Characterization of Humoral Immune Responses to Two Subunit Malaria Vaccine Candidates in Humans

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ZUSAMMENFASSUNG

Malaria ist eine der wichtigsten Infektionskrankheiten und betrifft weltweit mehrere Millionen Menschen. Eine wirksame Impfung gegen Malaria ist momentan noch nicht verfügbar, wäre aber eine wertvolle Ergänzung zu den bereits bestehenden Malaria-Kontroll-Strategien. Obwohl davon ausgegangen wird, dass Antikörper (AK) die natürlich erworbene partielle Immunität gegen den komplexen Malariaerreger vermitteln, bleiben der genaue AK-vermittelte Mechanismus bzw. die immunologischen Zusammenhänge, die zum Schutz gegen schwere Malaria führen, unklar. Standardisierte Versuche zur AK-Messung fehlen ebenfalls, weshalb keine Einigkeit darüber besteht, welche Methode am besten geeignet ist, um Antikörper gegen Malaria zu quantifizieren, zu beschreiben und zu interpretieren. In diesem Zusammenhang ist es das Ziel dieser Dissertation robuste standardisierte Verfahren für Messungen der *Plasmodium*-spezifischen AK einzuführen und die AK-Avidität als einen potenziellen Marker des impfvermittelten Schutzes gegen Malaria zu untersuchen.

Im ersten Teil wird eine neue zytometriebasierte Immunfluoreszenzmethode, bei der ganze Parasiten verwendet werden, beschrieben. Auf diese Weise wird versucht den Schutz vor Malaria mit der AK-Antwort zu erklären. Ein neu entwickelter Subtraktionsalgorithmus (overlap substraction algorithm (OSA)) ermöglicht die untersucherunabhängige Analyse der Daten. Im Rahmen der Dissertation wurden Messungen an Proben von Kindern und Erwachsenen, die Teilnehmer in einer klinischen Studie Phase I für den Malaria-Impfkandidaten GMZ2 waren, vor (D0) und nach (D84) der Impfung durchgeführt. Die Ergebnisse zeigen, dass Kinder, die mit der höchsten GMZ2-Dosis (100µg) geimpft wurden, einen 1,33-fachen Anstieg des Anteils fluoreszierender Zellen (percent positive fluorescent cells (PPFC; p=0,003)) an Tag 84 im Vergleich zu Tag 0 aufweisen. An Tag 84 konnte ein impfinduzierter verstärkender Effekt auf die bereits existierende anti-parasitäre Immunität (1,23-facher Anstieg in der mittleren Fluoreszenzintensität (mean fluorescent intensity (MFI), p=0,03) in semi-immunen Erwachsenen nachgewiesen werden.

Im zweiten Teil dieser Arbeit wurde eine modifizierte ELISA-basierende Methode implementiert, um den Aviditäts-Index (AI) von anti-Circumsporozoite Protein (CSP) AK von zwei unterschiedlichen Immunisierungsschemata (0-1-2 Monate und 0-1-7 Monate) mit dem Malariaimpfstoff RTS,S in einer Kohorte von gesunden afrikanischen Säuglingen zu untersuchen. Die Analysen zeigen, dass die Avidität der anti-CSP AK nach RTS,S Impfung wie erwartet ansteigt, die absolute AI die Impfeffektivität aber nicht vorhersagt. Die AIs der AK waren in beiden Immunisierungsplänen vergleichbar. Hervorzuheben ist, dass die Änderung der anti-CSP AK Titer (dCSP) und des Aviditäts-Indexes (dAI) zwischen der zweiten und der dritten Immunisierung mit 77% und 54% Reduktion des Risikos zur Entwicklung einer Malaria, assoziiert ist. Die Entwicklung der Avidität von CSP-spezifischen AK sollte in weiteren Studien untersucht werden, um zu sehen ob sie ein Marker für die Wirksamkeit von RTS,S ist.

Zusammenfassend lässt sich sagen, dass standardisierte neue Instrumente entwickelt wurden, um parasitenspezifische AK-Antworten zu untersuchen und die detaillierte Erforschung von anti-CSP AK-Avidität erweitert das momentane Verständnis der AK-vermittelten Immunität gegen Malaria. Diese Untersuchungen können als Basis für zukünftige Arbeiten zur AK-basierter Immunität für Malaria dienen und zur Entwicklung und der Evaluation von funktionellen anti-Malaria Impfungen der zweiten Generation beitragen.

SUMMARY

Malaria remains a major public health scourge affecting millions of people worldwide. An effective antimalarial vaccine is currently lacking and if available would add to other malaria control strategies. Although antibodies (Abs) are thought to mediate protective immunity to malaria, the exact Ab-mediated mechanisms or immunological correlates of protection against the complex plasmodial parasite remain unclear. Standardized assays for Ab measurement are also lacking and therefore no consensus exists on the best approach to quantitate, report and interpret antimalarial Abs. In an attempt to address some of these hurdles, this dissertation aims to implement robust standardized assays for measurements of *Plasmodium*-specific Abs and to investigate Ab avidity as a potential surrogate marker of vaccine-mediated protection against malaria.

In the first study, a novel cytometric based immunofluorescence assay technique is described that improves the detection of anti-plasmodial Abs using whole parasites and may be suitable for investigating Ab-based correlates of protection. An overlap subtraction algorithm (OSA) developed in parallel eliminates the investigator-dependent effects and thus facilitates the data analysis process. The workflow was applied to pre- (D0) and post-vaccination (D84) clinical samples from children and adult participants of Phase 1 trials of the malaria vaccine GMZ2. The results demonstrate that children vaccinated with the highest GMZ2 dose (100μg) showed a 1.33-fold increase in percent positive fluorescent cells (PPFC; p=0.003) on D84 compared to D0. Meanwhile, on D84, a vaccine-induced boosting effect of pre-existing anti-parasitic immunity (1.23-fold increase in mean fluorescent intensity; MFI, p=0.03) was observed in semi-immune adults.

In a second study, a modified ELISA-based method to assess the avidity index (AI) of anti-circumsporozoite protein (CSP) Abs elicited by two immunization (0-1-2 month and 0-1-7 month) schedules with the malaria vaccine RTS,S in a cohort of healthy African infants was used. The analyses revealed that the avidity maturation of anti-CSP Abs following RTS,S vaccination occurred as expected, although absolute AI did not predict vaccine efficacy. The AIs of Abs were found to be similar in both immunization

schemes. Interestingly, the change in anti-CSP Ab titer (dCSP) and avidity index (dAI) between second and third immunization was associated with 77% and 54% risk-reduction to develop clinical disease, respectively. Avidity maturation of vaccine-specific Abs deserves further investigation as surrogate marker of protective efficacy.

Together, standardized new tools for investigating parasite-specific Ab responses were developed and the detailed investigation of anti-CSP Ab avidity expands contemporary understanding of Ab-mediated indicators of protective immunity against malaria. These studies might serve as a basis for further work on Ab-based immunity to malaria and contribute to the development and evaluation of functional second-generation antimalarial vaccines.

LIST OF PAPERS

This doctoral dissertation is based on the following two original papers, which will be referred to by their Roman numerals:

I. **Ajua A**, Engleitner T, Esen M, Theisen M, Issifou S, Mordmüller B. A flow cytometry-based workflow for detection and quantification of anti-plasmodial antibodies in vaccinated and naturally exposed individuals.
Malaria Journal 2012; 11:367.

II. **Ajua A**, Lell B, Agnandji ST, Asante KP, Owusu-Agyei S, Mwangoka G, Mpina M, Salim N, Tanner M, Abdulla S, Vekemans J, Jongert V, Lievens M, Cambron P, Ockenhouse CF, Kremsner PG and Mordmüller B. The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01$_E$ on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants.
Malaria Journal 2015; 14:72.

Contributions to other publications not included in this dissertation:

III. Mamo H, Esen M, **Ajua A**, Theisen M, Mordmüller B, and Petros B. Humoral immune response to *Plasmodium falciparum* vaccine candidate GMZ2 and its components in populations naturally exposed to seasonal malaria in Ethiopia. *Malaria Journal* 2013; 12:51.

IV. Esen M, Forster J, **Ajua A**, Spänkuch I, Paparoupa M, Mordmüller B, and Kremsner PG. Effect of IL-15 on IgG versus IgE antibody-secreting cells in vitro.
Journal of Immunological Methods 2012; 375(1-2): 7-13.

LIST OF ABBREVIATIONS

Abs	Antibodies
ADCI	Antibody-dependent cellular inhibition
AI	Avidity index
AMA	Apical membrane antigen
$AS01_E$	Adjuvant system associated with RTS,S malaria vaccine candidate
ASCs	Antibody secreting cells
CD	Cluster of differentiation
CHMI	Controlled human malaria infection
CSP	Circumsporozoite protein
DCs	Dendritic cells
DDT	Dichlorodiphenyltrichloroethane
EBA	Erythrocyte-binding protein
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded program on immunization
FMP	Falciparum malaria protein
GIA	Growth inhibition assay
GLURP	Glutamate rich protein
GMZ2	Blood-stage malaria vaccine candidate
HBV	Hepatitis B virus
HPV	Human papillomavirus
IFA	Immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITNs	Insecticides-treated mosquito nets
ME-TRAP	Multiple-epitope–thrombospondin-related adhesion protein
MFI	Mean fluorescent intensity
MSP	Merozoite surface protein
NHP	Non-human primates
NK	Natural killer

OSA	Overlap subtraction algorithm
*Pf*RH5	*Plasmodium falciparum* reticulocyte-binding protein homologue 5
*Pf*SPZ	*Plasmodium falciparum* sporozoite
PPFC	Percent of positive fluorescence cells
PPRs	Pattern recognition receptors
RBC	Red blood cell
RTS,S	Pre-erythrocytic stage malaria vaccine candidate
TCM	Central memory T cells
TEM	Effector memory T cells
Th	T helper
TLRs	Toll-like receptors
Tregs	Regulatory T cells
TNF	Tumor necrosis factor
WHO	World Health Organization

1. GENERAL INTRODUCTION

1.1 Malaria: causative agent, epidemiology and control

Malaria is an infectious disease caused by obligate intracellular protozoal parasites which belong to the genus *Plasmodium*. The parasite is transmitted from human to human by infected female *Anopheles* mosquitoes. Currently, five plasmodial species are able to infect humans, namely *P. falciparum*, *P. vivax*, *P. ovale, P*. *malariae*, and *P. knowlesi*. In tropical Africa, *P. falciparum* is the most common species and is responsible for nearly 225 million cases of malaria and almost a million deaths annually, mostly among children below 5 years of age and pregnant women [1]. Meanwhile, in Asia and Latin American countries most of the remaining malaria cases are caused by *P. vivax*. The temperate regions (USA, Canada and Europe) that were also endemic for malaria are now free of malaria today due to successful implementation of malaria control including insecticide programs using dichlorodiphenyltrichloroethane (DDT) (**Figure 1**) [2].

The continued large-scale implementation of malaria control strategies in Africa, such as insecticides-treated mosquito nets (ITNs) and highly effective artemisinin-based therapies, has led to a significant reduction in malaria case incidence (reviewed in [3]). Nevertheless, malaria transmission is difficult to control in most endemic countries, partly due to the rise of insecticide and antimalarial drug resistance, even to the highly effective artemisinin-based therapies [4, 5]. This underscores the urgent need to develop more reliable interventional tools, including efficacious antimalarial vaccines to curb the disease.

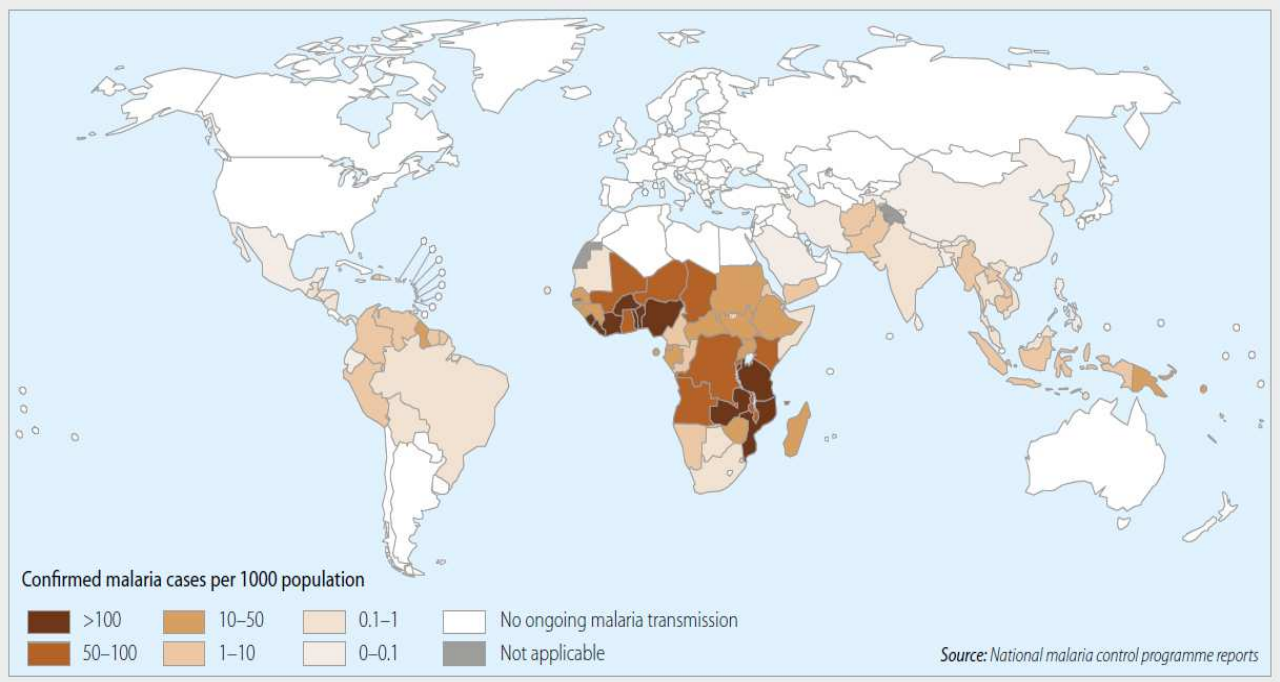

Figure 1. Intensity of malaria in countries with ongoing malaria transmission, 2013. Source: WHO World Malaria Report, 2014 [2].

1.2 Life cycle of *Plasmodium* spp.

The malaria life cycle is complex with different parasite stages developing in both the human and mosquito hosts (**Figure 2**). The cycle begins following the mosquito's injection of saliva containing infective sporozoites into human skin. The sporozoites exit the skin tissues and travel through the bloodstream and ultimately invade liver cells, multiply and differentiate asexually as exoerythrocytic stage parasites (liver-stage). During development in hepatocytes, the host presents no clinical symptoms of the disease.

Depending on the parasite species, maturation of liver-stages takes five to ten days. One infected liver cell releases thousands of merozoites into the blood stream that invade erythrocytes, grow, multiply and ultimately lead to the infection of up to ten new erythrocytes (blood-stage). This asexual blood stage results in a feed-forward loop with exponential growth of the parasite population if it is not controlled by immunity,

metabolic restriction or antimalarial treatment. It is therefore responsible for the symptoms and pathology of malaria. The erythrocytic cycle follows a variable periodic pattern: one day for *P. knowlesi*; two days for *P. falciparum*, *P. ovale* and *P. vivax* or three days in the case of *P. malariae* (reviewed in [3]).

During the erythrocytic cycle, some merozoites do not multiply but instead differentiate into the male and female gametocytes. When taken up by the mosquito during feeding, these sexual forms may fertilize within the mosquito's midgut to form a zygote, which matures to become an ookinete and subsequently the oocyst, which ultimately releases sporozoites, the infective form of the parasite. The sporozoites migrate to the mosquito's salivary glands and become available to infect the next host, thus completing the parasite's transmission cycle.

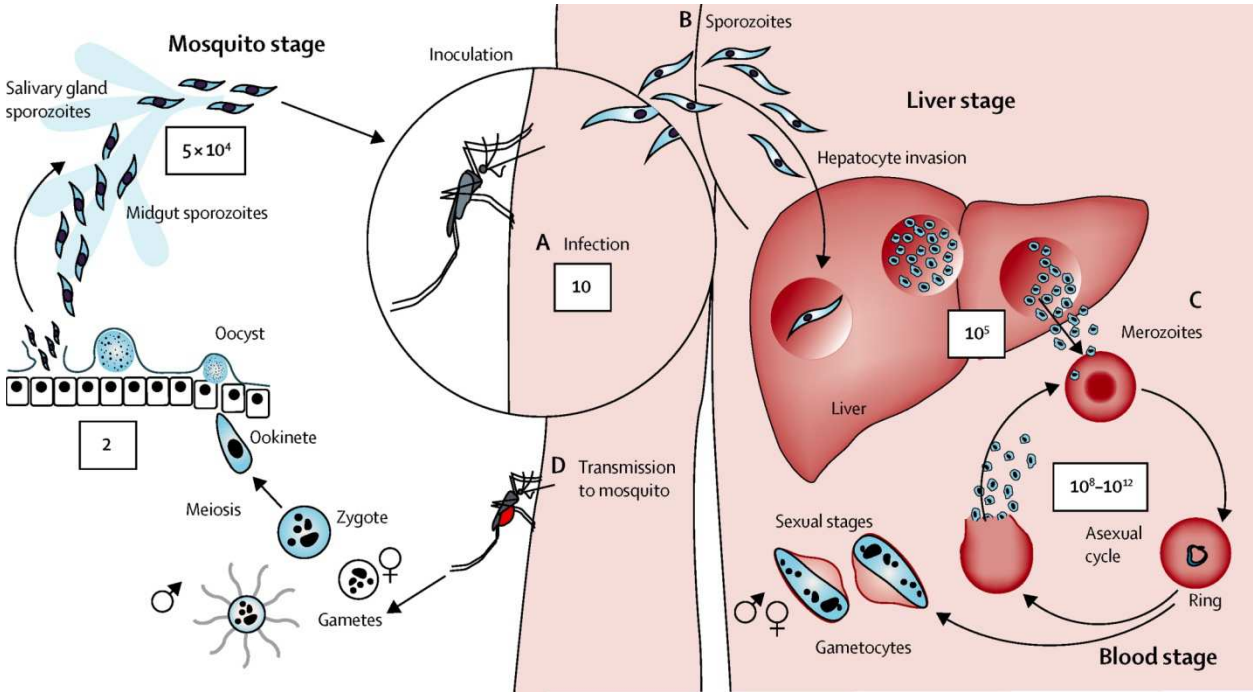

Figure 2. Life cycle of *Plasmodium* spp. in the human and mosquito hosts. Numbers in square boxes represent crude estimates of malaria parasites in the respective stage. Reprinted with permission from Elsevier Limited.
Source: White *et al*. 2014 [3].

