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ANTIBODY RESPONSES TO *Plasmodium falciparum* AS MARKERS OF EXPOSURE AND TOOLS TO MONITOR MALARIA TRANSMISSION

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Antibody responses to *Plasmodium falciparum* as markers of exposure and tools to monitor malaria transmission

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Diana, Lars, and Ingrid

ABSTRACT

Plasmodium falciparum malaria has the highest death toll of all human parasitic diseases and nearly half of the global population is living at risk of infection. Reducing the malaria burden with the goal of achieving elimination will require sustained commitment for control and better monitoring tools that can guide efforts to limit transmission. An effective malaria vaccine could significantly accelerate progress towards elimination but incomplete understanding of malaria immunity hampers vaccine development. Antibodies are key components of immunity to malaria and can also serve as sensitive markers of exposure. Data on the dynamics and specificity of the antibody response in natural *P. falciparum* infection could improve our understanding of the acquisition and maintenance of immunity, and be used to develop better serological tools for transmission surveillance.

In study **I**, we examined the temporal trends in malaria transmission over a period of 25 years in a closely monitored population in a rural area in Tanzania. We detected a gradual reduction in parasite prevalence starting prior to large-scale interventions and found that evaluation of spleen rate and haemoglobin levels were complementary to microscopy and molecular methods for estimates of malaria burden in this area of initially very high transmission. In study **II**, we developed new models for serological surveillance of malaria transmission based on cross-sectional data on age-specific antibody levels and evaluated their performance by further examining the transmission trends observed in study **I**. We demonstrated that these models are robust and improve precision in serological transmission estimates based on cross-sectional antibody data. In study **III**, we conducted a longitudinal follow-up of travellers treated for malaria in Sweden. We provided quantitative estimates of the dynamics and the longevity of malaria-specific antibodies and antibody secreting cells in absence of re-exposure. In study **IV**, we examined the antibody responses to 111 *P. falciparum* antigens in the longitudinally followed travellers and identified novel candidate serological markers of recent exposure that warrant further evaluation. Together these studies contribute to our overall understanding of the acquisition and maintenance of the antimalarial antibody response. The results help to improve current methods for serological malaria transmission surveillance and provide new information on antibody responses to *P. falciparum* that should be explored as markers of exposure.

LIST OF SCIENTIFIC PUBLICATIONS

The thesis is based on the following papers which will be referred to in the text by their corresponding roman numerals:

I. Färnert A, Yman V, Vafa Homann M, Wandell G, Mhoja L, Johansson M, Jesaja S, Sandlund J, Tanabe K, Hammar U, Botai M, Premji Z G, Björkman A, Rooth I
Epidemiology of malaria in a village in the Rufiji River Delta, Tanzania: declining transmission over 25 years revealed by different parasitological metrics
Malaria Journal, 2014, 13, 459

II. Yman V, White MT, Rono J, Arcà B, Osier F H A, Troye-Blomberg M, Boström S, Ronca R, Rooth I, Färnert A
Antibody acquisition models: A new tool for serological surveillance of malaria transmission intensity
Scientific Reports, 2016, 6, 19472

III. Yman V, White M T, Asghar M, Sundling C, Sondén K, Draper S J, Osier F H A, Färnert A
Dynamics of antibody responses to Plasmodium falciparum merozoite antigens after a single infection: Longevity explained by previous exposure and antibody secreting cell profiles
Manuscript, Submitted

IV. Yman V, Tuju J, White M T, Kamuyu G, Mwai K, Kibinge N, Asghar M, Sundling C, Sondén K, Bottai M, Murungi L, Kiboi D, Kimathi R, Chege T, Chepsat E, Kiyuka P, Nyamako L, Osier F H A, Färnert A
Serological signatures of recent and cumulative exposure to Plasmodium falciparum infection
Manuscript

The following papers and manuscripts were authored or co-authored during the course of PhD education but are outside the scope of this thesis:

1. Homann MV, Noushin Emami S, Yman V, Stenström C, Sondén K, Ramström H, Karlsson M, Asghar M, Färnert A
   Detection of malaria parasites after treatment in travellers: A 12-months longitudinal study and statistical modelling analysis
   EBioMedicine, 2017, 25:66-72

2. Asghar M, Yman V, Homann MV, Sondén K, Hammar U, Hasselquist D, Färnert A
   Cellular ageing dynamics after acute malaria infection: A 12 months longitudinal study
   Aging Cell, 2018. 12702

3. Parigi SM, Czarnewki P, Das S, Steeg C, Brockman L, Fernandez-Gaitero S, Yman V, Forkel M, Höög C, Mjösberg J, Westerberg L, Färnert A, Huber S, Jacobs T, Villablanca EJ
   Flt3 ligand expands in bona fide innate lymphoid cell precursors in vivo
   Scientific Reports, 2018, 8, 18283

4. Yman V, Wandell G, Mutemi D, Hammar U, Miglar A, Asghar A, Karolsson M, Lind I, Nordfjell C, Rooth I, Ngsala B, Homann MV, Färnert A
   Persistent transmission of Plasmodium ovale and *Plasmoidum malariae in an area of declining Plasmodium falciparum transmission*
   Manuscript

5. Eldh M, Felger I, Hammar U, Arnot D, Beck HP, Liljander A, Mercereau-Puijalon O, Migot-Nabias C, Mueller I, Ntoumi F, Ross A, Smith T, Sondén K, Yman V, Färnert A
   Number of clones in asymptomatic Plasmodium falciparum infections and risk of clinical malaria: A systematic review and pooled analysis of individual participant data
   Manuscript

6. Sundling C, Rönnberg C, Yman V, Jahnmatz P, Achour A, Tadepally L, Sondén K, Asghar M, Persson K, Brodin P, Färnert A
   B cell population dynamics in patients with malaria reveals enhanced expansion of CD11c expressing B cells in previously exposed individuals
   Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ACT	Artemisinin based combination therapy
AIC	Akaike information criterion
AMA	Apical membrane antigen
ASC	Antibody secreting cell
AUC	Area under the ROC curve
CHMI	Controlled human malaria infection
CI	Confidence interval
CrI	Credible interval
CSP	Circumsporozoite protein
EBA	Erythrocyte binding antigen
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GAMA	GPI-anchored micronemal antigen
GPI	Glycosylphosphatidylinositol
gSG6	*Anopheles gambiae* salivary gland protein 6
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITN	Insecticide treated nets
MFI	Median fluorescent intensity
MSP	Merozoite surface protein
OD	Optical density
OR	Odds ratio
PCR	Polymerase chain reaction
*Pf*EMP-1	*Plasmodium falciparum* erythrocyte membrane antigen 1
*Pf*SEA-1	*Plasmodium falciparum* schizont egress antigen 1
RH	Reticulocyte binding protein homologue
ROC	Receiver operating characteristic

RON	Rhoptry neck proteins
SCR	Seroconversion rate
SD	Standard deviation
TNF	Tumour necrosis factor
TTd	Tetanus toxoid

1 INTRODUCTION

1.1 MALARIA

Malaria is a mosquito-borne potentially life-threatening disease caused by protozoan parasites of the genus *Plasmodium* (1). The main six species that cause disease in humans are *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* spp. (*P. ovale curtisi* and *P. ovale wallikeri*), and *P. knowlesi* (2). *Plasmodium knowlesi* is a monkey malaria parasite of Southeast Asian macaques that is able to infect humans (3). The burden of malaria falls predominantly on sub-Saharan Africa where more than 90 per cent of the 216 million cases (95% confidence interval [CI]: 196–263) occurred in 2016 (1). This thesis focuses on *P. falciparum*, which is estimated to be responsible for more than 90 per cent of all malaria related morbidity and mortality (Figure 1) (4).
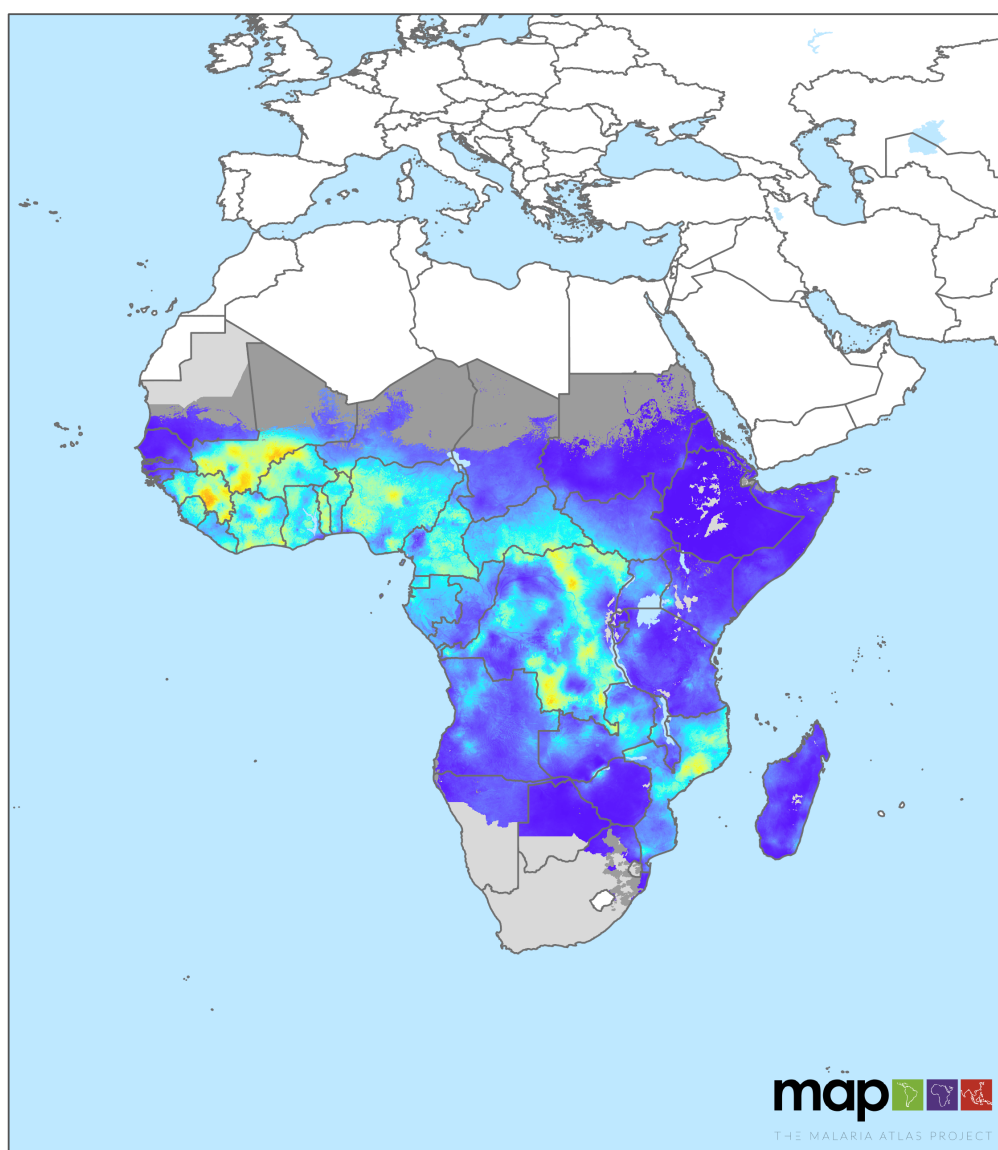

Figure 1. Spatial distribution of *P. falciparum* infection prevalence in African children (age 2-10) in 2015.
(Malaria Atlas Project (5), available from: https://map.ox.ac.uk, reproduced with permission)**.**

1.1.1 The parasite and its life cycle

The *P. falciparum* parasite is a complex organism with a large genome, containing approximately 5300 genes distributed across fourteen chromosomes. The parasite requires two fundamentally different hosts to complete its life cycle. The female *Anopheles* mosquito is the disease vector and the definitive host, in which the parasite reproduces sexually, and the human is the intermediate host where asexual reproduction occurs (Figure 2) (6).
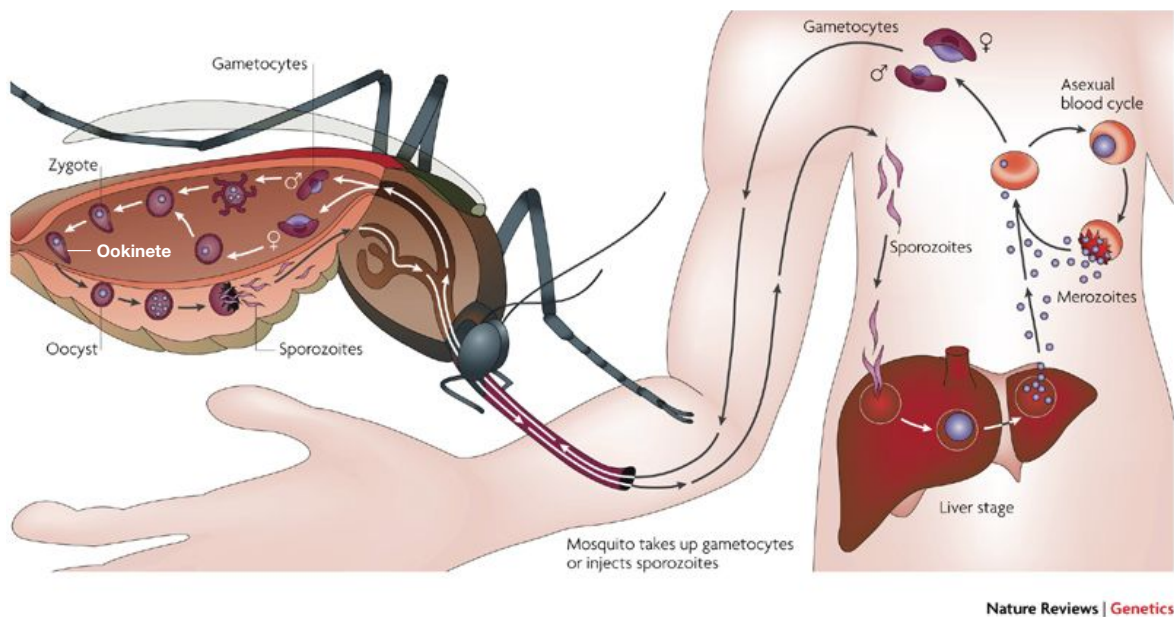

Figure 2. The life cycle *Plamsodium falciparum* (Su et al. 2007 (7), reproduced with permission from Nature publishing group).

Infection in the human host is initiated when a female *Anopheles* mosquito injects saliva containing *P. falciparum* sporozoites into the skin of the human during a blood meal (6). The motile sporozoites migrate to the liver where proteins on the surface of the sporozoite, e.g. circumsporozoite protein (CSP), mediate binding and invasion of liver cells (8). Following liver cell invasion the parasite undergoes asexual development and replication over a period of approximately 5-15 days. This process generates up to 40,000 parasite daughter cells, so called merozoites (9). This part of the life cycle, which is clinically silent, is referred to as the pre-erythrocytic stage of infection. At the end of this stage, a merosome, i.e. a form of parasite filled vesicle, bud off from the infected liver cell and transports the merozoites to the blood stream (10). Here, the merosome ruptures and the merozoites are released (11). The blood stage of infection is subsequently initiated when a merozoite invades an erythrocyte through a rapid but complex process (12,13). There is a high degree of redundancy in the mechanisms by which the merozoite can invade the erythrocyte and the parasite may utilise alternative pathways. Briefly, the process begins with an initial contact between the merozoite and the erythrocyte, in which merozoite surface proteins (MSP) are likely to play an important role (13). The remainder of the process requires regulated secretion of proteins to the merozoite surface from specialised apically located secretory organelles (14).

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After an apical re-orientation of the merozoite, a tight irreversible attachment is formed to the erythrocyte with the involvement of two parasite protein families, the erythrocyte binding antigens (EBA) and the reticulocyte binding protein homologues (RH). The interaction between RH5 and basigin on the erythrocyte surface has been demonstrated to be essential for erythrocyte invasion (15). The reorientation and attachment is followed by the formation of a moving junction, in which apical membrane antigen 1 (AMA-1) interact with rhoptry neck proteins (RON), thereby mediating entry of the parasite into the erythrocyte (16,17). Within the erythrocyte the parasite undergoes asexual development and replication during a period of approximately 48 hours after which the erythrocyte ruptures, releasing around 20 daughter merozoites. These merozoites go on to invade new erythrocytes, thereby establishing a cyclic blood-stage infection where parasite numbers increase exponentially with the completion of each cycle (18). A small proportion of merozoites are triggered to undergo sexual development after erythroycte invasion (6). This leads to the formation of male and female gametocytes that can be ingested by blood feeding *Anopheles* mosquitos. Within the midgut of the mosquito, the haploid male and female gametocytes develop into gametes and fuse to form a diploid zygote that further differentiates into a motile ookinete (6). The ookinete traverses the mosquito midgut wall, forming an oocyst within which haploid sporozoites develop. The oocyst ruptures, releasing new sporozoites that migrate to the mosquito salivary gland from where they can be transmitted to a human host (19).

