UNIVERSITAT DE BARCELONA

Understanding the immunogenicity of RTS,S/AS0 vaccination in African children: antigen glycosylation, booster dose and off-target antibody responses

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UNDERSTANDING THE IMMUNOGENICITY OF RTS,S/AS0 VACCINATION IN AFRICAN CHILDREN: ANTIGEN GLYCOSYLATION, BOOSTER DOSE AND OFF-TARGET ANTIBODY RESPONSES

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List of abbreviations

Abs	Antibodies
ACTs	Artemisinin-based Combination Therapies
ADCC	Antibody-Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
AL	Artemether-Lumefantrine
AMA1	Apical Membrane Antigen
APCs	Antigen-Presenting Cells
ART	Antiretroviral Treatment
AS+AQ	Artesunate-Amodiaquine
AS+SP	Artesunate+Sulfadoxine-Pyrimethamine
ASMQ	Artesunate-Mefloquine
ASPY	Artesunate-Pyronaridine
BCRs	B Cell Receptors
BM	Bone Marrow
bnAbs	broadly neutralizing Antibodies
BSV	Blood-Stage Vaccines
CCR7	Chemokine Receptor type 7
CelTOS	Cell-Traversal protein for Ookinetes and Sporozoites
CHMI	Controlled Human Malaria Infection
COVID-19	Coronavirus Disease 2019
CSP	Circumsporozoite Surface Protein
CSR	Class-Switch Recombination
DAMPs	Damage-Associated Molecular Patterns
DCs	Dendritic Cells
DDT	Dichlorodiphenyltrichloroethane
DHAP	Dihydroartemisinin-Piperaquine
DSB	Double-Strand Breaks
EBA	Erythrocyte-Binding Antigen
Fab	Antigen-binding fragment

Fc	Crystallizable fragment
FCM	Flow Cytometric
FDCs	Follicular Dendritic Cells
GC	Germinal-centre
GLURP	Glutamate Rich Protein
GPI	Glycosylphosphatidylinositol
GSK	GlaxoSmithKline
HBsAg	Hepatitis B surface antigen
HCs	Heavy Chain
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HRP2	Histidine-Rich Protein 2
Hz	Haemozoin
IF	Interfollicular
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-6	Interleukin 6
IPTp	Intermittent Preventive Treatment in pregnancy
IPTsc	Intermittent Preventive Treatment of Malaria in school-aged children
iRBCs	Infected Red Blood Cells
IRS	Indoor Residual Spraying
ITNs	Insecticide-Treated Nets
LAMP	Loop-mediated isothermal amplification
LCs	Light Chains

LLPC	Long-lived plasma cells
LPS	Lipopolysaccharide
LS	Liver stage
LSA1	Liver Stage Antigen 1
MalERA	Malaria Eradication Research Agenda
MALT	Mucosal-associated lymphoid tissue
MBCs	Memory B cells
MDA	Mass Drug Administration
MHC	Major Histocompatibility Complex
MS	Mass Spectrometry
MSP	Merozoite Surface Protein
TBV	Transmission-Blocking Vaccine
MW	Molecular Weight
MZ	Marginal Zone
NAI	Naturally Acquired Immunity
NHEJ	Non-Homologous end Joining
NK	Natural Killer
NO	Nitric Oxide
PAMPs	Pathogen-Associated Molecular Patterns
PDMC	Post-Discharge Malaria Chemoprevention
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein 1
pLDH	Plasmodium Lactate Dehydrogenase
PMC	Perennial Malaria Chemoprevention
PRRs	Pattern Recognition Receptors
PVM	Parasitophorous Vacuole Membrane
RBCs	Red Blood Cells
RDTs	Rapid Diagnostic Tests
RNA	Ribonucleic Acid
RSS	Recombination Signal Sequences
SERA5	Serine repeat antigen 5
SHM	Somatic Hypermutation

SLPCs	Short-lived Plasma Cells
SMC	Seasonal Malaria Chemoprevention
SP	Sulfadoxine-Pyrimethamine
SP-IPTi	Intermittent Preventive Treatment in infants
SP-IPTp	Intermittent Preventive Treatment in pregnancy
TCR	T Cell Receptor
TD	Thymus-Dependent
TFH	T follicular helper
TGF-β	Transforming Growth Factor beta
Th1	Type 1 T helper
TI	Thymus-Independent
TLRs	Toll-like Receptors
TNF-α	Tumor Necrosis Factor-alpha
TRAP	Thrombospondin Related Adhesion Protein
TSR	Thrombospondin-like type I repeat
VIMT	Vaccines that Interrupt Malaria Transmission
VLP	virus-like particle
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research

List of publications

This thesis is presented in a compendium of articles format and it consists of three objectives and three articles:

Article 1

Jairoce C, Macià D, Torres-Yaguana JP, Mayer L, Vidal M, Santano R, Hurtado-Guerrero R, Reiter K, Narum DL, Lopez-Gutierrez B, Hamerly T, Sacarlal J, Aguilar R, Dinglasan RR, Moncunill G, Izquierdo L, Dobaño C. **RTS, S/AS02A malaria vaccine-induced IGG responses equally recognize native-like fucosylated and nonfucosylated plasmodium falciparum circumsporozoite proteins.** The Journal of Infectious Diseases. 2024 Mar 15;229(3):795-9.

Impact factor (2023): 5.0 Quartile: 1 Category: Infectious Disease

Article 2

Sanchez L, Vidal M, **Jairoce C,** Aguilar R, Ubillos I, Cuamba I, Nhabomba AJ, Williams NA, Díez-Padrisa, N, Cavanagh D, Angov E, Coppel RL, Gaur D, Beeson JG, Dutta S, Aide P, Campo JJ, Moncunill G, Dobaño, C. **Antibody responses to the RTS, S/AS01E vaccine and Plasmodium falciparum antigens after a booster dose within the phase 3 trial in Mozambique.** NPJ Vaccines. 2020;5(1).

Impact factor (2020): 7.344. Quartile: 1. Category: Immunology

Article 3

Dobaño C, Ubillos I, **Jairoce C,** Gyan B, Vidal M, Jiménez A, Santano R, Dosoo D, Nhabomba AJ, Ayestaran A, Aguilar R, Williams NA, Díez-Padrisa N, Lanar D, Chauhan V, Chitnis C, Dutta S, Gaur D, Angov E, Asante KP, Moncunill G. **RTS, S/AS01E immunization increases antibody responses to vaccine-unrelated Plasmodium falciparum antigens associated with protection against clinical malaria in African children: a case-control study.** BMC medicine. 2019 Dec;17:1-9.

Impact factor (2019): 6.782. Quartile: 1. Category: Medicine

Resum

Títol: Immunogenicitat de la vacuna de la malària RTS,S/AS0 en nens africans: glicosilació de l'antigen, dosi de reforç i respostes d'anticossos enfront antígens no inclosos en la vacuna.

Introducció

La malària, causada pel *Plasmodium falciparum*, continua sent una preocupació important de salut pública, especialment en nens menors de 5 anys a l'Àfrica subsahariana. La RTS,S/AS01E és la primera de dues vacunes contra la malària aprovades per l'OMS (l'altra és R21/Matrix-M). Ambdues s'estan implementant actualment en nens africans però són parcialment efectives i generen una immunitat de curta durada. RTS,S genera anticossos IgG enfront la proteïna de superfície de l'esporozoït anomenada circumsporozoït (CSP), resposta que s'ha vist associada a la protecció enfront la malària. Comprendre el mode d'acció, els correlats immunològics de protecció i els factors que afecten la immunogenicitat i l'eficàcia de la vacuna RTS,S és essencial per guiar el disseny de vacunes contra la malària de nova generació més duradores i efectives.

Hipòtesis

La hipòtesi general d'aquesta tesi doctoral és que la resposta immunitària a la vacuna RTS,S és més complexa que la resposta estudiada fins ara d'IgG anti-CSP. Aquesta depèn del disseny de l'immunogen, de diferents subclasses d'IgG induïdes en les dosis primàries o per la dosis de reforç i afecta els anticossos adquirits de manera natural.

1. Els anticossos IgG induïts per RTS,S, on la CSP no està fucosilada, reconeixen pitjor la CSP fucosilada nativa en el paràsit, cosa que pot afectar la protecció.

2. Una quarta dosi de RTS,S (de reforç) està associada a canvis en els perfils de subclasses d'anticossos enfront antígens de la vacuna i de la malària, cosa que podria estar relacionada amb l'eficàcia de la vacuna i la seva durada.

3. RTS,S afecta l'adquisició d'anticossos contra antígens de *P. falciparum* no relacionats amb la vacuna i aquestes respostes impacten la immunitat protectora general enfront la malària.

Objectius

L'objectiu general és augmentar la comprensió de la immunogenicitat de la vacuna RTS,S en nens africans i la seva relació amb la protecció. Específicament:

1. Avaluar la unió dels anticossos induïts per RTS,S, on l'immunògen no està O-fucosilat , a la CSP fucosilada i no fucosilada i avaluar la rellevància d'aquesta modificació post-traduccional en la protecció contra la malària.

2. Caracteritzar la magnitud, tipus i longevitat de les respostes d'anticossos enfront els antígens de la vacuna RTS,S i altres de *P. falciparum* no inclosos a la vacuna, després de la vacunació primària i de reforç.

3. Comprendre la interacció entre la vacunació RTS,S i les respostes d'anticossos adquirides de manera natural a la malària i identificar els correlats immunològics de protecció.

Mètodes i resultats

Els resultats d'aquesta tesi es presenten com un compendi de tres articles publicats. Hem aplicat uns assajos serològics multiplex per mesurar els nivells d'anticossos específics a antígens relacionats i no relacionats amb la vacuna. En l'article 1, vam analitzar mostres de nens/es de Moçambic d'un estudi clínic de fase 2. Contràriament a la nostra hipòtesi, vam demostrar que després de la vacunació primària, les IgG induïdes per RTS,S reconeixen de manera similar els antígens CSP nadius fucosilats i els no fucosilats similars a la vacuna. A l'article 2, vam caracteritzar la magnitud, el tipus i la longevitat de les respostes d'anticossos enfront els antígens de la vacuna RTS,S i altres proteïnes del paràsit no relacionades amb la vacuna després de la immunització primària i la de reforç, a l'assaig clínic de fase 3 de la RTS,S en nens Africans. Els nivells de les subclasses IgG1, IgG3 i IgG4, però no IgG2 ni IgM, contra antígens de la vacuna, varen augmenten un mes després de la quarta dosi. En general, les respostes d'anticossos a la dosi de reforç van ser inferiors a la resposta màxima inicial a la immunització primària i el perfil de subclasses diferent. A més, els nens/es més petits/es tenien nivells d'IgG i IgG1 més baixos que nens/es més grans. Finalment, en els articles 2 i 3, pretenïem entendre la interacció entre la vacunació RTS,S i les respostes d'anticossos adquirides de manera natural enfront la malària i identificar els correlats immunològics de protecció. Vam trobar que la immunització amb RTS,S afecta l'adquisició d'IgG i IgM als antígens de *P. falciparum* que no formen part de la formulació de la vacuna de diferents maneres. Anticossos contra antígens molt immunogènics i coneguts com a

marcadors d'exposició a la malària disminueixen amb la vacunació, mentre que d'altres no es veuen afectats i d'altres augmenten, i aquests darrers anticossos estan associats a la protecció enfront la malària.

Conclusions

1. Les modificacions post-traduccionals per O-fucosilació a CSP present a *P. falciparum* no afecten la unió a l'antigen dels anticossos IgG induïts per la CSP no fucosilada inclosa a la vacuna RTS,S. A més, les IgGs generades de manera natural, presumiblement a CSP fucosilada en esporozoïts, reconeixen de forma similar l'antigen no fucosilat. Malgrat això, no podem descartar cap benefici potencial d'una vacuna alternativa RTS,S fucosilada.

2. La dosi de reforç RTS,S indueix respostes que difereixen de la resposta immune primària de la vacuna. Tot i que el reforç va augmentar els nivells d'anticossos IgG, IgG1 i IgG3 per a tots els antigens de la vacuna, que van disminuir substancialment amb el temps, el pic va ser inferior a la resposta màxima de la vacunació primària. A més, la dosi de reforç no va augmentar els nivells d'IgG2, com es va veure després de la vacunació primària, destacant els canvis en les subclasses d'anticossos després d'una dosi de reforç.

3. La vacunació RTS,S altera la immunitat adquirida de manera natural enfront la malària, disminuint els nivells d'IgG contra els antígens immunogènics de *P. falciparum*, que són marcadors d'exposició a aquesta malaltia infecciosa, alhora que augmenta els nivells d'IgG contra alguns antígens de malària no presents a la vacuna. L'augment dels nivells d'IgG per la vacunació RTS,S s'associa a una major immunitat i pot ser un nou correlat de la protecció induïda per RTS,S.

Introduction

Malaria

A Brief History of the discovery of malaria parasites

The term malaria was introduced into English by John MacCullogh in 1827 (1). It is derived from the Italian 'mal'aria', which means 'bad air', from the early association of the disease with marshy areas. Malaria is a severe and life-threatening infectious disease caused by *Plasmodium* parasites transmitted through infected Anopheles mosquito bites (2). The condition is regarded as an ancient disease that is preventable and curable. Its prevention and treatment using ancient remedies and modern pharmaceutical agents have been targeted in science and medicine for centuries. However, our understanding of the *Plasmodium* parasites that cause the disease had started on the 6th of November 1880 when Charles Louis Alphonse Laveran, a French army surgeon, noticed for the first time the *Plasmodium spp.* in the blood of a patient suffering from malaria, at Constantine in Algeria (1,3). On 20 August 1897, Ronald Ross made a landmark discovery of the malaria parasite while dissecting the stomach tissue of *Anopheles* mosquitoes fed previously on a malarious patient (1,4,5). Later, he unraveled the mystery of malaria transmission and elucidated that the malaria parasites could develop in the mosquitoes and migrate to the insect salivary glands, allowing the mosquitoes to infect birds during subsequent blood meals (6). Another pivotal milestone in the history of malaria was made in 1897 when William MacCallum discovered the sexual stages in the blood of infected birds (1,3,7). In 1898, the Italian malariologists Giovanni Battista Grassi and his associates demonstrated conclusively that human malaria could only be transmitted by *Anopheles* mosquitoes, particularly females, and they described for the first time the entire developmental cycle of the human malarial parasites in these insects (3,8). Cyril Garnham and Henry Shortt discovered the pre-erythrocytic stages of *P. cynomolgi* in the liver of a Rhesus monkey in 1948 (9). Another event of great biological and medical significance was the discovery of malarial hypnozoites by Wojciech A. Krotoski and colleagues in 1980 (10). Over the years, much has been done to understand the malaria parasite's life cycle and the mosquito's biology. The publication of complete genome sequences for the human parasite, the apicomplexan *P. falciparum,* and the mosquito *Anopheles gambiae* in 2002 was a notable advance in recent malaria research (11).

1. The global burden of the disease

Malaria's ongoing transmission occurs in 85 countries and territories (12), and it happens mainly in the world's poor tropical and subtropical areas (13–15). Human malaria is caused by five parasite species, including *P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi* (16), and two of these species, *P. vivax and P. falciparum*, pose the considerable threat globally (17). *P. falciparum* is the most fatal and most prevalent in sub-Saharan Africa (18), accounting for over 90% of all malaria infections in this region (14). *P. vivax* is the most widespread and dominant in most countries outside of sub-Saharan Africa. Particularly with a high prevalence of infection occurring in the Western-Pacific and Southeast Asian regions and a relatively high endemicity prevailing in much less densely populated areas of the Americas (18–20).

Many factors play a role in Africa's high disease burden. The abundance of dominant vector species, including the *Anopheles gambiae* complex, is partly responsible for the high and efficient spread of the predominant parasite species, *P. falciparum* (13,21,22). The transmission occurs year-round due to local weather conditions suitable for the life cycle and development of the parasites that cause malaria (13). These favorable conditions include rainfall, temperature, and humidity, which are known to impact the bionomics of malaria vectors and eventually determine malaria transmission intensity (23). Temperature is particularly critical, as the transmission is less intense and seasonal in cooler regions (15). For instance, below 20°C, *P. falciparum* cannot complete its development cycle in the *Anopheles* mosquito and cannot be transmitted (15). In many African countries, malaria control activities have been hindered due to scarce resources and socio-economic instability (13). Between 2000 and 2015, there was a progressive reduction in the burden of malaria. Malaria incidence was diminished by 27%, while the mortality rate was fell by 60%, thanks to the scale-up of standard control interventions. After that period, progress stalled and even reversed in some countries (12,24).

According to the World Health Organization (WHO), nearly 249 million malaria cases were reported globally in 2022 in 85 malaria-endemic countries (including the territory of French Guiana), with an increase of 5 million malaria cases from the preceding year (12). However, 608,000deaths were reported in the same year, a slight reduction of about 0.32% from 2021 (12). Between 2019 and 2020, an estimated increase of 55,000 deaths was reported (12), which was attributed to the disruption to the provision of malaria services due to the coronavirus disease 2019 (COVID-19) pandemic and other humanitarian emergencies, as most malaria-endemic countries experienced moderate disruptions (12). Climate change has also contributed to fluctuations in transmission patterns and the malaria burden globally by producing conditions that favor vector breeding (24).

The WHO African Region carries a high share of the world's malaria burden, accounting for about 93.6% of cases and 95.4% of deaths in 2022 (12). Malaria fatalities in the WHO African Region increased from 552,000 to 604,000 between 2019 and 2020. Although the estimated number of cases did not change, the estimated number of deaths reduced to 580,000 in 2022 (12). Between 2019 and 2022, seven countries in the WHO African Region had substantial increases in the estimated number of cases: Mozambique (1 million), Mali (1.1 million), Tanzania (1.3 million), Uganda (1.3 million), Madagascar (1.5 million), Ethiopia (2.4 million), and Nigeria (5.3 million) (12).

Children under five years of age are at higher risk, accounting for approximately 78.1% of malaria deaths in this region (12), because they have not yet developed immunity to the disease. Pregnant women are also another risk group as their immunity might be decreased, especially during first-time gestation (13,25). Moreover, individuals living with Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS), as well as people with low immunity traveling to areas with intense malaria transmission, including migrant workers, mobile populations, and travelers, are also at considerably higher risk of contracting malaria and developing severe and potentially fatal disease (18).

Although the prevalence of non-falciparum malaria represents only a small percentage of infections, their geographical ranges overlap (26). They increasingly contribute to infection, morbidity, and mortality in some African countries and become important sources of ongoing transmission (27).

In endemic regions, malaria accounts for 39% of public spending on health (19), up to 50% of outpatient visits and 30% to 50% of hospital admissions (28,29). The disease debilitates the workforce, contributes to children's school absenteeism, prevents pregnant mothers from effectively taking care of themselves and their families, and increases the risk of adverse consequences on birth outcomes (28). Moreover, it devastates health systems, economic development, and growth (27,28).

2. The parasite and the life cycle (*P. falciparum*)

The protozoan genus *Plasmodium* is a member of the phylum Apicomplexa, order *Haemosporida,* and family *Plasmodiidae* (30). It comprises unicellular eukaryotes that are obligate intracellular parasites of vertebrates (31). Over 270 species of *Plasmodium* have been formally described based on parasite morphology, host range (30) and other characteristics of interest. By far, *P. falciparum* is well known for being a devastating scourge of human health, resulting in hundreds of thousands of fatalities per year.

The life cycle of *P. falciparum* (**Figure 1**) in the human host begins with a silent **pre-erythrocytic stage** that includes both the sporozoite invasion of the human host and the liver *stage* (32) within 6–7 days (33).

A female Anopheles mosquito injects *Plasmodium* sporozoites into the dermis during blood feeding, and the injected sporozoites could take 1–3h to exit the site (34,35). The lymphatics drain and destroy the remaining sporozoites in the skin, generating the immune response (35). The sporozoites that successfully exit the injection site actively enter the vasculature, migrate to the liver, and enter the hepatocytes for replication (35). They form a parasitophorous vacuole membrane (PVM) and undergo schizogony over the subsequent 2–10 days, where the sporozoite transforms to a liver stage (LS) (35). In the liver cells, the sporozoites multiply asexually, causing no symptoms (36). The development culminates in releasing up to 40,000 daughter merozoites per hepatocyte into the bloodstream in packets called merosomes (37).

The following stage is the **blood stage** when malaria-related symptoms manifest (38,39), and the merozoites invade erythrocytes, developing either a 48-h asexual or sexual cycle (40).

In the blood stage **asexual cycle**, free merozoites invade erythrocytes in a fast, dynamic, and multi-step process that includes pre-invasion, active invasion, echinocytosis and develop through ring, trophozoite, and schizont stages (41). The process is complete within approximately 48 h before new merozoites are released at schizont egress and reinvade new erythrocytes (34,35)

In the blood stage **sexual cycle**, a small proportion of asexually reproducing merozoites develop into sexual forms called gametocytes (34,35). Within 15 days, gametocytes sequester, develop within the bone marrow, and enter the dermis after maturation, where another female mosquito takes them up during the blood meal. They emerge as extracellular male and female gametes in the mosquito midgut (35).

Inside the mosquito, the sporogonic cycle occurs through a fusion of micro and macrogamete to form a zygote, transforming over 24 h into an ookinete. The ookinete migrates through the mosquito midgut epithelium and encysts in the layer out of the midgut, where asexual sporogenic replication occurs to become an oocyst (35). Motile infectious sporozoites are released into the hemocoel by oocyst rupture and pass into salivary glands for transmission into another human host (35). Developmental time in the mosquito is typically in the order of 7–14 days (42). Contrary to the human host, the mosquito vector does not suffer from the presence of parasites (39).

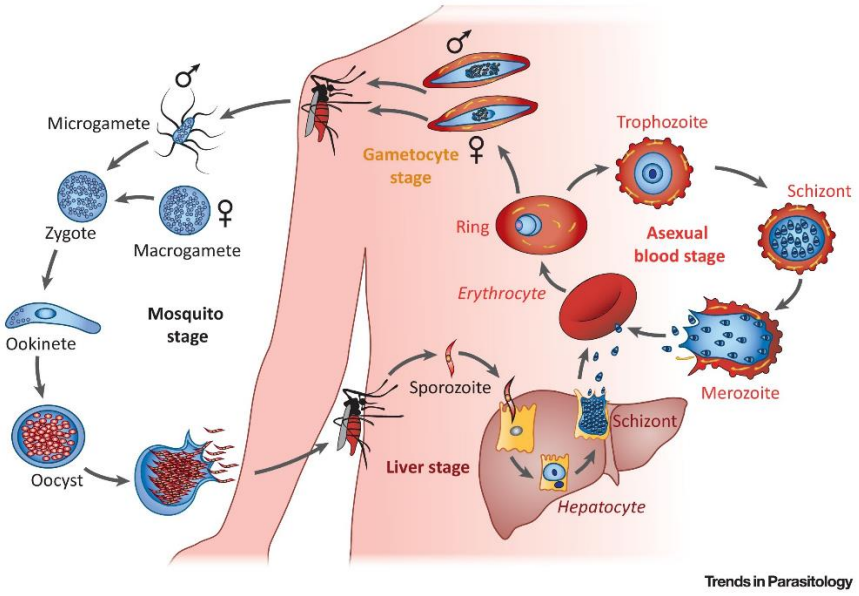

Figure 1. The *Plasmodium falciparum* life cycle in the human host *(43)*.

3. Pathogenesis

The intraerythrocytic stage and schizogony cause the clinicopathological features of malaria, where the rupture of erythrocytes is associated with spiking fevers and chills (44). In most infected patients, the first rupture and invasion are usually silent. However, in the next 24 to 48 h, the asexual cycle repeats itself, resulting in high parasitemia and increasing the host immune response (45). Fever and illness are caused by the release of proinflammatory cytokines (particularly tumor necrosis factor [TNF]) and other inflammatory mediators, including related pyrogenic cytokines interleukin 1 (IL-1) and IL-6 (44,46).

The pathology of severe falciparum malaria is complex. It probably results from a combination of host inflammatory responses, namely cytokine and chemokine production and cellular infiltrates, together with parasite-specific factors, including adhesion and sequestration in the microvasculature and the release of bioactive molecules (47). However, the most important virulence determinant in *P. falciparum* infection is the ability of parasite-infected erythrocytes containing a mature form to adhere to the vascular endothelium (cytoadhesion/sequestration) (44,45,48) or to surrounding uninfected red blood cells (rosetting) (49). These phenomena are mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family on the surface of the infected erythrocytes (48,50), which enable infected erythrocytes to engage multiple receptors that are expressed by vascular endothelial cells (50). The cascade of events of the pathology of severe falciparum malaria begins with the release of malarial antigens during the rupture and reinvasion of red blood cells (RBCs), particularly those having glycophosphatidyl inositol (GPI) membrane anchors, stimulating the

production of TNF (51) and IL-1 by macrophages (52). The production of IFN-γ by T cells primed by previous exposure to either malaria or cross-reacting antigens also contributes to the production of a pathologically high level of TNF (51). TNF induces the expression of adhesion molecules in vascular endothelial cells, including intercellular adhesion molecule-1, thrombospondin, vascular cell adhesion molecule-1, E-selectin, and chondroitin sulfate A (51).

P. falciparum trophozoite proteins interact with the plasma membrane of the infected erythrocytes to form knob-like protrusions and other membrane abnormalities (51). Infected RBC with altered membrane surfaces adhere to upregulated endothelial adhesion molecules (51). Trapped infected erythrocytes are sequestered away from the destruction by the immune system (53) and from splenic clearance (51). However, cytoadherence and rosetting contribute to inflammatory pathology, microcirculation obstruction, impaired tissue perfusion, lactic acidosis, and compromise of regional metabolism, leading to end-organ damage (42,44,53,54). This phenomenon is most evident in the brain, resulting in cerebral malaria, and it also occurs in other vital organs (44).

The release of bioactive molecules also plays a partial role in severe malaria. The malaria toxin hemozoin (Hz), also known as malaria pigment, is a by-product of intra-erythrocytic parasite replication, released in the circulation after schizont rupture (55,56). Hz has been reported to accumulate in large amounts inside multiple organs and is associated with malaria-associated pathologies (55,56). Phagocytosed Hz inhibits the differentiation and maturation of host phagocytes (57), regulates the synthesis of several host cytokines, and has been associated with the disease severity (58). Hz-containing peripheral white blood cells are a marker for disease severity (59), and Hz accumulation in brain micro-vessels results in cellular dysfunction and toxicity (56). Overall, Hz seems to be highly immunosuppressive (60,61).

The nitric oxide (NO) induction synthesis by endothelial cells also seems to contribute to inflammatory lesions in the brain (51). However, other critical immunoregulatory molecules are also produced, including IL-10, potentially downregulating the Type 1 T helper (Th1) immune response and TNF production, limiting the inflammatory response (51).

The virulence of *P. falciparum* is mainly associated with immune response-evading ability. *Plasmodium* has developed a range of biomolecular strategies across its whole life cycle based on a range of genetic changes, including extensive allelic variation, intracellular replication, and sequestration. These strategies contribute to the ability of the malaria parasite to evade the immune response and to guarantee its survival within both mosquitoes and the vertebrate host (62–65).

4. Symptomatology

The incubation period for *P. falciparum* (the interval between infection and the onset of clinical illness) is 6-14 days (66). Children living in endemic areas are more likely to develop severe malaria, resulting in higher mortality than adults who have gained protective immunity and maintain it so long as they continue to be exposed (44). The frequency of episodes and severity of clinical malaria depends on the age of the individual (**Figure 2**) and transmission intensity, as repeated exposure results in acquired immunity that develops first against severe malaria, followed by uncomplicated malaria but rarely, if ever, involving resistance against infection (67,68).

Low-grade infections occur with few or no clinical symptoms due to non-sterile immunity conferred by repeated exposure to *Plasmodium* (42,44) and asymptomatic parasitemia is a major cause of chronic anemia and splenomegaly in young children (44). However, most episodes of infection with *P. falciparum* in malaria-endemic areas lead to mild clinical symptoms (uncomplicated), such as fever, general malaise, headache (42,69), chills, night sweats, nausea, vomiting and body aches (69). Severe malaria complications evolve in a proportion of cases if untreated (70) or in non-immune subjects (71). Clinical manifestations of severe *P. falciparum* infection are variable in nature and severity (42) and are remarkably similar to many other conditions (46). Cerebral malaria, metabolic acidosis, and kidney dysfunction were found to be independent predictors of mortality in both adults and children (72,73). Cerebral malaria syndrome is associated with high mortality, usually with abnormal behavior, impairment of consciousness, seizures, coma, or other neurologic abnormalities (69).

Renal dysfunction or failure may occur as a direct effect of the parasites in the kidney, which trigger an inflammatory process resulting in different types of glomerulonephritis (74).

Other poor prognostic indicators in severe malaria include hypoglycemia, circulatory collapse and shock, disseminated intravascular coagulation, and spontaneous bleeding.

Severe anemia caused by the destruction of RBCs further compromises oxygen delivery, causing respiratory distress and hemoglobinuria (42,69,75).

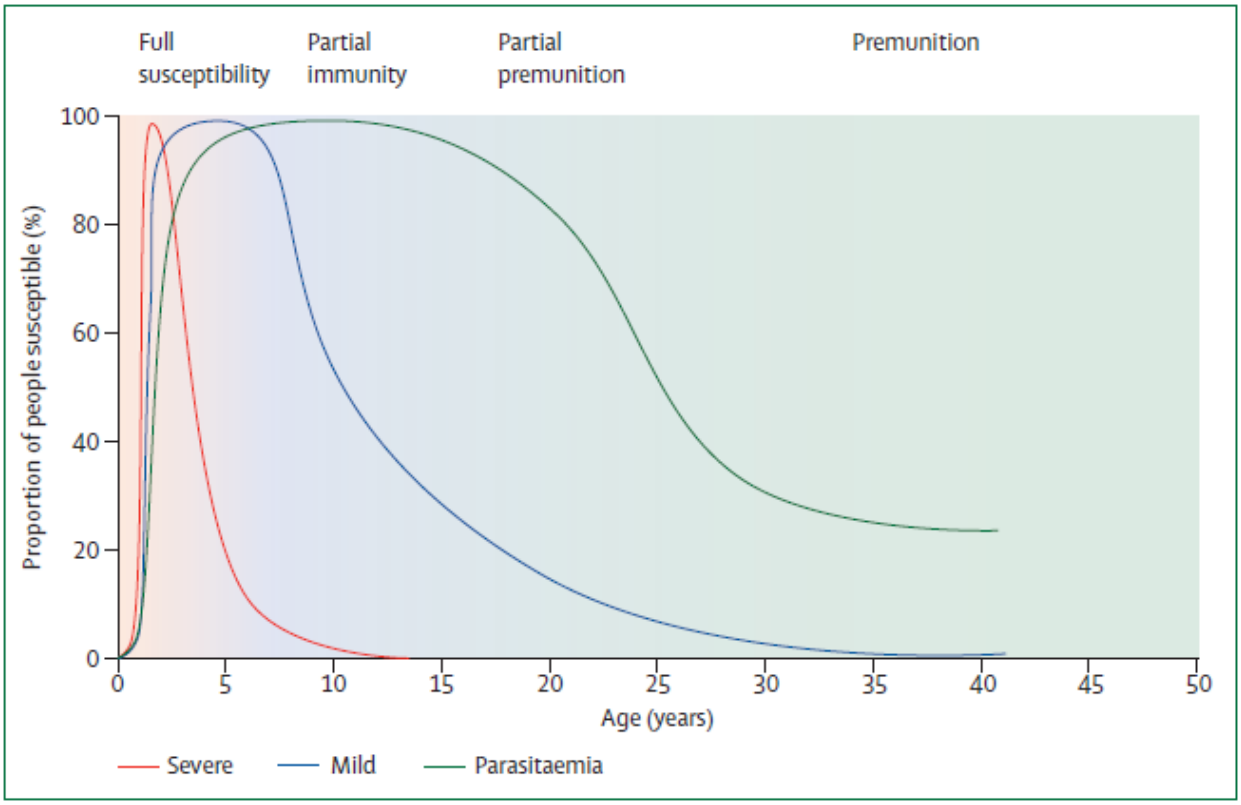

Figure 2. Relationship between the age of the individual and malaria severity in areas of moderate malaria transmission intensity *(67)*.

5. Diagnosis

5.1 Clinical Diagnosis

Clinical malaria diagnosis is based on the patient's signs, symptoms, and physical findings at examination (76,77). Malaria is considered a potential medical emergency and should be treated accordingly (76), as prompt diagnosis and correct treatment of malaria reduce disease, prevent deaths, and contribute to lowering transmission (18). The WHO recommends that clinicians in endemic areas suspect malaria in any patient who reports having fevers or has a current temperature ≥ 37.5 °C without any other apparent cause (78). Children with signs of anemia: pale palms or a laboratory test showing hemoglobin concentration of <8 g/dL should similarly be suspected of malaria (78). In areas with little to no malaria, the testing is only recommended for people with possible exposure to malaria (typically traveling to a malaria-endemic area) and unexplained fever (78). A laboratory test for malaria should always confirm clinical findings due to non-specific malaria symptoms and variable nature at the early stage (76,77). In addition to requesting malaria-specific diagnostic tests, it is recommended for healthcare providers to order complementary diagnostic tests, particularly a full blood count and a routine chemistry panel, as these tests can detect severe anemia,

hypoglycemia, renal failure, hyperbilirubinemia, and acid-base disturbances (77). In severely ill patients, additional analyses such as blood gases, blood culture, lactate, and clotting analysis are advisable (45).

5.2 Laboratory Diagnosis

In all settings, WHO recommends that all suspected malaria cases be confirmed using parasite based diagnostic testing (microscopy or a rapid diagnostic test [RDT]) (45). Conventional microscopic analysis of thick and thin peripheral blood smears remains the gold standard technique for laboratory confirmation of malaria (44,45,77). Before the examination, the specimen is stained with the Giemsa (most often) to give the parasites a distinctive appearance (44,77). The examination under the microscope uses a thick and thin smear and is a highly sensitive and specific laboratory technique. The thick smear is used for quick identification and parasitemia quantification, and the thin smear is used to determine the *Plasmodium* parasite species. The generated results are critical pieces of information when selecting therapeutic options (44,79). However, the technique is labor-intensive and ill-suited for high-throughput use, and species identification at low parasite density is still challenging (76).

Moreover, it also depends on the quality of the reagents, the microscope, electricity and the experience of the microscopists (44,77,79) and it involves relatively high costs for training and supervision (79).

Immunochromatographic RDTs detect malaria parasite-specific antigens or enzymes that are either genus or species-specific in finger-prick blood samples using test strips, cassettes, or cards impregnated with specific antibodies (44,79). Cassettes and cards tend to be simpler to use but more expensive (80). These are based either on the detection of histidine-rich protein 2 (HRP2), which is specific for *P. falciparum* or species-specific *Plasmodium* lactate dehydrogenase (pLDH) or species-specific aldolase (44,79). Some tests are designed to detect only one species (*P. falciparum*); others are designed to detect more human malaria species by detecting various other antigens (44,80).

Contrary to microscopy, RTDs are relatively simple to perform and interpret. They allow the rapid provision of results, do not require electricity or special equipment (269,271), and can be used in places without a skilled microscopist (44). They also have potential disadvantages, including the cost of the test kits, the inability to determine species of malaria and parasite load and the potential lack of sensitivity at very low parasitemias (44). Therefore, the use of RDTs should be reserved for settings where quality microscopy is not immediately accessible and should be followed as soon as possible by microscopy to confirm the results, identify the species, quantify the parasitemia, and monitor treatment (44,76).

Moreover, PfHRP2-based RDTs cannot distinguish new infections from recently and effectively treated infections due to the persistence of PfHRP2 in the blood for 1–5 weeks (79). Another major concern is the

emergence of the HRP2-negative *P. falciparum* strains that have been reported to be non-reactive to HRP2-based RDT, leading to false-negative results (81).

Developments in immunodiagnostic and molecular diagnostic tools include polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques, which have allowed an extensive characterization of the *Plasmodium* parasite and are generating new strategies for malaria diagnosis (76). Despite most of these advanced technologies being highly sensitive and specific, particularly nucleic acid-based techniques, they do not have a role in routine clinical management (79). However, immunodiagnostic tools are suitable for epidemiologic surveys, and molecular biology techniques can be used for research. They are all helpful in determining, confirming, and quantifying the infecting species and evidence of drug resistance.

6. Control strategies

In recent decades**,** expanded access to WHO-recommended malaria prevention tools and strategies, comprising effective vector control, diagnosis, surveillance, prevention, and treatment, has significantly reduced the global burden of malaria.

6.1 Malaria vector control

Mosquito control is a fundamental element of malaria control, elimination and eradication strategies as it is highly effective in averting infection and reducing the levels of disease transmission (18,82). At present, the WHO recommends two core interventions for large-scale deployment, either insecticide-treated nets (ITNs) or indoor residual spraying (IRS), for vector control in most endemic areas (82). In many settings, the WHO policy recommends two main classes of ITNs, either pyrethroid-only LLINs (recommended for deployment as a core intervention) or pyrethroid-piperonyl butoxide (PBO) net, a combination of pyrethroid insecticide and the synergist PBO, recommended for deployment when the malaria vector exhibit pyrethroid resistance (83,84). ITNs have been the foundation of malaria prevention efforts in sub-Saharan Africa and the most significant contributor to the drops in malaria incidence. The percentage of the entire at-risk population sleeping under an ITN increased exponentially from 2% to 43% from 2000 through 2020 (19,82).

IRS demands the application of a long-lasting insecticide to the inner side of the walls of houses and other structures where people sleep, aimed at killing mosquitoes when they rest on sprayed surfaces (85,86). The most recommended insecticides are pyrethroids and dichlorodiphenyltrichloroethane (DDT) (85,86). However, DDT has been known to pose a risk to human health (87). Currently, its use is restricted or only recommended if no equally effective alternative is available. The use of the IRS has declined, and the

percentage of the population at risk protected by the IRS fell from 5.8% in 2000 to 2.6% in 2020 (19,82). This decline in use in rent years is partly attributable to logistic challenges, lack of commitment and financing from governments for long-term sustainability, concerns about insecticide resistant mosquitoes, and fear of its possible detrimental effects on human health and the environment (86).

The effectiveness of malaria vector control relies on the systematic collection and interpretation of data on local vector species, the potential invasion by vectors from other geographical areas, their susceptibility to insecticides, vector and human behaviors and ongoing monitoring of the coverage, usage, quality and durability of vector-control interventions (82).

The progress in global malaria control is threatened by widespread resistance to insecticides, particularly pyrethroids, among *Anopheles* mosquitoes (18,82,83,88), as unchecked, insecticide resistance could lead to a considerable increase in malaria cases and mortality (82). Depending on the setting, circumstances, and available resources, ITNs or IRS may be supplemented by additional interventions (82,83). These include larviciding with chemical or biological insecticides (83), personal protection measures, area spraying, sterile male mosquitoes release, genetic modification of malaria vectors (88) and modifications to people's houses to prevent mosquito exposure (89), targeting livestock, mass drug administration (MDA) and sugar feeding (90).

6.2 Drugs

The primary aim of malaria treatment is to ensure the rapid and complete elimination of parasites, preventing uncomplicated malaria from progressing to severe disease or fatality. The WHO recommends the use of artemisinin-based combination therapies (ACTs) as the first and second line treatment for uncomplicated *P. falciparum* malaria (18). The ACTs combine an artemisinin derivative (including artesunate, artemether and dihydroartemisinin) with a partner drug with a longer half-life (91,92). The function of the artemisinin compound is to decrease the number of parasites in the first three days of treatment (rapid reduction of parasite biomass), while the partner drug ensures the clearance of the remaining parasites (cure) (91,92), hence reducing the likelihood of developing resistance to antimalarials.

WHO currently recommends six different ACTs with potential for deployment based on the latest available evidence on safety and efficacy, including artemether-lumefantrine (AL), artesunate-amodiaquine (AS+AQ), artesunate+sulfadoxine-pyrimethamine (AS+SP), artesunate-mefloquine (ASMQ), artesunate-pyronaridine (ASPY), and dihydroartemisinin-piperaquine (DHAP) (91,92). ACTs are also recommended for treating mixed

infections, as they have been shown to be efficacious against non-falciparum malaria (54). Atovaquone-proguanil, quinine sulfate plus (doxycycline, tetracycline, clindamycin), mefloquine, and chloroquine are other treatment options, although not as effective as ACTs. They can be used in adult and pediatric populations, with doses adjusted by the patient's weight (45).

In case of severe malaria, injectable AS is recommended as the first-line treatment in all patients (comprising children, lactating women, and pregnant women in all trimesters) (54,92). If it is not available, the recommended drug is artemether in preference to quinine (45,92).

The parenteral antimalarial therapy with AS or artemether should be followed by a full regimen of ACTs when the patient can tolerate oral treatment (45,54,91,92). In a low transmission setting, the addition of single-dose primaquine to ACTs is recommended for patients with *P. falciparum* malaria (except pregnant women, infants aged < 6 months, and women breastfeeding infants aged < 6 months). The combination was shown to rapidly reduce the infectivity of gametocytes to mosquitoes, thus reducing malaria transmission compared to ACTs alone (92). In case of recrudescence, which is the failure of resolution of fever and parasitemia or their recurrence within four weeks of treatment due to inadequate or ineffective treatment, the recommended second-line treatment alternative drugs known to be effective in the region should be given (93).

6.3 Preventive chemotherapies

Preventive chemotherapy is defined as using a full treatment course of antimalarial medicine (alone or in combination) to prevent malaria infections and their negative consequences at designated time points during the most significant malarial risk period, regardless of whether the receiver is infected with the malaria parasite. There are different types of preventive chemotherapy, including MDA, seasonal malaria chemoprevention (SMC), perennial malaria chemoprevention (PMC), intermittent preventive treatment of malaria in pregnancy (IPTp), infants (IPTi) and school-aged children (IPTsc) and post-discharge malaria chemoprevention (PDMC) (18). In Africa, the WHO recommends the use of SP for IPTp as part of antenatal care in endemic areas and in infants (SP-IPTi) (< 12 months of age) in moderate-to-high malaria transmission. Furthermore, it is recommended for SMC, particularly in the sub-Sahel region of Africa, with monthly AQ + SP for all children aged <6 years during each transmission season (92). The drugs used for preventive chemotherapies should not be used as a component of first-line treatments in the same country or region, and if the patient has taken chemoprophylaxis, the same medicine should not be used for treatment to prevent the emergence of resistance (92).

6.4 Antimalarial drug resistance

Inappropriate widespread use of antimalarial drugs exerts an intense selective pressure with genetic changes (mutations or gene amplifications) that confer the malaria parasite high resistance levels (92). Resistance has been reported in all classes of antimalarial medicines, including the artemisinin derivatives, and it is a significant threat that could derail global malaria control efforts (92). Antimalarial drug resistance has emerged over the last decade in the Greater Mekong subregion, Southeast Asia, and recent evidence shows drug-resistant malaria in Africa (18). Despite the drug resistance remaining a major concern, all strains of *P. falciparum* malaria globally can currently be treated with at least two ACTs (94). Genetic markers for artemisinin resistance and several other partner drugs have been established and have been helpful in tracking the emergence and geographical spread of resistance (95,96). New regimens and strategies using current antimalarial medicines will be required until novel compounds can be deployed (96). Moreover, regular monitoring of drug efficacy is required to inform treatment policies in malaria endemic countries and to ensure prompt detection of and response to drug resistance (18).

The immune system

1. Overview of the human immune system

The immune system is the collection of cells, organs, tissues, and molecules that mediate resistance to infection, including eliminating any foreign/non-self-substances (antigens). The immune system's orchestrated and tightly controlled reaction is called the immune response. Two distinct yet interrelated defense mechanisms are activated when an antigen attacks the host system: the innate (non-specific) and the adaptive (specific) immune response (97,98). Specific physiological mechanisms in both immune systems enable the host to recognize foreign materials to itself and neutralize, eliminate, or metabolize them (98).

Although all mature blood cell types originate from the same self-renew precursor hematopoietic stem cells in the bone marrow, their sites of maturation and residence differ (99,100). Many different types of innate immune cells typically reside in the blood and tissues (99).

The cells of the acquired immune system recombine their immune receptors and mature in the thymus (T cells) and bone marrow (B cells), known as primary immune organs (99). T and B cells with mature receptors then migrate through lymphatic vessels to lymph nodes, awaiting activation signals. The secondary immune

organs (also called the peripheral lymphoid organs) where lymphocytes reside include the lymph nodes, spleen, Peyer's patches, the appendix, tonsils, adenoids, and other mucosal-associated lymphoid tissue (MALT) (99,101).

Unlike innate immunity, adaptive immune responses are slow acting, possess exquisite antigen specificity and immunological memory, and respond more vigorously to repeated exposures, thus providing longer-lasting protection (102). There are two broad classes of adaptive immune responses, humoral (antibody-mediated) and cell-mediated immune responses, carried out by B cells and T lymphocytes, respectively (103,104). Notably, most immune responses involve the interplay and activity of both the humoral and the cell-mediated immune responses (98,105). Moreover, the adaptive immune system also frequently incorporates cells and molecules of the innate system (98). The activation of Pattern Recognition Receptors (PRRs) on Antigen-Presenting Cells (APCs), including macrophages and dendritic cells (DCs), enhance antigen-presenting activity and TLR mediated control of adaptive immune responses (106,107).

1.1 The innate immune system

The innate immune system aims to prevent infection and eliminate invader pathogens, and it represents the fast and first line of defense against germs that bypass the body's natural physical and chemical barriers (97). It was assumed that innate immunity has no immunological memory for a long time. However, recent evidence suggests that innate immune system activation can enhance responsiveness to subsequent antigen encounters. This de facto innate immune memory is known as 'trained immunity' or innate immune memory, and its induction is shaped by the innate stimuli, and epigenetic and metabolic reprogramming events (108–110). For instance, monocytes and macrophages can adapt their response to repeated pathogen encounters via cell intrinsic modifications that do not involve permanent genetic changes such as mutations and recombination, which are essential for adaptive immunity (110).

Innate immunity uses a combination of cellular and molecular attacks, and it comprises defensive barriers (e.g., skin ciliated epithelia and mucous membranes), physiological (e.g., temperature, low pH), inflammation-related serum proteins (e.g., complement, C-reactive protein, lectins), cytokines and many other inflammatory mediators (98,111). The innate system is also made of effector cells that participate in both phagocytosis and inflammatory processes, encompassing phagocytic cells (e.g., neutrophils, monocytes/macrophages), mast cells, basophils, DC, eosinophils, innate lymphoid cells, natural killer (NK), and platelets (111). The cells of the innate immune system express germline-encoded receptors that do not vary from cell to cell and the cells can respond immediately when triggered by antigens to provide a first line defense and stimulate the

subsequent acquired immune response by secreting chemokines and cytokines aiming to recruit and activate additional cells (112).

When innate mechanisms are unable to clear an infection, the adaptive immune response is mobilized.

1.2 Adaptive immunity with a focus on antibody-mediated responses

The components of the adaptive immune system have slower temporal dynamics that can take days to fully develop, with high specificity and a more potent secondary response due to the development of long-lasting immunological memory (98,99). B and T lymphocytes are responsible for carrying out the (1) humoral (antibody-mediated responses) and (2) cell-mediated responses, respectively (98). T lymphocytes recognize and attack their targets directly or indirectly by attracting the help of other immune cells. In contrast, antibody-mediated responses are mainly characterized by the secretion of immunoglobulins (Ig) by plasma cells, which are the B lymphocyte progeny (113). Antibody-mediated immunity is mediated by macromolecules in extracellular fluids (humor or body fluids), including secreted antibodies, cytokines and complement proteins and immune effector cells (98,99).

Mechanistically, the antibody-production pathway starts when a naïve B-cell antigen-binding receptor recognizes and binds to an antigen at the boundary of the T and B regions of the secondary lymphoid organ. These antigenically stimulated B cells, depending on the existing signals, may undergo (1) rapid clonal expansion in the extrafollicular foci to produce short-lived isotype-switched antibody-secreting plasmablasts (SLPCs), (2) interact with $CD4^+$ T follicular helper (T_{FH}) cells in a germinal-centre (GC)-dependent or GC-independent process to produce long-lived plasma cells (LLPC) or memory B cells (MBC) or (3) or short-lived anergic B cells (hyporesponsive state).

During the process, plasma cells may undergo isotype switching to produce antibodies that can perform different functions in response to the same antigens. The secreted antibodies bind to antigens labelling them for destruction through various mechanisms, including complement activation, opsonin promotion of phagocytosis, and elimination of pathogens by immune effector cells (104).

1.2.1 B cell or B lymphocyte

The abbreviation “B,” in B cell, comes from the bursa of Fabricius in birds (bursa-derived cells), where the B cells develop and mature (98,114). Contrary to birds, human B-cell development occurs primarily in the bone marrow in adults (114) or the liver in fetuses (115). They constitute approximately 15% of peripheral blood leukocytes (116), emerge from hematopoietic stem cells in the bone marrow and mature in secondary lymphatic organs such as the spleen and lymph nodes (117,118). Although B cells have been classically

associated with humoral immunity, growing evidence shows they are equally critical to cellular immunity. They participate in T-cell activation *via* antigen presentation, co-stimulation, and cytokine production. Additionally, they affect antimicrobial defenses and tissue inflammation and serve as regulatory cells modulating both cellular and humoral responses (113).

1.2.2 B-Lymphocyte development

The development of B cells within born marrow progresses through the rearrangement of Ig, leading to the assembly, expression, and signaling of the B cell antigen receptor (BCR) (119). It progresses sequentially from pro-B to pre-B to immature B cells, terminating in the expression of IgM mature BCR on the cell surface capable of binding antigens (113). The production of a highly diversified repertoire of functional antigen-binding regions of Ig found in B cells (BCRs) occurs through a mechanism of somatic recombination known as V(D)J recombination. V(D)J recombination occurs in a nearly random fashion and rearranges the variable (V), diverse (D), and joining (J) segments of the IG gene, resulting in Ig proteins composed of heavy (Ig-HCs) and light (LCs) chains (120).

This V(D)J recombination of the Ig loci is initiated by double-strand breaks (DSB), and a pair of recombination-activating genes called RAG1/RAG2 (RAG) recombinase catalyzed it at specific recombination signal sequences (RSS) (121,122). This random gene rearrangement process of Ig genes during B-cell development and maturation ensures the generation of more than 10^{10} B cells with a vast repertoire of BCRs able to recognize a huge diversity of antigens (98,113). Notably, the gene rearrangement provides a single epitope-binding specificity to the respective B cell (123). The immature B cells in the BM are later released into the circulation and subsequent completion of differentiation into mature B cells occurs in peripheral lymphoid organs (113). However, this process is sensitive and highly error-prone, resulting in the inherent generation of B cells that recognize various self-antigens (113). To avoid that, the developing B cells pass through selection processes in the BM and spleen that serve as checkpoints in eliminating autoreactive clones and establishing self-tolerance (98,113,114).

1.2.3 B cell terminal differentiation

Mature B lymphocytes are divided into two major sub-lineages known as B1 lymphocytes and B2 lymphocytes (124). Most B1 B lymphocytes arise from B1 progenitors in the fetal liver with little input from bone marrow beyond the perinatal period (113). On the other hand, the B2 lymphocytes consist of marginal zone (MZ) and follicular (FO) lineages that occur in the spleen and are classified based on their ontogeny

and anatomic localization (113). In the spleen, immature B cells differentiate into transitional B cells known as transitional 1 and 2 B cells (113,125). Transitional B cells of type 1 (T1) arrive in the spleen from the bone marrow; however, they are excluded from entering the lymph node due to a lack of homing receptors (125). In the spleen, these cells develop into the transitional B cells of type 2 (T2), which are cycling and found exclusively in the spleen primary follicles (125). Depending on the signals they receive through the BCR and other receptors, these transitional B cells of type 2 (T2) subsequently differentiate into MZ or FO lineages. These are considered mature B cells or naïve B cells (113,125).

Each lineage has distinct and overlapping functions in recognizing antigens via T-independent and T-dependent pathways, production of rapid IgM, and long-lasting IgG antibody responses (113). MZ B cells are retained in the spleen, where they are ideally placed to encounter blood-borne pathogens and particulate antigens (113,126). B1 and MZ B cells respond like innate cells since they express Toll-like Receptors (TLRs). They can readily respond to pathogen-associated or endogenous TLR ligands, with or without antigen recognition, *via* their BCR. Thus allowing them to mediate rapid IgM antibody responses (approximately 1–3 days) (113) and class-switched IgG (127), bridging the temporal gap in immunity against infections until the emergence of FO B cell–derived IgG antibodies (about 7 days).

Moreover, MZ B cells play a crucial role in recognizing T-independent carbohydrate and phospholipid antigens (113). MZ B cells also participate in responses to the T-dependent protein antigens pathway by generating high-affinity isotype-switched antibodies. This pathway was previously attributed solely to FO B cells (113).

Finally, FO B cells are the conventional B lymphocytes of the adaptive immune system and the dominant subset, and they are localized in the lymphoid follicles of the spleen and recirculate and populate various secondary lymphoid tissues (e.g., lymph nodes, tonsils, and gut-associated lymphoid tissues, such as Peyer patches) (113,126). They are primarily responsible for generating long-lasting, high-affinity IgG antibodies with the help of T lymphocytes, critical for classic humoral immunity mediating protection after infection or vaccination. They also participate in T-independent IgM responses (113,126).

1.3 Activation of B-cells

B cells can recognize and respond to soluble (cognate antigen in its native form) and membrane-associated antigens, and they respond rapidly to antigenic stimulation through several mechanisms (128,129). Through unique antibodies expressed on their cell surface, B cells are able to recognize antigens directly without the

need for APCs (104). However, B cell recognition of membrane-bound antigens plays a vital role in efficient B cell activation and affinity maturation, which selects high-affinity B cells (129).

B-cell activation is triggered when the antigen engages with the BCR and other co-receptors through which signals from helper T cells or pathogen-associated molecular patterns are delivered (130). The nature of the antigens determines the pathway by which the B cell activation and initiation of humoral response proceed. Thymus-dependent (TD) antigens refer to the antigens that require both B and T cell involvement to induce specific antibodies.

In contrast, non-protein antigens can engage B cells, inducing specific antibodies in the absence of T cell help, and these are so-called Thymus-independent (TI) antigens (131). Both TD and TI antigens elicit vigorous antigen-specific IgM responses and induce the production of isotype-switched antibodies (132). Moreover, growing evidence suggests that both TD and TI induce specific MBC development (133); however, it seems that the canonical T cell-dependent MBCs are generated more efficiently than in TI (134).

1.3.1 Thymus-dependent B cell responses

The production of antibody secreting cells in response to TD antigens occurs in the context of Major Histocompatibility Complex (MHC) class II molecules (135) and involves the interaction of cognate antigen, B-cells, professional APC, and $CD4^+$ T cells in the secondary lymphoid tissues (136). It is a two-step process, with the first resulting in the generation of short-lived plasmablasts for immediate protection, known as "extrafollicular response", and the second resulting in long-lived plasma cells and MBC providing lasting protection "(GC) response" (126).

In secondary lymphoid organs, the B lymphocytes re-circulate between B-cell-rich compartments (follicles or B zones), surveying for antigens (137). The cells can encounter soluble antigens accessible to B cells as free-diffusing antigens (138). However, recent evidence suggests that the relevant mode of antigen recognition by B cells may be of antigens that are attached to the surface of neighboring APCs such as macrophages, or DC (138,139), or antigens retained in follicular dendritic cells (FDCs) that serves as a reservoir of antigen during the GC formation and affinity maturation (138,140).

1.3.1.1 The signal model for B-cell activation

B-cell activation by a TD antigen consists of a sequence of events, including ***signal 1,*** antigen binding to BCRs; ***signal 2,*** costimulation supplied by an activated Th cell specific for the same antigen; and ***signal 3,*** Th cell-derived cytokines (**Figure 3**) (141,142).

Signal 1 is generated when the antigen crosslinks the BCRs, leading to increased expression of class II MHC and costimulatory B7 ligands CD80 and CD86 that are expressed on the surface of APCs, including B cells (141,143). Antigen–BCR complexes follow a cascade of intracellular signaling leading to internalization by receptor-mediated endocytosis, and the antigen is degraded to peptides (141,144). The T-cell epitope is bound by class II MHC and presented on the membrane as peptide–MHC complexes, which activate $CD4^+$ Th cells plus the costimulatory B7-CD28 signal between B-T cells (141). This interaction between the antigen-specific B and Th cells is essential for T-cell–dependent B-cell activation as it results in up-regulating the expression of CD40L (CD154), which is a $CD4^+$Th membrane protein expressed on activated T cells that then interacts with CD40 on B cells to provide an essential for B-cell activation (141) ***signal 2***.

The B–T cell interaction also depends on costimulatory molecules, where the major costimulatory signal is delivered by the B7 ligands CD80 and CD86 that are expressed on the surface of APCs that specifically interact with CD28 on the T cell (143). Meanwhile, $CD4^+$Th cells release cytokines (***signal 3***) such as IL-4, IL-5, and IL-6 in a directed manner, sending signals supporting the progression to later stages of B-cell activation and differentiation into plasma and MBC (141,144). The interaction of antigen-specific B cells and T cells results in a bifurcation of B-cell differentiation. Some activated B cells undergo rapid proliferation and differentiation as antigen-experienced B cells or MBCs or differentiate into plasmablasts (145) in the outer follicular areas (145,146). However, a fraction of activated B lymphoblasts relocate to the primary follicles attached to their respective helper T-cells, initiating their differentiation, and they become the precursors of GC B cells and T_{FH} cells (*pre-GC T cell*/B cell) (136,147) that will form the germinal center. At the individual B cell level, the initial affinity for the antigen and abundance of epitopes govern the decision between extrafollicular and GC differentiation. B cells react against either high affinity or abundant epitopes toward extrafollicular plasma cell differentiation, whereas responding clones with weaker antigen reactivity are primarily directed to GC, where they undergo affinity maturation (148).

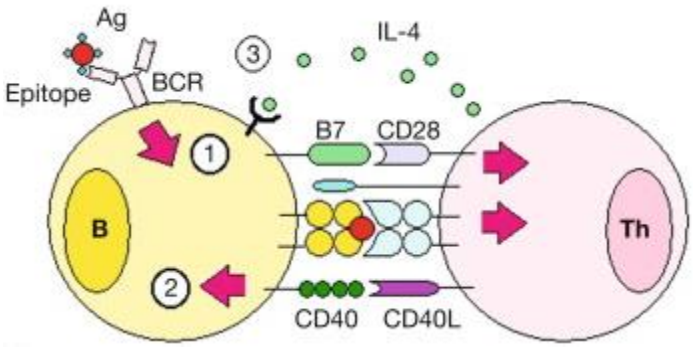

Figure 3. The T-cell–dependent B-cell three-signal activation model. B-cell activation requires three signals: binding of soluble or membrane-bound antigen peptides to BCR provides **signal 1**; interaction between CD40-CD40L is a key co-stimulatory pathway that provides **signal 2**; Cytokines, represented by IL-4 secreted by $CD4^+$ Th cells, provide **signal 3** *(141)*.

1.3.1.2 The extrafollicular response

After B cell activation, the T-cell help during the interaction of antigen-specific B cells rapidly drives B-cell differentiation into B lymphoblasts that may undergo Ig class-switch recombination (CSR) and differentiation into short-lived plasmablasts that secrete antibodies (**Figure 4**) (149). In secondary lymphoid organs, the B lymphoblasts that have been induced to become plasmablasts travel from the T zone to the local site of extrafollicular growth. During this extrafollicular growth and the subsequent differentiation to plasma cells, although there is no contact with Th cells, it appears that association with $CD11c^{high}$ DC is required for the full differentiation of plasmablasts to plasma cells (149). A fraction of the plasma cells produced in extrafollicular responses get access to stroma that secures their extended survival (149). However, certain antigens induce extrafollicular antibody responses without T-cell help, and in these responses, B cells also pass through the same sequence of microenvironments (149). The extrafollicular response is the source of most early protective antibodies produced (126). Nevertheless, the extrafollicular response exhibits low-level somatic hypermutation (SHM); therefore, the resulting antibody affinity tends to be moderate and unchanging (126). Despite earlier work suggesting that MBCs development depends on the classical GC pathway, more recent corroboration suggested GC-independent formation of non-switched IgM^+ and class-switched IgG^+ effectors or MBCs (150).

1.3.1.3 GC reaction response

The production of antigen-specific antibodies in GCs is precisely regulated by the cellular interactions among antigen-specific T_{FH} cells, antigen-specific GC B cells, and antigen-bearing FDCs (**Figure 4**) (151). Some activated B cells lymphoblasts that are initially activated outside the follicle re-enter the B cell follicle of secondary lymphoid organs attached to their respective helper T-cells, initiating their differentiation and becoming the precursors of GC B cells and T_{FH} cells (136,147), in association with FDCs (152,153). That is followed by vigorous proliferation to form GC (152–155). GCs are highly specialized and dynamic microenvironment that gives rise to secondary B cell follicles during an immune response (126). Their induction begins when a few antigen-specific activated B cells upregulate their expression of CXCR5 and migrate toward B-cell follicles. This migration is facilitated by the attraction of CXCL13-expressing FDCs (117). The FDCs functions include the production of B cell chemokines and the capture/retention of antigen complexes for extended periods, which GC B cells compete to bind and internalize through their surface Ig receptors (117,155).

Chemokines produced by FDCs attract activated antigen-specific B cells as well as T_{FH} into GCs (117,155). After binding of the B-cell receptor to protein antigens that are associated with the surface of FDCs, it follows a cascade of intracellular signaling leading to antigen internalization in intracellular sites whereby it is degraded and returned to the B-cell surface as peptides bound to MHC class II molecules (156). The peptide: MHC class II complex can be recognized by antigen-specific T_{FH} cells through their T-cell receptor (TCR) (156,157). Following TCR-MHC-II-peptide binding, T cells express the protein CD40L on their surface as well as cytokines such as IL-4 and IL-21 (157). CD40L serves as a necessary co-stimulatory factor for B cell activation by binding the B cell surface receptor CD40 (157). The cellular interactions result in the proliferation, survival, and selection of B cells with the highest possible antigen-specific affinity (117). This process of clonal selection selects GC B cells with the highest affinities for the antigen to mature into LLPC and MBC (116,117,126,155,158), providing effective protection against current and future infections. The combination of costimulation molecules, including CD40L, IL-4, and IL-21 in different ratios, seems to be the primary mix of T cell help signals that control B cell massive clonal proliferation, class switching recombination, somatic hypermutation, and differentiation (157). This phase occurs in parallel with the protective and rapid extrafollicular phase (126).

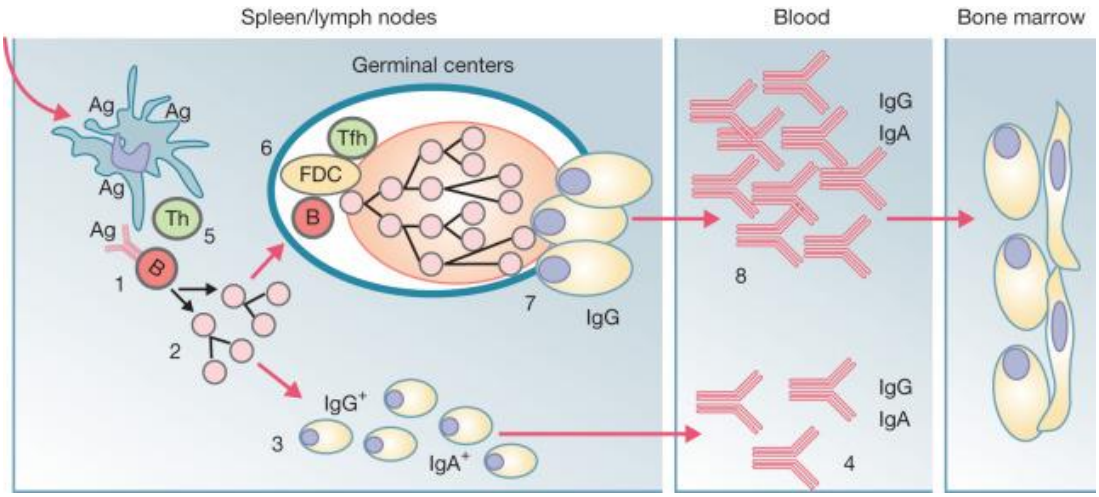

Figure 4. Extra follicular and germinal center responses to protein antigens in TD B cell response (117). **Extrafollicular responses to protein antigens (1-4).** *(1)*-B cells capable of binding to this antigen with their BCRs in response to a protein antigen reaching lymph nodes or spleen; (2)-undergo brisk activation and clonal expansion in an extrafollicular reaction; (3) B cells rapidly differentiate in plasma cells; (4)- low-affinity antibodies (of the immunoglobulin [Ig] M ± IgG/IgA isotypes) are produced days after infection. **Germinal Center responses to protein antigens (5-8).** (5)-Antigen-bearing dendritic cells (DCs) activate Antigen-specific T-helper (Th) cells; (6)- Some antigen-specific B cells are trigged to migrate toward follicular dendritic cells (FDCs) to initiate the germinal center (GC) reaction; (7)- In the GCs, follicular cells (Tfh) deliver additional signals to B cells and undergo a massive clonal expansion, class-switch from IgM toward IgG, IgA, or IgE; undergo affinity maturation; (8)- differentiation into plasma cells secreting large amounts of antibodies. A few plasma cells exit nodes/spleen and migrate to the survival niche microenvironment primarily located in the bone marrow at the end of the GC reaction, where they survive through signals provided by supporting stromal cells.