1.3 Immunity to malaria

During malaria parasite development in the vertebrate host, many antigens produced by the different parasite stages are capable of provoking the host's innate, humoral and cellular immune responses. Successful activation of innate, humoral and cellular immune mechanisms can induce a complex network of defense machineries that lead to the release of immune mediators that can limit further growth and development of the parasite (reviewed in [6]) as described below or result in immunopathology including sepsis-like syndromes and other life-threatening complications.

1.3.1 Innate immunity to malaria

The innate immune system provides the first-line of defense against *Plasmodium* infections after the induction of non-specific immune effector cells that are capable of targeting and damaging the malaria parasite (reviewed in [6]). Pre-existing cells of the innate immune system are able to sense infection using pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) expressed in various cell types including dendritic cells (DCs) [7, 8]. Once sporozoites are injected into the host's skin, they could be detected and engulfed by DCs, monocytes, macrophages, natural killer (NK) cells or other cells of the innate immune system present near the inoculation site. The plasmodial antigens are processed and presented to T cells in association with MHC class II molecules [9, 10], which may result in proliferation of antigen-specific T cells. A successful immune response (i.e. a response that results in clearance of the pathogen) depends on multiple factors, including the strength of stimulation, type of infected cells and quality of the antigen recognition. Apart from resolving the infection, some immune cells are instrumental in fine-tuning the magnitude and quality of the ensuing antibody- or cellular-mediated immune responses [10-12].

Due to the large antigen load blood-stage parasites can initiate strong innate immune responses via the generation of interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-12 by different cell types (reviewed in [6]). However, studies in animal

models report that the parasite's liver-stages could equally induce potent innate immune responses mediated by IFN-γ secretion [13] when stimulated appropriately.

1.3.2 Antibody-mediated immunity to malaria

The humoral immune response against asexual blood stage parasites is marked by the generation of a large repertoire of Immunoglobulin G (IgG) secreting cells. So far, the role of IgM, IgA, and IgE is not well described [14]. However, antibodies are important in mediating protective immunity to malaria, as has been clearly shown in passive transfer experiments where hyper-immune serum [15] and purified total IgG [16, 17] were used to control blood-stage clinical malaria. Similar studies in non-human primates (NHPs) confirmed that protective antibodies are critical for clearance of asexual blood-stage malaria infections [18]. Additional studies further demonstrated that transfer of antibodies to the circumsporozoite protein (CSP) of *P. falciparum* can protect mice from subsequent sporozoite challenge [19-21].

The IgG-mediated protection is largely dependent on the breadth of specific cytophilic IgG1 and IgG3 antibodies (reviewed in [22]). Though parasite-specific IgE antibodies also increase during malaria infections, it is not clearly known how they contribute to antimalarial immunity [14]. In general, antibodies exert protective functions by inhibiting hepatocytes invasion (reviewed in [6]), merozoite invasion of erythrocytes [23], blocking parasite adherence and sequestration to host's tissues to avoid clearance in the spleen [24], and elimination of parasite-infected red cells by phagocytosis [25]. Antibody-dependent cellular inhibition (ADCI) has been proposed as a mechanism by which antibodies to glutamate rich protein (GLURP) and merozoite surface protein (MSP)-3 may confer protection [26]. Although IgG1, IgG3, tumor necrosis factor (TNF) and other molecules have been reported to play a role in ADCI [27], the IgG3 antibodies against an MSP3-derived peptide (LR55) have recently been identified as the major inducer of ADCI activity [28]. The relative contribution of the different antibody-mediated activities to control malaria following naturally acquired infection and vaccine-induced immunity are not known.

1.3.3 Cell-mediated immunity to malaria

As described earlier, during the pre-erythrocytic phase of development, neutralizing antibodies can block hepatocyte invasion by sporozoites. Subsequently, the parasite develops within liver cells, a compartment that is difficult to access by antibodies. Hence, parasite-infected liver cells seem to be targeted by CD8+ T cells via secretion of the cytolytic factors perforin and granzyme B (reviewed in [29]). This could explain why higher levels of granzyme B-producing CD8 T cells have been associated with protection from clinical malaria following challenge of human volunteers with sporozoite-infected mosquitoes [30].

Apart from CD8+ T cells, specific CD4+ T cells are also associated with a successful immune response against pre-erythrocytic stages. For example, a recent study reported the strongest association of protection with cytotoxic CD4 T cells in humans following sporozoites challenge via the bites of the mosquito vector [30]. Four main subsets have been reported based on their cytokine patterns, namely, T helpers (Th) 1, Th2, Th17 and Tregs. Th1 cells secrete inflammatory cytokines such as IL-2, IFN-γ, and TNF-α, which activate macrophages and other cells to produce their own mediators. Th2 cells produce mainly IL-4, IL-5 and IL-13, which regulate the humoral immune response by activating B cells to differentiate into antibody secreting cells (ASCs) and produce antibodies. In addition, CD4+ T cells potentiate and sustain CD8+ T cells responses as well as regulate the cytokine produced by Th1 and Th2 cells to equilibrium levels (reviewed in [31]), a balance that is likely to determine the immunological control of malaria parasites. The initial pro-inflammatory response following malaria infection is crucial for the control of parasite replication as well as clearance of infected red blood cells (RBCs). A lack of an effective pro-inflammatory response could lead to unrestricted proliferation of the parasite. Conversely, failure to regulate the inflammatory response may result in the development of severe malaria (reviewed in [32]). In addition to the cell-mediated responses described, antibody-mediated anti-parasitic activity is crucial for the final control and clearance of malaria parasites (reviewed in [6]).

1.3.4 Naturally acquired immunity and immunological memory to malaria

It is generally believed that naturally-acquired immunity to malaria is short-lived and requires repeated exposure to parasite antigens to generate and sustain an effective memory response. Immunological memory, which builds up over time is orchestrated by memory T and B cells. These cells are known to rapidly proliferate and differentiate into T and B effector cells upon pathogen encounter, where they play key roles in protective immunity to malaria. For instance, accumulated long-lived memory B cells rapidly secrete specific high-affinity and high-titered antibodies to the most frequently encountered parasite antigens that are instrumental for control of blood-stage parasite load (reviewed in [6, 33]).

Notwithstanding, memory responses to plasmodial antigens seem not to be very effective and likely attributable to the seasonal nature of malaria transmission in endemic areas, the complexity of the pathogen and its life cycle and the constant exposure of humans to persistent or intermittent malaria infection (reviewed in [34]). Other challenges include the fact that naturally-induced responses are mostly antigen- and stage-specific, the difficulty of inducing high amounts of antibodies of the required quality and the development of parasites in different intracellular host's systems shields them from recognition and attack by antibody-mediated mechanisms further limit the generation of potent long-term memory responses to malaria (reviewed in [6]).

The provision of long-lasting protection is important for successful immunization programmes and many vaccines are optimized to generate a robust, long-lived immunologic memory. In this light, successful vaccines, such as those against yellow fever, smallpox, polio, tetanus toxoid and measles are prime examples as they provide up to lifelong protection against re-infection through antibody- and cell-mediated mechanisms ([35], reviewed in [36]).

Although no vaccine is currently licensed for use against malaria, an efficient antimalarial vaccine may need to induce robust and strong immune responses in order to confer protection against clinical malaria [37]. Antibodies alone may not suffice to achieve high levels of protective efficacy. Hence, the induction of potent vaccine-

specific T cell responses may also be crucial to confer protective immunity to malaria (reviewed in [6]), especially when the pre-erythrocytic stages of the plasmodial life-cycle is targeted.

1.4 Malaria vaccine development

Throughout history, vaccination has been one of the most successful strategies of controlling human and animal diseases. Until today, all successful vaccines were developed empirically, with limited understanding of how they induce protective immunity. This makes it very difficult nowadays to apply the same approach to design highly efficacious vaccines for emerging diseases [38]. The same is true for malaria vaccine development, where so far no reliable protective mechanisms or immune correlates of protection have been identified. Compared with some viruses and bacteria that are controllable through vaccination, malaria parasites are much larger and complex, as the parasite contains approximately 5000 diverse proteins, some of them highly polymorphic. This complicates the task of developing highly protective subunit malaria vaccines and further explains why until date no malaria vaccine is available for clinical use (reviewed in [6, 39, 40]). Notwithstanding, intensive research is ongoing to develop stage-specific vaccines targeting the three major parasite developmental stages; sporozoites and infected hepatocytes (pre-erythrocytic stage), merozoites and infected erythrocytes (asexual blood stage), as well as gametocytes (**Figure 3**). The results so far are promising, as a good number of candidates described below are currently in clinical development (**Figure 4**). The present work will focus on the RTS,S and GMZ2 vaccine candidates since the author took part in their development.

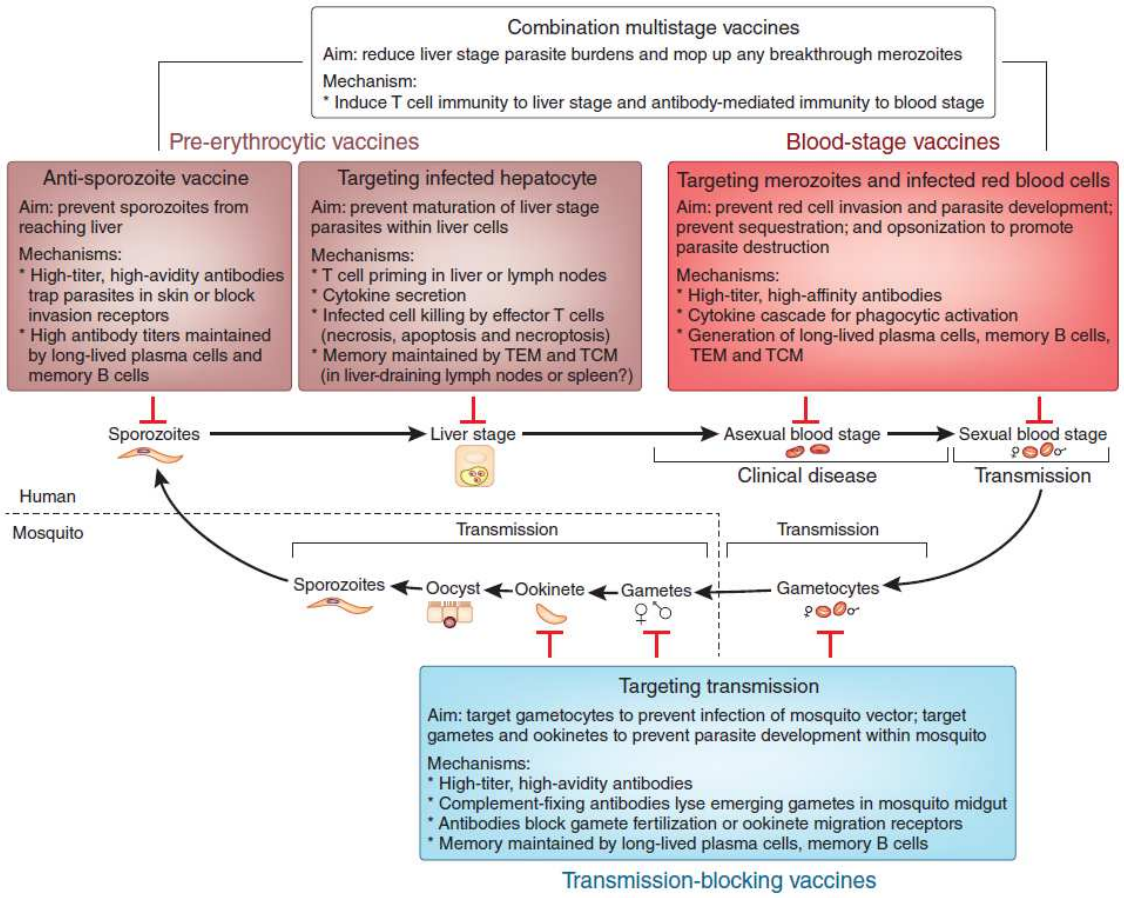

Figure 3. Malaria vaccine approaches: aims and required immune responses. TEM, effector memory T cells; TCM, central memory T cells.

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Source: Riley and Stewart, 2013 ([6]).

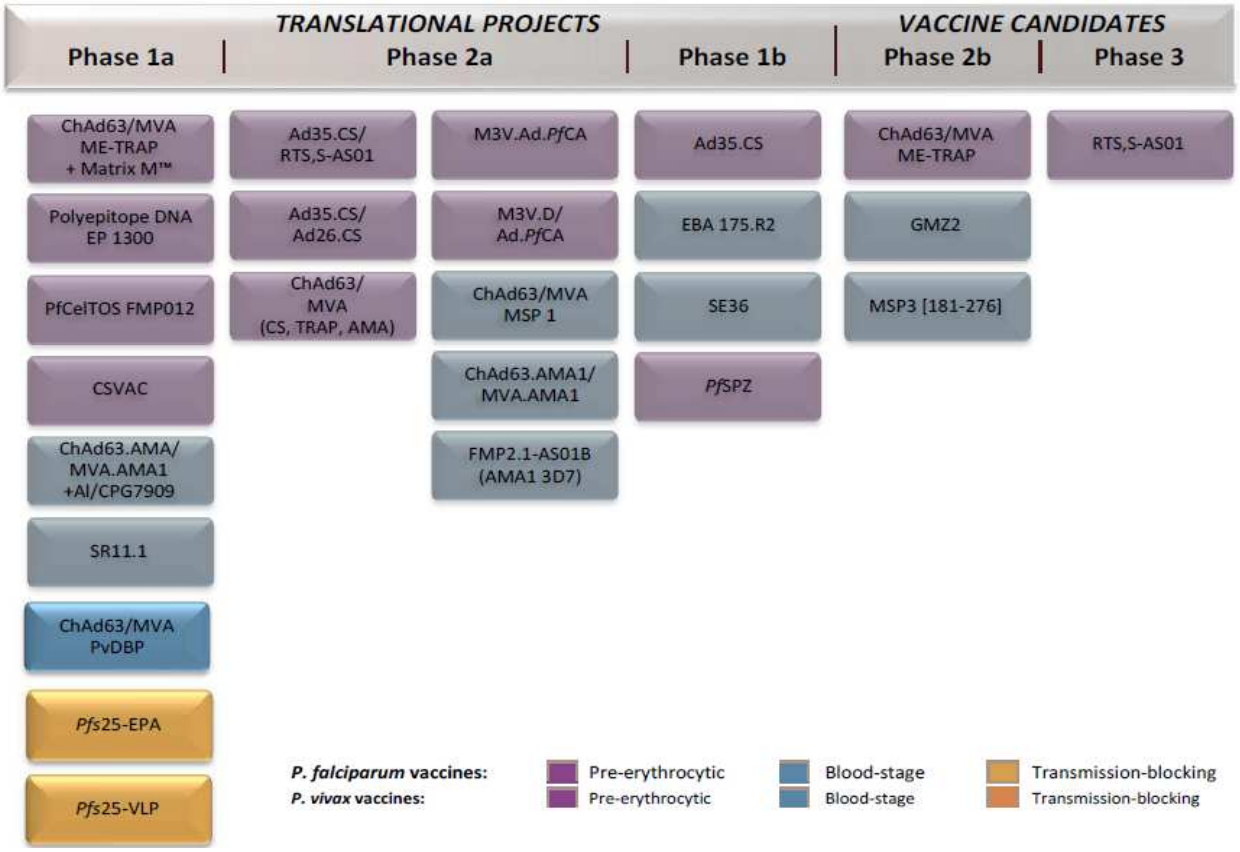

Figure 4. Global malaria vaccine development pipeline.
Source: WHO, 2015 [41].

1.4.1 Pre-erythrocytic stage vaccines

As depicted in **Figure 3**, the pre-erythrocytic stage vaccines are designed to block infection of liver cells by sporozoites as well as prevent completion of their development within hepatocytes, if infection occurs [6]. Targeting these parasite forms is advantageous but also difficult as only few sporozoites are normally transmitted to humans when the vector takes a blood meal [42]. To be efficacious, a pre-erythrocytic malaria vaccine must entirely block transition of parasites to the asexual blood stage parasites. If successful, this would also prevent transmission and hence could be used in malaria elimination programs [39]. On the other side, only one successful passage through the liver suffices to cause disease since the mechanism of pre-erythrocytic

immunity is different from the naturally acquired immunity against asexual blood stage parasites (reviewed in [43]). The feasibility of a pre-erythrocytic vaccine stem from studies where experimental vaccines based on radiation-attenuated *Plasmodium* sporozoites conferred sterile protection against malaria in different host systems (for example, [44-46]). Parasite-specific neutralizing antibodies as well as T cells (CD4+ and CD8+ T cells) have been identified as major players mediating protection to the pre-erythrocytic parasite-stages in experimental animals ([47]; reviewed in [6, 48, 49]).

More than 10 pre-erythrocytic vaccine candidates have progressed through preclinical studies and are being tested in clinical trials (reviewed in [50]; **Figure 4**). The most successful candidate so far is RTS,S. It targets an immunodominant fragment of *P. falciparum* circumsporozoite protein (CSP), which is composed of the central repeat region (R) that contains the B-cell and T-cell epitopes (T) of CSP. In RTS,S CSP is fused to the hepatitis B virus (HBV) surface (S) antigen and co-expressed in *Saccharomyces cerevisiae* with an additional HBV S-antigen, hence the name "RTS,S" (**Figure 5**). This means that RTS,S is a combination vaccine against malaria and hepatitis B. RTS,S is adjuvanted with the liposome-based Adjuvant System (AS) 01, which contains the immunostimulants monophosphoryl lipid A and a purified *Quillaja saponaria* saponin (QS-21) (reviewed in [51, 52]).