1.1.2 Pathogenesis

Symptoms of malaria occur only during the blood stage of the infection (20,21). A key component in the pathogenesis during *P. falciparum* infection is the ability of the parasite to express its own proteins on the surface of the infected erythrocyte (22). These proteins are the so called variant surface antigens, among which *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1) is the most well described (23). Towards the end of each asexual blood stage cycle, these proteins, particularly *Pf*EMP-1, mediate the binding of the infected erythrocytes to deep vasculature endothelial cells (sequestration) and to uninfected erythrocytes (rosetting). Thereby the infected erythrocytes avoid entering the spleen where they would otherwise be destroyed (24,25). However, sequestration within the deep vasculature of the brain, heart, liver, lungs, kidney, subcutaneous tissues, and during pregnancy within the placenta, leads to microvasculature obstructions and consequently to reduced tissue blood flow and oxygen delivery causing local inflammation and tissue damage (26,27). During the blood stage of infection a strong systemic inflammatory response, similar to that observed in bacterial sepsis, is also induced (28). Multiple parasite pathogen-associated molecular pattern molecules trigger inflammation through pattern recognition receptors, e.g. toll-like receptors, on a wide range of innate immune cells (29–32). This early immune activation causes a massive production and release of pro-inflammatory cytokines, e.g. interleukin (IL) IL-1β, IL-6, IL-8, IL-12, interferon-γ (IFN-γ), and tumour necrosis factor-α (TNF-α) (33–35). Furthermore, both parasite erythrocyte destruction and inflammation-induced suppression of erythropoiesis contribute to the development of malarial anaemia (36).

1.1.3 Clinical presentation and treatment

Fever is perhaps the most classical symptom of malaria, but the clinical presentation of *P. falciparum* infection may range from severe life-threatening syndromes to asymptomatic carriage of infection. Disease severity depends on a large number of host and pathogen factors, e.g. prior immunity, age, genetic resistance, parasite virulence, duration of infection etc. (37,38). In non-immune individuals infection with *P. falciparum* is almost always symptomatic. Patients often present with intermittent fever and non-specific symptoms, e.g. headaches, malaise, abdominal pain, muscle aches and diarrhoea (39). In absence of prompt diagnosis and treatment the disease can progress to a severe and complex life-threatening syndrome affecting multiple organ systems. Children, pregnant women, and individuals with immunosuppression or chronic diseases (e.g. diabetes) are at highest risk of severe disease (12,40). The clinical signs and symptoms of severe disease include (but are not limited to) prostration, impaired consciousness and coma, convulsions, shock, hypovolaemia, tachypnoea and respiratory distress, hypoxia, hypoglycaemia, jaundice and hemoglobinuria (41). Particularly in children, severe malaria can be categorised into three main syndromes, i) cerebral malaria, of which the hallmark symptoms are unrousable coma and multiple convulsions, ii) severe malarial anaemia (haemoglobin levels <5 g/dL), and iii) metabolic acidosis (41,42). In uncomplicated (i.e. non-severe) malaria the recommended first line treatment consists of an artemisinin based combination therapy (ACT). In addition to a full course of ACT, severe cases should also be treated with intravenous injections of artesunate (39).

1.2 ACQUIRED IMMUNITY TO MALARIA

In areas where *P. falciparum* transmission is high or moderate, disease incidence is highly age-dependent. Severe and life-threatening disease occurs primarily in children less than five years of age. Incidence of clinical malaria declines gradually with age, reflecting the gradual acquisition of immunity (Figure 3) (43). Immunity to clinical malaria is acquired slowly with repeated infections and requires continuous exposure in order to be maintained (Reviewed in: Marsh 2006, Langhorne 2008 and Crompton 2014) (20,44,45). In high transmission areas immunity to the most severe forms of disease is acquired roughly within the first five years of life. Individuals remain susceptible to less severe forms of febrile malaria until late childhood or early adolescence. Thereafter they acquire the ability to control also the milder forms of the disease and therefore seldom experience symptoms of clinical malaria. However, complete resistance to infection is rarely if ever achieved, and also in high endemic areas asymptomatic blood-stage infections remain common in individuals of all ages (46).
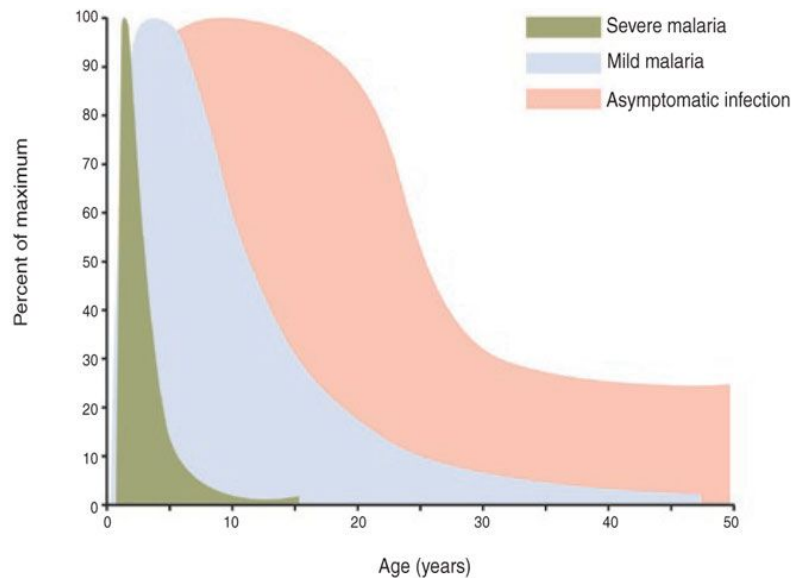
Figure 3. Schematic representation of the relative age-specific incidence of severe malaria, mild malaria and asymptomatic *P. falciparum* infection in individuals living in a malaria endemic area. (Langhorne et al. 2008 (45), reproduced with permission from Nature Publishing Group).

1.2.1 Antibodies and their role in protection against clinical malaria

Antibodies are critical components of the naturally acquired protective immune response to malaria and are particularly important during the blood stage of the infection. This was demonstrated already in the 1960's through the seminal work of Cohen et al. in which transfer of immunoglobulin G (IgG) from highly immune Gambian adults to children with acute clinical malaria was shown to limit disease severity and lead to rapid parasite clearance and resolution of symptoms (47). Important targets for immunity appear to include antigens on both the surface of the merozoite and on the infected erythrocyte (48,49). Parasite specific antibodies are likely to mediate protection through multiple mechanisms. For example, binding of antibodies to merozoite surface and secreted proteins may block erythrocyte invasion pathways and merozoite growth (50), inhibit parasite egress from schizonts (51), promote merozoite lysis due to complement activation (52), and stimulate phagocytosis and neutrophil respiratory burst attack through opsonisation (53,54). Furthermore, antibody binding of parasite proteins on the surface of infected erythrocyte may prevent sequestration, stimulate phagocytosis of infected erythrocytes as well as mediate agglutination of infected erythrocytes, thereby improving splenic clearance (55,56).

High titre antibody response to a large number of parasite antigens (including MSPs, and members of the EBA and RH protein families) have been correlated with clinical protection in cohort studies (48,57,58). However, discriminating responses that reflect a high degree of prior exposure from those actually mediating protection have proven difficult and robust serological correlates of protection are currently lacking. Identifying such correlates would require a detailed characterisation of the functional basis, as well as the target epitopes, of any protective effect associated with the magnitude of an antigen-specific antibody response (53,54,59,60).

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1.2.2 Acquisition and maintenance of antibody mediated immunity

In general terms, antibody mediated immunity to malaria requires the acquisition of sufficient levels of high-affinity inhibiting antibodies that are maintained over time. Circulating antibodies are produced by antibody secreting cells (ASCs), which are terminally differentiated cells of B-cell lineage, specialised in antibody secretion. The ASCs can be divided into two distinct categories i) short-lived ASCs (short-lived plasma cells/plasma blasts) and ii) long-lived ASCs (long-lived plasma cells) (61–63). Although, the short-lived ASCs are responsible for the bulk of the antibody secretion during and shortly after infection, the longevity of an antibody response is primarily determined by the generation and survival of long-lived ASCs (63–65). These long-lived ASCs reside in the bone marrow or secondary lymphoid organs where they continue to secrete antibodies for the duration of their life (64). Because of the physical niches they occupy, the long-lived ASCs are difficult to study. However, the numbers of ASCs in the bone marrow are highly correlated with the levels of circulating antibodies (66,67), and modelling of longitudinal antibody data using an antibody dynamics model has been used to estimate the dynamics of antibodies and both short- and long-lived ASCs (68). Memory B-cells are also important for the antibody-mediated immunity and respond rapidly to new infections by efficiently proliferating and differentiating in to ASCs (69–71). It has been demonstrated that malaria-specific memory B-cells can be maintained independently of antibody responses for several years in the absence of re-exposure (72,73).

For many viral and bacterial infections, protective and long-lived antibody responses are acquired after a single exposure (74). In contrast, the acquisition of antibody mediated immunity to malaria requires multiple repeated infections (20,44). Although protective antibodies are acquired with time, the process is remarkably slow (47,75,76) and the antibody response appears to be comparatively short-lived, particularly in children (68,77,78). The reasons for the slow acquisition of immunity remain incompletely resolved but several mechanisms have been proposed. Given the redundancy in host-cell invasion pathways (13), the high degree of antigen polymorphisms (79), and the clonal variation in erythrocyte surface antigens (23), it has been suggested that a large number of genetically diverse infections would be required to induce an antibody response with sufficiently broad specificity to provide protection (20). However, there is growing evidence that the immune environment induced during acute *P. falciparum* may be sub-optimal for the induction of a high quality and long-lived immune response. Impaired T-cell help and germinal centre formation (80,81) lead to a dysregulation of the B-cell response, with preferential induction of short-lived ASCs and the generation of so called atypical memory B-cells hampering development of both long-lived ASCs and immune memory (82–84).

Although, the antimalarial antibody response is often considered short-lived, the dynamics of antibodies and ASCs in response to infection remain poorly described and only a few studies have provided quantitative estimates on the longevity of antibodies (77,78) or ASCs (68).

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The dynamics of the response is likely to be antigen-specific and, in addition to prior exposure, also depends on a large number of individual-specific immune factors that govern the response longevity (85). A detailed characterisation of the antigen-specific dynamics of antibodies and ASCs, and of the individual and exposure related factors that determine the longevity of the response, could greatly improve our understanding of the acquisition and maintenance of antibody mediated immunity (75,86).

1.2.3 Malaria vaccines

The fact that functional and protective antibody responses are acquired following both natural and experimental infections has inspired efforts to develop a highly effective malaria vaccine (47,87,88). Despite its sophisticated immune evasion mechanisms, the parasite exposes large numbers both polymorphic and conserved proteins to the immune system (57). As described above, many of these proteins are involved in processes that are essential for the survival of the parasite, e.g. host-cell binding and invasion, and either have been or could be considered as candidate antigens for a malaria vaccine (88,89). Despite years of research, a highly protective vaccine is yet to be developed. Several approaches and potential vaccines based on different stages of the parasites life cycle are currently being explored. Among these there are i) pre-erythrocytic vaccines that aim to protect humans from acquiring infections all together, ii) blood-stage vaccines that aim to limit parasite replication, facilitate clearance and reduce the severity of disease manifestations, and iii) so called transmission blocking vaccines, targeting the sexual stages of the parasite, with the aim to prevent transmission back to mosquitoes (90,91).

A limited number of candidate vaccines representing each of these stages are currently undergoing clinical trials (92). Pre-erythrocytic vaccines in clinical trials are either whole sporozoite vaccines or subunit vaccines. The whole sporozoite vaccines consist of either live attenuated sporozoites or live sporozoites given under chemoprophylactic treatment (93–95). The most advanced malaria vaccine to date, RTS,S is a subnunit vaccine based on the sporozoite protein CSP. Despite a limited efficacy, pilot implementation of the RTS,S will be initiated in three African countries during 2018 (96,97). Several blood-stage antigens (e.g. MSP-1, MSP-2, MSP-3, and glutamate rich protein) have previously been evaluated as candidate subunit blood stage vaccines but with limited success (88). Currently clinical trials are ongoing for RH5, which is a leading blood stage vaccine candidate, and AMA-1 as well as for *Pf*EMP-1_VAR2CSA, a vaccine candidate specifically aimed at preventing pregnancy-associated malaria (92,98,99). The transmission blocking subunit vaccines currently being evaluated are based on sexual stage antigens *Pf*S25 or *Pf*S230 (92,100). Malaria vaccine responses in many cases appear to decay rapidly over time. For RTS,S, vaccine efficacy waned rapidly as antibody levels declined (97). Vaccine development would be greatly accelerated by a better understanding of the dynamics of the immune response, including a better understanding of the factors that determine the response longevity (85,86,101).

1.3 EPIDEMIOLOGY AND CONTROL

A highly effective malaria vaccine would greatly improve the prospects for control and eventual elimination of malaria (102,103). In absence of such a vaccine, the cornerstones of malaria control are still i) vector control, ii) prevention of disease in "at-risk" populations through chemoprophylaxis, iii) rapid and accurate diagnosis and treatment of malaria, and iv) sustained malaria surveillance (5).

Distribution of insecticide treated bed nets (ITNs) is the most widely adopted strategy for vector control and is highly accepted as ITNs also provide personal protection for the individuals using them (104). Other vector control strategies include indoor residual spraying (IRS) with insecticides and the use of larvicides to kill mosquito larvae and the pupae (105). Protection of at-risk individuals through chemoprophylaxis has previously mainly been practiced in the form of intermittent preventive treatment during pregnancy (IPTp) to reduce placental malaria, anaemia, neonatal mortality and low birth weight (106,107). However, since 2012, seasonal malaria chemoprevention has also been recommended for children aged 3-59 months living in areas of highly seasonal malaria transmission (1). Malaria rapid diagnostic tests (RDTs) have been implemented to improve access to accurate malaria diagnostics and furthermore, the prompt use of ACTs for treatment has been widely promoted (39,108).

Since year 2000 there has been a substantial reduction in the global malaria burden with an estimated reduction of more than 50 per cent in both prevalence of infection and clinical malaria incidence (1,5,109). It has been estimated that the global scale-up in malaria control activities have contributed to averting 663 million cases (credible interval [CrI]: 542– 753) of malaria between 2000 and 2015. The greatest effect in reducing transmission has been attributed to the use of ITNs which have been estimated to account for 68 per cent (CrI: 62-72%) of the reduction (5). Due to the overall reduction in transmission, several countries are currently approaching a state where malaria elimination appears feasible (110,111). However, despite the overall encouraging reduction in disease transmission, not all countries experience the same positive trends. Fifteen countries are currently estimated to account for approximately 80 per cent of all malaria cases globally, with Nigeria and the Democratic Republic of Congo accounting for 27 and 10 per cent of all cases, respectively (1). Furthermore, there are worrying reports of a widespread mosquito-resistance to the pyrethroid-containing insecticides that are used on ITNs and for IRS (112), as well as of the emergence of parasites resistant to ACT in Southeast Asia (113).

1.4 MONITORING MALARIA TRANSMISSION INTENSITY

In order to further reduce the global malaria burden and actually achieve regional elimination, sustained high quality transmission monitoring and disease surveillance are imperative (114–116). Due to the complex transmission cycle of *P. falciparum,* involving both the human and the mosquito, actually quantifying the intensity of transmission is far from trivial. Various surrogate metrics have traditionally been used to estimate the transmission intensity of *P. falciparum* (Reviewed in Tusting et al. 2014) (117). Among others, these metrics include evaluation of spleen rate and parasite prevalence in children (2–10 years old), estimates of malaria disease incidence, entomological inoculation rates (EIR), and the force of infection (FOI). All of these metrics are subject to varying degrees of uncertainty specifically related to the data on which they are based.