1.3.2 Thymus-independent B cell responses

These responses are elicited by non-protein antigens and are recognized by subsets of innate B cells, which do not reside in the lymphoid follicles, such as MZ B cells and B-1 cells (159). These responses can be subdivided into TI type 1 and TI type 2 based on different mechanisms of activating B lymphocytes. TI type 1 response is elicited by microbial ligands, such as lipopolysaccharide (LPS), via TLRs and can directly activate B cells, stimulating their polyclonal proliferation (159,160). On the other hand, TI type 2 responses occur when repeating polymers, such as polysaccharides, which have highly repetitive structures, cause extensive simultaneous cross-linking of specific BCRs, inducing proliferative signals (159,160).

TI responses generally lead to robust primary antibody production that develops faster than the TD responses, accounting for host protection in the early phases of infections (161). However, the generated

antibodies are less variable and less functionally versatile than those induced with T-cell help (149,156), and the responses are not known to generate MBCs (133).

1.4 The immunoglobulin structure and function

1.4.1 The basic Immunoglobulin structure

Immunoglobulins are glycoproteins secreted by terminally differentiated B lymphocytes, also known as plasma cells, upon specifically binding to an antigen. They are one of the principal effectors of the adaptive immune system and contribute to the neutralization, fixation, and clearance of pathogens (98,162–164).

The basic structure of a human antibody consists of two functional domains linked by a hinge region. The first domain, the antigen-binding fragment (Fab), is responsible for binding to antigens, thereby determining the antibody's specificity. The second domain, the crystallizable fragment (Fc), binds to host sensors to deploy effector functions. This clear division of labor within the antibody structure ensures its effective function in the immune system (113,163,165,166).

A single antibody molecule is composed of four polypeptides comprising two identical copies of both HCs (∼55 kD) and LCs (∼25 kD) that are joined together by disulfide and noncovalent bonds (113,163). The resulting molecule is represented by a schematic Y-structured molecule of ∼150 Kd (**Figure 5**) (163,165). These chains are further stratified into variable (VH or VL) domains and constant (CH or CL) domains, which form the Fab and the Fc domains (165). Fc domain diversity is generated during an immune response through the selection of different antibody isotypes, subclasses, and post-translational glycosylation profiles (165).

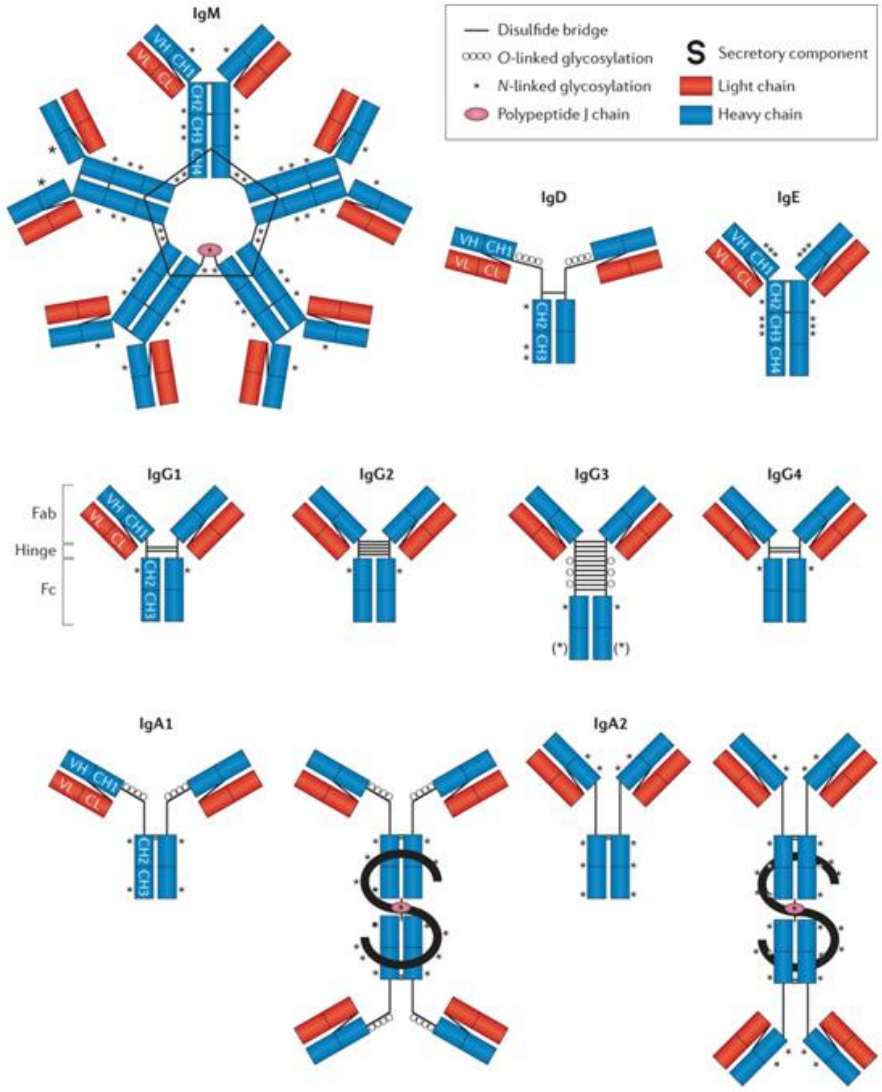

Figure 5. Antibody isotypes and subclasses *(165)*. The basic structure of a human antibody comprises two identical heavy chains (VH and CH) (blue) and two identical light chains (VL and CL) (red) represented by a schematic Y-structured molecule. The domains include an antigen-binding fragment (Fab) domain that binds to antigens, composed of one variable domain from each heavy and light chain (VH and VL). The heavy chain's constant domains CH2 and CH3 comprise the crystallizable fragment (Fc) domain that binds to host sensors mediating effector functions. Five human antibody isotypes (IgM, IgD, IgG, IgA, and IgE) and six subclasses (IgG1–4, IgA1, and IgA2) are shown. Each isotype or subclass exists as monomers and/or multimers, and it is linked by disulfide bridges, polypeptide J chains, or the secretory component associated with secreted dimeric IgA. The hinge length and flexibility, the number and location of disulfide bridges, and O-linked or N-linked glycosylation are structural features that influence and impact antibody–receptor and even antigen binding.

Five isotypes or classes of antibodies exist in humans (IgG, IgM, IgA, IgD, and IgE) with HCs denoted by the greek letters γ, μ, α, δ, and ε respectively, and six subclasses (IgG_{1-4} and IgA_{1-2}) (165). The stratification is based on differences in the amino acid sequences in the glycosylated conserved (C-terminus) regions of the HCs of the crystallizable fragment (113,163,166). IgAs, IgDs, and IgGs have three constant (C) and one variable (V) domains, whereas IgEs and IgMs have one variable and four constant domains. The isotypes IgG, IgD, and IgE and serum IgA are monomeric (a monomer is defined here as a pair of HC-LCs) (166). On the other hand, the secretory IgA and IgM isotopes have an additional J-chain, allowing them to assemble as dimers and pentamers, respectively (166). Ig class determines the immune response's type and temporal nature (163). Apart from Ig existing in secreted form, B cells also express a membrane-bound Ig (mIg) form as part of the BCR, which is indispensable for their differentiation, survival, and activation (162). The hinge constant structural features influence the antibody flexibility and conformation, impacting antibody–receptor and even antigen binding (165). Moreover, changes in isotype or subclass and glycosylation profile may also influence the capacity of any antibody to interact with innate immune receptors and thereby deploy distinct innate immune effector functions (165).

1.4.1.1 Antibody class switch recombination

Class switch recombination (CSR) involves DNA recombination events in response to antigen stimulation and co-stimulatory signals. Activated naïve B cells can rapidly switch from expressing IgM and IgD on their surface to expressing IgG, IgE, or IgA (167,168) and subclasses of these isotypes (IgG_{1-4}, IgA_{1-2}) (169). After activation of B cells by their cognate antigen, IgD expression is lost, and cells can either become IgM secretors or undergo CSR to the downstream isotypes (167). IgD CSR is rare, and its regulation is still poorly understood (170).

During the isotype switching, the IgM-defining constant (C)μ (mu) CH region is replaced with Cα (alpha), Cγ (gamma), or Cε (epsilon) heavy chain constant regions, coding for the constant regions of IgA, IgG, or IgE isotypes, respectively (98,167,171,172).

CSR occurs by deletional recombination when two different switch (S) regions upstream of an HC constant (C_H) region undergo switch-region recombination, removing the preceding C_H regions from the Ig locus (167,172). It is driven by B cell-specific enzyme activation-induced cytidine deaminase (AID) that catalyzes the conversion of cytidine nucleotides to uracil (172). Next, uracil removal by uracil N-glycosylase, which, along with endonucleases and the mismatch repair machinery, leads to the generation of programmed double-stranded DNA breaks at the Ig heavy chain (*Igh*) locus (172). Finally, the main steps involved in CSR

include the removal of the intervening DNA as excision circles and repairing the DNA double-stranded breaks, primarily by non-homologous end joining (NHEJ) (172). Thus, the CSR machinery preserves the variable region of the heavy chain without affecting the antigen specificity (98,171,172). CSR can be triggered by TD and TI antigens (121,173).

CSR plays a critical role in adaptive immune responses by changing the effector functions of antibodies (121). It generally occurs 7–10 days after exposure to antigens before the formation of germinal centers (146). This evidence suggests that CSR starts before SHM of variable region [V(D)J] genes (167).

In humans, the pathways of CSR are organized into two steps: (1) naïve classes (IgM/IgD) switch predominantly to proximal classes such as IgG3, IgG1, or IgA1, and (2) proximal classes may subsequently switch to distal classes, such as IgG2, IgG4, or IgA2. IgG1 and IgA1 seem to be central intermediates linking naïve to distal classes (174). Moreover, a preferential switch pathway was suggested from IgG2 to IgA2 (174). The evolution of IgG subclass CSR is modulated by interaction with Th cells and follows a 1-way direction (IgG3 → IgG1 → IgG2 → IgG4) (175).

Switching from IgM to different isotypes is mainly governed by two variables 1) the nature of antigen (TD or TI mode of B cell activation) and 2) the cytokines produced by other immune cell types (121,176) (**Table 1**). Moreover, the interactions engaging activated B cells with other immune cells, including APCs, helper T cells, and dendritic cells, also play a role (121,176). The immune reaction into preferential patterns of class switching is steered by the route an antigen enters our body and its chemical composition (177). Although the GC reaction is important for CSR, it is not absolutely required (178). CSR was suggested to proceed at comparable rates in GC and *extrafollicular* regions (179). It is well documented that IgG3 can be formed in the MZ through TI antigen stimulation (180,181). Polysaccharide antigens may induce class switching, particularly to IgG2, without T-cell help (177). On the other hand protein antigens usually trigger B-cells receiving T-cell help via MHC-class II expressed by the B-cell, and they typically induce class switching that shows a tendency to be IgG1 or IgG3 but can also be IgG4 or IgE (177).

Table 1. Cytokines responsible for the in vitro class switching in purified human B cells

Cytokine	Effect on B cells	Ref
IL-2	○ Induces CSR, preferentially to IgG1 and IgA	(182)
IL-4	○ Induces CSR, preferentially to IgG4, IgE and IgG1	(168,182–184)
IL-5	○ Enhanced IL-4-dependent IgE secretion	(185)
IL-6	○ Enhanced IL-4-dependent IgE secretion	(185)
IL-10	○ Induces CSR, preferentially to IgG1, IgA, IgE and IgG3 ○ Co-operates with TGF-β in promoting switching to IgA ○ Promotes differentiation of B cells to become plasma cells secreting IgM, IgG, IgA	(182,186–188)
IL-12	○ Inhibits IL-4-dependent IgE production	(189)
IL-13	○ Induces CSR, preferentially to IgG and IgE ○ Effects largely overlap with those of IL-4	(183,190–192)
IL-15	○ Potent inducer of IgG1, and IgA secretion, by CD40L-stimulated B cells	(193)
IL-21	○ Induces CSR, preferentially to IgG1, IgG3, and IgA1 ○ Promotes differentiation of B cells to become plasma cells secreting IgM, IgG (predominantly IgG_3), IgA (mostly IgA_1) ○ Synergizes with IL-4 for CSR to IgG and secretion of IgE	(169,194,195)
IFNα, IFNγ	○ Inhibit IL-4-dependent IgE secretion	(182,196)
TGFβ	○ Inhibits IL-4-dependent IgE secretion ○ Strongly suppressive for IgG4 and IgE production ○ Co-operates with IL-10 in promoting switching to IgA	(182,185,187)

1.4.1.2 Somatic hypermutation

Before exposure to antigen, the V(D)J recombination in early B-lymphocyte development produces an extensive repertoire of antibodies that can recognize vast and diverse antigen determinants (197,198). However, following specific antigen recognition by its cognate B lymphocyte and subsequent costimulation by helper T lymphocytes, the B lymphocyte migrates and enters the germinal center of peripheral lymphoid organs to become a centroblast B cell (171). Aiming to generate high-affinity antigen binding sites and

sometimes changes in fine specificity, the B-cells undergo a second wave of programmed and stepwise antibody diversification process called SHM (98,197,199). SHM can result in antibodies with increased affinity for antigens and may continue as long as B cells are activated (167). The mechanism occurs at a rate 10^6-fold greater than the normal rate of mutation observed in other genes (98,197). The process randomly introduces mutations into the Ig V regions, altering the affinity for antigens while maintaining the same Ig C isotype (172). However, the SHM does not discriminate between favorable and unfavorable mutations and can produce antibodies with lower affinity for antigen or no change in affinity for antigen (197).

1.4.1.3 Affinity maturation

The molecular mechanism and gene conversion of CSR and SHM show many similarities and share several enzymes. Both can increase the affinity of Ig to recognize and bind with various antigens; however, their regulation remains poorly understood (122). Most antibodies that are produced in a primary response to most antigens are high molecular weight (MW) immunoglobulins (IgM) with a low average affinity for the antigen (200). However, the antibodies produced later during the course of the response are lower MW classes (e.g., IgG and IgA) with increased average affinity or maturity for that antigen (200). These changes, often called affinity maturation, take place mainly in the GC in the context of TD responses within secondary lymphoid tissues. The proliferating antigen-stimulated B cells express the highly mutagenic cytidine deaminase that mediates Ig class-switching and sequence diversification of the Ig variable domains of antigen-binding receptors on B cells (BCR) (121,153,200). The degree of affinity maturation is inversely correlated to the dose of antigen administered, meaning that as the antigen becomes limiting, the clones with the higher affinity will possess a selective advantage. The positively selected B cells, with higher-affinity B-cell receptors, are expected to outcompete lower-affinity B cells progressively. Ultimately, MBC and LLPC are produced, effectively protecting against future reinfection (121,153).

1.4.2 Functional properties of antibodies

The antibodies exert their effector functions in three main ways: 1) They neutralize pathogens (e.g., attach to a virus and prevent it from entering a cell and replicating on it). Moreover, they similarly bind to toxins and block their ability to enter cells. 2) Through opsonization of pathogens that replicate outside of cells and via the activation of macrophages and other immune cells by binding to Fc receptors (FcRs) that recognize the constant regions of specific antibody classes, mediating the ingestion by the phagocytic cell and pathogen destruction. 3) Via the complement systematic activation. In activating the complement system via the classic pathway, pathogens bound by antibodies are recognized by the C1q protein, which triggers the complement

cascade that results in pathogen phagocytosis and lysis (113,201). The heavy-chain isotype and binding affinities of activating and inhibitory FcR on immune cells determine which effector mechanism dominates (**Table 2**) (113).

Table 2. Structure and functions of different main isotypes.

Feature	Immunoglobulin Isotypes						
	IgM	**IgA**	**IgE**	**IgG1**	**IgG2**	**IgG3**	**IgG4**
Heavy chain	μ	α	ε	γ_1	γ_2	γ_3	γ_4
Structure	Pentamer	Monomer, Dimer	Monomer	Monomer	Monomer	Monomer	Monomer
Molecular mass, kDa	970	160	188	146	146	165	146
Serum level (mean adult), mg/ml	1.5	2.1	5×10^{-5}	9	3	1	0.5
Half-life (days)				21	21	7.1	21
Half-life in serum (days)	10	6	2	21	20	7	21
Response to polysaccharide antigens	++	++	++	+	+++	+/−	+/−
Response protein antigens	+	+	+	++	+/−	++	++
Placental transfer	–	–	–	+++	+	++	–/+
Neutralization	+	++	–	++	++	++	++
Classic pathway of complement activation	++++	–	–	++	+	+++	–
Sensitization for killing by natural killer cells	–	–	–	–	++	–	++
Binding to macrophage and phagocyte Fc receptors	–	+	+	+	–	+	–/+
Binding to mast cells and basophils	–	–	+++	+	–	+	–

+ depict relative presence and – depict the relative absence of response to the type of antigen or specified feature. Adapted from (113,202,203).

IgD

The function of the membrane-bound form of and circulation IgD remains poorly understood (165,202). Most IgD^+ B cells also co-express IgM, and both participate in BCR signaling, which initiates early B-cell response (202).

IgM

IgM dominates the early phase of antibody response, playing a pivotal role in controlling infections. Therefore, it is frequently used to diagnose acute exposure to an immunogen or pathogen (202,204). However, some IgM is also produced in secondary and subsequent responses and after somatic hypermutation, even though other isotypes dominate the later phases of the antibody response (204).

The early IgM antibodies tend to be of low affinity because they are produced before the B cells have undergone somatic hypermutation (204). IgM is associated with transmembrane invariant accessory chains as a monomer, forming the B cell receptor. However, upon maturation and antigenic stimulation, it is secreted as multimeric (usually pentameric) IgM (165,204). The pentameric structure of IgM molecules, whose 10 antigen-binding sites can bind simultaneously, compensates for the relatively low affinity of the IgM monomers. This multipoint binding confers high overall avidity, especially if that antigen contains multiple repeating epitopes (202,204). Due to its pentameric large size, the IgM is mainly found in the blood and, to a lesser extent, the lymph. Furthermore, the pentameric structure renders IgM very efficient in opsonizing antigens for destruction and makes it especially effective in activating the complement system (202,204,205). Of note, in *P. falciparum*, the conventional model of IgM kinetics by which IgM peaks and wanes rapidly during a primary response and that the long-lived immunity was predominantly of IgG isotype has been challenged (206). IgM to merozoite surface was found to persist after experimental or natural exposure to a level similar to IgG following treatment, with levels associated with protection against clinical malaria (207).

IgG

IgG is the most abundant isotype, accounting for about 10–20% of plasma protein in human serum, and it has the most extended serum half-life of all Ig isotypes (177,202). It is the principal isotype in the blood and extracellular fluid, where accessory cells and molecules are available. It efficiently opsonizes pathogens for engulfment by phagocytes and activates the complement system (204). IgG can be further subdivided into four subclasses that are named in order of decreasing abundance, IgG1>IgG2>IgG3>IgG4, which are highly conserved, being more than 90% identical on the amino acid level (177). However, they differ in their constant region or heavy chain structure, particularly in their hinges and upper CH2 domains (177,208). As a result,

each subclass has a unique profile and different effector functions, both in terms of triggering FcγR-expressing cells, resulting in phagocytosis or antibody-dependent cell-mediated cytotoxicity, and in terms of their ability to activate the complement system (177,209).

The Fc-regions also contain a binding epitope for the neonatal Fc receptor (FcRn), which is responsible for the extended half-life, active transport of maternal IgG to the fetus during pregnancy, and bidirectional transport of IgG to mucosal surfaces (177). The best described human activating FcγRs among six subtypes (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, FcγRIIIB) are FcγRIIA and FcγRIIIA (208). FcγRIIA is primarily expressed in monocytes and macrophages, whereas FcγRIIIA is dominantly expressed in NK cells (208). There are also defined differences in the affinity to the three classes of FcγR (I, II and III) (202). IgG1 and IgG3 bind to all three FcγR classes (202). IgG4 binds only FcγRII and III, albeit significantly weaker than the binding of IgG1. And IgG2 binds only to FcγRII (202). IgG4 is the least potent human IgG subclass for the FcγR-mediated antibody effector function (210).

IgG1 is the predominant isotype of serum IgG (constituting 60%–75%) and has a longer half-life (21 days) (113), and it has the highest FcγR-binding affinity, followed by IgG3, IgG2, and IgG4 (208). IgG1, as well as IgG3, bind FcγR most efficiently, mediating the opsonization of invading microorganisms, leading to clearance of the pathogen by phagocytes, and also activating the complement cascade more efficiently among IgG isotypes, contributing to their associated proinflammatory effects (113,209).

The affinity for C1q, the first component of the complement pathway that binds to the CH2 domain of IgG, differs between members of the other three IgG subclasses. The complement activating order is (IgG3>IgG1>IgG2) (202,209). Of note, the IgG2 isotype requires a relatively high antigen concentration to activate complement (211), and it dominates in TI responses (209). The IgG4 is the only subclass that fails to trigger the complement cascade (202). IgG is able to cross the human placenta, mediated by the neonatal Fc receptors (FcRn) (209) and IgG1 and IgG3 are transported more efficiently across the placenta (212).

IgA

There are two known subclasses of IgA, IgA1 (>90% of serum IgA) and IgA2, whose structures differ mainly in their hinge regions (202). IgA is the principal isotype in secretion, the most important being those found in mucus epithelium of the intestinal and respiratory tracts where complement and phagocytes are generally absent. Therefore, being less potent opsonin, it is also a weak complement activator and functions chiefly as a neutralizing antibody (204). However, recent advances have shown that IgA can also activate the complement system (205). The IgA receptor is also expressed on neutrophils, which may be activated locally

to mediate Antibody-dependent cellular cytotoxicity (ADCC) (202). It provides protection in the blood as a monomer and in mucosal tissues as dimer secretory IgA (165).

IgE

IgE is present only at very low blood or extracellular fluid levels. It binds with extremely high affinity to the FcεRI, which is expressed in mast cells, basophils, Langerhans cells, and eosinophils (202). Antigen binding to this IgE triggers mast cells to release potent chemical mediators that induce local defense reactions and drive persistent antibody effector function for weeks to months against allergens and parasites, particularly helminths (165,202,204).

Host immune response to *P. falciparum*

1. Innate immunity to *P. falciparum*

The PRRs initially recognize microbial structures conserved among microbial species called PAMPs (213,214). They also recognize endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs) (213,215,216). The TLR family, among other family members of PRRs, is important for *P. falciparum* PAMPs recognition (50). Specialized immune cells, including macrophages, DCs and B cells, highly express TLRs. These cells have essential roles in clearing the initial burden of infectious organisms and in the induction of adaptive (antigen-specific) immune responses (217).

In the **pre-erythrocytic stage** of the infection, the innate immunity to sporozoites is limited due to the ability of the sporozoites to invade hepatocytes within minutes after entering the bloodstream and the lack of potent TLR agonists, which complicates the immune recognition and elimination (112,218). During the passage from the skin to the liver to blood—γδT cells present in the tissues (both tissue-resident and circulating γδT cells) could recognize parasites and become activated. However, it remains unclear whether they become activated in the skin, the liver or the lymphoid organs (203). *Plasmodium*-specific $CD8^+$ T cells primed by DCs ultimately target infected hepatocytes at this stage (201).

In the ***erythrocytic stage*** *of infection, P. falciparum* parasites are protected from the innate immune response, including phagocytosis by neutrophils and monocytes, while developing inside the infected red blood cells (iRBCs). However, after schizont rupture and merozoite release, parasite components are accessible to circulating leukocytes (112). Lymphocyte subsets, including αβT cells, γδT cells and NK cells, were shown to contribute to an early initial IFN-γ response in vitro stimulation of iRBCs and naïve human volunteers (219,220). IFN-γ released by γδ T cells would activate neutrophils, monocytes, macrophages and DC, thereby augmenting phagocytosis and secretion of additional cytokines (112). The additional secreted

cytokines also activate NK cells, inducing granulysin and IFN-γ release, contributing to parasite clearance and further stimulating the innate and adaptive responses (112).

The innate immune cells sense the intact parasites, but isolated parasite molecules, such as DNA and RNA, can induce strong responses (221). For example, the activation of TLR8 by *PfRNA* in human blood-stage malaria induces the secretion of IL-12 and bioactive IL-18 by monocytes, mediating the activation of NK cells (222). Upon phagocytosis of sporozoites, debris from infected hepatocytes, merozoites, and iRBCs, the DCs process and present *Plasmodium* antigens to activate naive $CD8^+$ and $CD4^+$ T cells, playing a critical role in bridging innate and adaptive immune responses during malaria (223).

2. Humoral immunity to *P. falciparum* (antibody response).

Naturally Acquired Immunity (NAI) to falciparum malaria refers to the development of adaptive immunity against *Plasmodium* infection that protects individuals routinely exposed to clinical and severe disease and death (224,225). It is a slow process that may take years to develop, even in high transmission areas, despite repeated exposure to the parasite (38). It does not lead to long-lasting sterile immunity (221). However, at least sterilizing immunity can be induced by sporozoites in the absence of pre-existing immunity under experimental conditions in humans (226).

Antibody responses are a central element of NAI following natural exposure to *P. falciparum* parasites (227), yet humoral immune responses are often either suboptimal or not efficiently induced (228). Both humoral and cellular immune responses to the parasite's pre-erythrocytic stages in the blood and liver are weaker compared with the immune response to asexual erythrocytic-stage merozoites, presumably owing to the very small numbers of *Plasmodium* sporozoites transmitted by mosquito bites during naturally occurring exposure (226).

The development of NAI is age dependent, with children under five more susceptible to developing the clinical disease (229). On the other hand, NAI may be diminished in older people due to immunosenescence (gradual deterioration of our immune system associated with natural age advancement) (229). Furthermore, antimalarial immunity is also influenced by human genetics, pregnancy, nutritional status and co-infections (230,231)

Immunity to *Plasmodium* is essentially strain, stage, and antigen specific (232), suggesting that an individual becomes immune after exposure to diverse strains circulating in the community. Additionally, the NAI that develops against the erythrocytic stage of the infection is ineffective against the sporozoite or intrahepatic stage (38,225). Immunity to various parasite life-cycle stages may contribute to protection (233).

2.1 Antibody-mediated response to pre-hepatic *P. falciparum* infection stage

Human immunization experiments with attenuated *P. falciparum* malaria have successfully protected against sporozoite challenges in individuals living in endemic areas (234). However, antibodies on their own cannot always be considered to be effective in preventing *P. falciparum* sporozoites from invading hepatocytes, even if the antibodies are eventually produced in large quantities (38). Antibodies to the sporozoite in pre-erythrocytic stages could potentially protect either through opsonization, leading to clearance before reaching the hepatocyte or blocking the invasion of hepatocytes (228,235,236).

Naturally occurring and non-natural parasite exposures induce antibody responses against several *P. falciparum* sporozoite surface proteins in humans, particularly the circumsporozoite surface protein (CSP) antigen, Thrombospondin-Related Adhesion Protein (TRAP), Cell-Transversal protein for Ookinetes and Sporozoites (CelTOS), and Liver Stage Antigen 1 (LSA1), which have been considered leading vaccine candidates.

IgG antibodies to CSP, LSA-1, and TRAP, primarily those of the IgG1 and IgG3 subclasses, were associated with protection against *P. falciparum* infection and a decreased risk of clinical malaria, presumably by limiting the initial burden of infected hepatocytes (237,238). Anti-CelTOS antibodies elicited by vaccination or passive immunization inhibit sporozoite and ookinete infection and correlate with partial protection (35).

2.2 Antibody-mediated response to liver stage antigens of *P. falciparum*

Antibodies generated against liver-stage antigens can activate complement and mediate phagocytosis and lysis by cytotoxic NK and NKT cells (62). In addition, antibodies recognize parasite neo-antigens at the surface of infected liver cells and mediate killing through an antibody-dependent cell-mediated mechanism by Kupffer cells and NK cells (62). However, the primary mediators at this stage are IFN-γ producing $CD8^+$ T cells ($CD4^+$ dependent), mainly involved in killing intrahepatic parasites (62). Other IFN-γ secreting cells, including NK cells, NKT and γδ T cells, also kill infected liver cells (235).

2.3 Antibody-mediated response to the erythrocytic stage of *P. falciparum* infection

It is widely recognized that antibodies play a significant role in controlling the erythrocytic stage by targeting both free merozoites and infected erythrocytes (228). They can neutralize, opsonize and initiate complement activity directed against free merozoites or iRBCs (228). Moreover, antibodies mediate cellular killing, block the adhesion of iRBCs to the endothelium, and neutralize parasite toxins, thus preventing the induction of

excessive inflammation (62). Antibodies directed against blood-stage antigens likely function to reduce parasite burden and limit malaria disease severity (228).

Antibodies against merozoites, particularly IgG3 to MSP1, MSP2, and MSP3, among others, have been identified as important targets of antibody-mediated complement-dependent inhibition, thus preventing invasion of erythrocytes. Moreover, they were also found to contribute to protection against clinical malaria (239,240).

The vast majority of blood-stage vaccines (BSV) initially developed targeted the merozoite surface proteins (MSP1, MSP3), apical membrane antigen (AMA1), and erythrocyte-binding antigens (EBA)-175 (241). These vaccines aimed to induce high titer antibodies against merozoite surface antigens that would impair parasite invasion (235) or mediate antibody-dependent cellular inhibition (ADCI) in the case of MSP3 (242). However, most of these candidates showed negligible evidence of protection against naturally occurring or controlled human infection (243).

The main challenges of developing anti-merozoite vaccines include (1) the short time (seconds) when merozoites progress between erythrocytes and are accessible to antibodies, (2) high degree of antigenic polymorphism, (3) redundant invasion pathways, and (4) the large number of malaria parasites that need to be targeted (241).

Searching beyond merozoite targets, PfEMP1 at the surface of iRBCs is regarded as one of the major targets of the immune response. Antibodies against PfEMP1 grant protection by blocking sequestration (rosetting and cytoadherence), inducing antibody-dependent cellular-inhibitory effect and opsonizing iRBCs for phagocytosis (244). The development of PfEMP1-based vaccines has been impeded by its highly polymorphic sequence, large size, and cysteine-rich conformational structure (241). However, a distinctly structured PfEMP1 family member, the VAR2CSA, used by the parasite to sequester in the placenta, was developed into a vaccine. PRIMVAC, a subunit vaccine based on VAR2CSA, has been shown to be safe, immunogenic, and induced functional antibodies in humans (241).

Immune response to blood-stage infection is also characterized by upregulation of innate immune response and inflammation-related cytokines (IL-1β, IL-6, TNF, and IFN-γ), which enhances phagocytosis and killing of iRBCs by macrophages, generation of reactive oxygen intermediates and L-arginine-derived NO, and stimulating the proliferation of T cells (245,246). However, the overproduction of these cytokines contributes to malarial immune-related pathology. The downregulation of inflammation by IL-10 and *TGF-β* cytokines seems necessary for suppressing severe pathology during *Plasmodium* infection (247).

2.4 Antibody-mediated response to gametocyte stage of *P. falciparum* infection

When the gametocytes are not taken up by mosquitoes in the human host, they die, releasing intracellular proteins/antigens into the host circulation. APC then process and present these antigens to effector cells, eventually eliciting humoral immune responses (248). Antibodies against gametocytes are produced after natural infection (249–251). They may control the development and survival of gametocytes in circulation or interrupt gamete development and fertilization in the mosquito midgut following ingestion (250). To date, extensively studied antigens include gametocyte/gamete proteins such as the Pfs45/48 and Pfs230 and the zygote/ookinete proteins Pfs25 and Pfs28, but other antigens may be involved. Antibodies against Pfs230 and Pfs45/48 target the pre-fertilization phase and seem to be markers of recent exposure to gametocytes (251), whereas anti-Pfs25 and anti-Pfs28 antibodies represent the post-fertilization (248). Antibody targets of naturally acquired immunity against gametocytes can be instrumental in discovering novel antigens, leading, to the development of more effective Transmission-blocking vaccines (TBV) (250).

2.5 The role of other antibody isotypes/subclasses in immunity against *P. falciparum* infection

Most studies on human immunity to malaria have focused on total IgG. However, evidence suggests that other isotypes and subclasses contribute to malaria immunity. Merozoite-specific IgM was persistent, similar to IgG in *P. falciparum* infected children and adults with lifetime exposure. Moreover, apart from blocking merozoite invasion of RBC in a complement-dependent manner, merozoite-specific IgM was also associated with a significantly reduced risk of clinical malaria in children (252). It was also reported that exposure to *P. falciparum* sporozoites through infection or immunization triggers a functional IgA response. The CSP N terminus was identified as a target of protective IgA (253). Several findings suggest that IgE could play a harmful role during malaria disease development (254). IgG1, IgG2 and IgG3 against *P. falciparum* blood stages in individuals naturally exposed were associated with asymptomatic and uncomplicated malaria, whereas IgG4, IgE and IgM antibodies were pronounced among individuals with complicated malaria (255). Several studies have classically associated cytophilic IgG1 and IgG3 with protection (256–258), whereas non-cytophilic IgG2 and IgG4 have been related to the risk of malaria (255,257). However, other studies found gG2 and IgG4 associated with protection (255,259).

Malaria vaccines: rationale for development

Immunization is one of the most successful and cost-effective public health interventions for preventing diseases and death (260,261). It has led to the eradication of smallpox, the elimination of polio in many countries, and the control of other diseases, including diphtheria, tetanus, pertussis, rubella, and hepatitis B (261). The new Technology Roadmap updated in 2013 established strategic goals: by 2030, licensed malaria vaccines should provide at least a protective efficacy of 75% against clinical malaria, reduce parasite transmission, and be suitable to be deployed in mass campaigns (262). Over the past decades, malaria vaccinology's expiring goal has been developing an immunization strategy that mounts protection superior to NAI (263). However, several challenges have tempered the hope for an effective and widely available vaccine. The complexity of the malaria parasite is the major challenge for vaccine development. The parasite has approximately 5,300 protein-encoding genes (264) and hundreds of potential targets and vaccine candidates over different life stages (265). Different immune system arms are required for different parasite stages depending on their extracellular or intracellular location and distinct immunogenic properties; for example, the protective antibodies against sporozoites fail to recognize merozoites (266).

The current vaccine strategies are broadly grouped by the malaria parasite targets in the life cycle (**Figure 6**).

Pre-erythrocytic vaccines. They target the surface antigens in sporozoites, which are inoculated by a mosquito in the skin, through induced antibody-mediated inhibition, clearing sporozoites from the skin or bloodstream or blocking their invasion of hepatocytes (241,265–267). They are considered more attractive than other stage vaccines as they can prevent initial blood-stage infection, thereby preventing clinical disease and malaria transmission (241,265,267). In the beginning, the parasite infects only a small number of hepatocytes, and it takes about 7 days to complete this phase, allowing enough time for a vaccine to act (266). In addition, the infected hepatocytes express parasite antigens that can induce T-cell responses that can target and kill these infected cells, thus preventing merozoite release into the blood (241). The leading vaccine candidates in this category target sporozoites (CSP and whole sporozoites) and liver stage (TRAP, CSP, CelTOS) (265). The **RTS,S/AS01 vaccine (Mosquirix™)**, a pre-erythrocytic based on CSP, is the first malaria vaccine recommended for widespread use in African children by WHO (19). The **R21/Matrix-M** pre-erythrocytic vaccine is a virus-like particle (VLP), also based on CSP, and the second to be recommended by WHO in sub-Saharan Africa following RTS,S, as it was proven safe and effective in preventing malaria in children (268). Whole sporozoite vaccines using radiation and chemically or genetically attenuated strains of *P. falciparum* sporozoites are also being investigated (266).

Erythrocytic vaccines or blood-stage vaccines. They produce antibodies that generally target the merozoite form and may also target the variant surface antigens of the erythrocyte membrane PfEMP1 (265). They aim to prevent replication and the development of clinical disease and malaria transmission by reducing or clearing blood-stage parasitemia. The road to asexual blood-stage vaccine development has been challenging, with most clinical trials showing limited efficacy, primarily due to high polymorphisms in vaccine antigens, poor understanding of targets of NAI, the involvement of functionally redundant merozoite invasion pathways, and, in some cases, safety concerns (269,270). In this category, the leading vaccine candidates target are AMA1, MSP2, Rh5, MSP3, GLURP, SERA5 and EMP1-VAR2CSA (265).

Transmission-blocking vaccines (TBVs). They target the sexual (gametocytes) and parasite stages (gametes and ookinetes) in the mosquito midgut to prevent infection of mosquitoes and subsequent transmission of sporozoites to another human host (265,266), aiming to effectively block malaria transmission at the population level, thereby contributing to malaria elimination (271). However, the success of this strategy has not yet been demonstrated in clinical trials or human population studies (265,267). These vaccines generate antibodies that prevent the sexual reproduction of the malaria parasite by blocking either the gametes fertilization or the zygote's development into sporozoites (272,273). The four leading candidates have been grouped as gamete surface proteins in human blood, such as Pfs230 and Pfs48/45 of *P. falciparum* (271) and zygote surface proteins expressed in the mosquito host, such as Pfs25 and Pfs28 (274). Antibodies to these antigens were alone or synergistically found to block transmission. For example, cytophilic (IgG1) and IgG3 antibodies to Pfs230 were found to be a major target of complement-fixing antibodies, which may be necessary for antibody-mediated transmission-blocking immunity (275).

Multi-component vaccines (polyvalent vaccines). The antigenic diversity and strain specificity are challenges in developing a single-target malaria vaccine. Polyvalent vaccines targeting several stages of the parasite life cycle or multiple *Plasmodium* species have been proposed, with the potential for additive or synergistic improvements in protective efficacy compared to single-target vaccines (276).

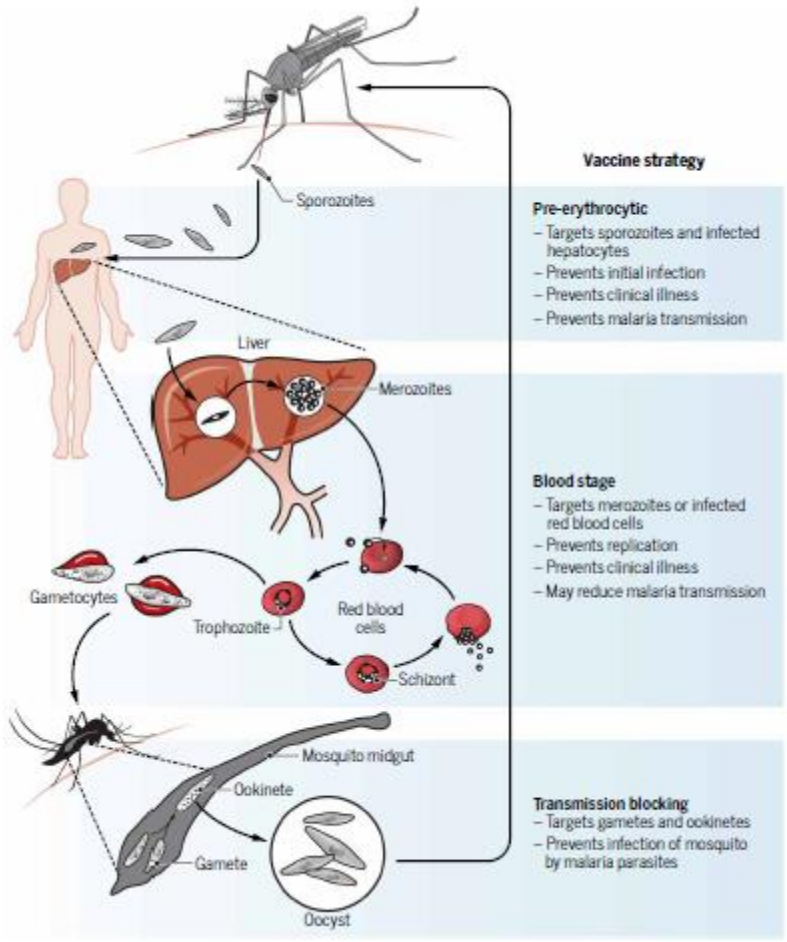

Figure 6. Life cycle and vaccine targets and immune mechanisms involved in protection *(265)*.

The RTS,S malaria vaccine

1. A brief history of RTS,S malaria vaccine

Potential malaria vaccines have been an area of intense research, and efforts to create effective vaccines have spanned decades. Starting from the late 1960s, different research groups have shown that immunization with radiation-attenuated sporozoites could confer protection against malaria in animals and humans (277–279). However, developing a vaccine based on attenuated sporozoites was shown to be technical and logistically challenging at that time. After vaccination with radiation-attenuated sporozoites, the CSP antigen was identified as an immune response target (278). The CSP was subsequently cloned and sequenced (280). Efforts to produce a full-length CSP construct vaccine were proved unsuccessful then. However, a series of alternative constructs were produced (281–283).

As an alternative, in 1984, the Walter Reed Army Institute of Research (WRAIR) teamed up with scientists from GlaxoSmithKline (GSK) Biologicals (Rixensart, Belgium) on the development of a subunit malaria vaccine, using GSK's *Escherichia coli* expression systems (284,285). Four vaccines were developed and tested in animals, but only the R32tet32 formulated with alum (FSV-1) advanced to clinical testing in humans. The vaccine was shown to be safe. However, it was poorly immunogenic (285). It was suggested that the poor immunogenicity in the R32tet32 was due to a lack of recognizable T-helper epitopes (286).

Over the next several years, multiple attempts to advance a standalone CSP recombinant vaccine continued, but unfortunately, none showed significant clinical efficacy (285). GSK scientists pioneered the use of hepatitis B surface antigen (HBsAg) as a carrier matrix for the *P. falciparum* CSP central repeat region, resulting in hybrid protein particles with more improved CSP antigen presentation (286). The initial experimental recombinant malaria vaccine was R16-HBsAg, which contained the last 16 NANP repeats of the central domain fused to HBsAg produced by yeast cells. It was abandoned in favor of a more promising construct after the phase 1 clinical trial (287). A more promising CSP–HBsAg fusion protein was developed, incorporating the CSP C-terminal region containing B- and T-cell epitopes into a chimeric gene expressed in *Saccharomyces cerevisiae* and the construct was named "RTS,S" (284,288). Despite self-assembly into HBsAg VLPs, non-adjuvant RTS,S was shown to exhibit inferior anti-CSP antibody and cellular responses over adjuvanted RTS,S (289).

Since then, many adjuvants have been developed and have demonstrated promising results, sparking hope in the field of vaccine development. These adjuvants, such as MPL in the early 1980s, QS-21 in the late 1980s, and CpG identified in 1995, have been discovered and tested in humans, showing significant potential due to their immuno-enhancing properties (290).

However, the striking change was the introduction of the novel concept of Adjuvant Systems, which combine various molecules (3-O-desacyl-4'-monophosphoryl lipid A [MPL], QS-21, CpG) with classical adjuvants (aluminium, liposomes, oil-in-water emulsions), resulting in an additive effect on the adaptive immune response and potentially an impact on the breadth of the immune response (cellular as well as humoral components) (290). In that regard, multiple novel adjuvant systems developed at GSK have been tested with RTS,S, and adjuvant systems that were eventually selected and used in most clinical trials belong to the AS01 and AS02 families (291). Both include the immune response enhancers MPL and QS21 and are formulated either as an oil-in-water emulsion for AS02 or a liposome-based adjuvant in the case of AS01 (291).

2. The RTS,S malaria vaccine design

The basic organizational structure of CSPs between *Plasmodium* ssp. are similar (292,293). However, their primary amino acid sequences differ (293) (**Figure 7**). The full-length CSP encompasses an N-terminus that encodes a signal peptide sequence, binds heparin sulfate proteoglycans (Region I), and is based on a short and a conserved five amino acid (KLKQP) proteolytic cleavage site sequence and Pexel motifs (292,293). The middle third consists of tandem, species-specific amino acid repeats that are B-cell immunodominant epitopes (280,294). The end comprises a positively charged C-terminus domain placed at the C-terminal ends of the molecule (295). The C-terminus domain contains a thrombospondin-like type I repeat domain (TSP) with cell adhesive properties (Region II) and a canonical glycosylphosphatidylinositol (GPI) anchor addition sequence (293). Moreover, it has three known T-cell epitopes: (1) a highly variable $CD4^+$ T-cell epitope before the TSP, (2) a highly variable $CD8^+$ T-cell epitope within the TSP, and (3) a "promiscuous" $CD4^+$ T-cell epitope whose structure is conserved among all parasite isolates (293).

On the other hand, the RTS,S/$AS01_E$, contains only the last 18 NANP repeats of the central domain (hence the "R") and the C-terminus exclusive of the GPI anchor addition sequence that contains T-cell epitopes separated by immunodominant $CD4^+$ and $CD8^+$ T cell epitopes (Th2R and Th3R), (hence the "T"), all based on the *P. falciparum* NF54 strain, genetically fused to N-terminal HBsAg, (thus the "S" (surface). These molecules form a single fusion protein ('RTS') when co-expressed in *Saccharomyces cerevisiae* yeast cells yielding a VLP, displaying both CSP and S at their surfaces. The second "S" portion is an unfused or free HBsAg that spontaneously assembles to the RTS component, hence the name RTS,S. The resulting VLP comprises 25% of the CSP-HBsAg fusion protein and 75% HBsAg (296–298) and it is formulated with a potent liposomal formulation AS01, consisting of immunostimulants, QS-21 and MPL (293,299).

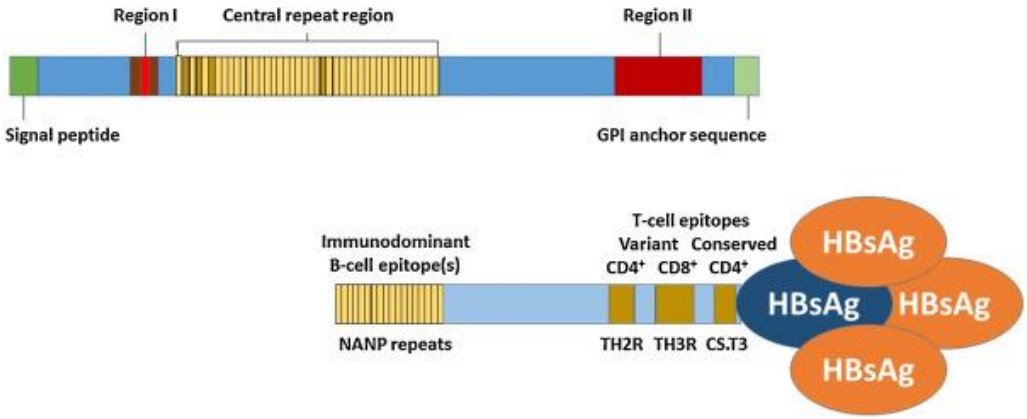

Figure 7. *Plasmodium falciparum* circumsporozoite protein (CSP) and RTS,S vaccine basic organizational structures *(293)*.

3. O-fucosylation of CSP and its interactions with the host

The CSP thrombospondin type 1 repeat (TSR) domain has an O-fucose consensus sequence that is amenable to protein post-translational modification termed O-fucosylation (295,296). Protein O-fucosyltransferase 2 adds O-linked fucose, which interacts with underlying amino acids, stabilizing the folded TSR domain (300). This protein in the RTS,S vaccine produced in yeast is not O-fucosylated (301). The role of O-fucosylation in the *P. falciparum* life cycle was recently identified to ensure efficient infection of both the mosquito vector and human hepatocytes (302).

Glycoconjugates in many pathogens are important mediators of host-pathogen interactions and have been shown to be important in invasion, cellular growth, host immunity regulation, and differentiation (303–305). The protein glycosylation has been implicated in many aspects of adaptive immune activation as it can greatly influence antigen uptake and proteolytic processing (304). Growing evidence shows that antigen post-translational modifications are recognized by T cells (306). Some studies have shown that O-linked glycosides remain intact during processing by the immune proteasomes and can survive lysosomal degradation. The resulting glycopeptides can be presented in a MHC-restricted manner (307,308). However, glycosylation can have a detrimental effect as reported in hepatitis C virus (HCV) envelope-associated glycans that play a crucial role in masking functionally important neutralizing epitopes in certain antigens (309).

The glycosylation of protein antigen is expected to be relevant for vaccine development. For example, immunization with the glycosylated tripartite vaccine was superior in tumor-associated mucin MUC1 prevention, eliciting specific cytotoxic T cells and more lytic antibodies compared to the unglycosylated control (310). In addition, the observation that many infected patients eventually develop highly potent broadly neutralizing antibodies against surface glycoproteins has greatly stimulated the vaccine design efforts against HIV (311).

In *P. falciparum*, glycoproteins were initially studied in intraerythrocytic stages (312–314). For example, the presence of glycoproteins on MSP1 was described, and the incomplete protection elicited by recombinant *P. falciparum* MSP1 was proposed to be due to a lack of N and O-glycoproteins (315). Recent evidence shows that the two leading vaccine candidates, antigens, CSP and TRAP, are glycosylated in their TSR domains (316). However, it remains largely unclear how this modification in the CSP interacts with the human host and its relevance to vaccine design. Understanding such protein modifications is crucial for target selections

in designing effective antibody-based vaccines, as glycosylation significantly alters *the* recognition of epitopes (316).

4. Overview of RTS,S malaria vaccine phases 1 and 2

The drug development process encompasses extensive pre-clinical studies before clinical trials are undertaken for potential medical products. These studies aim to determine whether the drug has scientific merit for further testing in people. The studies *in vitro* (test tube or cell culture) and *in vivo* (animal model) provide detailed dosing, efficacy, toxicity levels and pharmacokinetic information. After answering these fundamental questions about drug safety, clinical trials are ready to be undertaken. The drug development process is commonly classified into four phases: (1)- Safety and dosage, (2)- Efficacy and side effects, (3)- Efficacy and monitoring of adverse reactions and 4- Safety and efficacy in a real-world setting. The drugs that successfully pass phases 1, 2, and 3 and have approval from relevant national and international authorities for use in the general population undergo phase 4 trial “post-marketing” or 'surveillance' studies aiming to monitor safety over several years (317).

The phases 1 and 2 clinical trials allowed an initial assessment of the RTS,S sole candidate vaccine’s safety and efficacy profile. Initially, the vaccine was tested in adult naïve volunteers in the United States (US) and Belgium who were challenged with mosquito-borne homologous *P. falciparum* malaria, followed by semi-immune adults, adolescents, and children. Later, the vaccine was assessed in infants living in malaria-endemic Africa exposed to diverse *P. falciparum* strains (288,297,318–322).

Multiple adjuvant systems formulations were used with RTS,S and tested in animals for immunogenicity by GSK scientists (284,323). However, among the promising, AS04 (SBAS4), AS03 (SBAS3), AS02 (SBAS2), AS02A and AS01B have been further tested in humans and shown to improve the protection provided by RTS,S (323,324). The RTS,S/AS04 was a formulation containing alum and MPL, and the RTS,S/AS03 consisted of RTS,S in an oil-in-water emulsion; and the RTS,S/AS02 consisted of an oil-in-water *emulsion* plus the immune stimulants MPL and QS21 (318).

The clinical trial testing these three vaccines, followed by the sporozoite challenge, demonstrated that the RTS,S/AS02 formulation enhanced protection against Controlled Human Malaria Infection (CHMI) (318). The original AS02 containing thimerosal, a mercury-based reagent used as a preservative, was renamed AS02A, a new adjuvant containing lactose as cryopreservant over concerns of potential neurotoxicity, although no increased toxicity was proven (323).

When the study team was ready to launch the phase 2b proof of concept trial with RTS,S/AS02, the GSK scientists reported problems of deterioration of the vaccine in real-time stability studies. They carried out an

intensive program to develop a more stable formulation that resulted in the development of a lyophilized RTS,S formulation for reconstitution with AS02A vialed separately instead of the initial single-vial liquid vaccine antigen, and all subsequent trials of RTS,S have used this two-vial system (325).

The pediatric clinical development program for RTS,S underwent a series of age de-escalation and dose-finding studies in the first phase, which resulted in a phase 2b proof-of-concept efficacy trial in young children in endemic Africa (325). The second phase included a series of confirmatory safety and/or efficacy studies in an expanded population of children, including studies that age-de-escalated the vaccine to infants. They further evaluated the vaccine with or without the Expanded Program of Immunization (EPI) vaccine co-administration (325).

During these phases, the vaccine also underwent significant formulation changes that resulted in the development of a new adjuvant system known as AS01, also GSK property, also comprised of MPL and QS-21, in which liposomes replaced the oil-in-water emulsion of AS02. That provided the opportunity to improve the RTS,S immunogenicity (323). A comparison between RTS,S/AS01, and RTS,S/AS02 in malaria-naive adults followed by malaria challenge showed somewhat better efficacy, higher anti-CSP IgG titers, and higher $CD4^+$ T cell responses (326). Further on, a comparative field trial in Kenyan adults confirmed the findings (327).

AS01 and AS02 have been formulated in both adult ($AS01_B$, $AS02_A$) and pediatric ($AS01_E$, $AS02_D$) dosages, as age de-escalation process within the RTS,S clinical development plan. They found a trend towards better anti-CSP and anti- HBsAg responses with RTS,S/$AS01_E$ than RTS,S/$AS02_D$ (328). This formulation, RTS,S/$AS01_E$, was advanced for testing as part of phase 2 clinical trials and setting the stage for subsequent phase 3 clinical trials (**Table 3**) (327–332).

Table 3. The RTS,S malaria vaccines in phase 1 and 2 trials using the most advanced formulations AS02 and ASO1

Formulation and study objective	Clinical testing	Location	Age enrollment	Main findings	Ref
RTS,S/AS02$_A$* To compare liquid and lyophilized formulations	Phase 1/2a	USA	Malaria-naïve adults	○ Both were well tolerated, safe and similarly immunogenic.	(319)
RTS,S/AS02 Immunization schedules (0, 1, and 3 months and 0, 7, and 28 days)	Phase 2a	USA	Malaria-naïve adults	○ 0, 1, and 2-month schedules demonstrated a favourable safety and efficacy profile.	(333)
RTS,S/AS02$_A$*and RTS,S/AS01$_B$ Evaluation**	Phase 2b	Kenya	Healthy adults aged 18 to 35 years	○ Both candidates were well tolerated over a 12-month surveillance period. ○ A more favorable immunogenicity profile for RTS,S/AS01$_B$ than RTS,S/AS02$_A$	(327)
Efficacy of RTS,S/AS02	Phase 2b	The Gambia	Men aged 18–45 years	○ RTS,S/AS02 was safe, immunogenic, and was the first to show significant protection against natural infection with *P. falciparum*.	(288)
Efficacy of the RTS,S/AS02$_A$*	Phase 2b	Mozambique	1-4 years.	○ Safe, well tolerated, and immunogenic.	(320)
Duration of protection of RTS,S/AS02$_A$*	Phase 2b	Mozambique	1–4 years	○ Reduced risk of clinical malaria (vaccine efficacy of 35%) and severe malaria (vaccine efficacy of 49%) over 18 months.	(321)

Safety of the RTS,S/AS02$_D$*	Phase 1/2b	Mozambique	10, 14, and 18 weeks of age	○ Risk of infection reduced by 65% over 3 months after the 3rd and final dose, and risk of clinical disease by 35% over 6 months following the 1st dose ○ safe, well tolerated, and immunogenic in young infants.	(322)
RTS,S/AS01$_E$or RTS,S/AS02$_D$***	Phase 2	Gabon	18 months to 4 year	○ A trend towards better anti-CSP and anti-HBs responses with RTS,S/AS01$_E$ better than RTS,S/AS02$_D$ ○ Both formulations similarly well-tolerated and immunogenic	(328)

*oil in water emulsion-based adjuvant system; **Liposome-based adjuvant system

5. Overview of the RTS,S/AS01$_E$ malaria vaccine pediatric phase 3 Clinical Trial

The RTS,S/AS01$_E$ phase 3, double-blind, individually randomized controlled trial, three-arm trial, enrolled 15,459 infants and young children and was conducted between 2009 and 2014 at 11 sites in 7 countries of sub-Saharan Africa, all situated in areas with different intensities of malaria transmission and seasonalities (**Figure 8**) (334).

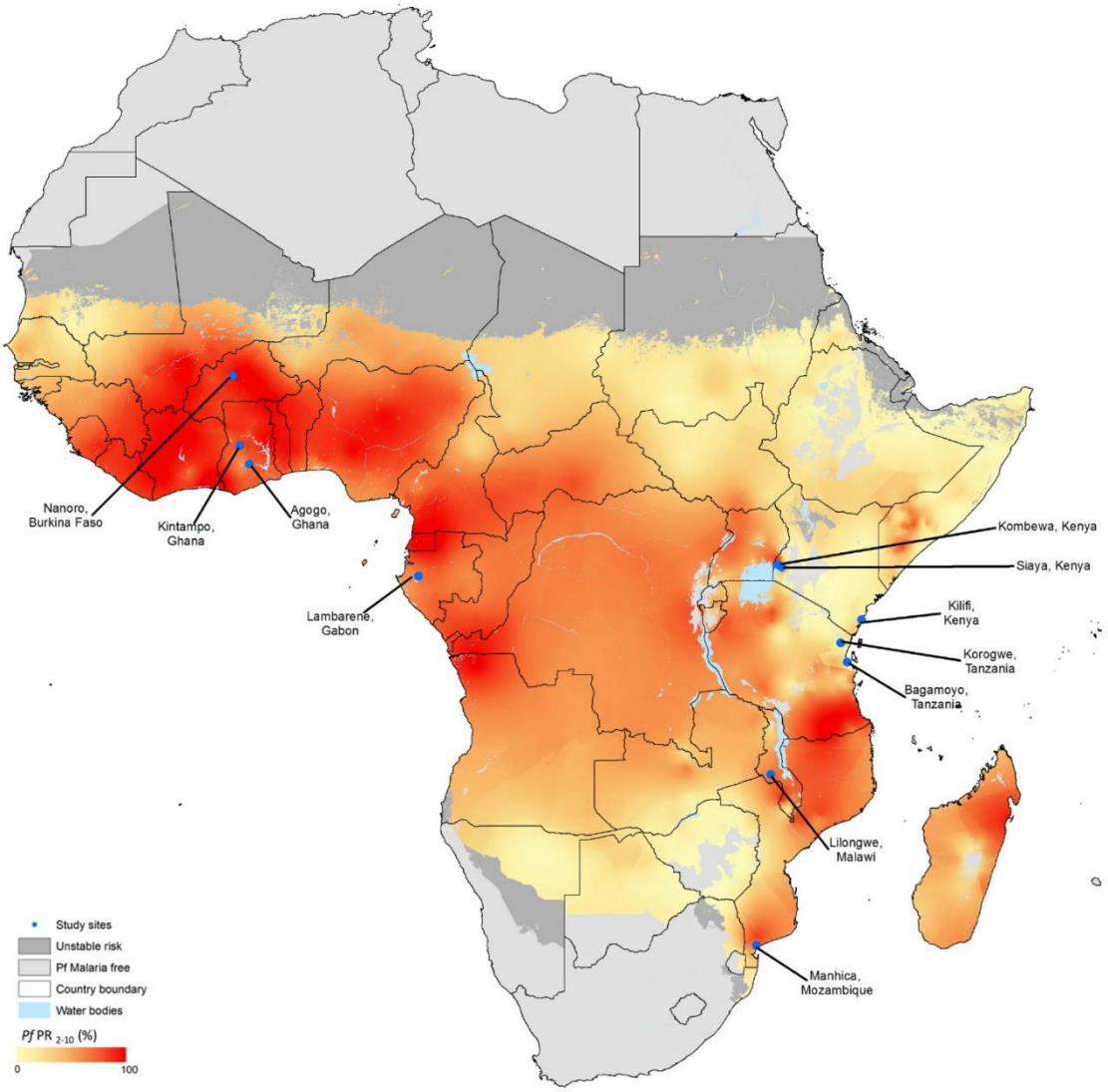

Figure 8. Study sites and malaria endemicities *(335)*.

The countries included (one site in Mozambique, Burkina Faso and Gabon, two in Tanzania and Ghana and three in Kenya), and the trial was registered under ClinicalTrials.gov, NCT00866619.

The participants were males and females in both age cohorts (infants 6-12 weeks of age and children 5-17 months) at enrolment. The subjects were randomly assigned (1:1:1) at first vaccination by block randomization with minimization by the center and received either three doses of RTS,S/AS01$_E$ once per month at months 0, 1, and 2 (primary schedule) and a booster dose at 20 months (R3R group); three doses of RTS,S/AS01$_E$ vaccine and a dose of comparator vaccine at 20 months (R3C); or three doses of a comparator vaccine at months 0, 1, 2, and a booster dose at 20 months (C3C) (**Figure 9**).

The comparator vaccines used were meningococcal serogroup conjugate vaccine: Menjugate (Novartis), in the 6-12 weeks age category. For the 5-17 months age category, the comparator vaccine was the rabies vaccine (VeroRab, Sanofi-Pasteur). The study was designed to initially asses the vaccine efficacy (VE), safety, and immunogenicity during 32 months of follow-up. However, the protocol was amended to extend (the median observational period to 48 months for children aged 5–17 months, whereas for infants aged 6–12 weeks, the median follow-up period to 38 months). The definitions, procedures and study design are described in detail elsewhere (334–338).

The vaccine reduced clinical and severe malaria episodes in children and showed a favorable safety profile (336,337). Over a 12-month follow-up period after a 3-dose primary vaccination, in the 5–17-month age category, the efficacy against the first or only episode of malaria was estimated at 55.8% (97.5% confidence interval [CI] 50.6–60.4) (337) and 31.3% (23.6–38.3) in infants age 6–12 weeks (336).

VE and immunogenicity were lower in young infants than children and waned over time in both age groups (335–337). Further results of the vaccine with or without a booster dose have revealed a substantial reduction in clinical malaria cases, and the efficacy was enhanced by administering a booster dose (334). Additional analyses were published on the trial's secondary, tertiary and exploratory aims, including the co-administration with other routinely administered vaccines, safety and efficacy in HIV-positive participant. In 2015, the vaccine received a positive scientific opinion of the vaccine by the European Medicines Agency (EMA) (339). After a subsequent recommendation for pilot implementation in Ghana, Kenya, and Malawi under the auspicious of the WHO, the vaccine made history in 2021 as the first malaria vaccine recommended for widespread use in African children (19).

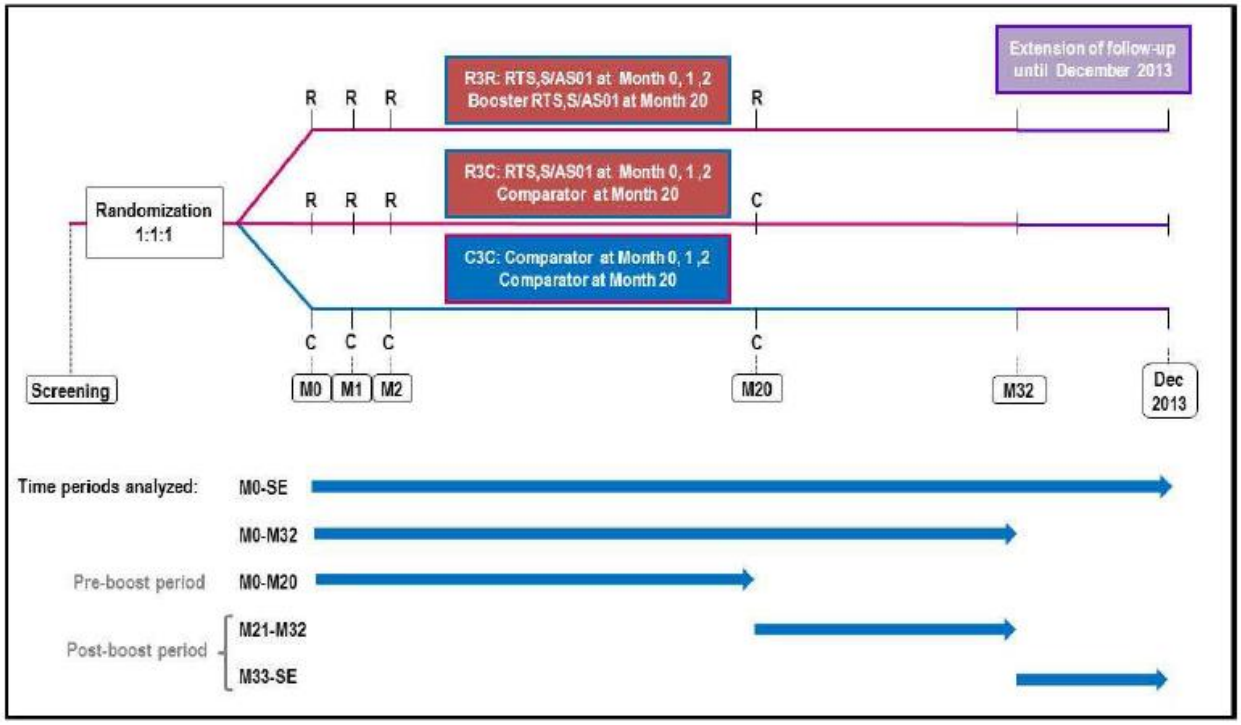

Figure 9. RTS,S/AS01$_E$ malaria vaccine phase 3 trial design. *(334)*. R3R: 3 doses of RTS,S/AS01$_E$ at months,0,1,2 plus a 4th dose of RTS,S/AS01$_E$ at month 20; R3C: 3 doses of RTS,S/AS01$_E$ at months,0,1,2 plus 4th dose of comparator vaccine at month 20; C3C: 3 doses of comparator vaccine at months,0,1,2 plus a 4th doses of a comparator vaccine at month 20; The median follow-up time after dose 1 was approximately 38 months in 6-12 weeks and 48 months for the 5-17 months age group and the follow-up time had depended on their enrolment date.