After many years of clinical development and extensive testing of RTS,S in populations living in different transmission settings, the vaccine was found to be safe, well tolerated and efficacious. It consistently showed moderate protective efficacy against infection and clinical disease over one year (for example, reviewed in [6, 39, 40, 50-52]). These findings constituted the driving force behind the implementation of large scale phase III efficacy trials in seven African countries [39]. The results from such trials have shown that RTS,S is able to reduce the rates of clinical as well as severe malaria by about 30% in African infants 6-12 weeks old [53]. Earlier findings from the same trials in children of aged 5–17 months reported that the protective efficacies of RTS,S against both clinical and severe malaria vaccine ranged from 50 to 56% [54]. With protective efficacy of 30-50%, RTS,S may be licensed and deployed as the world's first generation malaria

vaccine for use in sub-Saharan Africa where the disease burden remains relatively high (reviewed in [39]). Notwithstanding, research is on-going to improve the efficacy of RTS,S by including additional antigens in order to create a multi-stage, multi-antigen RTS,S-based vaccine [55].

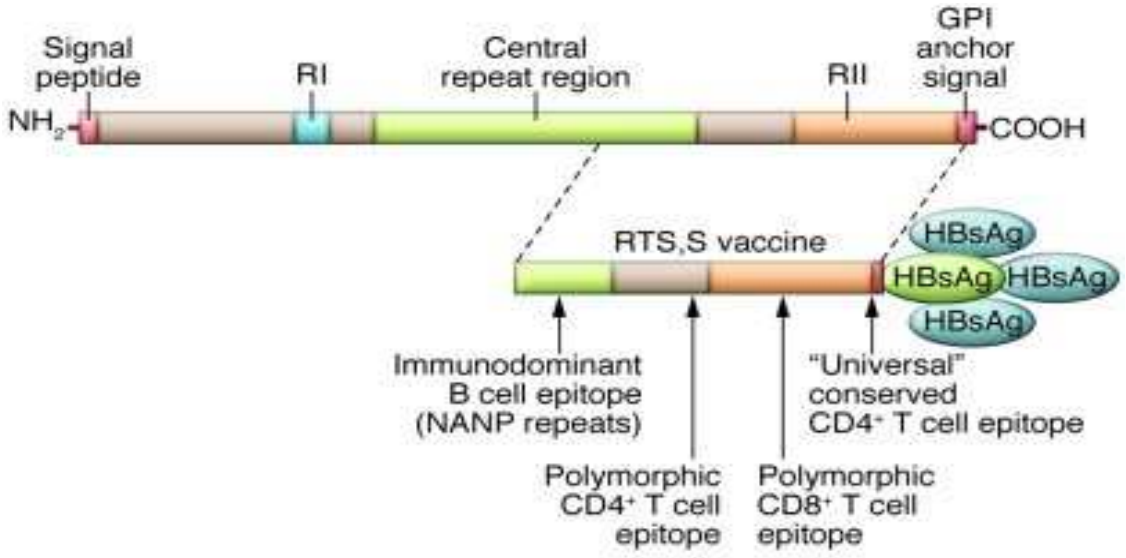

Figure 5: The CSP of *P. falciparum* and the protein region incorporated into the RTS,S vaccine.
Reprinted with permission from the American Society for Clinical Investigation.
Source: Crompton *et al*. 2010 [52].

1.4.2 Blood-stage vaccines

The scientific rationale for developing blood-stage vaccines stem from the fact that clinical illness, complications and fatality associated with malaria occurs during blood-stage infection. As such, an effective blood-stage vaccine would have the greatest impact by reducing malaria parasitemia, clinical symptoms and complications, hence such vaccines are intended for use in reducing morbidity due to malaria in endemic areas [56].

This vaccination approach is based on the concept that following repeated malaria attacks, persons living in malaria-endemic countries often develop acquired immunity that often targets blood-stage antigens. Infected persons are therefore able to control parasite density to below fever thresholds [57]. It may therefore be possible to mimic and accelerate the development of anti-disease immunity through a blood-stage malaria vaccine [52].

Several blood-stage vaccines are composed of merozoite surface-derived antigens (reviewed in [58]) that lead to the production of antibodies that block invasion and replication of merozoites within red cells (**Figure 3**). Such neutralizing antibodies may also target parasite-infected red cells preventing their adherence to different host tissues and reducing their numbers to levels that do not cause clinical disease or death (reviewed in [6, 39, 40]).

Clinical evaluation of previous candidates, such as merozoite surface protein (MSP)1-, MSP2- and apical membrane antigen (AMA)1-based vaccines, showed no protective efficacy, although some showed signs of allele-specific protection (reviewed in [6]). As a consequence, further clinical development of these candidates slowed down (reviewed in [40, 59]). The lack of MSP1-based vaccine efficacy may be due to genetic diversity of MSP1 protein (reviewed in [39]) and for the AMA1-based vaccine candidate, efficacy was mostly strain-specific [60]. Currently, a few other promising candidates (**Figure 4**) are being evaluated in field trials, including the erythrocyte-binding protein 175 (EBA175), $MSP3_{181-276}$, and GMZ2 (reviewed in [59]).

With advances in knowledge and technology, new antigens are being discovered and further developed as blood-stage vaccines candidates [61, 62]. One such antigen is the *P. falciparum* reticulocyte-binding protein homologue 5 (*Pf*RH5). *Pf*RH5, a merozoite protein located in the neck of rhoptries has been identified as essential for erythrocyte invasion [63]. *Pf*RH5-induced inhibitory antibodies have been shown to block the invasion of RBCs in animal models as well protect NHPs against clinical disease following challenge with a heterologous *P. falciparum* strain [62, 64].

Although the antigen is not a major target of naturally acquired antibodies to *P. falciparum*, naturally acquired anti-PfRH5 antibodies have been shown to predict

protection from clinical malaria [65]. Thus, *Pf*RRH5 constitute an attractive blood-stage vaccine candidate [62], and two PfRH5–based vaccine candidates (ChAd63 RH5 and MVA RH5) are currently undergoing human Phase 1a clinical trials [66].

Among those, GMZ2 is the most advanced candidate. It is a combination vaccine composed of the N-terminal region of glutamate rich protein ($GLURP_{27–500}$) (non-repeat R0 region and major B-cell epitope) and the conserved C-terminal fragment of $MSP3_{212-380}$ and expressed in *Lactococcus lactis* [67].

The discovery and further clinical development of GMZ2 was based on the ability of MSP3 and GLURP to induce high titers functional antibodies assessed by antibody growth inhibition assay (GIA) and ADCI [26, 68]. GIA measures the ability of purified IgG or serum antibodies to inhibit the invasion and subsequent growth of malaria parasites in human red cells *in vitro*. On the other hand, the ADCI assesses the antibody-dependent cellular activity of monocytes against malaria parasites (reviewed in [69]).

In pre-clinical studies, GMZ2 induced broader antibodies to both GLURP and MSP3 than co-administration of the antigens alone or a mix of both. The protective effects of anti-GMZ2 IgG antibodies was demonstrated by *in vitro* parasite-growth inhibition [67]. In further studies in NHPs, the vaccine was found to induce partial protection against *P. falciparum* [70]. These findings showed that GMZ2 could be a valuable malaria vaccine candidate and pave the way for its further clinical development [67, 70].

In Phase I trials of GMZ2 adjuvanted with aluminium hydroxide, high levels of vaccine antigen-induced antibodies and GMZ2-specific memory B-cells were detected in malaria-naive Germans as well as in malaria-exposed adults and children. The vaccine was found to be safe and well tolerated. These results supported further clinical testing of GMZ2 in a much larger population (reviewed in [59]). The recently completed multi-center Phase IIb efficacy trials enrolled almost 2000 children aged 1-5 years in study sites in Burkina Faso, Gabon, Ghana and Uganda.

1.4.3 Transmission blocking vaccines

As the name suggests, such vaccines are designed to target the gametocytes, gametes and ookinetes, preventing them from maturing, infecting and developing in the mosquito vector. Such candidates are not meant to provide individual level protection, as in the case of pre-erythrocytic and blood-stage vaccines, but instead to stop malaria transmission at the community level. The hope is that in the long-term this approach could be helpful in the elimination and even eradication of malaria from certain areas (reviewed in [71]). Knowledge about how this type of vaccine induces immunity is very limited, but it is widely believed that antibodies, cytokines and complement taken up by the vector in the course of feeding on blood hamper the development of sexual stages into infective sporozoites (reviewed in [6, 40]).

The development of malaria transmission blocking vaccines proceeds at a slower pace compared with the other types of malaria vaccines. However, two major gametocyte-specific surface antigens, Pfs48/45 and Pfs230, and two ookinete-specific surface coat proteins, Pfs25 (*P. falciparum*) and Pvs25 (*P. vivax*), constitute the main focus of anti-transmission vaccines (reviewed in [72]). So far, the leading candidates (Pfs25 and Pfvs25), formulated in Montanide ISA 51, have been tested in Phase I trials. Safety concerns led to modification of the Pfs25 vaccine antigen, as well as replacement of the adjuvant. Use of the reformulated Pfs25 conjugated to recombinant *Pseudomonas aeruginosa* ExoProtein A (Pfs25-EPA) and adjuvanted with alhydrogel has given satisfactory results in Phase 1a trials (reviewed in [59]).

1.4.4 Multi-stage, multi-antigen and whole parasite vaccines

Most current candidate vaccines under development aim to target just one stage of the parasite's life cycle. Considering the vast number of antigens expressed by the four human parasite stages (sporozoites, liver stage, merozoites and gametes), it is proposed that next-generation malaria vaccines be designed to have wider coverage by targeting multiple parasite developmental stages (reviewed in [73, 74]), and as such could induce multi-immune responses to many different parasite antigens that may

provide better protection [75]. The greatest challenge of this strategy is to identify and choose the optimal antigen combination that would induce the most robust and desired immune responses. However, adding other components (FMP1; falciparum malaria protein 1) to the most clinically advanced malaria vaccine (RTS,S/AS02A) did not show an improvement in the level of protection [55]. Other multi-component vaccines, for example the multiple-epitope–thrombospondin-related adhesion protein (ME-TRAP) and polyepitope DNA-based vaccines have also been developed and are currently been tested in field trials (reviewed in [59]).

Apart from multistage and multi-antigen vaccines, developing a whole parasite vaccine based on attenuated sporozoites is been actively pursued. The approach had proved to be highly successful since the 1970s [76]. Whole organism vaccines are so far the only vaccines that have reached >90% protective efficacy against malaria in humans [44, 77, 78]. The downside of this approach is the need for hundreds of infected mosquitoes for vaccination. Sanaria Inc., an U.S. based company has recently developed methods to purify and cryopreserve fully infectious and attenuated *P. falciparum* sporozoites (*Pf*SPZ). Vaccination by intravenous injection of radiation-attenuated *Pf*SPZ (*Pf*SPZ Vaccine) led up to 100% protective efficacy in a small number of human volunteers receiving controlled human malaria infection (CHMI) [79], which further confirms the potency of this vaccination concept. However, the issue of the need of intravenous administration, scalability and maintenance of cold chain are critical issues that still may limit the large-scale applicability of this strategy for immunization of infants, should the *Pf*SPZ vaccine becomes adopted in future as a vaccine against malaria.

1.5 Antibodies as correlate or surrogate markers of protection

An immune correlate of protection as defined by Plotkin [36] is an immune response closely related to protection or that provides protection. Immune correlates are considered to be of prime importance during efficacy trials of vaccine candidates (reviewed in [36]).

In circumstances where the exact correlates of protective immunity are unknown, unavailable or hard to measure, useful surrogate or substitute markers - mostly serum antibodies - are measured mostly by enzyme-linked immunosorbent assay (ELISA) and associated with vaccine-induced protection in clinical studies (reviewed in [36]). Plotkin [80] further defined a surrogate marker as an immune response that substitutes for the true immunologic correlate of protection, which may be unknown or not easily measurable. In general, most successful vaccines are known to induce high levels of antigen-specific neutralizing antibodies of defined protective threshold which serve as biomarkers of protection against disease (reviewed in [38, 80]).

A direct cause-and-effect relationship between vaccine-induced antimalarial antibodies and protection from malaria disease is yet to be established. As a consequence, there are currently no validated immunological markers or correlates of protection against malaria, either by naturally-acquired anti-disease immunity or elicited by the most advanced malaria vaccine candidate RTS,S [55]. Studies have shown that the induction of robust CSP-specific antibodies by the RTS,S vaccine is associated with better protection from clinical malaria (reviewed in [6, 81]), although the data are inconsistent.

In the case of blood-stage vaccines where antibodies are considered as the effective agent or crucial for protection against disease, two promising candidates (AMA-1 and FMP-1) both failed to provide a meaningful protective immunity [82, 83]. Nevertheless, certain neutralizing antibodies subclasses (IgG1 and IgG3) specific for some blood-stage vaccine antigens have been associated with protection against malaria disease [84]. As such, malaria-specific antibody responses are frequently considered as surrogate marker of protection to malaria disease [85].

2. AIMS OF THE PRESENT WORK

Our research team has over the years been involved in the clinical development of different malaria vaccine candidates, including the most advanced pre-erythrocytic vaccine (RTS,S) and blood-stage vaccine (GMZ2) candidates. With the aim to expand current knowledge of vaccine immunogenicity and contribute to the search for antibody-based correlates of protection against malaria, I worked on serum samples collected from three clinical trials of the afore-mentioned vaccine candidates to characterize the development of antibody-based immune responses in different populations of vaccinees presented in this dissertation.

In order to achieve these goals, two separate but related immunological studies were performed. First, a cytometry-based immunoassay was set up to standardize the quantification of anti-plasmodial IgG antibodies in the serum of GMZ2-vaccinees. Second, the avidity index (AI) of antibodies to the CSP repeats induced by RTS,S was evaluated in vaccine recipients.

The specific objectives of the work presented in this dissertation are as follows:

- To develop and implement a quantitative flow cytometry based assay for high throughput measurement of anti-plasmodial and vaccine-induced IgG antibodies to whole malaria parasite antigens (**Paper I**).

- To evaluate the change in the avidity of anti-CSP antibodies over time in a cohort of infants who underwent two separate immunization schedules of RTS,S and explore the effect of anti-CSP IgG avidity on RTS,S vaccine efficacy (**Paper II**).

3. RESULTS

A summary of the results obtained in each of the papers is presented here. Reference is made to the tables and figures in the different publications.

3.1 Paper I

A flow cytometry-based workflow for detection and quantification of anti-plasmodial antibodies in vaccinated and naturally exposed individuals

***Anthony Ajua**, Thomas Engleitner, Meral Esen, Michael Theisen, Saidou Issifou and Benjamin Mordmüller.*

***Malaria Journal 2012; 11:367**.*

Antibodies are known to play an important role in anti-malarial immunity but the exact antibody-mediated correlates of protection remain elusive. Highly standardized assays that may allow comparability of antibody measurements are also lacking and so the best way to measure, report and interpret antimalarial antibody responses is unknown.

In this study, the standard microscopy-based immunofluorescence assay (IFA) was transformed into a standardized, investigator- and bias-free, high throughput-amenable cytometry-based assay to assess antibody reactivity against whole parasites.

The novel cytometry-based assay was validated using semi-immune serum samples from Gabonese adults (Figure 2, Figure 3 – Paper I) and applied to sera from adult and pediatric participants of two GMZ2 Phase Ib trials.

Baseline anti-plasmodial antibodies and the effect of vaccination on the anti-GMZ2 antibody response were evaluated. Upon vaccination, children vaccinated with the

highest GMZ2 dose (100µg) showed a 1.33-fold increase in percent positive fluorescent cells (PPFC; p=0.003) on D84 compared to D0 (Figure 7 – Paper I). Adults showed a boost of pre-existing anti-parasitic antibodies resulting in improved parasite recognition (increased MFI; 1.23-fold change; p=0.03) (Table 3 – Paper I).

During analysis, we observed that available statistical approaches (e.g. model-based gating algorithms) to analyze flow cytometry data were performing worse than bias-prone manual gating strategies (Table 1, Figure 4, Figure 5, Table 2 – Paper I). A new method for data-driven gating, the overlap subtraction algorithm (OSA), was developed and tested against other strategies. OSA-derived results correlated well with those derived by expert manual gating and showed improved characteristics of performance compared to other frequently used strategies when applied to data of the novel assay (Table 1, Figure 4, Figure 5, Table 2, Figure 6 – Paper I).

Taken together, standardized new tools are presented that could be useful for characterizing antibody-mediated immune responses and identifying vaccine-induced correlates of protection against malaria.

3.2 Paper II

The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01$_E$ on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants

***Anthony Ajua**, Bertrand Lell, Selidji Todagbe Agnandji, Kwaku Poku Asante, Seth Owusu-Agyei, Grace Mwangoka, Maxmilliam Mpina, Nahya Salim, Marcel Tanner, Salim Abdulla, Johan Vekemans, Erik Jongert, Marc Lievens, Pierre Cambron, Chris F. Ockenhouse, Peter G. Kremsner and Benjamin Mordmüller.*

***Malaria Journal 2015; 14:72**.*

RTS,S is known to induce antibodies against the *P. falciparum* circumsporozoite protein (CSP). While higher levels of RTS,S-induced anti-CSP antibodies have been associated with protection against infection and episodes of clinical malaria, it is unknown if anti-CSP antibody avidity (strength of antibody binding) predicts RTS,S-induced protection.

In this second study, anti-CSP antibody avidity was measured during two different three-injection vaccination schedules to assess if it predicts vaccine efficacy in infants immunized with RTS,S. The clinical Phase II trial included one arm receiving the standard regimen (0-1-2 month) and the other arm received a delayed third dose (0-1-7 month) vaccination schedule of RTS,S/AS01$_E$ [86, 87].

The findings indicated that post dose 3, the antibody concentration and absolute AI were similar ($p>0.05$) in both immunization schedules (Figure 2 – Paper II). Meanwhile, an increase in AI (dAI) between the second and third vaccine doses was observed in the two different schedules, though the increment was modestly higher for the extended vaccination schedule (7.1-fold increase) compared to the standard schedule (4.2-fold increase) (Figure 3 – Paper II).