Evaluation of the spleen rate (i.e. the proportion of a population with palpable spleen enlargement) was the first method established for estimating the burden of malaria transmission (118,119). However, the spleen rate is not an entirely malaria-specific metric and has largely been replaced by more specific methods, e.g. evaluation of parasite prevalence, which has become the most frequently collected transmission metric (120). In areas where malaria is endemic, both the spleen rate and parasite prevalence have traditionally been used to categorise the intensity of transmission into four endemicity-levels: holoendemic >75%, hyperendemic 51–75%, mesoendemic 11–50%, and hypoendemic <10% (121).

Although useful and well established, the evaluation of parasite prevalence has several limitations with regards to quantifying the intensity of transmission. The prevalence of infection tends to saturate when transmission intensity is high, partly due to acquired immunity within the population and heterogeneous biting by the mosquito vectors (122). In high transmission settings parasite prevalence therefore underestimates the intensity of transmission and has limited possibility to detect transmission changes (117). Parasite densities also vary substantially during the course of an infection (e.g. due to sequestration, the age of the infection, the individual level of acquired immunity) and may fluctuate between undetectable and detectable levels. Samples collected at a single time-point may therefore underestimate the level of transmission in the population (123,124). Furthermore, microscopy, which has traditionally been the standard method to evaluate parasite prevalence, lacks the sensitivity to detect very low-density infections. These so called sub-microscopic infections can be detected with more sensitive molecular methods, e.g. polymerase chain reaction (PCR), and such methods may be a valuable alternative for evaluation of parasite prevalence in particular in low transmission settings (125). However, when transmission is low and the true prevalence of infection is less than approximately five per cent, the utility of prevalence surveys is limited due to the difficulty in sampling sufficient numbers of parasite positive individuals to obtain reliable estimates of the parasite prevalence (123,125).

Estimates of malaria disease incidence provide a more direct indicator of transmission burden and are more sensitive than parasite prevalence when transmission is low (126). Such estimates are often also easier to obtain as they can be based on passively collected data from hospital clinical records reported through healthcare surveillance systems. However, data on clinical incidence are often unreliable because there tends to be a substantial overdiagnosis of clinical cases at the health facilities while cases occurring in the community are missed due to low care-seeking rates. Furthermore, in many malaria endemic countries there is a large discrepancy between the number of cases diagnosed at health facilities and the number of cases actually reported to surveillance system (1,4,5,43).

The collection of entomological parameters such as the EIR, i.e. the average number of infectious mosquito bites received per individual and year, provides a way to directly measure the intensity of transmission. The EIR has therefore often been considered the gold-standard method for estimating malaria transmission intensity (119). However, data on EIR, traditionally based on human landing catches, are notoriously difficult, expensive, and labour intensive to collect (127). Furthermore they are often imprecise, particularly in low transmission settings, because of heterogeneity in both malaria transmission and vector distribution (128–130).

For many infectious diseases, the most direct measure of transmission intensity is often the force of infection, i.e. the rate at which susceptible individuals become infected (131). However, for a disease such as malaria where there is a long latency period between infection and onset of symptoms and where both asymptomatic and multiclonal infections are common, estimating the force of infection is difficult (132). Obtaining reliable estimates of the force of infection of *P. falciparum* requires both high-frequency active longitudinal sampling and the use of sensitive molecular techniques that can distinguish super-infections from old infections (131,133). Even if this can be achieved, the highest intensity that can be accurately estimated will be determined by how often the samples are collected.
As for the other metrics discussed above, estimates of the force of infection will also be affected by the sensitivity of the parasite detection method and by fluctuations in parasite densities during the course of infection (117).

1.5 SEROLOGY AS AN EPIDEMIOLOGICAL TOOL

As previously described antibodies are key components of the effective antimalarial immune response. However, given that antibodies are maintained beyond the length of an actual infection, they may serve as a serological footprint of prior infections (134). Serology has become an increasingly important tool for the surveillance of a wide range of infectious diseases in both humans and animals and can provide alternative means of estimating the force of infection (135). In the context of malaria, not only the parasite proteins, but also proteins in mosquito saliva that are injected into the skin during mosquito blood-feeding induce strong antibody responses (136–138). Parasite and mosquito serology can therefore be used to evaluate malaria and vector exposure, respectively.

A serological sample taken at a certain point in time provides information on whether the individual has been exposed prior to that time-point. Antibodies in plasma are generally measured in a semi-quantitative fashion. However, in seroepidemiological studies, the quantitative measurement is often dichotomised by comparing it to a predetermined threshold in order to define whether the individual has been previously exposed or not (i.e. is seropositive or seronegative) (135). If a disease is endemic, knowledge about the rate at which individuals in a cohort pass from seronegative to seropositive, i.e. the rate of seroconversion (SCR), provides information on the force of infection. In theory this could be accurately estimated from a longitudinal study in which a population is followed from birth and the rate at which people get infected and thus seroconvert is identified (139).

For example, let us consider that a cohort of malaria naïve children who live in an area where the disease is endemic (technically at endemic equilibrium) is sampled at birth for the measurement of malaria specific antibodies. For the sake of simplicity we assume that they have no maternal antibodies and that they are therefore all seronegative. During their first year of life a proportion of the children in the cohort will have malaria. If we conduct a serological follow-up study in the same children after a year, we will find that a proportion of the children are now seropositive as a reflection of having been infected during their first year of life. If we conduct a new follow-up study after another year, an additional number of children will have acquired the infection and therefore seroconverted. If the duration of seropositivity is sufficiently long, the proportion of seropositive children will increase with time as a consequence of exposure.

Longitudinal serological cohort studies are often difficult or even impossible to conduct due to time and resource constraints, however cross-sectional serological population surveys may provide much similar information and are therefore commonly used as an alternative way to study the epidemiology infectious diseases (135,140,141). The conceptual similiarities between a longitudinal serological study following a cohort from birth and cross-sectional serological population survey are illustrated in Figure 4.
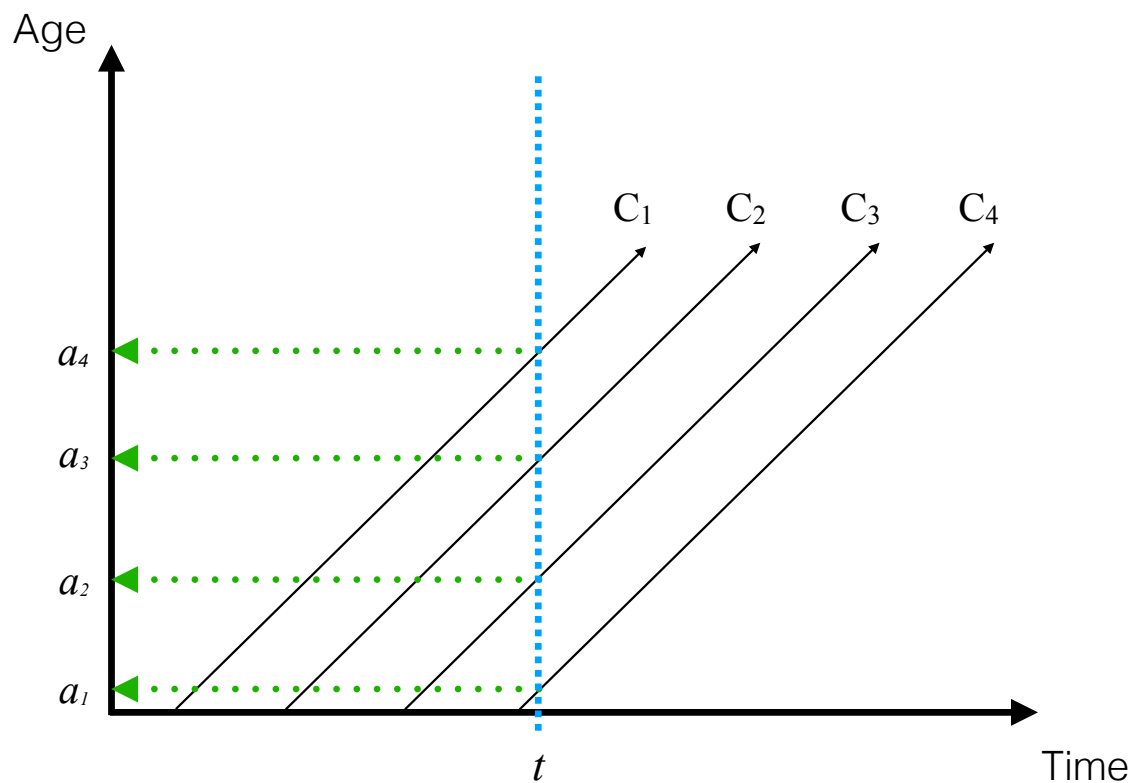
Figure 4. Schematic illustration of conceptual differences and similarities in data from longitudinal serological cohort studies and cross-sectional serological population surveys. (Adapted from Hens et al. 2012 (135), reproduced with permission from Springer Science + Business Media)

As illustrated in Figure 4, a population can be thought of as consisting of multiple cohorts (C_1-C_4) of individuals born at different points in time. As time passes the individuals within each of these cohorts will become older, as illustrated by the solid diagonal lines. In a longitudinal serological study we would follow one of the cohorts, e.g C_1, from birth (i.e. along the diagonal line) and examine the rate at which individuals seroconvert as a consequence of exposure. However, a cross-sectional sample of the population at time t (indicated by the vertical dashed blue line) can be considered to capture all of the cohorts (C_1-C_4) at a certain point in time, and thereby the individuals within each cohort at a certain age a (indicated by the horizontal dotted green line). The proportion of seropositive individuals of each age (a_1-a_4) at time t could thus be considered to represent the cumulative exposure within each of the cohorts (C_1-C_4) that they represent. The rate of increase in seroprevalence with age will provide information of how fast individuals in the population seroconvert and can therefore be used as a marker of the force of infection.

1.5.1 Estimating seroconversion rates from cross-sectional data

A common pattern in the age-specific prevalence of antibodies to malaria in a population living in areas where the disease is endemic is illustrated in Figure 5.
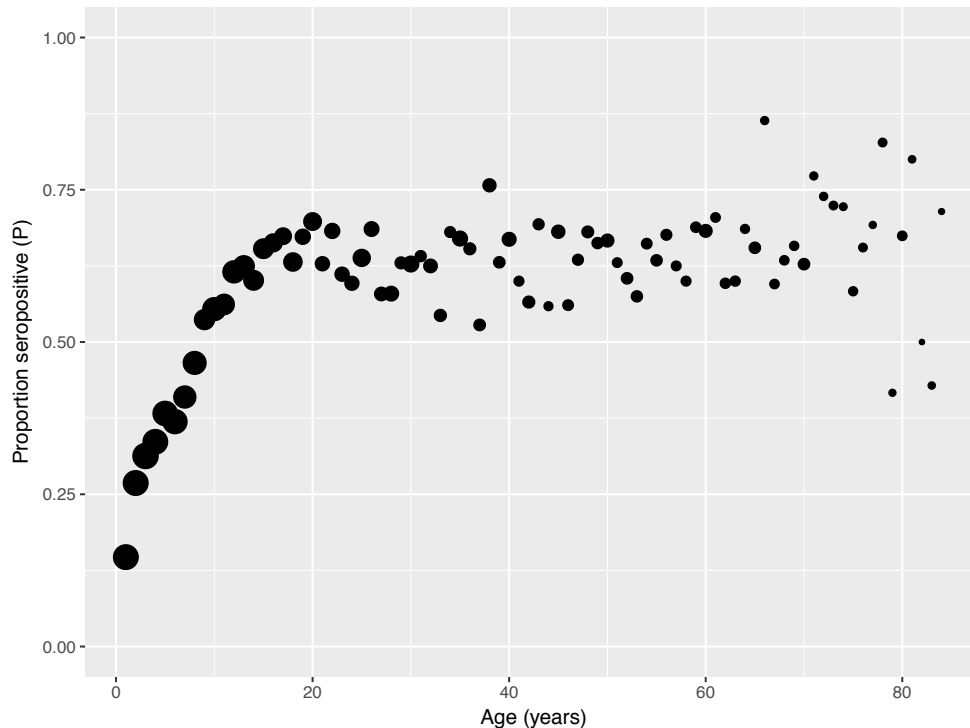

Figure 5. Typical age pattern in prevalence of malaria specific antibodies in a population living in an endemic area. Data on seropositivity to MSP-1 or AMA-1 from 17,503 individuals participating in baseline surveys of the REDHOT cluster randomised trial (Bousema et al. 2016 (142)). Each dot represents the seroprevelence within a one-year age stratum. The size of the dots represents the number of individuals in each age stratum. Data is publicly available through the Dryad data repository https://doi.org/10.5061/dryad.nr8d8.

As exemplified in Figure 5, seroprevalence often increases with age until reaching a plateau where it saturates in the population. This saturation implies one or more of the following: i) not all individuals in the population become exposed, ii) for a given antigen, not all individuals will seroconvert upon exposure, or iii) antibody responses once acquired can also be lost (143). The loss of antibodies leading to a transition from seropositive to seronegative is referred to as seroreversion. The increase in seroprevalence in the population with age can, as previously described, be described using a reversible catalytic seroconversion model (serocatalytic model) (139,141,144–146).

If seronegative individuals become seropositive at a rate $\lambda(t)$, and if seropositive individuals revert to seronegative at a rate ρ, the proportion of seropositive individuals in a cohort P is described by the following differential equation:

$$\frac{dP}{dt} = \lambda(t)(1-P) - \rho P \tag{1}$$

Assuming constant intensity in transmission this equation can be solved to give estimates of the proportion of individuals of age a that are seropositive:

$$P(a) = \frac{\lambda}{\lambda+\rho}(1 - e^{(\lambda-\rho)a}) \tag{2}$$

where $P(a)$ is the proportion of individuals of age that are seropositive, λ is the rate of seroconversion, ρ the seroreversion rate and a the age of the individual at the time when the sample was collected. The model parameters (λ and ρ) are estimated by fitting the model to cross-sectional data on age-specific antibody prevalence. By allowing the seroconversion rate to vary in the population over time, the model can be extended to evaluate temporal changes in the force of infection (143,147,148).

The seroconversion rate provides a robust surrogate marker of the force of infection of malaria and has been previously validated across a wide range of transmission settings (146). Furthermore, seroconversion rates for a number of *P. falciparum* pre-erythrocytic (e.g. CSP and *Pf*CelTOS) (149) and blood stage antigens (e.g. MSP-1, MSP-2, and AMA-1) (146,150,151) have been estimated to examine the magnitude and temporal trends in transmission intensity in different geographical areas (152–157). Besides malaria, catalytic models based on age-specific seroprevalence have been applied to estimate the force of infection of a number of parasitic infections (e.g. toxoplasmosis (158), and onchocerciasis (159)), as well as both viral infections (e.g. dengue (160,161), hepatitis A and B (162,163), rubella (164), measles (165), respiratory syncytial virus (166)) and bacterial infections (e.g. Chlamydia trachomatis (167)).

As described above, antibody responses are generally measured in a semi-quantitative fashion. However, the use of serocatalytic models to estimate the seroconversion rates from cross-sectional data requires the quantitative antibody measurements to be dichotomised into simply seropositive or seronegative. This process of dichotomisation leads to a substantial loss of information contained in the quantitative antibody measurement (168). Furthermore defining the threshold of seropositivity is complicated and an inappropriate threshold may lead to spurious estimates of the seroconversion rate (151,169,170). It is therefore likely that a method that makes use of the information provided by the quantitative antibody measurements, thereby avoiding dichotomisation, could improve the precision in transmission estimates.

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1.5.2 Longitudinal data on individual antibody dynamics

In an individual plasma sample collected in a cross-sectional survey the level of pathogen-specific antibody can often give some indication of how recently the individual was infected. Higher levels tend to indicate a more recent exposure but can also reflect a greater cumulative exposure (171,172). However, there are distinct temporal dynamics in the magnitude of specific antibody responses after infection (68). If these temporal dynamics are sufficiently predictable both within and between individuals, the dynamics of the response can be exploited to estimate when an individual was last infected with a specific pathogen. Given that the dynamics can be accurately described, cross-sectional data on antibody responses can theoretically provide a direct measurement of the force of infection (131). In such cases, a reliable model of the dynamics in antibodies after infection must first be created. Given a measured antibody level, this model can then be used to back-calculate the time since infection, which in turn can be used for incidence estimation (131).