4.1 Antibody response to RTS,S malaria vaccine and correlates of protection

Early studies from clinical trials in adult malaria-naïve volunteers (318,319,333,340) and African field trials in children and semi-immune adults (316) suggested that the RTS,S induces strong anti-CSP IgG responses to repeat sequences of NANP region. The RTS,S/AS01 vaccine-induced anti-CSP antibody titers have been correlated with protection against infection and clinical malaria (323,341). Moreover, the anti-CSP antibody titers have been proposed as a surrogate of protection. However, no protective threshold value has been determined (341), indicating the situation may be more complex. Antibody magnitude was inconsistently associated with protection across studies. Morever, the exact mechanism(s) by which vaccine-induced antibodies provide protection remains to be elucidated (342). The recent evidence proposed that although anti-CSP titers are important, they are not enough to explain the VE, suggesting that the quality of antibodies, including their fine specificity and functional activity, may play a significant role in protection (343).

As explained in the sections above, many properties determine antibody function, including the availability of effector molecules, the isotype, the subclass, the post-translational modification, and the affinity/avidity of antibodies (344). The avidity, defined as the overall strength of interaction between antigen-antibody, may also impact protective efficacy as only a few sporozoites circulate for a short time after an infectious mosquito bite, likely requiring fast and strong binding properties (344,345). A challenge trial found that individuals with a high antibody titer response that also showed high avidity against the NANP repeat region provided near perfect protection upon first challenge (98.2% [95% Credible Interval 91.6–99.7%]) (345). Another CMHI study conducted as part of phase 2 trials found that the anti-CSP IgG avidity against the NANP repeat region was associated with protection between the second and third dose of vaccination (344); however, after the last vaccine injection, it was not associated with protection (344). Our groupfound that avidity to C-terminus post-vaccination significantly associated with VE phase 3 field trial (346).

IgG subclass are also important to assess since their levels and effector mechanisms are different, with subsequent impact on malaria protection (346). In fact, cytophilic IgG (IgG1 and IgG3) responses to the C-terminal and NANP repeat regions of CSP and anti-HBsAg antibodies post RTS,S/AS01$_E$ vaccination were found to be associated with malaria protection in prior studies from our group, whereas the non-cytophilic (IgG2 and IgG4) were associated with malaria risk over 1-year follow-up (347). Furthermore, a higher ratio of cytophilic IgG1 and IgG3 to non-cytophilic IgG2 and IgG4 antibodies was found to be associated with antimalarial immunity (347).

Using applied systems serology, qualitative functional humoral features, rather than antibody concentration, represented critical predictors of protection from infection with *Plasmodium* spp (342). Various functions of RTS,S-induced antibodies have been identified to predict protection against CHMI, including antibody-mediated complement fixation (348), phagocytosis (342), FcγR-binding (342,348) and opsonization (343,349). The available evidence suggests that the protective immunity afforded by the RTS,S/AS01 can be achieved via multiple immune mechanisms (343,350). Both titer and Fc effector functions of antibodies are worth exploring (351).

Hypothesis-generating omics and systems vaccinology analyses and assessment of pre-immunization correlates have the potential to identify in-depth novel correlates of protective immunity (352). However, the effect of vaccination on the acquisition of clinical immunity (353) and the diversity of immunological assays (352) remain key barriers to identifying robust immunological correlates of RTS,S/AS01 VE.

4.2 Factors affecting the magnitude and duration of RTS,S antibody-induced immunity

The analysis of the dynamics of anti-CSP IgG antibodies exhibits a biphasic exponential distribution pattern characterized by a rapid waning in the first 6 months after vaccination followed by slower waning over the subsequent years up to 4-7 years (341,354). It was proposed that anti-CSP antibody titers might also predict the duration of efficacy against clinical malaria, with or without a booster dose, across different age categories, transmission intensities and pre-exposure. RTS,S/AS01 is more immunogenic in children than infants (341,355). In infants, high pre-vaccination anti-CSP antibody titers were associated with low anti-CSP antibody titers post-vaccination (341), suggesting maternal antibodies or fetal exposure to malaria parasites might hinder immunogenicity (355).

The immunogenicity of the booster dose is strongly associated with immunogenicity after primary vaccination (341), presumably reflecting the maturation of the immune system and boosting of memory responses.

Immunogenicity has also been demonstrated to depend on transmission intensity. The efficacy wanes more rapidly at higher transmission intensity, with the rate at which anti-CSP antibodies wane similar to the rate of efficacy decline (356).

A booster dose given 18 months after the primary schedule vaccination provided a low anti-CSP IgG response peak compared with primary immunization. This is contrary to the classic immunological picture of vaccine-induced responses where booster doses induce higher levels than primary vaccination (341). This finding might indicate shorter than usual half-lives for MBC or helper $CD4^+$ T cells (341).

Anti-CSP and anti-*P. falciparum* antibody levels pre-immunization were associated with increased susceptibility to malaria over 1-year follow-up (356), presumably because such responses also correlate with prior exposure to malaria. Measures of an individual's previous malaria exposure are likely to predict their level of future exposure (258) and, thus, their relative chance of vaccine failure (352). Prior malaria exposure may induce functional exhaustion of lymphocytes, de-regulated immune system, potentially impeding optimal control of infection (357) or suppressing vaccine-mediated protection (347,352,358). In addition, the induction of functional antibodies was found to be reduced in children with higher malaria exposure (359).

Other factors also seem to affect the protection afforded by vaccination. Baseline antibody responses against HBsAg were found to be predictive of protection. However, the pathway(s) underlying this association remain unclear (347). Being HIV positive was also found to be associated with reduced immunogenicity (341,360,361). However, the vaccine still provides some protection against malaria in HIV-infected children on ART (361).

4.3 The relationship between RTS,S/AS01 vaccine-induced CSP-specific antibodies and CD4+ T cells

Both RTS,S/AS01-induced anti-CSP antibody titers and CSP-specific CD4+ T cells have been identified as immunological surrogates of protection against infection and clinical malaria (326,331,341,350,362,363). However, whether the CD4+ T cells act as direct effectors or indirectly through modulation of antibody responses remains unclear (341). Some studies have shown that RTS,S/AS01 induces CSP-specific CD4+ T cells producing IL-2, TNF, IFN-γ and CD40L in infants and children (364,365). The relation between antibody titers and the number of IL-2-producing CD4+ T lymphocytes has been reported (366,367). Data from malaria-naïve adults in phase 2a found that RTS,S/AS induced CSP-specific CD4+ effector/effector memory ($T_{E/EM}$) and CD4+ central memory (T_{CM}) cells and that both subsets generated strong IL-2 responses (366). The frequency and total numbers of IL-2-producing CD4+ $T_{E/EM}$ and CD4+T_{CM} cells were higher in protected than non-protected volunteers (366). Importantly, IL-2-producing memory CD4+ T cells correlated with the CSP-specific antibody titers within corresponding individuals (366).

IL-2-producing CD4+ T may directly induce the differentiation of CSP-specific B-cell responses since IL-2-producing CD4+ T cells express CD40L (326), which could bind to CD40 receptors on B cells (368). Similarly, IL-2-producing CD4+ T cells may provide helper T-cell signalling critical for GC formation. CD4+ T cells may interact with other cells, including B cells and dendritic cells, promote antibody class switch (368–370), antibodies affinity maturation (344,369,370), induction of MBC (369–371) and maintenance of circulating anti-CSP antibodies (367).

Hypotheses

The **overall hypothesis** of this PhD thesis is that the RTS,S vaccine immune response is more complex than the previously studied anti-CSP IgG response; it depends on the immunogen design, and different IgG subclasses predominate at the primary versus the booster doses, and affects naturally acquired antibodies.

The specific hypotheses are:

1. IgG antibodies induced by RTS,S immunization, where CSP is not fucosylated, have lower recognition activity for the native-like fucosylated CSP, which may impact protection.

2. A fourth RTS,S dose (booster) is associated with changes in the IgG subclass antibody profiles against vaccine and malaria antigens, which could be associated with the duration of the vaccine efficacy.

3. RTS,S primary immunization affects the acquisition of antibodies to vaccine-unrelated *P. falciparum* antigens and such responses impact overall malaria protective immunity.

Objectives

The **overall aim** of this PhD thesis is to increase our understanding of the immunogenicity of RTS,S vaccination in African children, including the impact of biochemical composition of the malaria immunogen during primary immunization, the qualitative profile of antibody responses to booster vaccination, and the effect of vaccination on antibody responses to vaccine-unrelated *P. falciparum* protein antigens.

The specific objectives are:

1. To assess the binding of antibodies induced by RTS,S, in which CSP *O*-fucosylation post-translational modification is absent, to fucosylated and non-fucosylated CSP and to assess the relevance of this modification in malaria protection.

2. To characterize the magnitude, type and longevity of antibody responses against RTS,S vaccine antigens and vaccine-unrelated antigens after primary and booster vaccination.

3. To understand the interplay between the RTS,S vaccination, and naturally acquired immune responses to malaria and to identify immune correlates of protection.

Article 1

This article addresses the first objective of assessing the binding of antibodies induced by RTS,S, in which CSP *O*-fucosylation post-translational modification is absent, to fucosylated and non-fucosylated CSP, and assessing the relevance of this modification in malaria protection. The results of this work have been published in the following peer-reviewed journal article:

Jairoce C, Macià D, Torres-Yaguana JP, Mayer L, Vidal M, Santano R, Hurtado-Guerrero R, Reiter K, Narum DL, Lopez-Gutierrez B, Hamerly T, Sacarlal J, Aguilar R, Dinglasan RR, Moncunill G, Izquierdo L, Dobaño C. **RTS, S/AS02A malaria vaccine-induced IGG responses equally recognize native-like fucosylated and nonfucosylated plasmodium falciparum circumsporozoite proteins.** The Journal of Infectious Diseases. 2024 Mar 15;229(3):795-9.

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BRIEF REPORT

RTS,S/AS02A Malaria Vaccine-Induced IgG Responses Equally Recognize Native-Like Fucosylated and Nonfucosylated *Plasmodium falciparum* Circumsporozoite Proteins

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The RTS,S/AS02A malaria vaccine is based on the *Plasmodium falciparum* circumsporozoite protein (PfCSP), which is *O*-fucosylated on the sporozoite surface. We determined whether RTS,S/AS02A-induced immunoglobulin G (IgG) antibodies recognize vaccine-like nonfucosylated PfCSP better than native-like fucosylated PfCSP. Similar to previous vaccine trials, RTS,S/AS02A vaccination induced high anti-PfCSP IgG levels associated with malaria protection. IgG recognition of nonfucosylated and fucosylated PfCSP was equivalent, suggesting that PfCSP fucosylation does not affect antibody recognition.

Clinical Trials Registration. NCT00197041.

Keywords. RTS,S; antibodies; immunogenicity; *Plasmodium falciparum*; circumsporozoite protein (PfCSP); fucosylation; malaria.

RTS,S/AS01E is the first malaria vaccine recommended for widespread use by the World Health Organization in African children. The vaccine has a favorable safety profile and reduces episodes of both clinical and severe malaria in children [1]. Data from the phase 3 trial showed that vaccine efficacy against clinical malaria is modest 12 months after a 3-dose primary vaccination, estimated at 55.8% (97.5% confidence interval [CI], 50.6%–60.4%) in children aged 5–17 months [1], and 31.3% (97.5% CI, 23.6%–38.3%) in infants aged 6–12 weeks [2], and the duration of protection wanes over time [1, 2]. The development of a more efficacious vaccine remains a high priority for malaria control and elimination. Multiple factors may contribute to the suboptimal efficacy of adjuvanted recombinant protein subunit vaccines like RTS,S/AS01E, and the absence of native glycosylation residues may be one important element [3]. The vaccine, produced by GlaxoSmithKline Biologicals (Belgium), contains the hepatitis B virus surface antigen (HBsAg) genetically fused to a fragment of the *Plasmodium falciparum* circumsporozoite protein (PfCSP) including the last 18 NANP repeats of the central domain and its C-terminal end, and is formulated as virus-like particles with a liposome-based adjuvant (AS01E) [4]. We have previously shown that the avidity of antibodies to the C-terminus of PfCSP correlates with protection against malaria following vaccination with RTS,S/AS01E [5].

The PfCSP C-terminus comprises a domain with homology to the thrombospondin type-1 repeat superfamily (TSR), which in its native form contains an *O*-fucosylation motif [6], and a fucose monosaccharide modification was recently identified on PfCSP from salivary gland sporozoites [7]. *O*-fucosylation is a simple posttranslational modification that facilitates protein folding and trafficking and may affect antigenicity [6]. Recent evidence suggests that *O*-fucosylation of TSR domains in *P. falciparum* proteins is important for liver infection and may also be relevant in the mosquito stages of the life cycle [8]. Moreover, the glycosylation profile on *P. falciparum* surface proteins such as PfCSP has the potential to influence parasite-specific humoral and cellular immune responses. We, therefore, hypothesized that the antibodies induced by the RTS,S vaccine, in which PfCSP is not fucosylated, might not properly recognize the native fucosylated PfCSP antigen on the sporozoite, which could compromise the overall protective efficacy. To test this hypothesis, we used immune sera from young children enrolled in the pivotal Mozambique phase 2b trial of the RTS,S vaccine formulated with a previous version of the adjuvant (AS02A) [9], and compared the levels of vaccine-induced immunoglobulin G (IgG) that bound to fucosylated versus nonfucosylated PfCSP.

METHODS

Study Participants

Participants in this phase 2b clinical trial of the malaria vaccine candidate RTS,S/AS02A in the Manhiça District, southern

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Mozambique (ClinicalTrials.gov registry No. NCT00197041) were children aged 1–4 years at first vaccination. The samples analyzed in this study were from children in the Manhiça cohort 1 (n = 708) (Supplementary Figure 1). The clinical trial definitions, study design and population, procedures, and interventions have been described elsewhere [9].

Ethics

The clinical trial research protocols were approved by the National Mozambican Ethics Review Committee, the Hospital Clínic of Barcelona Ethics Review Committee in Spain, and the PATH-Malaria Vaccine Initiative Research Ethics Committee, and all parents or legal guardians of the participants provided written informed consent.

Antigens

The recombinant full-length PfCSP was expressed using the *Pichia pastoris* expression system, and the TSR C-terminal domain of PfCSP was expressed in *Escherichia coli*. Both PfCSP TSR and full-length PfCSP fucosylated domains were produced using the human protein *O*-fucosyltransferase 2 (huPoFUT2). The details of the antigens production, purification and sequences can be found in Supplementary Materials and Methods and Supplementary Figure 2.

Antibody Assays

An in-house multiplex bead-based antibody assay was performed to assess IgG responses against fucosylated and nonfucosylated versions of the PfCSP full-length and the C-terminal TSR domain (Supplementary Materials and Methods and Supplementary Figure 2). The assay applied the xMAP technology (Luminex Corporation), previously standardized and optimized [10]. Briefly, antigens were coupled to magnetic beads, multiplexed, and incubated with 50 µL of the test, negative control, or positive control serum samples. Next, beads were washed and incubated with a biotinylated secondary antibody, washed again, and incubated with streptavidin-R-phycoerythrin. Finally, using a Luminex xMAP 100/200 analyzer, at least 50 microspheres per analyte were acquired per well. The IgG levels were measured as median fluorescence intensity [10] and transformed to levels in arbitrary units using the modelled calibration curves from serial dilutions of a positive reference sample (Supplementary Figure 3).

Statistical Analysis

Antibody levels were compared with boxplots showing geometric means, medians, and interquartile ranges. Fold-changes in IgG level geometric means between time points or between vaccination groups were estimated by calculating average differences in $\log_{10}$-transformed measurements. The 95% CI and *P* values were obtained from 2-tailed 2-sample *t* tests for comparisons between vaccination groups and 2-tailed paired *t* tests for comparisons between time points. Through exponentiation of the $\log_{10}$-transformed level differences and their corresponding 95% CI, we obtained the desired IgG level fold-change estimates.

To study the relationship between the levels of IgG binding to fucosylated and nonfucosylated antigens, we used scatter plots to capture the bivariate distribution, acknowledging that the closer the points to the diagonal, the closer the relationship to that of perfect identity. We also calculated the Pearson correlation coefficients to assess the strength of this relationship. Statistics and plots were always conducted or shown in $\log_{10}$-transformed levels or scales to stabilize the variance.

RESULTS

The baseline characteristics comparing vaccination groups (Supplementary Table 1) and prevaccination antibody levels (month 0) were similar for both fucosylated and nonfucosylated versions of PfCSP full-length (Figure 1*A*) and C-terminus TSR domain-only antigens (Figure 1*B*). Primary vaccination (month 3) anti-PfCSP IgG reactivity was strong, with fold-increases in geometric mean levels >1000 for RTS,S vaccinees as compared to the comparator group. These data are well aligned with previous estimates using enzyme-linked immunosorbent assay (ELISA) from a larger longitudinal study of the same clinical trial [11] and with other RTS,S clinical trials measuring anti-NANP IgG [12]. Anti-TSR IgG responses were somewhat weaker than the full-length PfCSP IgG responses, with increase estimates between vaccination groups at month 3 that were 5- to 6-fold lower for TSR. Also, as expected, PfCSP full-length and TSR antibody levels waned between month 3 and month 8 (reductions by 5- and 3.5-fold, respectively), closely replicating previous estimates of IgG decay rates [11]. Contrary to our expectation, we observed nearly identical responses when we compared IgG levels bound to fucosylated versus nonfucosylated PfCSP full-length and TSR antigens. Figure 1*C* and 1*D* shows anti-PfCSP fucosylated versus not fucosylated IgG levels, yielding a bivariate distribution that approached perfect correlation.

Next, we examined the children's characteristics affecting increases in IgG antibody levels reacting to fucosylated PfCSP constructs relative to prevaccination. We observed no significant differences in the rise of IgG geometric means between pre- and postvaccination time points against the fucosylated PfCSP proteins between age groups or sexes in the RTS,S vaccinees. However, we observed a lower increase of IgG to fucosylated TSR in older children (2–4 years) compared to younger (1–2 years) after RTS,S vaccination (month 3) ($P = .02$; Table 1). As expected, higher increases in anti-PfCSP full-length and TSR IgG levels were associated with protection from disease. Thus, RTS,S-vaccinated children who did not develop clinical malaria within the first year of follow-up had significantly higher anti-PfCSP IgG increases from month 0 to month 3 ($P = .042$; Table 1) as well as a trend towards higher anti-TSR IgG increases ($P = .2$). Differences were larger and

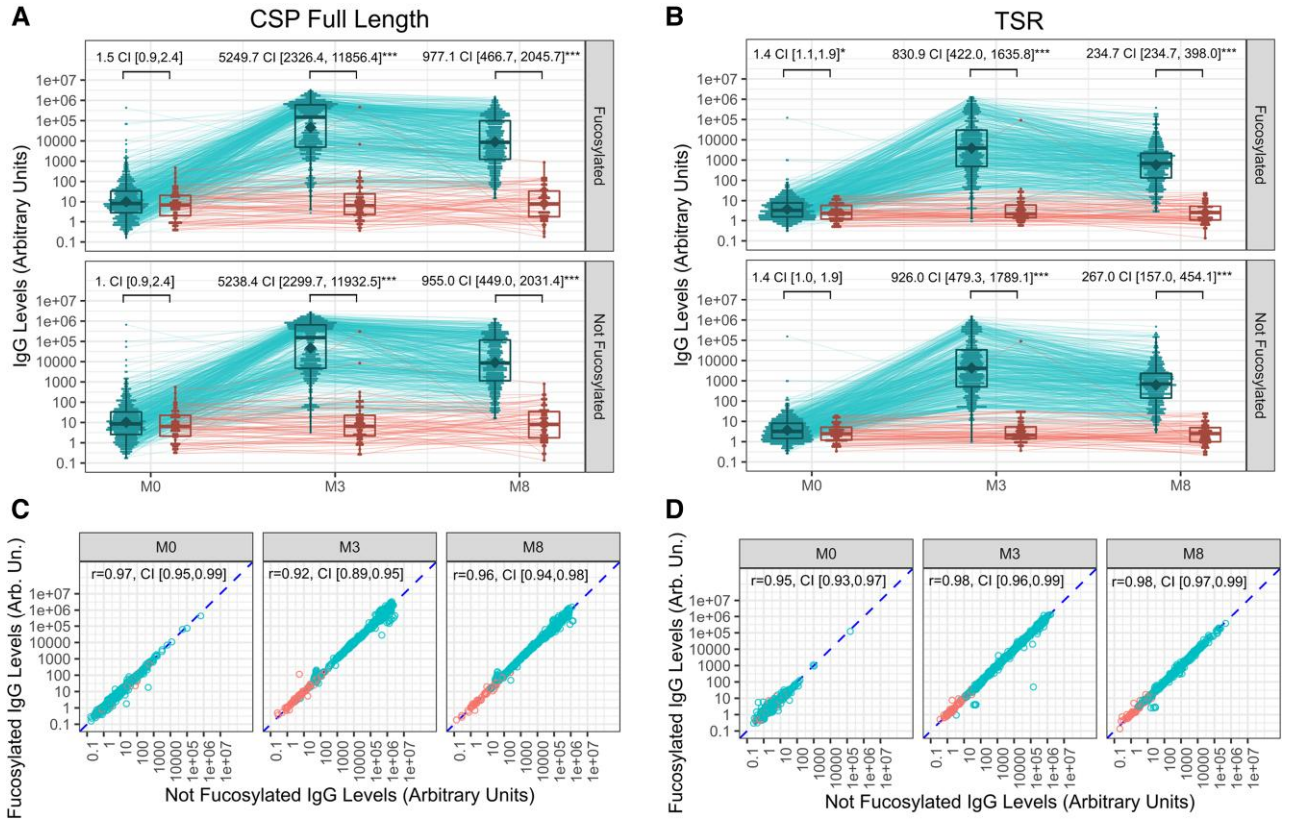

Figure 1. RTS,S/AS02A induces strong IgG responses that equally recognize fucosylated and nonfucosylated *Plasmodium falciparum* CSP and C-terminal TSR antigen constructs. *A* and *B,* Levels of IgG antibodies binding to fucosylated (y-axis) and nonfucosylated (x-axis) PfCSP full-length (*A*) and C-terminus TSR (*B*) at different time points (month 0, 3, and 8). Boxplots represent the geometric mean (diamond), median (central lines), quartiles (box limits), and estimated range (whiskers extending 1.5 times the interquartile range). IgG levels from the same individual across time points are linked with thin lines (longitudinal trajectories). Vaccination groups were compared through a 2-sample *t* test on the $\log_{10}$-transformed levels and statistical significance indicated by asterisks (*$P < .05$; **$P < .01$, ***$P < .001$). *C* and *D,* Scatter plots comparing IgG antibody levels binding to the fucosylated antigen (y-axis) versus those binding to the nonfucosylated equivalent (x-axis). The comparison is repeated for all time points (month 0, 3, and 8) for PfCSP full-length (*C*) and TSR (*D*). The Pearson correlation coefficient is computed across all observations. Abbreviations: AU, arbitrary unit; CI, 95% confidence interval; CSP, circumsporozoite protein; IgG, immunoglobulin G; PfCSP, *Plasmodium falciparum* circumsporozoite protein; TSR, thrombospondin type-1 repeat.

more clearly significant for anti-PfCSP IgG level increases from month 0 to month 8 for both full-length ($P = .0004$) and TSR ($P = .005$) (Table 1). Associations between IgG levels against nonfucosylated PfCSP full-length and TSR antigens with age, sex, and clinical malaria were identical to the results reported for fucosylated antigens (Supplementary Table 2).

To determine whether malaria endemicity could influence the binding of vaccine or naturally induced IgG to fucosylated versus nonfucosylated PfCSP antigens, another set of samples (n = 124) from Ilha Josina, a trial site of higher transmission intensity (cohort 2) [9], was also tested. Again, no difference was noted in recognition of fucosylated compared to nonfucosylated PfCSP antigens (Supplementary Figure 4), suggesting that the binding is independent of malaria exposure.

DISCUSSION

To our knowledge, no previous study has directly tested whether RTS,S vaccine-induced IgG antibodies bind less to a native-like fucosylated PfCSP (present on *P. falciparum* sporozoites) [7] than to nonfucosylated PfCSP (as present in the RTS,S vaccine). Of note, while our results show that RTS,S-induced antibodies recognize similarly fucosylated and nonfucosylated PfCSP constructs, we observed the same phenomenon in prevaccination and comparator postvaccination samples. This suggests that even IgG induced by natural exposure, presumably to fucosylated PfCSP on sporozoites, efficiently recognized the target antigen epitopes regardless of fucosylation.

Because our assay was multiplexed and tested the same sample for each individual against 2 slightly different PfCSP presentations, potential differences in the binding of any relatively important subpopulation of polyclonal IgG would have implied a divergence in the paired antigen-specific IgG levels, which was not observed.

Nevertheless, we cannot rule out a potential benefit of an alternative RTS,S vaccine containing fucosylated PfCSP. To establish this, fucosylated and nonfucosylated PfCSP-based

Table 1. Factors Affecting Increases in IgG Antibody Levels Reacting to Fucosylated PfCSP Constructs

	Month 3/Month 0		Month 8/Month 0	
Compared Conditions	Fold Difference (95% CI)	*P* Value	Fold Difference (95% CI)	*P* Value
Antigen PfCSP full length				
Increase ratio estimate	5079.1 (3833.1–6730.01)	<.0001***	829.2 (631.9–1088.2)	<.0001***
Old/young	0.65 (.34–1.25)	.20	0.61 (.32–1.14)	.12
Female/male	1.04 (.59–1.83)	.89	0.83 (.48–1.43)	.48
P. falciparum not infected/infected	1.96 (1.02–3.75)	.042*	3.07 (1.64–5.73)	.0004**
Antigen PfCSP TSR				
Increase ratio estimate	778.4 (625.7–968.3)	<.0001***	153.3 (128.0–183.5)	< .0001***
Old/young	0.56 (.34–.92)	.02*	0.7 (.46–1.06)	.09
Female/male	1.1 (.71–1.7)	.68	0.85 (.59–1.21)	.36
P. falciparum not infected/infected	1.39 (.84–2.31)	.2	1.8 (1.18–2.72)	.005*

Associations are reported as ratio estimates of month 3/month 0 or month 8/month 0 between groups of interest (compared conditions).

Abbreviations: CI, confidence interval; PfCSP, *Plasmodium falciparum* circumsporozoite protein; TSR, thrombospondin type-1 repeat.

*$P < .05$; **$P < .01$, ***$P < .001$.

vaccines should be tested head-to-head to compare overall immunogenicity (humoral and cellular responses) and vaccine efficacy. The concentration and quality of immunoglobulins might differ when induced by a fucosylated PfCSP, particularly IgG subclasses, their avidity, and their neutralizing and nonneutralizing functional capacities. It is also possible that a fucosylated PfCSP protein-based vaccine could lead to broader glycan-dependent T helper and cytotoxic cell responses, as it can be presented on both MHC-I and MHC-II molecules, and the resulting complex can be recognized by T cells. Recent advances in human immunodeficiency virus (HIV-1) [13] and hepatitis C virus (HCV) [14] vaccine development have been stimulated by a better understanding of the HIV-1 envelope spike and HCV E1 and E2 glycan composition and their interactions with the human immune responses. These advances highlight increasing evidence that glycans are important antigenic determinants in immune responses to various pathogens. Their relevance for prophylactic and therapeutic vaccine design and development is worth exploring in more depth.

Limitations of our study include the lack of data on IgG subclasses, which have different affinities for cellular Fc receptors, thus mediating different antiparasite effector functions, and functional antibody measures. In addition, we used PfCSP, recombinantly expressed in *P. pastoris* instead of native PfCSP, which might replicate better the complexity of the sporozoite surface. However, obtaining sufficient quantities of native PfCSP poses significant technical challenges and the potential divergent outcomes remain uncertain, considering our robust data that show a similar recognition of fucosylated and nonfucosyltated protein. In contrast, recombinant PfCSP can be readily overexpressed in *P. pastoris*, its production has been optimized, and it is well characterized [15], possibly mirroring native PfCSP conformation.

In conclusion, our data suggest that posttranslational modification by *O*-fucosylation in PfCSP, absent in the RTS,S vaccine, does not affect antibody-antigen binding both for IgG induced by vaccination (not fucosylated) and natural infections (fucosylated). This negative result does not weigh in favor of the hypothesis that a fucosylated PfCSP-based vaccine could lead to improved vaccine efficacy. However, this result alone is insufficient to rule it out and further experiments are required.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Author contributions. R. R. D., G. M., L. I., and C. D. contributed conceptualization. C. J., J. P. T.-Y., L. M. and M. V. performed experimental work. R. A., C. D., G. M. supervised laboratory work. D. M. and R. S. performed data curation and analysis. J. S. coordinated the clinical trial. C. J., D. M., R. A., G. M., L. I., and C. D. wrote the original draft. R. H.-G. A., K. R., D. L. N., B. L.-G., T. H., and R. R. D. performed antigen expression and modification. All authors critically read, revised, and approved the final manuscript.

Disclaimer. The content and interpretation are solely the responsibility of the investigators and do not necessarily represent the official views of the funders. GSK was provided with the opportunity to review a draft of this manuscript.

Data availability. The datasets generated and/or analyzed in the current investigation are available from the corresponding author upon reasonable request.

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Supplementary materials, methods and results from Article 1

Article 1

Jairoce C, Macià D, Torres-Yaguana JP, Mayer L, Vidal M, Santano R, Hurtado-Guerrero R, Reiter K, Narum DL, Lopez-Gutierrez B, Hamerly T, Sacarlal J, Aguilar R, Dinglasan RR, Moncunill G, Izquierdo L, Dobaño C. **RTS, S/AS02A malaria vaccine-induced IGG responses equally recognize native-like fucosylated and nonfucosylated plasmodium falciparum circumsporozoite proteins.** The Journal of Infectious Diseases. 2024 Mar 15;229(3):795-9.

Impact factor (2023): 5.0 Quartile: 1 Category: Infectious Disease

SUPPLEMENTARY MATERIAL

RTS,S/AS02A malaria vaccine-induced IgG responses equally recognize native-like fucosylated and non-fucosylated *Plasmodium falciparum* circumsporozoite proteins

Supplementary Materials and Methods

Antigens

Full-length PfCSP (PfCSP) was expressed and produced at Narum's lab using a *Pichia pastoris* expression system, as described [1,2]. Purified full-length PfCSP containing O-linked mannosylated and non-mannosylated forms were separated on a Jupiter 5 µm, 300Å, 250 x 10mm column (Phenomenex). Up to 20 mg of protein was injected per run with an elution gradient of A: water + 0.1% TFA (w/w) and B: Acetonitrile + 0.1% TFA (w/w). The mannosylated and non-mannosylated forms of full-length PfCSP eluted as two separate peaks, and the fractions were collected and fully characterised.

The TSR C-terminal domain of PfCSP was produced at Hurtado-Guerrero's lab, with huPoFUT2 obtained from James M. Rini's lab. TSR fucosylation reactions were incubated with 150mM NaCl, 20mM Tris pH7.4, 0.2µM huPoFUT, 0.05µM TSR-PfCSP and 150µM of GDP-Fucose (GDP-Fuc) for 24 h at 37ºC. PfCSP fucosylation reactions were performed with 150mM NaCl, 20mM Tris pH7.4, 0.2-0.4µM huPoFUT2, 0.5-1µM PfCSP and 500µM of GDP-Fuc at 37ºC for 24 h. Different time point samples were analysed by LC-MS using an Agilent 6550 Q-TOF mass spectrometer to monitor the reaction **(Figure S2).**

The PfCSP fragment, expressed in *P. pastoris*, is indicated underlined in the native PfCSP sequence (amino acids 73 to 383). The *O*-fucosylated Threonine residue is indicated in bold.

MMRKLAILSVSSFLFVEALFQEYQCYGSSSNTRVLNELNYDNAGTNLYNELEMNYYGKQENWY
SLKKNSRSLG<u>ENDDGNNEDNEKLRKPKHKKLKQPADGNPDPNANPNVDPNANPNVDPNANPN
VDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNA</u>

NPNANPNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNAN

PNANPNANPNANPNANPNANPNANPNKNNQGNGQGHNMPNDPNRNVDENANANSAVKNNN

NEEPSDKHIKEYLNKIQNSLSTEWSPCSV**T**CGNGIQVRIKPGSANKPKDELDYANDIEKKICKME

KCSSVFNVVNSSIGLIMVLSFLFLN

The TSR domain sequence, expressed in *Escherichia coli*, is shown below (the *O*-fucosylated Threonine residue is indicated in bold)

EPSDKHIKEYLNKIQNSLSTEWSPCSV**T**CGNGIQVRIKPGSANKPKDELDYANDIEKKICKMEKC

Below, underlined the TSR domain in the PfCSP protein (amino acids 310 to 374):

MMRKLAILSVSSFLFVEALFQEYQCYGSSSNTRVLNELNYDNAGTNLYNELEMNYYGKQENWY

SLKKNSRSLGENDDGNNEDNEKLRKPKHKKLKQPADGNPDPNANPNVDPNANPNVDPNANPN

VDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNA

NPNANPNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNAN

PNANPNANPNANPNANPNANPNANPNKNNQGNGQGHNMPNDPNRNVDENANANSAVKNNN

NEEPSDKHIKEYLNKIQNSLSTEWSPCSV**T**CGNGIQVRIKPGSANKPKDELDYANDIEKKICKME

KCSSVFNVVNSSIGLIMVLSFLFLN

Supplementary Results

TABLE S1 | Baseline characteristics of the study population

	Comparator N=64	**RTS,S N=644**	**p-value overall**
Sex:			0.398
Female	33 (51.6%)	291 (45.2%)	
Male	31 (48.4%)	353 (54.8%)	
Age group:			0.811
12<24 months	14 (21.9%)	155 (24.1%)	
24<60 months	50 (78.1%)	489 (75.9%)	
Height (cm) [median (IQR)]	87.0 [80.0;94.6]	88.0 [80.8;96.0]	0.687
Weight (Kg) [median (IQR)]	12.8 [11.0;14.5]	12.6 [10.8;14.7]	0.872
WHZ score [median (IQR)]	0.13 [-0.37;0.83]	-0.03 [-0.85;0.65]	0.186
Hematocrit (%)[median (IQR)]	34.2 [32.7;35.8]	34.3 [32.1;36.1]	0.873
Distance to the health centre (Km)	1.50 [1.01;2.30]	1.70 [1.04;2.42]	0.410

Abbreviations: IQR=Interquartile range; Kg=kilograms; cm=centimeters, Km= kilometre, WHZ= Weight-for-Height Z-score.

Table S2. Factors affecting increases in IgG antibody levels reacting to non-fucosylated PfCSP constructs. Associations are reported as ratio estimates of month (M)3/M0 or M8/M0 between groups of interest (compared conditions). Odds ratios are very close to those from Table 1 in the manuscript which are computed using fucosylated PfCSP constructs (their CI largely overlap). Additionally, we conducted a two-way repeated ANOVA using fucosylation and our condition of interest (rows in the table) to assess whether differences in the association were different between fucosylated or not fucosylated measures (test on the interaction term). All tests were not significant.

		M3/M0		M8/M0	
Antigen	**Compared conditions**	**Fold Difference**	**p-value**	**Fold Difference**	**p-value**
PfCSP full-length	Increase Ratio Estimate	4910.0 CI [4003.8, 6089]	<0.0001***	796.3 CI [604.6, 1048.7]	<0.0001***
	Old/Young	0.66 CI [0.36, 1.21]	0.17	0.61 CI [0.32, 1.15]	0.12
	Female/Male	1.13 CI [0.66, 1.92]	0.65	0.9 CI [0.52, 1.57]	0.50
	Pf not infected/infected	1.6 CI [0.85, 3.02]	0.14	3.56 CI [1.84, 6.89]	0.00015**
PfCSP TSR	Increase Ratio Estimate	831.0 CI [668.2, 1030.6]	<0.0001***	169.3 CI [137.7, 207.76]	< 0.0001***
	Old/Young	0.61 CI [0.32, 1.15]	0.089	0.65 CI [0.4, 1.05]	0.07
	Female/Male	1.13 CI [0.66, 1.92]	0.65	0.9 CI [0.52, 1.57]	0.71
	Pf not infected/infected	1.23 CI [0.69, 2.19]	0.47	2.42 CI [1.48, 3.97]	0.0004**

Abbreviations: M0, month 0; M3, month 3; M8, month 8, PfCSP, *Plasmodium falciparum* Circumsporozoite protein; TSR,Thrombospondin type-1 repeat; Pf, *Plasmodium falciparum*.
*p<0.05; **p<0.01, ***p<0.001.

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Figure S1. Flowchart of the study population

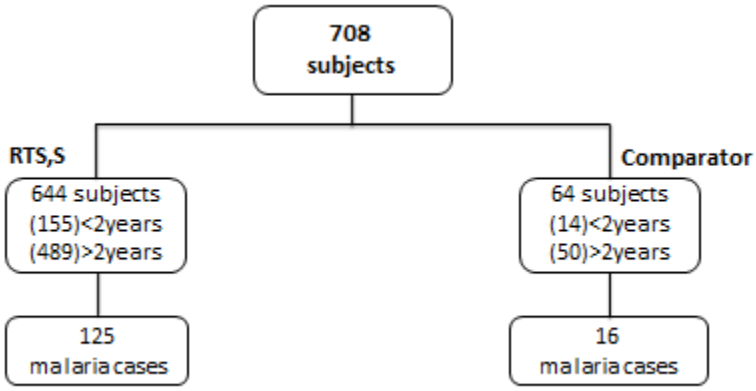
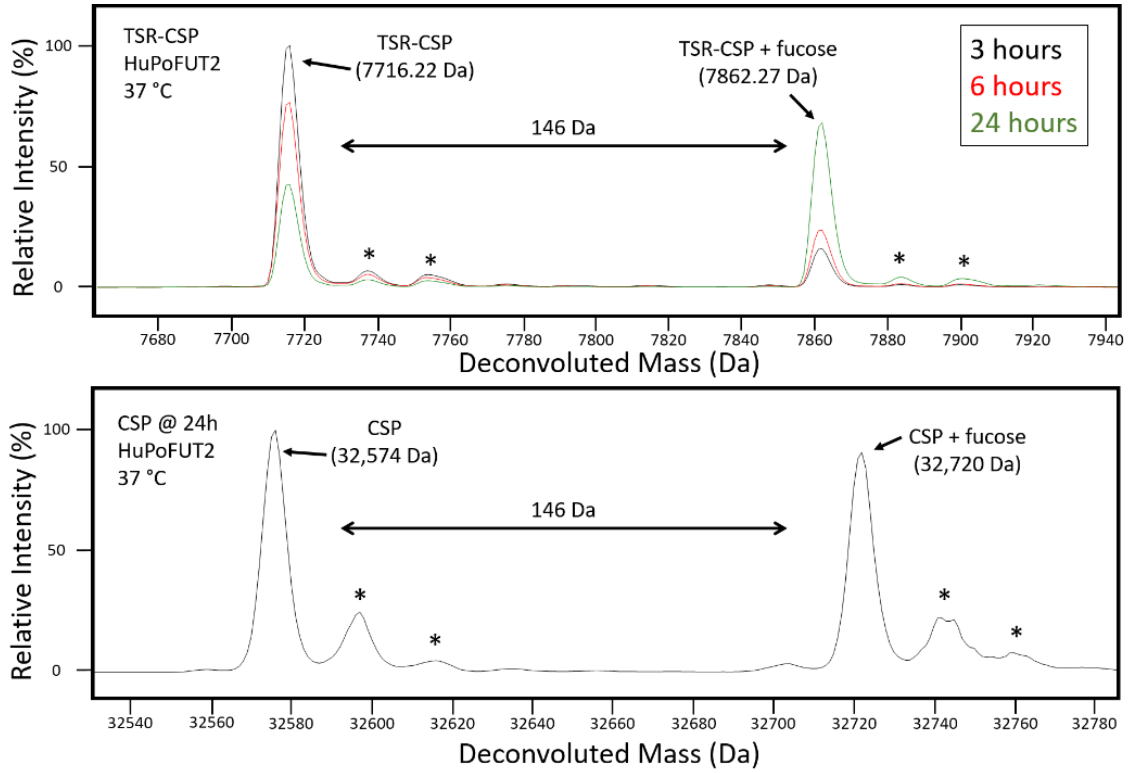
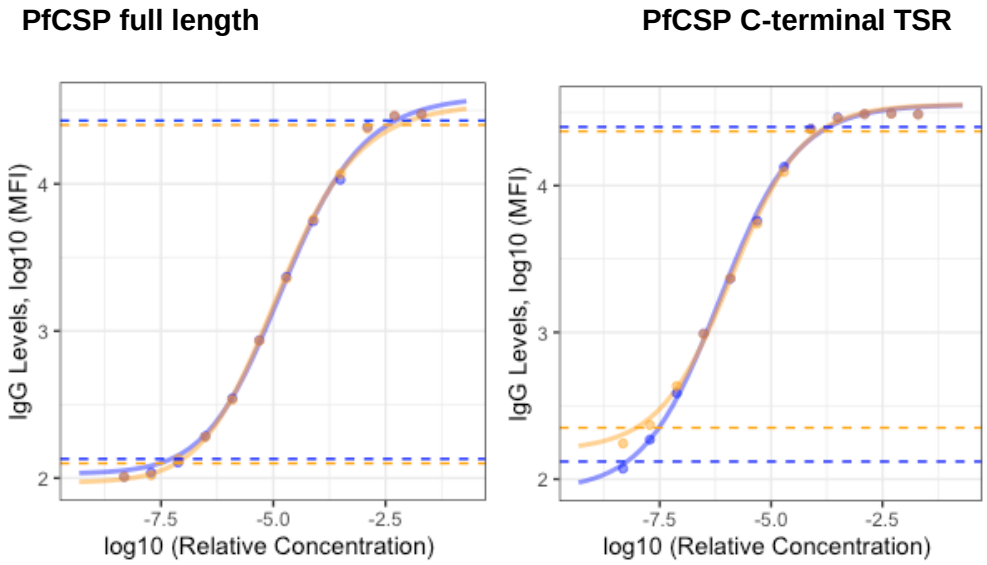


Figure S2. PfCSP TSR and full-length fucosylation by human protein *O*-fucosyltransferase 2 (huPoFUT2).



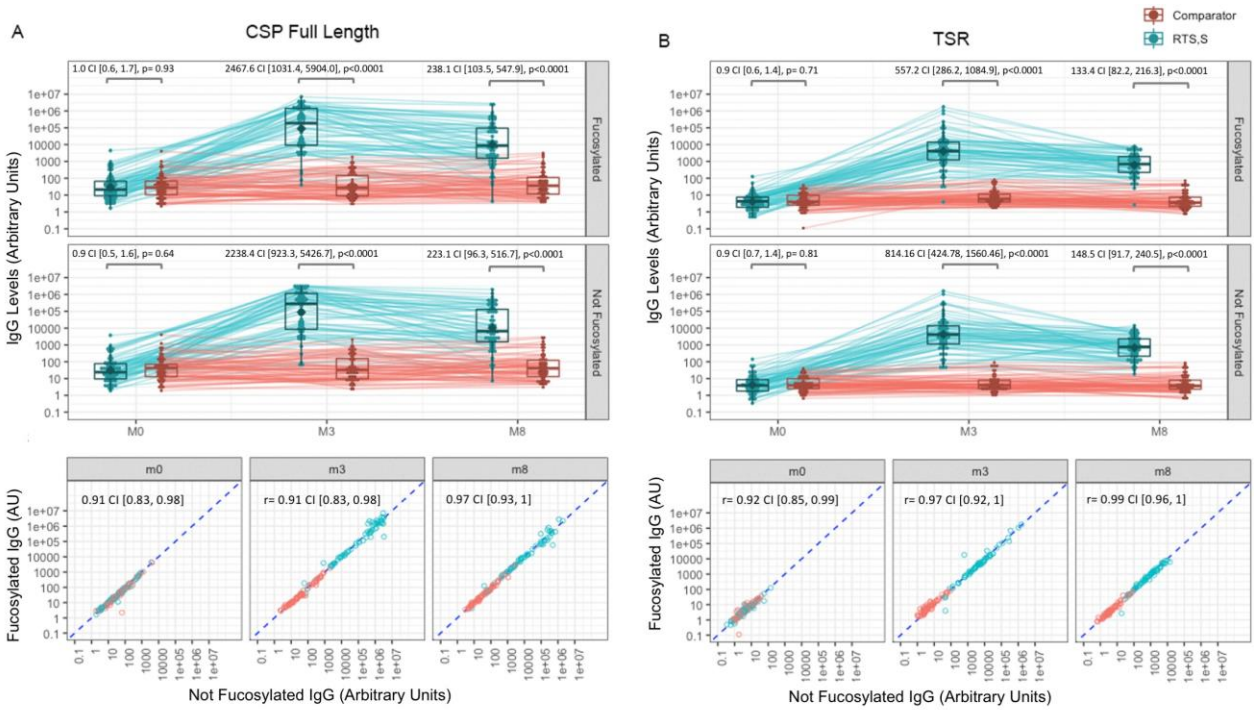
Deconvoluted mass spectra showing non-fucosylated and fucosylated TSR domain from PfCSP (TSR-CSP) (Top panel) and full-length PfCSP (Bottom panel). Reactions were performed using huPoFUT2 at 37 °C in the presence of GDP-fucose and analysed by LC-MS using an Agilent 6550 Q-TOF mass spectrometer. In both the TSR-PfCSP and PfCSP full length, a mass shift of 146 Daltons (Da) was observed, correlating to the addition of a fucose residue. For the TSR-PfCSP, three time points were taken over 24 hours, with an increase in the fucosylated protein signal seen as time progressed. The high resolving power of the Q-TOF mass spectrometer enables the differentiation of protein species with 12C (major peaks with labels) from those with a small amount of naturally occurring 13C (denoted with a *).

Figure S3. Comparison of IgG antibody Luminex calibration curves.



Calibration curves for a given plate were done using a positive control sample which has been increasingly diluted to cover the whole range of quantification. We used 5 parameter logistic curves to fit the sigmoid trajectories. Lower asymptotes were constrained to correspond to measurements from blank spots for each plate. Only the displacement parameter in the logistic curve was allowed to differ to account for plate variability, but we kept the other parameters fixed for each analyte to avoid overfitting. The top and bottom limits of quantification were determined based on a minimum median fluorescence intensity (MFI) signal fold-change response of 0.2 for a unit fold-change in IgG level. That is 20% of what would be expected if linearity existed (slope of 1 in the log-log scale). Beyond these top and bottom limits, where the curve is too flat for a reliable quantification, no further modelling of the flattening curve was conducted. When measurements happened to occur there, we transformed MFI measurements into levels based on the minimum slope at the quantification limits. Yellow colours correspond to fucosylated antigens and blue to non-fucosylated.

Figure S4. RTS,S/AS02A IgG responses to fucosylated and non-fucosylated *Plasmodium falciparum* CSP and C-terminal TSR antigen constructs in Ilha Josina.



RTS,S/AS02A induces strong IgG responses that equally recognize fucosylated and non-fucosylated *Plasmodium falciparum* CSP and C-terminal TSR antigen constructs in a high malaria transmission intensity site. Panels A and B show levels of IgG antibodies binding to fucosylated (y-axis) and non-fucosylated (x-axis) PfCSP full-length (panel A) and C-terminus TSR (panel B) at different time points (Month [M]0, M3, and M8). Boxplots represent the geometric mean (diamond), median (central lines), quartiles (box limits) and estimated range (whiskers extending 1.5 times the interquartile range). IgG levels from the same individual across time points are linked with thin lines (longitudinal trajectories). Groups were compared through a two-sample t-test on the $\log_{10}$-transformed levels. Panels C and D show scatter plots comparing IgG antibody levels binding to the fucosylated antigen (y-axis) vs. those binding to the non-fucosylated equivalent (x-axis). The comparison is repeated for all time points (M0, M3 and M8) for PfCSP full-length (panel C) and TSR (panel D). Observations are colored according to the vaccination groups. The

Pearson correlation coefficient is computed across all observations. PfCSP, *Plasmodium falciparum* Circumsporozoite protein; TSR, Thrombospondin type-1 repeat.

Article 2

This article addresses the second objective of characterizing the magnitude, type and longevity of antibody responses to RTS,S vaccine antigens and vaccine-unrelated antigens after primary and booster vaccination. The results of this work have been published in the following peer-reviewed journal article:

Sanchez L, Vidal M, **Jairoce C,** Aguilar R, Ubillos I, Cuamba I, Nhabomba AJ, Williams NA, Díez-Padrisa, N, Cavanagh D, Angov E, Coppel RL, Gaur D, Beeson JG, Dutta S, Aide P, Campo JJ, Moncunill G, Dobaño, C. **Antibody responses to the RTS, S/AS01E vaccine and Plasmodium falciparum antigens after a booster dose within the phase 3 trial in Mozambique.** NPJ Vaccines. 2020;5(1).
Impact factor (2020): 7.344. Quartile: 1. Category: Immunology

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ARTICLE OPEN

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Antibody responses to the RTS,S/AS01$_E$ vaccine and *Plasmodium falciparum* antigens after a booster dose within the phase 3 trial in Mozambique

Lina Sánchez[1,2], Marta Vidal[1], Chenjerai Jairoce[1,3], Ruth Aguilar[1], Itziar Ubillos[1], Inocencia Cuamba[3], Augusto J. Nhabomba[3], Nana Aba Williams[1], Núria Díez-Padrisa[1], David Cavanagh[4], Evelina Angov[5], Ross L. Coppel[6], Deepak Gaur[7,8], James G. Beeson[9], Sheetij Dutta[5], Pedro Aide[3], Joseph J. Campo[1,3], Gemma Moncunill[1,3,10]✉ and Carlota Dobaño[1,3,10]✉

The RTS,S/AS01$_E$ vaccine has shown consistent but partial vaccine efficacy in a pediatric phase 3 clinical trial using a 3-dose immunization schedule. A fourth-dose 18 months after the primary vaccination was shown to restore the waning efficacy. However, only total IgG against the immunodominant malaria vaccine epitope has been analyzed following the booster. To better characterize the magnitude, nature, and longevity of the immune response to the booster, we measured levels of total IgM, IgG, and IgG$_{1-4}$ subclasses against three constructs of the circumsporozoite protein (CSP) and the hepatitis B surface antigen (HBsAg, also present in RTS,S) by quantitative suspension array technology in 50 subjects in the phase 3 trial in Manhiça, Mozambique. To explore the impact of vaccination on naturally acquired immune responses, we measured antibodies to *P. falciparum* antigens not included in RTS,S. We found increased IgG, IgG1, IgG3 and IgG4, but not IgG2 nor IgM, levels against vaccine antigens 1 month after the fourth dose. Overall, antibody responses to the booster dose were lower than the initial peak response to primary immunization and children had higher IgG and IgG1 levels than infants. Higher anti-Rh5 IgG and IgG$_{1-4}$ levels were detected after the booster dose, suggesting that RTS,S partial protection could increase some blood stage antibody responses. Our work shows that the response to the RTS,S/AS01$_E$ booster dose is different from the primary vaccine immune response and highlights the dynamic changes in subclass antibody patterns upon the vaccine booster and with acquisition of adaptive immunity to malaria.

npj Vaccines (2020)5:46; https://doi.org/10.1038/s41541-020-0192-7

INTRODUCTION

Despite the great reduction in malaria cases in the last 15 years, thanks to the combination of multiple control measures, it is estimated that 219 million malaria cases and 435,000 deaths occurred in 2017, mostly associated with *Plasmodium falciparum*[1]. Importantly, 90% of these deaths concentrated in sub-Saharan Africa and a large proportion occurred in children under 5 years. Owing to the concerning rise of parasite resistance to antimalarial drugs and vector resistance to insecticides[1,2] and stalling progress in reducing malaria since 2016[1,2], integration of a malaria vaccine with other preventive measures will be a useful addition to control disease burden in the future.

Currently, the pre-erythrocytic RTS,S/AS01$_E$ vaccine is the most advanced, having shown consistent but partial vaccine efficacy (VE) that wanes over time and is less effective in infants compared to children[3]. RTS,S/AS01E contains a fusion protein including the central tandem repeat (NANP) and the C-terminal (C-term) regions of the *P. falciparum* circumsporozoite protein (CSP), and the hepatitis B virus surface antigen (HBsAg). It is expressed together with HBsAg, and injected in combination with the AS01 adjuvant system[4]. The vaccine was tested in a phase 3 clinical trial of a 3-dose immunization schedule (month [M] 0, M1 and M2) with a fourth dose 18 months after primary vaccination (M20)[3], with the booster dose partly restoring the waning VE. Specifically, VE for the 3-dose immunization schedule was 35.2% in children and 20.3% in infants up to M32 of the study, but VE waned over time with a VE of 16.1 and 7.6%, respectively, when considering only the period from M20 to M32. In children and infants who received the booster dose, waning VE was restored to overall levels of 43.9 and 27.8%, respectively[3]. In order to understand why protection offered by RTS,S is suboptimal and continue efforts to improve it, there is a need to decipher the mechanisms of protection elicited by the vaccine. It has been shown that antibody levels are involved in the vaccine-induced immunity, but they do not fully explain the protective effect of the vaccine[5,6]. Thus far, the study of antibody response in trials performed in endemic areas has been largely focused on IgG levels against the NANP repeat region of CSP, with the exception of our previous work assessing more generally subclass responses to NANP and to other antigens after primary vaccination in the phase 3 trial[7–9].

Characterizing responses by other antibody isotypes, subclasses, and responses to different epitopes may provide in depth understanding of the immune response to the vaccine and the mode of action. Antibody levels are not the sole means to determine vaccine mechanisms of action. Characteristics like the balance between isotypes or subclasses of the antibodies are

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important because of their varying effector functions[10]. For instance, some IgG subclasses act as cytophilic while others have non-cytophilic functions[10], influencing the roles of Fc-mediated functions such as complement fixation and phagocytosis[11]. Determining which type of response is detrimental or beneficial could further inform which responses could be modified to enhance the efficacy of the vaccine.

The epitope specificity of the antibody response is also relevant. There is clear evidence that NANP is related to VE[6] but other regions could also mediate protection. Avidity of IgG to the CSP C-term has been associated with protection in African children[12], and C-term and not the NANP-repeat-specific antibodies have been reported to be the main mediators of phagocytic activity in naive adults[13]. Furthermore, antibodies to both C-term and NANP-repeat can mediate complement fixation in children, suggesting both regions are important for functional activity[14,15].

Additionally, studying the response to *P. falciparum* blood stage antigens not present in the vaccine is relevant to determine the effect of the vaccine on naturally acquired immunity (NAI), developed from continuous parasite exposure. It has been hypothesized that vaccination could (1) decrease NAI by reducing the exposure to the parasite, which could mean individuals are left vulnerable in the long term due to the waning efficacy of the vaccine[3], as predicted for other malaria prevention tools[16], or (2) increase NAI by allowing subclinical exposure to the parasite due to the partial efficacy of the vaccine[9,17].

Here, we used samples from the phase 3 trial at the time of the booster dose (M20) and onwards from a subgroup of subjects in Manhiça, Mozambique, to characterize the effect of the RTS,S/AS01$_E$ booster dose on different antibody responses. We evaluated total IgM, IgG and IgG$_{1\text{-}4}$ subclasses to vaccine and vaccine-unrelated *P. falciparum* blood stage antigens. Data were combined with those from the primary vaccine response previously assessed[7–9] to display the kinetics from baseline (M0) until M32.

RESULTS

Short- and long-term booster immunogenicity

The RTS,S/AS01$_E$ booster dose increased IgG, IgG1, IgG3, and IgG4 levels against all vaccine antigens 1 month (M21) after its administration (M20), but it did not increase IgG2 nor IgM levels (Figs. 1 and 2; and Supplementary Table 1). The increase in antibody levels was significant both when comparing the levels pre-boost at M20 and M21 of the same individual, and when comparing the levels at post-boost M21 of the RTS,S booster group (R3R) to those of the individuals who did not receive a booster (R3C), except for IgG3 CSP NANP and IgG3 CSP full length (FL) for the latter comparison. At M21, the highest levels were against FL CSP, followed by the CSP NANP region, the CSP C-term and HBsAg. The predominant subclass was IgG1 followed by IgG3, then lower levels of IgG2 and least for IgG4.

Longer-term immunogenicity was measured 1 year after the administration of the booster (M32). IgG and IgG1 (but not IgG3) levels against vaccine antigens in the R3R group remained above the R3C group, except for IgG1 NANP (Figs. 1 and 2; and Supplementary Table 1). Similar to the pattern at M21, IgG2 and IgM levels were not higher in R3R at M32. For IgG4, levels were significantly higher in R3R compared to R3C only for CSP C-term and HBsAg. In comparison to the group that did not receive any RTS,S dose (C3C), the R3R and R3C groups levels at M32 remained higher for most antigens and IgG subclasses, except HBsAg IgG2 and IgG3, and NANP IgG3.

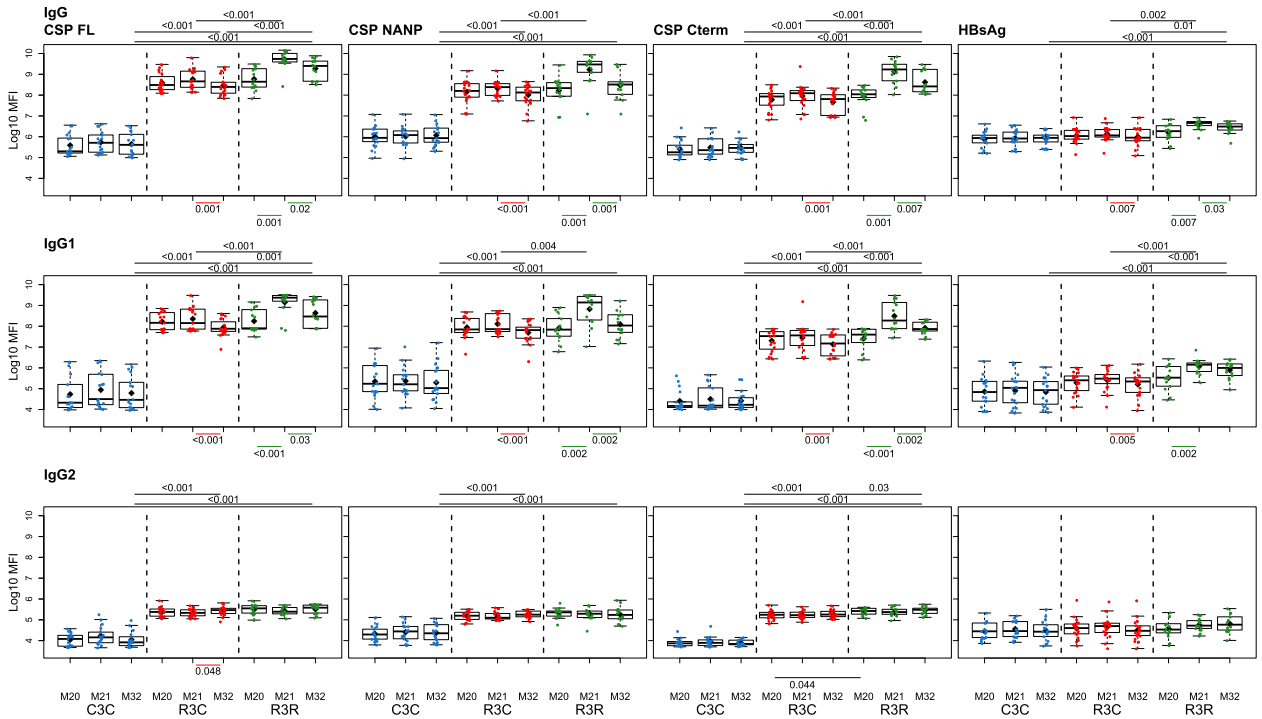

Fig. 1 RTS,S/AS01$_E$ booster and long-term immunogenicity against vaccine antigens: total IgG, IgG1-2 subclasses for CSP constructs and HBsAg at month (M) 20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and log$_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs. M21) and the long-term immunogenicity (M21 vs. M32), as well as to compare the R3C and R3R groups at each timepoint. Only *p*-values < 0.05 after adjustment for multiple testing are shown. The *y*-axis is in logarithm 10 scale. R3R (green): three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C (red): three doses of RTS,S/AS01$_E$ and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.

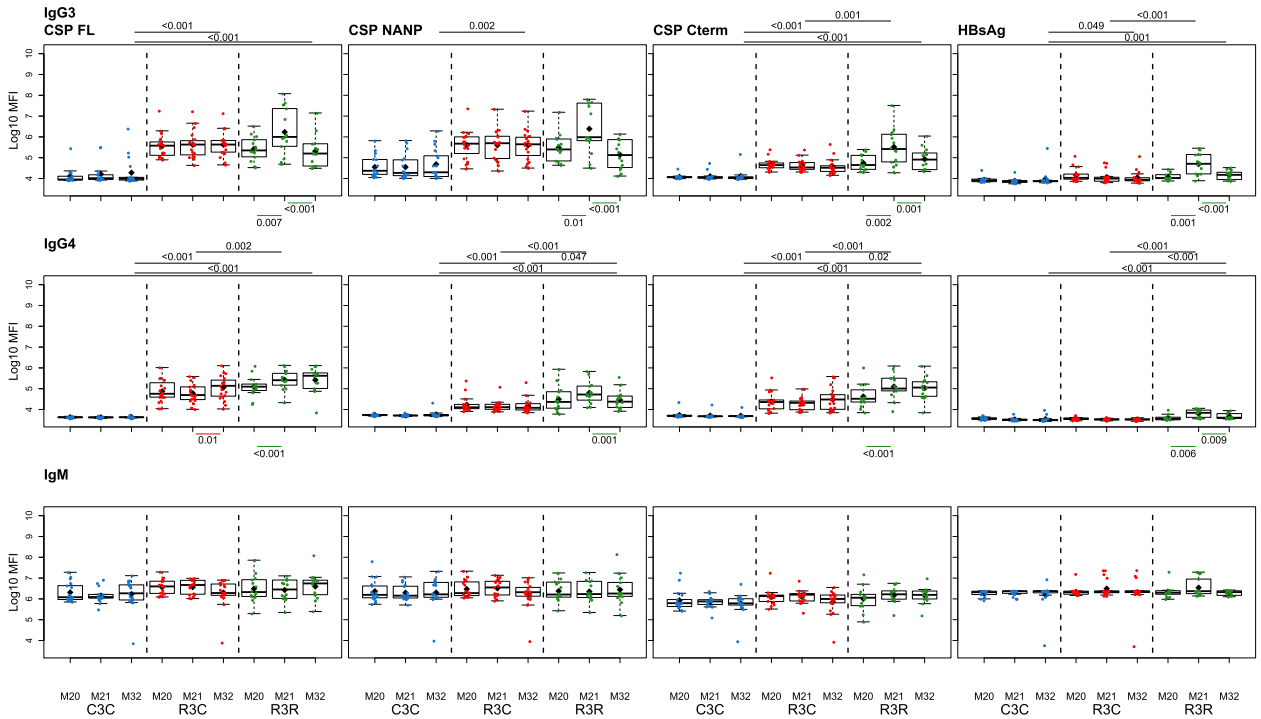

Fig. 2 RTS,S/AS01$_E$ booster and long-term immunogenicity against vaccine antigens: IgG3-4 subclasses and IgM for CSP constructs and HBsAg at month (M) 20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs. M21) and the long-term immunogenicity (M21 vs. M32), as well as to compare the R3C and R3R groups at each timepoint. Only *p*-values < 0.05 after adjustment for multiple testing are shown. The *y*-axis is in logarithm 10 scale. R3R (green): three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C (red): three doses of RTS,S/AS01E and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.

Antibody kinetics through the entire study follow-up

When comparing the booster response (M21) to the primary vaccination response (M3), the group that received the booster had a lower peak in IgG, IgG1 and IgG3 levels after the booster than after primary vaccination (Figs. 3 and 4; and Supplementary Figs. 1 and 2). In contrast, IgG4 levels against CSP constructs showed higher levels after the booster dose than after primary vaccination, and overall levels increased in time. The opposite happened with HBsAg where IgG4 levels decreased with time and were higher at M3 than at M21. Although primary vaccination increased IgG2 and IgM levels, the booster dose did not increase them.

The decrease in anti-CSP levels from primary vaccination to M20 was larger for IgG3 than for IgG and IgG1; the mean decreases for anti-NANP IgG and IgG1 was around 1.5 $\log_{10}$ median fluorescent intensity (MFI) while for IgG3 it was around 2.5 $\log_{10}$MFI. IgG2 levels remained more stable after the primary vaccination and did not increase or decrease vastly after M3. Remarkably, IgG4 levels against CSP FL and C-term at M20 were slightly higher than levels at M3 although this effect was not observed in the C3C group.

The levels of IgG and IgG$_{1\text{-}4}$ to CSP in RTS,S/AS01$_E$ vaccination groups (R3), with or without booster, were higher for most post-vaccination time points than the levels in the comparator group. IgM levels were higher only at M3 in the R3 groups compared to the C3C group.

Factors affecting immunogenicity

Age. Children who received RTS,S/AS01$_E$ either with or without a booster had higher IgG and IgG1 levels against CSP antigens than infants throughout the study period (Supplementary Figs. 1–6 and Supplementary Table 2). Without booster, IgG3 levels to NANP and FL CSP, but not C-term, were higher in children than infants. In contrast, we did not detect differences in IgG3 levels between age groups after booster immunization. Most of the differences observed were not statistically significant but there were consistent patterns, e.g., for the same isotype/subclass and antigen, levels were lower in infants than children and all comparisons were $p < 0.05$ before adjustment for multiple testing. We did not detect a significant influence of age on IgG2, IgG4, or IgM levels in any group after the booster, except for NANP IgG4 levels in the R3R group that were higher in children. Likewise, we did not detect significant differences in antibody levels against HBsAg between age groups.