AI, dAI and change in median anti-CSP titers (dCSP) were evaluated as biomarkers for RTS,S-mediated protection. It was observed that AI after the third dose was not

associated with a significant reduction in the risk of developing malaria. Furthermore, the dCSP and dAI datasets were divided on the median and volunteers classified as “high” and “low” responders. Compared with the “low-dCSP” group, classification as “high-dCSP” responder was associated with a significant risk reduction (77%) to develop clinical malaria. Similarly, classification as “high-dAI” group member was associated with a 54% risk reduction (Figure 4 – Paper II).

This study suggests that an increase in anti-CSP IgG concentration and avidity between second and third vaccine injection is associated with a strong risk-reduction for malaria after immunization.

4. GENERAL DISCUSSION AND CONCLUSIONS

Antibodies elicited through pathogen infection or by vaccination constitute an essential component of the humoral immune response in humans and anti-parasitic antibodies play an important role in protective immunity to malaria [15-19, 21]. Despite this fact, the exact antibody responses, their targets and functional activities that are required to mediate protection are largely unknown [85]. Moreover, there are no reliable means to adequately distinguish protective from non-protective antibodies [88]. These intellectual and experimental challenges might contribute to the multiple unsuccessful attempts to develop a highly potent antimalarial vaccine [89]. A clear understanding of the mechanisms of protective immunity and identification of immune correlates of protection against malaria could be a way to revolutionize the development and introduction of vaccines with greater efficacy [6, 89]. Such knowledge may further pave the way for detailed quantitative evaluation of current and next-generation vaccines as well as serve as a measure for estimating efficacy, duration of protection or immunological memory following vaccination without the need of large and expensive trials in vulnerable populations with the clinical endpoint malaria [90].

As malaria-specific antibodies may change following infection [88] or after immunization, accurate quantification becomes critical for the assessment of immunogenicity and investigation of antibody correlates [91]. However, there is currently no consensus on the choice of assay(s) that could be employed to measure antibody responses or investigate potential markers of protection [6, 92-94]. The development and implementation of standardized methods will facilitate the accurate and reproducible detection of specific vaccine-induced immune responses and guide the vaccine development process [93, 95]. Moreover, the use of robust standardized assays for antibodies assessment would allow better comparison of vaccine immune responses

especially in the context of multicenter clinical studies and might aid in the identification of novel correlates of protection [93].

Most vaccine candidates under clinical development are evaluated for immunogenicity using IgG-based assays, such as ELISA and the indirect immunofluorescence assay (IFA), as they are considered good markers for predicting protective malaria immunity [93]. Although these assays are rapid and easy to perform, they have important limitations. For instance, coating of antigen onto a solid support, as is normally done in the ELISA-based system may alter the antigen structure and hence affect its reactivity [92, 96]. Moreover, the technique only measures the antibody response to single antigens at a time and thus requires larger volumes of serum [97], when antibody responses to multiple antigens are being tested. On the other hand, the IFA is an important immune-epidemiological tool [98, 99] and also has great value in vaccine-induced antibody functionality studies [98, 100], considering that whole parasite antigen can be used for antibody detection. The downside of IFAs are their low throughput, dependence on the investigator and poor standardization, which limits their widespread applicability in biological and clinical research [101]. Each of the two studies presented in this dissertation addresses a major gap in knowledge that affects malaria research in general and malaria vaccine development in particular.

In a first study, we developed and validated a new high throughput flow cytometry-based IFA assay and tool for rapid and reliable measurement and analysis of anti-plasmodial antibodies in human serum [102]. This new workflow was applied to evaluate the effect of vaccination on antibody responses using residual serum samples and clinical data from participants who completed two Phase 1 clinical trials of GMZ2 candidate malaria vaccine [103, 104].

For antibody detection, matured *P. falciparum* schizonts served as an antigen source for performing the assay. Following cultivation, whole schizont parasites were fixed using a combination of paraformaldehyde and glutaraldehyde as described [105], which better preserve the antigen structure [106] and might facilitate the occurrence of an anti-parasitic reaction. Employing fixed and intact parasites makes it possible that large

number of samples such as those from immune-epidemiology and multicenter clinical studies can be consistently analyzed over an extended time period, especially when a loader-equipped flow cytometer is used. A data analytical tool (OSA), developed and incorporated into the assay setup reduced bias and facilitated analysis of large flow cytometric datasets.

Recent findings indicate that the host's previous encounters with malaria antigens could affect the evaluation of vaccine-induced effects [85]. Similarly, maternally-derived pre-existing antibodies have been shown to interfere with the development of antibody responses following immunization of mice with an MSP1-based vaccine [107]. Our analyses revealed an increase in vaccine-induced anti-plasmodial antibodies response (increase in PPFC; percent of positive fluorescent cells) in children with no prior or very limited pre-existing malaria immunity. In contrast, a vaccine-mediated boosting of pre-existing anti-parasitic immune response (increase in MFI; mean fluorescent intensity) was observed in the semi-immune adults. The pattern of reactivity showed that the assay is able to capture the level and time of exposure to malaria by comparing baseline values of antibodies in malaria-exposed children to adults. This could help analysis and interpretation of immunogenicity data following vaccination in highly endemic regions because it allows incorporation of previous exposure into the analysis [85].

Moreover, reliable quantification of the cumulative antibody responses to all accessible whole parasite antigens, instead of using single parasite proteins may better predict *in vivo* protection [91]. Our assay may be very useful in this regard, as it potentially measures both naturally-acquired and vaccine-induced anti-plasmodial antibodies to parasite antigens in populations with varying degree of immunity.

Efficacy studies of the RTS,S vaccine candidate have shown that the induction of high titers of CSP-specific antibodies partially predicts the protective efficacy of the vaccine [87, 108, 109]. This implies that apart from antibody amounts, other characteristics of antibody, such as isotype, subclass, functional properties, ability of vaccine-induced antibodies to bind to intact parasites, or affinity and/or avidity of antibodies, may be

important determinants of antibody function ([110], reviewed in [111]). It is very difficult to systematically investigate all these parameters in the same study, due to the restricted sample volumes available for immunological studies. However, high titers and avidity antibodies have been proposed as the leading antibody-based mechanisms (**Figure 3**, page 18) by which vaccine-induced protective immunity to malaria can be achieved by the different vaccine types (reviewed in [6]).

Interestingly, antibody avidity (AI), a marker of antibody quality, has also been identified as an important marker of efficacy for some licensed vaccines ([112, 113], reviewed in [80]). So far, antibody avidity has not been extensively investigated in the framework of malaria vaccine development ([92]), and only very few biological studies have assessed the avidity of antibodies in humans [114-117] and in a mouse model of malaria [118]. Together, these studies have suggested that high avidity of naturally-acquired antibodies to blood-stage antigens could predict antimalarial immunity and protection from clinical disease. In terms of pre-erythrocytic stage antigens, studies in mice have associated high anti-CSP antibody affinity with protection from subsequent sporozoite challenge [21, 119].

Although these findings may be encouraging, there are no data from clinical studies of malaria vaccine candidates. Therefore, I chose to explore as part of this dissertation the avidity of antibodies induced by the CSP-based candidate vaccine RTS,S for a number of reasons. First, the number of sporozoites deposited into the human skin is typically relatively small (median: 15 sporozoites) [42]. Moreover, sporozoites are known to be poorly immunogenic, as they only circulate for a brief period of time (reviewed in [6]) and migrate from the mosquito's injection site on the skin to the liver in less than 15 minutes [120]. As such, sporozoites may be less prone to exposure and damage by antibodies. Hence, the availability of high amounts of sporozoite-specific antibodies during the pre-erythrocytic infection phase (reviewed in [121]), combined with the high speed and strength of antibody binding to sporozoites may be critical to confer protection.

In the second study, we evaluated the change in antibody avidity over time and explored the contribution of AI to the protective efficacy induced by two immunization

schedules of the RTS,S vaccine. For this first investigation of its kind, we used serum samples and clinical data from multicenter Phase 2b trials [86, 87] that evaluated the safety, immunogenicity and clinical efficacy of RTS,S when co-administered with vaccines routinely administered through the World Health Organization's Expanded Program on Immunization (EPI). Antibody responses induced by the vaccine had been assessed by a standard ELISA technique [122]. The same assay was adapted for the measurement of the AI of anti-CSP antibodies in the current study. In terms of vaccine outcome, both the 0-1-2 month and the 0-1-7 month vaccination schedules reportedly showed comparable vaccine efficacy. In addition, one year after the third vaccine dose, high vaccine-induced anti-CSP antibody titers were associated with a significant reduction (48%) in the risk to develop clinical disease [87]. This therefore offered an excellent opportunity for us to attempt investigations of possible biomarkers to predict vaccination outcome.

A number of factors, such as the nature and dose of vaccine antigen, certain adjuvants and carrier proteins as well as the interval between vaccine doses, can modulate the avidity of antibodies ([123, 124], reviewed in [125]). Interestingly, the analyses revealed that after the second and third vaccine doses, AI was similar between the two vaccine schedules. This implies that delaying the third vaccine dose does not improve the avidity of antibodies strongly as would be expected if longer interval between vaccination favored the induction of long-lived anti-CSP antibodies [121] and affinity maturation of antibodies (reviewed in [125]). Our observation is nevertheless notable as it supports the adoption of the 0-1-2 month vaccination schedule of RTS,S for further clinical evaluation, which can be easily integrated into the EPI vaccine schedules used in developing countries. A similar study recently reported that spacing either the second (0-6 month) or third (0-1-6 month) dose of the human papillomavirus (HPV) vaccine does not seem to increase the magnitude of antibody avidity in vaccine recipients [126].
As expected, avidity increased in the two vaccine groups between the second and last vaccine dose. This reflects the sequential acquisition of somatic mutations and hence affinity maturation of B cells in the germinal centers following repeated immunization with the same vaccine antigen [127].

Although antibody avidity has been proposed as an important correlate of protective efficacy for several vaccine types [112, 113, 128], we observed no significant association between the avidity of anti-CSP antibody and RTS,S-mediated protective efficacy, even after adjustment for possible confounding variables as site, schedule and anti-CSP antibody concentrations. This could mean that avidity is not an important determinant of RTS,S vaccine efficacy but it should be noted that analysis of the effect of anti-CSP antibody avidity on protective efficacy was purely exploratory and not prospectively planned in the original study. We were nevertheless able to demonstrate in this study that the increase of antibody titer (dCSP) and avidity (dAI) between the second and third vaccine doses greater than the median were significantly associated with 77% and 54% reduction in the risk to develop clinical malaria, respectively.

CONCLUSIONS

In the first part of this dissertation, the development and validation of a novel, non-biased, cytometry-based immunoassay that improves the detection of anti-plasmodial antibodies in malaria-exposed and non-exposed populations is described. The new approach can therefore be reliably used to reproducibly assess possible antibody-mediated correlates or surrogates of protection against clinical malaria.

In the second study, affinity maturation of anti-CSP antibodies elicited by the RTS,S candidate vaccine in infants was investigated in samples from a trial designed to measure clinical vaccine efficacy. Avidity after three RTS,S doses did not predict protection, but an increase of avidity between second and third RTS,S injection greater than the median was associated with a 54% risk-reduction to develop malaria. Additional studies are proposed to further explore the suitability of anti-CSP antibody avidity kinetics as a surrogate marker of RTS,S-mediated protection.

Taken together, the studies presented in this dissertation provide a reliable mean of quantifying antimalarial antibodies and advance current understanding of antibody-

mediated immunity to malaria and constitute an important step towards the development of highly effective antimalarial vaccines.

5. PERSONAL CONTRIBUTIONS

My personal contributions to the two papers presented in this thesis are as follows:

Paper I: (*Malaria Journal* Published): A flow cytometry-based workflow for detection and quantification of anti-plasmodial antibodies in vaccinated and naturally exposed individuals.

- ✓ Contributed to the study design,
- ✓ Established the flow cytometry-based IFA,
- ✓ Performed the laboratory experiments,
- ✓ Analyzed and interpreted the datasets, and
- ✓ Drafted and reviewed the manuscript for publication.

Paper II: (*Malaria Journal* Published): The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01$_E$ on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants.

- ✓ Contributed to the study conception,
- ✓ Organized, cleaned, analyzed and interpreted the data, and
- ✓ Prepared, revised and approved manuscript for publication.

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List of Publications

1. **Ajua A**, Lell B, Agnandji ST, Asante KP, Owusu-Agyei S, Mwangoka G, Mpina M, Salim N, Tanner M, Abdulla S, Vekemans J, Jongert E, Lievens M, Cambron P, Ockenhouse CF, Kremsner PG and Mordmüller B. The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01$_E$ on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants. Malaria Journal 2015; 14:72.

2. Ali A, Netongo PM, Ngongang EO, **Ajua A**, Atogho-Tiedeu B, Achidi EA, Mbacham WF. Amodiaquine-artesunate versus artemether-lumefantrine against uncomplicated malaria in children less than 14 years in Ngaoundere, North Cameroon. Efficacy, Safety and Baseline drug resistant mutations in pfcrt, pfmdr1 and pfdhfr genes. Malaria Research and Treatment 2013; 2013:234683.

3. Mamo H, Esen M, **Ajua A**, Theisen M, Mordmüller B, Petros B. Humoral immune response to Plasmodium falciparum vaccine candidate GMZ2 and its components in populations naturally exposed to seasonal malaria in Ethiopia. Malaria Journal 2013; 12:51.

4. **Ajua A**, Engleitner T, Esen M, Theisen M, Issifou S, Mordmüller B. A flow cytometry-based workflow for detection and quantification of anti-plasmodial antibodies in vaccinated and naturally exposed individuals. Malaria Journal 2012; 11:367.

5. Esen M, Forster J, **Ajua A**, Spänkuch I, Paparoupa M, Mordmüller B, Kremsner PG. Effect of IL-15 on IgG versus IgE antibody-secreting cells in vitro. Journal of Immunological Methods 2012; 375(1-2): 7-13.

6. Mbacham WF, Evehe MSB, Netongo PM, Ateh IA, Mimche PN, **Ajua A**, Nji AM, Echouffo-Tcheugui JB, Tawe B, Hallett R, Roper C, Targett G, Greenwood B. Efficacy of amodiaquine, sulphadoxine-pyrimethamine and their combination for the treatment of uncomplicated Plasmodium falciparum malaria in children in Cameroon at the time of policy change to artemisinin-based combination therapy. Malaria Journal 2010; 9:34.

7. Achidi EA, Apinjoh TO, Mbunwe E, Besingi R, Yafi C, Wenjighe Awah N, **Ajua A**, Anchang JK. Febrile status, malarial parasitaemia and gastrointestinal helminthiases in school children resident at different altitudes, in south-western Cameroon. Annals of Tropical Medicine and Parasitology 2008; 102 (2): 103 -118.

8. Achidi EA, **Ajua A**, Kimbi KH, Sinju CM. In vivo efficacy study of quinine sulphate in the treatment of uncomplicated Plasmodium falciparum malaria from South Western Cameroon. East African Medical Journal 2005; 82(4): 181 – 185.

9. APPENDIX: PUBLICATIONS I and II

MALARIA JOURNAL

METHODOLOGY Open Access

A flow cytometry-based workflow for detection and quantification of anti-plasmodial antibodies in vaccinated and naturally exposed individuals

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Abstract

Background: Antibodies play a central role in naturally acquired immunity against *Plasmodium falciparum*. Current assays to detect anti-plasmodial antibodies against native antigens within their cellular context are prone to bias and cannot be automated, although they provide important information about natural exposure and vaccine immunogenicity. A novel, cytometry-based workflow for quantitative detection of anti-plasmodial antibodies in human serum is presented.

Methods: Fixed red blood cells (RBCs), infected with late stages of *P. falciparum* were utilized to detect malaria-specific antibodies by flow cytometry with subsequent automated data analysis. Available methods for data-driven analysis of cytometry data were assessed and a new overlap subtraction algorithm (OSA) based on open source software was developed. The complete workflow was evaluated using sera from two GMZ2 malaria vaccine trials in semi-immune adults and pre-school children residing in a malaria endemic area.

Results: Fixation, permeabilization, and staining of infected RBCs were adapted for best operation in flow cytometry. As asexual blood-stage vaccine candidates are designed to induce antibody patterns similar to those in semi-immune adults, serial dilutions of sera from heavily exposed individuals were compared to naïve controls to determine optimal antibody dilutions. To eliminate investigator effects introduced by manual gating, a non-biased algorithm (OSA) for data-driven gating was developed. OSA-derived results correlated well with those obtained by manual gating (r between 0.79 and 0.99) and outperformed other model-driven gating methods. Bland-Altman plots confirmed the agreement of manual gating and OSA-derived results. A 1.33-fold increase (p=0.003) in the number of positive cells after vaccination in a subgroup of pre-school children vaccinated with 100 μg GMZ2 was present and in vaccinated adults from the same region we measured a baseline-corrected 1.23-fold, vaccine-induced increase in mean fluorescence intensity of positive cells (p=0.03).

Conclusions: The current workflow advances detection and quantification of anti-plasmodial antibodies through improvement of a bias-prone, low-throughput to an unbiased, semi-automated, scalable method. In conclusion, this work presents a novel method for immunofluorescence assays in malaria research.

Keywords: Malaria, Flow cytometry-based IFA, Algorithmic data analysis, Anti-malarial antibodies, Human serum

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Background

Malaria is a major cause of morbidity and mortality in endemic countries with African children carrying the major burden of the disease. An efficacious malaria vaccine would be a cost-effective and easy-to-implement intervention to complement current control strategies, but until today no malaria vaccine is registered for routine use [1], although one product – RTS,S/AS01 – has shown promising results in a clinical phase III study [2]. In contrast to vaccines containing pre-erythrocytic antigens, such as RTS,S, vaccines directed against the asexual blood stage are thought to act mainly through antibodies (Abs). Hence, it is hypothesized that anti-plasmodial Ab concentrations similar to those acquired upon natural exposure are required to attain semi-immunity, a type of non-sterile but robust immunity that protects from clinical complications and excessive parasite replication [1,3]. The main evidence for the role of Abs in semi-immunity comes from studies where purified Abs from African malaria-immune adults were successfully used to treat non-immune malaria patients [4,5] within Africa or, as an extension of this, in South-East Asia [5]. The mechanisms, properties, and specificities of Abs that mediate protection in malaria, however, remain unknown [3].