For many pathogens, including *P. falciparum*, however, the lack of accurate quantitative estimates of how the magnitude of the antigen-specific individual-level antibody response changes over time after infection is a common and important limitation for developing models that can reliably predict the time since infection (131). Such quantitative estimates are preferably obtained through the longitudinal study of the dynamics of antibodies after infection. However, it is difficult to obtain reliable quantitative estimates for *P. falciparum* by studying individuals living in areas where malaria is endemic. Studies are hampered both by the latency between infection and symptom onset and the overall high frequency of low-density asymptomatic infections which make it difficult to determine when an infection actually started (173). Furthermore, because of a continuous risk of re-infection during follow-up that can lead to antibody boosting, the decay in antibody levels is difficult to accurately characterise (68). For *P. falciparum*, The situation is further complicated by the fact that individuals may harbour infections with multiple parasite clones by which they have been infected at different points in time (174,175).

Experimental challenge studies of controlled human malaria infection (CHMI), in which the exact time-point of infection is known, could be used to partially address these issues (35). However, participants in CHMI trials are typically treated at microscopic patency of blood-stage infection, which in many cases occurs before the onset of symptoms (176,177). The dynamics of the antibody response following CHMI may therefore not reflect the dynamics of the response following a symptomatic natural blood-stage infection in which parasitaemia is higher and the inflammatory response more pronounced (35).

However, if studying individual-level dynamics of antibody responses in travellers returning with malaria to a malaria free country after short-term travel it would be possible to significantly narrow down the time-point when infection occurred. A follow-up of malaria infected travellers in a malaria free country would also make it possible to study the decline in antibody levels in complete absence of re-exposure, allowing for a detailed characterisation of the dynamics of the response.

If such a study includes individuals with varying levels of prior malaria exposure it would also be possible to identify whether the response to some antigens provides information on recent exposure while the response to others reflects the cumulative magnitude of prior exposure.

2 AIM

The overall aim was to investigate how malaria exposure affects the acquisition and maintenance of the antimalarial antibody response with the goal to improve our understanding of antimalarial immunity and develop new methods for monitoring malaria transmission.

Our specific aims were to:

I.   Evaluate the temporal trends in malaria transmission over 25 years in a Tanzanian village using estimates of parasite prevalence obtained with different methods and to assess the impact of the presence of a research team.

II.   Evaluate temporal trends in malaria transmission using serology and develop new mathematical models that can improve serological surveillance of transmission intensity.

III.   Characterise the dynamics and longevity of antibodies and antibody secreting cells following a single malaria infection.

IV.   Identify serological signatures of recent and cumulative malaria exposure.

3 MATERIALS AND METHODS

Brief overviews of the most important materials and methods are described in this section. More detailed descriptions are presented within the corresponding papers or manuscripts.

3.1 STUDY POPULATIONS

3.1.1 Nyamisati Tanzania (Study I and II)

Studies **I** and **II** were conducted within a longitudinal project on the epidemiology of malaria in Nyamisati, a rural fishing village situated in the Rufiji river delta in coastal Tanzania, approximately 150 km south of Dar es Salaam (S 7°47'43'', E 39°16'31''). Malaria transmission in the area is perennial with seasonal increases following longer periods of rain occurring twice yearly. The predominating vectors in the area belong to the *Anopheles gambiae* complex and are *An. gambiae* sensu strictu, *An. arabiensis,* and *An. merus* (178,179). The research project was established in 1985 and a research team, who lived in the village, maintained a primary health care unit and monitored malaria cases through a passive case detection system operational from 1986-88 and 1993-1999. Furthermore, repeated cross-sectional surveys, in which all villagers were invited to participate, were conducted in 1986-1988, 1993-1999, and in 2010. The surveys consisted of a clinical examination of health status including evaluation of spleen size according to Hackett's score (118), measurement of haemoglobin levels, collection of blood slides as well as of venous blood samples stored separated as packed cells and plasma. Insecticide treated nets (ITNs) were distributed to pregnant women and mothers with small children after the survey in 1993 and to all survey participants after the survey in 1999. Long-lasting insecticidal nets were distributed to all survey participants after the survey in 2010. Other vector control measures such as IRS or larviciding have not been deployed in the area. Study **I** includes all available survey data on health status, spleen size, haemoglobin levels, and parasite positivity by microscopy as well as data on malaria incidence recorded through the passive case surveillance system 1986-1988 and 1993-1999. Available samples collected in surveys conducted in 1994-1995, 1999, and 2010 were selected for analysis by parasite-specific PCR. For study **II**, plasma samples collected from children 1-16 years old who participated in the cross-sectional surveys conducted in 1999 and 2010 were selected for the analysis of antibody responses.

3.1.2 Swedish travellers cohort (Study III and IV)

Studies **III** and **IV** were conducted within a prospectively followed cohort of travellers. Sixty-five adult travellers diagnosed with *Plasmodium falciparum* malaria at Karolinska University hospital were enrolled in the study upon hospitalisation. Venous blood samples were collected at enrollment and study participants were invited to provide follow-up samples after ten days, and one, three, six and twelve months. A questionnaire detailing country of origin, previous countries of residence, medical and travel history as well as prior malaria exposure was administered to all study participants at enrollment and at the end of follow-up. Study participants differed in terms of their prior malaria exposure.

Twenty-one individuals were born in a malaria-free country and reported no prior malaria exposure, three individuals who were born in a malaria-free country reported, or had a confirmed, single prior episode of clinical *P. falciparum* malaria, and forty-one individuals who were born, or had lived a substantial period of time, in a malaria endemic country, reported multiple prior clinical malaria episodes. Study **III** was restricted to include subjects with either no prior or multiple prior episodes of clinical malaria while study **IV** included all subjects in the cohort.

3.1.3 Negative controls

For studies **II** and **III,** plasma samples from 20 Swedish donors who had never travelled to a malaria endemic area were analysed as negative controls. For study **IV**, a different set of plasma samples from 42 malaria naïve European donors were analysed as negative controls.

3.2 ETHICAL CONSIDERATIONS

Studies **I** and **II** were granted ethical approval by the Nyamisati village board, the Ethical Review board of the National Institute of Medical Research in Tanzania, the Regional Ethical Committee at Karolinska Institutet, and the Regional Ethical Review Board in Stockholm. Studies **III** and **IV** were approved by the Regional Ethical Review Board in Stockholm. Informed consent was obtained from all study participant or, when applicable, the study participant and/or their guardians.

3.3 PARASITE DETECTION

3.3.1 Microscopy (Study I)

Examination of Giemsa-stained thick and thin films was performed using light microscopy. Parasite densities were estimated per microlitre of blood and were enumerated against the number of leukocytes by assuming there are 8000 leukocytes per microlitre. Blood films were considered negative if no parasites were detected following examination of 100 fields of the thick film.

3.3.2 Polymerase chain reaction (PCR) (Study I)

Detection of *P. falciparum* parasites by PCR was performed in study **I** to assess the prevalence of infection. Briefly, DNA was extracted from packed cells in EDTA using Qiagen blood mini kit (Qiagen, Germantown, MD, USA), phenol-chloroform extraction, or using an automated Qiagen BioRobot® M48 (Qiagen). A plasmodium species-specific real-time PCR assay targeting the multicopy 18S rRNA gene was performed using an ABI TaqMan 7500 instrument (Applied Biosystems, Foster City, CA, USA) (180). In addition, a nested PCR assay targeting the two allelic types of the polymorphic single copy *msp2* gene was performed using fluorescently labelled primers and fragment sizing by capillary electrophoresis (181,182).

3.4 ANTIBODY ASSAYS

3.4.1 *Anopheles gambiae* salivary gland protein 6 ELISA (Study II)

An enzyme-linked immunosorbent assay (ELISA) protocol was used to evaluate the antibody response towards the recombinant *Anopheles gambiae* salivary gland protein 6 (gSG6) (137,183). Microtitre plates (Costar® Corning, Tewksbury, MA, USA) were coated overnight at 4°C with 25 µl per well of recombinant gSG6, at 5 µg/ml in sodium-carbonate buffer. After washing and blocking, 25 µl of test plasma at 1:100 dilution were added and incubated at 37°C for 1 hour. After washing, IgG was detected using 25 µl per well of alkaline-phosphatase conjugated goat anti-human IgG (Mabtech, Nacka, Sweden). The assay was developed with p-nitrophenyl phosphate disodium substrate (Sigma-Aldrich, St. Louis, MO, USA), and after stopping the reaction with 1M hydrochloric acid, optical densities (OD) were read at 405 nm using a VmaxTMKinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3.4.2 Schizont extract ELISA (Study III)

An ELISA was used to quantify total IgG levels to schizont extract according to a previously described protocol (184). Each well of microtitre plates were coated overnight at 4°C with 100 µl of *P. falciparum* schizont extract (3D7 clone) at 2 µg/ml. After blocking and washing, plates were incubated with 100 µl of test plasma at 1:1000 dilution overnight at 4°C. Bound IgG was detected by horseradish-peroxidase conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark). Plates were developed with o-phenylenediamine dihydrochloride and the reaction stopped with 2M H_2SO_4. ODs were read at 492 nm using a VmaxTMKinetic microplate reader (Molecular Devices).

3.4.3 Bead-based immunoassays (Study II and III)

In studies **II** and **III**, a multiplex bead-based immunoassay was used for the simultaneous quantification of IgG antibody responses to multiple recombinant *P. falciparum* antigens as previously described (185). Briefly, each antigen was covalently coupled to a spectrally unique set of carboxylated paramagnetic beads (Bio-Rad Laboratories, Hercules, CA, USA). Antigen coupled beads were mixed and incubated with test plasma at room temperature. Bound antigen-specific antibodies were detected using R-phycoerythrin conjugated F(ab')$_2$ goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and the assay readouts were obtained using a Bio-Plex 200 instrument (Bio-Rad Laboratories). For study **II,** the assay antigens included the 19 kDa fragment of merozoite surface protein 1 (MSP-1$_{19}$) (186), and two allelic variants of each of MSP-2 (187), MSP-3 (188), and apical membrane antigen 1 (AMA-1) (189). For study **III** the assay was expanded to also include the RH5 antigen (190,191) and was repeated for quantification of the response of each of the four IgG subclasses (IgG$_{1-4}$) in addition to total IgG, as previously described (192). Furthermore, for study **III**, an additional monoplex bead-based immunoassay was used for detection and quantification of total IgG antibodies to tetanus toxoid (TTd) according to a previously described protocol (193).

3.4.4 Antibody microarray (Study IV)

The KILchip v1.0 protein microarray (Kamuyu et al., *Manuscript under review*) was used in study **IV** to simultaneously screen plasma samples for antibody reactivity towards 111 *P. falciparum* antigens corresponding to 88 unique proteins. The selection, expression and purification of antigens have been previously described (48,51,187,188,194–198). Out of the 111 antigens, 82 were full-length ectodomains or, if multimembrane proteins, corresponded to the largest predicted extracellular loop while 29 were protein fragments of 8 unique proteins (i.e. MSP-1, MSP-2, MSP-3, MSPDBL1, MSPDBL2, *Pf*SEA-1, PF3D7_06293500 and Surfin 4.2) (187,188,194–196). Out of the 29 fragments, 16 were derived from the 3D7 sequence while 13 were derived from non-3D7 sequences (i.e. cho150/9, Dd2, K1, MAD20, PaloAlto, RO33, and Wellcome). Plasma samples were assayed in 1:400 dilution and bound antibody was detected using AlexaFluor[647] conjugated donkey anti-human IgG. The processed microarray slides were read at 635 nm using a GenePix® 4000B scanner (Molecular Devices) and results obtained using the GenePix® Pro 7 software (Molecular Devices). In order to account for day-to-day and slide-to-slide variation in assay signal intensity, normalisation was performed using robust linear models as previously described by Sboner et al. (199).

3.4.5 Defining thresholds of seropositivity

For all immunoassays, thresholds of seropositivity were defined for each antigen as the mean antigen-specific reactivity of malaria unexposed negative controls + 3 standard deviations (SD).

3.4.6 Converting assay signal intensity to relative antibody concentration

In all bead-based immunoassays and ELISAs, a serially diluted standard calibrator of either purified IgG from malaria immune donors (multiplex bead-based assay, schizont extract ELISA), recombinant human IgG (gSG6 ELISA), or pooled plasma from highly tetanus immune donors (tetanus toxoid assay) was assayed on each plate on each day of experiment to provide a standard calibrator curve used to control for day-to-day and plate-to-plate assay variation. Each assay OD or MFI was converted to a relative concentration in arbitrary units by interpolation from the corresponding standard calibrator curve using a five-parameter sigmoidal curve fitting.

3.5 STATISTICAL ANALYSIS AND MATHEMATICAL MODELLING

Statistical analysis and mathematical modelling were performed using Stata v12 to v14 (Stata Corp., College Station, TX, USA) and R v3.2.2 to v3.4.4 (R Core Team, Vienna, Austria).

3.5.1 Logistic regression models (Study I)

In study **I**, multivariable generalised estimating equation logistic regression models were used to evaluate the temporal trends in parasite prevalence, determined by microscopy and each of the two PCR methods, and anaemia prevalence while adjusting for known and potential confounders, e.g. age and sex. Age was treated as a categorical variable with 5 categories. An interaction effect between age and survey year was included in all models.

3.5.2 Serocatalytic models for antibody prevalence (Study II)

To evaluate temporal trends in malaria transmission intensity using serology, a previously described reversible catalytic seroconversion (serocatalytic) model was used to estimate the annual rate of seroconversion (SCR) from cross-sectional data on age-specific seroprevalence (146). The serocatalytic model was fitted separately to seroprevalence data for each antigen but jointly to data from both cross-sectional surveys included in study **II**. Three different models, representing three possible malaria transmission intensity patterns over time, i.e. constant transmission (M1), single sharp stepwise reduction (M2), or continuous linear reduction (M3) in transmission intensity, were evaluated. For each antigen, the best performing model, and thus most likely transmission pattern, was identified in terms of the lowest Akaike Information Criterion (AIC) value (200).

3.5.3 Antibody acquisition models for antibody levels (Study II)

We developed novel models for estimation of transmission intensity, referred to as antibody acquisition models, based on cross-sectional data on age-specific antibody levels. The antibody acquisition models, which incorporate insights from longitudinal antibody dynamics, assume that antibody levels increase as a function of age and therefore that the rate at which they are acquired can be used as an alternative marker of transmission intensity (Figure 6) (68,141).