Malaria episodes. We compared the antibody levels at M20, M21 and M32 in individuals who had either presented or not with clinical malaria before M20 (Figs. 5–7 and Supplementary Table 3). None of the comparisons were statistically significant after adjusting for multiple testing. At M20 we did not detect any significant difference between individuals who presented or not with prior clinical malaria in the RTS,S vaccinees. In the R3R group at M21 there was a pattern for lower anti-CSP FL and anti-C-term IgG, IgG1, IgG3, IgG4 and IgM levels, and anti-NANP IgG4 levels ($p < 0.05$ before adjustment) in individuals who had clinical malaria. For the R3C group at M21, individuals who presented with clinical malaria before M20 had lower anti-CSP IgG and IgG1 mean levels against CSP antigens, and lower IgG3 levels against CSP FL ($p < 0.05$ before adjustment). In contrast, IgM levels were higher in plasma from previous malaria cases but this was not statistically significant. In the C3C group, IgG and IgG$_{1\text{-}3}$ to FL CSP and NANP were higher in the subjects with previous malaria cases but this difference was not statistically significant after adjusting for multiple testing. Lower levels of IgG, IgG1, and IgG3 to HBsAg were also observed in R3C at M20, M21, and M32 in the previous

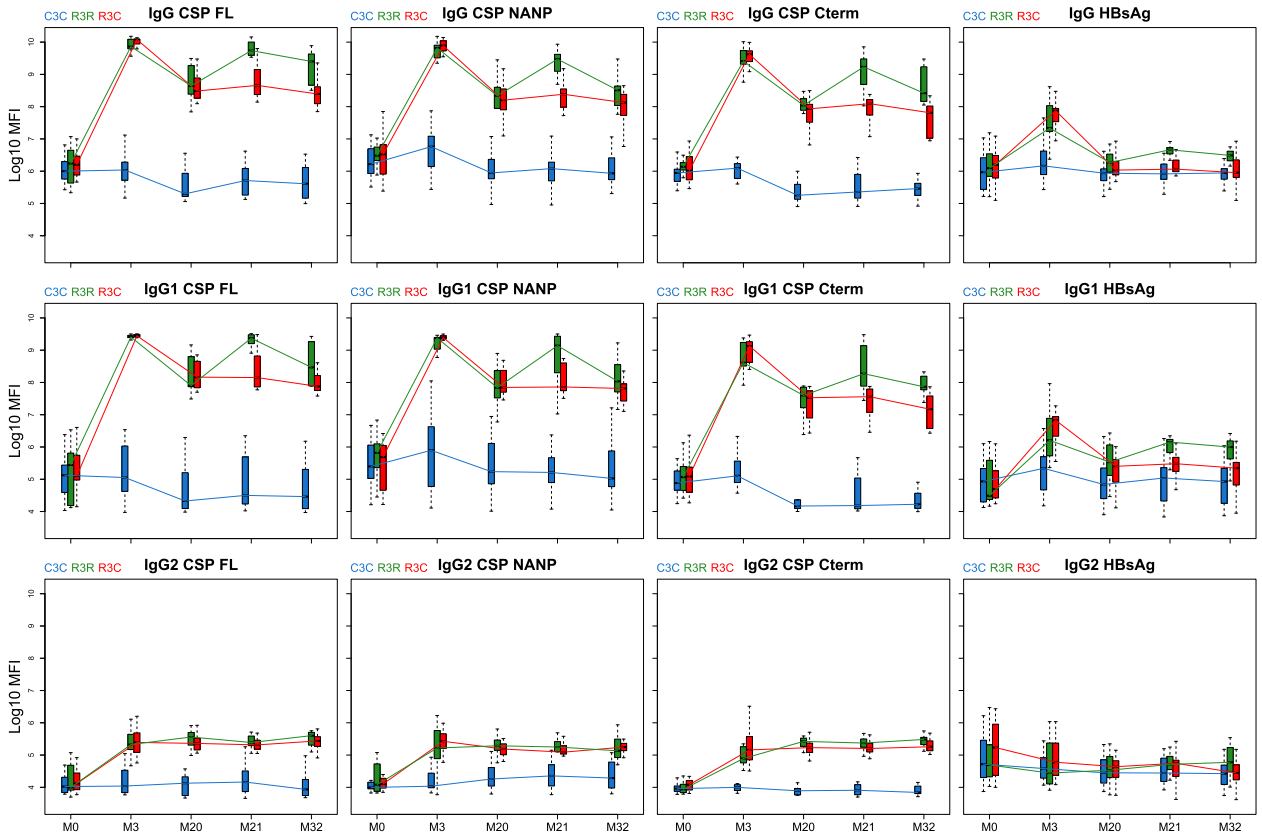

Fig. 3 Antibody responses against vaccine antigens for months (M) 0, 3, 20, 21, and 32 for IgG, IgG1, and IgG2. Boxplots with median, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, and lower whisker as the largest between minimum × value and Q1 − 1.5*IQR. The *y*-axis is in logarithm 10 scale. Data from months 0 and 3 were obtained from a previous study in the same individuals[7], thus a batch effect might be present. R3R (green): three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster at month 20. R3C (red): three doses of RTS,S/AS01$_E$ and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.

malaria cases (Supplementary Fig. 7), but these differences were not statistically significant.

The study was not designed to assess associations with future malaria risk but we had some provisional findings. IgG2 and IgG3 to vaccine antigens in the R3R group at M21 were higher in those subjects with subsequent clinical malaria, but this difference was not statistically significant. Only for anti-NANP IgG2 and anti-HBsAg IgG2 and IgG3 levels, differences had $p < 0.05$ before adjusting for multiple testing (Figs. 8–11 and Supplementary Table 3). In contrast, IgG1 and IgG4 levels to NANP and CSP FL in the R3R group at M21 were lower in malaria cases, but not significantly. An opposite pattern consisting of higher levels in malaria cases was observed in the R3C group. Fold-change in IgM levels against CSP constructs from M20 to M21 in the R3R group was lower in malaria cases ($p < 0.05$ before adjustment) (Supplementary Figs. 8 and 9). This contrasted to what was observed in the R3C group who had higher fold-change in IgM levels in malaria cases. Additionally, the fold-change in anti-HBsAg IgG3 levels was higher in malaria cases ($p < 0.05$ before adjustment). In most cases, there was no statistically significant difference between subjects presenting with clinical malaria after M21 and those who did not.

Effect of RTS,S booster vaccination on antibodies to blood stage antigens

For most of the blood stage antigens we studied, we could not detect differences in antibody levels before and after the booster dose, nor when comparing the R3R, R3C, and C3C groups. There were some differences ($p < 0.05$ before adjustment) in antibody levels at M3 and/or M21 for MSP5, MSP1$_{42}$, MSP1-BL2, Rh4.2, EBA140 and EBA175 (Supplementary Figs. 10–15 and Supplementary Table 4). Interestingly, Rh5 antibodies showed a consistent change in levels after the RTS,S booster for IgG and all IgG subclasses, with higher levels in the R3R group (Figs. 12 and 13; Supplementary Fig. 16 and Supplementary Table 4). In the case of IgG, IgG1 and IgG2 the differences were significant both in the short (M21) and long (M32) term, while for IgG3 and IgG4 differences were only at M21 with $p < 0.05$ before adjustment. Curiously, overall levels diminished over follow-up with the exception of IgG4.

Age did not have a significant effect on the antibody levels against the studied blood stage antigens (Supplementary Figs. 16–23 and Supplementary Table 5). Individuals who were classified as having had a case of clinical malaria before M20 tended to have higher levels of antibody to blood stage antigens at M20-32 but this difference was not statistically significant (Supplementary Figs. 24–30 and Supplementary Table 6). The most remarkable difference was the levels of MSP1$_{42}$ that acted as a marker for malaria exposure, showing higher levels in those who had clinical malaria, in particular for IgG, IgG1 and IgG2. Overall, the responses showed a general pattern of higher levels at all time points for all vaccination groups in individuals who subsequently presented with a malaria case but it was not significant (Supplementary Figs. 31–37 and Supplementary Table 6).

DISCUSSION

This study confirms that the RTS,S/AS01$_E$ booster dose increases total IgG levels against vaccine antigens and elucidates its

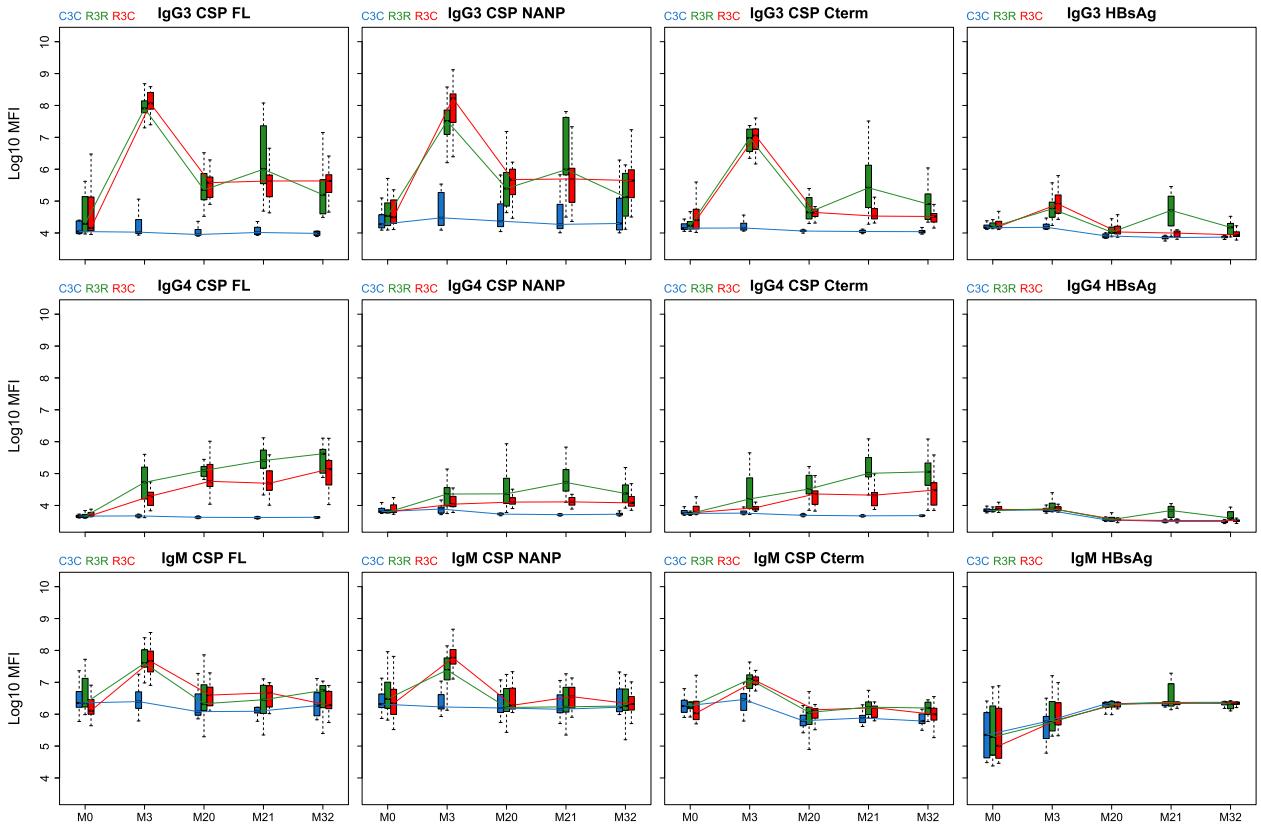

Fig. 4 Antibody responses against vaccine antigens for months (M) 0, 3, 20, 21, and 32 for IgG3, IgG4 and IgM. Boxplots with median, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, and lower whisker as the largest between minimum × value and Q1 − 1.5*IQR. The *y*-axis is in logarithm 10 scale. Data from months 0 and 3 were obtained from a previous study in the same individuals [7], thus a batch effect might be present. R3R (green): three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster at month 20. R3C (red): three doses of RTS,S/AS01$_E$ and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.

differing effect on IgG subclasses and IgM not previously studied. We describe for the first time the long-term RTS,S/AS01$_E$ antibody response to different antigens and CSP epitopes. The booster dose increased total IgG, IgG1, IgG3, and IgG4 for all vaccine antigens compared to pre-booster levels, and they remained above the levels of non-vaccinated individuals during the entire follow-up period. Remarkably, the fourth dose did not induce an increase in IgG2 levels (although the primary vaccination did) but it increased IgG1 and IgG3 levels, which may explain how the booster led to higher efficacy overall. IgG1 and IgG3 can effectively fix complement and promote interactions with Fcγ-receptors on phagocytes[10], which could be contributing to RTS,S-induced protection. IgG2 and IgG4, on the contrary, are non-cytophilic antibodies unable to fix complement and to interact with Fcγ-receptors[10].

The profile of antibody responses seems to be epitope-specific. Previously, the bulk of studies had only evaluated NANP antibodies and have provided clear evidence that NANP antibodies are associated with protection[6], being the established immunodominant region of the vaccine antigen[4]. However, there is evidence that antibodies against C-term are involved in phagocytic activity in US naive adults[13], and RTS,S vaccine-induced antibodies to the C-term among children can promote complement fixation[14]. Also, in our previous work we have found that post-primary vaccination, the avidity of the IgG response to CSP C-term was associated with protection[12]. Here, we show that the booster dose increases levels of antibodies against both NANP and the C-term, and that the responses against these two regions may behave differently. Antibody levels to NANP were higher compared to C-term, but the proportional increase 1 month after the booster dose was not different.

Previously it was reported that the IgG levels to NANP were increased by the booster dose, but the peak post-booster levels were lower than following primary vaccination[6]. In this study, we found similar results for IgG subclasses—there was boosting but levels overall were lower than following primary vaccination[7]. Remarkably, IgG4 levels against CSP kept increasing with time. The RTS,S pattern differs to other vaccines in which the peak for the booster response is higher than the peak for the primary vaccination[18]. The unusual response to the booster dose could be caused by different factors. These factors include the response to primary vaccination[19], but also the booster dose or the primary vaccination schedule[20,21]. For instance, it has been reported that for some vaccines high residual levels of vaccine antibodies have a negative effect on the post-booster response[19]; however, our study did not find evidence for a negative correlation between M20 and M21 antibody levels (Supplementary Figs. 38 and 39). There is also evidence from the response to a Meningococcal conjugated vaccine that primary vaccination administered with a short interval doses might lead to higher antibodies at the primary vaccination peak, but a higher number of doses lead to a lower post-booster response[20]. The effect of the dosing interval on the responses to the RTS,S vaccine was observed on a phase 2 trial that compared a 0, 1, 2 months vs. 0, 1, 7 months schedule and showed that the highest peak was observed following the 0, 1, 2 month schedule[21]. Also, the booster dose might induce different IgG subclass patterns to primary vaccination because it is acting on immune memory cells such as B memory cells and it might be inducing class switch and increasing antibody affinity[19,22].

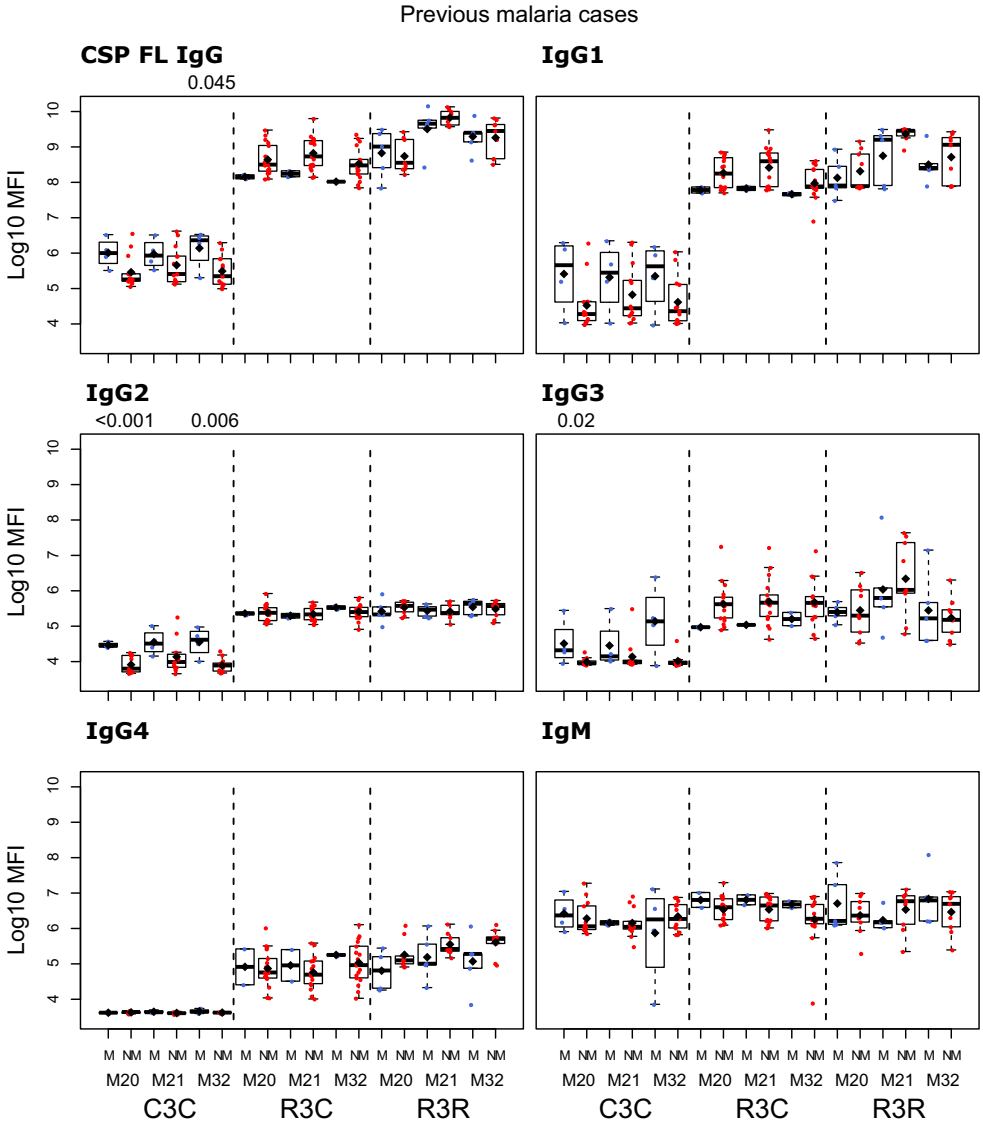

Fig. 5 Immunogenicity for CSP FL stratified by previous clinical malaria: total IgG, IgG1-4 subclasses and IgM at month (M)20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M = blue) and subjects without malaria (NM = red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs. NM). *p*-values were adjusted for multiple comparisons, but none was significant. Only *p*-values < 0.05 before adjustment are shown. The *y*-axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C: three doses of RTS,S/AS01$_E$ and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

The AS01$_E$ adjuvant and the innate response and cytokine milieu that it elicits likely affect the evolution of antibody subclass patterns observed. In addition, it may be influenced by differences in immune development due to older age and environmental exposures[19]. For instance, it has been reported that malaria transmission affects the antibody subclasses patterns in subjects who had malaria episodes[23]. Nevertheless, it is important to note that because primary and booster antibody data were not analyzed at the same time, the comparison of antibodies between time points in our study should be interpreted carefully.

We are reporting for the first time the antibody booster response against HBsAg. Although the booster dose did increase IgG, IgG1, IgG3 and IgG4 antibodies, the increments were lower than those of anti-CSP antibodies, and were similar to the response induced by primary vaccination in the Manhiça site[7]. Interestingly, the results of a trial that analyzed the response to a hepatitis B vaccine booster after RTS,S primary vaccination showed an increased response in HBsAg IgG concentrations compared to primary vaccination[24]. The responses to HBsAg may be an indication of the quality of the immune response or the immune status of the child and the capacity to respond to vaccination and it requires further investigation.

It has been previously reported that antibodies after primary vaccination are lower in the infant group than in the children group. This may be due to the interference of maternal antibodies against CSP or intrinsic differences in the developing and functional competence of the immune system[5]. Antibodies seem to be lower in infants than children also at months 20, 21, and 32 but only for total IgG and IgG1 against CSP epitopes, not for HBsAg responses, even though maternal antibodies are no longer present at this age. At the time of the booster, both age groups have passed the critical age of 2 years for immune ontogeny and

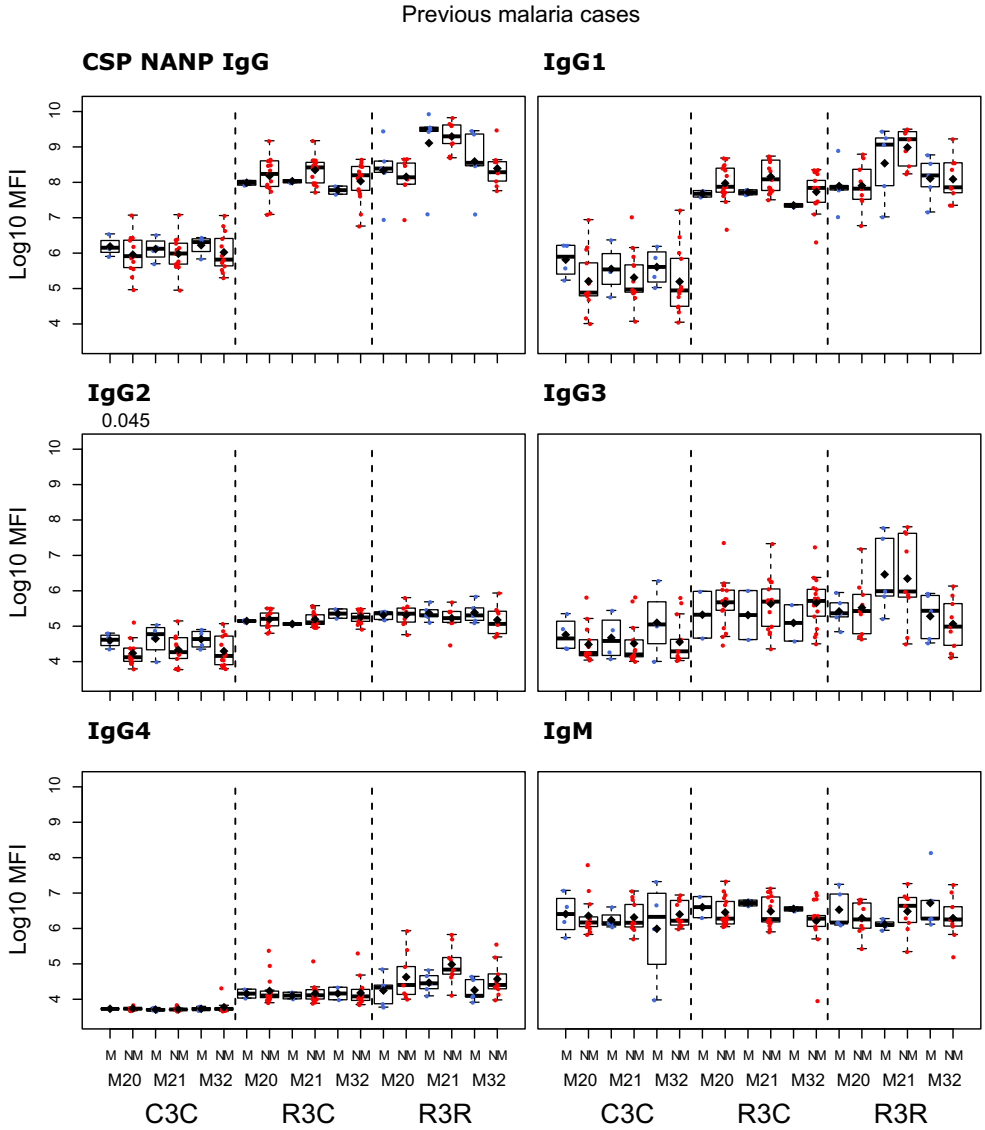

Fig. 6 Immunogenicity for CSP NANP stratified by previous clinical malaria: total IgG, IgG1-4 subclasses and IgM at month (M)20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M = blue) and subjects without malaria (NM = red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs. NM). *p*-values were adjusted for multiple comparisons, but none was significant. Only *p*-values < 0.05 before adjustment are shown. The *y*-axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C: three doses of RTS,S/AS01$_E$ and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

their immune system has achieved a more adult status[25,26], therefore, major differences driven by intrinsic changes in the immune system are less likely at this timepoint. Instead, differences detected may reflect a distinct establishment of a memory response in infants and children after primary vaccination that differentially affects the boosting of responses.

One of the factors identified thus far in this and our previous studies[7], with an impact on vaccine responses, is prior malaria exposure. Preceding malaria cases were associated with lower IgG, IgG1, and IgG3 levels to CSP constructs at the time points studied. How malaria episodes may affect immunization outcomes is another key issue, since it may affect any malaria vaccine and needs to be addressed in follow-up studies.

The study was not powered to assess association of antibodies with malaria risk but we obtained some preliminary findings. Interestingly, in children with malaria episodes post-booster dose, the IgG2 and IgG3 levels at M21 were higher than non-malaria controls, while IgG, IgG1, and IgG4 levels were lower, although with the available sample size this was not statistically significant for most comparisons. These results are in line with the findings of our previous study[7] in which IgG2 levels to RTS,S antigens were higher in malaria cases than in controls, whereas IgG1 levels were lower. The booster dose might be increasing protection not only by increasing total IgG levels, but also by not inducing IgG2 that could be detrimental, or not protective according to our previous results. However, the tendency for malaria cases to be associated with higher levels of IgG3 and lower levels for IgG4 to CSP constructs was opposite to what was reported to occur after primary vaccination[7] where a higher ratio between cytophilic (IgG1 and IgG3) to non-cytophilic (IgG2 and IgG4) CSP antibodies was associated with protection. The association between IgG4 levels and protection is consistent with findings of Chaudhury

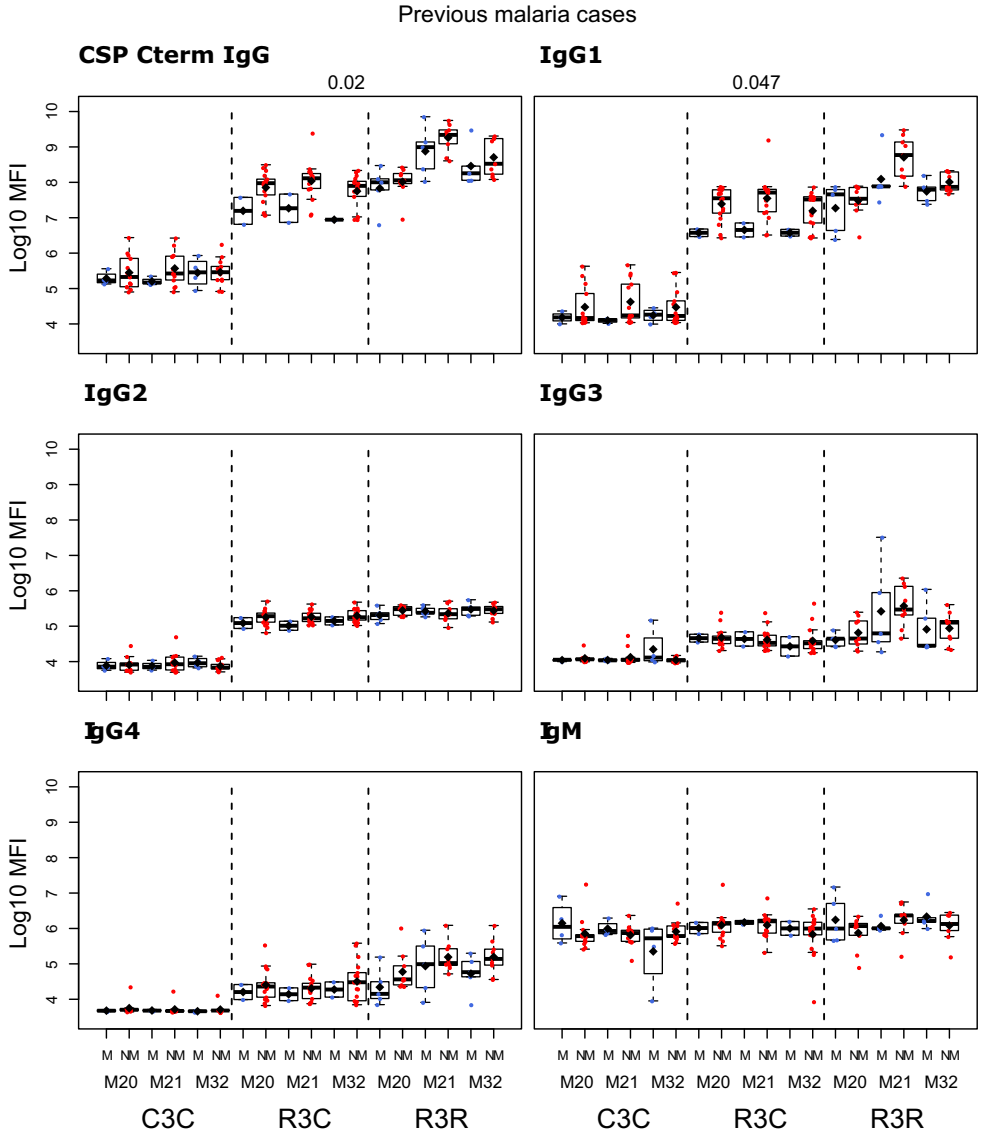

Fig. 7 Immunogenicity for CSP C-term stratified by previous clinical malaria: total IgG, IgG1-4 subclasses and IgM at month (M)20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M = blue) and subjects without malaria (NM = red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$ (geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs. NM). *p*-values were adjusted for multiple comparisons, but none was significant. Only *p*-values < 0.05 before adjustment are shown. The *y*-axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C: three doses of RTS,S/AS01$_E$ and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

et al.[27] who reported that only IgG4 levels were positively associated with increased vaccine efficacy in malaria-naive adults under the fractional dose regime. We have also previously found an association between IgG4 responses to non-RTS,S antigens after primary vaccination and protection[8]. IgG4 antibodies are associated with repeated or long-term exposure to antigens and have been linked to induction of tolerance, for instance higher IgG4/IgE ratios are associated with better food tolerance, as IgG4 competes with IgE[28]. In the context of helminth infections, high IgG4 is associated with asymptomatic infection for some parasites[10]. However, there is conflicting evidence on the role of IgG2 and IgG4 on protection against malaria, whilst more information exists on the protective role of IgG1 and IgG3. In the context of naturally acquired immunity, the ratio of cytophilic (IgG1 + IgG3) to non-cytophilic subclasses (IgG2 + IgG4) is generally higher in subjects with uncomplicated malaria compared to subjects with complicated malaria, and higher in subjects protected from malaria[7,29]. Additionally, it has been reported that the IgG2/IgG4 ratio is higher in subjects with uncomplicated malaria[30]. However, it has also been observed that IgG2 and IgG4 with high avidity are found in subjects with uncomplicated malaria compared to complicated malaria[29]. In contrast, the Chaudhury et al.[27] study assessing avidity and opsonization reported that RTS,S protection was mediated by IgG4 against the C-term of CSP. All of this evidence indicates that not only antibody levels are important for protection but also the balance between subclasses.

We note that the pattern of higher IgG, IgG1, and IgG4 levels to CSP FL and C-term in non-malaria cases was not apparent for NANP.

We previously observed that after primary vaccination, HBsAg antibody responses were associated with malaria protection[7,12].

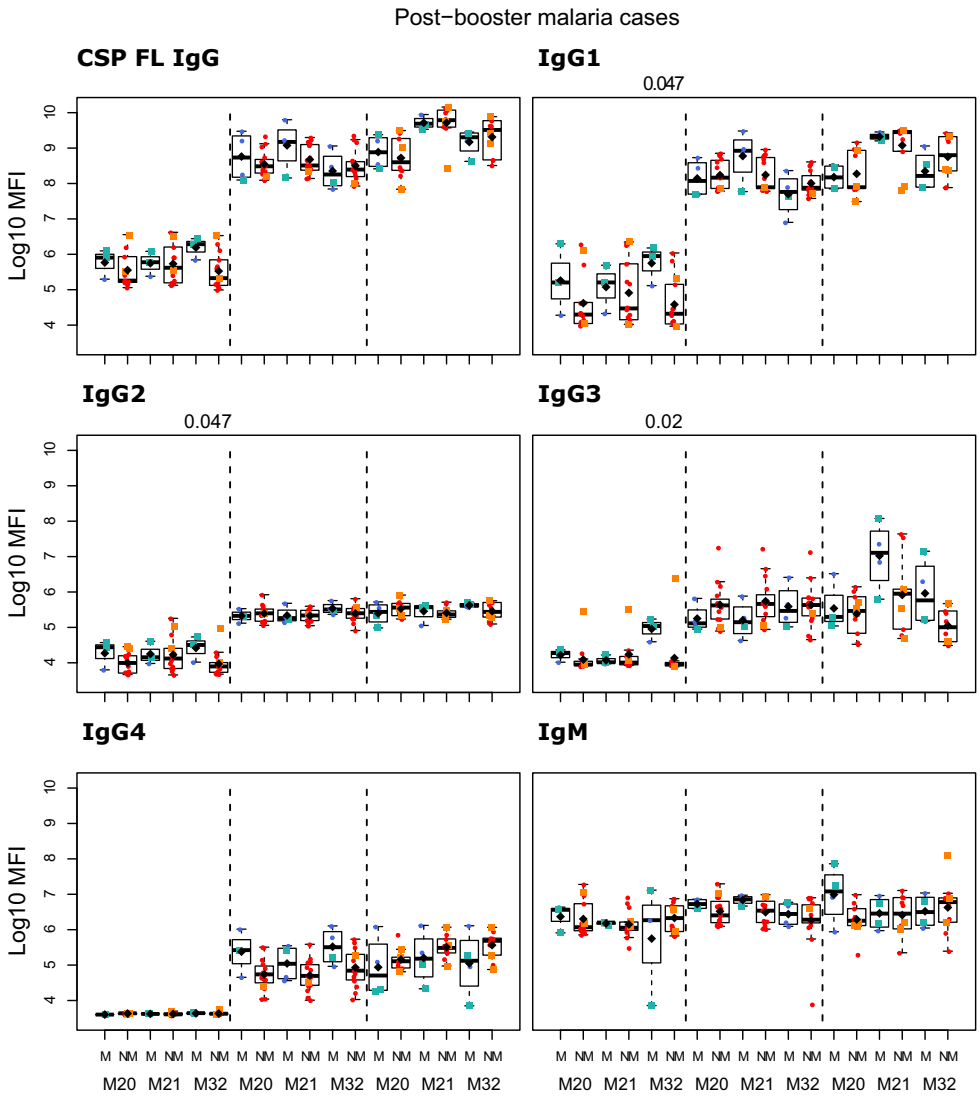

Fig. 8 Immunogenicity stratified by clinical malaria after M21: total IgG, IgG1-4 subclasses and IgM for CSP FL at month (M) 20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M = blue) and subjects without malaria (NM = red). Subjects who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$ (geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs. M). *p*-values were adjusted for multiple comparisons, but none was significant. Only *p*-values < 0.05 before adjustment are shown. The *y*-axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C: three doses of RTS,S/AS01$_E$ and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

On the contrary, 1 month after receiving the RTS,S booster dose, anti-HBsAg IgG2 and IgG3 levels appeared as risk factors for future malaria episodes, further indicating that the nature and role of responses may differ following a primary and a booster immunization.

The analysis of the phase 3 clinical trial found that children who did not receive the booster dose were at higher risk of severe malaria than the comparator (non-RTS,S) group[3]. It was hypothesized that the primary vaccination had prevented vaccinees from acquiring natural immunity, as has been predicted for other malaria prevention tools[16], increasing the risk of severe malaria in those individuals in whom infection reached the erythrocytic stage. However, on a longer follow-up study of up to 7 years on 3 of 11 sites, no increased risk was found for severe malaria between those groups that received the RTS,S/AS01 vaccine and the control group[31]. Antibody responses to asexual blood stage antigens have been studied previously with samples from phase 2 clinical trials and showed a reduced antibody response in RTS,S vaccinees, but these trials did not include a booster dose[17,32,33]. We detected a decreased antibody response to certain vaccine-unrelated *P. falciparum* antigens after primary vaccination in the phase 3 trial as well[8,9]. However, we also observed an induction of antibody responses to other *P. falciparum* antigens following RTS,S vaccination (MSP1-BL2, EBA140, EBA175, and Rh4.2), which could contribute to malaria protection[8,9]. Here, we did not find consistent differences in the NAI response between booster groups except for Rh5. Interestingly and in line with our previous results, Rh5 IgG and IgG1-2 levels were higher in the RTS,S booster group than in the comparator vaccine groups at M21 and M32, and R3C levels were either higher or did not differ from C3C.

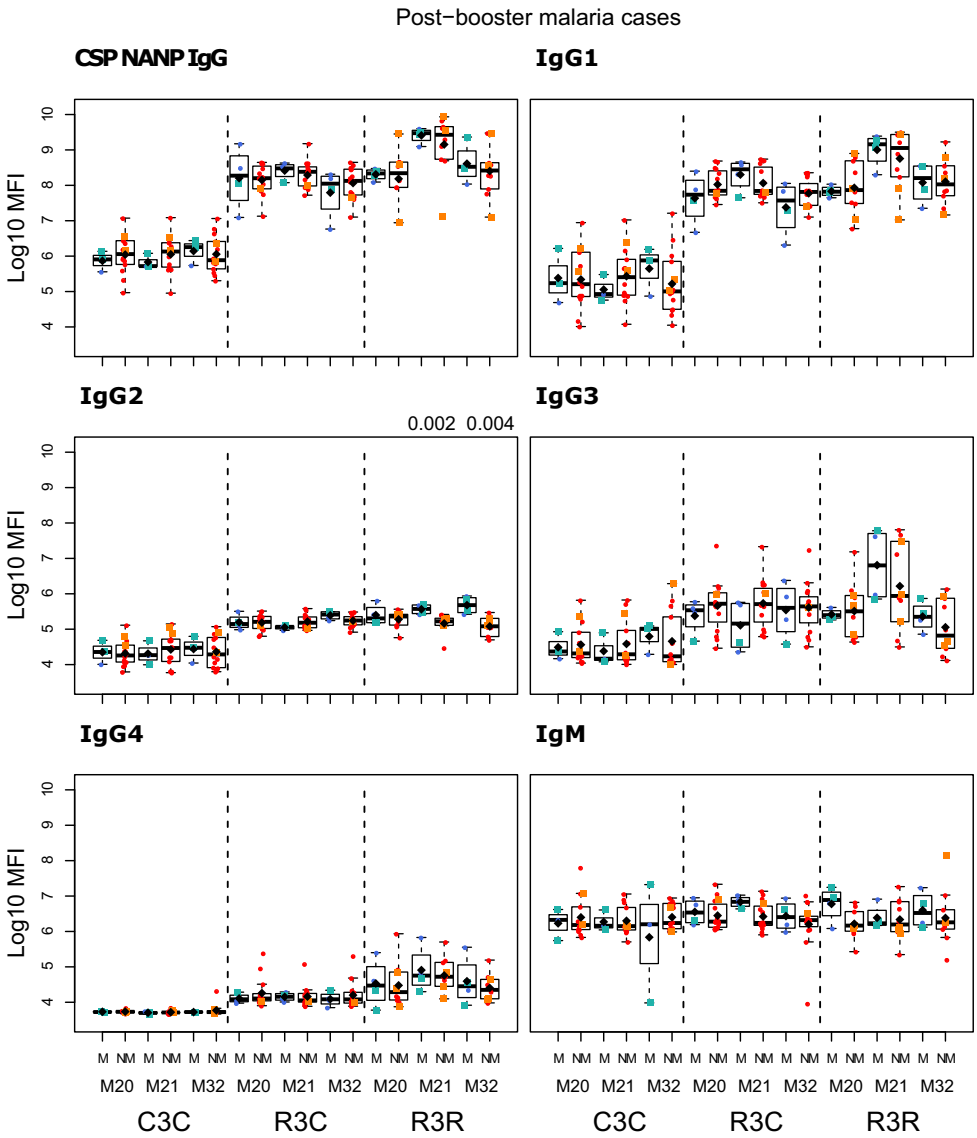

Fig. 9 Immunogenicity stratified by clinical malaria after M21: total IgG, IgG1-4 subclasses and IgM for CSP NANP at month (M) 20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M = blue) and subjects without malaria (NM = red). Subjects who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs. M). *p*-values were adjusted for multiple comparisons, but none was significant. Only *p*-values < 0.05 before adjustment are shown. The *y*-axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C: three doses of RTS,S/AS01$_E$ and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

This finding requires future investigation to understand the basis and clinical relevance of this effect, especially since Rh5 is a leading vaccine candidate[34], and because Rh5 antibody concentrations need to be very high to actually confer protection[35]. However, these observations are important as they may explain why an anti-sporozoite infection vaccine also protects against clinical disease in the parasite blood stage, considering that Rh5 plays an essential role during erythrocyte invasion by *P. falciparum* merozoites[36–38]. Additionally, IgG to MSP5 showed higher levels after RTS,S booster dose compared to the comparator booster group, but this was not statistically significant.

Our findings are limited because of a small sample size and because data were obtained only for Manhiça. Therefore, a larger longitudinal study with samples from different sites is necessary to corroborate these data. This is particularly important in our case because there are some special considerations about Manhiça that limits the generalization of these findings: (1) at the time of the study malaria transmission was low[3,39], (2) there were unexpected results of VE in the phase 3 clinical trial, i.e., VE was lower in the R3R than in the R3C group, contrary to most sites, and (3) Manhiça has a high HIV prevalence[40]. HIV infection was associated with a reduced immunogenicity to the vaccine in a phase 3 trial exploratory analysis but it was concluded that HIV-infected children should not be excluded from RTS,S vaccination[41].

Despite the constraints, our study provides new and interesting clues to the immune response elicited by the RTS,S booster dose. Additionally, avidity and functional antibody responses should be assessed, and these results integrated with cellular data to address memory responses induced by the booster. This information is necessary for a deeper understanding of the mechanisms of

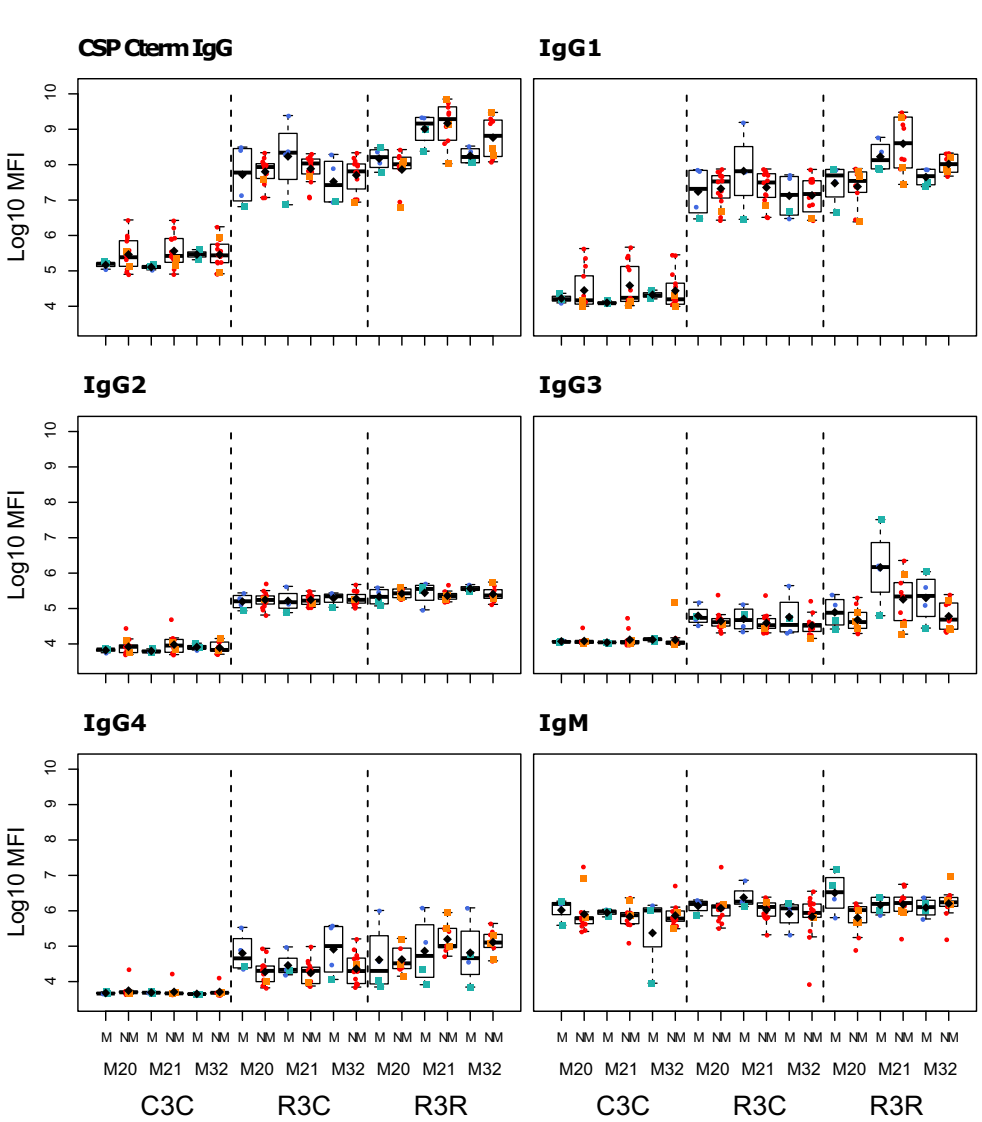

Fig. 10 Immunogenicity stratified by clinical malaria after M21: total IgG, IgG1-4 subclasses and IgM for CSP C-term at month (M) 20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M = blue) and subjects without malaria (NM = red). Subjects who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs. M). *p*-values were adjusted for multiple comparisons, but none was significant. Only *p*-values < 0.05 before adjustment are shown. The *y*-axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C: three doses of RTS,S/AS01$_E$ and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

action of the vaccine, as well as the determination of the factors causing partial and short VE. Results of these studies are required for the rational design and deployment of improved CSP-based vaccines and other malaria vaccines with an increased and long-term efficacy.

METHODS

Population and study design

This study was performed using plasma samples previously collected from subjects in Manhiça, Mozambique, a site of low malaria transmission intensity[3,39], as part of the MAL067 study ancillary to the phase 3 randomized clinical trial MAL055 (NCT00866619)[3]. A subset of 50 individuals (24 children 5–17 months and 26 infants 6–12 weeks) was selected from those previously analyzed[7–9] who had available antibody data from M0 (baseline) and M3 (one month after third dose) and plasma samples for M20 (booster dose), M21, and M32 (Supplementary Table 7). The subjects had either received three doses of the RTS,S/AS01$_E$ vaccine and a RTS,S/AS01$_E$ booster (R3R, $n = 14$) at M20, three doses of RTS,S/AS01$_E$ and a comparator booster (R3C, $n = 19$), or three doses and a booster of a comparator vaccine (C3C, $n = 17$) (Supplementary Fig. 40). The comparator vaccines used in the primary series were a Meningococcal C Conjugate Vaccine (Menjugate™) in the 6–12 weeks age category, and a cell-culture rabies vaccine (VeroRab™) in the 5–17 months age category. The booster comparator was Menjugate™ for both age groups. Clinical malaria cases were detected by passive case detection and defined as fever of at least 37.5 °C and any asexual *P. falciparum* parasitemia by microscopy[3]. The prevalence of HIV infection in the Manhiça area was around 40% in adults[40]. HIV infection was not a protocol exclusion/inclusion criteria, but only healthy children were included in the study. HIV testing was not a trial procedure. The study protocol was approved by the Ethics Committees of

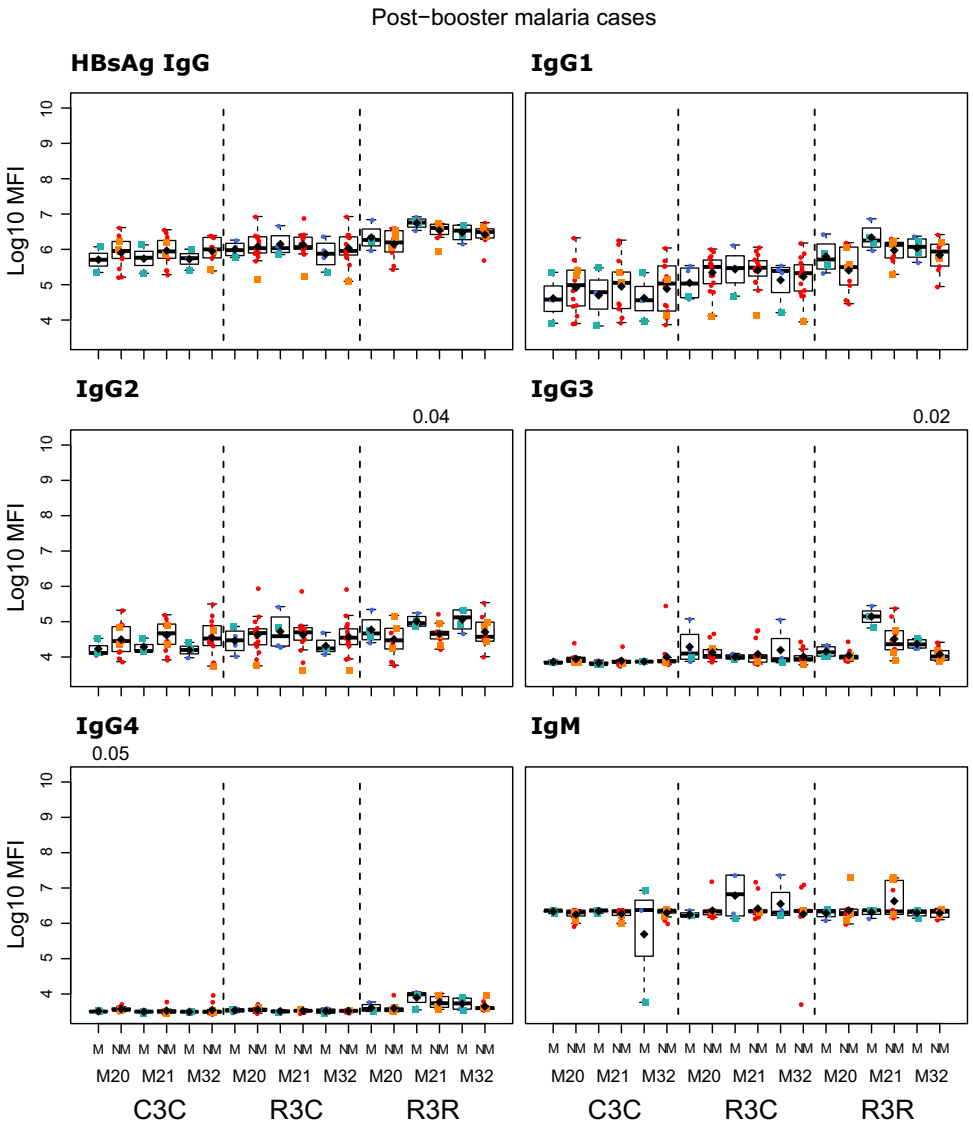

Fig. 11 Immunogenicity stratified by clinical malaria after M21: total IgG, IgG1-4 subclasses and IgM for HBsAg at month (M) 20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M = blue) and subjects without malaria (NM = red). Subjects who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs. M). *p*-values were adjusted for multiple comparisons, but none was significant. Only *p*-values < 0.05 before adjustment are shown. The *y*-axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C: three doses of RTS,S/AS01$_E$ and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

PATH-MVI (REC) in the US, Hospital Clínic in Spain (CEIm) and the CNBS in Mozambique, and written informed consent was obtained from parents or guardians before recruitment.

Antibody luminex assays

Antibody response was analyzed using a quantitative suspension array technology (qSAT). MAGPlex beads were coupled separately to: three CSP constructs (FL, C-term, NANP-repeat region) and HBsAg that are antigenic components of the RTS,S vaccine; seven *P. falciparum* blood stage antigens (MSP1 [block 2 and MSP1$_{42}$ fragments, 3D7 strain], MSP5, EBA140, EBA175 region 3-5, Rh4.2 and Rh5) that were shown to be affected by vaccination in our previous studies[7–9] and/or that are leading vaccine candidates; and Glutathione S-transferase (GST) as a control for antigens co-expressed with a GST tag (Supplementary Table 8)[42]. The coupling of the beads to the antigens was performed as described previously[43].

Antigen-coupled beads were added to a 96-well µClear® flat bottom plate (Greiner Bio-One) in multiplex (1000 microspheres/analyte/well) resuspended in 50 µL of PBS, 1% BSA, 0.05% Azide pH 7.4 (PBS-BN). Fifty microliters of sample, negative or positive control were added to wells and incubated overnight at 4 °C in a shaker protected from light. Plates were washed three times with 200 µL/well of wash buffer (PBS-Tween 20: 0.05%) using a manual magnetic washer. Then, 100 µL of biotinylated secondary antibody were added diluted in PBS-BN: anti-human IgG 1/2500 (B1140 Sigma), anti-human IgG1 1/4000 (ab99775 Abcam), anti-human IgG3 1/1000 (B3523 Sigma), and anti-human IgM 1/1000 (B1265 Sigma). For IgG2 and IG4, mouse anti-human IgG2 1/500 and IgG4 1/500 (MA1-34755 and MA5-16716 Thermo Fisher), respectively, were added, followed by biotinylated goat anti-mouse IgG 1/40,000 for IgG2 and 1/10,000 for IgG4 (B7401 Sigma) in PBS-BN. All antibody incubations were performed for 45 min, at room temperature, in agitation and protected from light. Again, plates were washed as before and 100 µL/well streptavidin-R-

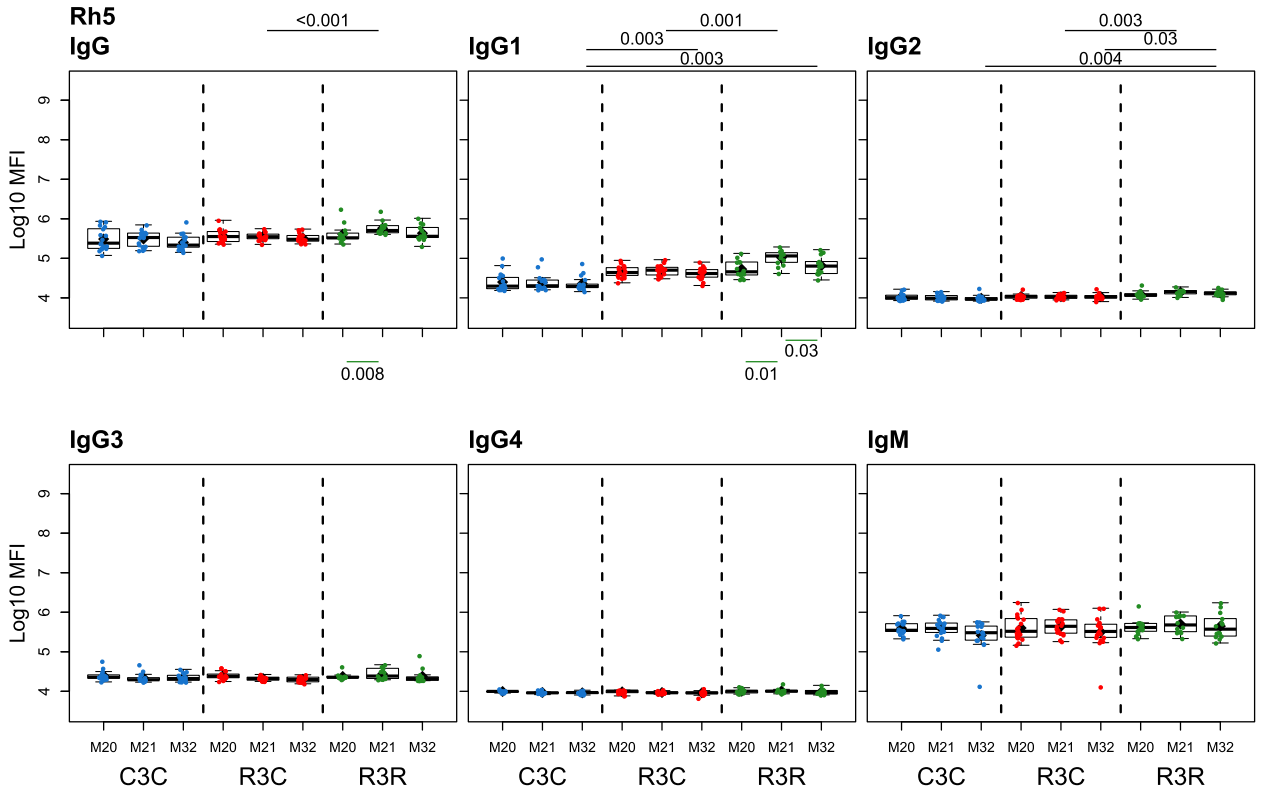

Fig. 12 RTS,S/AS01$_E$ booster and long-term immunogenicity against the blood stage antigen Rh5: total IgG, IgG1-4 subclasses and IgM at month (M) 20, 21, and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR, lower whisker as the largest between minimum × value and Q1 − 1.5*IQR, and $\log_{10}$(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs. M21) and the long-term immunogenicity (M21 vs. M32), as well as to compare the R3C and R3R groups at each timepoint. Only *p*-values < 0.05 after adjustment for multiple testing are shown. The *y*-axis is in logarithm 10 scale. R3R (green): three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster. R3C (red): three doses of RTS,S/AS01$_E$ and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.

phycoerythrin 1/1000 (42250 Sigma) in PBS-BN was added to all wells and incubated 30 min, at room temperature, in agitation and protected from light. Plates were washed as before and resuspended in 100 µL/well of PBS-BN. Plates were stored at 4 °C overnight protected from light and read the next day using the Luminex xMAP® 100/200 analyzer; at least 50 microspheres per analyte were acquired per sample and Report Gain was set as High PMT.

For IgG, IgG1, IgG3, and IgM, 20 serial dilutions 1:2 of a positive control were used to perform antigen-subclass-specific standard curves. For IgG2, 16 serial dilutions 1:2 were used. For IgG4, no standard curve was performed and only one positive control dilution was included. The positive control consisted of a WHO Reference Reagent for anti-malaria *P. falciparum* human serum (NIBSC code: 10/198)[42,44] at 1:50 mixed with a pool of plasmas from RTS,S/AS02 vaccinated children[42,45] with high IgG levels against CSP at 1:200. Blanks were added to each plate in duplicates and two negative controls samples from malaria-naive adults were added in each plate. Test samples were assayed at three dilutions for IgG (500, 20,000, 500,000), IgG1, IgG3 (100, 2500, 100,000) and IgM (100, 1000, 25,000) to ensure that at least one dilution lie in the linear range of the respective standard curve, i.e., close to the highest slope between two dilution points. Owing to the low levels previously observed in these samples for IgG2 and IgG4 in Ubillos et al.[7] only one dilution (1/50) was used. Sample distribution across plates was designed to ensure a balanced distribution of vaccination groups, sex, age cohorts, and malaria cases. The three time points for each individual and the respective dilutions were placed on the same plate. Data were captured using xPonent software, and antibody levels were measured as MFI.

Data pre-processing. The standard curve for each antigen-isotype/subclass-plate was estimated using the drLumi R package flow[46], fitted in a 4- or 5-parameter logistic (4-PL or 5-PL) regression model, and data points logarithmically transformed. To select the sample dilution for IgG, IgG1, and IgG3 in the linear part of the sigmoidal curve (antigen, isotype/subclass and plate specific), an algorithm that detects the two points with the highest slope between them was used. The slope was computed as: $m = (\mathrm{MFI_i} - \mathrm{MFI_{i+1}})/(\mathrm{dilution\_factor_i} - \mathrm{dilution\_factor_{i+1}})$. The mean MFI value of the two points was computed and used as the reference value, but the standard curves were visually inspected and if the model did not converge, the $R^2 < 0.9$ or the curve maximum values were < 15,000 MFI, a 15,000 MFI reference value was set instead of the highest slope criteria. The nearest MFI of the test sample to the reference value was determined and the corresponding dilution was selected. Since only one dilution was used for IgG2 and IgG4, the standard curves were not used to select a dilution. The MFI measurement of the selected dilution was corrected multiplying by its corresponding dilution factor and transformed to $\log_{10}$ scale to stabilize the variance. Blank and GST signals were not subtracted. Blanks were used to measure background signal, and GST to assess for unspecific binding to the GST-fused antigens (CSP FL, CSP C-term, and CSP-NANP). Background values were below 500 MFI, and no correlation was found between IgG to GST and IgG to GST-fused antigens (Supplementary Figs. 41 and 42).

Statistical analysis

Descriptive comparisons of Ig isotype/subclass levels to specific antigens ($\log_{10}$ transformed MFI) at each visit were done by boxplots with $\log_{10}$(geometric mean), medians, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR and lower whisker as the largest between minimum × value and Q1 − 1.5*IQR. Wilcoxon-Signed Rank Test between M20 and M21 for the R3R vaccination group, and between M21 and M32 for the R3R and R3C vaccination groups were performed to determine if the antibody levels changed significantly 1 month and 1 year after the booster. Additionally, Mann–Whitney tests were performed to compare the R3R and R3C groups at each timepoint.

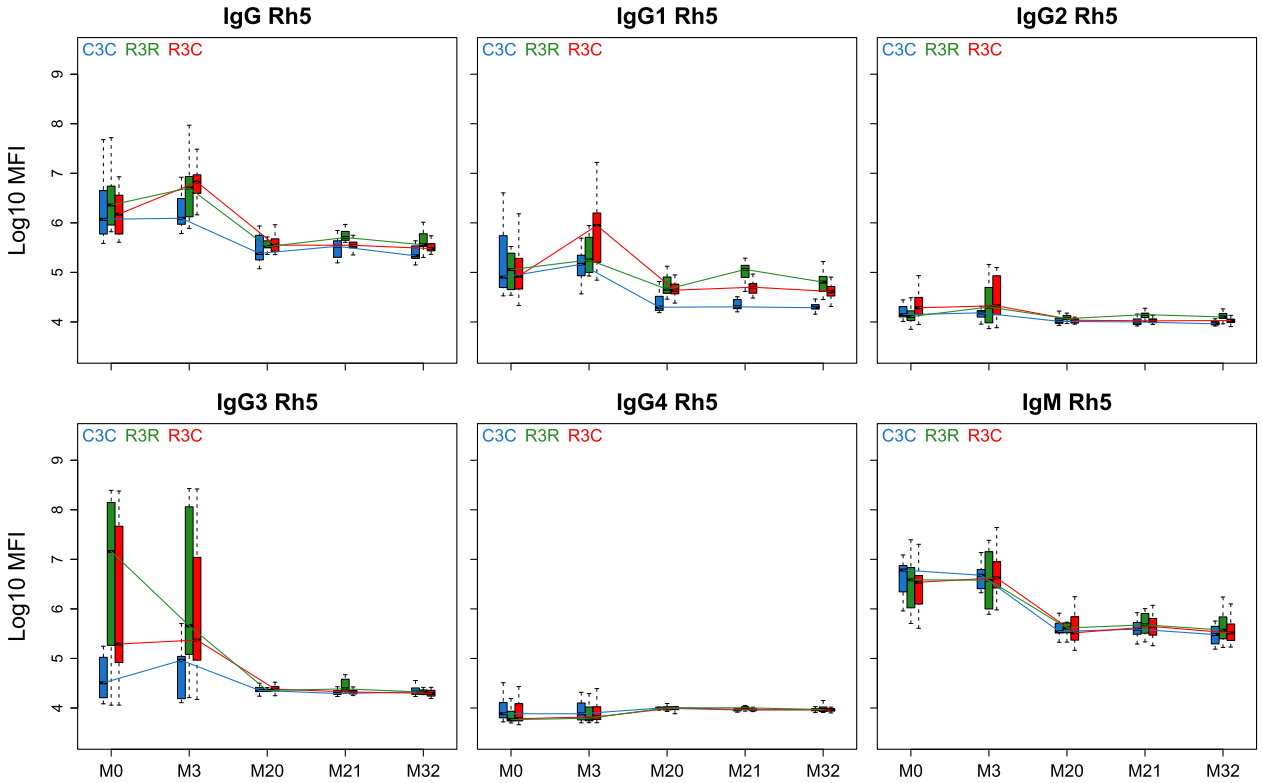

Fig. 13 Antibody responses against the blood stage antigen Rh5 for months (M) 0, 3, 20, 21, and 32 for IgG, IgG1, IgG2, IgG3, IgG4 and IgM. Boxplots with median, interquartile ranges (IQR), upper whisker as the smallest between maximum × value and Q3 + 1.5*IQR and lower whisker as the largest between minimum × value and Q1 − 1.5*IQR. The *y*-axis is in logarithm 10 scale. Data from months 0 and 3 were obtained from a previous study in the same individuals [7], thus a batch effect might be present. R3R (green): three doses of RTS,S/AS01$_E$ and a RTS,S/AS01$_E$ booster at month 20. R3C (red): three doses of RTS,S/AS01$_E$ and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.

p-values were adjusted for multiple testing using p.adjust on R[47] by the Holm approach for IgG and for IgM to control for family wise error, and by the Benjamini–Hochberg approach for IgG1-4, altogether to control for the false-discovery rate since there were more tests. The comparisons between time points were corrected separately from the comparisons between vaccination groups, likewise the comparisons for vaccine antigens were corrected separately from blood stage antigens. Data for M0 and M3 from our previous study[7] was used to analyze the kinetics of the antibody response throughout the 5 time points in the clinical trial. Adjusted *p*-values were considered significant when <0.05. The qSAT assay of M20, M21, and M32 samples was performed in the same laboratory using the same reagents and under similar conditions as the assay of the M0 and M3 samples, but they were not executed at the same time and a smaller set of antigens was used.