During clinical development of the malaria vaccine candidate GMZ2 [6-8], it was noted that current assays to monitor immunogenicity and pre-existing immunity to malaria with intact parasites are bias-prone and difficult to standardize. Conventionally, most approaches are based on enzyme-linked immunosorbent assay (ELISA) using recombinant proteins or synthetic peptides as bait antigen [9]. These could differ from their corresponding native parasite counter-parts in their folding and post-translational modifications, potentially altering the target protein's antigenic properties [3]. In addition, the degree of parasite antigen exposure to the immune system (e.g. the effects of localization in protein complexes or organelles) may be crucial for an effective anti-parasitic reaction or as a correlate for successful vaccination. This becomes even more important as second-generation, multi-subunit and whole cell vaccines enter clinical development [10]. As such, the use of microscopic immunofluorescent antibody assay (IFA) to study Ab concentrations against total parasite proteins expressed in mature blood stage schizonts and merozoites using native parasites [9,11] may provide important insights into the Ab-mediated anti-plasmodial immune response.

Microscopic IFA however, has many setbacks; quantification is done by determination of titers and quality control remains problematic due to poor assay standardization and potential investigator bias. Additionally, the assay is not scalable and, therefore, investigation of larger cohorts proves prohibitive [12]. On the other side, in skillful hands, microscopic IFA is highly sensitive and specific and provides information about the ability of vaccine-induced Abs to bind to native parasite molecules [9]. This being known, a scalable, sensitive, reproducible, and quantitative assay based on flow cytometry, a well-established and automatable technology, which is widely available in developing countries [13], was proposed to improve microscopy-based assays and allow for high throughput measurements [14-16]. A major drawback of this approach is that flow cytometry data are routinely analysed by manual gating, which is potentially biased and inconsistent [15]. To overcome these challenges, a data-driven algorithm was developed to automatically analyse flow cytometric data and a novel workflow for a medium-throughput, sensitive, and reliable flow cytometry-based immunoassay for the detection and quantification of anti-plasmodial antibodies in human serum is presented.

Methods

Study populations and serum samples

Serum samples from Day 0 (before vaccination) and Day 84 (4 weeks after the last of three vaccine administrations) were collected from two clinical trials of GMZ2. Details of the volunteers and vaccination schedules are described elsewhere [7,8]. In brief, two double-blind, randomized phase Ib clinical trials of GMZ2 were performed in Lambaréné, Gabon; one enrolled adults [8], the other pre-school children [7]. The trial involving healthy Gabonese adults took place between July 2007 and August 2008. Twenty participants received 100 μg GMZ2 adjuvanted with aluminium hydroxide (alum) subcutaneously on Days 0, 28 and 56, whereas the 20 participants in the control group received rabies vaccine intramuscularly at the same time points (Days 0, 28, and 56). The pediatric trial took place from September 2008 to October 2009 and involved 30 healthy pre-school children aged 1 to 5 years. The children received three doses of either rabies control vaccine (n = 10), 30 μg GMZ2 (n = 10) or 100 μg GMZ2 (n = 10). The 3 doses were administered one month apart (Days 0, 28 and 56) by intramuscular injection.

Both studies were reviewed by the regional ethics committee (*Comité d'Ethique Régional Indépendant de Lambaréné*; CERIL) and followed Good Clinical Practice guidelines as defined by the International Conference on Harmonization. All studies were conducted according to the principles of the Declaration of Helsinki in its 5th revision.

Plasmodium falciparum culture, synchronization and enrichment for late stages

The laboratory-adapted *P. falciparum* strain 3D7A, obtained from the Malaria Research and Reference

Reagent Resource (ATCC, Virginia, USA) was cultured in complete medium (RPMI 1640, 25 mM HEPES, 2.4 mM L-glutamine, 50 μg/mL gentamicin and 0.5% w/v Albumax). Confirmatory experiments were done using the *P. falciparum* strain Dd2 obtained from the same source. All cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 5% O_2, with daily changes of medium at 5% haematocrit and dilution with red blood cells when the parasitaemia exceeded 5%.

Parasite cultures were synchronized at early ring stage by treatment with 5% D-sorbitol (Sigma, St. Louis, USA) for 10 min at 37°C. Isolation of synchronized *P. falciparum* parasites (late trophozoite and schizont) was performed using LD-MACS magnetic columns (Miltenyi Biotec, Gladbach, Germany), as described previously, at a parasitaemia of about 5% [17]. Following enrichment, the purity of the parasite preparation was verified by light microscopy and by flow cytometry after DNA staining with Hoechst 33342. In later experiments, Vybrant DyeCycle violet stain (Invitrogen, Germany) replaced Hoechst 33342.

Flow cytometry-based immunofluorescence assay to detect anti-plasmodial antibodies

Preparation of parasites for cytometry was based on a previously described fixation protocol [18]. Briefly, *P. falciparum* culture enriched for late developmental parasite stages were washed once in phosphate buffered saline (PBS) and fixed by incubation in a combination of PBS with 4% EM grade paraformaldehyde (Merck, Germany) and 0.0075% EM grade glutaraldehyde (Sigma-Aldrich, Germany) for 30 min. Fixed cells were washed again in PBS and permeabilized for 10 min in PBS/0.1% Triton-X-100 (TX100) (Sigma-Aldrich, Germany). After another PBS wash step, free aldehyde groups were reduced by incubating cells for 10 min in PBS with 0.1 mg/ml sodium borohydride (Merck, Germany). The preparation was washed again with PBS and cells blocked in PBS/3% BSA. The cells were counted using a haemocytometer (Neubauer–counting chamber) and the pellet reconstituted in PBS to standardize the number of cells used in the assay. As a modification of the original protocol, all subsequent handling of cells in 1.5 ml sample tubes (Eppendorf, Hamburg, Germany) was performed in 96-well round-bottom plates (Corning, NY, USA) instead. To detect parasite-specific immunoglobulin G (IgG), parasite suspension (2 μl of approx. 5.0 x 10^7 cells per ml) was added into each well of the 96-well plate resulting in a total volume of 100 μl of test sera and control samples (each diluted in PBS/3%BSA) and allowed to bind for 1 h at RT on a plate shaker. After incubation, the cells were washed thrice with 150 μl of PBS to remove excess unbound primary antibody. Subsequently, pellets were resuspended in 100 μl AlexaFluor 488 goat anti-human IgG (Molecular Probes, Germany), diluted in PBS/3%BSA, and incubated in the dark for 1 hour. Following three washes with PBS, cells were stored at 4°C in the dark prior to cytometric analysis.

Antibody dilutions of both primary and secondary antibodies used in the assay were pre-determined through checkerboard titration experiments. The combination of antibody dilutions that gave the best separation between negative and positive fluorescent parasites was selected and used in subsequent experiments. Furthermore, different dilutions of three second-step AlexaFluor-conjugated goat anti-human IgG antibodies as well as a non-conjugated anti-histidine rich protein 2 (HRP2) monoclonal IgM (used as positive control) were tested. In addition, the shelf-life of parasite preparations was estimated by re-assaying at Days 0, 3, 7, and 14, since measurements from large clinical trials may take more than one day and it would be preferable to be able to use one parasite batch for such extended analyses.

Assay controls

Parasites stained i) without primary Ab and ii) with serum from malaria naïve donors followed by the fluorescently labelled secondary antibody were used as negative controls. Positive control serum came from a pool of serum from malaria-exposed semi-immune adults living in Lambaréné, Gabon. As an additional positive control, infected RBCs were stained for HRP2 with a mouse monoclonal Ab (55A, anti-PfHRP2; Immunology Consultants Laboratories, Newberg, USA) at a 20 μg/ml concentration. Detection was performed using a 1/3,000 dilution of AlexaFluor 633 goat anti-mouse IgM (Invitrogen, Germany). Before analysing the cells with a flow cytometer, fluorescence microscopy was done to verify the effectiveness of the fluorescence stains and to verify the cellular localization of Ab-bound parasite proteins.

Flow cytometry data acquisition and analysis

Parasite-infected cells were measured on a Becton Dickinson FACS Canto II flow cytometer equipped with the FACSDiva software version 6.1.2 (BD Biosciences, San Jose, USA) and an attached Carousel loader in high throughput mode. Relative fluorescence intensity of each event was analysed using FACSDiva software version 6.1.2 (BD Biosciences, San Jose, USA). Ab-reactivity was expressed as percentage of positive fluorescent cells (PPFC) and mean fluorescent intensity (MFI). Data acquisition was stopped after 50,000 events for each serum sample tested.

Model-based analysis of flow cytometry data

Several model-based algorithms have been developed to automate the gating process thereby directly addressing several inherent limitations in gating-based analysis [19].

Some of these methods, including two popular model-based approaches, k-means [20] and an implementation of the Expectation Maximization algorithm (EM) [21] were tested on two experimental datasets. As part of this work, the Overlap Subtraction Algorithm (OSA) was developed and compared with model-based approaches. All described methods were benchmarked using manual gating as a gold standard. The OSA is implemented in the programming language R and is available from the authors.

Design and mode of operation of the overlap subtraction algorithm

The algorithm effectively mimics manual gating whenever the gate is set with respect to an internal control. It detects overlapping areas of two datasets (e.g. between a control and the measurement of interest) in the two-dimensional space and sets a gate at the border of the overlap. Currently, the algorithm is able to process one colour staining, though it can be easily extended to process multicolour staining. The algorithm accepts files in the flow cytometry standard (FCS) 2.0 and 3.0 formats. MFI and PPFC are computed and reported as output.

With flow cytometry typically a fixed number of cells (e.g. 50,000) C are measured and analysed for each sample. Depending on the nature of the experiment, for each measured cell $c_i \in C$ a vector of attributes $a_1 \ldots a_n$ can be assigned, e.g., colour intensities for different dyes, forward scatter (FSC), side scatter (SSC), etc. Generally, each cell is represented by a data point in the two-dimensional space, defined by the attributes a_1 and a_2.

The algorithm starts by partitioning the whole value range for each attribute a_i of interest in β equidistant intervals, resulting in the vectors A_1 and A_2 of length β. The next step is to define two $| A_1 | \times | A_2 |$ matrices T and C for the test and control sample respectively. Then the values for T_{ij} and C_{ij} are calculated according to:

$$\begin{aligned} T_{ij} &= |c| \geq A_{1i} \wedge |c| < A_{1i+1} + |c| \geq A_{2j} \wedge |c| < A_{2j+1} \\ C_{ij} &= |c| \geq A_{1i} \wedge |c| < A_{1i+1} + |c| \geq A_{2j} \wedge |c| < A_{2j+1} \end{aligned} \quad (1)$$

Each entry in the matrices T and C stores the number of data points $|c|$ whose values for the attributes a_1 and a_2 lie within a certain interval defined by the two vectors A_1 and A_2. Next, the percentage of data points coming from the test sample is determined according to the following formula:

$$R_{ij} = T_{ij} / (C_{ij} + T_{ij}) \quad (2)$$

Following this calculation, positive entries are selected, i.e. entries in R that exceeds a certain threshold λ. To

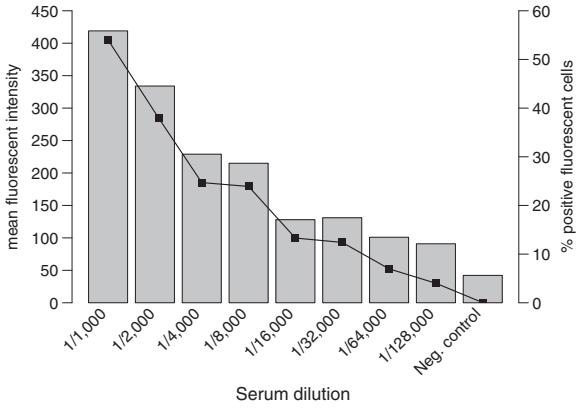

Figure 2 Dose–response relationship in pooled serum. Dilution series using a semi-immune serum pool. Bars show mean fluorescence intensity (MFI) and connected squares percentage of positive fluorescent cells (PPFC).

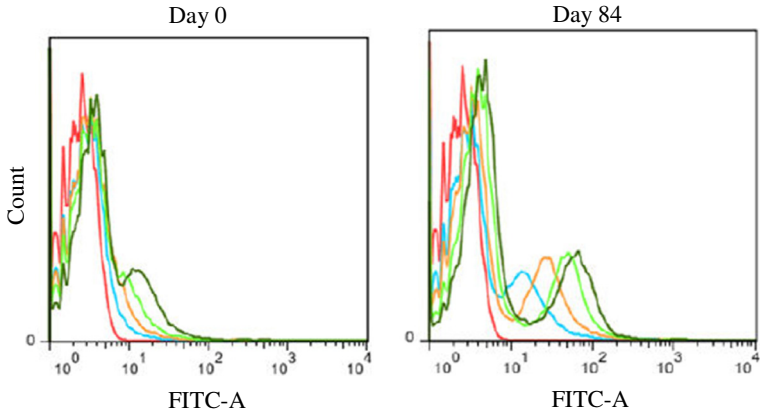

Figure 1 Representative overlay showing the anti-plasmodial Ab responses of a semi-immune individual vaccinated with GMZ2. The best separation between the negative and positive fluorescent cells is obtained when serum was diluted at 1/4,000. Test and control samples were treated as described in the methods. Note the increase in fluorescence intensity as shown by the shift to the right when parasites were incubated with serum diluted 1/32,000 (blue curve), 1/16,000 (orange curve), 1/8,000 (light green curve), 1/4,000 (green curve) or the control (red line) and the overall higher response after vaccination (Day 84).

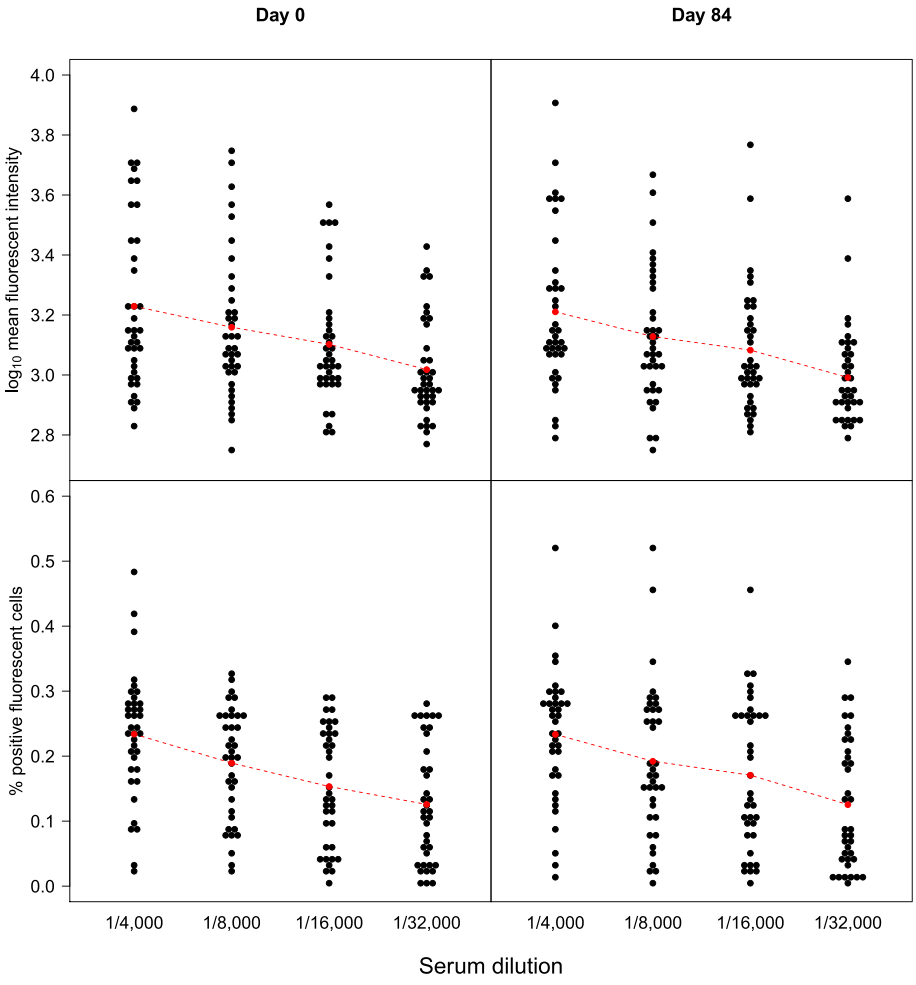

Figure 3 Dose–response relationship in individual samples from semi-immune donors. A set of 40 paired Day 0 (left panel) and Day 84 (right panel) sera from the same semi-immune population as in Figure 2. Dose-dependent responses can be seen for both the mean fluorescence intensity (MFI, upper panel) and percentage of positive fluorescent cell (PPFC, lower panel).

achieve a high specificity, λ is set to 0.99 by default, meaning that 99 percent of the data points that were counted for a particular entry come from the test sample. The correct gate is then set by finding the ω^{th} occurrence of an entry with:

$$Z_{ij} \leq \lambda \qquad (3)$$

The parameter ω controls the sensitivity of the method. In practice it is used to fine-tune the gate's distance to the negative control. By using low values of ω the gate is set close to the border of the negative control sample. Higher values of ω tend to produce gates that have a bigger gap from the control sample. After selection of relevant entries, the final gate is determined by Loess Regression through the selected coordinates.

Statistical analysis of datasets from different populations

To detect differences in the MFI between groups due to vaccination, a linear regression model was used. To account for baseline differences on Day 0, it was included as covariate in the model (see Formula 4). Raw MFI measurements were $\log_{10}$ transformed before use in further analysis.

$$\mathrm{MFI}_{\mathrm{day84}} = \beta_0 + \beta_1 \cdot \mathrm{MFI}_{\mathrm{day0}} + \beta_2 \cdot \text{vaccine group} \qquad (4)$$

For PPFC measurements, which cannot be assumed to follow a normal distribution, standard transformations to achieve normality as proposed by Ahrens *et al.* [22] did not work for both datasets. Therefore, $\log_2$ fold changes between Day 0 and Day 84 were calculated.