If an individual's antibody level is boosted at a rate α and decays at a rate r, the antibody levels can be described by the following differential equation:

$$\frac{dA}{dt} = \alpha(t) - rA \tag{3}$$

By allowing α to vary over time we evaluate different scenarios for a change in transmission intensity. The models were fitted to the data analysed in study **II** in the same way as described for the serocatalytic models. The same three alternative transmission patterns were evaluated and the most likely pattern was identified based on the model AICs.
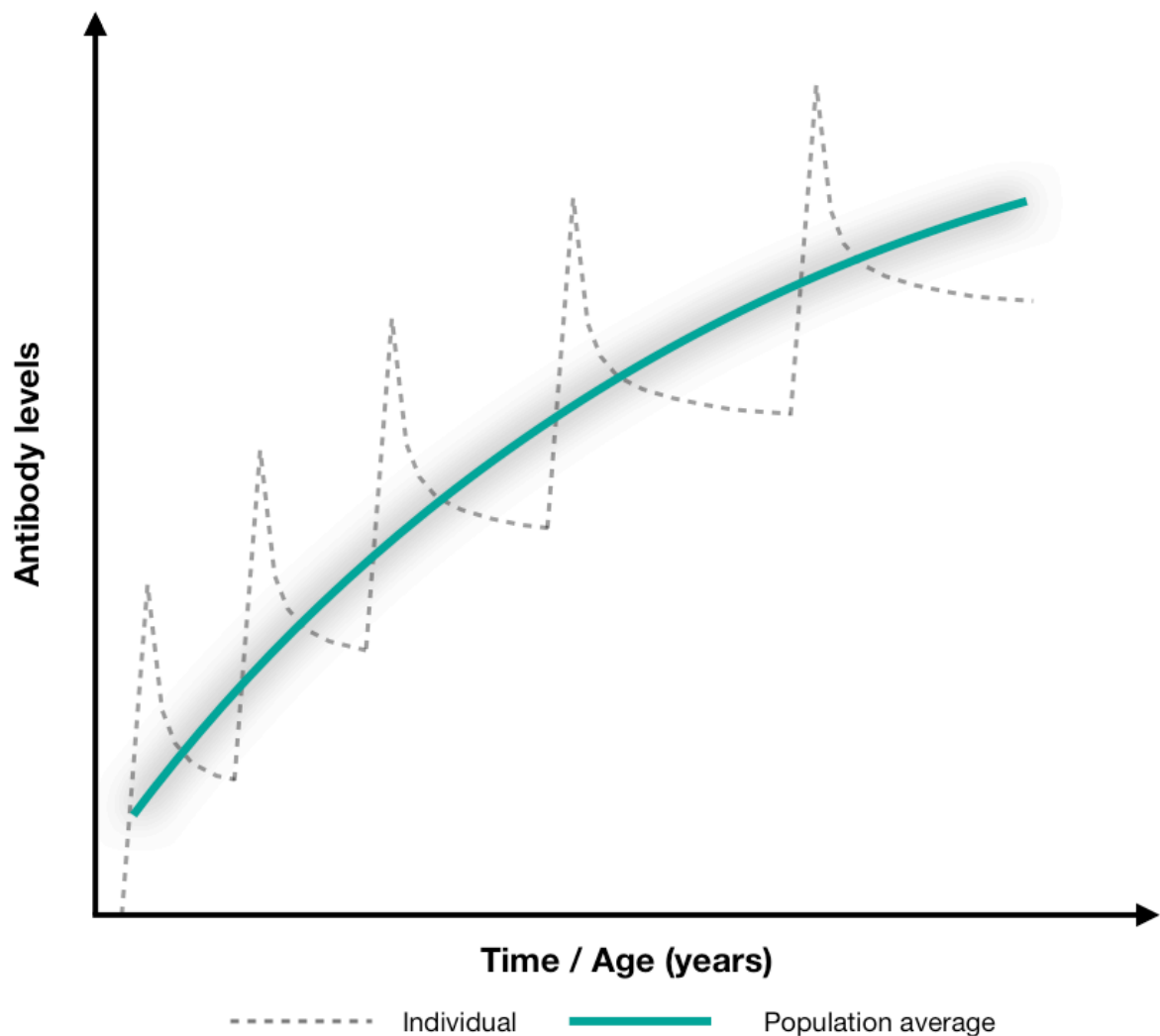
Figure 6. Schematic representation of how the antibody acquisition model incorporates insights from longitudinal antibody dynamics. The dashed line provides an illustration of how a naïve individual is assumed to acquire an antibody response with time. Each new infection is assumed to cause a consequent increase in antibody levels until reaching a plateau where no further increase is possible (68,141). The solid green line is the corresponding average increase in the population antibody levels with time. In the context of cross-sectional data, the analogous scenario can be represented by the level of antibodies at each age. We assume that the average annual rate of increase in antibody levels with age can be used as a marker of transmission intensity.

3.5.4 Antibody dynamics model (Study III)

In study **III,** the longitudinal data on antibody responses was analysed using a mathematical model that captures the dynamics of the antibody response while simultaneously allowing estimation of the longevity of both antibodies and antibody secreting cells. The model is an extension of the antibody dynamic model, previously described by White et al., that incorporates the possibility to account for differences in the individual level of prior exposure (68).

The rise and fall in antibody levels after infection at time τ_0 can be represented by the following equation:

$$A(t) = A_0 e^{-r_l(t-\tau_0)} + \left((1-\rho) \frac{e^{-r_s(t-\tau_0)} - e^{-r_a(t-\tau_0)}}{r_a - r_s} + \frac{e^{-r_l(t-\tau_0)} - e^{-r_a(t-\tau_0)}}{r_a - r_l} \right) \qquad (4)$$

As further illustrated in Figure 7, the model assumes that in previously naïve individuals, infection at time τ_0, leads to the proliferation and differentiation of B-cells generating an amount β of ASCs that secrete IgG. A proportion of the ASCs (ρ) are long-lived and decay at rate r_l while a proportion $(1-\rho)$ are short-lived and decay at rate r_s causing a biphasic decay in antibody levels. All ASCs produce antibodies that decay at rate r_a. Individuals who have had prior *P. falciparum* infections may maintain a level of pre-existing antibodies (A_0), generated during previous infections, that decay at rate r_a and are maintained by old long-lived ASCs, which decay at rate r_l. Naïve individuals, who suffer a primary *P. falciparum* infection, are assumed to have no pre-existing antibodies or ASCs at the onset of infection ($A_0 = 0$). The above model is valid for all t greater than τ_0.

The model accounts for exposure related differences in the dynamics of the response by allowing for differences in the magnitude of antibody increase (i.e. boosting) upon infection and in the proportion of long-lived ASCs that are generated and maintained.
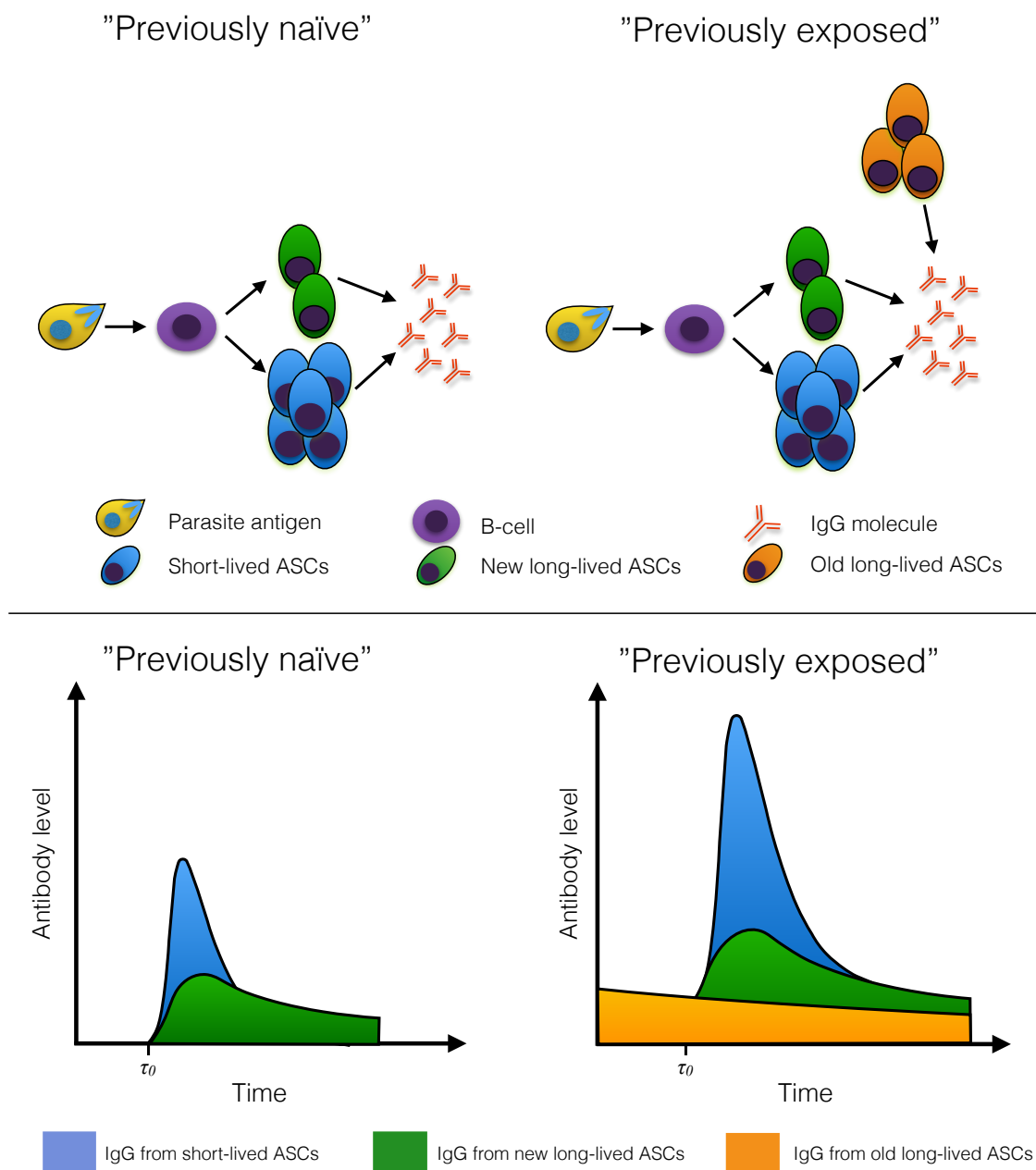
Figure 7. Schematic representation of the antibody dynamics model. The top row represents how the model captures the underlying immunological processes depending on prior exposure and the bottom row depicts the change in antibody levels over time.

3.5.5 Decay in antibody reactivity (Study IV)

In study **IV**, the antigen-specific rate of decay in antibody reactivity was estimated using mixed effects models as previously described (78,201). Antibody levels were assumed to decay exponentially over time, corresponding to a linear decay in log antibody levels. Models were fitted to the log-transformed MFI data that had been curated to include only data from the decay phase of the response towards each antigen. The rate of decay was expressed as the half-life of the antibody response (78,201).

3.5.6 Antibody responses predictive of recent exposure (Study IV)

In study **IV** we used binary classification analysis to evaluate if the antibody response to a *P. falciparum* antigen was predictive of whether a sample was collected from a recently infected individual. A recent infection was defined as an infection having occurred within 90 days of sample collection. Univariable logistic regression models were fitted to data for each of the antigens individually. Receiver operating characteristic (ROC) analysis was used to evaluate the classification performance of each model and a cross-validation was performed for each classifier using repeated random sub-sampling in order to evaluate the classifier performance outside of its training sample (202). To evaluate if classification could be improved by incorporating data on the response to multiple antigens, we first performed a feature selection with the Boruta algorithm, a wrapper method built around a random forest classification algorithm, as previously described (203). The Boruta algorithm was fitted jointly to antibody data for all immunogenic antigens and antibody responses identified as contributing significant information to classification of recent exposure were selected for further evaluation. Multivariable logistic regression models were fitted to all possible two- to five-way combinations of antibody responses to these selected antigens. In order to evaluate whether a combination of responses could improve performance of classification of recent infection we examined the classifier cross-validated area under the ROC curve (AUC).

4 RESULTS

4.1 STUDY I

In order to characterise the temporal trends in malaria transmission in Nyamisati, Tanzania, we analysed data on spleen rate, parasite prevalence, and haemoglobin levels collected in repeated cross-sectional surveys conducted between 1985 and 2010. Parasite prevalence was evaluated using microscopic examination of blood slides as well as two different PCR methods. During the 1980's, efforts to limit malaria transmission consisted of providing prompt diagnosis and antimalarial treatment. Insecticide treated nets (ITNs) were first introduced after the cross-sectional survey in 1993, when the research team distributed 300 nets to pregnant women and families with small children. An additional round of 900 ITNs were distributed after the survey in 1999. Assuming one net on average provides protection for 1.8 individuals, these interventions were estimated to have achieved a bed net coverage of approximately 45% in 1993 and 100% in 1999 (204).

Between 1985 and 2010, we observed a substantial decline in all evaluated malaria transmission metrics. The reduction started before the introduction of ITNs and a decline in the malaria burden was first observable in the spleen rate, in which a decline was discernible already during the 1980's. This was later followed by a reduction in parasite prevalence as well as a concurrent decline in the prevalence of both severe and moderate anaemia. The overall parasite prevalence by microscopy, which was 70% in 1985 declined, gradually during the 1990's to 39% in 1994 and 27% in 1999, reaching an estimated 5% in 2010 (Figure 8).
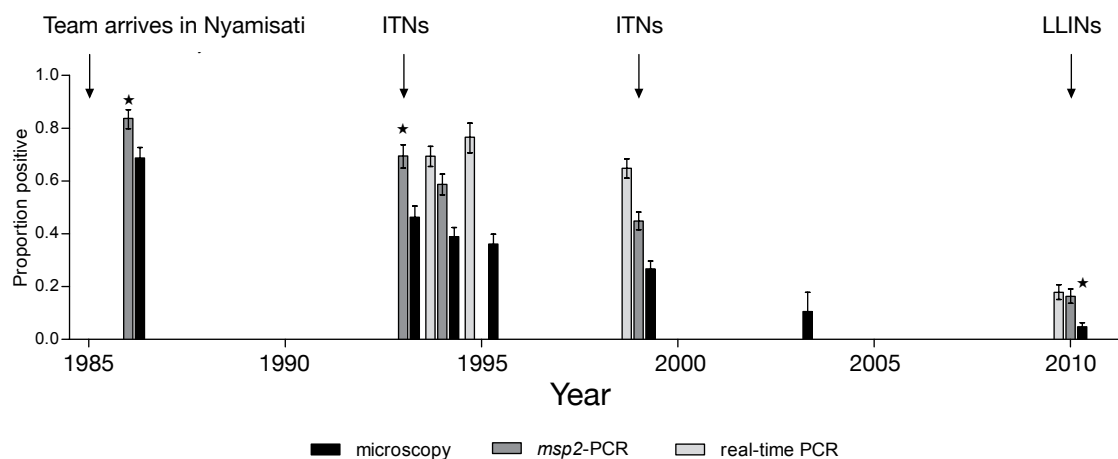

Figure 8. Temporal trends in parasite prevalence. Bars denote the all-age prevalence for each method and the error bars the 95% CI. * indicates that data has been estimated from corresponding microscopy or PCR data using the prevalence estimation tool developed by Okell et al. (125). ITNs and LLINs indicate the distribution of insecticide treated nets and long-lasting insecticidal nets, respectively, after the survey.

The observed PCR prevalence, which was consistently higher than corresponding estimates based on microscopy, declined substantially between 1994 and 2010 from 69.4% to 17.8% and 59% to 16% when evaluated by real-time PCR and *msp2*-PCR, respectively. The magnitude of the estimated annual reduction in PCR prevalence was age-specific and was greatest among children 5-8 years of age (real-time PCR, Odds ratio [OR]: 0.79, 95% CI: 0.76-0.83; *msp2*-PCR, OR: 0.80, 95% CI: 0.76-.85) and 9-12 years of age (real-time PCR, OR: 0.79, 95% CI: 0.76-0.83; *msp2*-PCR, OR: 0.82, 95% CI: 0.79-0.86). Over time this lead to a shift in the age-prevalence pattern and at the end of the study period the highest prevalence was observed among 13-16 years old children. Furthermore, there was a significant reduction in the prevalence of any level anaemia from 1994 to 2010 (OR: 0.32, 95% CI: 0.24-0.41).

4.2 STUDY II

In study **II,** we developed new antibody acquisition models for serological surveillance based on cross-sectional data on antibody levels with the aim to improve serological estimates of malaria transmission intensity. The new models make use of the data on individual antibody levels by assuming that antibody levels increase as a function of age. Further they assume that the rate at which antibody levels are boosted by exposure can be used as an alternative marker of transmission intensity. To evaluate the performance of the antibody acquisition models, we compared them to the previously validated serocatalytic models based on antibody prevalence (146) and applied both approaches to further examine the temporal transmission patterns in Nyamisati described in study **I.**

We analysed data on antibody responses to *P. falciparum* and *An. gambiae* antigens measured in children 1-16 years old who participated in the cross-sectional surveys conducted in Nyamisati village in 1999 (n = 313) and 2010 (n = 355). For each individual antigen, i.e. MSP-1$_{19}$, two allelic variants of each of MSP-2, MSP-3 and AMA-1, and gSG6, serocatalytic and antibody acquisition models were fitted jointly to data from both cross-sectional surveys. Based on the known reduction in parasite prevalence over time (study **I**), we evaluated a set of three models for each modelling approach, each assuming a different temporal pattern in transmission intensity, i.e. no change (M1), single sharp stepwise reduction (M2), or continuous linear reduction (M3).

We observed a significant reduction in both prevalence (χ^2-test: all *p<0.001*) and levels (Mann Whitney-U test: all *p<0.001*) of antibodies to all *P. falciparum* antigens and the gSG6 from 1999 to 2010. Seroprevalence and levels of antibodies increased with age at both surveys except for MSP-1$_{19}$, and MSP-3 antigens, for which there was no age-trend in antibody levels at the 2010 survey, and for gSG6, for which neither prevalence nor levels displayed an age-trend at either survey. The serocatalytic models assuming constant transmission (M1) provided poor fit to data for all evaluated antibody responses but a substantial reduction in transmission intensity was identified by models assuming changes in the intensity of transmission (M2 and M3). For several antigens, the serocatalytic models assuming a stepwise reduction (M2) provided estimates of a 61 to 79% reduction in transmission intensity with a time-point of change between 1997 and 2000 while models assuming a linear decline in transmission intensity (M3) estimated a reduction of 64 to 95%. However, the models exhibited a considerable degree of uncertainty in parameter estimates for which confidence intervals were wide and for a majority of antigens the serocatalytic models were unable to determine whether a stepwise (M2) or a linear reduction (M3) had been most likely (comparing M2 to M3: ΔAIC <2).