Stratified analyses and Mann–Whitney tests for independent groups and Wilcoxon-Signed Rank Tests for paired samples were performed between age groups and between malaria cases and controls, for each timepoint. *p*-values were adjusted following the same strategy as above. There were no reported malaria cases between M20 and M21. The change in antibody levels between M20 and M21 was calculated as $\log_{10}$MFI(M21) − $\log_{10}$MFI (M20) and compared between individuals who either did or did not present with clinical malaria after M21. All data analysis and plots were performed using R packages gridExtra[48], dplyr[49], ggplot2[50], tidyr[51], and psych[52].

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The datasets generated in the current study are fully available at the Dipòsit Digital de la Universitat de Barcelona at http://hdl.handle.net/2445/164777.

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AUTHOR CONTRIBUTIONS

Designed the immunology study and protocols: C.D., J.J.C., G.M., R.A.; performed the clinical trial: P.A.; processed samples and/or data management: C.J., I.C., A.J.N., M.V., I.U., G.M., J.J.C., C.D.; managed the study: N.A.W., N.D., C.D.; produced antigens: D.C., E.A., R.L.C., D.G., J.G.B., S.D.; performed experiments: L.S., M.V.: supervised lab work: R.A.; performed statistical analysis: L.S. Wrote the first draft: L.S., G.M., C.D. Reviewed and approved the manuscript: all.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Supplementary materials, methods and results from Article 2

Article 2

Sanchez L, Vidal M, **Jairoce C,** Aguilar R, Ubillos I, Cuamba I, Nhabomba AJ, Williams NA, Díez-Padrisa, N, Cavanagh D, Angov E, Coppel RL, Gaur D, Beeson JG, Dutta S, Aide P, Campo JJ, Moncunill G, Dobaño, C. **Antibody responses to the RTS, S/AS01E vaccine and Plasmodium falciparum antigens after a booster dose within the phase 3 trial in Mozambique.** NPJ Vaccines. 2020;5(1).

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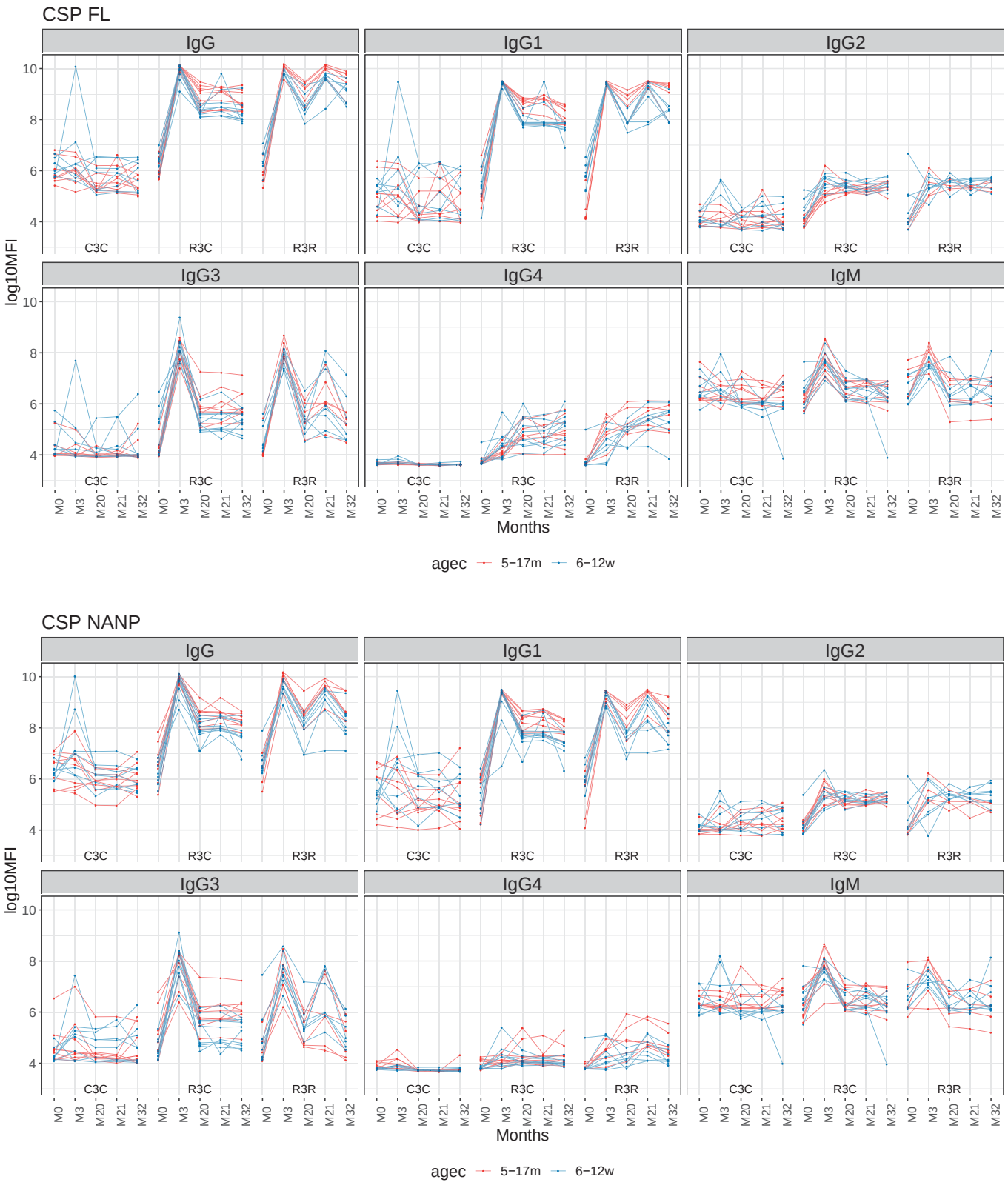
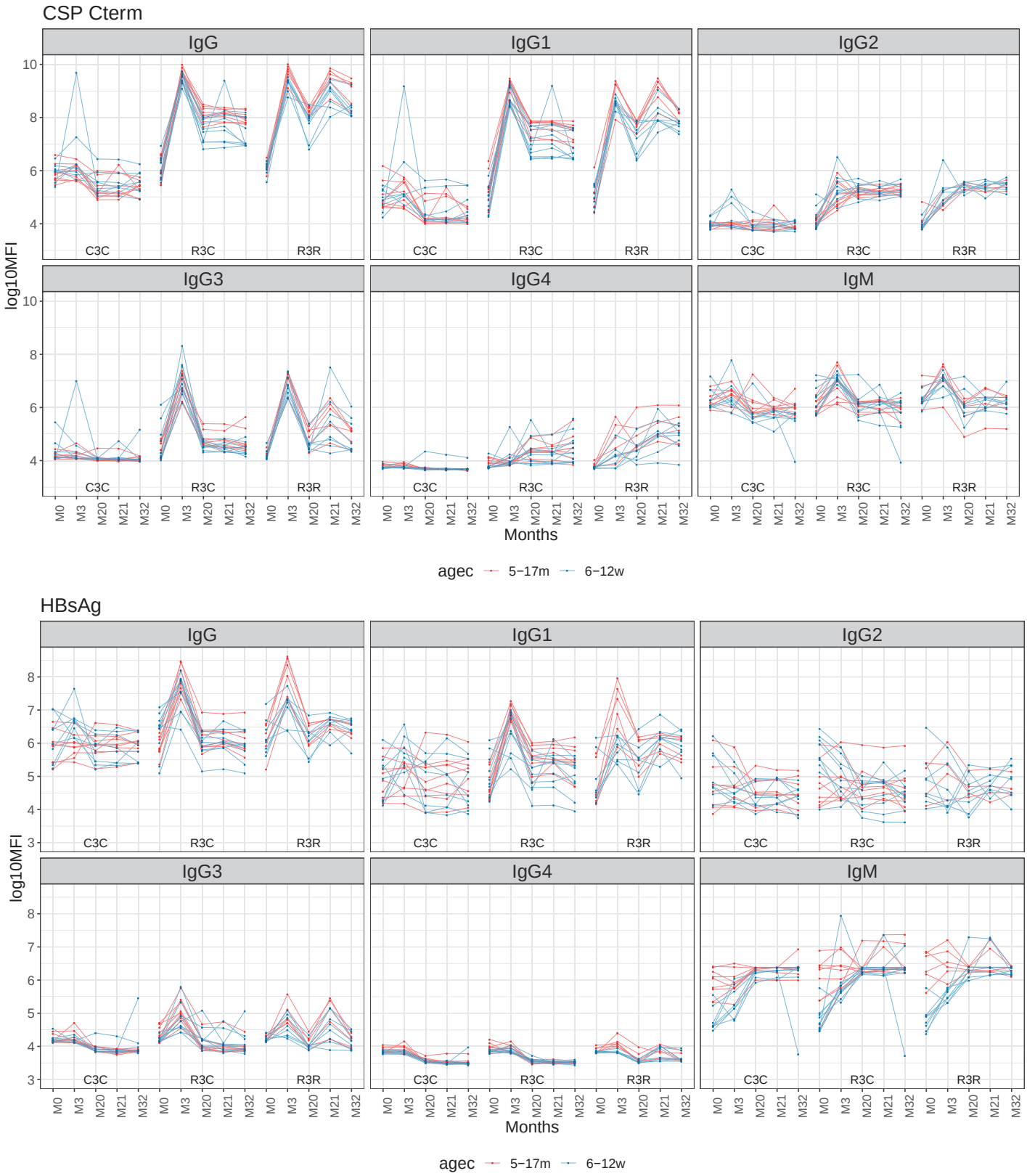
Supplementary Figures and Tables

Supplementary Table 1. RTS,S/AS01E booster and long-term immunogenicity for total IgG, IgG1-4 subclasses and IgM against vaccine antigens: Non-parametric test p-values for comparison of the booster response (M20 vs M21) and the long-term immunogenicity (M21 vs M32), as well as to compare the R3C and R3R groups at each timepoint. for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Adjusted p values are shown in parenthesis. P-values <0.05 before adjustment are highlighted in yellow and P-values <0.001 before adjustment in green. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

		Comparison between vaccination groups					Comparison Time points		
		R3R vs R3C			R3R vs C3C	R3C vs C3C	M20vsM21	M21vsM32	
Isotype	Antigen	M20	M21	M32	M32	M32	R3R	R3R	R3C
IgG	CSP_FL	1	<0.001	<0.001	<0.001	<0.001	0.001	0.02	0.001
IgG	Cterm	1	<0.001	<0.001	<0.001	<0.001	0.001	0.007	0.001
IgG	HBsAg	1	0.002	0.01	<0.001	1	0.007	0.03	0.007
IgG	NANP	1	<0.001	0.19	<0.001	<0.001	0.001	0.001	<0.001
IgG1	CSP_FL	0.63	<0.001	0.001	<0.001	<0.001	<0.001	0.03	<0.001
IgG1	Cterm	0.59	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.001
IgG1	HBsAg	0.38	<0.001	<0.001	<0.001	0.14	0.002	0.08	0.005
IgG1	NANP	1	0.004	0.1	<0.001	<0.001	0.002	0.002	<0.001
IgG2	CSP_FL	0.17	0.21	0.32	<0.001	<0.001	0.84	0.27	0.048
IgG2	Cterm	0.044	0.05	0.03	<0.001	<0.001	0.88	0.63	0.26
IgG2	HBsAg	1	0.41	0.09	0.07	0.96	0.08	1	0.29
IgG2	NANP	0.16	0.09	0.79	<0.001	<0.001	0.75	0.87	0.08
IgG3	CSP_FL	0.81	0.13	0.1	<0.001	<0.001	0.007	<0.001	0.96
IgG3	Cterm	0.95	0.001	0.08	<0.001	<0.001	0.002	0.001	0.26
IgG3	HBsAg	1	<0.001	0.1	0.001	0.049	0.001	<0.001	0.30
IgG3	NANP	0.41	0.08	0.15	0.08	0.002	0.01	<0.001	0.41
IgG4	CSP_FL	0.23	0.002	0.08	<0.001	<0.001	<0.001	1	0.01
IgG4	Cterm	0.16	<0.001	0.02	<0.001	<0.001	<0.001	0.75	0.057
IgG4	HBsAg	0.54	<0.001	<0.001	<0.001	0.17	0.006	0.009	0.88
IgG4	NANP	0.21	<0.001	0.047	<0.001	<0.001	0.08	0.001	0.95
IgM	CSP_FL	1	1	1	1	1	1	1	0.96
IgM	Cterm	1	1	1	0.09	1	1	1	1
IgM	HBsAg	1	1	1	1	1	1	1	1
IgM	NANP	1	1	1	1	1	1	1	1



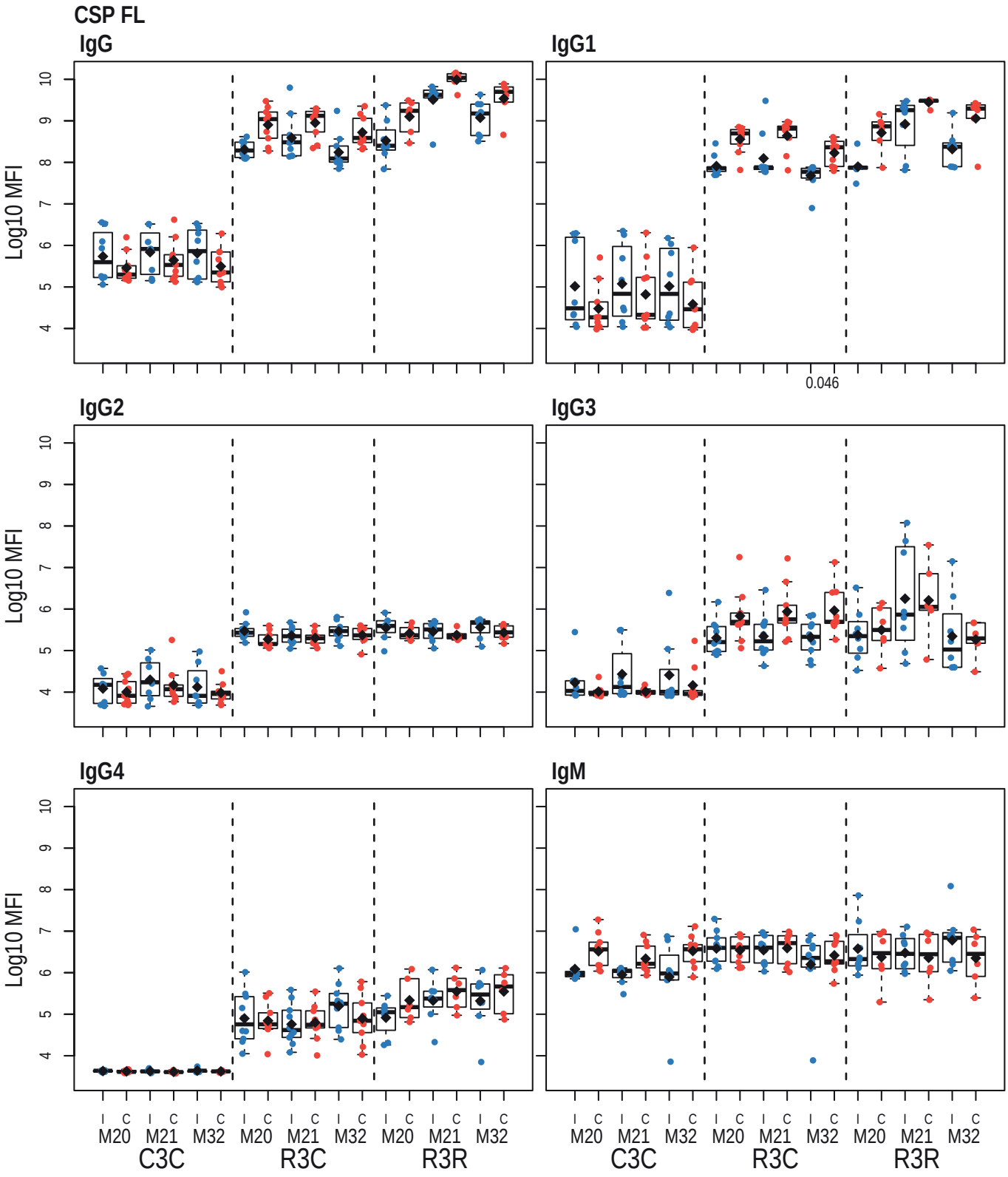
Supplementary Figure 1. Antibody responses against vaccine antigens CSP FL and CSP NANP for months (M) 0, 3, 20, 21 and 32 for IgG, IgG1, IgG2, IgG3, IgG4 and IgM. Line time plots connecting levels for each individual. The y axis is in logarithm 10 scale. Data from months 0 and 3 were obtained from a previous study in the same individuals [5], thus a batch effect might be present. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster at month 20. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster. Children age 5-17 months are shown in orange and infants age 6-12 weeks at first immunization are shown in blue.



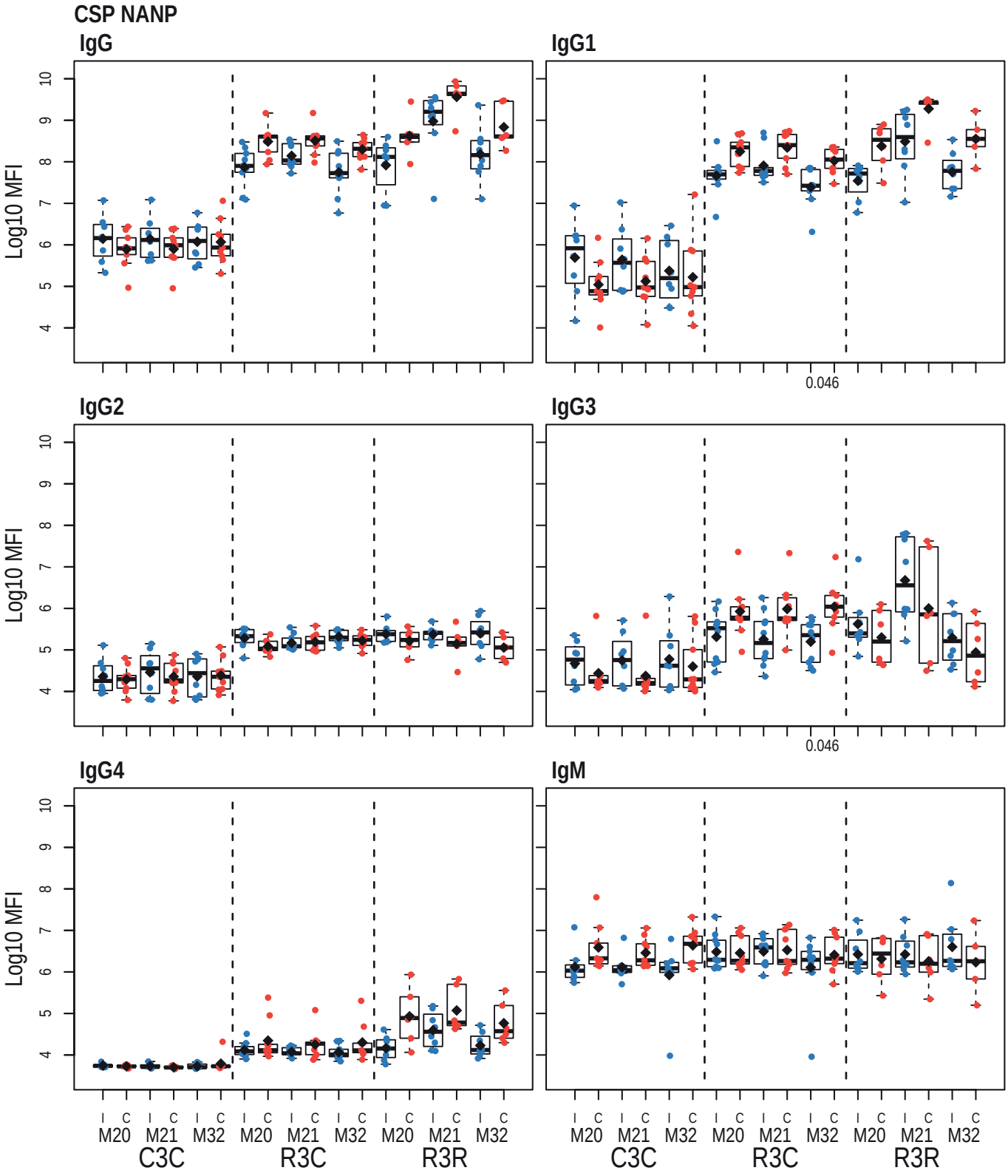
Supplementary Figure 2. Antibody responses against vaccine antigens CSP Cterm and HBsAg for months (M) 0, 3, 20, 21 and 32 for IgG, IgG1, IgG2, IgG3, IgG4 and IgM. Line time plots connecting levels for each individual. The y axis is in logarithm 10 scale. Data from months 0 and 3 were obtained from a previous study in the same individuals [5], thus a batch effect might be present. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster at month 20. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster. Children age 5-17 months are shown in orange and infants age 6-12 weeks at first immunization are shown in blue.

Supplementary Table 2. RTS,S/AS01E booster and long-term immunogenicity for total IgG, IgG1-4 subclasses and IgM against vaccine antigens stratified by age: Non-parametric test p-values for comparison of the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants), adjusted p values are shown in parenthesis. RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Children were age 5-17 months and infants 6-12 weeks at the time of first vaccination. P-values <0.05 before adjustment are highlighted in yellow.

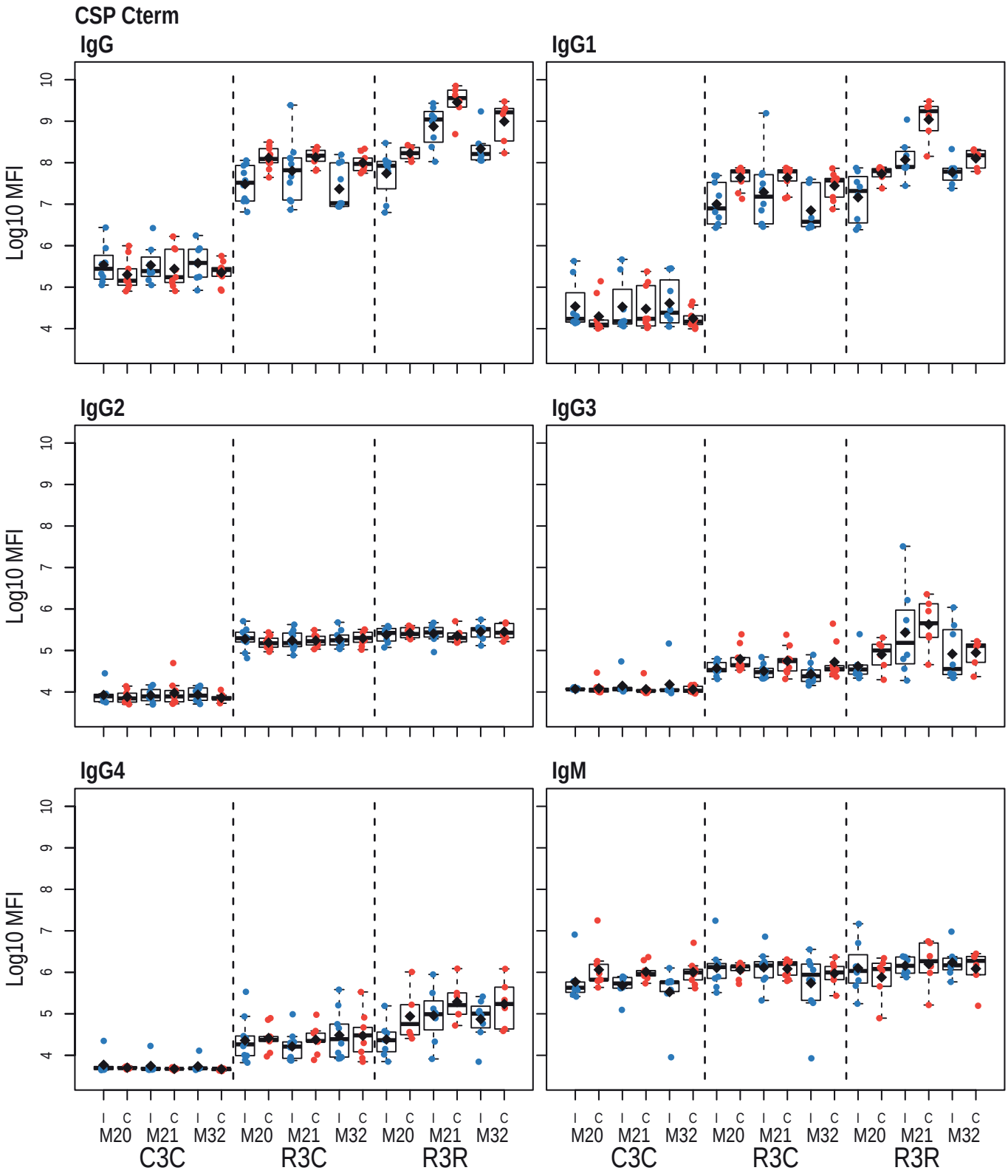
		Comparison between timepoints						Age comparison					
		M20vsM21		M21vsM32				Children vs Infants					
		R3R		R3R		R3C		R3C			R3R		
Isotype	Antigen	Infants	Children	Infants	Children	Infants	Children	M20	M21	M32	M20	M21	M32
IgG	CSP_FL	0.17	0.44	0.44	0.44	0.09	0.44	0.07	0.63	0.20	0.39	0.17	0.39
IgG	Cterm	0.25	0.44	0.44	0.44	0.18	0.20	0.05	0.63	0.39	0.39	0.36	0.36
IgG	HBsAg	0.35	0.44	0.44	0.44	0.44	0.44	1	1	1	1	1	1
IgG	NANP	0.17	0.44	0.17	0.44	0.09	0.17	0.07	0.36	0.33	0.39	0.36	0.39
IgG1	CSP_FL	0.06	0.08	0.15	0.08	0.07	0.06	0.05	0.13	0.046	0.09	0.12	0.13
IgG1	Cterm	0.06	0.08	0.09	0.08	0.09	0.06	0.07	0.16	0.1	0.24	0.09	0.20
IgG1	HBsAg	0.08	0.08	0.45	0.08	0.08	0.18	0.16	0.38	0.29	0.68	0.84	0.68
IgG1	NANP	0.09	0.08	0.09	0.08	0.06	0.06	0.07	0.13	0.046	0.12	0.09	0.11
IgG2	CSP_FL	0.86	0.93	0.54	0.59	0.16	0.28	0.15	0.74	0.73	0.45	0.64	0.45
IgG2	Cterm	0.93	0.45	0.99	0.18	0.65	0.40	0.55	0.99	0.74	0.76	0.64	0.98
IgG2	HBsAg	0.26	0.45	0.86	0.59	0.71	0.34	0.73	0.74	0.85	0.76	0.98	0.68
IgG2	NANP	0.99	0.71	0.79	0.45	0.09	0.71	0.21	1	0.46	0.64	0.35	0.24
IgG3	CSP_FL	0.11	0.12	0.06	0.08	0.93	1	0.12	0.12	0.12	0.76	0.84	0.98
IgG3	Cterm	0.11	0.08	0.08	0.08	0.38	0.79	0.24	0.29	0.21	0.40	0.73	0.91
IgG3	HBsAg	0.08	0.08	0.06	0.08	0.65	0.45	0.45	0.59	0.68	0.45	0.51	0.76
IgG3	NANP	0.08	0.59	0.06	0.08	0.27	1	0.15	0.11	0.046	0.73	0.40	0.59
IgG4	CSP_FL	0.06	0.08	0.99	1	0.07	0.51	0.91	0.85	0.55	0.45	0.68	0.73
IgG4	Cterm	0.06	0.08	0.86	0.82	0.09	0.45	0.68	0.29	1	0.20	0.64	0.51
IgG4	HBsAg	0.06	0.27	0.26	0.08	0.93	0.99	0.35	0.75	0.75	0.29	0.68	0.68
IgG4	NANP	0.11	0.71	0.08	0.08	1	0.99	0.59	0.46	0.45	0.12	0.40	0.16
IgM	CSP_FL	1	1	1	1	1	1	1	1	1	1	1	1
IgM	Cterm	1	0.75	1	1	1	1	1	1	1	1	1	1
IgM	HBsAg	1	1	1	1	1	1	1	1	1	1	1	1
IgM	NANP	1	1	1	1	1	1	1	1	1	1	1	1



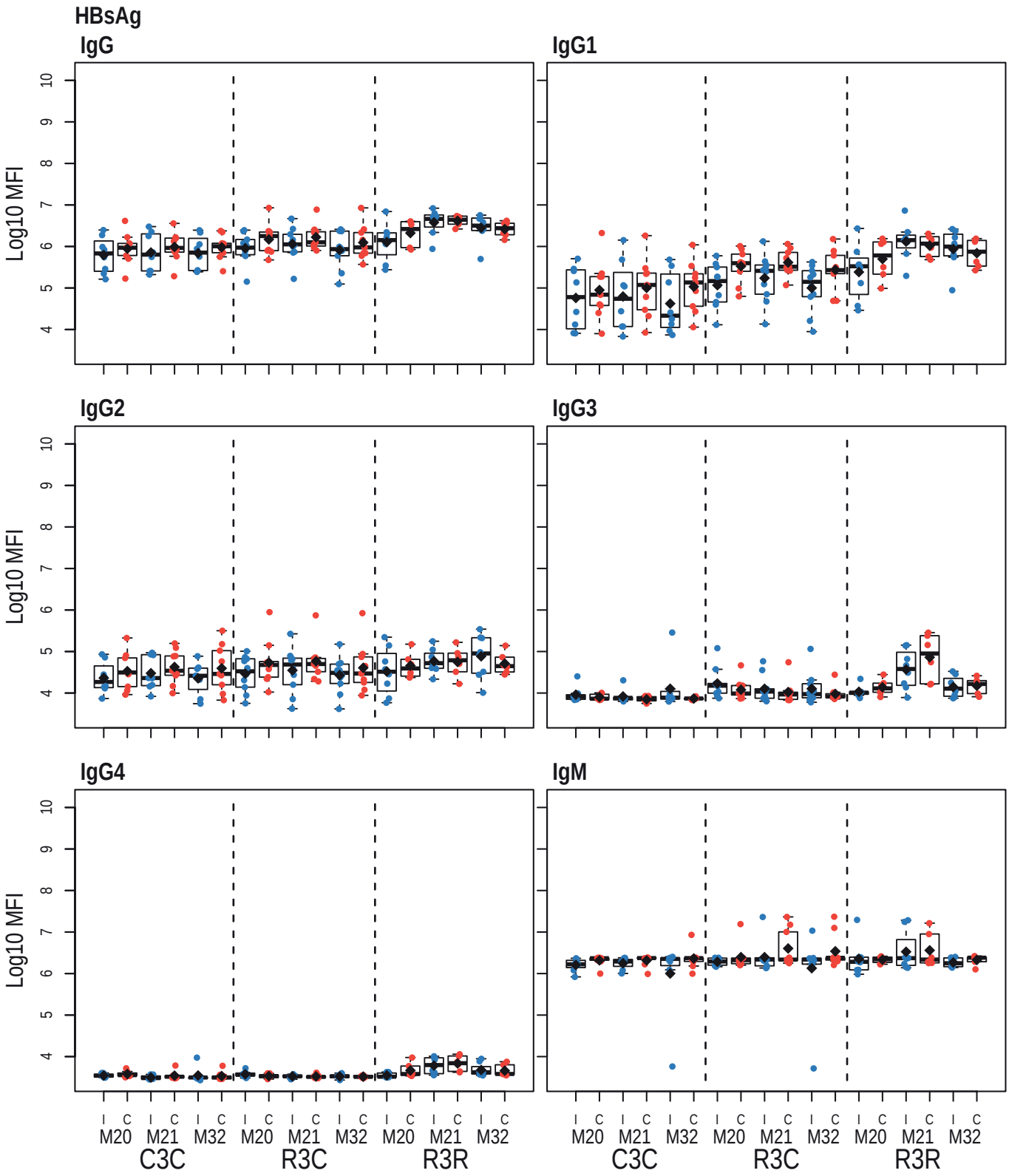
Supplementary Figure 3. RTS,S/AS01E booster and long-term immunogenicity stratified by age: Total IgG, IgG1-4 subclasses and IgM for CSP FL at month (M)20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) 5-17 months (red) and infants (I) 6-12 weeks (blue) at the moment of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P values are reported in supplementary tables. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



Supplementary Figure 4. RTS,S/AS01E booster and long-term immunogenicity stratified by age: Total IgG, IgG1-4 subclasses and IgM for CSP NANP at month (M)20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) 5-17 months (red) and infants (I) 6-12 weeks (blue) at the moment of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P values are reported in supplementary tables. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



Supplementary Figure 5. RTS,S/AS01E booster and long-term immunogenicity stratified by age: Total IgG, IgG1-4 subclasses and IgM for CSP Cterm at month (M)20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) 5-17 months (red) and infants (I) 6-12 weeks (blue) at the moment of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P values are reported in supplementary tables. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

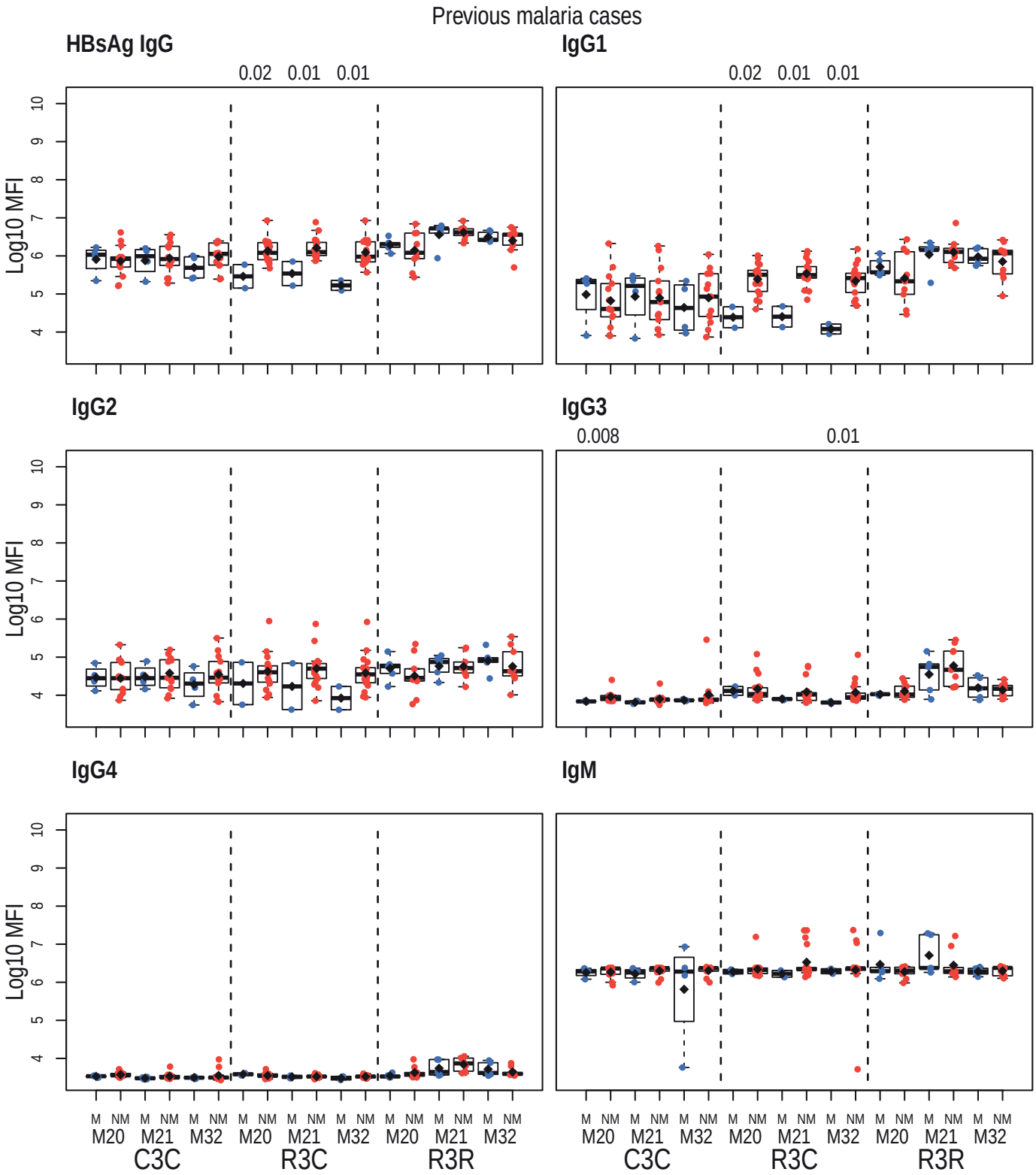


Supplementary Figure 6. RTS,S/AS01E booster and long-term immunogenicity stratified by age: Total IgG, IgG1-4 subclasses and IgM for HBsAg at month (M)20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) 5-17 months (red) and infants (I) 6-12 weeks (blue) at the moment of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P values are reported in supplementary tables. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

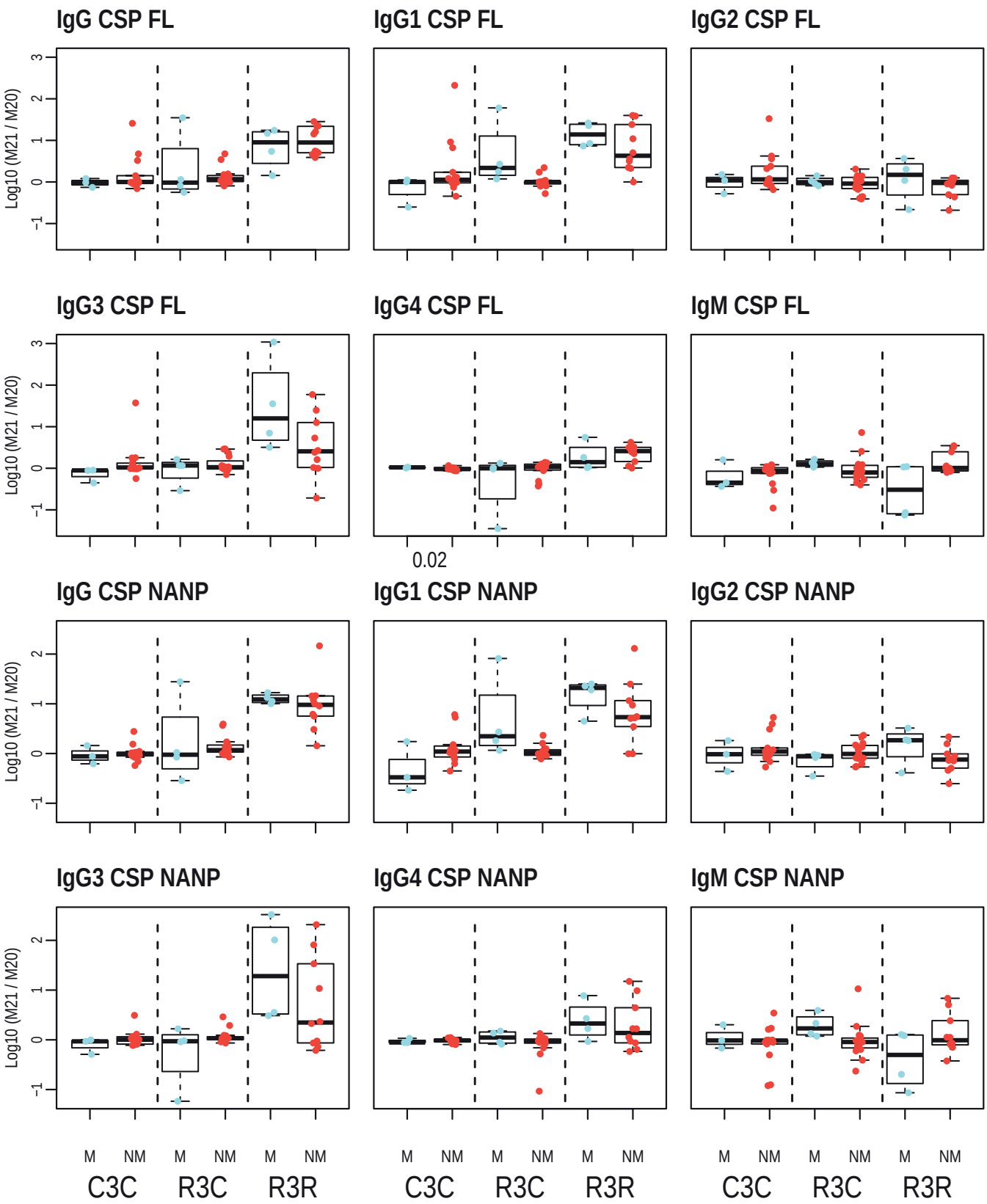
Supplementary Table 3. Immunogenicity to vaccine antigens stratified by previous clinical malaria and by clinical malaria after a booster dose (M21): Non-parametric test p-values for comparison of levels with or without clinical malaria (M vs NM) for IgG, IgG1-4 subclasses and IgM at month (M)20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). P-values <0.05 are highlighted in yellow. P-values were adjusted for multiple comparisons, but none was significant and data are not shown.

		Prebooster cases: Malaria vs No Malaria								
		C3C			R3C			R3R		
Isotype	Antigen	M20	M21	M32	M20	M21	M32	M20	M21	M32
IgG	CSP_FL	0.06	0.30	0.045	0.07	0.07	0.14	0.70	0.44	0.90
IgG	Cterm	0.87	0.16	0.87	0.11	0.07	0.02	0.61	0.30	0.44
IgG	HBsAg	0.55	0.70	0.25	0.02	0.01	0.01	0.70	0.61	0.70
IgG	NANP	0.41	0.55	0.35	0.57	0.42	0.35	0.61	0.90	0.70
IgG1	CSP_FL	0.25	0.55	0.30	0.14	0.14	0.14	0.61	0.15	0.70
IgG1	Cterm	0.78	0.06	0.70	0.07	0.047	0.29	0.80	0.06	0.19
IgG1	HBsAg	0.62	0.87	0.48	0.02	0.01	0.01	0.44	0.80	0.70
IgG1	NANP	0.1	0.70	0.20	0.29	0.19	0.11	0.90	0.52	0.90
IgG2	CSP_FL	<0.001	0.08	0.006	0.84	0.95	0.35	0.44	1	0.61
IgG2	Cterm	1	0.70	0.19	0.35	0.14	0.42	0.30	0.61	0.90
IgG2	HBsAg	0.96	0.62	0.30	0.75	0.65	0.07	0.44	0.90	0.70
IgG2	NANP	0.045	0.30	0.16	0.65	0.55	0.57	0.90	0.36	0.30
IgG3	CSP_FL	0.02	0.08	0.16	0.07	0.19	0.23	1	0.61	0.84
IgG3	Cterm	0.19	0.78	0.30	0.84	0.84	0.75	0.52	0.44	1
IgG3	HBsAg	0.008	0.07	0.73	0.57	0.46	0.01	1	0.44	0.80
IgG3	NANP	0.16	0.70	0.48	0.65	0.65	0.29	1	0.90	0.61
IgG4	CSP_FL	0.46	0.09	0.17	0.95	0.75	0.65	0.24	0.30	0.19
IgG4	Cterm	0.28	0.57	0.23	0.65	0.57	0.84	0.11	0.70	0.24
IgG4	HBsAg	0.13	0.19	0.82	0.26	0.89	0.39	0.31	0.29	0.59
IgG4	NANP	0.95	0.53	0.73	0.95	0.95	0.75	0.30	0.06	0.30
IgM	CSP_FL	0.62	0.30	0.87	0.42	0.42	0.35	0.80	0.30	0.70
IgM	Cterm	0.30	0.35	0.35	0.95	0.84	1	0.80	0.24	0.61
IgM	HBsAg	0.48	0.1	1	0.65	0.11	0.42	0.90	0.24	0.80
IgM	NANP	0.78	0.96	0.70	0.42	0.49	0.29	0.36	0.15	0.30

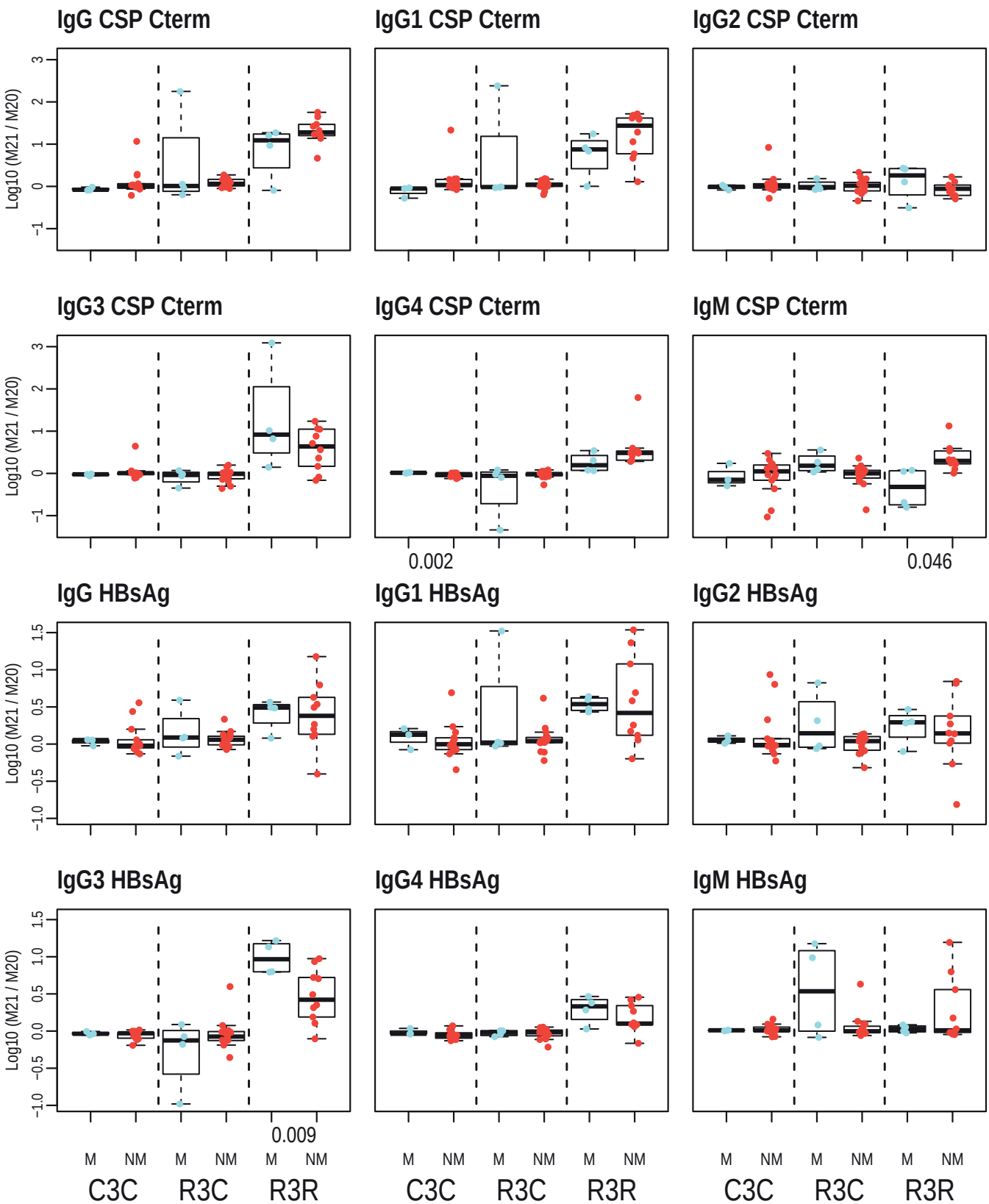
		Postbooster cases: Malaria vs No Malaria								
		C3C			R3C			R3R		
Isotype	Antigen	M20	M21	M32	M20	M21	M32	M20	M21	M32
IgG	CSP_FL	0.36	0.86	0.09	0.81	0.36	0.53	0.64	0.54	0.45
IgG	Cterm	0.43	0.07	0.86	0.89	0.18	0.60	0.37	0.45	0.14
IgG	HBsAg	0.51	0.36	0.30	0.53	0.74	0.47	0.73	0.14	0.73
IgG	NANP	0.43	0.43	0.77	0.81	0.36	0.60	0.95	0.95	0.84
IgG1	CSP_FL	0.20	0.68	0.047	0.41	0.18	0.47	0.95	0.64	0.37
IgG1	Cterm	0.95	0.20	0.77	0.89	0.31	0.66	0.54	0.30	0.19
IgG1	HBsAg	0.59	0.66	0.68	0.22	0.96	1	0.37	0.19	0.45
IgG1	NANP	1	0.43	0.43	0.41	0.74	0.36	0.95	1	1
IgG2	CSP_FL	0.16	0.86	0.047	0.53	0.89	0.26	0.64	0.64	0.54
IgG2	Cterm	0.38	0.30	0.57	0.81	0.81	0.74	0.54	0.30	0.11
IgG2	HBsAg	0.36	0.24	0.20	0.66	0.89	0.31	0.37	0.04	0.14
IgG2	NANP	0.86	0.68	0.86	0.96	0.19	0.1	0.64	0.002	0.004
IgG3	CSP_FL	0.07	0.85	0.02	0.26	0.22	0.89	0.95	0.19	0.14
IgG3	Cterm	0.90	1	0.12	0.31	0.53	0.60	0.45	0.14	0.19
IgG3	HBsAg	0.09	0.34	0.90	0.66	0.73	1	0.19	0.054	0.02
IgG3	NANP	1	0.68	0.51	0.36	0.18	1	0.95	0.45	0.64
IgG4	CSP_FL	0.07	0.75	0.34	0.08	0.36	0.1	0.45	0.45	0.45
IgG4	Cterm	0.34	0.19	0.21	0.08	0.53	0.22	0.73	0.64	0.30
IgG4	HBsAg	0.05	0.80	0.75	0.65	0.51	0.88	0.78	0.32	0.72
IgG4	NANP	1	0.57	0.95	0.89	0.74	0.96	0.95	0.73	0.84
IgM	CSP_FL	0.95	0.36	1	0.41	0.15	1	0.19	1	0.84
IgM	Cterm	0.51	0.36	0.77	0.26	0.08	0.96	0.054	1	0.64
IgM	HBsAg	0.68	0.77	0.77	0.22	0.81	1	0.84	0.54	0.64
IgM	NANP	0.95	0.95	0.95	0.47	0.1	0.89	0.11	0.64	0.54



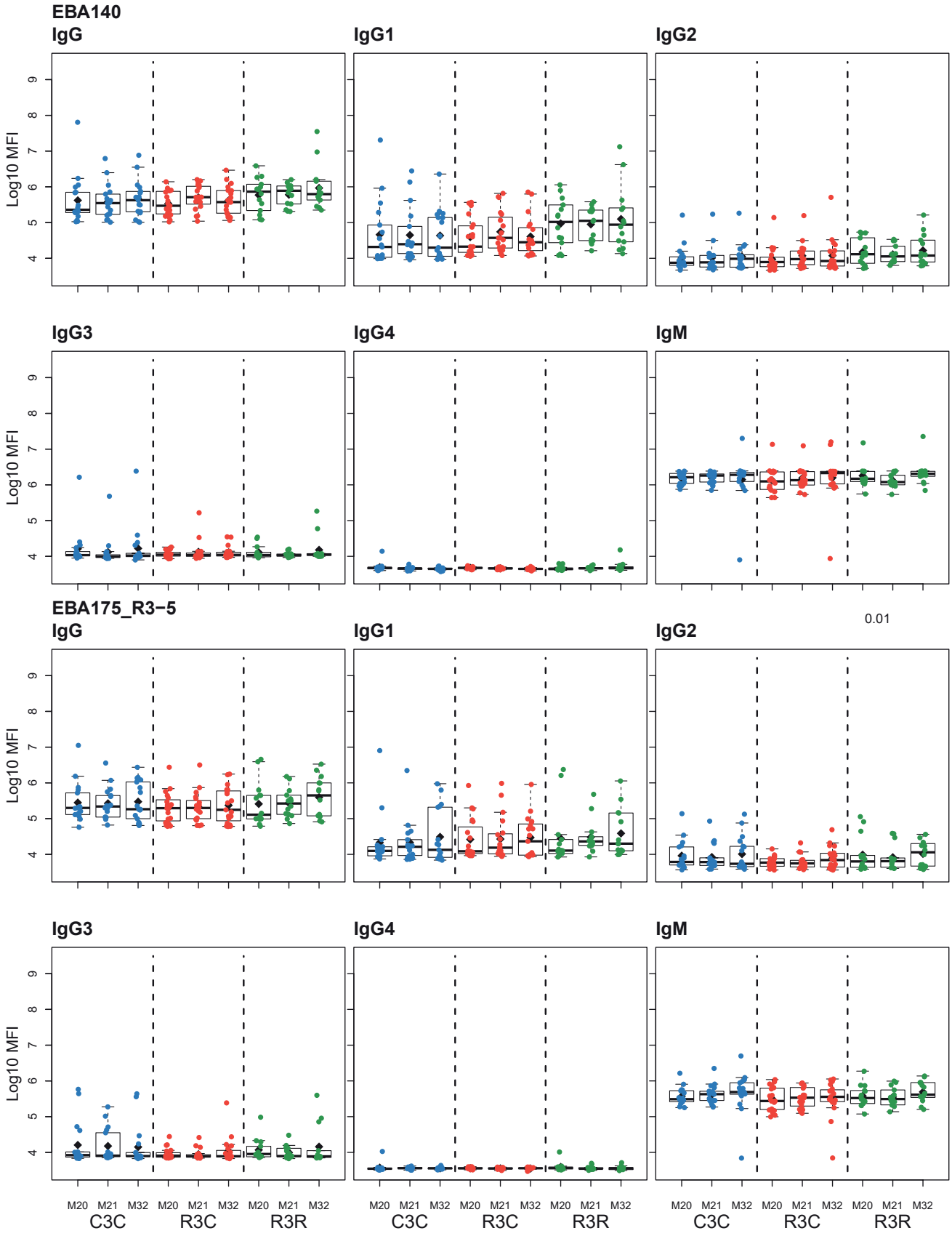
Supplementary Figure 7. Immunogenicity to HBsAg stratified by previous clinical malaria: IgG, IgG1-4 subclasses and IgM at month (M)20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



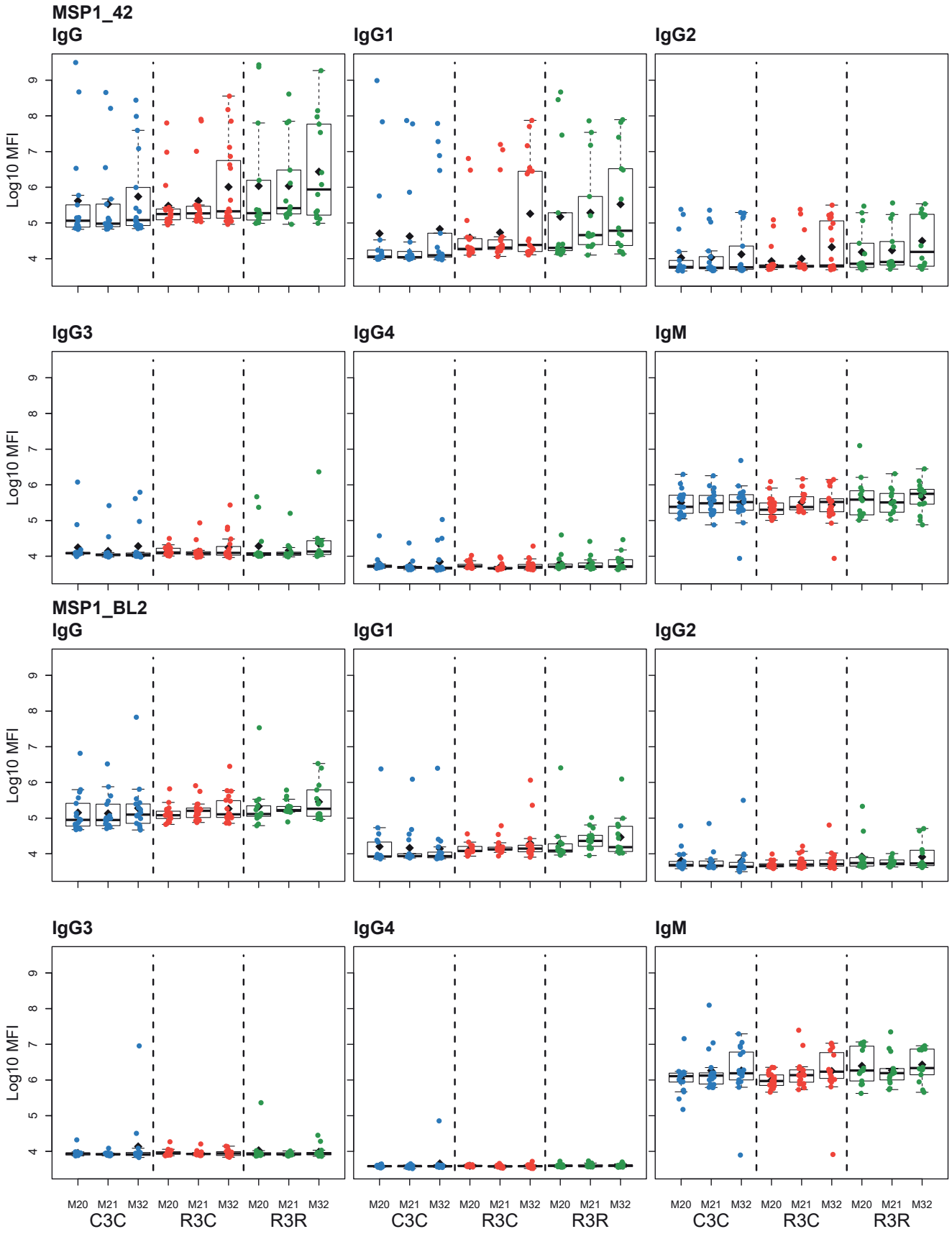
Supplementary Figure 8. Fold change in immunogenicity against vaccine antigens from month (M) 20 to M21 stratified by clinical malaria after M21: Antibody response, IgG, IgG1-4 subclasses and IgM, for CSP FL and CSP NANP, for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without malaria cases (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare fold change with or without clinical malaria. P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



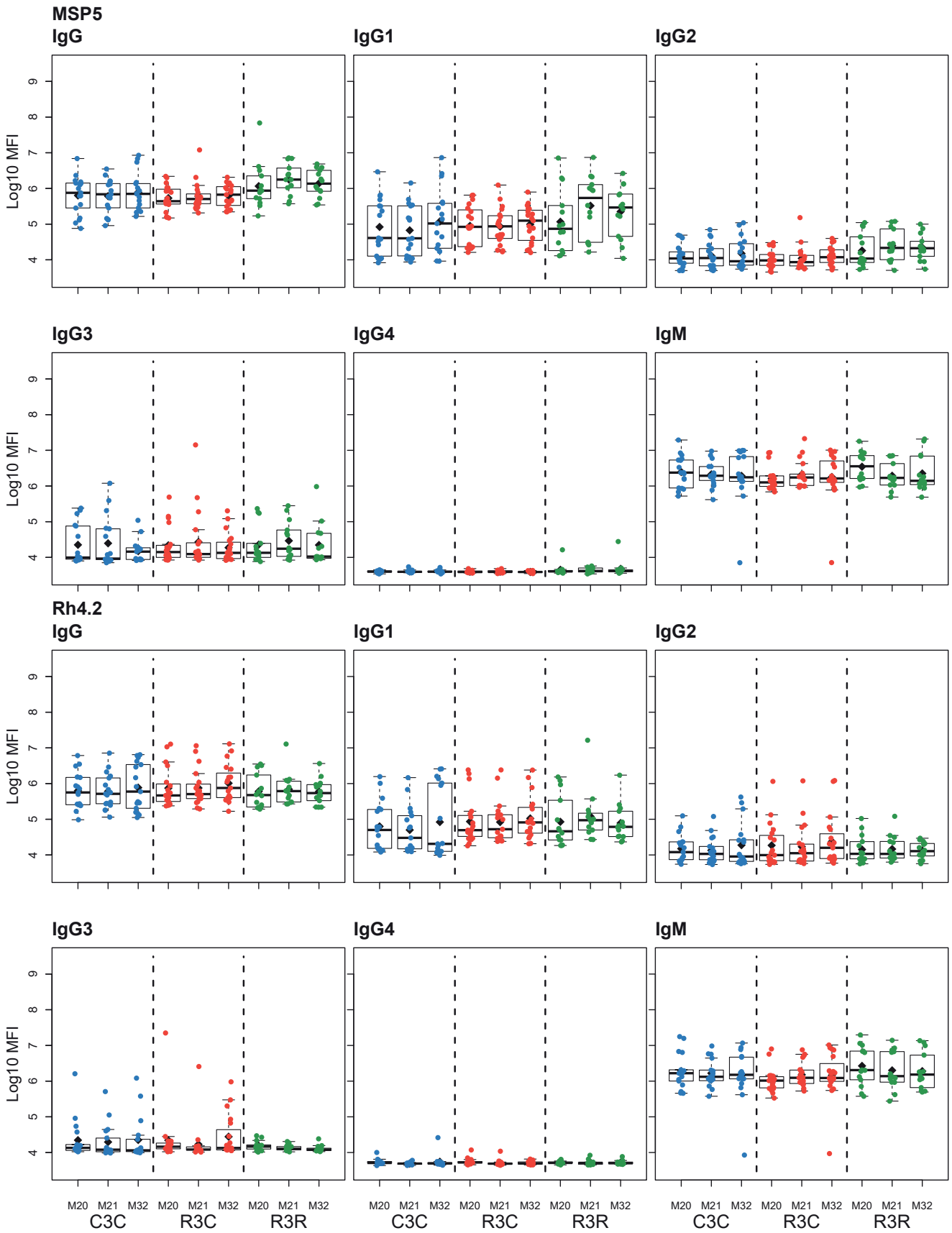
Supplementary Figure 9. Fold change in immunogenicity against vaccine antigens from month (M) 20 to M21 stratified by clinical malaria after M21: Antibody response, IgG, IgG1-4 subclasses and IgM, for CSP Cterm and HBsAg, for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without malaria cases (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare fold change with or without clinical malaria. P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



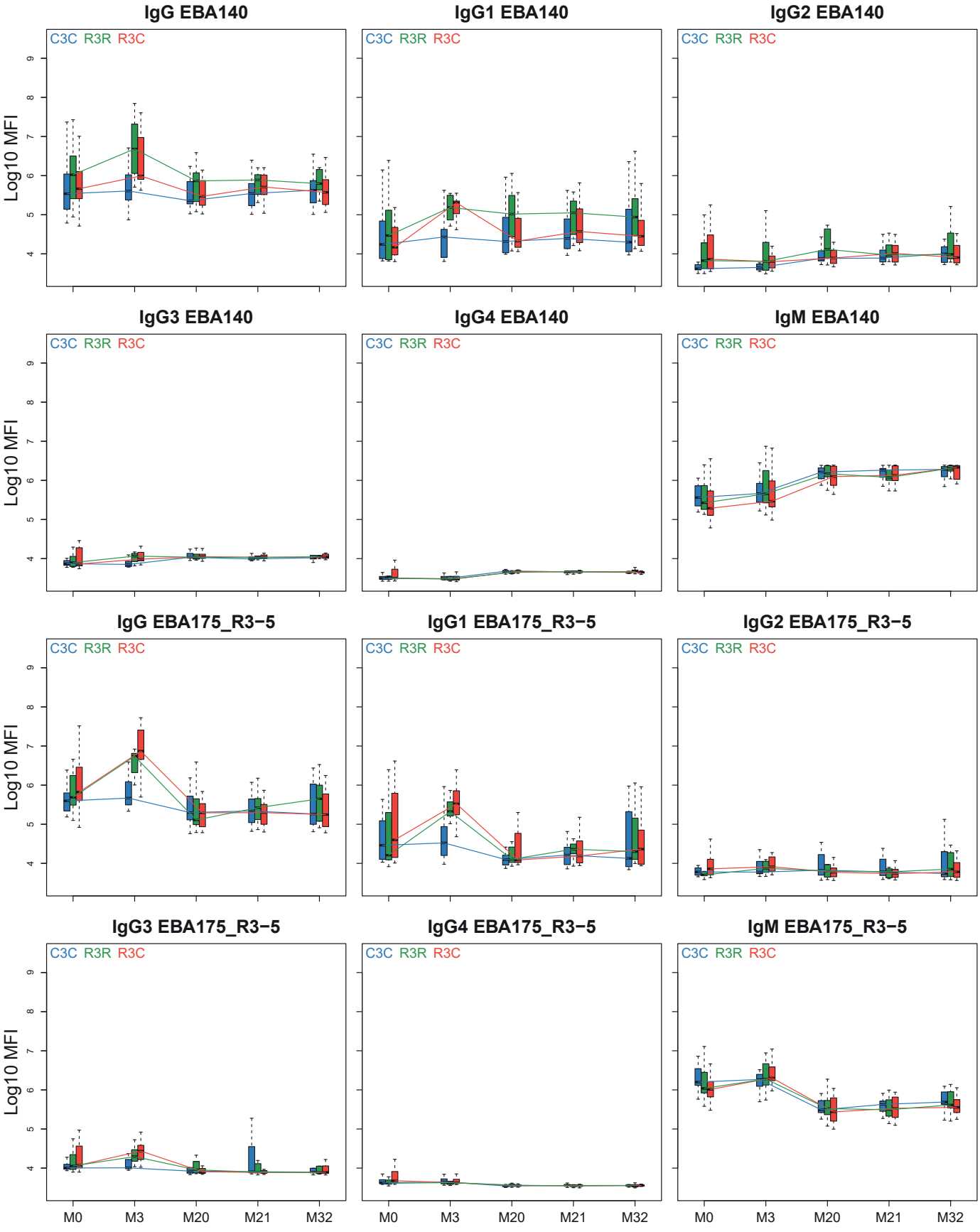
Supplementary Figure 10. RTS,S/AS01E booster and long-term immunogenicity against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for EBA140 and EBA175 R3-5 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21) and the long-term immunogenicity (M21 vs M32), as well as to compare the R3C and R3R groups at each timepoint. P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R (green): three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C (red): three doses of RTS,S/AS01E and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.