Between-group differences in the children dataset were tested by a one-way ANOVA followed by contrast

extraction for comparisons of interest. Effects of vaccination within groups were tested by Student's t-test.

Between-group comparisons and effects of vaccination in the adult dataset were tested using a non-parametric Wilcoxon test because even after transformation or calculation of ratios the data shows deviations from a normal distribution. To compare results derived manually as well as those obtained by automatic gating, Pearson's correlation coefficients were calculated using $\log_{10}$ transformed Ab data measured as MFI. For PPFC comparisons Spearman's rank correlation was used. Agreement between the methods was further evaluated with the Bland-Altman method [23]. The 95% confidence intervals for the mean difference are indicated for all Bland-Altman plots. All analyses were done with R v.2.13.0 [24] and statistical significance was defined as a two-sided $p<0.05$.

Results

Setup of assay parameters

To develop a standardized flow cytometric IFA to assess the Ab-reactivity to fixed *P. falciparum* parasites, a published fixation protocol [18] was adapted for use in flow cytometry. The basis for optimization was the best discrimination between positive and negative cells upon incubation with a serum pool from semi-immune individuals and preserved integrity and morphology of the cells. The final fixation and permeabilization conditions are given in the methods. Titration experiments showed that the use of semi-immune sera diluted at 1/4,000 followed by a 1/3,000 dilution of AlexaFluor 488 conjugated goat anti-human IgG best discriminated between negative and positive fluorescent cells (Figure 1).

Assay validation procedure

Following protocol development the new flow cytometry-based assay was validated using African semi-immune serum samples. These sera were selected on the basis of high anti-GMZ2 Ab-concentrations in ELISA. To assess concentration-dependent responses in antibody levels, a semi-immune serum pool diluted from 1/1,000 to 1/128,000 was used. Staining was specific (Figure 2) with only minimal cross-reaction to negative samples.

In addition, experiments were performed using a set of 40 Day 0 and Day 84 sera from the GMZ2 phase Ib trial in Gabonese adults serially diluted from 1/4,000 to 1/32,000. As expected, the PPFC and MFI values were dependent on the serum concentration (primary antibody) used in the assay and showed a consistent and obvious dose-dependent response relation on the different time points (Figure 3).

Application of model-based algorithms in flow cytometry data analysis

Model-based gating algorithms were tested on two datasets. Of these, only two methods (k-means and the EM algorithm) tend to produce results that were comparable to those obtained by manual gating. They were selected and their performance was further evaluated in comparison to the manual gating strategy. Considering the MFI, results from the two methods do significantly correlate ($p<0.001$) with those obtained manually in both datasets. In contrast, k-means produced non-significant results for PPFC on Day 0 and 84 in the population of Gabonese adults when compared to manual gating. In the pediatric

Table 1 Correlation of the four strategies employed for gating raw flow cytometry data

	Manual gating			
Gabonese adults (n = 37)[a]	MFI day 0	MFI day 84	PPFC day 0	PPFC day 84
k-means	$r = 0.95$	$r = 0.89$	$\rho = 0.04^{\S}$	$\rho = 0.14^{\S}$
	$r^2 = 0.91$	$r^2 = 0.79$		
EM*	$r = 0.92$	$r = 0.89$	$\rho = 0.89$	$\rho = 0.94$
	$r^2 = 0.85$	$r^2 = 0.80$		
Overlap subtraction	$r = 0.99$	$r = 0.98$	$\rho = 0.99$	$\rho = 0.99$
	$r^2 = 0.99$	$r^2 = 0.96$		
Gabonese children (n = 28)[b]	MFI day 0	MFI day 84	PPFC day 0	PPFC day 84
k-means	$r = 0.71$	$r = 0.76$	$\rho = -0.93$	$\rho = -0.88$
	$r^2 = 0.51$	$r^2 = 0.59$		
EM*	$r = 0.61$	$r = 0.64$	$\rho = 0.78$	$\rho = 0.81$
	$r^2 = 0.38$	$r^2 = 0.41$		
Overlap subtraction	$r = 0.79$	$r = 0.83$	$\rho = 0.94$	$\rho = 0.96$
	$r^2 = 0.62$	$r^2 = 0.69$		

*Expectation Maximization. r = Pearson's correlation coefficient. r^2 = Coefficient of determination. ρ = Spearman correlation coefficient. Ab (IgG) responses are expressed as mean fluorescence intensity (MFI) and percentage of positive fluorescent cell (PPFC). Correlations for MFI were calculated using $\log_{10}$ transformed data. [a]Data excluded for 3 participants due to problems with data acquisition and inability of some algorithms to set an appropriate gate. [b]Two participants have been excluded from analysis for the same reasons as above. All p-values are significant ($p < 0.001$) except for those marked with §.

dataset, k-means-based results for PPFC measurements were even negatively correlated with those derived by manual gating (ρ=-0.93 on Day 0, ρ=-0.89 on Day 84, both p<0.001) (Table 1). Figures 4 and 5 show correlation matrices from Gabonese adults and children comparing the different analytical approaches using Day 0 PPFC measurements. Despite the significant correlation in most comparisons, Bland-Altman analyses show considerable lack of agreement between k-means, EM and manual gating for both, MFI and PPFC (Table 2). In both datasets k-means tends to under-estimate whereas EM over-estimates the MFI using results from the manual gating as reference. With regards to the PPFC among the children population, k-means over-estimates it by 40% and 34% on Day 0 and Day 84 respectively when compared to the manual gating. The poor performance of these methods on the datasets therefore motivated the development of a new method for data-driven gating. Since the different statistical approaches were not well-suited for the data, an algorithmic approach (OSA) was tested. In general, the algorithm produced results, which compared well (p<0.0001) to manually gated data

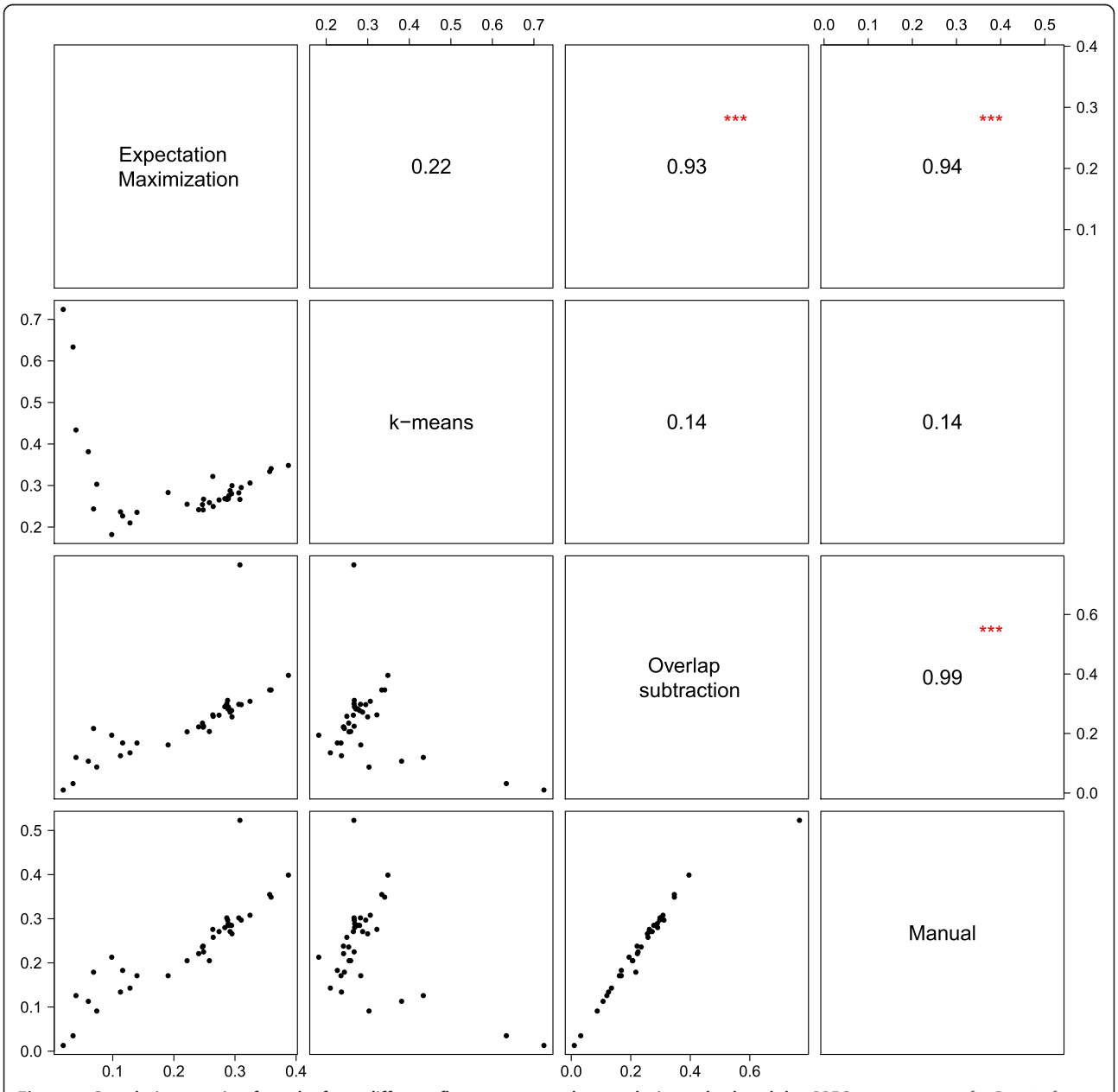

Figure 4 Correlation matrix of results from different flow cytometry data analysis methods: adults. PPFC measurements for Day 84 from Gabonese adults. The diagonal separates scatterplots (lower part) and the respective correlation coefficients (upper part).

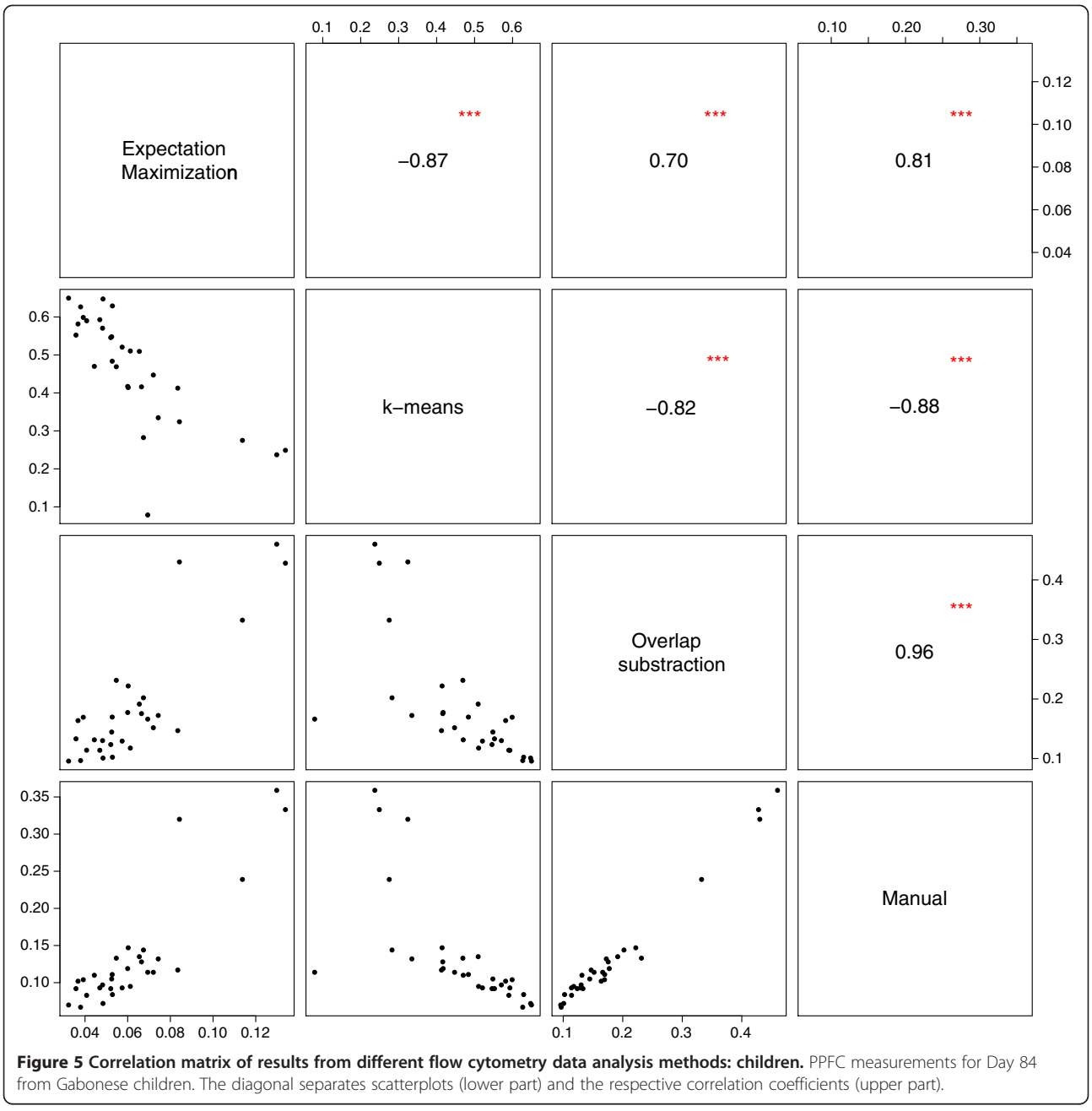

Figure 5 Correlation matrix of results from different flow cytometry data analysis methods: children. PPFC measurements for Day 84 from Gabonese children. The diagonal separates scatterplots (lower part) and the respective correlation coefficients (upper part).

(Table 1). In terms of MFI and PPFC for the different time points, the correlation appeared to be stronger for the adults ($r \geq 0.98$) than for the children ($r \geq 0.79$). In contrast to the other methods, OSA shows a high agreement with the results obtained from manual gating (Table 2). The expected absolute error for the PPFC in the semi-immune adults population is 30 and 60 times lower than for EM and k-means, respectively (Table 2). Figure 6 shows representative Bland–Altman plots with 95% limits of agreement (LOA). From all methods tested, OSA shows the smallest 95% LOA in terms of PPFC and MFI (Table 2).

Application of the cytometric IFA on sera from vaccinated subjects

The new method was applied to datasets from two GMZ2 phase Ib trials to detect possible effects of vaccination on Ab response. Each dataset consists of paired serum samples taken on Day 0 pre- and Day 84 post-vaccination. In total, 70 samples were analysed, 40 from semi-immune adults [8] and 30 from pre-school children [7], both from Gabon. Figure 7 illustrates the $\log_2$ fold changes in PPFC between Day 84 and Day 0 (baseline) responses of the different vaccine groups. Among children, most volunteers in the two subgroups

Table 2 Bland-Altman analyses of the different data gating strategies

	Manual gating			
Gabonese adults (n = 37)[a]	MFI day 0	MFI day 84	PPFC day 0	PPFC day 84
k-means	611	600.9	−0.04	−0.06
	(464.3, 757.7)	(350.5, 851.3)	(−0.09, 0.005)	(−0.12, -0.005)
Expectation	−388.3	−328.7	0.02	0.01
Maximization	(−600.2, -176.4)	(−604.8, -52.7)	(0.004, 0.04)	(−0.003, 0.03)
Overlap subtraction	−124.2	−98.1	0.006	−0.004
	(−160.3, -88.2)	(−143.4, -52.7)	(0.003, 0.009)	(−0.02, 0.001)
Gabonese children (n = 28)[b]	MFI day 0	MFI day 84	PPFC day 0	PPFC day 84
k-means	198.6	205.8	−0.4	−0.34
	(166.7, 230.6)	(176.8, 234.8)	(−0.49, -0.31)	(−0.42, -0.27)
Expectation	−208.9	−171.7	0.09	0.06
Maximization	(−290.4, -127.3)	(−214.5, -128.9)	(0.03, 0.14)	(0.05, 0.08)
Overlap subtraction	39.8	56.2	−0.04	−0.05
	(25.1, 54.5)	(43.6, 68.9)	(−0.03, -0.05)	(−0.06, -0.03)

Ab reactivity is expressed as mean fluorescence intensity (MFI) and percentage of positive fluorescent cell (PPFC). Data is given as mean differences of MFI and PPFC values (lower and upper 95% confidence interval) between the different approaches. [a]Data excluded for 3 participants due to problems with data acquisition and inability of some algorithms to set an appropriate gate. [b]Two participants have been excluded from analysis for the same reasons as above.

vaccinated with GMZ2 had a higher response on Day 84 (63% and 90% who received 30 μg and 100 μg GMZ2, respectively). Out of all volunteers vaccinated with GMZ2, only those who received 100 μg GMZ2 showed a significant increase (p=0.003) in their Ab reactivity (1.33-fold, 95% CI: 1.15, 1.55), while no significant increase was observed in the 30 μg group (1.01-fold, 95% CI: 0.81, 1.27). Interestingly, 33% of all participants in the rabies-vaccinated group had also a higher response on Day 84. However, the remaining six showed no or minimal increase in reactivity on Day 84. As a consequence, no significant increase in vaccine response was detected on Day 84 (1.09-fold, 95% CI: 0.94, 1.28). In contrast to the pre-school children, no significant treatment effect on Day 84 was detectable neither in the 100 μg GMZ2 (0.83-fold, 95% CI: 0.71, 0.99) nor in the rabies control group (1.08-fold, 95% CI: 0.97, 1.21) of the adult volunteers. In addition, no differences between the vaccine groups could be detected in both datasets. Interestingly, by applying a linear regression model (Table 3) to the $\log_{10}$ transformed MFI values, which adjusts for the Ab reactivity on Day 0 (baseline), significantly higher vaccine responses (p=0.03) were detected in the 100 μg GMZ2 group compared to the rabies group. In the pre-school children population no significant between-groups differences were detected.