The antibody acquisition models overall provided a better fit to data, more consistent estimates across antigens, as well as greater precision in parameter estimates. A model assuming a stepwise reduction (M2) in transmission intensity provided superior fit to data (i.e. with smaller AIC values) than a model assuming a linear reduction (M3) in transmission intensity for all *P. falciparum* antigens (Figure 9, Table 1).
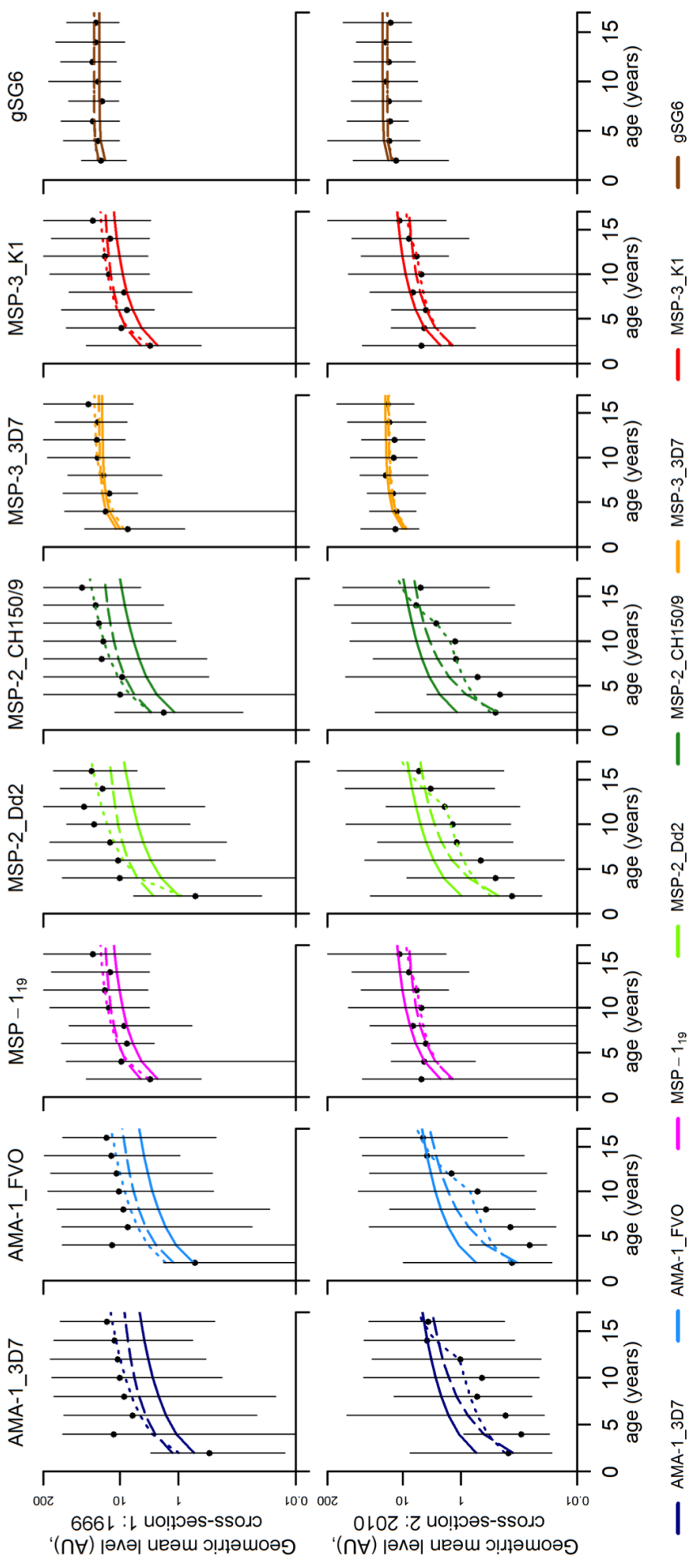
Figure 9. Best-fit antibody acquisition models. Black points denote geometric mean antibody levels in arbitrary units (AU) and vertical bars denote the 95% range of the data. M1: stable transmission (solid lines). M2: stepwise reduction in transmission (dotted lines). M3: linear reduction in transmission (dashed lines).

Table 1. Antibody acquisition model parameter estimates with 95% CI

Antigen	Model	α_0	Γ	r	t_c	σ	Log-likelihood	AIC
AMA-1_3D7	M1	0.27 (0.24, 0.34)	–	0.0 (0.0, 0.03)	–	2.18 (2.06, 2.29)	-2026.17	4058.35
	M2	0.89 (0.66, 1.32)	0.09 (0.07, 0.14)	0.0 (0.0, 0.05)	1998 (1997, 1999)	1.92 (1.82, 2.02)	-1942.73	**3895.45**
	M3	1.55 (0.91, 2.72)	0.009(0.001,0.04)	0.10 (0.01, 0.20)	–	1.97 (1.88, 2.08)	-1961.93	3931.85
AMA-1_FVO	M1	0.28 (0.24, 0.34)	–	0.0 (0.0, 0.02)	–	2.23 (2.11, 2.35)	-2052.93	4111.85
	M2	0.95 (0.66, 1.47)	0.08 (0.06, 0.12)	0.0 (0.0, 0.06)	1998 (1997, 2000)	1.93 (1.81, 2.06)	-1957.98	**3925.96**
	M3	1.48 (0.72, 2.53)	0.003(0.001,0.03)	0.08 (0.0, 0.18)	–	2.00 (1.91, 2.11)	-1980.09	3968.18
MSP-1_19	M1	0.47 (0.34, 0.71)	–	0.07 (0.0, 0.18)	–	1.67 (1.59, 1.77)	-1998.72	4003.44
	M2	7.8. (1.2, 45.5)	0.084 (0.016, 0.28)	0.23 (0.02, 0.45)	1991 (1988, 2002)	1.64 (1.55, 1.72)	-1987.69	**3985.38**
	M3	1.19 (0.71, 2.01)	0.29 (0.17, 0.49)	0.13 (0.05, 0.25)	–	1.65 (1.57, 1.74)	-1988.70	3985.40
MSP-2_Dd2	M1	0.50 (0.44, 0.59)	–	0.0 (0.0, 0.02)	–	2.03 (1.92, 2.14)	-2381.28	4768.56
	M2	1.96 (1.56, 2.92)	0.08 (0.06, 0.11)	0.0 (0.0, 0.05)	1997 (1996, 1998)	1.70 (1.61, 1.81)	-2263.92	**4537.84**
	M3	3.37 (2.02, 5.58)	0.002 (0.001,0.2)	0.12 (0.04, 0.22)	–	1.79 (1.69, 1.88)	-2296.06	4600.12
MSP-2_CH150/9	M1	0.58 (0.49, 0.71)	–	0.0 (0.0, 0.02)	–	2.26 (2.14, 2.39)	-2562.06	5130.12
	M2	1.95 (1.52, 3.28)	0.08 (0.06, 0.11)	0.0 (0.0, 0.06)	1998 (1997, 1999)	1.95 (1.84, 2.06)	-2463.86	**4937.71**
	M3	3.64 (2.13, 6.37)	0.0 (0.0, 0.02)	0.11 (0.02, 0.21)	–	2.01 (1.91, 2.11)	-2483.66	4975.31
MSP-3_3D7	M1	6.80 (4.53, 12.97)	–	0.34 (0.19, 0.72)	–	1.17 (1.12, 1.24)	-2976.95	5959.90
	M2	29.98 (3.0,100)	0.08 (0.07,0.15)	0.35 (0.31,0.48)	1992 (1990, 1993)	1.15 (1.10,1.22)	-2966.78	**5943.55**
	M3	10.59 (6.18, 22.8)	0.59 (0.42, 0.86)	0.38 (0.22, 0.79)	–	1.16 (1.10, 1.24)	-2973.43	5954.87
MSP-3_K1	M1	1.20 (0.84, 1.89)	–	0.06 (0.0, 0.17)	–	1.81 (1.71, 1.90)	-2706.36	5418.72
	M2	2.90 (1.87, 6.69)	0.28 (0.18, 0.39)	0.10 (0.03, 0.22)	1998 (1996, 2001)	1.73 (1.63, 1.82)	-2675.38	**5360.75**
	M3	4.99 (3.07, 9.0)	0.14 (0.07, 0.23)	0.17 (0.09, 0.33)	–	1.74 (1.65, 1.84)	-2680.85	5369.70
gSG6	M1	18.2 (12.7, 32.1)	–	0.82 (0.55, 1.50)	–	0.69 (0.65, 0.73)	-2677.39	5360.78
	M2	27.6 (17.1, 39.4)	0.64 (0.42,0.75)	1.02 (0.60,1.50)	2008 (1997, 2009)	0.66 (0.63,0.71)	-2651.88	5313.75
	M3	37.25 (23.9, 42.5)	0.46 (0.38, 0.55)	0.96 (0.65, 1.19)	–	0.66 (0.63, 0.71)	-2652.21	**5312.41**

Maximum likelihood parameter estimates and 95% confidence intervals for antibody acquisition models fitted to cross-sectional antibody levels data. α_0 is the rate of antibody boosting, γ ($= \alpha_t/\alpha_0$) is the reduction in transmission, r is the rate of antibody decay, t_c is the estimated time-point (calendar year) of drop in transmission, σ is the standard deviation of the antibody levels on the log scale, log-likelihood is the maximised log-likelihood of the model and AIC is the Akaike Information Criterion value. A bold font indicates the model with the smallest AIC for each of the antigens. M1, M2, and M3 denote the "no change", "stepwise change", and "linear change" models, respectively

Depending on antigen, the stepwise decrease model estimated a 72 to 92% reduction in transmission intensity and for a majority of antigens the reduction, corresponding to the largest single reduction event, was estimated to have occurred between 1997 and 2000 (Figure 10, Table 1).
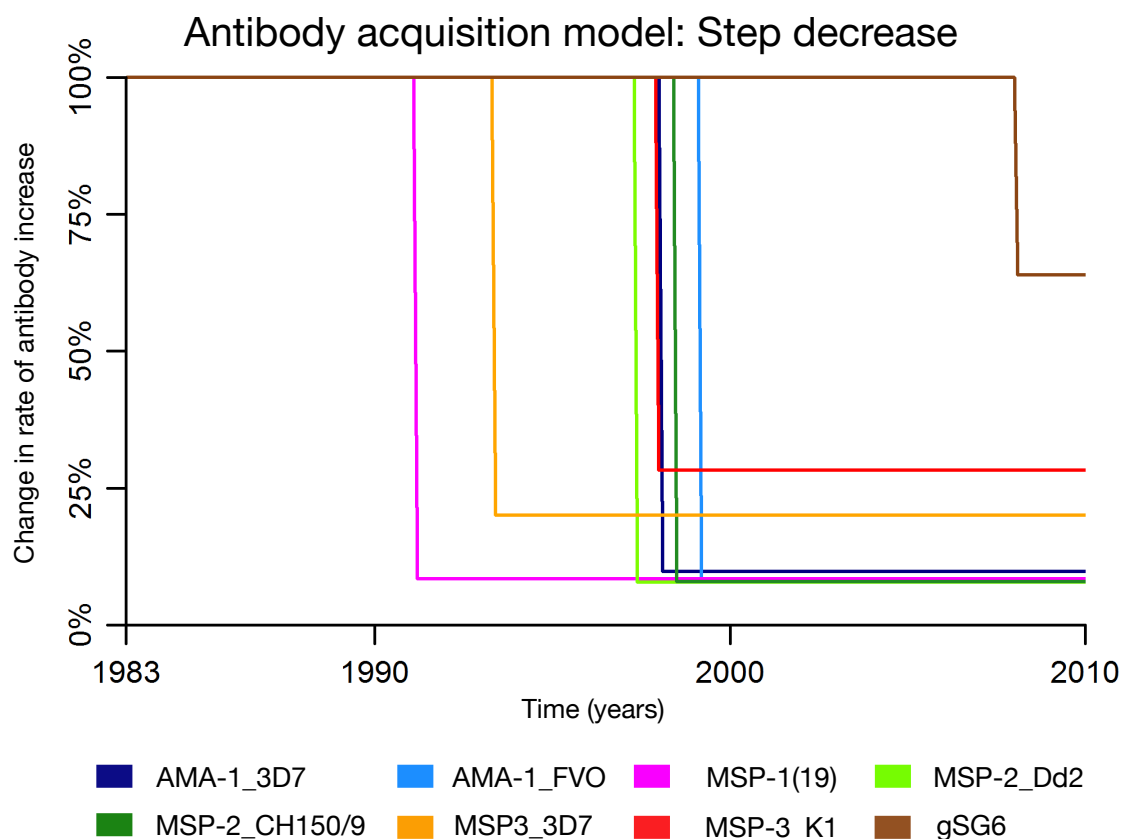

Figure 10. Estimated changes in transmission. Model estimates from antibody acquisition models assuming a stepwise reduction in transmission intensity. Lines represent the estimated magnitude and time-point of change from the model fitted to each of the antigens.

Furthermore, a complementary sensitivity analysis based on both simulated data (simulated using the antibody acquisition model) and the data for AMA-1_3D7, demonstrated that the antibody acquisition models are more robust than serocatalytic models when the sample size is small. Reducing the samples size to 50 or 100 samples per cross-section decreased both accuracy and precision of serocatalytic model estimates whereas it only slightly affected the precision of the antibody acquisition model.

4.3   STUDY III

In study **III,** we investigated the dynamics of the total IgG and IgG subclass response to *P. falciparum* schizont extract and recombinant vaccine candidate antigens (MSP-1$_{19}$, MSP-2, MSP-3, AMA-1, and RH5) in travellers followed prospectively, and in complete absence of re-exposure, after treatment of a clinical malaria episode. In total, 61 adult travellers, 41 previously malaria exposed and 20 previously malaria naïve, were enrolled in the study upon diagnosis and followed with repeated blood sampling during the subsequent year. We used mathematical models to examine the exposure dependent dynamics of the antibody response and to estimate the decay rates of antibodies and short- and long-lived ASCs, as well as the relative contribution from short- and long-lived ASCs to the overall response. To provide a validation of our estimates we also examined the dynamics of the antibody response to the unrelated but well characterised vaccine antigen TTd in the same individuals.

On average, the magnitude of the IgG antibody response towards each of the *P. falciparum* antigens increased in both exposure groups until reaching a maximum after approximately 14 days. After this, levels initially declined rapidly followed by a second phase of more slow decay. Geometric mean malaria-specific total IgG levels were consistently higher in individuals with prior malaria exposure for all antigens except MSP-3. The differences were particularly evident in the greater boost size and in the maintenance of higher plateau levels at the end of follow-up, where individuals with prior exposure maintained on average 2 to 9-fold greater antibody levels than previously naïve individuals (Figure 11).
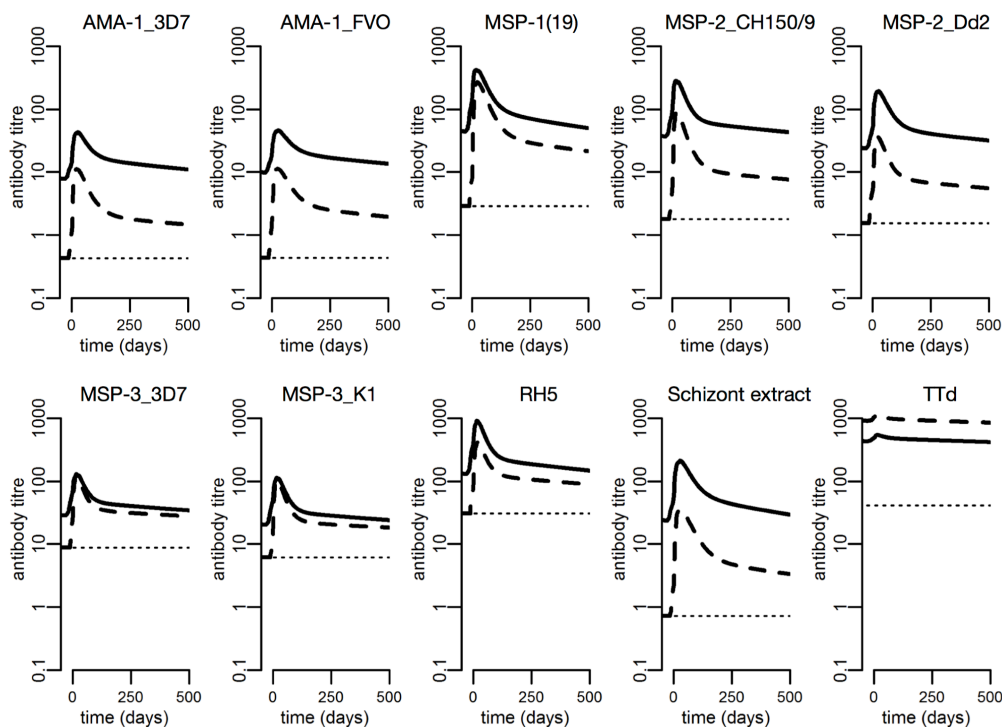

Figure 11. **Model predicted antibody dynamics.** The geometric mean antigen-specific total IgG levels over time for malaria antigens and TTd in each exposure group. Solid lines denote the previously exposed while dashed lines denote the previously malaria naïve. The dotted line represents the assay lower limit of quantification.