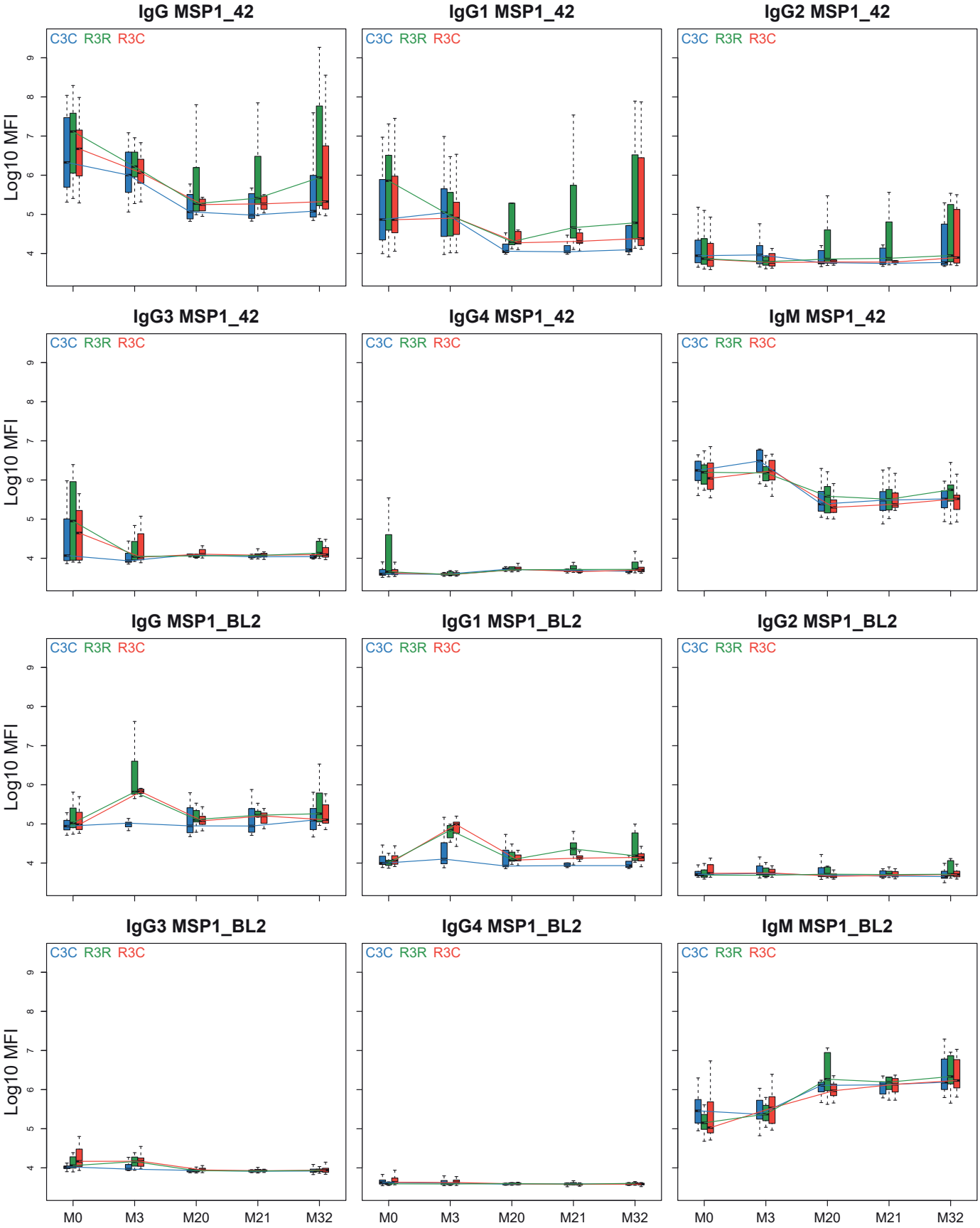
Supplementary Figure 11. RTS,S/AS01E booster and long-term immunogenicity against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for $MSP1_{42}$ and MSP1 Block2 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21) and the long-term immunogenicity (M21 vs M32), as well as to compare the R3C and R3R groups at each timepoint. P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R (green): three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C (red): three doses of RTS,S/AS01E and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.



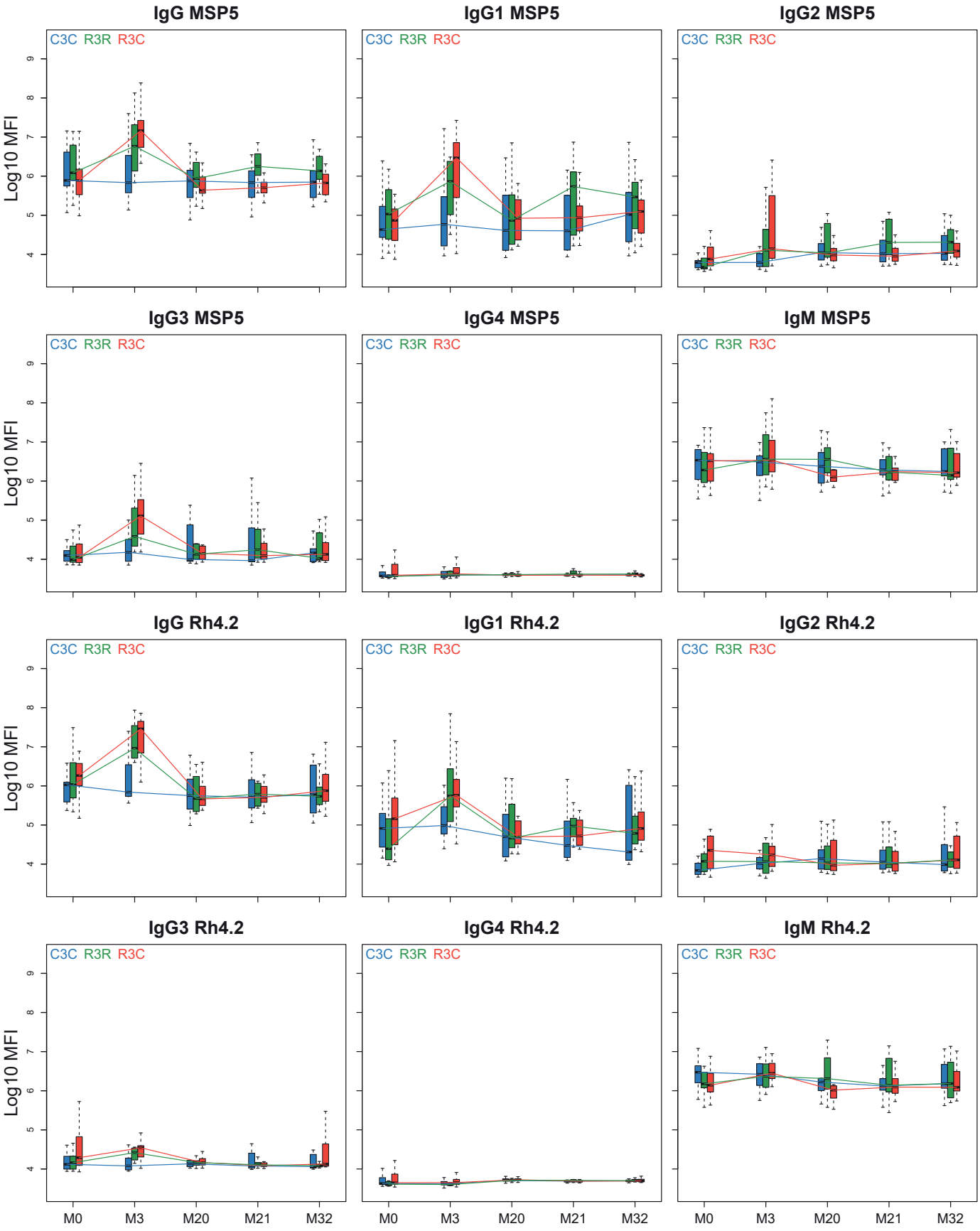
Supplementary Figure 12. RTS,S/AS01E booster and long-term immunogenicity against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for MSP5 and Rh4.2 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21) and the long-term immunogenicity (M21 vs M32), as well as to compare the R3C and R3R groups at each timepoint. P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R (green): three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C (red): three doses of RTS,S/AS01E and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.



Supplementary Figure 13. Antibody responses against blood stage antigens (EBA140 and EBA175 R3-5) for months (M) 0, 3, 20, 21 and 32 for IgG, IgG1-4 and IgM. Boxplots with median and interquartile ranges. The y axis is in logarithm 10 scale. Data from M 0 and 3 were obtained from a previous study in the same individuals [5], thus a batch effect might be present. R3R (green): three doses of RTS,S/AS01E and a RTS,S/AS01E booster at month 20. R3C (red): three doses of RTS,S/AS01E and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.



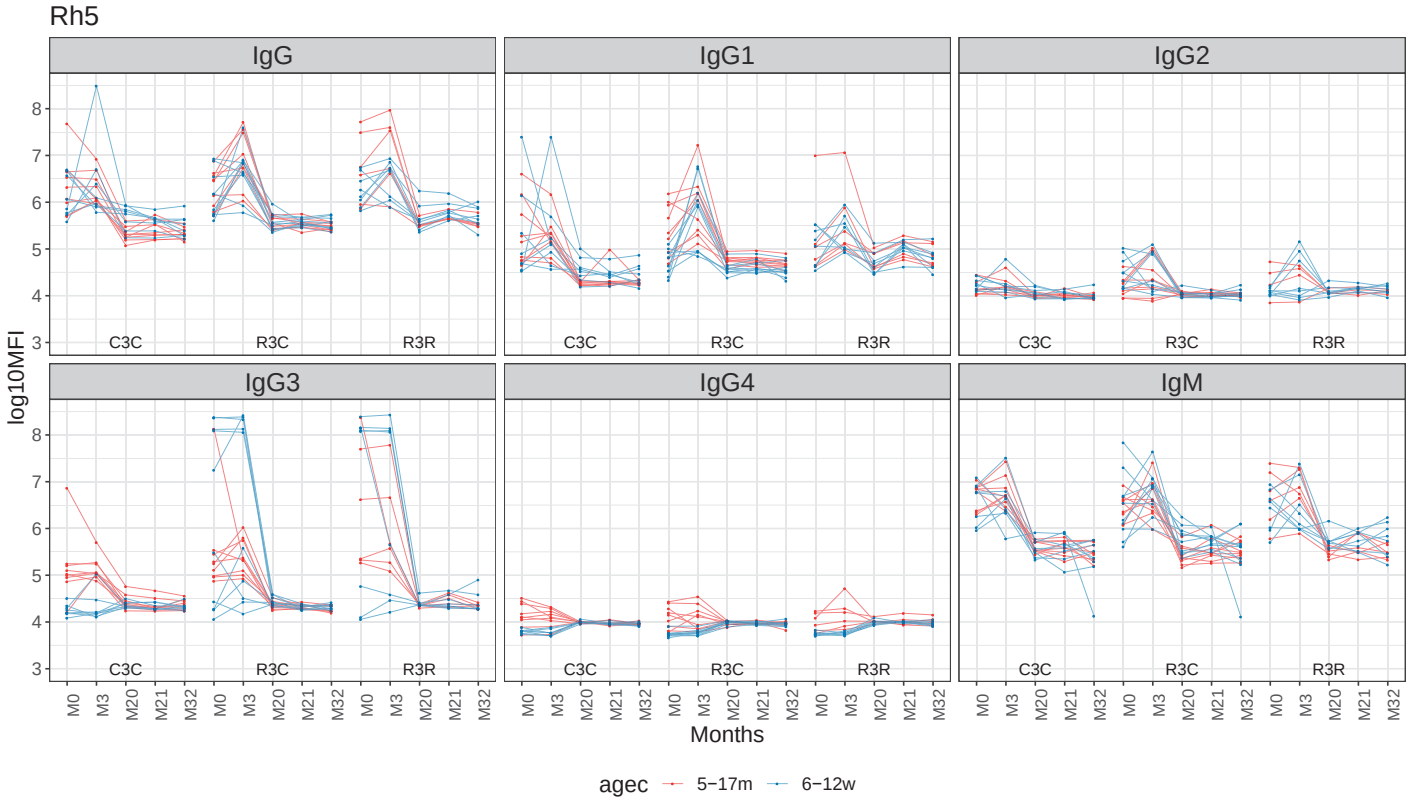
Supplementary Figure 14. Antibody responses against blood stage antigens ($MSP1_{42}$ and MSP1 Block2) for months (M) 0, 3, 20, 21 and 32 for IgG, IgG1-4 and IgM. Boxplots with median and interquartile ranges. The y axis is in logarithm 10 scale. Data from M 0 and 3 were obtained from a previous study in the same individuals [5], thus a batch effect might be present. R3R (green): three doses of RTS,S/AS01E and a RTS,S/AS01E booster at month 20. R3C (red): three doses of RTS,S/AS01E and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.



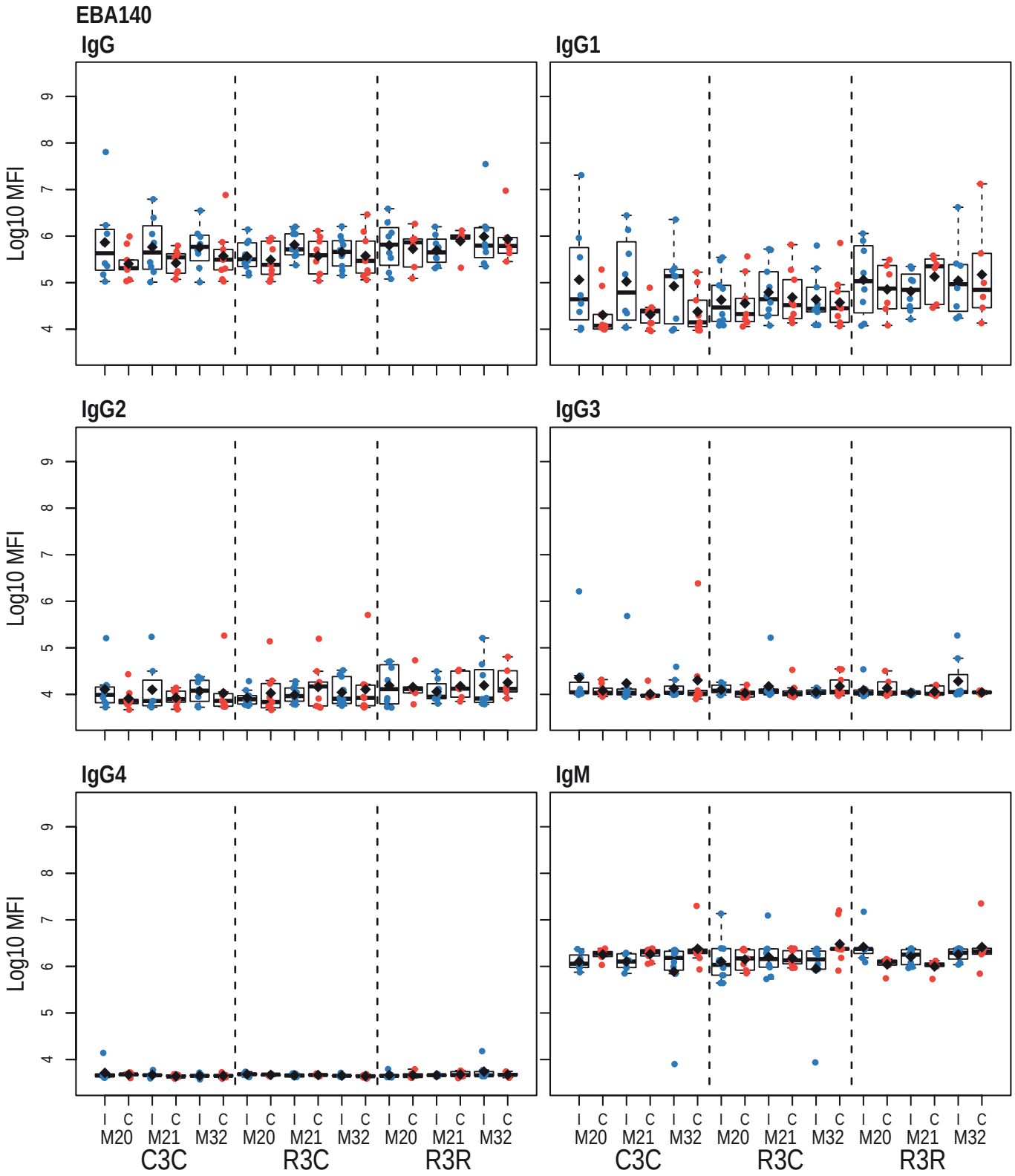
Supplementary Figure 15. Antibody responses against blood stage antigens (MSP5 and Rh4.2) for months (M) 0, 3, 20, 21 and 32 for IgG, IgG1-4 and IgM. Boxplots with median and interquartile ranges. The y axis is in logarithm 10 scale. Data from M 0 and 3 were obtained from a previous study in the same individuals [5], thus a batch effect might be present. R3R (green): three doses of RTS,S/AS01E and a RTS,S/AS01E booster at month 20. R3C (red): three doses of RTS,S/AS01E and a comparator booster. C3C (blue): three doses of a comparator vaccine and a comparator booster.

Supplementary Table 4. RTS,S/AS01E booster and long-term immunogenicity against Plasmodium falciparum blood stage antigens: Comparisons of overall antibody levels between vaccination groups and timepoints. Total IgG, IgG1-4 subclasses and IgM for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Non-parametric tests were used to compare the booster response (M20 vs M21) and the long-term immunogenicity (M21 vs M32), as well as to compare the R3C and R3R groups at each timepoint. Adjusted p values are shown in parenthesis. P-values <0.05 before adjustment are highlighted in yellow and P-values <0.001 before adjustment in green. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

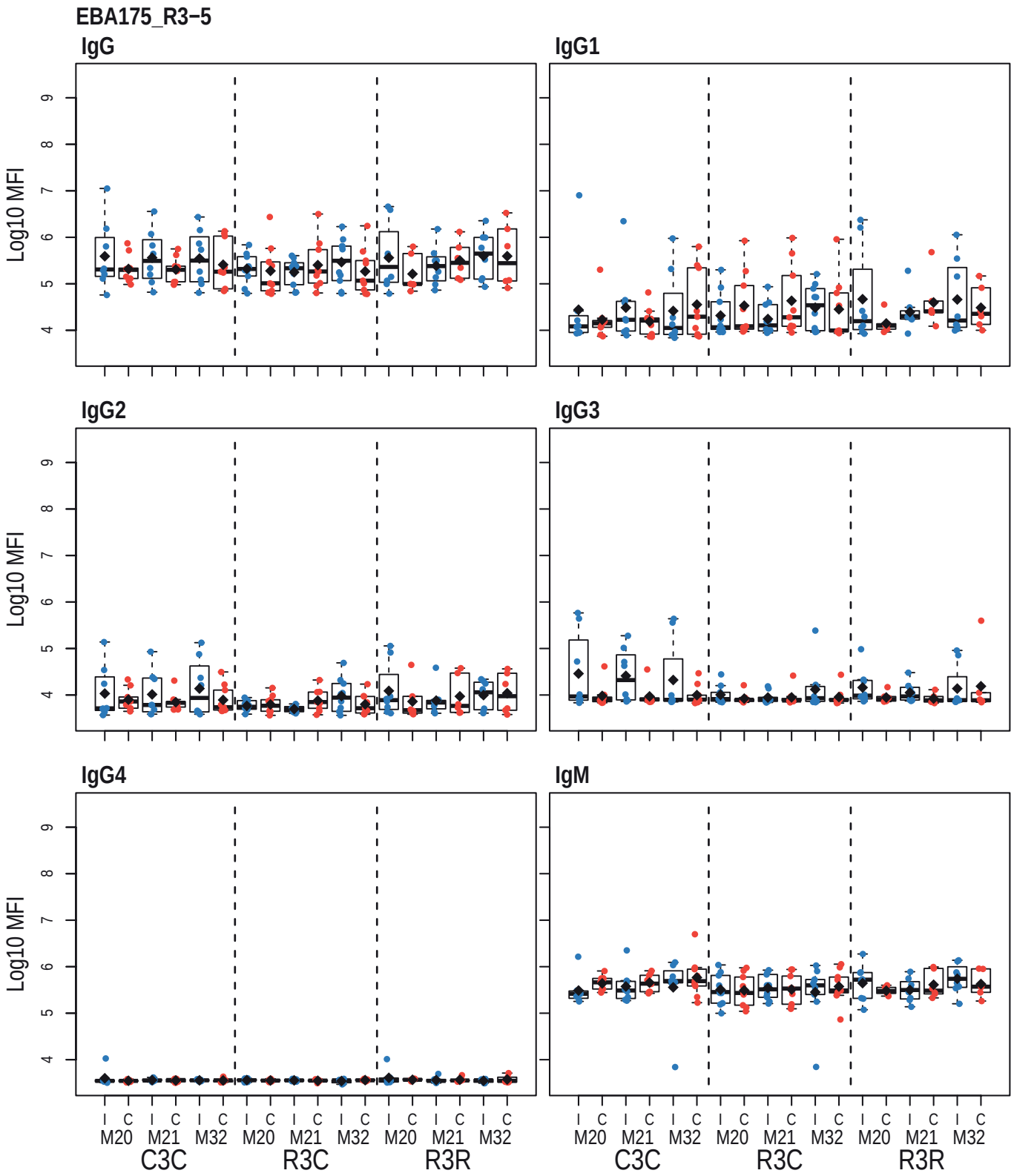
		Comparison between vaccination groups					Comparison Time points		
		R3R vs R3C			R3R vs C3C	R3C vs C3C	M20vsM21	M21vsM32	
Isotype	Antigen	M20	M21	M32	M32	M32	R3R	R3R	R3C
IgG	EBA140	1	1	1	1	1	1	1	1
IgG	EBA175_R3−5	1	1	1	1	1	1	1	1
IgG	MSP1_42	1	1	1	1	1	1	1	1
IgG	MSP1_BL2	1	1	1	1	1	0.33	1	1
IgG	MSP5	1	0.055	0.45	1	1	1	1	1
IgG	Rh4.2	1	1	1	1	1	1	1	0.65
IgG	Rh5	1	<0.001	1	0.13	0.64	0.008	0.33	0.93
IgG1	EBA140	0.56	0.56	0.31	0.33	0.87	0.95	1	0.86
IgG1	EBA175_R3−5	1	0.58	0.58	0.58	0.80	0.69	1	1
IgG1	MSP1_42	0.95	0.11	0.58	0.11	0.18	0.69	1	0.86
IgG1	MSP1_BL2	0.95	0.07	0.62	0.051	0.09	0.28	0.92	1
IgG1	MSP5	1	0.26	0.43	0.58	1	0.69	0.84	1
IgG1	Rh4.2	0.83	0.73	0.83	0.58	0.56	0.80	0.80	0.73
IgG1	Rh5	0.83	0.001	0.11	0.003	0.003	0.01	0.03	0.17
IgG2	EBA140	0.33	0.80	0.55	0.55	1	0.80	1	1
IgG2	EBA175_R3−5	0.87	0.65	0.58	0.92	0.85	0.80	0.86	0.80
IgG2	MSP1_42	0.33	0.11	0.55	0.21	0.56	0.86	0.86	0.69
IgG2	MSP1_BL2	0.21	0.80	0.75	0.22	0.46	1	0.86	1
IgG2	MSP5	0.55	0.11	0.28	0.52	1	0.69	0.86	0.86
IgG2	Rh4.2	1	0.87	0.93	0.93	0.80	0.92	1	0.69
IgG2	Rh5	0.11	0.003	0.03	0.004	0.11	0.34	0.80	1
IgG3	EBA140	1	0.87	1	0.56	0.80	0.80	0.80	0.80
IgG3	EBA175_R3−5	0.56	0.73	1	0.95	0.88	0.80	1	0.86
IgG3	MSP1_42	0.56	1	0.80	0.33	0.56	0.92	0.69	0.83
IgG3	MSP1_BL2	0.58	0.80	1	1	1	1	0.92	1
IgG3	MSP5	1	0.73	1	0.58	0.70	1	0.69	0.83
IgG3	Rh4.2	0.96	0.80	0.22	0.85	0.33	0.72	0.86	0.69
IgG3	Rh5	0.58	0.11	0.62	1	0.56	0.69	0.41	0.80
IgG4	EBA140	0.31	1	0.18	0.33	0.87	0.86	0.80	0.69
IgG4	EBA175_R3−5	0.83	1	1	1	1	0.80	0.86	0.97
IgG4	MSP1_42	1	0.17	0.69	0.55	0.68	0.86	1	0.69
IgG4	MSP1_BL2	0.93	0.58	0.55	0.68	0.95	1	1	0.92
IgG4	MSP5	0.55	0.48	0.11	0.49	0.58	0.89	1	0.83
IgG4	Rh4.2	0.69	0.73	0.80	0.85	1	0.92	0.89	0.80
IgG4	Rh5	1	0.15	0.75	0.83	1	0.95	0.69	0.86
IgM	EBA140	1	1	1	1	1	0.01	0.17	1
IgM	EBA175_R3−5	1	1	1	1	1	1	0.99	1
IgM	MSP1_42	1	1	1	1	1	1	1	1
IgM	MSP1_BL2	0.49	1	1	1	1	1	1	1
IgM	MSP5	0.80	1	1	1	1	0.17	1	1
IgM	Rh4.2	0.86	1	1	1	1	0.24	1	1
IgM	Rh5	1	1	1	1	1	1	1	1



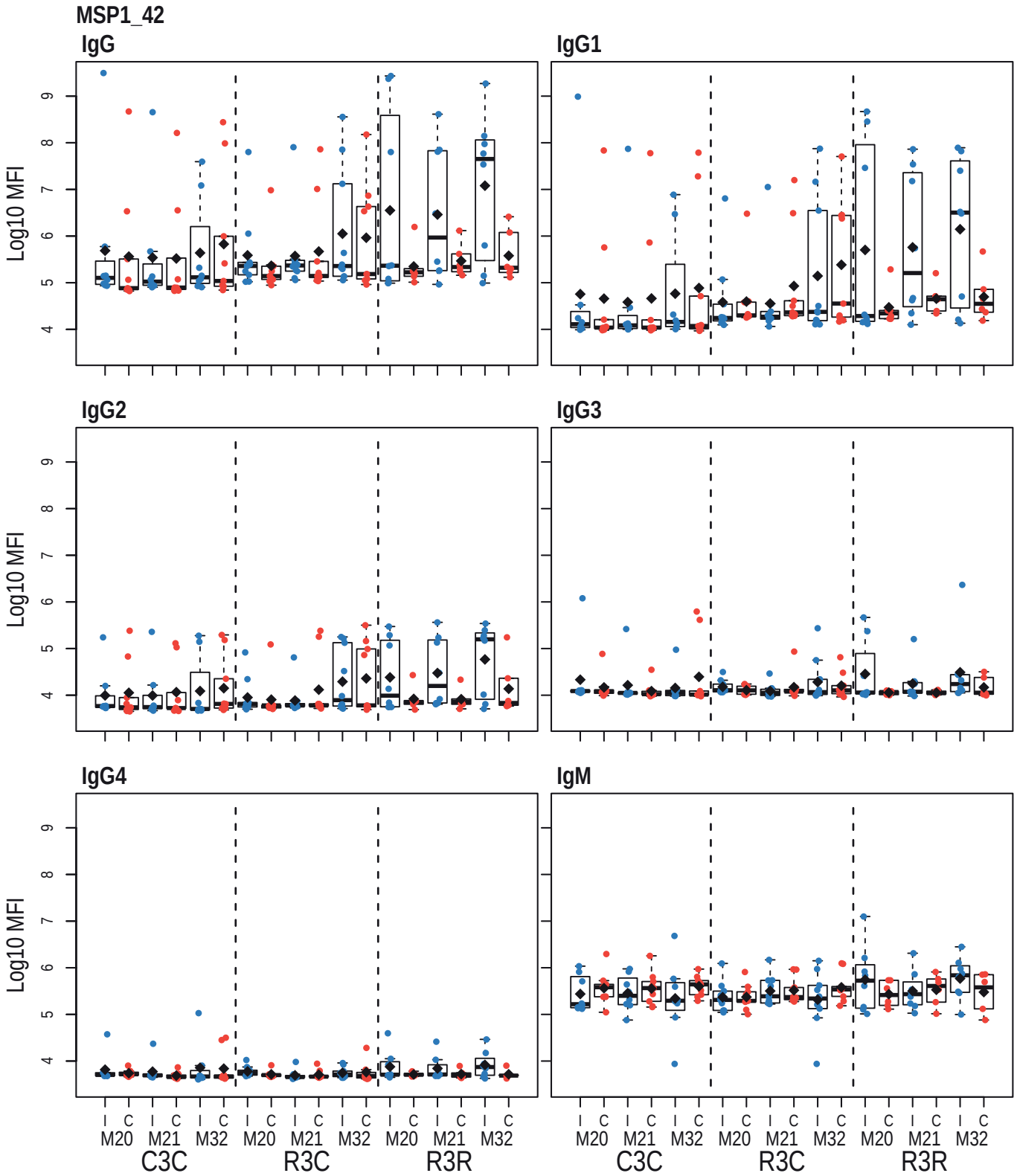
Supplementary Figure 16. Antibody responses against the blood stage antigen Rh5 for months (M) 0, 3, 20, 21 and 32 for IgG, IgG1, IgG2, IgG3, IgG4 and IgM. Line time plots connecting levels for each individual. The y axis is in logarithm 10 scale. Data from months 0 and 3 were obtained from a previous study in the same individuals [5], thus a batch effect might be present. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster at month 20. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster. Children age 5-17 months are shown in orange and infants age 6-12 weeks old at first immunization are shown in blue.



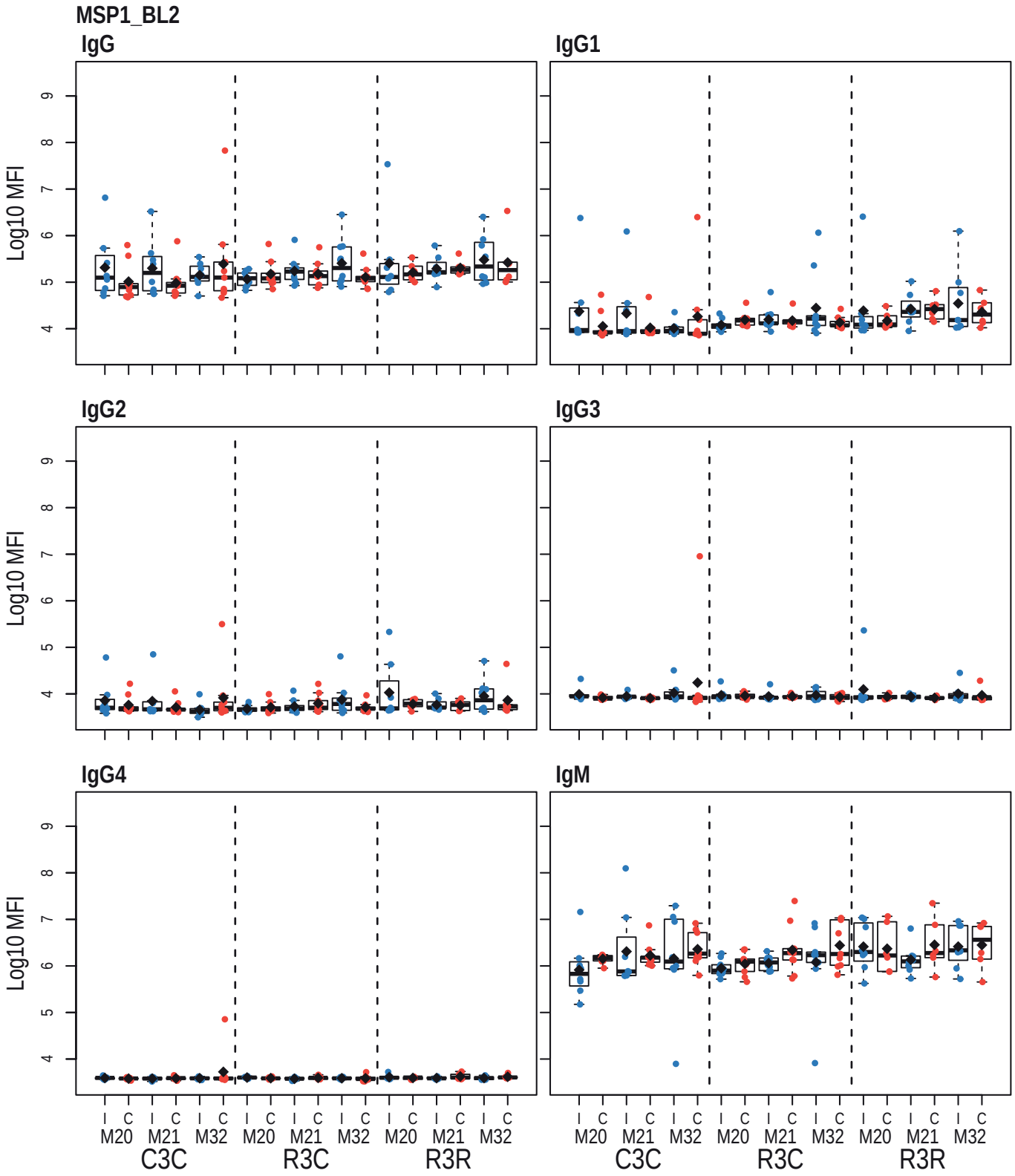
Supplementary Figure 17. RTS,S/AS01E booster and long-term immunogenicity stratified by age against blood stage antigens: IgG, IgG1-4 subclasses and IgM for EBA140 at month (M) 20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) age 5-17 months (red) and infants (I) age 6-12 weeks (blue) at the time of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



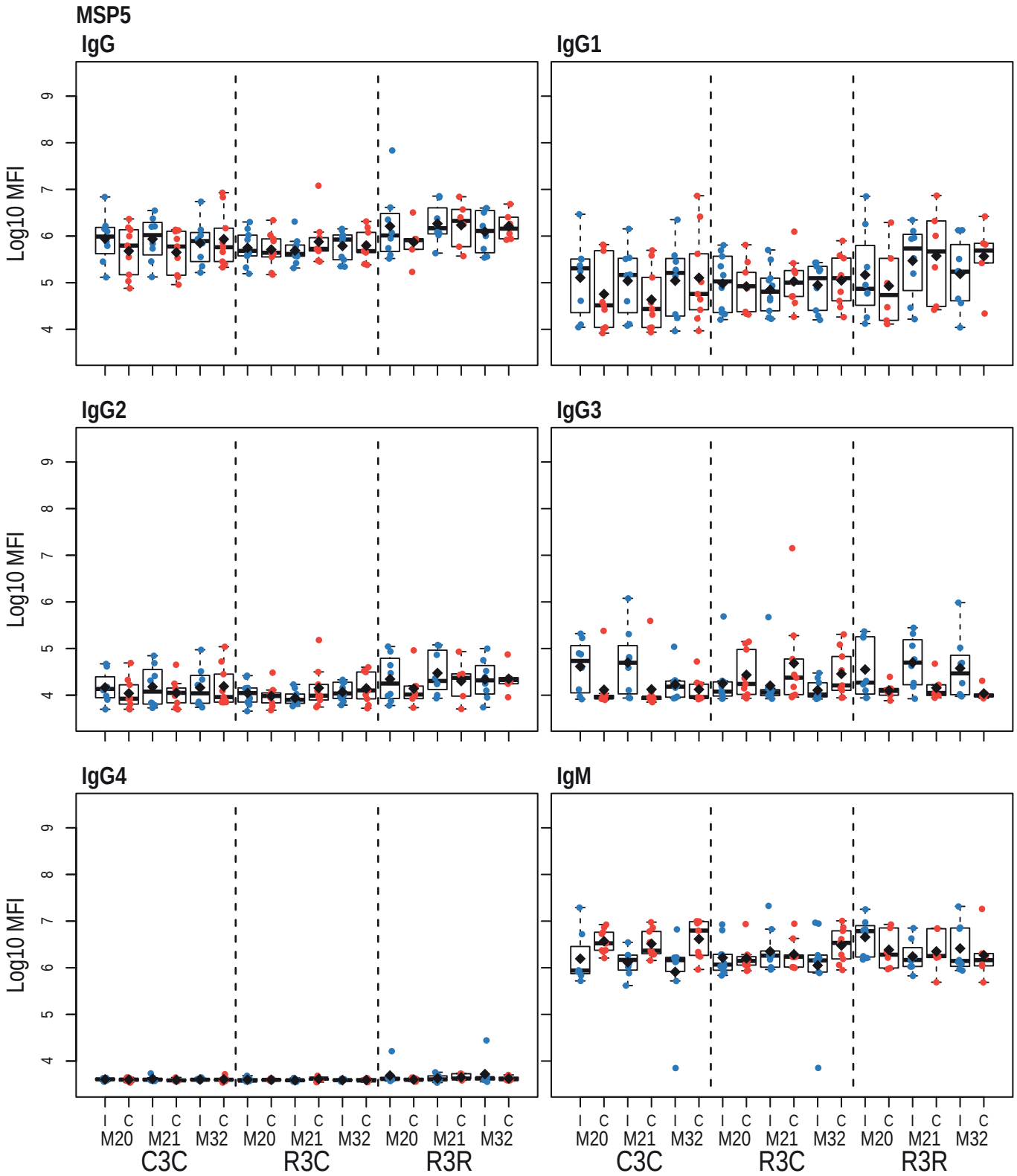
Supplementary Figure 18. RTS,S/AS01E booster and long-term immunogenicity stratified by age against blood stage antigens: IgG, IgG1-4 subclasses and IgM for EBA175 R3-5 at month (M) 20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) age 5-17 months (red) and infants (I) age 6-12 weeks (blue) at the time of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



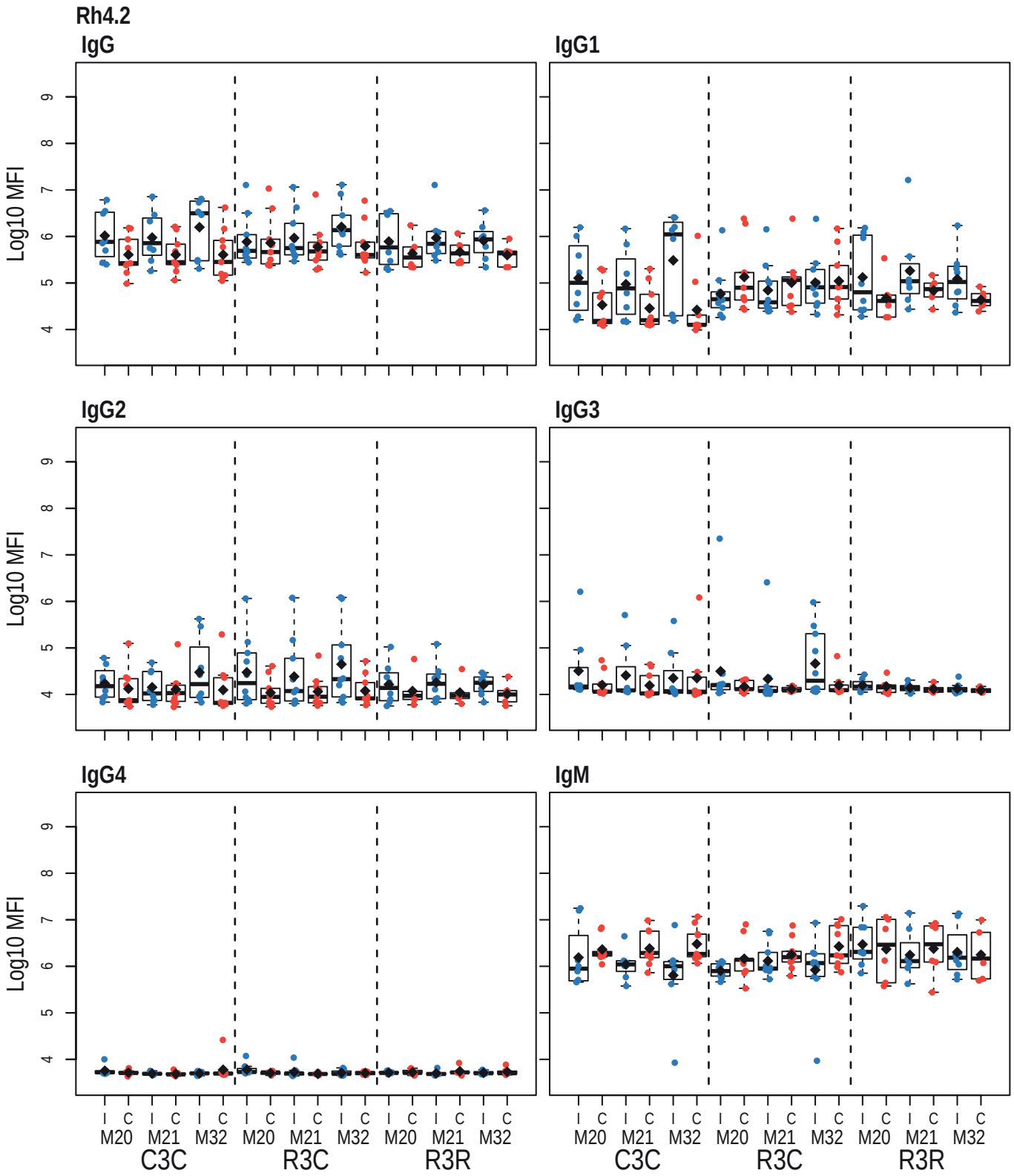
Supplementary Figure 19. RTS,S/AS01E booster and long-term immunogenicity stratified by age against blood stage antigens: IgG, IgG1-4 subclasses and IgM for $MSP1_{42}$ at month (M) 20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) age 5-17 months (red) and infants (I) age 6-12 weeks (blue) at the time of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



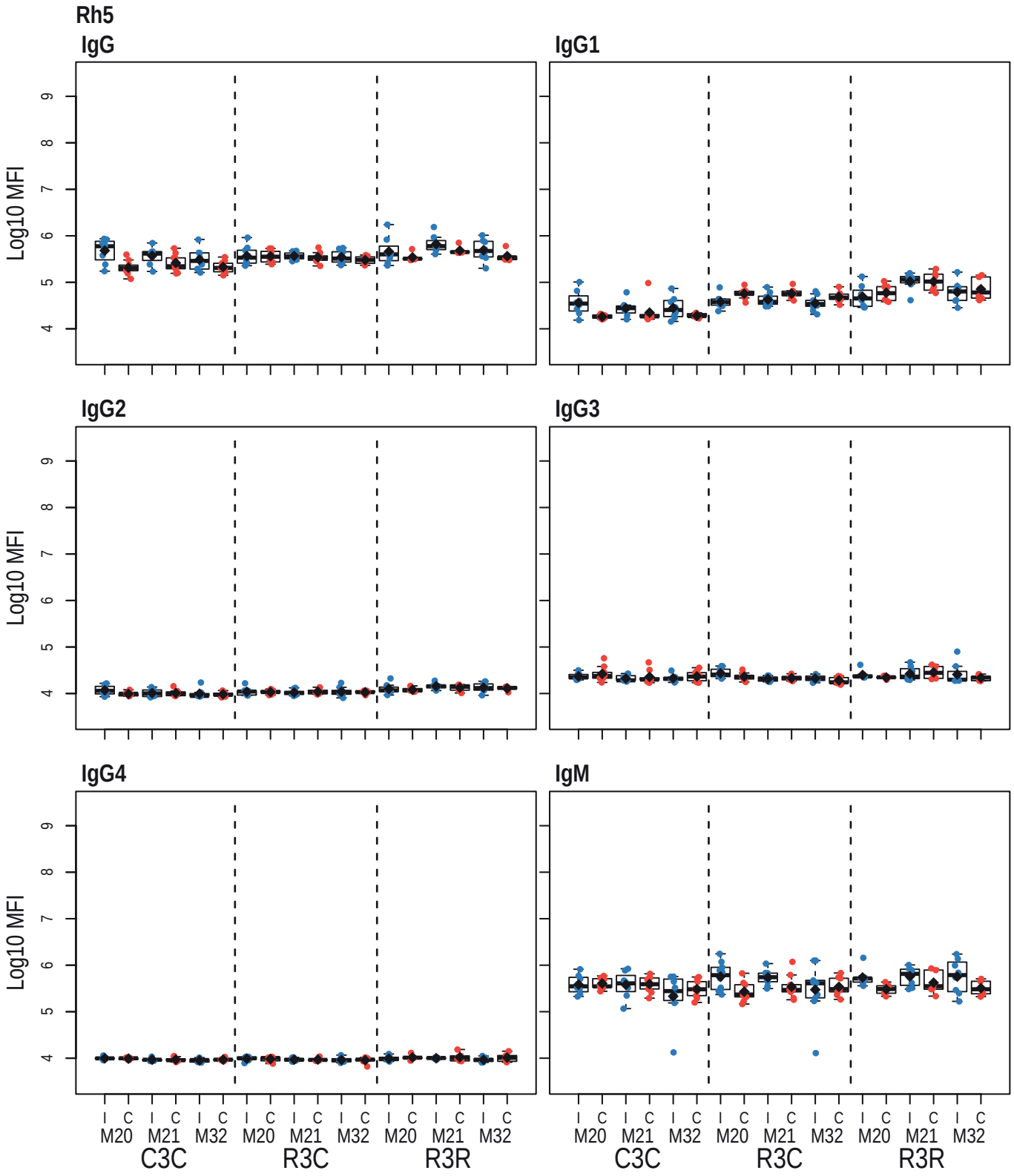
Supplementary Figure 20. RTS,S/AS01E booster and long-term immunogenicity stratified by age against blood stage antigens: IgG, IgG1-4 subclasses and IgM for MSP1 Block2 at month (M) 20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) age 5-17 months (red) and infants (I) age 6-12 weeks (blue) at the time of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



Supplementary Figure 21. RTS,S/AS01E booster and long-term immunogenicity stratified by age against blood stage antigens: IgG, IgG1-4 subclasses and IgM for MSP5 at month (M) 20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) age 5-17 months (red) and infants (I) age 6-12 weeks (blue) at the time of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



Supplementary Figure 22. RTS,S/AS01E booster and long-term immunogenicity stratified by age against blood stage antigens: IgG, IgG1-4 subclasses and IgM for Rh4.2 at month (M) 20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) age 5-17 months (red) and infants (I) age 6-12 weeks (blue) at the time of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



Supplementary Figure 23. RTS,S/AS01E booster and long-term immunogenicity stratified by age against blood stage antigens: IgG, IgG1-4 subclasses and IgM for Rh5 at month (M) 20, M21 and M32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children (C) age 5-17 months (red) and infants (I) age 6-12 weeks (blue) at the time of first vaccination. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). P-values were not significant after adjustment for multiple testing. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.

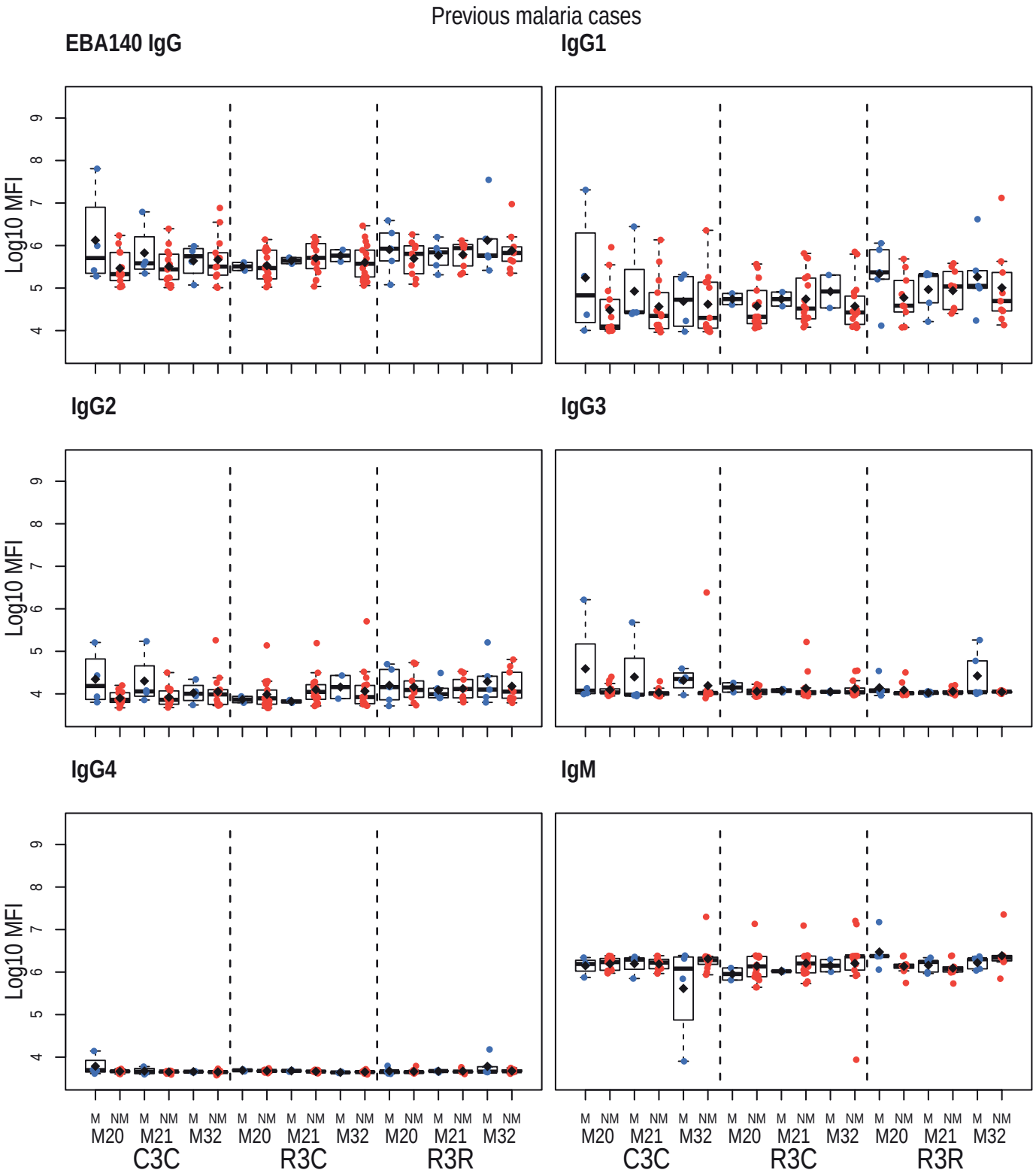
Supplementary Table 5. RTS,S/AS01E booster and long-term immunogenicity against Plasmodium falciparum blood stage antigens stratified by age: Comparisons of antibody levels between timepoints and age groups for total IgG, IgG1-4 subclasses and IgM. RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by age, children 5-17 months and infants 6-12 weeks at the time of first vaccination. Non-parametric tests were used to compare the booster response (M20 vs M21), the long-term immunogenicity (M21 vs M32), and the effect of age (children vs infants). Adjusted p values are shown parenthesis. P-values <0.05 before adjustment are highlighted in yellow.

		Comparison between timepoints						Age comparison					
		M20vsM21		M21vsM32				Children vs Infants					
		R3R		R3R		R3C		R3C			R3R		
Isotype	Antigen	Infants	Children	Infants	Children	Infants	Children	M20	M21	M32	M20	M21	M32
IgG	EBA140	1	1	1	1	1	1	1	1	1	1	1	1
IgG	EBA175_R3−5	1	1	1	1	1	1	1	1	1	1	1	1
IgG	MSP1_42	1	1	1	1	1	1	1	1	1	1	1	1
IgG	MSP1_BL2	1	1	1	1	1	1	1	1	1	1	1	1
IgG	MSP5	1	1	1	1	1	1	1	1	1	1	1	1
IgG	Rh4.2	1	1	1	1	0.08	1	1	1	1	1	1	1
IgG	Rh5	0.96	1	1	1	1	1	1	1	1	1	1	1
IgG1	EBA140	0.95	0.88	0.95	0.95	0.95	0.95	0.99	0.99	0.99	0.99	0.92	1
IgG1	EBA175_R3−5	1	0.65	0.95	0.91	0.91	0.74	0.99	0.99	0.99	0.99	0.99	0.99
IgG1	MSP1_42	0.95	0.68	0.95	0.91	0.95	1	0.92	0.92	0.99	1	0.99	0.98
IgG1	MSP1_BL2	0.78	0.65	1	0.91	0.88	0.74	0.92	1	0.99	0.99	1	0.99
IgG1	MSP5	0.91	0.68	0.84	1	0.95	0.95	1	0.99	0.99	0.99	0.99	0.99
IgG1	Rh4.2	0.91	0.88	0.95	0.74	0.68	1	0.99	0.99	1	0.99	0.99	0.92
IgG1	Rh5	0.65	0.65	0.65	0.65	0.78	0.65	0.92	0.92	0.92	0.99	1	0.99
IgG2	EBA140	0.91	0.91	1	1	0.95	1	0.99	0.99	0.99	0.99	0.99	0.99
IgG2	EBA175_R3−5	0.84	1	0.91	0.95	0.65	0.91	1	0.92	0.99	0.99	0.99	0.99
IgG2	MSP1_42	0.95	0.95	0.95	0.91	0.74	0.95	0.99	0.99	0.99	0.99	0.92	0.98
IgG2	MSP1_BL2	0.95	0.95	0.88	1	0.88	0.74	0.99	0.99	0.99	1	1	0.99
IgG2	MSP5	0.91	0.91	0.88	1	0.74	0.95	0.99	0.99	0.99	0.99	0.99	1
IgG2	Rh4.2	0.84	0.95	1	0.95	0.65	1	0.94	0.99	0.92	0.99	0.99	0.92
IgG2	Rh5	0.73	0.74	0.91	0.91	0.95	0.95	0.99	0.99	1	0.99	0.99	0.99
IgG3	EBA140	1	0.74	0.74	1	0.95	0.74	0.92	0.92	0.99	1	0.99	0.99
IgG3	EBA175_R3−5	0.95	0.84	1	0.95	0.95	0.91	0.99	0.99	0.99	0.98	0.92	1
IgG3	MSP1_42	0.95	0.95	0.88	0.91	0.78	0.95	0.99	0.99	0.99	0.99	0.99	0.99
IgG3	MSP1_BL2	0.91	0.91	0.95	0.95	0.95	0.91	1	0.92	0.99	0.99	0.99	0.99
IgG3	MSP5	0.95	0.91	0.91	0.74	0.95	0.88	0.99	0.99	0.92	0.99	0.92	0.92
IgG3	Rh4.2	0.91	0.84	0.95	0.95	0.74	0.95	0.99	0.99	0.92	0.99	0.99	0.99
IgG3	Rh5	0.95	0.74	0.84	0.68	0.95	0.65	0.92	0.99	0.99	0.94	0.99	1
IgG4	EBA140	0.95	0.95	0.74	0.95	0.95	0.65	0.99	0.99	0.99	0.99	1	1
IgG4	EBA175_R3−5	0.88	0.95	0.95	0.95	0.74	0.95	0.99	0.99	0.99	0.99	0.99	0.99
IgG4	MSP1_42	0.84	0.95	0.95	0.95	0.74	0.95	0.92	0.99	0.99	0.99	0.99	0.99
IgG4	MSP1_BL2	0.91	0.88	1	1	0.78	0.95	0.92	0.99	0.99	0.99	0.99	0.99
IgG4	MSP5	0.95	0.74	0.91	0.68	0.95	0.74	0.99	0.92	1	0.99	0.99	0.99
IgG4	Rh4.2	0.84	0.95	0.78	0.95	0.95	0.74	0.92	0.99	1	1	0.92	1
IgG4	Rh5	0.95	1	0.73	0.95	0.95	0.95	0.99	1	0.99	0.99	1	0.99
IgM	EBA140	1	1	1	1	1	1	1	1	1	0.19	1	1
IgM	EBA175_R3−5	1	1	1	1	1	1	1	1	1	1	1	1
IgM	MSP1_42	1	1	1	1	1	1	1	1	1	1	1	1
IgM	MSP1_BL2	1	1	1	1	1	1	1	1	1	1	1	1
IgM	MSP5	0.33	1	1	1	1	1	1	1	1	1	1	1
IgM	Rh4.2	0.33	1	1	1	1	1	1	1	1	1	1	1
IgM	Rh5	1	1	1	1	1	1	0.53	1	1	0.11	1	1

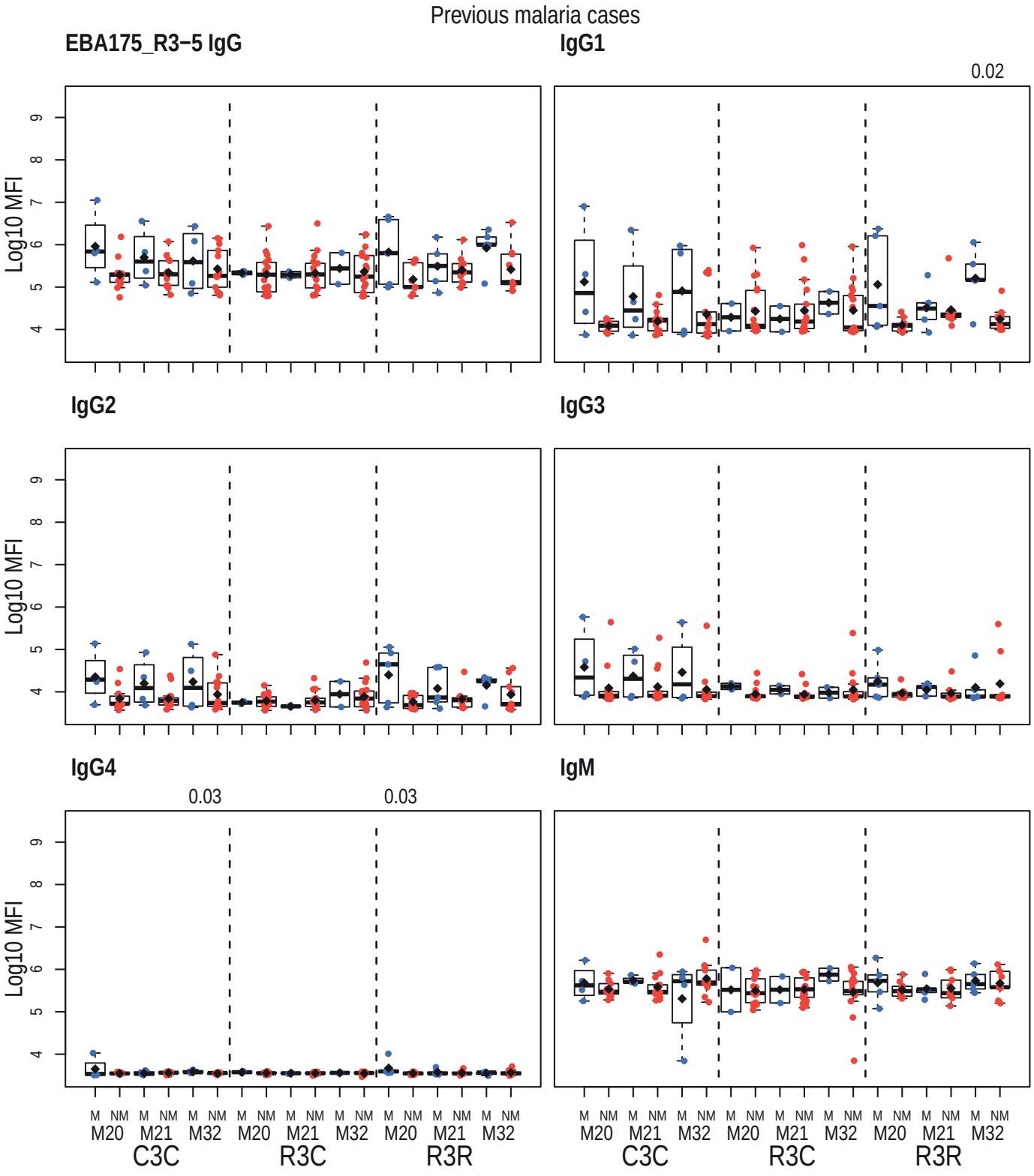
Supplementary Table 6. Immunogenicity to Plasmodium falciparum blood stage antigens stratified by previous clinical malaria (left) and by clinical malaria after booster dose (right): Non-parametric test p-values for comparison of levels with or without clinical malaria (M vs NM) for IgG, IgG1-4 subclasses and IgM at month (M)20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). P-values <0.05 are highlighted in yellow. P-values were adjusted for multiple comparisons, but none was significant and are not shown.

