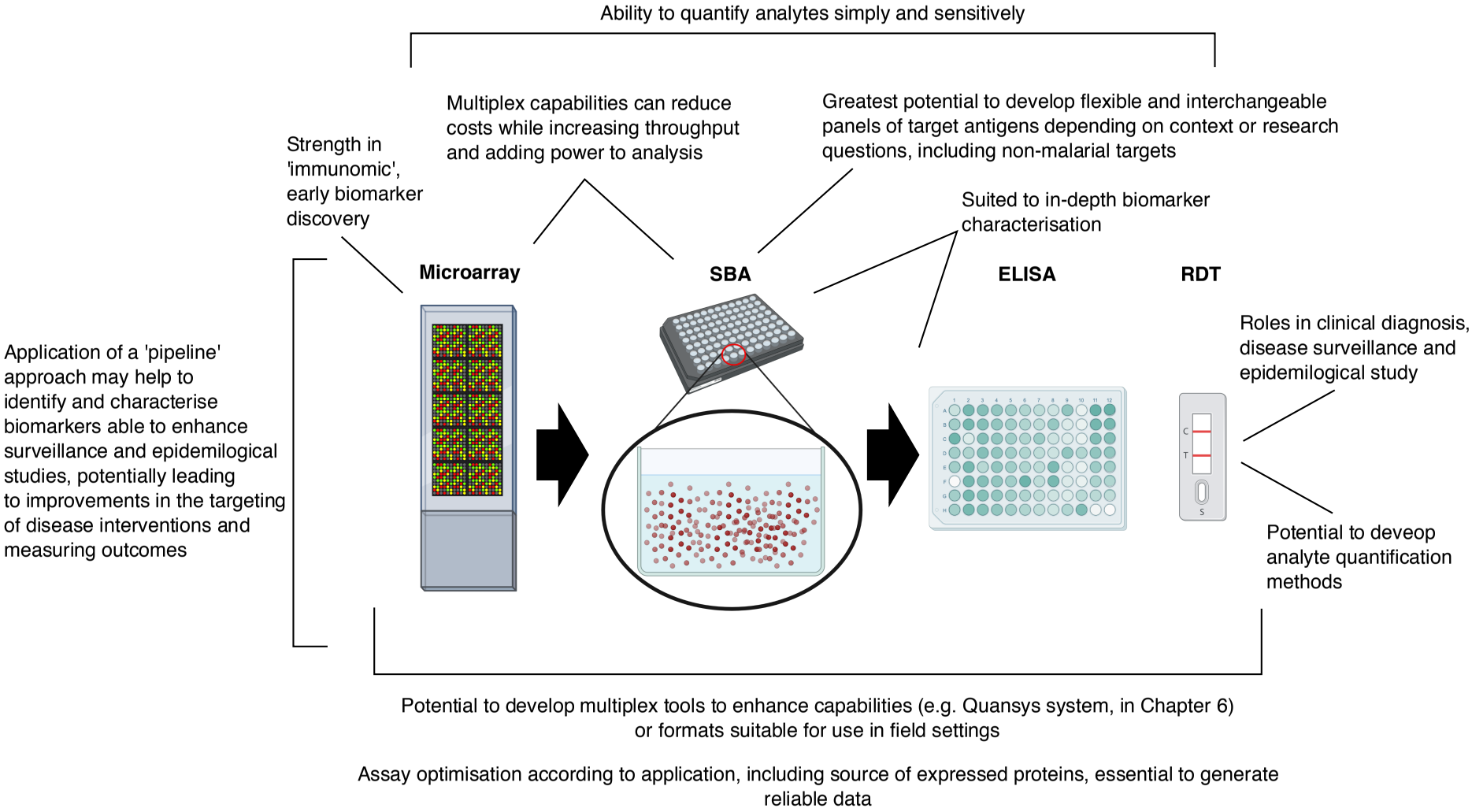


Figure 7.1. Key summary points surrounding assay application and development. Created with BioRender.com.

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