The half-life of IgG antibody molecules varied slightly with antigen specificity and ranged from 13 to 21 days. The half-lives of short-lived ASC ranged from 9 to 16 days and were shortest for ASCs producing antibodies specific for MSP-3 while longest for ASCs producing antibodies to AMA-1 (Figure 8B). The half-life of malaria-specific long-lived ASCs ranged from 610 to 1368 days (i.e. 1.7-3.7 years) and tended to be shorter for schizont extract and MSP-1$_{19}$ while longer for MSP-3. The half-life of long-lived ASCs producing IgG to TTd was considerably long in comparison and was estimated to 2704 days, i.e. 7.4 years (95% CrI: 5-11.4 years). The contribution by malaria-specific long-lived ASCs to the overall antibody response was estimated to be greater in individuals with prior malaria exposure, where it ranged from 10 to 57% depending on antigen, than among previously naïve individuals where it was estimated to be less than 10% for all antigens. For TTd the proportion of long-lived (expected to be 100%) was estimated to 96% and thus consistent with a non-boosted antibody response (Figure 12).

The dynamics of the IgG subclass responses were similar to those of the total IgG responses. Geometric mean antibody levels were consistently higher in previously exposed individuals for all antigens and IgG subclasses, except for IgG$_2$ to MSP-1$_{19}$ for which there was no difference between groups. The differences were particularly evident in the IgG$_1$ response to AMA-1 and in the IgG$_3$ response to MSP-2 antigens, where previously exposed individuals maintained 15.5 to 21.5-fold and 14.1 to 31.1-fold greater levels at the end of follow-up, respectively. On average, only previously exposed individuals mounted a detectable IgG$_4$ response and although levels were low, difference in geometric mean levels were substantial across all antigen specificities. Furthermore, estimates of decay rates based on IgG$_1$ and IgG$_3$ were similar to corresponding estimates based on total IgG. For IgG$_1$ and IgG$_3$, half-lives of long-lived ASCs ranged from 800 to 1424 days (i.e. 2.1-3.9 years). There was greater variability in half-lives of long-lived ASCs producing IgG$_2$ and IgG$_4$, for which estimates ranged from 729 to 2232 days (i.e. 2.0–6.1 years). Previously exposed individuals were estimated to have a greater contribution from long-lived ASCs for most subclass responses, however, there was substantial variability between subclasses and antigen specificities and the estimated proportion of long-lived ASCs ranged from 5% (95% CrI: 2 – 89) to 85% (95% CrI: 56 – 99). In previously naïve individuals, the estimated proportion of long-lived ASCs was similar across antigen specificities within each IgG subclass and ranged from 2% (95% CrI: 0 – 14) to 23% (95% CrI: 9 – 42).
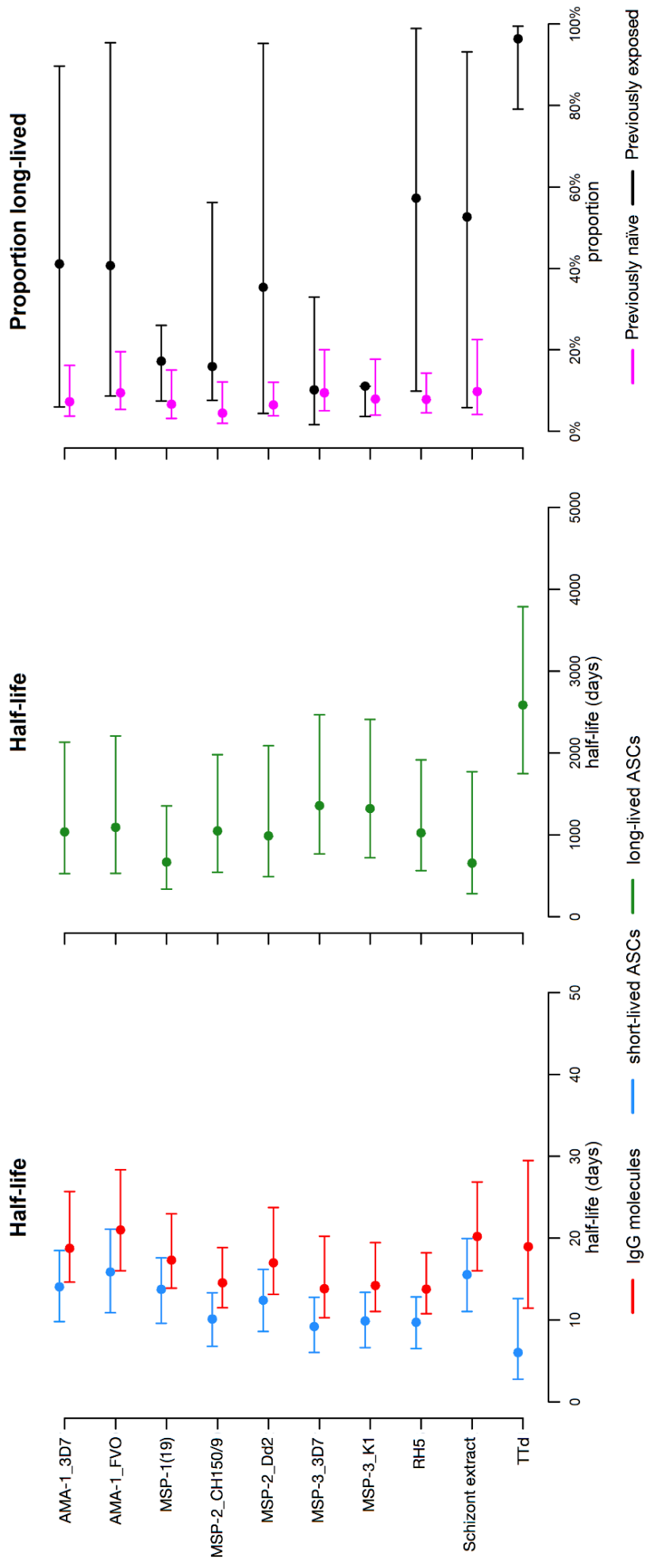
Figure 12. Parameter estimates for the dynamics of the total IgG response to *P. falciparum* antigens and TTd. Dots denote the model parameter estimates and capped error bars the corresponding 95% CrI.

4.4 STUDY IV

To identify novel candidate serological markers of recent and cumulative malaria exposure, we exploited the insights regarding antibody dynamics obtained from study **III**. We used a protein microarray approach to measure the antibody response to 111 *P. falciparum* blood-stage antigens in 65 adult travellers (21 without and 44 with prior malaria exposure) followed longitudinally for one year in a malaria free country after a successfully treated *P. falciparum* infection.

We identified 86 of the 111 tested antigens as immunoreactive in the cohort under study. Although the particular dynamics of the antibody response differed for each antigen, the majority of responses exhibited a substantial increase in magnitude after the acute infection, with a peak in the response around day 10, followed by a subsequent gradual loss of the response throughout the remainder of the follow-up period. Individuals with prior malaria exposure displayed greater magnitude and breadth of the response, in particular at the time of diagnosis. For MSP-1 and MSP-2 antigens, as well as MSP-4, MSP-10, AMA-1, EBA-175, and EBA181, individuals with prior exposure displayed a greater magnitude of the antibody response throughout the follow-up period, suggesting that the response to these antigens provide information on cumulative exposure. Antibody decay rates, estimated using linear mixed effects models, were antigen dependent and estimated to be highest for MSP2CH150/9DBL2, PF3D7_1136200, GAMA, MSP33D7, PF3D7_0206200, MSP8, and PTEX150 while lowest for PFA044W, CLAG3.2, RH2b, PFA0210c, and PF3D7_1460600(ISP3). The estimated half-lives of the response ranged from 129 days (95% CI: 113-150 days) for MSP2CHO150/9DBL2 to 795 days (95% CI: 425 – 6285 days) for PF0445w. The reactivity towards antigens for which the estimated half-lives were longest tended to be low. To evaluate whether the antibody responses to any of the antigens could be used to classify if a sample had been collected from an individual that had been recently infected (i.e. a recent infection defined as having occurred within 90 days of sample collection) we used binary classification based on logistic regression. First, we fitted univariable logistic regression classifiers to the antibody data for each antigen individually and compared the performance of the classifiers using cross-validated ROC analysis. In this univariable analysis the best classification performance was obtained using data for GAMA, PTEX150, PF3D7_1136200, and *Pf*SEA-1, for which the cross-validated AUCs all exceeded 0.8 (Figure 13). For a majority of responses, however, classification performance was relatively poor.

We then proceeded to evaluate whether combining data on antibody responses for up to five antigens could improve the classification performance of the logistic regression classifier. Because there are 34,826,302 ways to select a combination of five antigens out of 86, we used a feature selection algorithm based on a random forest classifier to reduce the number of antibody responses to be evaluated. We limited our evaluation to antigens that were determined by the feature selection algorithm to contribute significantly to classification of whether or not an individual had been recently infected and identified 24 antigens that were

selected for further evaluation. We tested all possible two to five-way combinations of antibody responses to these 24 antigens. We found that combining information on the antibody response to more than one antigen provided limited improvement of overall classifier performance as indicated by minor increase in cross-validated AUC. The cross-validated AUC of the best classifier increased from 0.83 to 0.84 with the sequential addition of up to five antibody responses. The best classifiers based on a combination of antibody responses all included the response to GAMA, however, these classifiers did not perform significantly better than a classifier based on the response to GAMA alone.
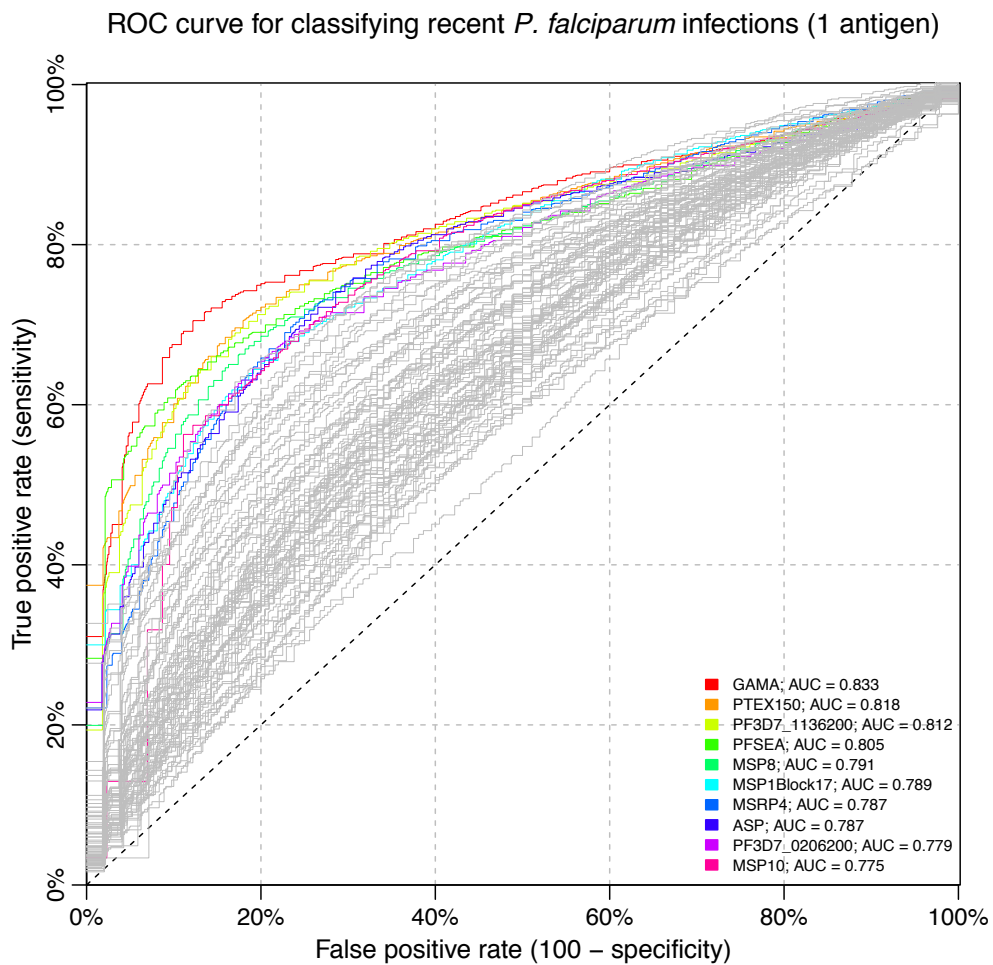

Figure 13. Antibody responses predictive of recent infection. Cross-validated ROC-curves of classifier performance for binary classifiers based on antibody data for each of the 86 immunoreactive antigens.

5 DISCUSSION

The studies presented within this thesis examine the temporal transmission patterns in a rural Tanzanian setting and present new methodology for the surveillance of infectious disease transmission. They also provide quantitative estimates of antibody dynamics, and characterise the broad specificity of the response following a single malaria infection. Together they contribute to our overall understanding of the acquisition and maintenance of the antimalarial antibody response and provide new information on how antibody responses to *P. falciparum* can be explored as markers of exposure.

In study **I,** data and samples from cross-sectional surveys conducted within a longitudinally followed cohort in Nyamisati, a rural Tanzanian village, were analysed. Study **I** provides important long-term data on the temporal trends in *P. falciparum* prevalence within a closely monitored population and contributes to the understanding of the changing malaria prevalence in East Africa. We identified a substantial reduction in the prevalence of *P. falciparum* infection from 1985 to 2010. The findings are consistent with reports from across the African continent, including Tanzania, where the decline in transmission intensity after year 2000 has been largely attributed to increasing coverage of ITNs (5,205). However, in our cohort, a decline in *P. falciparum* prevalence preceded the introduction of ITNs, and started shortly after the arrival of the research team in 1985. Similar observations have been reported from elsewhere in Tanzania as well as from Kenya and Senegal (206–208). Although several factors may have contributed to this initial decline, it is likely that the presences of a research and healthcare team in the village made a considerable impact. This further highlights the importance of prompt diagnosis and treatment in order to limit malaria morbidity as well as transmission, as has been previously described (5,110,206–208). ITNs were introduced in Nyamisati village at large scale in 1999, after which malariometric monitoring ceased when the research team moved from the village. A new cross-sectional survey, conducted in 2010, revealed a substantial further decline in the prevalence of *P. falciparum* infection in Nyamisati. In addition, we identified a substantial reduction in the overall prevalence of anaemia during the study period, consistent with the reduction in malaria burden and possibly a general improvement of health status (36). The lack of surveillance between 1999 and 2010 makes it difficult to evaluate which factors may have contributed to the change in malaria burden. Between year 2000 and the beginning of 2010 there was a substantial scale-up of the national malaria control efforts in Tanzania. This started with the introduction of IPTp in 2002, and was followed by a nation-wide distribution of ITN vouchers for pregnant women and infants beginning in 2004, the implementation of ACTs as first-line treatment from December 2006, and an expansion of the ITN program to under-fives in 2008 (205). However, the ITN campaigns did not reach Rufiji to any larger extent prior to 2010, and ACTs were not readily available in Nyamisati until 2009, and are thus not likely to have contributed substantially to the observed reduction in parasite prevalence between 1999 and 2010 (205).

Studies conducted at several sites in the coastal area of the Tanga region, where organised vector control efforts were limited, report substantial changes in abundance and composition of the vector populations during the corresponding time period (209,210). Unfortunately no data on vector populations were collected in Nyamisati during the project.