		Prebooster cases: Malaria vs No Malaria								
		C3C			R3C			R3R		
Isotype	Antigen	M20	M21	M32	M20	M21	M32	M20	M21	M32
IgG	EBA140	0.30	0.41	0.78	0.95	0.84	0.49	0.61	0.84	0.80
IgG	EBA175_R3−5	0.1	0.20	0.70	0.89	1	0.65	0.08	0.80	0.08
IgG	MSP1_42	<0.001	<0.001	0.006	0.19	0.19	0.65	0.03	0.11	0.004
IgG	MSP1_BL2	0.48	0.41	0.48	1	0.84	0.11	0.08	0.90	0.24
IgG	MSP5	0.01	0.03	0.70	0.65	0.49	0.49	0.61	0.61	0.61
IgG	Rh4.2	0.30	0.41	0.48	0.57	0.95	0.75	0.44	0.24	0.52
IgG	Rh5	0.48	0.13	0.61	0.42	0.57	0.49	0.61	0.70	0.19
IgG1	EBA140	0.34	0.23	0.62	0.49	0.65	0.23	0.15	1	0.52
IgG1	EBA175_R3−5	0.16	0.41	0.48	0.59	0.57	0.55	0.08	0.80	0.02
IgG1	MSP1_42	0.004	<0.001	0.006	0.84	0.95	0.95	0.004	0.11	0.004
IgG1	MSP1_BL2	0.33	0.39	0.70	0.1	0.55	0.46	0.08	0.80	0.02
IgG1	MSP5	0.08	0.30	0.87	0.29	0.23	0.65	0.19	0.70	0.44
IgG1	Rh4.2	0.96	0.62	0.70	0.01	0.19	0.23	0.52	0.30	0.19
IgG1	Rh5	0.73	0.09	0.70	0.01	0.07	0.29	0.36	0.90	0.42
IgG2	EBA140	0.16	0.19	1	1	0.29	0.46	1	1	0.80
IgG2	EBA175_R3−5	0.13	0.34	0.53	1	0.35	0.84	0.11	0.44	0.36
IgG2	MSP1_42	0.004	0.004	0.01	0.07	0.84	0.42	0.042	0.007	0.06
IgG2	MSP1_BL2	0.17	1	0.28	0.84	0.75	0.13	0.24	0.61	0.11
IgG2	MSP5	0.004	0.006	0.57	0.51	0.51	0.65	0.44	0.61	0.90
IgG2	Rh4.2	0.26	0.30	0.23	0.65	0.49	1	0.52	0.30	0.61
IgG2	Rh5	0.65	0.03	0.53	0.69	0.07	0.84	1	0.84	0.80
IgG3	EBA140	0.73	0.78	0.23	0.32	0.55	1	0.44	0.50	0.84
IgG3	EBA175_R3−5	0.16	0.69	0.73	0.1	0.13	0.95	0.36	0.08	1
IgG3	MSP1_42	0.14	0.03	0.36	0.03	0.07	0.95	0.01	0.08	0.02
IgG3	MSP1_BL2	0.73	0.86	0.14	0.21	0.16	0.04	0.44	0.80	0.46
IgG3	MSP5	0.13	0.21	0.87	0.65	0.95	0.29	0.80	0.90	0.84
IgG3	Rh4.2	0.16	0.13	0.62	0.23	0.1	1	0.19	0.44	0.42
IgG3	Rh5	0.69	0.73	0.87	0.35	0.054	0.32	0.39	0.61	0.79
IgG4	EBA140	0.26	0.78	0.73	0.59	0.55	0.64	0.95	0.59	0.79
IgG4	EBA175_R3−5	0.65	0.48	0.03	0.32	0.79	0.69	0.03	1	1
IgG4	MSP1_42	0.054	0.01	0.02	0.07	0.054	0.55	0.11	0.23	0.02
IgG4	MSP1_BL2	0.36	0.28	0.73	0.18	0.79	0.16	0.79	0.35	0.64
IgG4	MSP5	0.08	0.39	0.13	0.26	0.32	0.46	0.89	0.19	0.69
IgG4	Rh4.2	0.91	0.19	0.57	0.59	0.32	0.55	0.46	1	0.50
IgG4	Rh5	0.31	0.65	0.87	0.64	0.35	0.23	0.70	0.36	0.39
IgM	EBA140	0.62	0.78	0.55	0.42	0.42	0.49	0.15	0.90	0.36
IgM	EBA175_R3−5	0.62	0.1	0.70	1	0.95	0.14	0.44	1	0.90
IgM	MSP1_42	0.16	0.25	0.62	0.19	1	0.23	0.02	0.61	0.02
IgM	MSP1_BL2	0.62	0.87	0.03	0.75	0.95	0.84	0.30	0.61	0.70
IgM	MSP5	0.16	0.55	0.96	0.42	0.29	0.42	0.52	0.52	0.70
IgM	Rh4.2	0.35	0.35	0.30	0.42	0.35	0.95	0.80	0.44	1
IgM	Rh5	0.20	0.16	0.55	0.07	0.14	0.57	0.61	0.19	0.24

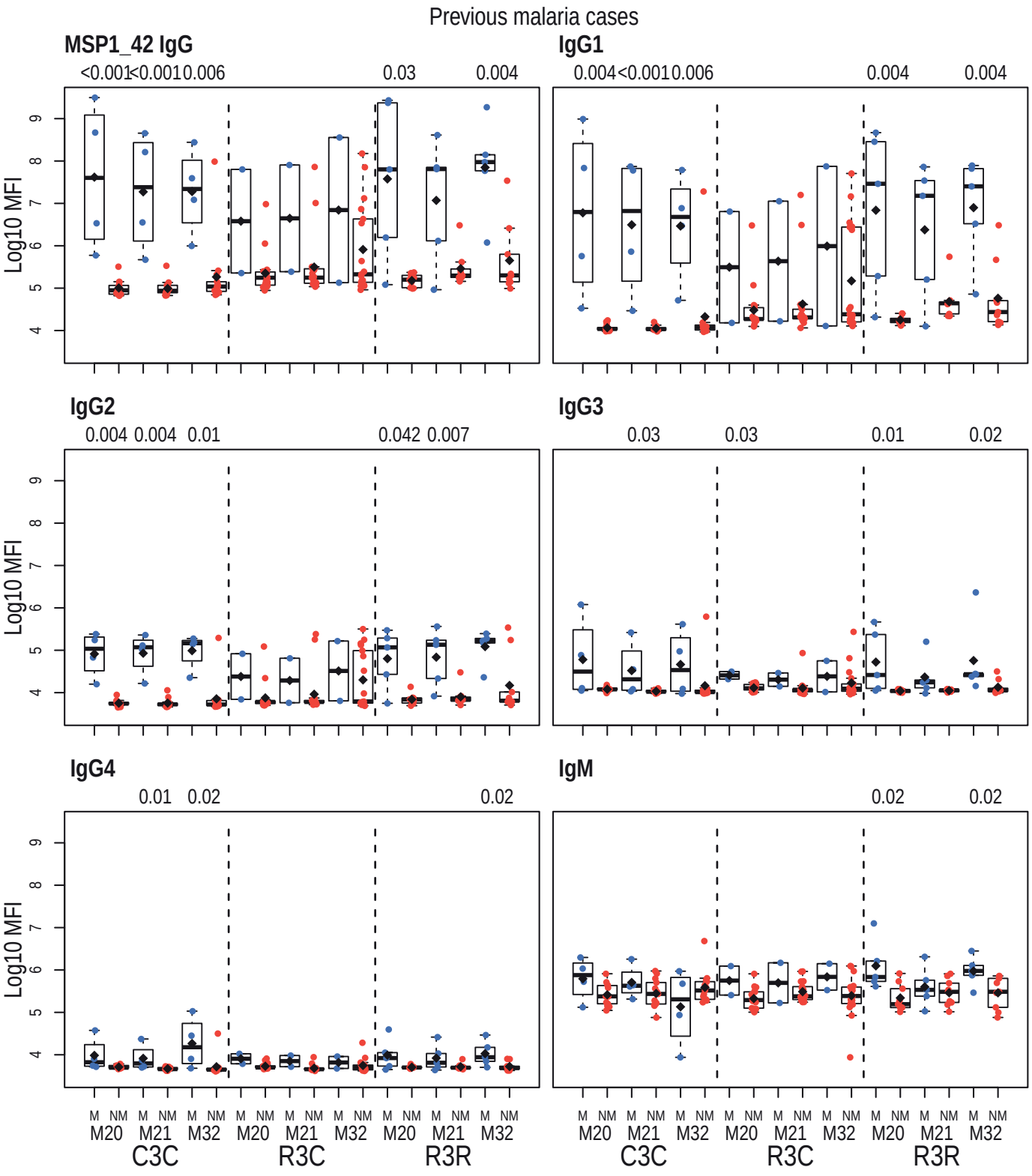
		Postbooster cases: Malaria vs No Malaria								
		C3C			R3C			R3R		
Isotype	Antigen	M20	M21	M32	M20	M21	M32	M20	M21	M32
IgG	EBA140	0.68	0.43	0.59	0.81	0.41	0.81	0.73	0.1	0.02
IgG	EBA175_R3−5	0.07	0.12	0.01	0.15	0.26	0.009	0.95	0.14	0.37
IgG	MSP1_42	0.047	0.047	0.03	0.22	0.89	0.08	0.73	0.37	0.054
IgG	MSP1_BL2	0.68	0.86	0.047	0.049	0.53	0.60	0.37	0.19	0.30
IgG	MSP5	0.16	0.51	0.16	0.41	0.81	0.08	0.64	0.08	0.64
IgG	Rh4.2	0.95	0.68	0.12	0.96	0.96	0.74	0.64	0.73	0.45
IgG	Rh5	0.24	0.16	0.15	0.53	0.41	0.1	0.37	0.30	0.73
IgG1	EBA140	1	0.90	0.59	0.74	0.96	0.36	0.64	0.64	0.30
IgG1	EBA175_R3−5	0.09	0.43	0.003	0.65	0.74	0.01	0.48	0.054	0.30
IgG1	MSP1_42	0.07	0.047	0.09	0.03	0.31	0.15	0.84	0.30	0.054
IgG1	MSP1_BL2	0.66	0.57	0.16	0.23	0.45	0.96	1	0.30	0.19
IgG1	MSP5	0.24	0.68	0.20	0.74	0.26	0.009	0.84	0.24	0.73
IgG1	Rh4.2	0.43	0.68	0.68	0.53	0.89	0.60	0.73	0.84	0.84
IgG1	Rh5	0.80	0.34	0.59	0.89	0.89	0.31	0.73	1	0.89
IgG2	EBA140	0.43	0.75	0.77	0.73	0.41	0.40	0.54	0.26	0.01
IgG2	EBA175_R3−5	0.07	0.15	0.03	0.25	0.31	0.01	0.95	0.04	0.04
IgG2	MSP1_42	0.04	0.051	0.07	0.23	0.84	0.15	0.95	0.19	0.002
IgG2	MSP1_BL2	0.21	0.75	0.03	0.62	0.96	0.84	0.11	0.04	0.11
IgG2	MSP5	0.17	0.30	0.23	0.58	0.34	0.06	0.73	0.04	0.24
IgG2	Rh4.2	0.90	0.24	0.49	0.41	0.60	0.31	0.73	0.30	0.04
IgG2	Rh5	0.57	0.07	0.49	0.55	0.48	0.73	0.72	0.29	0.008
IgG3	EBA140	0.53	0.75	0.07	0.21	0.76	0.69	0.84	0.44	0.006
IgG3	EBA175_R3−5	0.12	0.34	0.02	0.02	0.45	0.02	0.54	0.62	0.72
IgG3	MSP1_42	0.41	0.16	0.044	0.34	0.66	0.12	0.84	0.48	0.054
IgG3	MSP1_BL2	0.85	0.85	0.051	1	0.76	1	0.64	0.84	0.29
IgG3	MSP5	0.95	1	0.41	0.41	0.80	0.1	1	0.30	0.72
IgG3	Rh4.2	0.047	0.03	0.01	1	0.88	0.12	0.64	0.95	0.44
IgG3	Rh5	0.28	0.61	0.01	0.47	0.80	0.29	0.04	0.04	0.14
IgG4	EBA140	0.75	0.01	0.66	0.32	0.04	0.03	0.48	0.16	0.02
IgG4	EBA175_R3−5	0.25	0.24	0.06	0.34	0.84	0.1	0.67	0.32	0.52
IgG4	MSP1_42	0.71	0.31	0.16	0.29	0.45	0.051	0.78	0.12	0.08
IgG4	MSP1_BL2	0.08	0.41	1	0.31	0.13	0.12	0.57	0.57	0.26
IgG4	MSP5	0.53	0.41	0.07	0.19	0.19	0.29	0.72	0.054	0.12
IgG4	Rh4.2	0.70	0.66	0.31	0.92	0.057	0.29	0.62	0.67	1
IgG4	Rh5	0.07	0.75	0.43	0.58	0.51	0.41	0.37	0.73	0.72
IgM	EBA140	0.86	0.16	0.43	0.1	1	0.31	0.11	0.19	0.84
IgM	EBA175_R3−5	0.95	0.36	0.36	0.74	0.96	0.66	0.054	0.04	0.054
IgM	MSP1_42	0.95	1	0.95	0.74	0.18	0.1	0.45	0.37	0.64
IgM	MSP1_BL2	0.95	0.51	0.09	0.66	0.53	0.47	0.24	0.24	0.95
IgM	MSP5	1	0.86	0.43	0.53	0.53	0.96	0.30	0.64	0.054
IgM	Rh4.2	0.95	0.77	0.95	0.31	0.18	0.60	0.14	0.19	0.19
IgM	Rh5	0.36	0.30	0.95	0.31	0.22	0.15	0.08	0.24	0.02



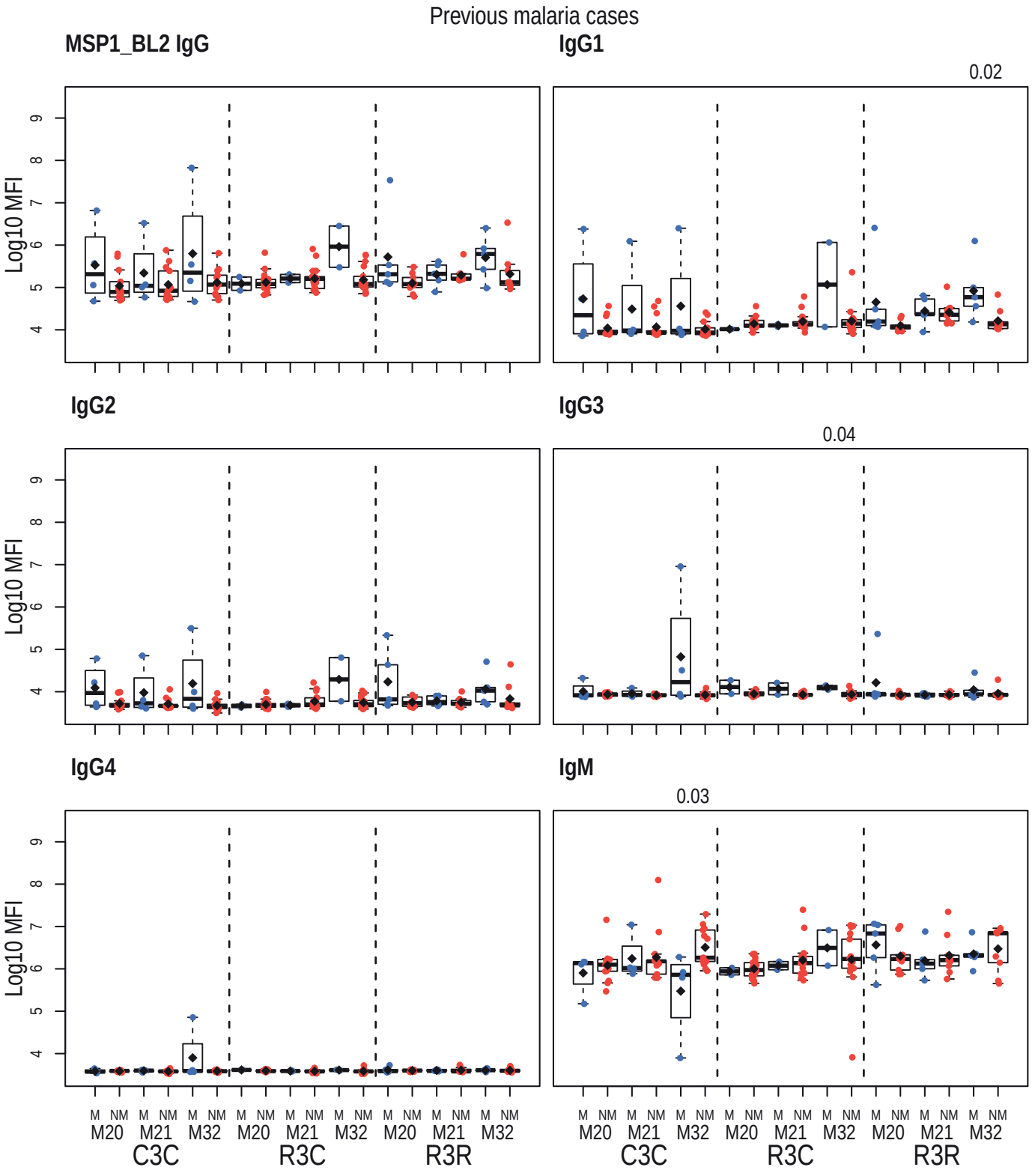
Supplementary Figure 24. Immunogenicity stratified by previous clinical malaria against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for EBA140 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



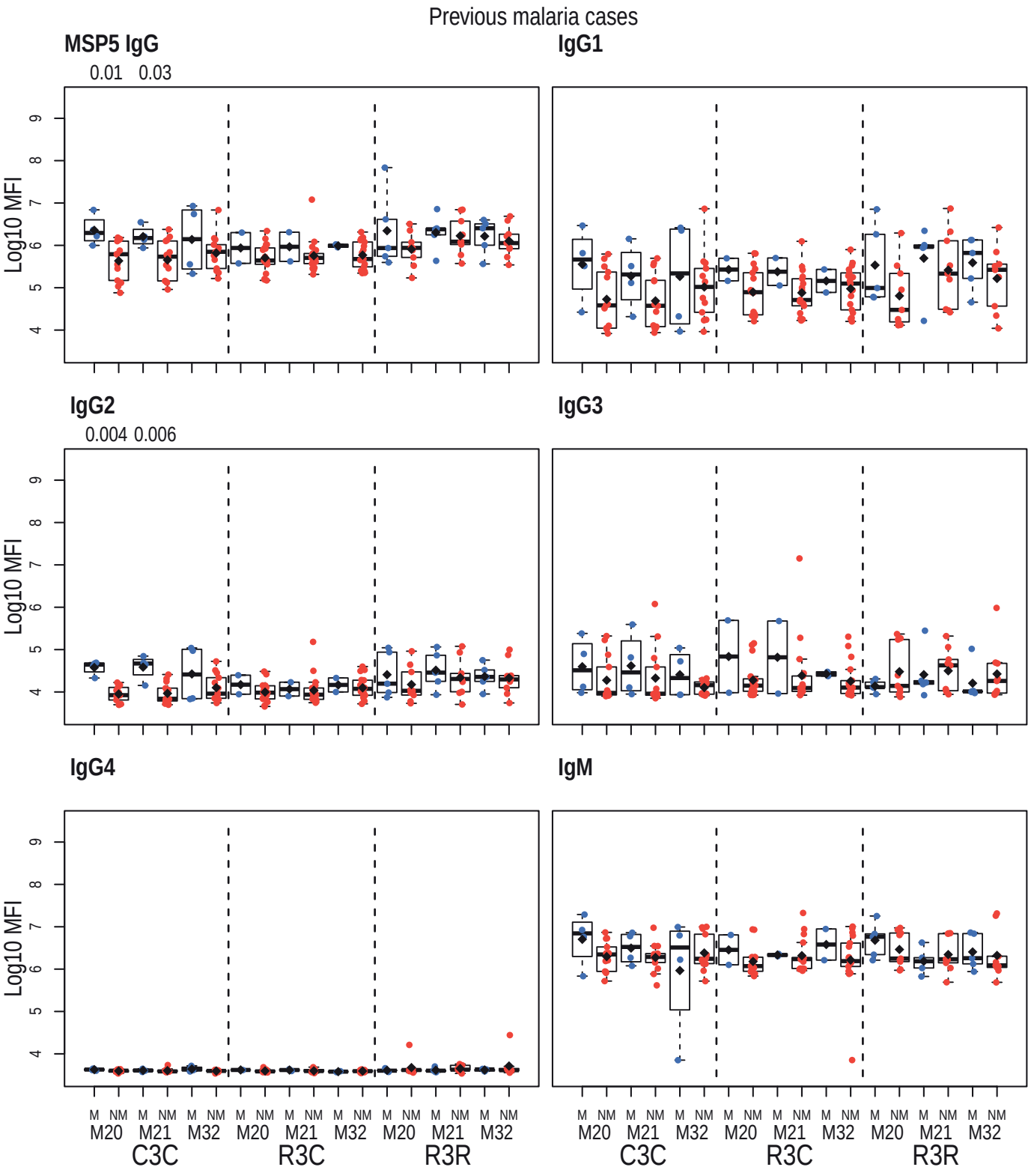
Supplementary Figure 25. Immunogenicity stratified by previous clinical malaria against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for EBA175 R3-5 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



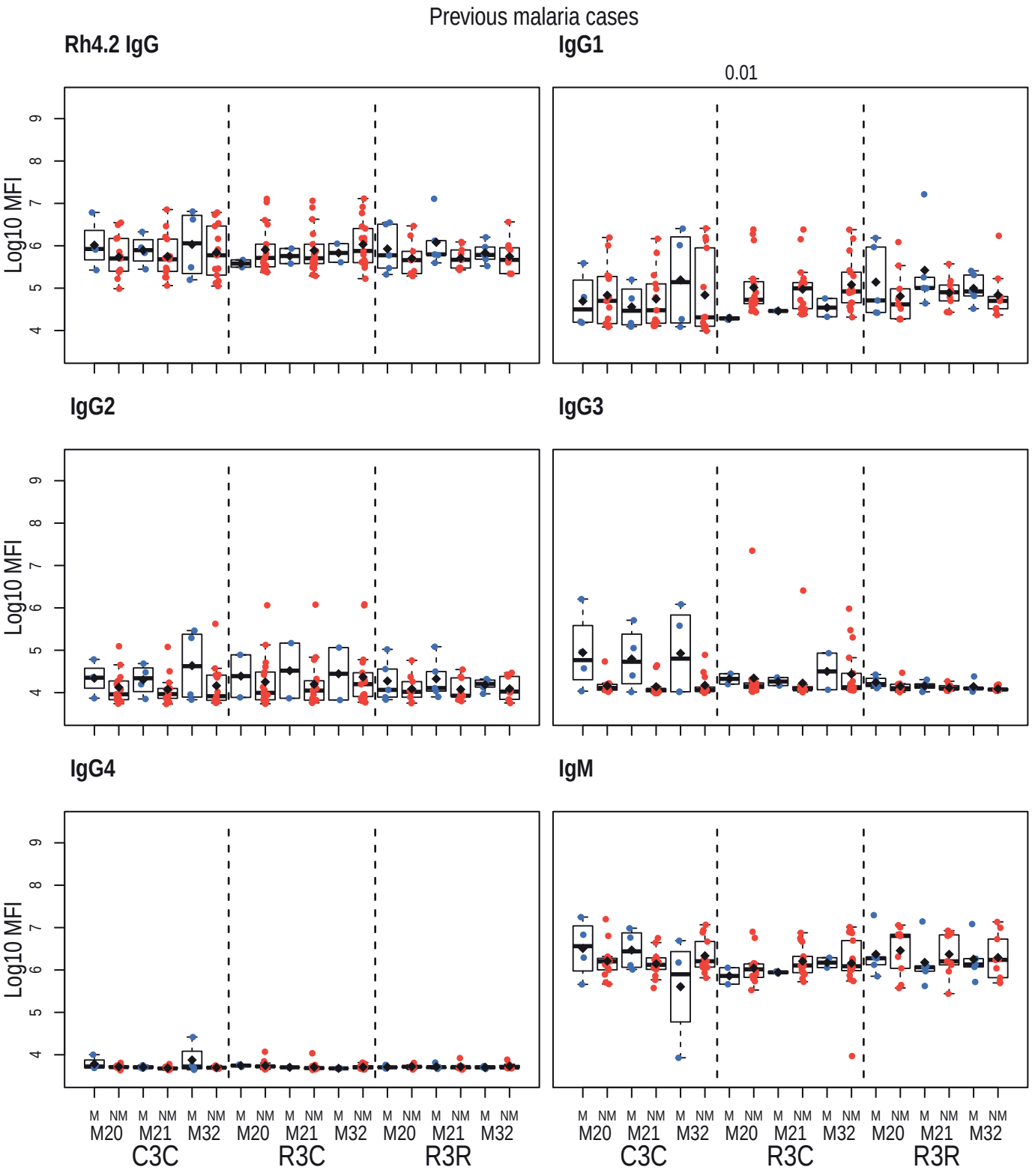
Supplementary Figure 26. Immunogenicity stratified by previous clinical malaria against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for $MSP1_{42}$ at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



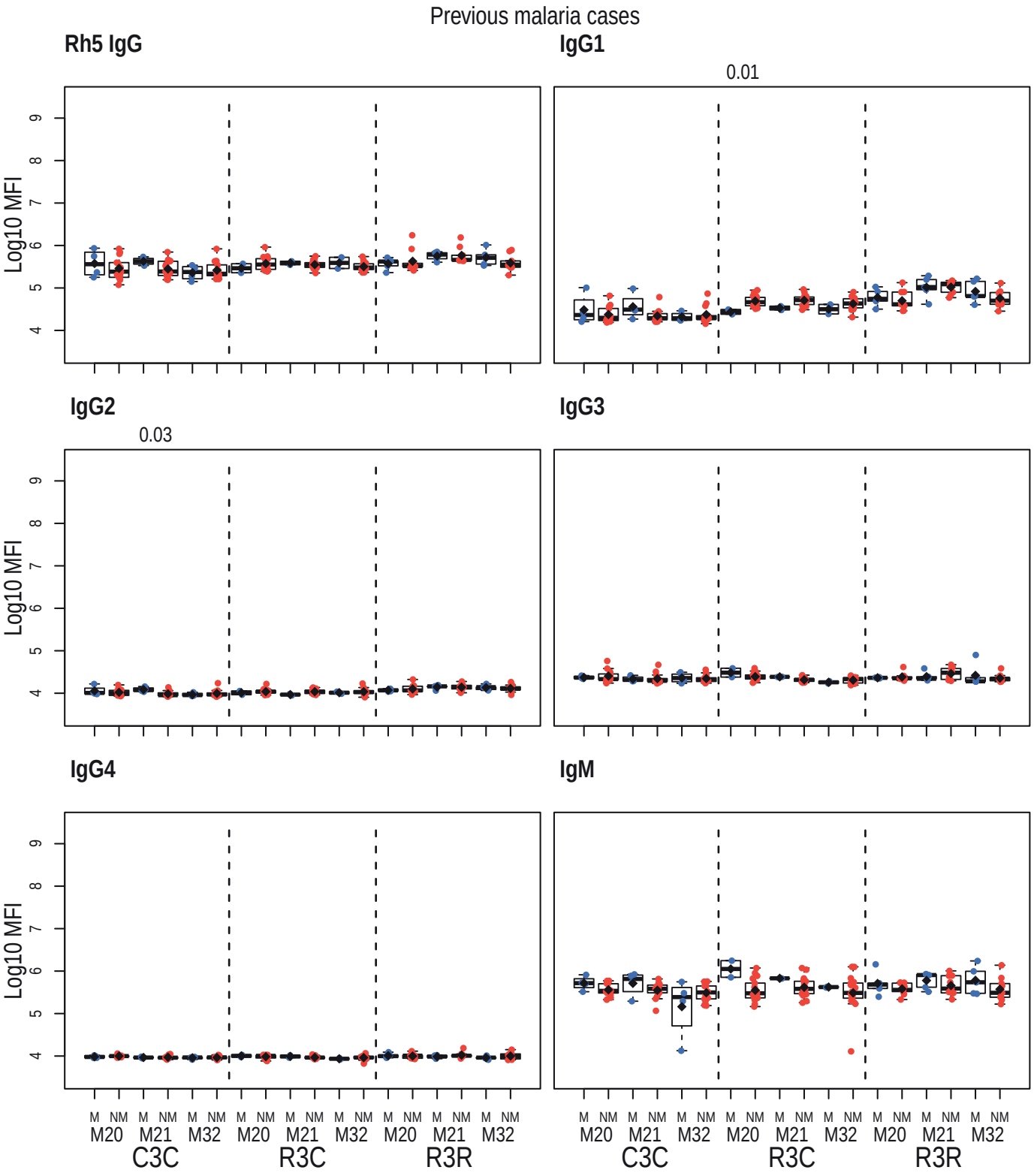
Supplementary Figure 27. Immunogenicity stratified by previous clinical malaria against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for MSP1 Block2 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



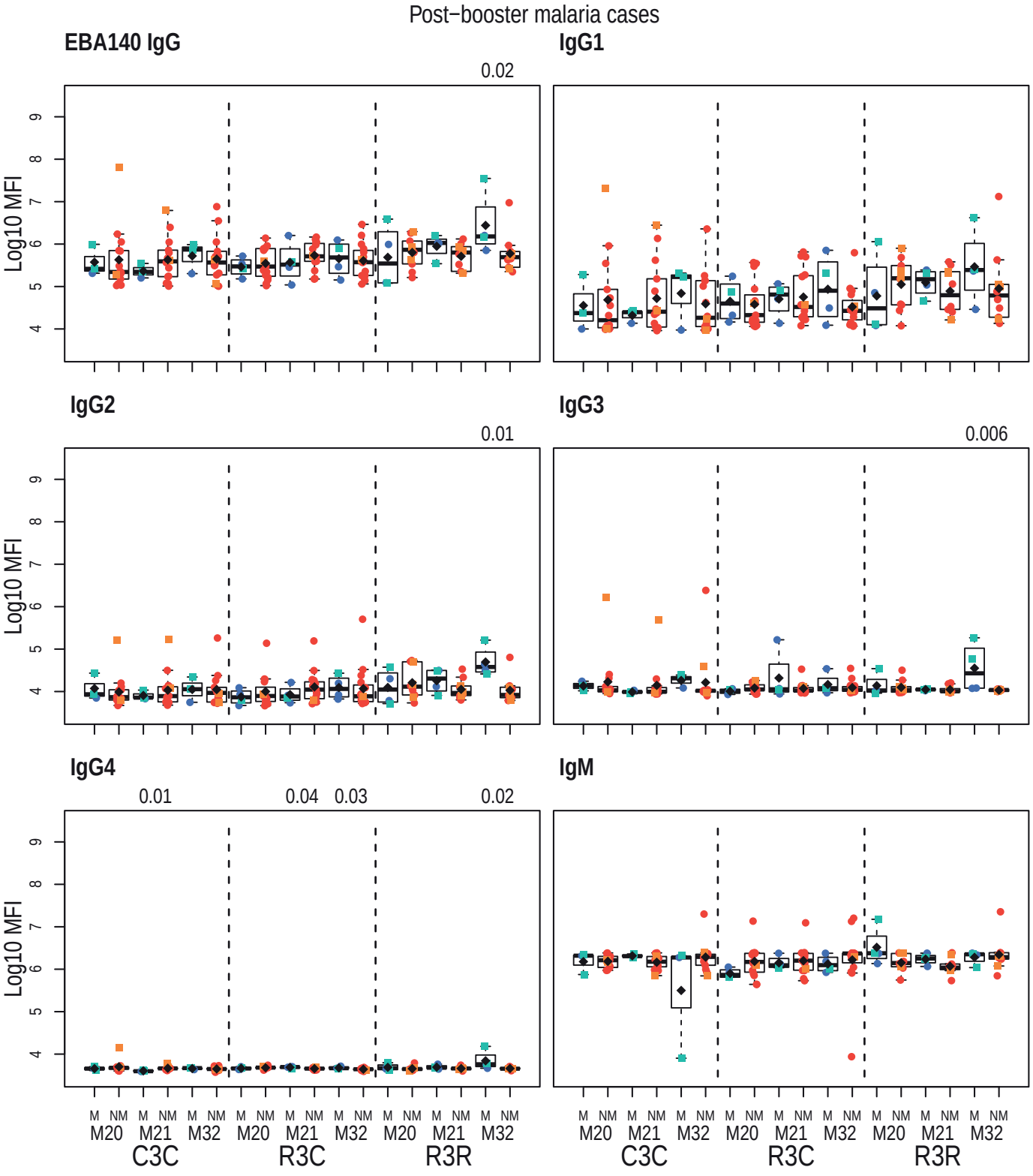
Supplementary Figure 28. Immunogenicity stratified by previous clinical malaria against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for MSP5 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



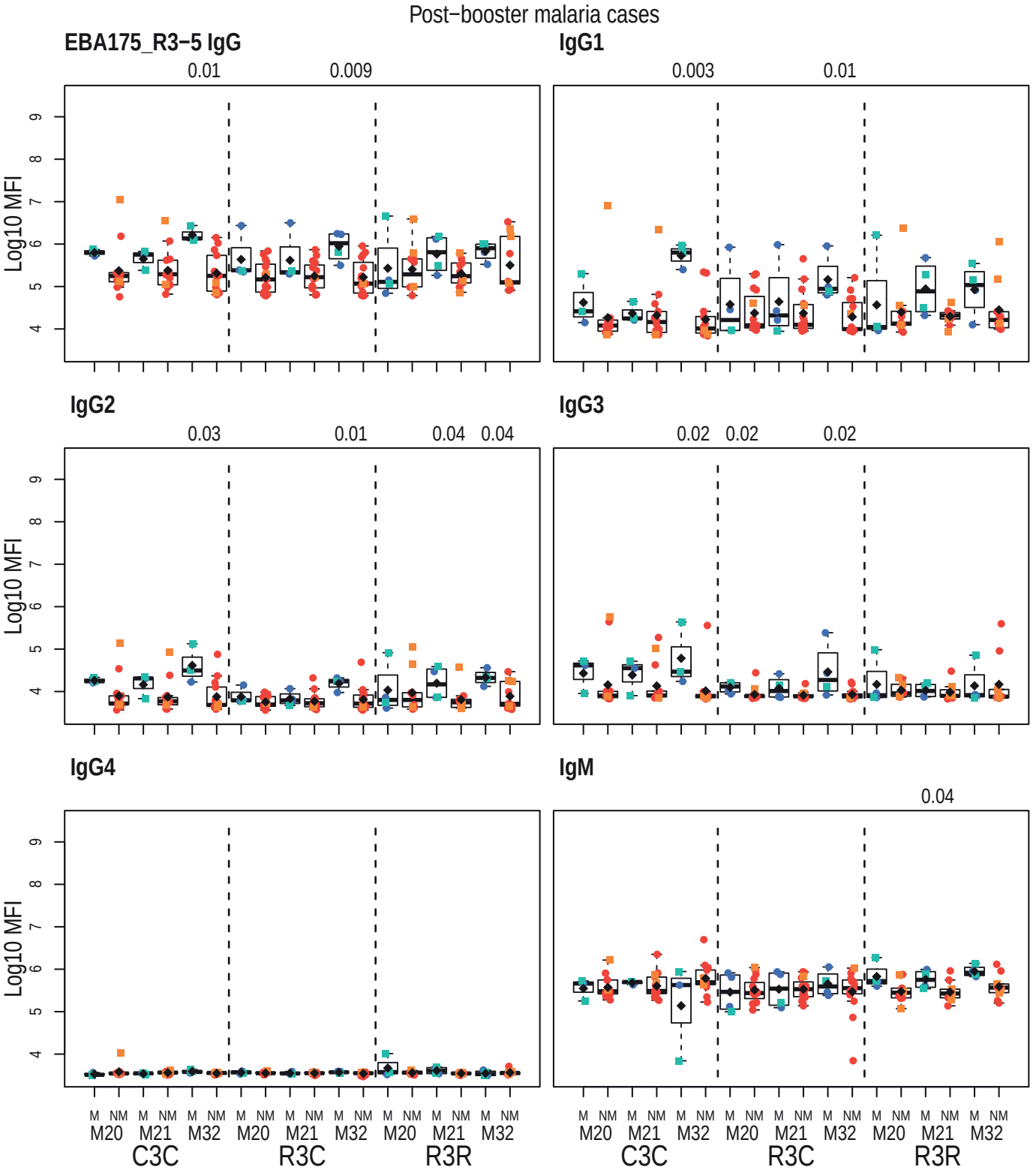
Supplementary Figure 29. Immunogenicity stratified by previous clinical malaria against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for Rh4.2 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



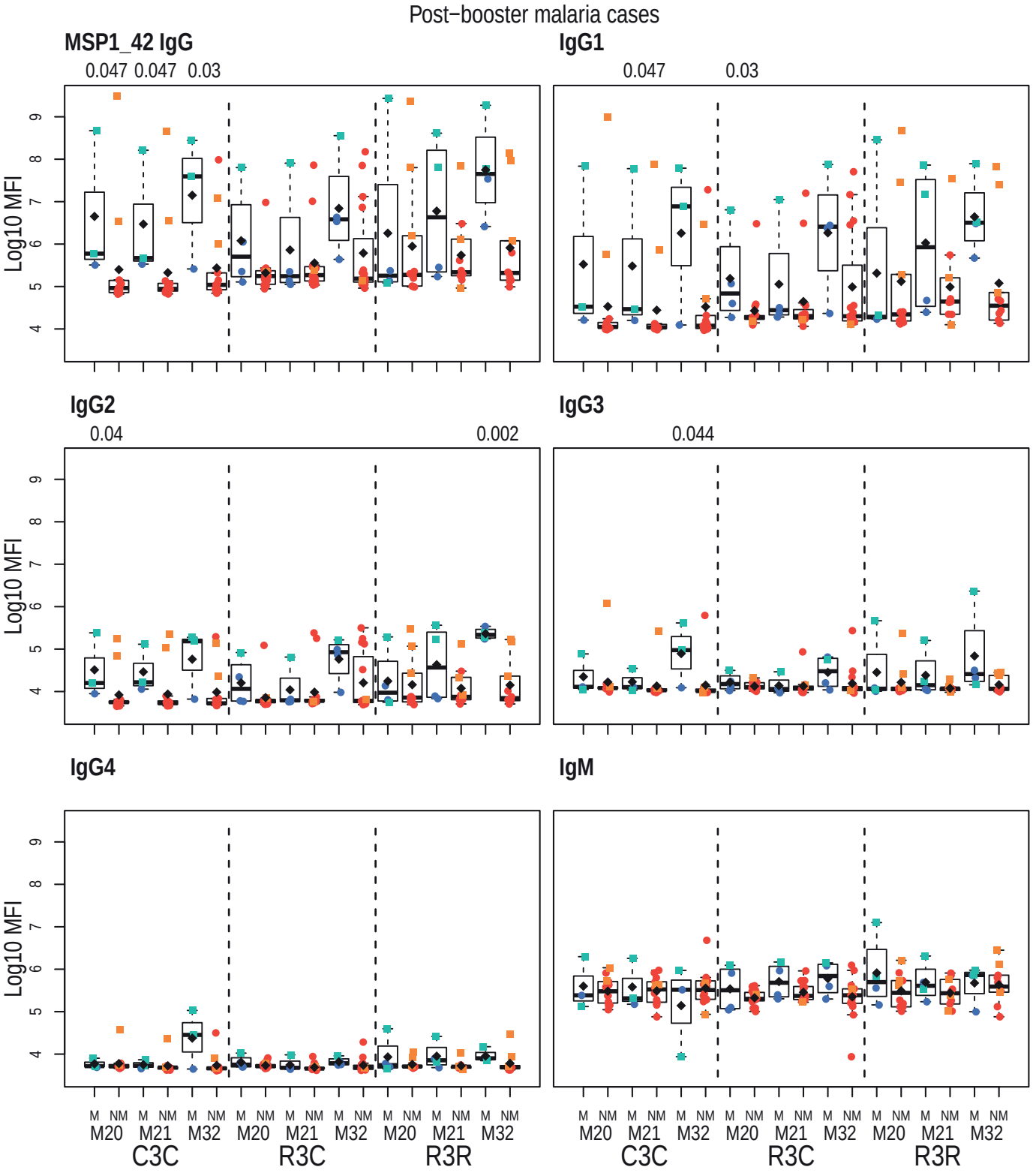
Supplementary Figure 30. Immunogenicity stratified by previous clinical malaria against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for Rh5 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria cases before M20, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (M vs NM). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



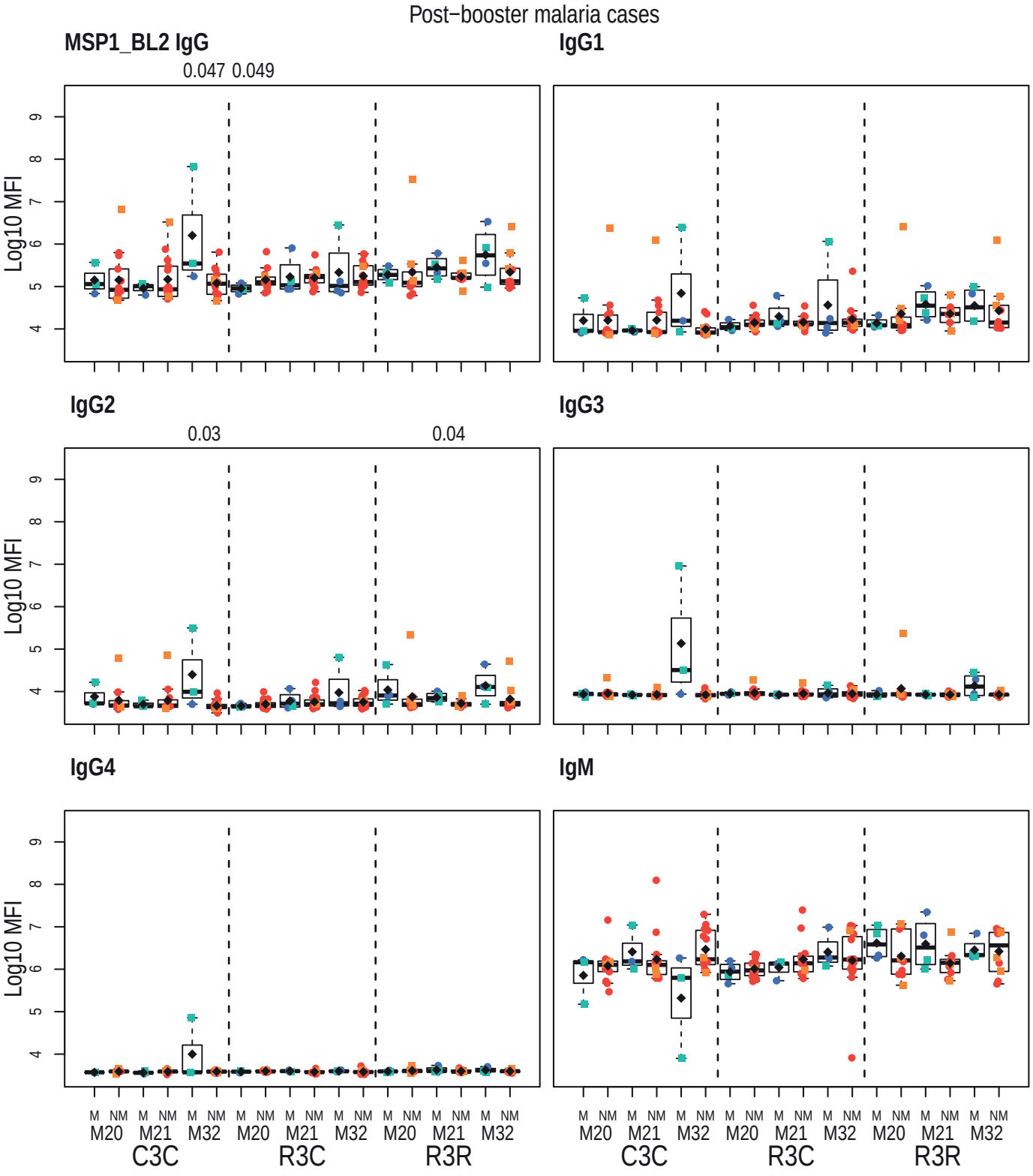
Supplementary Figure 31. Immunogenicity stratified by clinical malaria after M21 against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for EBA140 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Those who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs M). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



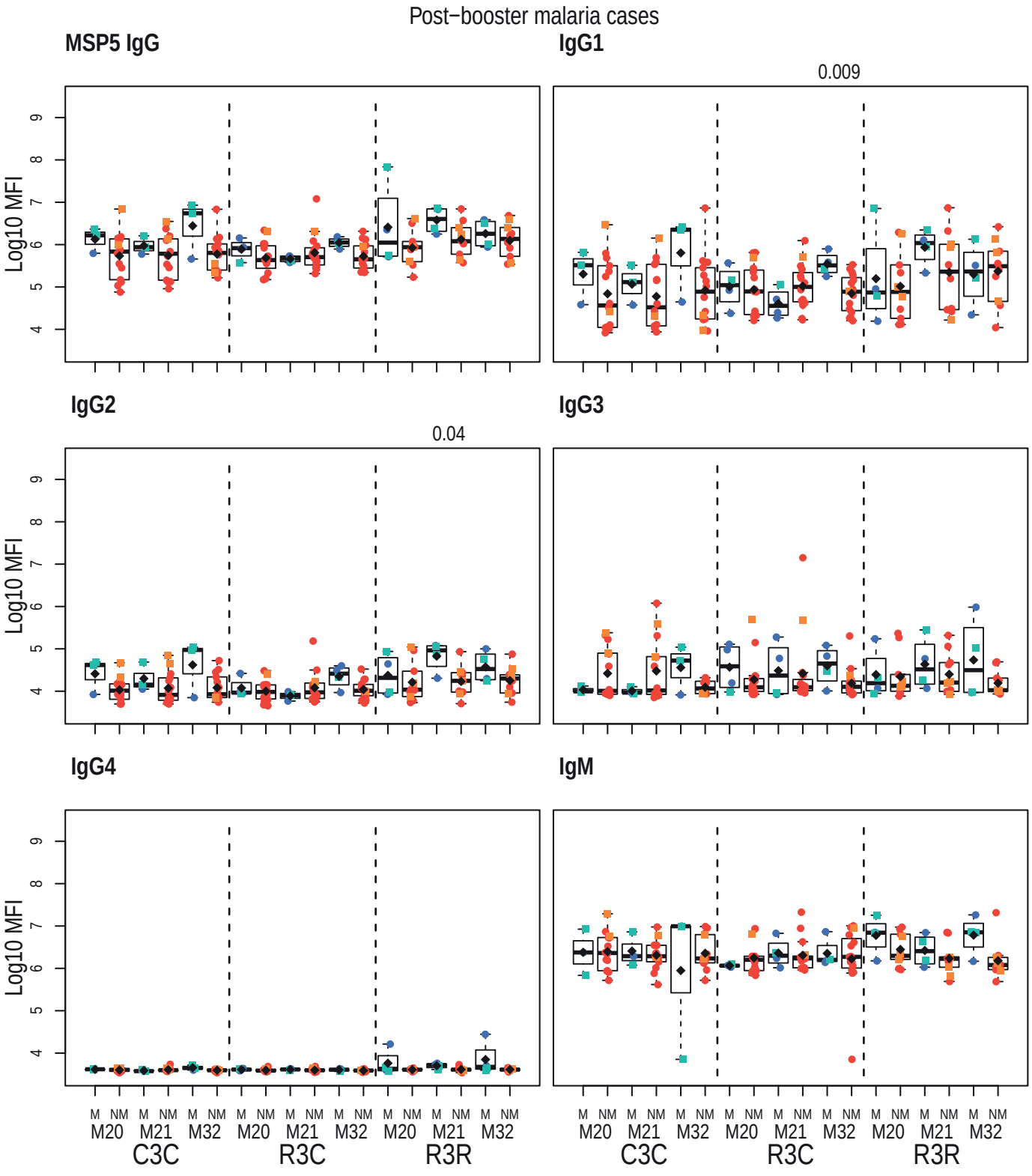
Supplementary Figure 32. Immunogenicity stratified by clinical malaria after M21 against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for EBA175 R3-5 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Those who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs M). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



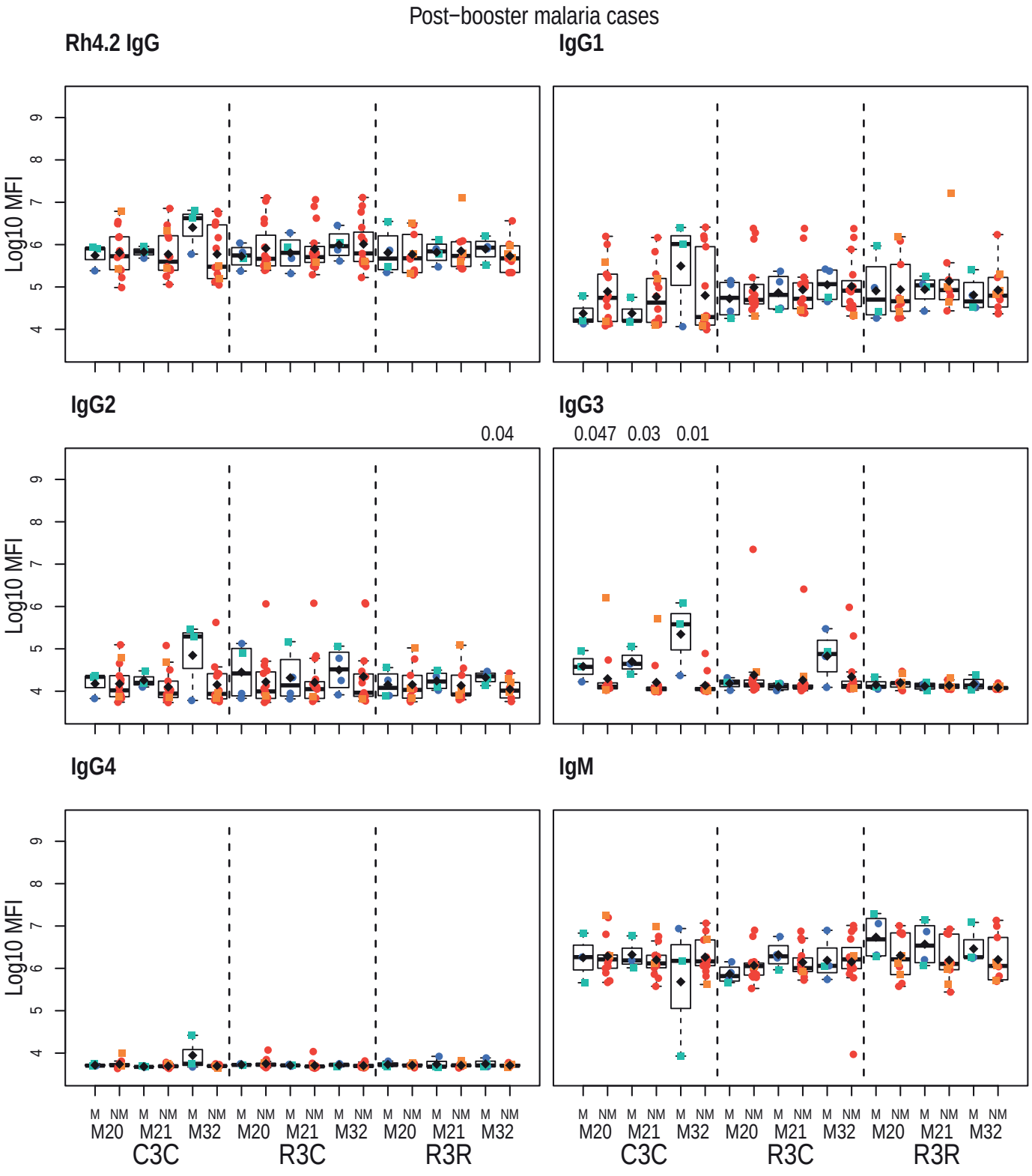
Supplementary Figure 33. Immunogenicity stratified by clinical malaria after M21 against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for $MSP1_{42}$ at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Those who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs M). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster-



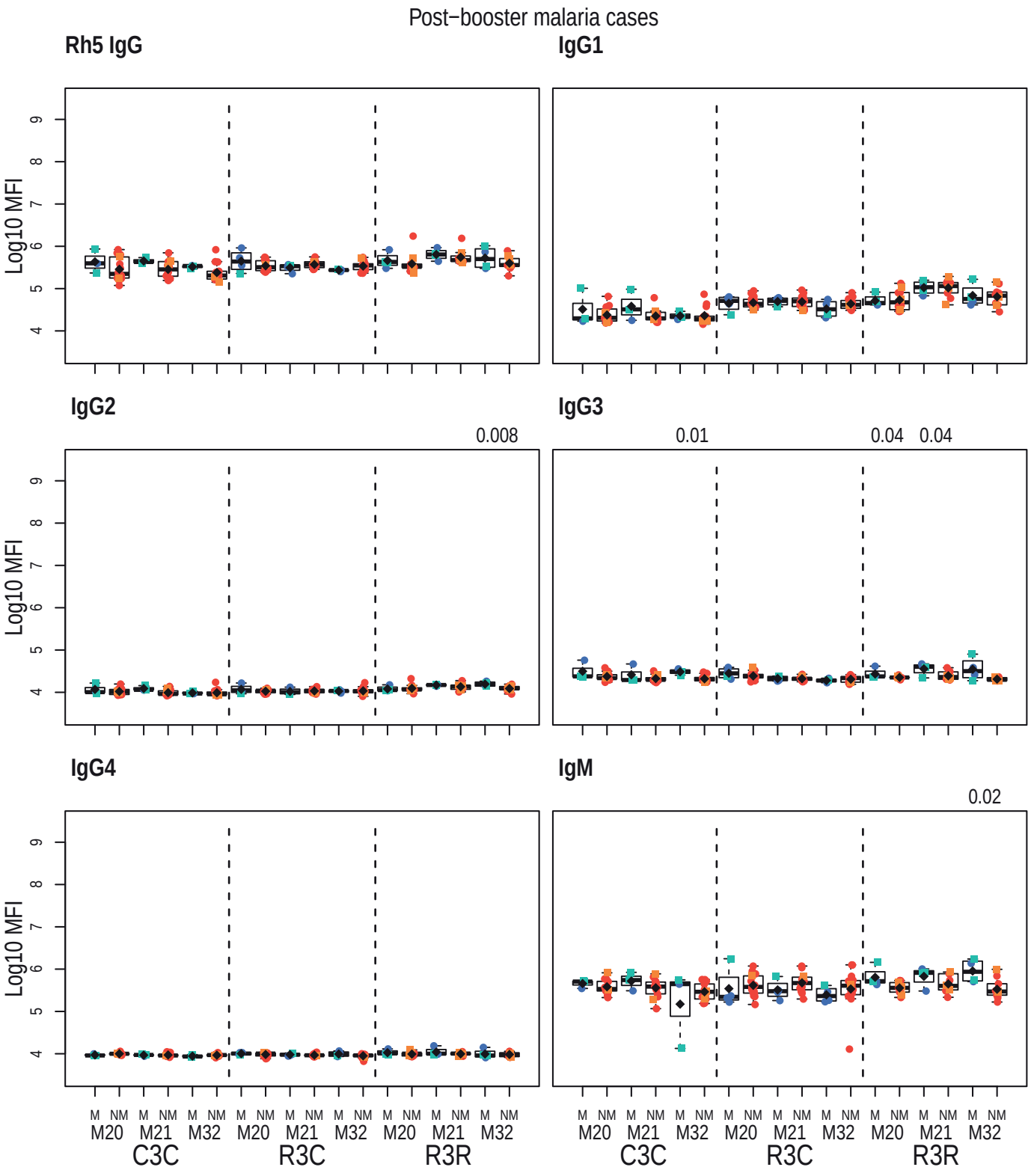
Supplementary Figure 34. Immunogenicity stratified by clinical malaria after M21 against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for MSP1 Block 2 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Those who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs M). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



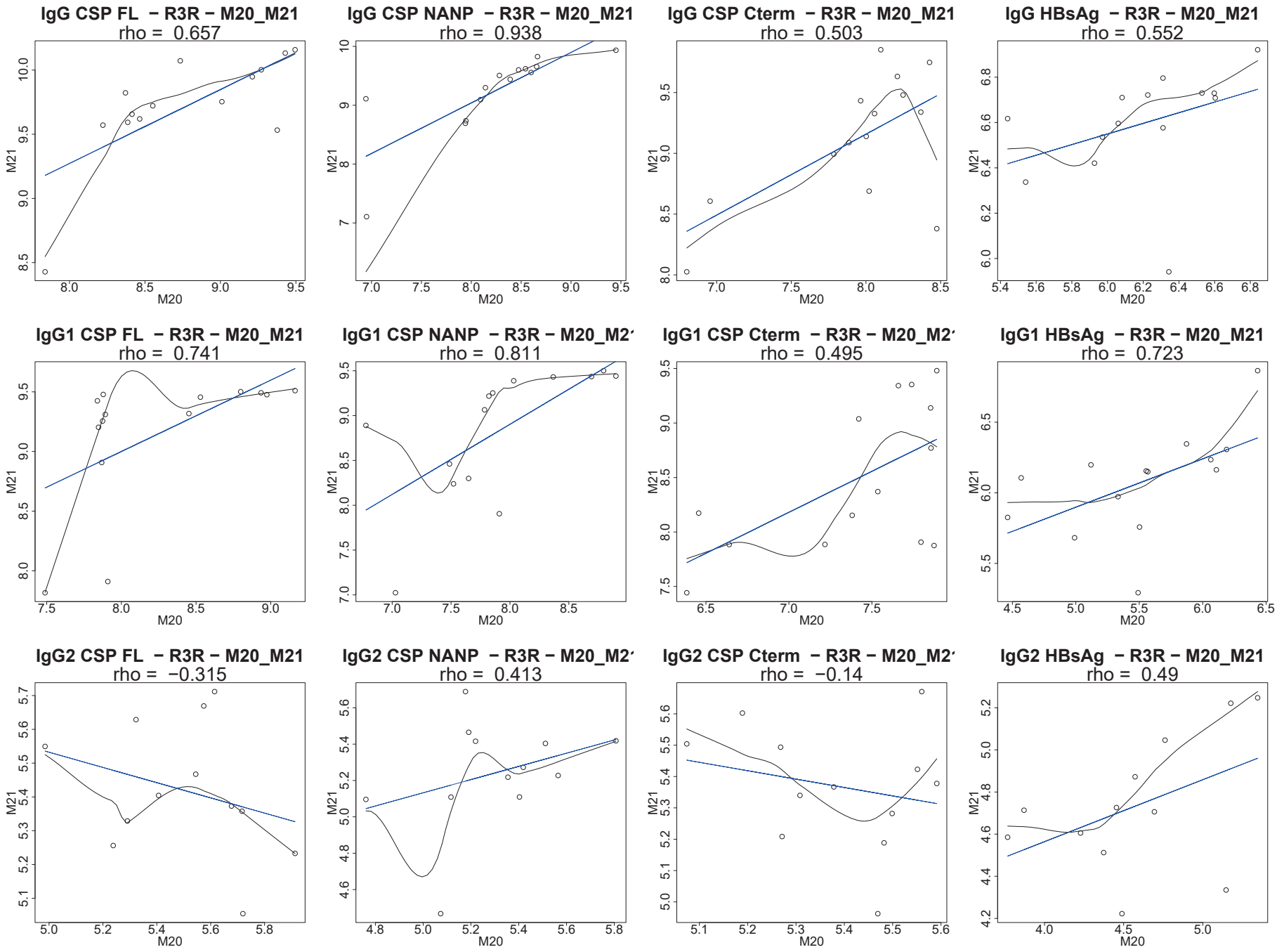
Supplementary Figure 35. Immunogenicity stratified by clinical malaria after M21 against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for MSP5 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Those who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs M). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



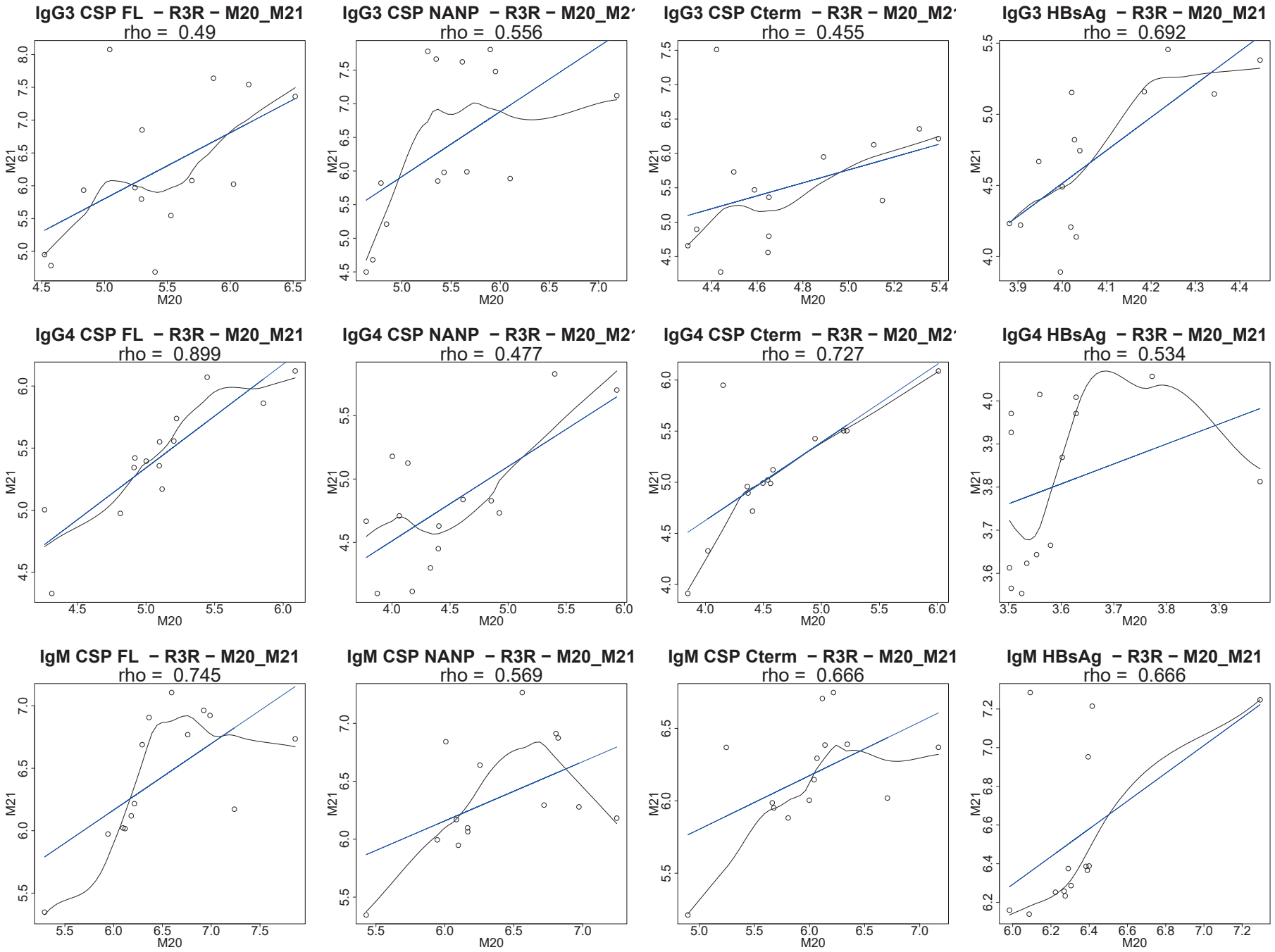
Supplementary Figure 36. Immunogenicity stratified by clinical malaria after M21 against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for Rh4.2 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Those who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs M). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster.



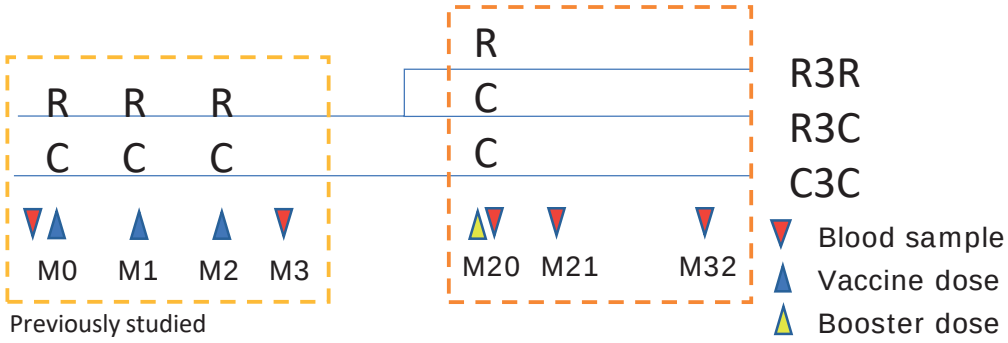
Supplementary Figure 37. Immunogenicity stratified by clinical malaria after M21 against blood stage antigens: Total IgG, IgG1-4 subclasses and IgM for Rh5 at month (M) 20, 21 and 32 for RTS,S/AS01 vaccinees with (R3R) and without (R3C) booster, and comparator (C3C). Stratified analysis by malaria after M21, subjects who presented with clinical malaria (M=blue) and subjects without clinical malaria (NM=red). Those who presented with clinical malaria before M20 are represented with green and orange squares. Boxplots with medians, interquartile ranges (IQR), upper whisker as the smallest between maximum x value and Q3 + 1.5 * IQR, lower whisker as the largest between minimum x value and Q1 – 1.5 * IQR, and log10(geometric mean(MFI)) (diamond). Non-parametric tests were used to compare levels with or without clinical malaria (NM vs M). P-values were adjusted for multiple comparisons, but none was significant. Only p-values <0.05 before adjustment are shown. The y axis is in logarithm 10 scale. R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster .



Supplementary Figure 38. Correlation plots between M20 and M21 for CSP constructs and HBsAg for IgG, IgG1-2: : linear regression (blue line), LOESS curve fitting (black line).



Supplementary Figure 39. Correlation plots between M20 and M21 for CSP constructs and HBsAg for IgG3-4 and IgM: : linear regression (blue line), LOESS curve fitting (black line).



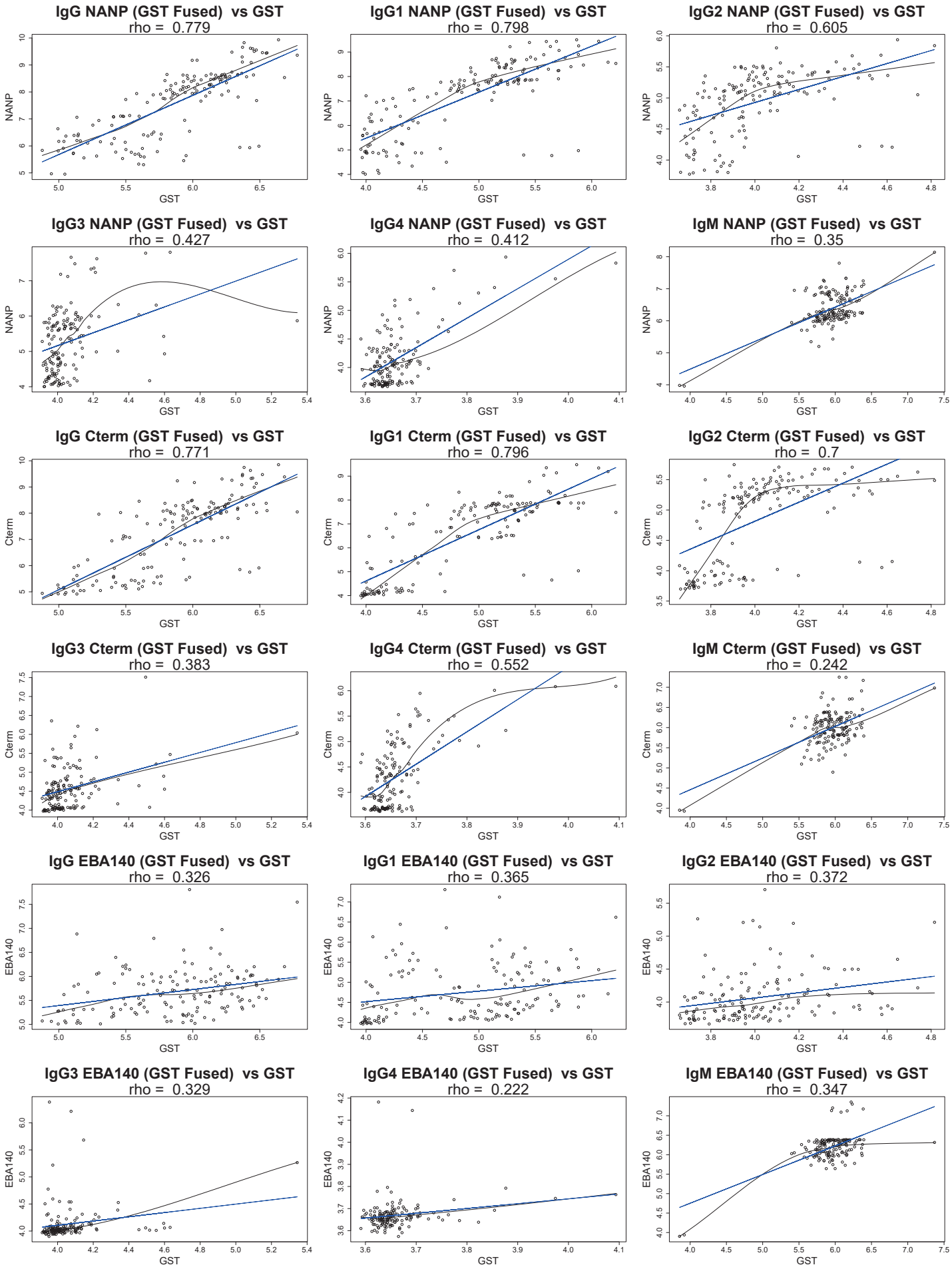
Supplementary Figure 40. Study design, vaccination and blood sampling schedule.

Supplementary Table 7. Study population demographic characteristics.