Discussion

A well-studied reaction of the immune system to malaria or vaccination with malaria vaccine candidates is the induction of antigen-specific antibodies [25]. Implementation of assays that adequately detect levels of antibodies induced by natural exposure or vaccination is critical for monitoring immunogenicity. In this respect, flow cytometric-based IFA techniques similar to the approach described here have extensively been employed to assess total IgG antibodies in the sera of humans infected with protozoan parasites different from *Plasmodium* [26-32]. With human malaria, some studies have adapted related techniques - mainly to analyse responses against plasmodial variant surface antigen [12,33-36], which may have a role in parasite virulence or be used as vaccine candidates.

Here, a novel approach for immunofluorescence assays, which incorporates flow cytometry and offers a rapid and reliable method of measuring total anti-plasmodial Ab in human serum, is presented. In contrast to conventional methods which utilize recombinant or synthetic peptides as antigen to assess Ab responses [9], the improved workflow has several advantages: i) *Plasmodium* parasites can be routinely maintained in continuous *in vitro* cultures to produce enough material for medium- to high-throughput assays; ii) the use of whole-cell preparations of *P. falciparum* may preserve the target protein's antigenic properties better compared to soluble antigens [3], which could be essential for an effective anti-parasitic reaction to occur; and iii) the protein of interest is presented in its native context. Since fixed parasites remained intact and stable for more than 2 weeks when stored at 4°C, it is possible to analyse large sample numbers over an extended period of time. Furthermore, data acquisition using a flow cytometer equipped with a carousel or plate loader in high-throughput mode ensures rapid and consistent analysis of samples

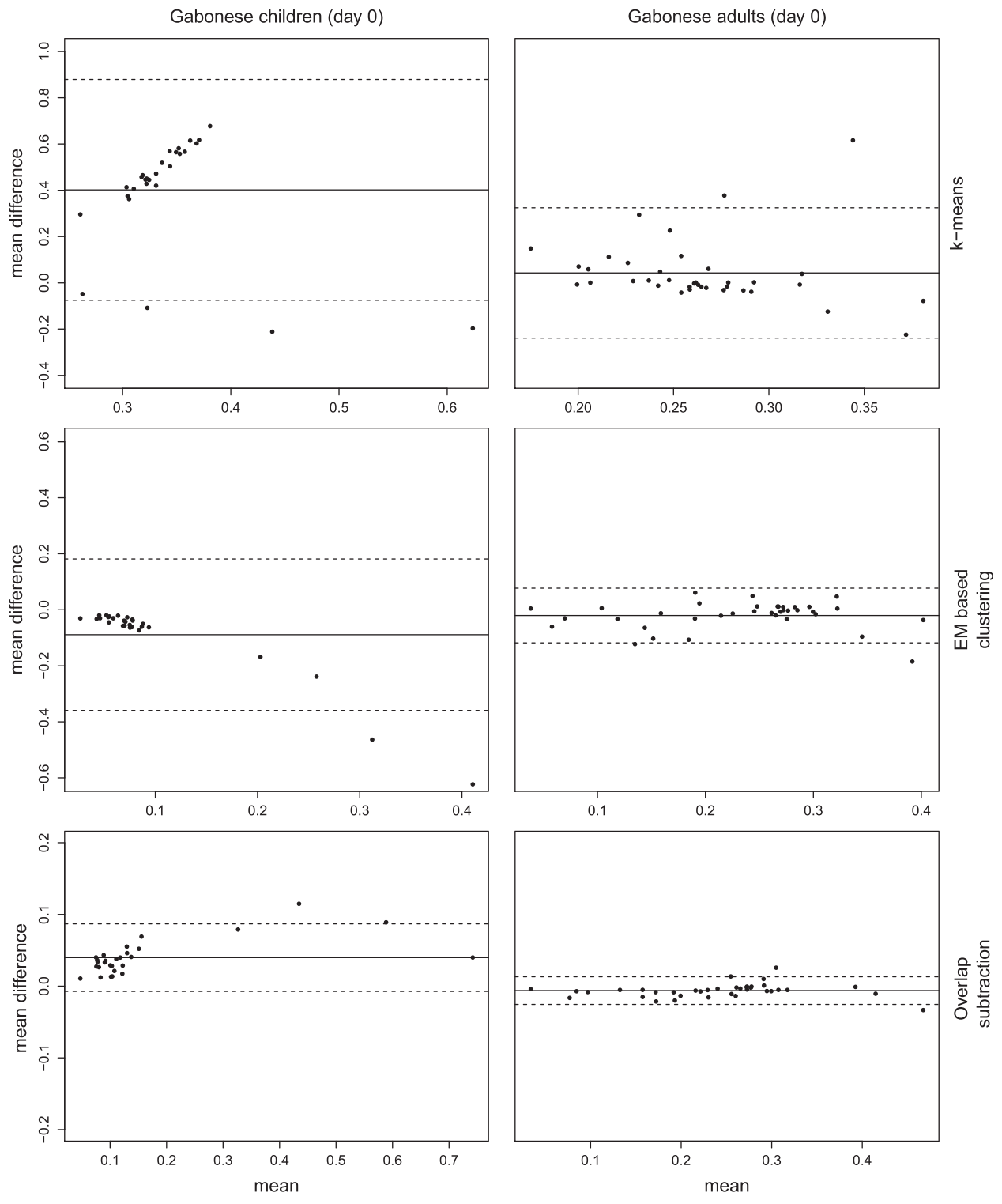

Figure 6 Representative Bland-Altman plots obtained by comparing different analytical approaches. The *x*-axis shows the mean of both computationally and manually derived estimates for the PPFC and the *y*-axis the difference between them. The inner solid line represents the mean difference for PPFC values while the outer dotted lines denote the lower and upper 95% limits of agreement between the different strategies.

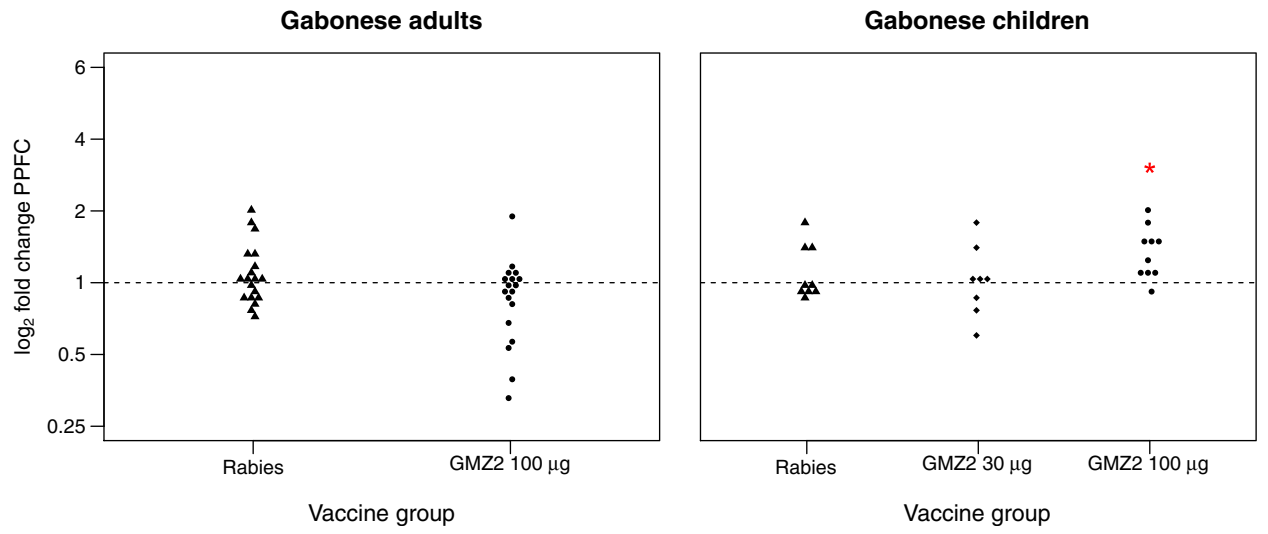

Figure 7 Changes in Ab levels of Gabonese adults and children following immunization with GMZ2. Data is expressed as $\log_2$ fold change in PPFC between Day 0 and Day 84. P-values were obtained by a one-way ANOVA and the Wilcoxon test for the children and adults' data respectively.

thereby reducing sample processing time and handling variations. This greatly improves the assay reliability when compared to the microscopic IFA technique, where the effort is limited by the microscopist's experience and speed and where substantial variation among microscopists is common. The level of standardization and throughput that is possible using fully automated synthetic or recombinant peptides cannot be attained with such an approach.

The conventional method of manual gating of flow cytometry data is often investigator-dependent and difficult to standardize. To overcome these shortcomings several statistical methods have been proposed in the literature. After applying them to two study datasets, even the best performing ones (k-means and EM) showed high error rates when compared to expert manual gating. This disadvantage was remedied by the development of a new algorithm (OSA), which, in contrast to model-based methods, does not make any assumption on the data distribution and mimics manual gating strategies. OSA-derived results correlate well with those derived by manual gating. As a data-driven algorithm, OSA may not perform equally well in other experimental setups as it depends heavily on the data structure.

Table 3 Fold-changes in Ab reactivity after GMZ2 immunization of Gabonese adults and children

Study populations	Mean (95% CI)	P-value	Comparison
Gabonese adults	1.23 (1.02, 1.48)	0.03	GMZ2 100 µg/Rabies
Gabonese children	1.04 (0.92, 1.17)	0.52	GMZ2 30 µg/Rabies
	1.04 (0.93, 1.16)	0.48	GMZ2 100 µg/Rabies
	1.0 (0.89, 1.13)	0.98	GMZ2 30 µg/100µg

Ab reactivity is presented as mean fluorescent intensity (MFI). Data is shown as mean fold changes of the different comparisons (95% confidence interval). P-values for MFI comparisons derived by linear regression model.

The whole workflow (cytometric IFA plus OSA) was validated using samples from two vaccine studies in malaria exposed adults and children who profoundly differ in their baseline anti-plasmodial immunity and showed a significant increase in specific Ab-reactivity against the GMZ2 vaccine after vaccination [7,8]. By applying the workflow, a moderate but significant increase in vaccine-induced Abs response was observed based on the PPFC, one month after a full immunization schedule (Day 84) in a subgroup of children who received the highest dose of GMZ2 (100 µg). Meanwhile, the effect induced by a lower dose of the vaccine (GMZ2 30 µg) was small and no significant treatment effect was detectable with this approach. A larger sample size may be required to detect a significant effect in this subgroup. In contrast to GMZ2-specific ELISA, which distinguishes GMZ2- from control-vaccinated children consistently, cytometric IFA results represent the integrated reactivity against all accessible parasite antigens after cell permeabilization. This decreases the ability to detect a specific signal but adds information about the size of the effect in the context of naturally acquired immunity and consequently complements antigen-specific methods.

Based on the PPFC outcome measure, no treatment effect was observed in semi-immune adults immunized with 100 µg GMZ2 (Figure 7). In contrast, a significant vaccination effect was detected between the two subgroups in the adult dataset when considering the MFI (Table 3). From the statistical point of view, a possible explanation for the contrasting observations in the two outcome measures (MFI and PPFC) may relate to the fact

that outlying data points have a greater influence on MFI than PPFC. Consequently, PPFC is the more conservative measure and should be preferred in case of discordant results when no mechanistic explanation is present. In the present study, two different populations, which largely differ in their response pattern after vaccination were investigated. Children with no or very little immunity develop anti-plasmodial antibodies upon vaccination (increase in PPFC), whereas in semi-immune adults a vaccine-mediated boost of pre-existing anti-parasitic immune response that translates into improved parasite recognition (increased MFI) is expected. Therefore, the results are in line with the mechanistic concept of vaccination in naïve and pre-exposed populations, respectively.

The relatively high pre-vaccination antibody levels with specificities to different malaria parasite antigens reported in the adults population [8] contribute much to the large variation in the data. Therefore it is not surprising that a response to a single antigen is difficult to detect. Nevertheless, results from this investigation illustrate that a vaccine-induced increase in Ab- binding to fixed *Plasmodium* parasites is detectable by this methodology, demonstrating their potential functional properties [34]. However, the assay may need further adaptation for its use in subjects with no previous exposure to malaria and low immune responses as was observed in pilot experiments. IgG subclass-specific Ab responses, especially the cytophilic antibodies known to be associated with reduced risk of malaria [37,38], have not been addressed in the present study but can be integrated rather easily.

In summary, a new flow cytometry-based immunofluorescence assay is presented. It is a cheap, reliable and rapid method to detect and quantify anti-plasmodial antibodies in human sera and may be of value in malaria research. As a next step this workflow will be applied to samples from clinical phase II/III trials of malaria vaccine candidates to characterize Ab-mediated immune responses and identify correlates of vaccine-induced protection against malaria. The non-biased data-driven computational analysis tool (OSA) integrated in this methodology will be provided under a general public license to the scientific community.

Abbreviations

RBCs: Red Blood Cells; Ab: Antibody; ELISA: Enzyme-linked immunosorbent assay; IFA: Immunofluorescent antibody assay; PBS: Phosphate buffered saline; HRP2: Histidine Rich Protein 2; EM: Expectation Maximization; MFI: Mean fluorescent intensity; PPFC: Percentage of positive fluorescent cells; OSA: Overlap subtraction algorithm; FSC: Forward scatter; SSC: Side scatter; FITC: Fluorescein isothiocyanate; LOA: Limits of agreement.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BM conceived the study and directed the experimental work. AA performed the experimental work shown in this paper. TE, BM and AA analysed the data. AA, TE and BM wrote the paper. ME collected samples and performed ELISA experiments. MT invented the GMZ2 vaccine and donated antigens for use in ELISA. SI contributed to the study design and reviewed the manuscript. All authors read and approved the final manuscript.

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MALARIA JOURNAL

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The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01$_E$ on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants

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Abstract

Background: The malaria vaccine RTS,S induces antibodies against the *Plasmodium falciparum* circumsporozoite protein (CSP) and the concentration of Immunoglobulin G (IgG) against the repeat region of CSP following vaccination is associated with protection from *P. falciparum* malaria. So far, only the quantity of anti-CSP IgG has been measured and used to predict vaccination success, although quality (measured as avidity) of the antigen-antibody interaction shall be important since only a few sporozoites circulate for a short time after an infectious mosquito bite, likely requiring fast and strong binding.

Methods: Quantity and avidity of anti-CSP IgG in African infants who received RTS,S/AS01$_E$ in a 0-1-2-month or a 0-1-7-month schedule in a phase 2 clinical trial were measured by enzyme-linked immunosorbent assay. Antibody avidity was defined as the proportion of IgG able to bind in the presence of a chaotropic agent (avidity index). The effect of CSP-specific IgG concentration and avidity on protective efficacy was modelled using Cox proportional hazards.

Results: After the third dose, quantity and avidity were similar between the two vaccination schedules. IgG avidity after the last vaccine injection was not associated with protection, whereas the change in avidity following second and third RTS,S/AS01$_E$ injection was associated with a 54% risk reduction of getting malaria (hazard ratio: 0.46; 95% confidence interval (CI): 0.22-0.99) in those participants with a change in avidity above the median. The change in anti-CSP IgG concentration following second and third injection was associated with a 77% risk reduction of getting malaria (hazard ratio: 0.23, 95% CI: 0.11-0.51).

Conclusions: Change in IgG response between vaccine doses merits further evaluation as a surrogate marker for RTS,S efficacy.

Trial registration: ClinicalTrials.gov Identifier NCT00436007.

Keywords: Malaria, RTS,S, Vaccine, *Plasmodium falciparum*, Antibody, Avidity, Correlate of protection

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Background

Malaria has an enormous public health impact and new preventive interventions are urgently needed. After more than 100 years of research on malaria vaccines, RTS,S was the first pre-erythrocytic vaccine candidate that entered phase III clinical development [1-3]. RTS,S contains hepatitis B surface antigen (HBsAg) together with a fusion protein of HBsAg and a carboxy-terminal fragment of *Plasmodium falciparum* circumsporozoite protein (CSP), co-expressed in yeast and formulated with a proprietary adjuvant (AS01). The exact mechanism of RTS,S-mediated protection is not known, although Immunoglobulin G antibodies (IgG) against the CSP repeat region are likely to play an important role since the concentration of anti-CSP IgG partly explains protection in most studies that assessed efficacy of RTS,S in African children [4-6]. In addition, passive transfer of anti-CSP IgG can protect animals from subsequent challenge [7,8]. Besides concentration, many other properties determine antibody function. Among them are availability of effector molecules, post-translational modification, isotype, subclass, affinity and avidity of antibodies. It is difficult to measure all these characteristics in one sample, particularly in the small sample volumes obtained during clinical trials in infants. Affinity, defined as the strength of interaction between an epitope and an antibody binding site, would be a particularly interesting variable to measure in the context of anti-CSP IgG-mediated immunity, since the time of interaction with the parasite is short (less than 30 minutes [9]), sporozoites are strongly diluted and few. In fact, only one successful hepatocyte infection is sufficient to initiate and maintain blood stage infection. Studies in mice have shown that high antibody affinity against a synthetic CSP immunogen is positively associated with protection [8,10] and most studies in humans indicate that anti-CSP IgG concentration explains only parts of the vaccine-mediated protection. Increase in antibody affinity after repeated antigen exposure is the result of affinity maturation due to somatic hypermutation. The rate and extent of maturation may be influenced by several factors, including nature, route and dose of the antigen, adjuvants and carriers as well as the immunization schedule. In the present study antibody avidity was measured. It is a representation of the strength of interaction between antibodies and antigens in a complex and besides antibody affinity, valences of antibodies and antigens as well as structural features of the complex are important determinants of avidity. For CSP, it has been shown that the use of some adjuvants can increase the avidity of anti-CSP IgG after vaccination of human volunteers [11]. In this study IgG avidity against the repeat region of CSP was measured after the second and third injection of RTS,S/AS01$_E$ in infants that received the vaccine as part of a phase IIb clinical trial to assess safety and efficacy of RTS,S/AS01$_E$ in the age-group targeted by the expanded programme on immunization (EPI) [5,12].