The long time intervals between the cross-sectional surveys and the absence of data on vector populations hinder efforts to elucidate the temporal transmission patterns and evaluate potential causes of the observed decline in parasite prevalence between 1999 and 2010. This highlights the need for alternative tools for surveillance in areas where only data from limited number of cross-sectional surveys are available (123,128,132).

Cross-sectional data on age-specific antibody responses to *P. falciparum* antigens have successfully been used to evaluate temporal trends in malaria transmission (146,170,171) and antibody responses to *Anopheles gambiae* salivary gland protein 6 (gSG6) has been highlighted as a useful surrogate marker for malaria vector exposure (136,138). In study **II**, we aimed to improve serological methods for transmission monitoring based on cross-sectional data by developing new antibody acquisition models which assume that the rate of increase in antibody levels with age can be used as an alternative marker of transmission intensity. Using both the previously validated serocatalytic models and the novel antibody acquisition models to further examine the transmission patterns in Nyamisati (described in study **I**) allowed us to compare their performance. Models were fitted to age-specific data on antibody responses to MSP-1, MSP-2, MSP-3, AMA-1, and gSG6 in 1-16 years old children participating in cross-sectional surveys conducted in 1999 and 2010. We demonstrated that the new antibody acquisition models, which avoid the loss of information that occurs when antibody levels are dichotomised, increased both the precision and power of transmission estimates (168). They enabled us to establish that a 72-92% stepwise decrease in transmission intensity occurring between 1997 and 2000 was likely to have been the single most important reduction event during the period under study. The serocatalytic models did not have the statistical power to determine whether a stepwise reduction in transmission was more likely than a continuous decline, and although similar point estimates for the time of change were obtained, these displayed considerable uncertainty. The estimated timing of the stepwise reduction in transmission intensity coincides roughly with the distribution of ITNs in Nyamisati that was conducted in 1999. Furthermore, the substantial reduction in both levels and prevalence of antibodies to gSG6 between 1999 and 2010 clearly indicated a reduction in vector exposure. However, the current modelling approach provided poor fit to the gSG6 data due to the absence of an age-trend in the antibody response. As demonstrated in study **I**, parasite prevalence in Nyamisati declined gradually during the 1990's, and although several factors may have contributed to the low level of parasite and vector exposure in 2010, the results provide strong support of the largest single reduction in transmission intensity being related to the distribution of ITNs in 1999. The antibody acquisition models performed better than the corresponding serocatalytic models in the context of high to moderate transmission intensity in which study **II** was conducted.

In such a setting the sensitivity and precision of serocatalytic models often are limited by a long duration of seropositivity and therefore by saturation in seroprevalence already among children (211–213). In study **II,** it is likely that this phenomenon contributed to the uncertainty of the serocatalytic model parameter estimates. Antibody levels do not display an age-dependent saturation to the same extent and furthermore, antibody levels have been shown to decline rapidly when exposure is reduced (68,77,78,171,214). These two factors contribute to the additional power of the antibody acquisition models to detect transmission changes, in particular when transmission intensity is high (214). In contrast, serocatalytic models may be more useful when transmission is low because data on seropositivity for multiple antigens can easily be combined to improve sensitivity to detect seroconversion events (215). In study **II**, antibody responses to AMA-1 provided most precise parameter estimates and most consistent transmission estimates between the two modelling approaches. There was little saturation in antibody levels to AMA-1 with increasing age and antibody levels to each of the two allelic variants of AMA-1 were highly correlated. This suggests that AMA-1 may be suitable as a serological marker for monitoring of medium to long-term transmission trends in a wide range of transmission settings.

The issue of parameter identifiability is a critical factor when modelling cross-sectional data on antibody responses to estimate the rate of seroconversion or antibody acquisition (216). This means that in cases where data are limited, it may be impossible to distinguish between a scenario in which individuals acquire and lose antibodies rapidly or the opposite in which antibodies are both acquired and lost slowly leading to unreliable estimates of transmission intensity (216). An additional challenge for serological surveillance, particularly in elimination settings, is the difficulty to differentiate recent from more distant exposure (132,217). Further improvement of serological tools for transmission monitoring therefore requires a better understanding of the factors that determine the acquisition and maintenance of the antimalarial antibody response. Reliable quantitative estimates of antigen specific antibody decay rates and identification of novel serological markers, or combinations of markers, that discriminate between recent or more distant exposure are also needed (132,217).

In study **III**, we provided a detailed characterisation of the dynamics of the antibody response to *P. falciparum* vaccine candidate antigens (MSP-1$_{19}$, MSP-2, MSP-3, AMA-1 and RH5) after a single clinical malaria episode in complete absence of re-exposure by studying malaria exposed travellers followed prospectively for one-year after treatment in Sweden. We used mathematical modelling of antibody dynamics to provide quantitative estimates of the longevity of antibodies and antibody secreting cells. The breadth and magnitude of the response was, as expected, greater among individuals with prior malaria exposure. For a majority of the evaluated antigens, differences in the magnitude between exposure groups were particularly evident in levels of IgG$_1$ and IgG$_3$**,** which have previously been associated with protection from clinical disease (53,218–220).

A greater boosting of antibody levels, as was observed in previously exposed individuals, corresponds to the generation of a greater absolute number of short-lived ASCs. This was likely due to the presence of a previously established memory B-cell response, rapidly proliferating and differentiating into short-lived ASCs upon re-infection (69–71).

When examining the antigen-specific total IgG response, we found that the half-lives of short-lived ASCs ranged from a few days to a few weeks, whereas the half-life of long-lived ASCs ranged from 2-4 years. These estimates were highly comparable with previous results from African children (68,77,78). Half-lives of IgG_1 and IgG_3 producing long-lived ASCs were similar to those estimated from the total IgG response, while estimates for long-lived ASCs producing IgG_2 or IgG_4 tended to be slightly longer. Interestingly, we found that individuals without prior malaria exposure did acquire long-lived ASCs following a primary malaria infection. However, in these individuals the numbers of acquired long-lived ASCs were relatively small and, in the case of total IgG responses, on average represented less than 10% of the ASCs generated. We showed that individuals with prior exposure generated and maintained a larger number of long-lived ASCs. This was supported both by an overall higher proportion of ASCs that were long-lived and by the maintenance of higher antibody levels at the end of follow-up, reflecting maintenance of a greater absolute number of long-lived ASCs. To provide a validation of the model structure and the reliability of model estimates, we fitted the same model to data on the dynamics of the response to tetanus toxoid in the same individuals. The longevity of the antibody response to TTd has been well characterised. We found that the estimated half-lives of long-lived tetanus-specific ASCs were highly consistent with previously published estimates (i.e. approximately 7-14 years) (74,221,222) and determined that this provided a validation of our model estimates for the *P. falciparum* antigens.

Accumulating evidence suggests that the immune environment induced during a *P. falciparum* infection inhibits the development of a long-lived antibody response. This has been proposed to be mediated through a dysregulation of the B-cell response in which impaired T-cell help and germinal centre formation (80,81) leads to preferential induction of short-lived ASCs and the generation of so called atypical memory B-cells (57,68,82,83,223). In study **III,** we found that the half-life of long-lived ASCs specific for the evaluated malaria vaccine candidate antigens was notably short in comparison with that of long-lived tetanus specific ASCs. In addition, we also found the acquisition of long-lived ASCs following primary malaria infection to be low.

Study **III** demonstrated that exposure dependent differences in antibody dynamics can be described by differences in the size of the antibody boost and the acquired proportion of long-lived ASCs. In light of these results, we propose that the relatively short-lived nature of the naturally acquired antibody response following primary malaria infection could be attributed to a poor acquisition and short half-life of long-lived ASCs. Furthermore, we propose that more long-lived antibody responses are acquired over time with repeated infections by small consecutive additions to the pool of long-lived ASCs with each new infection.

This is supported by data from Ghana and the Gambia, where the proportion of long-lived ASCs in children was estimated to increase with age (68,77), and could also partly explain the observation of a more rapid seroreversion in children, compared to adults, in endemic areas following interruption of transmission (172).

The results from study **III** contribute to our understanding of naturally acquired immune response to malaria and can be used to guide strategies for further development of both vaccines and serological tools to monitor exposure. To date a vast majority of successful vaccines against various infections induce protection mediated through antibodies that are maintained following just a few immunisations (101). Antibodies are likely to be important also for mediating protection in the context of a malaria vaccine (89). The findings in study **III** emphasise the importance of a vaccine response not only to mimic the naturally acquired immune response but also the need for a malaria vaccine to skew the humoral response towards generation of long-lived ASCs through improvement of delivery platforms, adjuvants and by optimisation of dosage and vaccine regimens (89).

The quantitative estimates of the antibody dynamics presented in study **III** can be used to improve serological estimates of transmission intensity based on cross-sectional data on the response to these antigens. However, further improvement of serological tools for disease surveillance will require identification of antibody responses to novel target antigens that change predictably over time, regardless of the individual's prior level of exposure. Data on the dynamics for such an antibody response can theoretically be exploited to estimate when the individual was last infected which would in turn allow for disease incidence to be estimated from cross-sectional antibody data (131).

In study **IV,** we used a protein microarray containing 111 *P. falciparum* antigens with the aim to examine differences in the specificity and dynamics of the response related to prior exposure, to characterise the rate of antibody loss following clearance of infection and to evaluate the predictive performance of the tested antigens as serological markers of either cumulative or recent exposure. For several antigens, in particular AMA-1, MSP-1, MSP-2, MSP-4, MSP-10, EBA-175, and EBA-181, the magnitude of the antibody response was substantially and significantly greater throughout the follow-up period in individuals with prior malaria exposure. This suggest that these antigens to a larger extent induce a memory response in the form of memory B-cells and long-lived plasma cells (63,65,70). In line with previous findings, the exposure related differences in the response to these antigens further suggest they may be informative of an individual's cumulative malaria exposure. This has been previously demonstrated for AMA-1, MSP-1, and MSP-2 (147,152,153), and to a lesser extent reported also for MSP-4, MSP-10, and EBA-181 (156,224,225). For a majority of the evaluated antigens, however, there were no significant differences in the magnitude of the response related to prior exposure.

Although we observed high levels of antibodies to several of these antigens, in particular towards GAMA, MSP-8, RAMA, *Pf*SEA-1, PF3D7_1136200, PF3D7_0206200, and PTEX150, the response to these antigens did not appear to be maintained over time. This was further supported by the higher rate of antibody decay estimated for these antigens, suggesting a poor induction of a long-lived plasma cell response (68,86).

Interestingly, there were overall substantial antigen dependent differences in the rate of antibody decay with estimated antibody half-lives ranging from 129 (95% CI: 113 – 150) to 795 (95% CI: 425 – 6285) days. We identified antibody response to 24 *P. falciparum* antigens that, either independently or in combination, provided information on whether an individual had been infected within the last three months. Among these 24 antigens, only MSP-1, MSP-2, and MSP-10 had previously been brought forward as useful markers of exposure (146,217,226). The 24 antigens were selected for a more in depth evaluation. Through binary classification using logistic regression we determined that for single antigens the best prediction of recent exposure was achieved using data on the response to GAMA, PTEX150, PF3D7_1136200, or *Pf*SEA-1, for which the cross-validated AUC of the binary classifiers exceeded 0.8. We further evaluated all possible combinations of antibody responses for up to five of the 24 selected antigens and found that combining data on the response to more than one of these antigens did not provide major improvements of the ability to predict a recent exposure. We found the response towards GPI-anchored micronemal antigen (GAMA), *Plasmodium* translocon of exported protein (PTEX150), PF3D7_1136200, and *P. falciparum* schizont egress antigen 1 (*Pf*SEA-1) to be the most informative. We believe that the responses to these antigens show promise as candidate serological markers of recent malaria exposure and should be further evaluated.

In summary, the studies presented within this thesis evaluate different methods to estimate malaria transmission intensity and examine multiple aspects of the antimalarial antibody response on both a population and individual level. The studies provide information that contributes to our understanding of the acquisition and maintenance of the antimalarial antibodies and will help improve serological methods for malaria surveillance. While many aspects of the antibody responses studied here are specific for malaria, the overall concepts are generally applicable with regards to antibody responses in infection. The results presented here may therefore provide guidance for future studies on antibody responses to other infectious diseases.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

- Parasite prevalence, measured by microscopy or PCR, is commonly used to estimate *P. falciparum* transmission but evaluation of spleen rate and haemoglobin levels remain useful complements to these methods for estimating the malaria burden in areas of intense transmission where infection prevalence is high.
- Antibody acquisition models can improve precision in estimates of transmission intensity based on cross-sectional serological data, particularly in settings of moderate and high transmission intensity where seroprevalence is high.
- Quantitative estimates of the immune parameters underlying the dynamics of the antibody response are imperative for understanding acquisition and maintenance of antibody-mediated immunity and for further improvement of serological methods for transmission surveillance.
- The overall short-lived nature of the antibody response to natural malaria infection can be attributed to a combination of an inefficient acquisition and a short half-life of long-lived ASCs.
- The dynamics of antimalarial antibody responses are antigen specific. For some antigens, a more long-lived response is acquired after repeated infections, while for others, this type of memory responses is not induced to the same extent.
- Identification of novel serological markers of recent malaria exposure, which can be used to estimate the time since the last infection, would greatly improve transmission surveillance. Antibody responses to *P. falciparum* antigens GAMA, *Pf*SEA-1, PF3D7_1136200, PTEX150 appear to be interesting candidate markers of recent malaria exposure that warrant further investigation.

While several countries are currently experiencing a situation where malaria transmission has declined to the extent that malaria elimination appears achievable, others provide concerning reports of resurgence in transmission after years of positive trends.

The most efficient way to further limit malaria transmission is likely through a highly effective vaccine. However, despite substantial efforts for development, such a vaccine remains elusive. The most advanced malaria vaccine to date, RTS,S, provides only partial protection and vaccine efficacy appears to wane quickly as antibody responses decline. Although our understanding of the antimalarial immune response to both natural infection and vaccination has improved significantly in recent years, the lack of reliable immunological correlates of protection hampers vaccine design efforts. Identification of correlates of protection would substantially accelerate vaccine discovery and should be explored through future studies taking a systems immunology, or systems serology, approach. These studies will need to simultaneously evaluate multiple immunological parameters by combining data on the magnitude as well as the functional aspects of the immune response.

Preferably these studies should also investigate the factors that cause the large inter-individual variation in the immune response to both natural infection and vaccination.

Independent of vaccine development, and irrespective of the current transmission situation, maintaining a robust system for disease surveillance is imperative in order to generate reliable information that can be used to prioritise resources for malaria control and limit transmission.

As previously discussed, serology is a tool that could be useful and help improve malaria surveillance particularly in low transmission settings where the prevalence of infection is low, or in cases where data on disease incidence is limited or unreliable, as is often the case in malaria endemic areas. In order to make optimal use of serology as a tool for malaria transmission surveillance there is a need for identification of better and more reliable serological markers of exposure. However, any serological markers that appear informative in explorative analyses will need to be rigorously evaluated and validated to confirm their usefulness prior to implementation as a surveillance tool. Such a process will need to include careful evaluation of the candidate marker in cohort studies conducted in different geographical areas and different transmission intensity settings.

If reliable serological markers can be identified and serology is to be implemented on a larger scale for coordinated surveillance of disease transmission, standardisation of the serological assays to be used is critical. This concerns the recombinant proteins used as antigens, as well as the assay procedures, and includes assuring that the measured quantities are comparable and reproducible across laboratories, platforms, and situations. To date, there have been some efforts to increase the degree of standardisation within the research community, e.g. through sharing of protocols and standard calibrator preparations between laboratories, and by WHO to provide a commercially available lyophilised malaria reference serum. However, common standards have not yet become widely adopted. Ideally the format of the immunoassay should also be adapted to allow for easy implementation in resource-poor settings without the need for access to more advanced laboratory equipment. This could be achieved e.g. by further development of a validated standard serological ELISA assay into a lateral flow immunoassay format.

Monitoring and surveillance systems in countries approaching malaria elimination are facing new challenges. Tools suitable for monitoring of a disease that is endemic may not necessarily be useful in a near elimination setting or in a scenario where there is need to confirm that elimination has been achieved and is effectively maintained. In such a scenario the goal will not be to estimate the force of infection but rather to confirm interruption of transmission. This will require readjusting surveillance systems for this task as well as developing new tools that can provide robust estimates of the absence of transmission to support the process of malaria elimination.

7 ACKNOWLEDGEMENTS

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