Methods

Clinical trial

The objective of the study was to explore the effect of anti-CSP IgG avidity on RTS,S vaccine efficacy in naturally exposed infants. Details of the clinical trial have been published previously [5,12]. Briefly, safety and efficacy of RTS,S/AS01$_E$ when given through the EPI was assessed in 511 children from Gabon, Ghana and Tanzania. Participants were randomly assigned to one of three intervention arms: 1) RTS,S/AS01$_E$ as three injections, one month apart (0, 1, 2 months schedule [012]; n = 170), 2) RTS,S/AS01$_E$ extended schedule (0, 1, 7 months schedule [017]; n = 170) or 3) control (EPI vaccines alone; n = 171). Malaria was defined as parasitaemia >500 parasites per μl and an axillary temperature >37°C. The efficacy of RTS,S against first malaria episodes, detected by passive case detection, was equivalent in the two schedules one year after the third injection. The study followed Good Clinical Practice guidelines, the Declaration of Helsinki (4th revision) and received approval from the appropriate local and national ethics committees of each site. In addition, ethical review by the ethics committees of the London School of Hygiene and Tropical Medicine Ethic Committee, the Swiss Tropical Institute Committee and the Western Institutional Review Board was sought. The trial is registered with ClinicalTrials.gov (NCT00436007).

Antibody measurements

Antibodies against CSP were measured by evaluating IgG responses against the CSP-repeat region, using a validated enzyme-linked immunosorbent assay (ELISA) with R32LR as the coating antigen [13]. An anti-CSP IgG titre of 0.5 ELISA units per millilitre (EU/mL) or greater was considered to be positive. For measurements of avidity of IgG against the repeat region of CSP, samples were evaluated as described [13], but in two different plates; one treated with a chaotropic agent and one untreated plate. As chaotropic agent a 1 M solution of ammonium thiocyanate (NH_4SCN) was added in the treatment plate while 0.05% Tween-20 in PBS was added in the untreated plate and both CSP ELISA plates were further washed and developed as described [13]. The avidity index (AI) was calculated as the ratio of the concentration of anti-CSP IgG (EU/ml) that remained bound to the coated antigen after treatment with NH_4SCN, divided by the concentration of IgG (EU/ml) that remained bound to the coated antigen in the untreated plate. Anti-CSP IgG quantification and avidity were measured at the Center for Vaccinology, Ghent University Hospital, Belgium.

For statistical modelling the logarithm of anti-CSP IgG concentration was used since previous data showed that log-transformation results in a better fit to the normal distribution. AI was analysed in the two RTS,S-vaccinated arms and after the second and third vaccination. Since the majority of infants before vaccination and those receiving control vaccine do not have measurable anti-CSP IgG, AI cannot be calculated. Delta AI (dAI) was defined as the difference in AI between the second and third vaccination. Similarly, delta CSP (dCSP) was defined as the difference in anti-CSP IgG concentration between the second and third vaccination.

Statistics

Analysis of the effect of IgG avidity on protective efficacy was exploratory and not detailed in the statistical analysis plan of the original study. IgG responses between the groups were analysed by descriptive statistics and represented as boxplots together with the individual measurements. The effect of anti-CSP IgG concentration and AI on risk of malaria was calculated using the according-to-protocol (ATP) dataset with a Cox proportional hazards model in R v2.15.2. For statistical modelling antibody concentrations were log-transformed. To calculate the effect of dAI and dCSP on the occurrence of malaria episodes with a Cox proportional hazards model, values were dichotomized on the median dAI or dCSP and labelled as 'high' and 'low', respectively. All models included the covariates schedule and site. If appropriate, other covariates were added as reported in the results section. A p-value below 0.05 was considered significant and 95% confidence intervals (95% CI) are given where appropriate.

Results

After screening 605 participants, 170 received RTS,S in the standard (012) and 170 in the extended (017) schedule, as depicted on the CONSORT flowchart of the primary study (Figure 1). Samples from 315 (300 ATP) participants were available for immunological analysis (012: n = 154 [148]; 017: n = 161 [152]). Paired immunological samples to calculate dAI were available from 187 (179 ATP) participants (012: n = 103 [100]; 017: n = 84 [79]).

As reported earlier [5], high anti-CSP IgG titres after three vaccine injections were associated with a reduction in subsequent incidence of clinical malaria: the hazard ratio of a ten-fold increase in anti-CSP IgG was 0.52 (95% CI: 0.34-0.81), which corresponds to a 48% risk reduction.

Absolute AI after two (012: 35.9, 017: 34.9; t-test p = 0.57) and three (012: 41.2, 017: 39.3; t-test p = 0.22) RTS,S injections were similar between the two vaccination schedules (Figure 2). As expected, an increase in AI between the second and third vaccination was present (Figure 3). Increase in delta AI (dAI) was slightly, albeit not statistically significant, higher in the 017 (7.1) group compared to the 012 (4.2) group (delta: 3.0; 95% CI: −0.3-6.1; t-test p = 0.08).

To explore the effect of AI, dAI and dCSP on malaria risk, three Cox proportional hazard models were defined and tested. AI after the third injection, corrected for site, schedule and anti-CSP IgG concentration, did not explain a significant reduction in risk of clinical malaria (Model 1; hazard ratio: 0.99, 95% CI: 0.97-1.02). Participants were then divided on the median in dCSP and dAI 'high' and 'low' responders and included as categorical variable in the model. Classification as 'high-dCSP' was associated with a significant risk reduction (77%) compared to the 'low dCSP' group in a model corrected for site and schedule (Model 2; hazard ratio: 0.23, 95% CI: 0.11-0.51). When dAI, corrected for site, schedule and dCSP was analysed, the hazard ratio between high and low responders separated by the median, was 0.46 (Model 3; 95% CI: 0.22-0.99; Wald test p = 0.049), hence classification as 'high dAI' group member is associated with a 54% risk reduction (Figure 4).

Discussion

The complex interplay of vaccine-primed immune mediators that define a successful response upon pathogen encounter is not well understood. Cellular and humoral components have important roles, although in various compositions, depending on the pathogen and the host. Antibodies are the prototypic vaccine-induced immune mediators and play an important role in anti-malarial immunity during the pre-erythrocytic [8,10] as well as the erythrocytic stage [14] of the disease, as shown by passive transfer experiments in mice and man. The sheer concentration of antigen-specific antibodies is normally used to measure immunization success and serves as a surrogate to estimate protective efficacy. The clinical development of RTS,S is a unique opportunity to investigate the effect of further variables such as antibody avidity, isotype or subclass on vaccine efficacy, since clinical (true) efficacy is known [5], being 57% (95% CI: 33–73) with the 012 schedule and 32% (95% CI: 16–45) following the 017 schedule.

Here, anti-CSP IgG avidity was measured to assess if it predicts vaccine efficacy in a phase II clinical trial of RTS,S independent of anti-CSP IgG concentration [5,12]. Regardless of the vaccination scheme and site, avidity did not improve prediction over anti-CSP IgG concentration alone. This may mean that: i) the assay is not sensitive enough to reflect avidity; ii) collinearity between antibody concentration and avidity blurs the effect of avidity; or, iii) that avidity is not an important determinant of vaccine efficacy. In this study IgG concentration and avidity was measured after the second and third

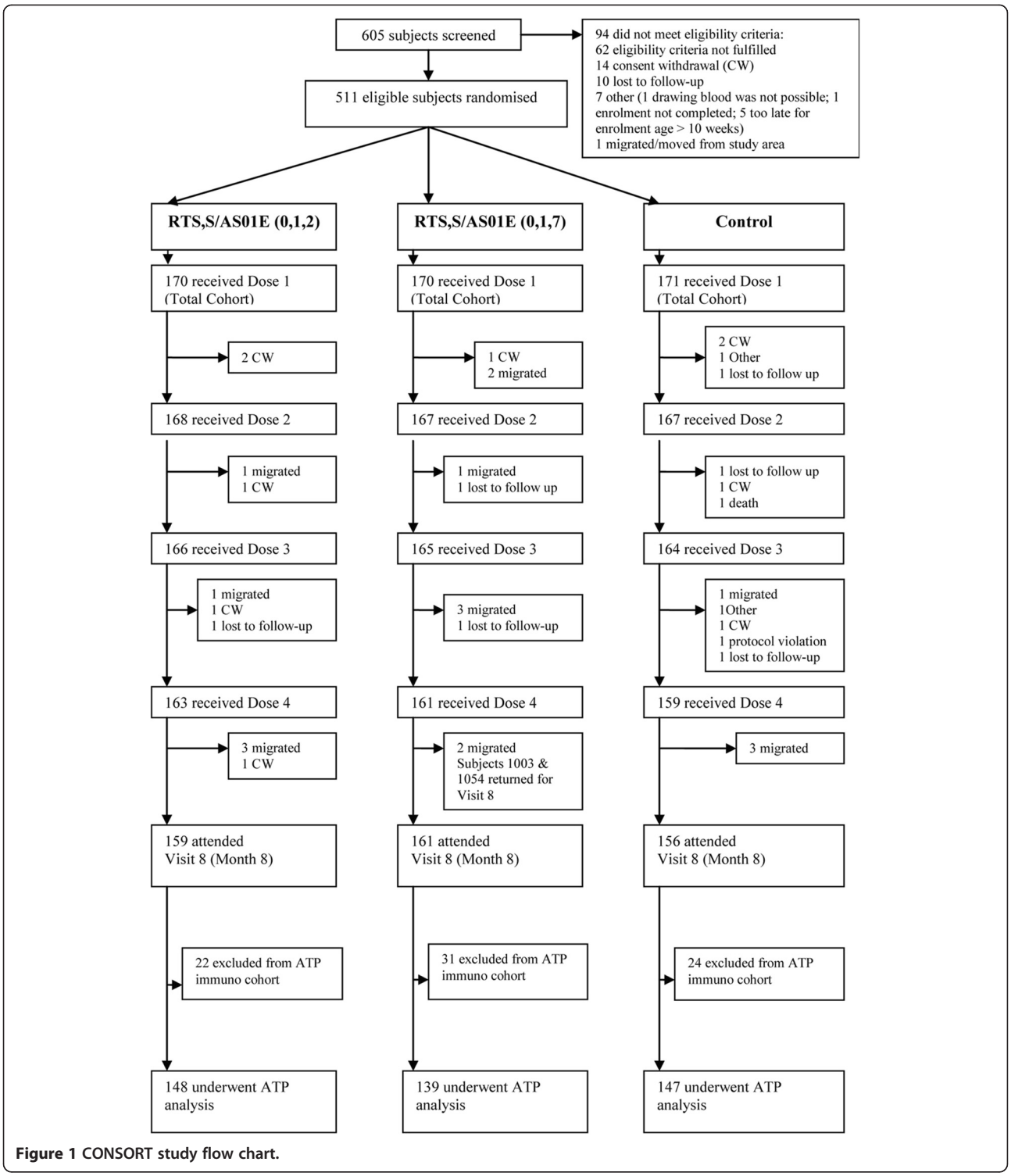

Figure 1 CONSORT study flow chart.

vaccine injection. This approach is valid to assess if the immune system reacted to vaccination successfully. Since kinetics of IgG vary over time and the study was performed under natural exposure to malaria parasites, the time of encounter with the parasite becomes an important variable. This is in contrast to controlled human malaria infection (CHMI) studies, where the time of infection is defined. Hypothetically, the difference in IgG concentration (and avidity) between second and third vaccination could be a better predictor of effective antibody-mediated protection than concentration after the third vaccine injection, because it better reflects the

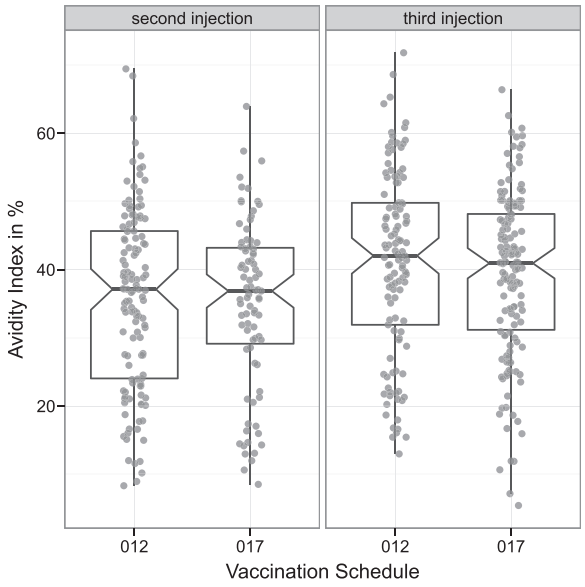

Figure 2 Box-plot and single measurements of absolute AI at second and third vaccination using two vaccination schedules (012 or 017).

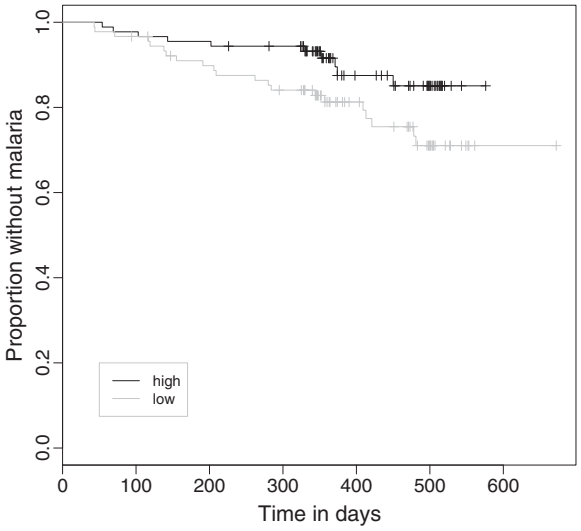

Figure 4 Kaplan Meier plot of malaria episodes over time in participants classified as having high (black) or low (grey) dAI.

further evolution of antibody responses until next parasite encounter. The present data argue for the use of this approach since it was shown that a high dCSP predicts protective efficacy and dAI explains part of the protection in the RTS,S vaccinated children (Model 3). How AI evolves over time and if it is a useful predictor of vaccine efficacy remains to be validated with further, independent and confirmatory studies.

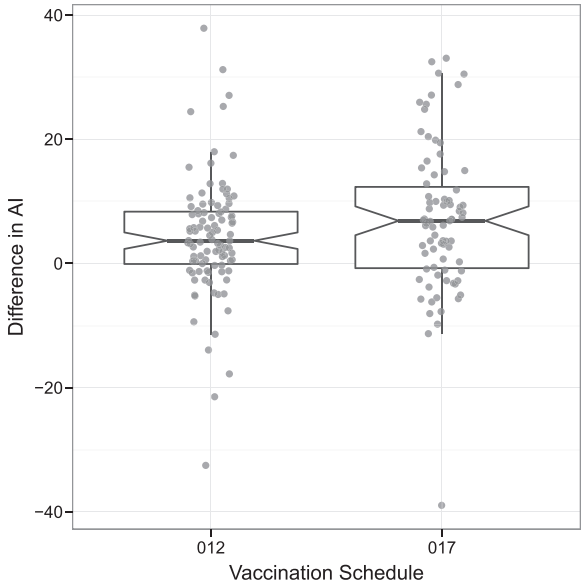

Figure 3 Box-plot and single measurements of difference in AI (dAI) between second and third vaccination using two vaccination schedules (012 or 017). Note that for the analysis of dAI only paired samples were used (n = 179).

Nevertheless, this observation adds a new component to the search of correlates of protection and the understanding of the immune responses elicited by pre-erythrocytic malaria vaccine candidates such as RTS,S. Since adjuvants also have a profound effect on the speed of avidity maturation [11], the effect of avidity on vaccine efficacy could even be analysed with interventional studies that assess the effect of timing between immunizations (as in this study) and different adjuvants on protective efficacy while direct measures of maturation of the immune system such as single-cell based sequencing of IgG genes of anti-CSP memory B-cells [15,16] are performed. This may be particularly interesting for antigens such as CSP that are not highly immunogenic *per se*, because highly immunogenic antigens often induce antibodies with strong avidity over a short period of time and a threshold antibody concentration is appropriate to predict their efficacy [17]. Other studies in the development of RTS,S (e.g., challenge experiments [18] and the recently completed phase III trial [1-3]) will certainly provide additional information and may establish the measurement of avidity as one biomarker for vaccine efficacy. Additionally, such knowledge may guide the design of next generation vaccines and administration schemes.

Conclusions

So far, the most robust correlate of protection for the malaria vaccine candidate RTS,S is anti-circumsporozoite (CSP) IgG concentration following immunization. Preclinical data and theoretical considerations suggest that avidity may have an additional impact on protective

efficacy. It is shown that an increase in anti-CSP IgG concentration and avidity between second and third vaccine injection is associated with a strong risk-reduction for malaria after immunization. This finding shall influence the way of analysis of immunological correlates of protection since using change in antibody concentration and avidity rather than single measurements enables improved modelling of immune-effector function at the time of pathogen encounter and hence more powerful prediction of vaccine efficacy.

Consent

Written informed consent was obtained from each child's parent(s). Illiterate parents were informed about the study in the presence of an impartial and literate witness and informed consent was documented by thumbprint of the parent and signature of the witness.

Abbreviations

AI: Avidity index; dAI: delta AI; CSP: *Plasmodium falciparum* circumsporozoite protein; dCSP: Delta CSP; ATP: according to protocol; IgG: Immunoglobulin G.

Competing interests

This study was funded by PATH-MVI and GlaxoSmithKline Biologicals SA. GM and MT report receiving funding for study-related travels. MT reports receiving financial compensation for activities outside the submitted work for board membership of the Optimus Foundation and the Novartis Institute for Tropical Diseases, having grants pending from both PATH-MVI and the Bill and Melinda Gates Foundation, and receiving travel reimbursements from PATH-MVI and Sanaria Corp. JV, EJ, ML, and PC are employees of the GlaxoSmithKline group of companies. JV, EJ and ML receive GlaxoSmithKline stock and/or options. CFO is an employee of PATH-MVI. Other authors report no conflicts of interest other than study funding.

Authors' contributions

AA and BM drafted the manuscript and performed the statistical analysis. BL, STA, KPA, SO-A, GM, MM, and NS collected the data and performed analyses. MT, SA, JV, EJ, ML, PC, CFO, and PGK conceived and supervised the study. All authors contributed to writing and review of the manuscript. All authors read and approved the final manuscript.

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