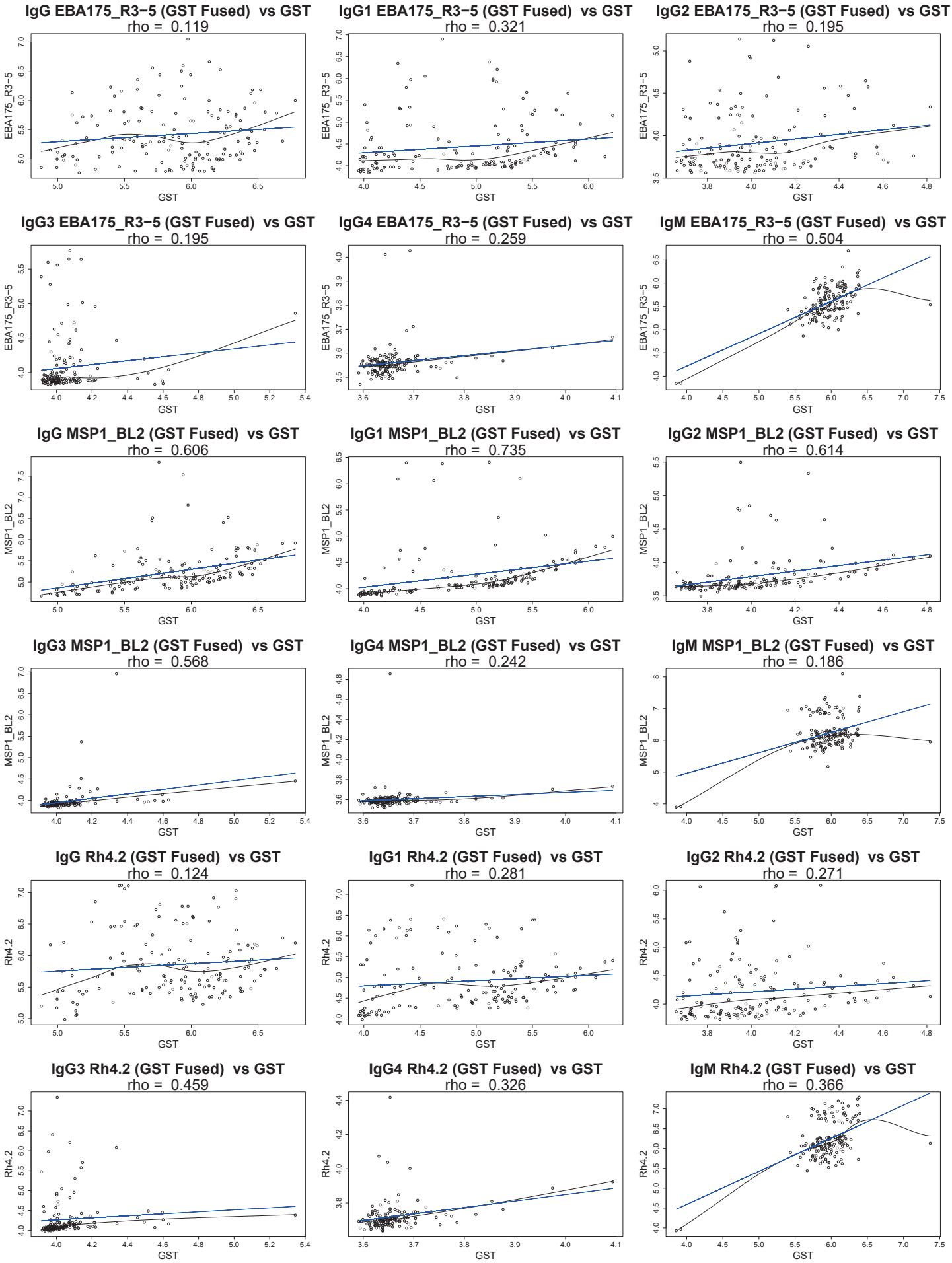
Study Population						
	R3R		**R3C**		**C3C**	
	N=14		N=19		N=17	
	n	%	n	%	n	%
Sex						
Female	4	28.6	7	36.8	9	52.9
Male	10	71.4	12	63.2	8	47.1
Age						
5-17 months	6	42.9	9	47.4	9	52.9
6-12 weeks	8	57.1	10	52.6	8	47.1
Malaria cases						
Before booster (pre-M20)	5	35.7	2	10.5	4	23.5
After booster (post-M20)	4	28.6	4	21.1	3	17.6
Malaria transmission in Manhiça was low [1,2].						
HIV infection was not a protocol exclusion/inclusion criterion, but only healthy children were included in the study. HIV testing was not a trial procedure. The prevalence of HIV infection in adults in the Manhiça area was around 40% [3].						
R3R: three doses of RTS,S/AS01E and a RTS,S/AS01E booster at month 20. R3C: three doses of RTS,S/AS01E and a comparator booster. C3C: three doses of a comparator vaccine and a comparator booster. M: month.						

Supplementary Table 8. Antigens coupled to the multiplex beads.

Antigen	Description		Sequence	Tag
CSP Full Length [4]	Circumsporozoite Protein	Vaccine components	YGSSSNTRVLNELNYDNAGTNLYNELEMNYYGKQENWYSLKKNSRSLGENDDGNNEDNEKLRKPKHKKLKQPADGNPDPNAN PNVDPNANPNVDPNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNAN PNANPNANPNKNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGIQ VRIKPGSANKPKDELDYANDIEKKICKMEK	Histidine
CSP C-terminus [5]			KNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSDKHIKEYQNKIQNSLSTEWSPCSVTCGNGIQVRIKPGSANKP KDELDYANDIEKKICKMEK	GST
CSP $NANP_6$ [6]			NANPNVDPNANPNVDPNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANP NANPNANPNANP	GST
HBsAg	Hepatitis B surface Antigen		Source: Abcam	
$MSP1_{42}$ [7]	Merozoite surface protein	Blood stage antigens	MAHHHHHHPGGSGSGTMAISVTMDNILSGFENEYDVIYLKPLAGVYRSLKKQIEKNIFTFNLNLNDILNSRLKKRKYFLDVLESDL MQFKHISSNEYIIEDSFKLLNSEQKNTLLKSYKYIKESVENDIKFAQEGISYYEKVLAKYKDDLESIKKVIKEEKEKFPSSPPTTPPSPAKT DEQKKESKFLPFLTNIETLYNNLVNKIDDYLINLKAKINDCNVEKDEAHVKITKLSDLKAIDDKIDLFKNPTDFEAIKKLINDDTKKDM LGKLLSTGLVQNFPNTIISKLIEGKFQDMLNISQHQCVKKQCPENSGCFRHLDEREECKCLLNYKQEGDKCVENPNPTCNENNGG CDADATCTEEDSGSSRKKITCECTKPDSYPLFDGIFCS	Histidine
Rh5 [8]	Reticulocyte binding protein		NAIKKTKNQENNLTLLPIKSTEEEKDDIKNGKDIKKEIDNDKENIKTNNAKDHSTYIKSYLNTNVNDGLKYLFIPSHNSFIKKYSVFNQ INDGMLLNEKNDVKNNEDYKNVDYKNVNFLQYHFKELSNYNIANSIDILQEKEGHLDFVIIPHYTFLDYYKHLSYNSIYHKSSTYGK CIAVDAFIKKINETYDKVKSKCNDIKNDLIATIKKLEHPYDINNKNDDSYRYDISEEIDDKSEETDDETEEVEDSIQDTDSNHTPSNKK KNDLMNRTFKKMMDEYNTKKKKLIKCIKNHENDFNKICMDMKNYGTNLFEQLSCYNNNFCNTNGIRYHYDEYIHKLILSVKSKN LNKDLSDMTNILQQSELLLTNLNKKMGSYIYIDTIKFIHKEMKHIFNRIEYHTKIINDKTKIIQDKIKLNIWRTFQKDELLKRILDMSNE YSLFITSDHLRQMLYNTFYSKEKHLNNIFHHLIYVLQMKFNDVPIKMEYFQTYKKNKPLTQ	Histidine
EBA175 region 3-5 [9]	Erythrocyte binding antigen		Amino acid residues 761–1298 (*Plasmodium falciparum* 3D7 genotype)	GST
EBA140 region 3-5 [9]	Erythrocyte binding antigen		Amino acid residues 770–1064 (*P. falciparum* 3D7)	GST
MSP5 [10]	Merozoite surface protein		MNILCILSYIYFFVIFYSLNLNNKNENFLVVRRLMNDEKGEGGFTSKNKENGNNNRNNENELKEEGSLPTKMNEKNSNSSDKQPN DISHDESKSNSNNSQNIQKEPEEKENSNPNLDSSENSSESATRSVDISEHNSNNPETKEENGEEPLDLEINENAEIGQEPPNRLHFD NVDDEVPHYSALRYNKVEKNVTDEMLLYNMMSDQNRKSCAINNGGCSDDQICININNIGVKCICKDGYLLGTKCIILNSYSCHPF FSILIYITLFLLLFV	Histidine
Rh 4.2 [11]	Reticulocyte binding protein		Amino acid residues 1277-1451 (*P. falciparum* 3D7)	GST
MSP1 Block 2 [12]	Merozoite surface protein		NEEEITTKGASAQSGASAQSGASAQSGASAQSGASAQSGASAQSGTSGPSGPSGT SPSSRSNTLPRSNTSSGASPPADAS	GST
GST	For fusion proteins	Tag	MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCP KERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLD AFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLEVLFQGPLGS	



Supplementary Figure 41. Correlation plots for antibody response to IgG, IgG1-4, IgM against GST fused antigens (CSP NANP,CSP Cterm, EBA140) vs GST: linear regression (blue line), LOESS curve fitting (black line).



Supplementary Figure 42. Correlation plots for for antibody response to IgG, IgG1-4, IgM against GST fused antigens (EBA175_R3-5, MSP1_BL2, Rh4.2) vs GST: linear regression (blue line), LOESS curve fitting (black line).

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Article 3

This article addresses the third objective of understanding the interplay between the RTS,S vaccination and naturally acquired immune responses to malaria and identifying immune correlates of protection. The results of this work have been published in the following peer-reviewed journal article:

Dobaño C, Ubillos I, **Jairoce C,** Gyan B, Vidal M, Jiménez A, Santano R, Dosoo D, Nhabomba AJ, Ayestaran A, Aguilar R, Williams NA, Díez-Padrisa N, Lanar D, Chauhan V, Chitnis C, Dutta S, Gaur D, Angov E, Asante KP, Moncunill G. **RTS, S/AS01E immunization increases antibody responses to vaccine-unrelated Plasmodium falciparum antigens associated with protection against clinical malaria in African children: a case-control study.** BMC medicine. 2019 Dec;17:1-9.

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RESEARCH ARTICLE Open Access

RTS,S/AS01E immunization increases antibody responses to vaccine-unrelated *Plasmodium falciparum* antigens associated with protection against clinical malaria in African children: a case-control study

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Abstract

Background: Vaccination and naturally acquired immunity against microbial pathogens may have complex interactions that influence disease outcomes. To date, only vaccine-specific immune responses have routinely been investigated in malaria vaccine trials conducted in endemic areas. We hypothesized that RTS,S/A01E immunization affects acquisition of antibodies to *Plasmodium falciparum* antigens not included in the vaccine and that such responses have an impact on overall malaria protective immunity.

Methods: We evaluated IgM and IgG responses to 38 *P. falciparum* proteins putatively involved in naturally acquired immunity to malaria in 195 young children participating in a case-control study nested within the African phase 3 clinical trial of RTS,S/AS01E (MAL055 NCT00866619) in two sites of different transmission intensity (Kintampo high and Manhiça moderate/low). We measured antibody levels by quantitative suspension array technology and applied regression models, multimarker analysis, and machine learning techniques to analyze factors affecting their levels and correlates of protection.

Results: RTS,S/AS01E immunization decreased antibody responses to parasite antigens considered as markers of exposure ($MSP1_{42}$, AMA1) and levels correlated with risk of clinical malaria over 1-year follow-up. In addition, we show for the first time that RTS,S vaccination increased IgG levels to a specific group of pre-erythrocytic and blood-stage antigens (MSP5, MSP1 block 2, RH4.2, EBA140, and SSP2/TRAP) which levels correlated with protection against clinical malaria (odds ratio [95% confidence interval] 0.53 [0.3–0.93], $p = 0.03$, for MSP1; 0.52 [0.26–0.98], $p = 0.05$, for SSP2) in multivariable logistic regression analyses.

(Continued on next page)

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Conclusions: Increased antibody responses to specific *P. falciparum* antigens in subjects immunized with this partially efficacious vaccine upon natural infection may contribute to overall protective immunity against malaria. Inclusion of such antigens in multivalent constructs could result in more efficacious second-generation multistage vaccines.

Keywords: Malaria, *Plasmodium falciparum*, Vaccine, RTS,S, Antibody, Pre-erythrocytic antigens, Blood-stage antigens, Naturally acquired immunity, Protection, Maternal antibodies

Background

Immunity against infectious diseases can be acquired by natural exposure to the microbe, or through intervention by vaccination, although in neither case is immunity necessarily sterile. Naturally acquired and experimentally induced immunity to infectious diseases are not necessarily mediated by the same host mechanisms, particularly in the case of pathogens that may have multiple and complex life cycle stages and where several potential immune effectors such as antibody vs cellular may act on distinct phases of infection. The way naturally acquired immunity (NAI) interacts with active immunization in clearing microbes in vaccinated subjects is multifaceted and not well understood. First, pre-existing immunity, as a result of prior exposure to infection and/or passively transferred maternal IgGs in the case of neonates and infants [1], may have an impact on response to vaccines and induction of protective efficacy. Second, immune responses induced by continuous exposure to pathogens before, during, and right after vaccination may also affect the type and magnitude of vaccine-induced immune responses and impact protective immunity against the disease and thus alter overall vaccine efficacy. Third, vaccination with partially or fully effective vaccines inducing moderate or strong immunity could decrease microbe exposure that is required for the induction and/or maintenance of NAI and this may result in a "rebound" in the incidence of disease if the vaccine is moderately efficacious and short-lived.

In the case of malaria caused by *Plasmodium falciparum*, in areas of heavy and continuous transmission, NAI is acquired with age and exposure and consequently the burden of disease is concentrated in children [2]. NAI is mediated mainly by IgG antibodies to antigens of the parasite asexual blood stage (BS) [3], but the specific epitope targets have not been unequivocally defined. The most advanced malaria vaccine globally, RTS,S/AS01E, has been tested in African subjects in phase 2 and 3 trials, showing consistent though moderate and waning efficacy against clinical malaria (range 55.8% in children to 31.3% in infants after 1 year of follow-up) [4, 5]. RTS,S elicits strong IgG antibodies to the circumsporozoite protein (CSP), the predominant protein of the *P. falciparum* pre-erythrocytic (PE) stage sporozoite, a response that has been implicated in vaccine-induced protection against malaria [6, 7], albeit inconsistently. Neither the effect that natural exposure and/or pre-existing immunity could have on RTS,S efficacy and longevity nor how vaccination affects NAI has been investigated in sufficient depth.

Based on clinical and immunogenicity data from previous phase 2b trials in Manhiça, Mozambique [8, 9], we proposed a model of development of protection to RTS,S that was dependent on the intricate interaction between vaccination and NAI, which might influence duration of vaccine efficacy [10]. We postulated that duration of vaccine efficacy depends on two distinct, but related, mechanisms: (1) initial partial PE protection via induction of vaccine-specific immune responses, which reduces the release of merozoites from the liver into the bloodstream, and (2) long-term protection resulting from enhancement of BS immunity facilitated through subclinical BS infection due to partial RTS,S protection (the "leaky vaccine" hypothesis). This represents a fourth, unexplored mechanism of vaccine interaction with NAI whereby reduced microbial burden resulting from partial vaccine efficacy may enhance NAI through a low-dose stimulus to the immune system [11]. Our previous analyses of Mozambican phase 2b trial samples measuring antibodies to a panel of *P. falciparum* antigens revealed that RTS,S vaccinees had similar or significantly lower IgG responses than comparator vaccines at 6 months after vaccination, particularly in younger children < 2 years of age [12]. Thus, there was no evidence of an enhancement of BS immunity through RTS,S vaccination. Rather, measured antibodies represented markers of exposure and reduction of antibody breadth and magnitude reflected vaccine efficacy [13].

In this study, using multiplex quantitative suspension array assays, we evaluated within the pediatric multicenter African RTS,S phase 3 clinical trial the impact of vaccination on NAI using an expanded panel of antigens that are putatively associated with malaria immunity and exposure. As NAI is dependent upon age and exposure, and may significantly affect vaccine efficacy, we included infant and children cohorts from two sites of different malaria transmission intensity (MTI). We investigated whether antibody responses 1 month post-immunization were modified by RTS,S and whether these responses contributed to malaria protective immunity.

Methods

Design

This study was carried out in two of the seven sites included in the multicenter immunology study MAL067, ancillary to the phase 3 randomized clinical trial MAL055 (NCT00866619): Kintampo in Ghana (moderate-high MTI) and Manhiça in Mozambique (low MTI) [6]. Briefly, our study included 109 infants aged 6–12 weeks and 86 children aged 5–17 months from the phase 3 trial who were randomly assigned to receive 3 doses of either the RTS,S/AS01E vaccine or a comparator vaccine (the meningococcal C conjugate in infants or rabies vaccines in children) during month (M) 0, 1, and 2. Subjects were followed up by passive case detection (PCD) starting at M0 and during the subsequent study months. For correlates of protection analyses in our study, we used a follow-up time of 12 months, when subjects were censored, and starting 14 days after sample collection at M3 (approximately 44 days after the third dose at M2). Subjects with ≥ 150 μL plasma/serum samples available at M0 (baseline) and M3 were selected. We included 129 RTS,S/AS01E- and 66 comparator-vaccinated children and infants from both sites. For the correlates of malaria protection/risk analysis, 78 children and infants were randomly selected from Kintampo, and 117 participants were selected from Manhiça according to a prior case-control study of cellular markers [14], and all were analyzed in a case-control design.

Antibody assays

Quantitative suspension array technology (qSAT) applying the xMAP™ technology (Luminex Corp., Texas) was used to measure antibody responses to 38 *P. falciparum* antigens including three CSP constructs (Additional file 1: Table S1). Antigens were selected on the basis of profiling BS immunity, but also for the effect of vaccination on PE immune responses to sporozoite (SSP2/TRAP and CelTOS) and liver stages (LSA1). Although some of the BS antigens have been characterized as markers of exposure, such as AMA1 and MSP1 [15], antigen selection was primarily directed toward prominent targets of immunity, vaccine candidates, or prior association with protection in seroepidemiological studies or animal models. Additionally, several antigens were specifically included with said characteristics and limited polymorphism (e.g., RH2, RH4, RH5, and EBA140). VAR2CSA, a pregnancy-restricted variant of *P. falciparum* erythrocyte membrane proteins, was included as a representation of maternally derived antibodies.

qSAT assays contained bovine serum albumin (BSA) and glutathione S-transferase (GST)-coupled beads for background determination and as a control for signal from non-specific binding of *P. falciparum* GST fusion proteins, respectively. *P. falciparum* proteins were covalently coupled directly to MagPlex beads and blocked with BSA. qSAT assays were previously standardized and optimized to control for sources of variability [16–18]. Briefly, antigen-coupled multiplex beads were mixed with 50 μL of test sample, negative or positive control [8, 19], at multiple dilutions (see Additional file 1). After incubation and washing, biotinylated secondary antibodies were added. Following streptavidin-R-phycoerythrin incubations, samples were acquired with a Luminex 100/200 analyzer and antibody levels measured as median fluorescence intensity (MFI). Data pre-processing is detailed in Additional file 1.

Data analysis

Comparisons of crude Ig levels ($\log_{10}$ MFI) across antigens and isotypes were done through boxplots with geometric means, medians, and interquartile ranges (IQR), by *t* tests, and *p* values adjusted for multiple comparisons by the Holm approach [20]. Analyses included either all subjects or separately by visit and by vaccination, and in some cases stratifying by site, by age, and by age group within a site. To evaluate factors affecting M3 Ig levels to each antigen, we fitted first univariable and next multivariable linear regression models (coefficient, 95% confidence interval [CI], *p* values) including the following predictors: vaccination, sex, malaria transmission season at M3, having clinical malaria episodes between M0 and M3, and baseline variables like age cohort, antibody levels (using the same antigen/Ig as the outcome variable at M3), hemoglobin (Hb) concentrations, weight-for-age *Z*-score (WAZ), and height-for-age *Z*-score (HAZ). Models were also fitted separately at pre-vaccination (M0). Malaria transmission season was defined as high between April and October for Kintampo and November and April for Manhiça; the remaining months were defined as low transmission season. Linearity of the associations with continuous covariates was evaluated through penalized splines in generalized additive models (GAM); variables were modeled as linear. A combination of backward and forward stepwise algorithm was used in multivariable models.

Analysis of antibody correlates of protection against clinical malaria (fever > 37.5 °C with any parasitemia in the 12 months after M3.5) was based on a case-control design. Logistic regression models (odds ratio [OR], 95% CI, *p* values) were fitted first univariable and next multivariable to obtain the effect of different predictors in the odds of having malaria. Main predictors included levels ($\log_{10}$ MFI) of antibodies at M3 and change in antibody levels from M0 to M3. The change in antibody levels from M0 to M3 was calculated as the difference between $\log_{10}$ MFI levels at M3 and $\log_{10}$ MFI levels at M0 ($\log_{10}$ fold change [FC]) for each antibody-antigen pair. The impact of the other covariates (same as above) on the association between antibody responses and malaria risk/protection was

also assessed. The linearity of the $\log_{10}$-transformed antibody levels was evaluated when the outcome was case-control. Multivariable models were obtained through the stepwise algorithm, R package MASS and function stepAIC. Both backwards and forward methods were combined to obtain the model with the minimum Akaike information criterion (AIC). All potential variables were proposed in the first step of the model, not only the significant ones.

Finally, we performed multimarker analysis by principal component analysis (PCA), correlation matrices, and machine learning partial least squares discriminant analysis (PLS-DA) using the R packages FactoMineR [21], Corrplot [22], and DiscriMiner [23], respectively. PLS-DA is a supervised method useful when there are many colinear variables. It is similar to PCA, but in addition it also takes into account a categorical response variable (clinical malaria or non-malaria in our case). Based on this, it creates new components with different loadings of the explanatory variables (antibody levels to the different antigens in our case) that better explain the response variable. For the PCA analysis, we included the $\log_{10}$-transformed levels of all antigen-isotype pairs at M3 to generate the principal components. We selected the first three principal components that best explained the variance of the data and tested these components on the variables of malaria, vaccination, age, and site. Correlations between IgG to all antigens were done by Spearman. For PLS-DA, the analysis was performed using the $\log_{10}$-transformed antibody levels of all antigen-isotype pairs at M3 for only RTS,S vaccinees. Then, we used the PLS-DA components to fit multivariable logistic regression models, including also age and site. Finally, we calculated the area under the curve (AUC) performance using the prediction of malaria outcome obtained with the PLS-DA and calculated overfitting parameters of the model (cumulative R^2) to determine its quality. To check for areas of amino acid similarity in antigens with CSP that might cause cross-reactivity of RTS,S-induced antibodies, we aligned sequences using BLAST queries of each full-length antigen to the CSP. Additionally, we assessed the similarity of specific peptides by aligning 25 amino acid (a.a.) sequences overlapping by 7 a.a. against full-length CSP. We considered any hits with E value < 1 as significant, but also report the hits for the default threshold E value < 10 in Additional file 1.

Results

Baseline characteristics

We analyzed a total of 195 subjects (78 in Kintampo, Ghana, and 117 in Manhiça, Mozambique) with samples available at both pre-vaccination (M0) and post-vaccination (M3) [7]. RTS,S/AS01E (n = 129) and comparator (n = 66) vaccinees were similar with regard to baseline characteristics (age, sex, WAZ, HAZ, other vaccinations, previous malaria, season, distance to health center, Hb concentration), and most participants (93%) completed the 12-month post-vaccination follow-up [7]. The median time to drop-out of the study was 113 days (range 21–276), and most were early terminations due to loss to follow-up (7 subjects) or migration (4 subjects). A total of 89 malaria clinical events were recorded during the follow-up period: 60 in Kintampo (36 in RTS,S and 24 in comparators) and 29 in Manhiça (18 in RTS,S and 11 in comparators). Thirty-five clinical malaria events (39%) were registered in the children age cohort (48% in Kintampo, 21% in Manhiça), and the remaining in the infant age cohort. Parasitemia of subjects who had clinical malaria was comparable between RTS,S and comparator vaccinees. The Kaplan-Meier median follow-up time was 365 days (IQR = 128 to 365).

P. falciparum antigens are grouped into decreasing, unaffected, and increasing antibody responses to RTS,S/AS01E vaccination

First, we aimed to assess the impact of RTS,S vaccination on the levels of IgG and IgM to vaccine-unrelated *P. falciparum* antigens 1 month post-vaccination (M3). We measured IgG and IgM levels (expressed as $\log_{10}$ MFI) (Additional file 1: Table S1) at M0 and M3 and analyzed the effect of vaccination in univariable and multivariable models adjusting for age, site, baseline levels at M0, and previous malaria episodes from M0 to M3. We defined three different groups of antibodies based on the three different patterns of antibody responses that emerged upon RTS,S immunization, primarily based on IgG levels (Additional file 1: Table S2 multivariable models and Table S3 univariable models): decrease in antibody levels ("group i" antigens, Fig. 1a); no change in antibody levels ("group ii" antigens, Additional file 1: Figure S1a); increase in antibody levels ("group iii" antigens, Fig. 1b). IgG levels to $MSP1_{42}$, EXP1, and AMA1 were significantly (or borderline statistically significant) lower in RTS,S vs comparators vaccinees at M3 (Additional file 1: Table S2). In contrast, IgG levels to EBA140, EBA175 R3–5, MSP1 Block (Bl) 2 (3D7, Well, RO33, and MAD20 strains), MSP5, MSP6, RH2 2030, RH4.2, RH5, and SSP2 (TRAP) were significantly higher in RTS,S vs comparators vaccinees at M3 (Additional file 1: Table S2) and/or vs baseline levels in RTS,S vaccinees only (Fig. 1b). More specifically, RTS,S vaccination increased from 1.52 to 6.55 times the M3 IgG levels against group iii antigens compared to comparators. IgM levels remained largely unaffected by RTS,S vaccination (Additional file 1: Figure S1b) except for $MSP1_{42}$ 3D7 that was significantly higher in comparators, as was seen for IgG to this antigen, and SSP2 that was significantly higher in RTS,S vaccinees (Additional file 1: Table S3a). To sum up,

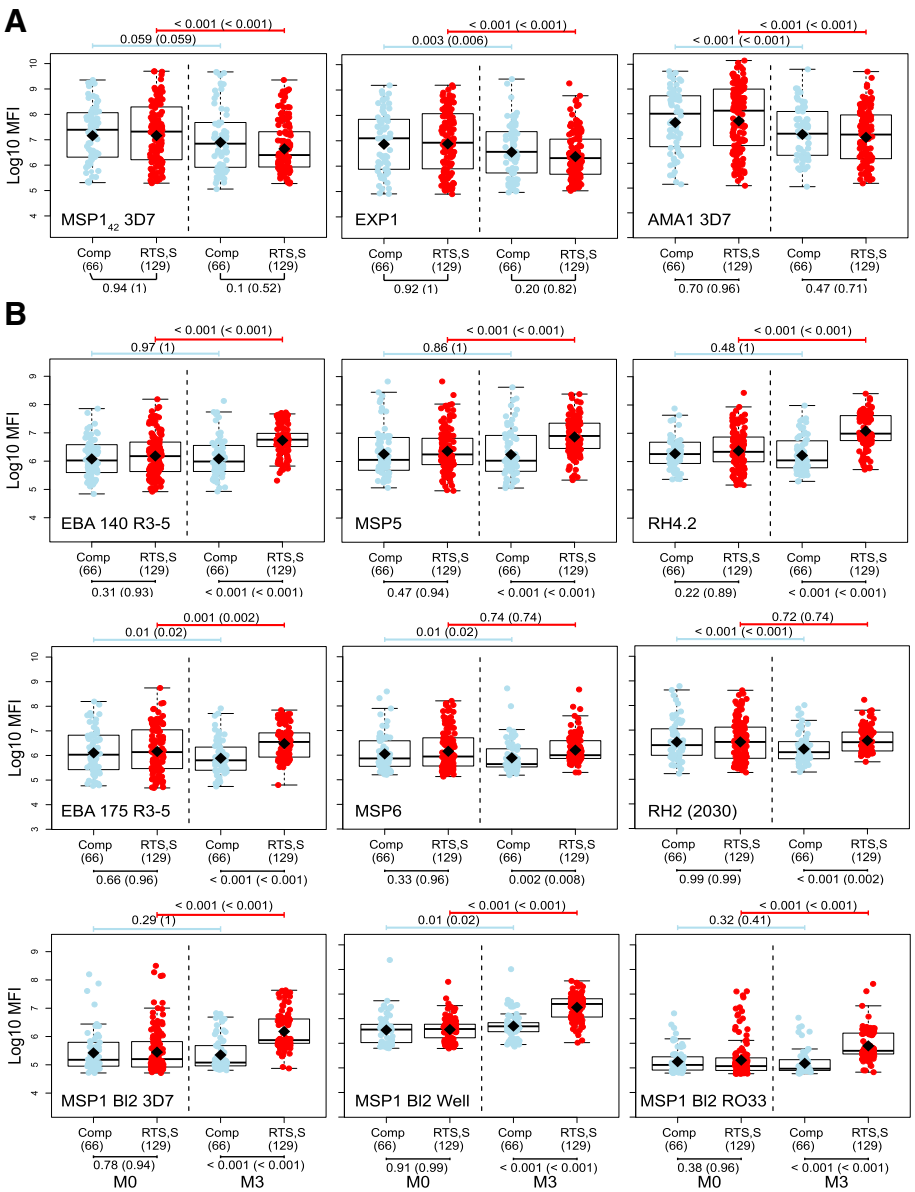

Fig. 1 Effect of RTS,S/AS01E vaccination on IgG levels to non-RTS,S *P. falciparum* antigens at month 3. **a** Group i: Antibody levels at month 3 (M3) were lower in RTS,S than in comparator vaccinees. **b** Group iii: Antibody levels were higher at month 3 in RTS,S than in comparator vaccinees. Some representative examples are shown. Group ii antigens are illustrated in Additional file 1: Figure S1. Boxplots illustrate the medians and the 25th and 75th quartiles, whiskers display 1.5 times interquartile ranges, and diamonds show the geometric mean. Groups were compared through *t* tests and *p* values corrected for multiple comparisons using the Holm approach. Raw p values and adjusted p values in parenthesis are shown. $\log_{10}$ MFI, $\log_{10}$ of the median fluorescence intensity levels measured by quantitative suspension array technology

RTS,S vaccination differently affected the IgG levels 1 month post-vaccination, depending on the antigens.

Effect of other variables on antibody responses to *P. falciparum* antigens

Next, we wanted to understand the effect of the study covariates on the M3 antibody levels measured, which could help explain the three patterns of responses that we observed upon vaccination. Comparing antibody levels over time, IgGs did not change markedly from M0 to M3. However, for some group i and ii antigens (AMA1, EXP1, MSP2, MSP3 3D7, EBA175 R2 F2, and the pregnancy-related VAR2CSA DBL1–2 and DBL3–4), IgG levels decreased with age from M0 to M3 (Fig. 1a) probably reflecting decay of maternal antibodies. Children had higher IgG levels than infants for most of the antigens at M3 in adjusted models (Additional file 1: Table S2), particularly for group ii (not affected by RTS,S)

and group iii (increased by RTS,S) antigens. At M0, IgG levels to group i antigens were higher in infants than in children in adjusted models, and there was a mixed pattern for group ii and iii antigens (Additional file 1: Table S4b). IgM levels to most antigens were significantly higher in children than in infants at M0 and were similar between age groups or higher in children at M3 (Additional file 1: Tables S2 and S3).

Regarding the effect of geographical location (site), M0 IgG levels for all antigens and IgM levels for most antigens were significantly higher in Kintampo than in Manhiça in adjusted models (Additional file 1: Table S4b), reflecting the higher MTI in Kintampo. However, many antibodies were not significantly different at M3 by site in adjusted models, and an inverse pattern was seen for IgG to many group iii antigens, MSP3 and LSA1, whereby levels were higher in Manhiça than in Kintampo (Additional file 1: Table S2).

Higher baseline Hb concentrations and WAZ (and HAZ to a lesser extent) were significantly associated with lower M0 IgM levels to most antigens in adjusted models, and some similar significant associations were found for IgG (Additional file 1: Table S4b). Sex had no significant associations with baseline IgG or IgM levels. The strongest and most consistent associations were for previous episodes of clinical malaria and higher M0 antibody levels (to the homologous antigen), both predicting higher M3 antibody levels (Additional file 1: Table S4). The magnitude of the associations, particularly for prior malaria episodes, was higher with greater significance for IgG than for IgM. Group i and ii antigens ($MSP1_{42}$, EXP1, MSP2) were more affected by previous malaria.

Overall, these results suggest that antigens from groups i and ii are more immunogenic and reflect better malaria exposure than antigens from group iii.

Correlations of RTS,S and non-vaccine-related antibody responses

We performed PCA of all M3 antibody responses, including IgG and IgM levels to RTS,S antigens (NANP, C-terminus, and CSP full length [7]), to reduce the dimensionality of the data and get insights into the relationship between vaccine and non-vaccine antibody responses. Study participants clustered by vaccine group (Fig. 2a) and principal component 2 (PC2 or dimension 2) separated both clusters. The 10 antibody responses contributing more to PC2 were, as expected, IgG against the three CSP constructs, IgM to CSP full length, but also group iii antigens: RH4.2 (that was highly correlated with CSP), four MSP1 Bl2 constructs, and EBA140 (Fig. 2a, b).

Correlations among IgG responses to non-RTS,S antigens of groups i, ii, and iii, in addition to RTS,S vaccine antigens, in RTS,S vaccinees, are depicted in Fig. 3. Three main patterns were observed: (1) most group iii antigens and CSPs significantly correlated among themselves, although to varying degrees (rho between 0.25 and 1); (2) $MSP1_{42}$, AMA1, MSP3 3D7, and VAR2CSA (DBL1–2 and DBL3–4) correlated negatively (rho between − 0.25 and 0) with CSPs, and together with MSP2 full-length Dd2 correlated negatively with MSP1 Bl2 Well (rho between − 0.5 and 0), and VAR2CSA (DBL1–2 and DBL3–4), MSP3 3D7, and AMA1 correlated negatively with most group iii antigens and some group ii antigens (rho between − 0.25 and 0); and (3) some group ii and all group i and iii antigens correlated positively among themselves (mainly with rho > 0.25) (Fig. 3). In addition, group ii antigen RH4.9 correlated negatively with CSP and some antigens of all three groups.

To assess whether antibody responses correlating with CSP could be an artifact of cross-reactive CSP antibodies, we aligned the *P. falciparum* 3D7 strain ortholog of each protein in this study with CSP to check for regions of local a.a. similarity. Among all proteins, including the GST tag, only SSP2 showed significant primary structure with *E* value < 1 for full-length queries (a.a. 249–286, % identity = 37.8, *E* value = 1.44E−05), aligning with the thrombospondin type 1 domain-containing CSP a.a. 330–374. At the peptide level (7 a.a. overlapping 25mers), two sequential SSP2 fragments had significant similarity corresponding to a.a. 235–259 (% identity = 90.9, *E* value = 0.002) and a.a. 253–277 (% identity = 66.7, *E* value = 0.17), the region of SSP2 containing a C-terminal thrombospondin type 1 domain. Additional hits using the default *E* value threshold of 10 are described in Additional file 1.

Antibodies to group i and ii antigens had no effect or were associated with increased risk of clinical malaria

We next wanted to decipher the role of the antibody responses measured in protection against clinical malaria. Having higher baseline IgM to most *P. falciparum* antigens, and higher baseline IgG to half of the antigens, was associated with occurrence of clinical malaria during the 12-month follow-up period after M3.5, as illustrated in Fig. 4 for IgG. In the univariable analysis, M3 antibodies to group i antigens and some group ii antigens that are considered markers of exposure (including maternal exposure) were higher in malaria cases than in controls (Fig. 4); stratified by age, this was more significant in children (Additional file 1: Figure S3). IgG levels to the above antigens that were significantly higher in infants than in children with malaria at baseline and in comparators were presumably derived from the mother (Additional file 1: Figure S3). In the multivariable analysis, these risk associations disappeared except for IgG to group i antigen AMA1 FVO (Table 1) and IgM to RH2 (2030) (OR = 3.66 [1.44; 10.03], $p = 0.008$).

Analysis of the change in IgG levels between M0 and M3 ($\log_{10}$ FC) showed that there were either no significant

Table 1 Effect of IgG levels to *Plasmodium falciparum* antigens on malaria protection

Antibody	Month 3 IgG levels		Age (5–17 months)		Site (Manhiça)		Baseline IgG levels		WAZ		Vaccine (RTS,S)	
Antigen	OR	*p*	OR	*p*	OR	*p*	OR	*p*	OR	*p*	OR	*p*
Group i antigens												
$MSP1_{42}$ 3D7	1.11 (0.7; 1.74)	0.65			*0.18 (0.08; 0.39)*	*< 0.001*	1.47 (0.96; 2.28)	0.08				
$MSP1_{42}$ FVO	0.87 (0.57; 1.29)	0.48	0.5 (0.23; 1.04)	0.07	*0.13 (0.05; 0.29)*	*< 0.001*	*1.47 (1.02;2.15)*	*0.04*	0.78 (0.56; 1.08)	0.13	0.59 (0.28; 1.2)	0.15
EXP1	0.8 (0.47; 1.33)	0.39			*0.19 (0.08; 0.43)*	*< 0.001*	*1.94 (1.24; 3.12)*	*0.005*	0.75 (0.54; 1.05)	0.1	0.53 (0.25; 1.1)	0.09
AMA1 FVO	*1.6 (1.15; 2.27)*	*0.006*			*0.13 (0.06; 0.25)*	*< 0.001*						
AMA1 3D7	0.98 (0.51; 1.83)	0.94			*0.14 (0.07; 0.28)*	*< 0.001*	1.54 (0.93; 2.67)	0.1	0.78 (0.56; 1.08)	0.14	0.56 (0.27; 1.15)	0.12
Group ii antigens												
pTRAMP	1.01 (0.51; 2.03)	0.98	*0.42 (0.2; 0.85)*	*0.02*	*0.12 (0.06; 0.25)*	*< 0.001*	*2.18 (1.16; 4.21)*	*0.02*			*0.54 (0.26; 1.11)*	*0.1*
EBA175 R2(F2)	0.96 (0.56; 1.64)	0.89	*0.49 (0.24; 0.98)*	*0.047*	*0.09 (0.04; 0.18)*	*< 0.001*						
PfRH1	1.58 (0.79; 3.19)	0.2	*0.4 (0.18; 0.83)*	*0.02*	*0.11 (0.05; 0.23)*	*< 0.001*			0.79 (0.56; 1.09)	0.15		
MSP3 3D7	1.32 (0.77; 2.3)	0.32	0.51 (0.25; 1.02)	0.06	*0.1 (0.05; 0.2)*	*< 0.001*						
Var2csa DBL3–4	1.03 (0.5;2.1)	0.94	0.5 (0.21; 1.13)	0.1	*0.09 (0.04; 0.2)*	*< 0.001*						
p41	1.41 (0.64; 3.16)	0.4	*0.46 (0.22; 0.94)*	*0.04*	*0.1 (0.05; 0.19)*	*< 0.001*						
MSP2 FL Dd2	0.89 (0.47; 1.61)	0.71			*0.25 (0.11; 0.57)*	*0.001*	*2.15 (1.31; 3.67)*	*0.003*	0.76 (0.54; 1.06)	0.11	0.52 (0.25; 1.07)	0.08
MSP2 FL CH150	1.02 (0.57; 1.83)	0.96			*0.25 (0.11; 0.58)*	*0.001*	*2.05 (1.31; 3.29)*	*0.002*	0.76 (0.54; 1.06)	0.11	0.44 (0.2; 0.92)	0.03
MSP1 BI2 hybrid	1.11 (0.68; 1.83)	0.67	*0.5 (0.24; 0.98)*	*0.049*	*0.09 (0.04; 0.19)*	*< 0.001*						
MSP3 3C	1.22 (0.74; 2.01)	0.43	*0.41 (0.19; 0.84)*	*0.02*	*0.1 (0.05; 0.22)*	*< 0.001*	1.67 (0.94; 2.99)	0.08			0.55 (0.27; 1.14)	0.11
PfRH2 b240	1.03 (0.49; 2.18)	0.94	*0.49 (0.24; 0.98)*	*0.049*	*0.09 (0.04; 0.19)*	*< 0.001*						
LSA1	0.88 (0.51; 1.49)	0.64	0.5 (0.24; 0.99)	0.051	*0.09 (0.04; 0.18)*	*< 0.001*						
MSP1 BI2 PA17	0.96 (0.53; 1.7)	0.89	*0.5 (0.24; 0.98)*	*0.048*	*0.09 (0.04; 0.18)*	*< 0.001*						
Var2csa DBL1–2	0.9 (0.37; 2.27)	0.82			*0.14 (0.07; 0.29)*	*< 0.001*	*2.06 (1.01; 4.26)*	*0.047*			0.57 (0.28; 1.16)	0.12
CyRPA	1.81 (0.76; 4.43)	0.18	*0.4 (0.18; 0.82)*	*0.02*	*0.1 (0.04; 0.19)*	*< 0.001*			0.79 (0.57; 1.09)	0.15	0.57 (0.28; 1.17)	0.13
CelTOS	1.55 (0.77; 3.13)	0.22	*0.41 (0.19; 0.85)*	*0.02*	*0.1 (0.05; 0.2)*	*< 0.001*			0.79 (0.57; 1.09)	0.16	0.58 (0.28; 1.18)	0.13
RH4.9	0.62 (0.3; 1.26)	0.19	0.53 (0.26; 1.05)	0.07	*0.08 (0.04; 0.16)*	*< 0.001*						
Group iii antigens												
DBL-α	1.35 (0.62; 2.91)	0.44	*0.45 (0.21;0.92)*	*0.03*	*0.12 (0.05; 0.24)*	*< 0.001*	1.9 (0.88; 4.22)	0.11				
RH5	1.93 (0.98; 4.01)	0.07	*0.38 (0.17; 0.79)*	*0.01*	*0.11 (0.05; 0.21)*	*< 0.001*			0.78 (0.56; 1.08)	0.15	0.51 (0.24; 1.07)	0.08
SSP2 (TRAP)	*0.52 (0.26; 0.98)*	*0.05*			*0.11 (0.05; 0.23)*	*< 0.001*	*2.29 (1.14; 4.8)*	*0.02*				
MSP1 BI2 Mad20	0.61 (0.13; 2.7)	0.52	0.55 (0.26; 1.13)	0.11	*0.14 (0.07; 0.3)*	*< 0.001*	*5.91 (1.5; 29.52)*	*0.02*	0.79 (0.57; 1.09)	0.15		
MSP6	1.19 (0.56; 2.61)	0.65	*0.47 (0.21; 0.98)*	*0.05*	*0.14 (0.06; 0.3)*	*< 0.001*	1.63 (0.89; 3.01)	0.12	0.78 (0.56; 1.07)	0.12	0.51 (0.23; 1.09)	0.08
RH2 (2030)	1.37 (0.72; 2.65)	0.35	0.51 (0.25; 1.01)	0.059	*0.1 (0.04; 0.19)*	*< 0.001*					0.55 (0.26; 1.16)	0.12
EBA175 R3–5	1.17 (0.71; 1.96)	0.54	*0.45 (0.21; 0.91)*	*0.03*	*0.1 (0.05; 0.2)*	*< 0.001*			0.79 (0.57; 1.09)	0.16	0.55 (0.25; 1.18)	0.13

Table 1 Effect of IgG levels to *Plasmodium falciparum* antigens on malaria protection *(Continued)*

Antibody	Month 3 IgG levels		Age (5–17 months)		Site (Manhiça)		Baseline IgG levels		WAZ		Vaccine (RTS,S)	
Antigen	OR	*p*	OR	*p*	OR	*p*	OR	*p*	OR	*p*	OR	*p*
MSP5	0.78 (0.48; 1.25)	0.3	0.49 (0.23; 1)	0.056	*0.12 (0.06; 0.24)*	*< 0.001*	*2.06 (1.26; 3.45)*	*0.005*				
EBA140 R3–5	1.21 (0.69; 2.14)	0.5	*0.47 (0.22; 0.94)*	*0.04*	*0.09 (0.04; 0.18)*	*< 0.001*					0.54 (0.24; 1.19)	0.13
MSP1 BI2 RO33	*0.53 (0.3; 0.93)*	*0.03*	0.53 (0.25; 1.07)	0.08	*0.1 (0.04; 0.21)*	*< 0.001*	1.64 (0.93; 3.03)	0.1				
MSP1 BI2 Well	*0.55 (0.3; 0.99)*	*0.05*	0.51 (0.23; 1.07)	0.08	*0.1 (0.05; 0.2)*	*< 0.001*	2.06 (0.95; 4.72)	0.08				
MSP1 BI2 3D7	0.63 (0.39; 1)	0.054	*0.47 (0.22; 0.96)*	*0.042*	*0.08 (0.04; 0.17)*	*< 0.001*			0.79 (0.56; 1.09)	0.16		
RH4.2	0.85 (0.52; 1.38)	0.51	0.53 (0.25; 1.06)	0.08	*0.09 (0.04; 0.18)*	*< 0.001*						

Each line shows the multivariable logistic regression model's results with IgG levels to each antigen (expressed as $\log_{10}$ of the median fluorescence intensity measured by quantitative suspension array technology) at month 3 as predictors and clinical malaria as outcome, adjusted by covariates. Columns show the odds ratio (OR) with 95% confidence intervals and *p* values (significant in italics) of the month 3 IgG levels and the covariables retained in the models for some antigens (in parenthesis the category that is compared to the reference). Variables that were not significant or did not improve the multivariable model are not shown.
WAZ weight-for-age *Z*-score, *FL* full length, BI2 block 2

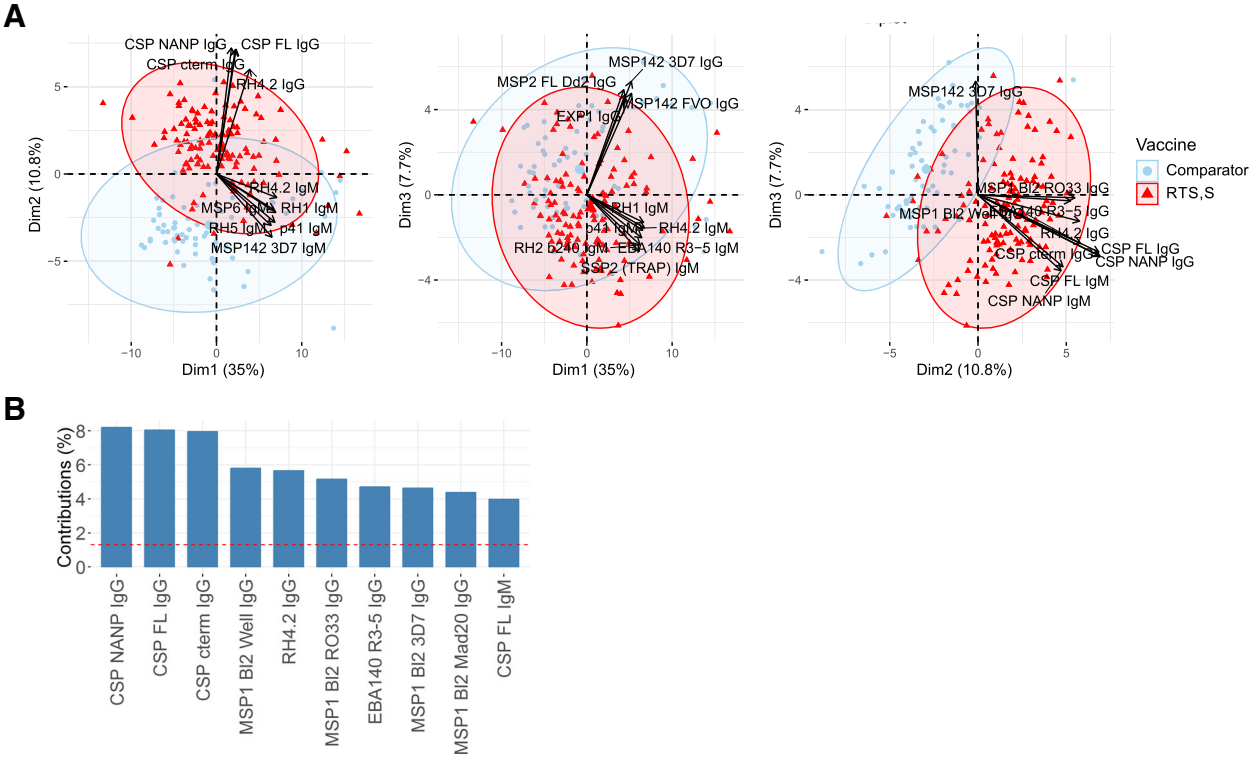

Fig. 2 Relationship among antibody responses to RTS,S and non-RTS,S antigens after RTS,S/AS01E vaccination. **a** Principal component analysis (PCA) plots of individuals clustered by vaccine type. The first three principal components (Dim 1, Dim 2, and Dim 3) that explained the highest percentage of the variance of the IgG and IgM data at month 3 (percentage in parenthesis) were chosen for representation. **b** Contribution of the top 10 variables to the PCA Dim 2. The red dashed line indicates the expected average contribution. Any variable with a contribution above this cutoff can be considered as important in contributing to the component

effects of $\log_{10}$ FC on malaria risk or, if there were, IgG levels declined from M0 to M3, and the decrease was lower in non-malaria controls than in malaria cases (Fig. 4). The drop in antibodies from M0 to M3, more pronounced in infants, and the lower decrease in protected subjects, could be due to more production and/or less antibody decay from M0 to M3. In fact, age-stratified analyses (Fig. 5 and Additional file 1: Figure S3) showed that malaria-protected children had an increase of IgG against EXP1 and MSP2 from M0 to M3, whereas children with malaria cases had a decrease and, therefore, significantly higher $\log_{10}$ FC in protected children. In multivariable analysis, IgG $\log_{10}$ FC was associated with malaria protection for EXP1 (OR = 0.6 [0.38; 0.91], $p = 0.02$), MSP2 Dd2 (OR = 0.54 [0.32; 0.87], $p = 0.02$), and MSP2 CH150 (OR = 0.58 [0.37; 0.88], $p = 0.01$, also for IgM).

Antibodies to group iii antigens were associated with reduced risk of clinical malaria

In contrast, higher M3 IgG levels to three group iii antigens, SSP2 and MSP1 Bl2 (strains RO33 and Well, borderline for 3D7), were significantly associated with reduced risk of clinical malaria (Table 1). In these multivariable analyses, RTS,S vaccination, children age cohort, Manhiça site, low baseline antibody levels, and high WAZ were associated (statistically significantly or not) with lower risk of malaria (Table 1). Sex, prior malaria episodes, season, HAZ, or Hb concentrations did not significantly contribute to the models for any antigen. In addition, non-malaria controls had significantly higher $\log_{10}$ FC than malaria cases (Fig. 4), unlike the pattern seen for some group i and ii antigens. Stratifying by age group and site, the protective association was seen only for children and mainly in samples from Kintampo (Fig. 5b). In the multivariable analysis, an association with protection between higher IgG $\log_{10}$ FC and malaria was significant for MSP5 (OR = 0.62 [0.41; 0.91], $p = 0.02$) in addition to SSP2 and MSP1 Bl2 RO33 and Well strains, for which M3 levels were also significantly associated with protection (Table 1), and additionally for MSP1 Bl2 Mad20 (OR = 0.3 [0.09; 0.88], $p = 0.03$).

Multimarker analyses of antibody levels and malaria protection

To perform multimarker analysis accounting for co-linearity in antibody responses, we performed PLS-DA of antibodies at M3 among RTS,S vaccinees, with malaria as a response variable (Fig. 6a, b), and identified two

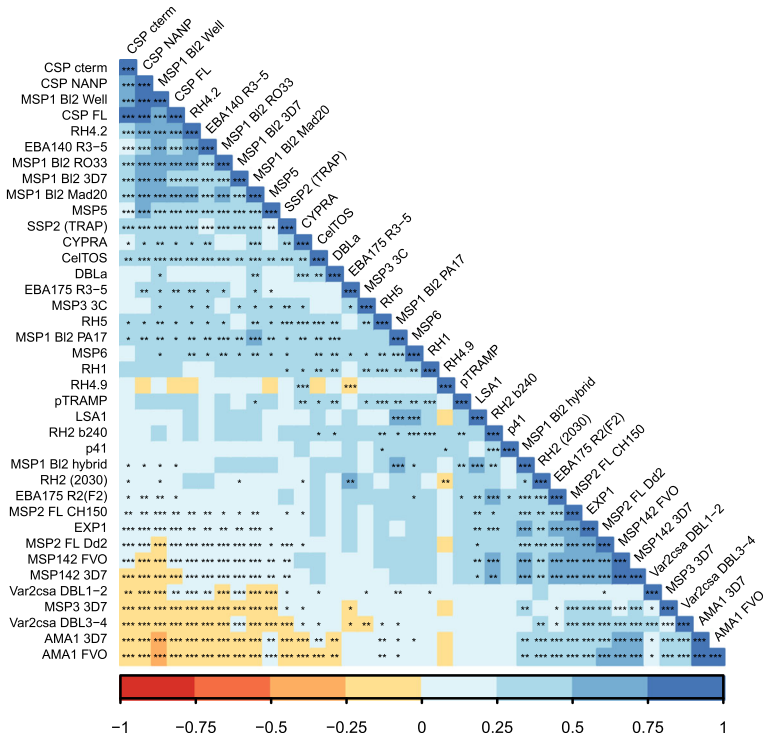

Fig. 3 Correlation among IgG responses in RTS,S vaccinees. Colors indicate Spearman coefficients (rho). *p* values: *< 0.05, **< 0.01, ***< 0.001

components associated with the outcome. Interestingly, the loadings of component 1 (Fig. 6b) showed a pattern in which roughly all group i and ii antigens and almost half of group iii antigens were associated with increased malaria risk. IgG levels to the other half of group iii antigens (SSP2, MSP5, EBA140, MSP1 Bl2 [RO33, Well, 3D7], RH4.2) and CSP (C-terminus, NANP, and full length) were associated with protection, whereas almost all IgM levels to the same antigens were associated with increased risk. In component 2, there was a mixed pattern for groups i and ii antigens, while IgG and IgM to exactly the same group iii antigens as component 1 were associated with protection. In a multivariable logistic model including these two PLS-DA components and adjusted by age and site, the PLS-DA components were independently associated with malaria (component 1: OR = 1.26 [1.09; 1.48], $p = 0.002$ and component 2: OR = 1.31 [1.06; 1.66], $p = 0.016$), while age was not (OR = 1.22 [0.38; 3.93], $p = 0.73$) and site was borderline (OR = 0.33 [0.11; 1.01], $p = 0.05$). The predictive ability of the model was moderate (33 out of 54 malaria cases and 64 out of 75 controls correctly predicted) with an AUC of 0.732 and a cumulative R^2 of 0.397.

A schematic summary of the characteristics of antibody responses to group i vs iii antigens is shown in Fig. 7. Antigens to which there were more maternally derived antibodies at M0 and increased with previous malaria at M3 (and by M0 levels), and for which levels were higher in Kintampo than in Manhiça (group i), were the ones that did not increase with RTS,S vaccination at M3, and vice versa. Table 2 shows a summary of the analyses of correlates with malaria risk and protection.

Discussion

Immunization with a partially effective PE malaria vaccine, RTS,S/AS01E, may affect the acquisition of IgG and IgM responses to multiple PE and BS *P. falciparum* antigens upon natural microbial exposure in young African hosts, some of whom have maternal IgGs. Our results reveal an association between these antibody levels and protection against clinical malaria, in two areas of different endemicity. Data from a phase 3 clinical trial showed that RTS,S vaccination can alter NAI antibodies to *P. falciparum* in two different ways: by decreasing antibody levels ("group i" antigens), as seen in prior phase 2b trials [12, 13, 24], or by increasing IgG levels ("group iii" antigens), which to our knowledge has not been reported before, likely due to the limitations of previous studies in their breadth of antibody screen and time points available for study. This study had the additional advantage of being able to draw on the association between antibodies to NAI antigenic targets and protection from, or risk of, clinical malaria measured prospective in a controlled multicenter trial.

On the one hand, RTS,S vaccination decreases exposure to the parasite by providing protection against malaria

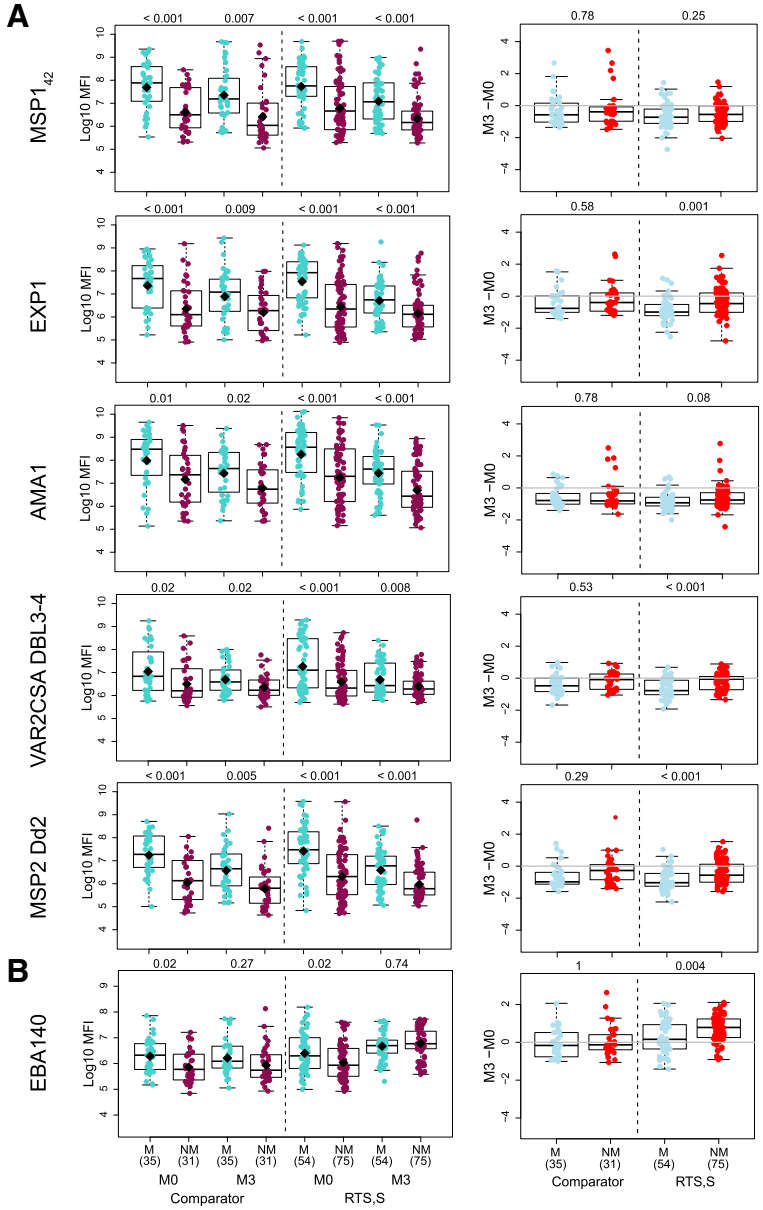

Fig. 4 Association between IgG responses to non-RTS,S antigens and malaria protection. **a** Antigen groups i and ii. **b** Antigen group iii. Levels of IgG and change from pre- (M0) to post-vaccination (M3). Some representative examples of the distinct patterns observed in each group are shown. Boxplots illustrate the medians and the 25th and 75th quartiles, whiskers display 1.5 times interquartile ranges, and diamonds show the geometric mean. Groups were compared through *t* tests and *p* values corrected for multiple comparisons using the Holm approach are shown. M malaria, NM no malaria, $\log_{10}$ MFI $\log_{10}$ of the median fluorescence intensity (MFI) levels measured by quantitative suspension array technology, M3-M0 change between M0 and M3 antibody levels expressed as $\log_{10}$ MFI

infection and this would be reflected in the reduction of antibody levels to *P. falciparum* antigens that are primarily markers of microbial exposure, even if functional antibody activity exists. This effect would be more distinct with time, as seen in prior phase 2b studies by Campo et al. 6 months after vaccination (M8.5 visit), particularly in younger children [12, 13]. In our phase 3 study, group i antigens that are considered and used as markers of MTI in seroepidemiological surveys [15] showed this pattern, specifically MSP1$_{42}$ and AMA1. Notably, as early as 1 month post-vaccination (M3), there were some significant differences in antibody levels as a result of reduced *P. falciparum* BS exposure associated with vaccine efficacy. Antibodies to MSP1$_{42}$, in particular, appear to be very fast and sensitive sensors to detect changes in parasite exposure. We predict that with time, antibody levels

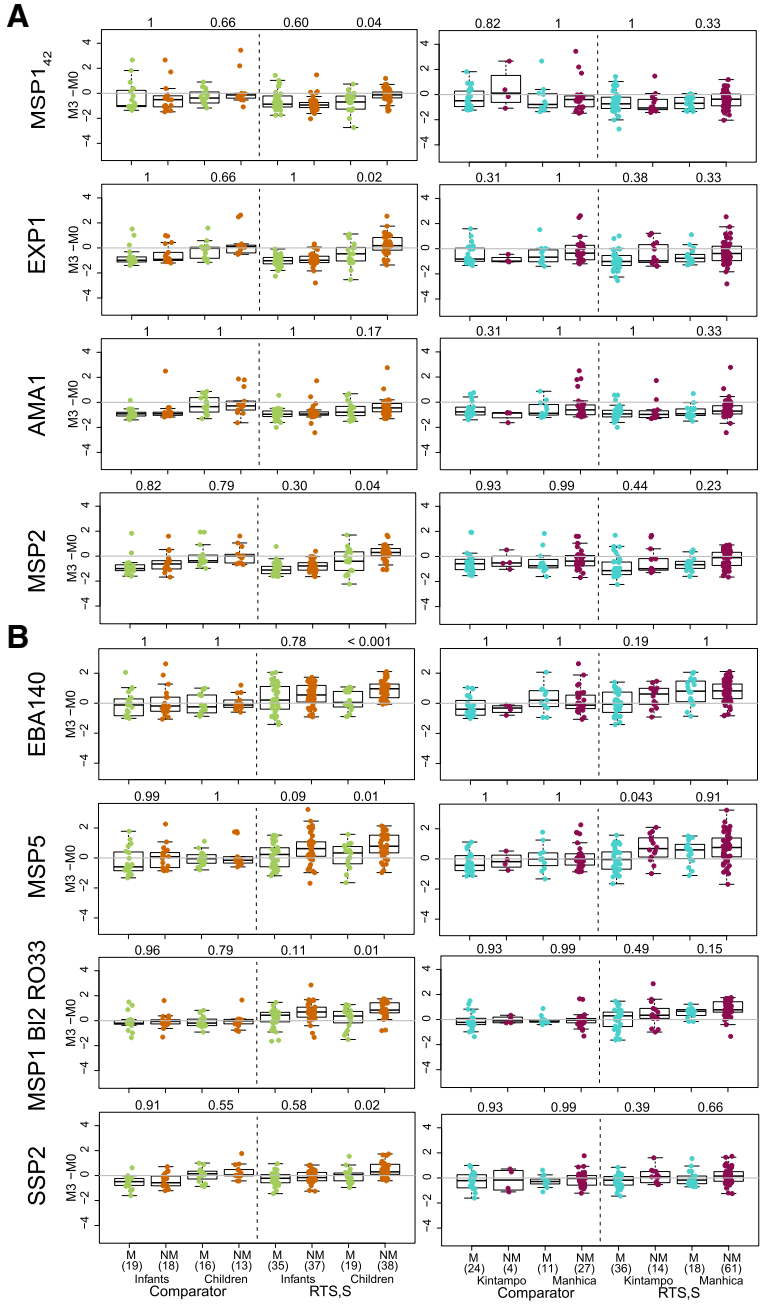

Fig. 5 Association between IgG levels to non-RTS,S antigens and malaria protection stratified by age and site. **a** Antigen groups i and ii. **b** Antigen group iii. Change from pre- (M0) to post-vaccination (M3) antibody levels. Some representative examples are shown. Boxplots illustrate the medians and the 25th and 75th quartiles, whiskers display 1.5 times interquartile ranges, and diamonds show the geometric mean. Groups were compared through *t* tests and *p* values corrected for multiple comparisons using the Holm approach are shown. M malaria, NM no malaria, M3-M0 change between M0 and M3 antibody levels expressed as $\log_{10}$ of the median fluorescence intensity (MFI) measured by quantitative suspension array technology

to most group ii antigens would also be reduced as a result of RTS,S vaccination, similar to the Campo et al. studies. Antibodies to group i and ii antigens behave as markers of exposure based on multiple criteria: (a) they are significantly higher in Kintampo (higher MTI) than in Manhiça (lower MTI), (b) increase significantly if the subject had prior malaria episodes, (c) M3 levels strongly correlate with M0 levels, and (d) point toward an increased malaria risk (AMA1). Furthermore, IgG levels for many of those antigens are elevated at baseline, higher in infants than in children, and significantly decay from M0 to M3, indicating a predominantly maternal origin. Transplacental

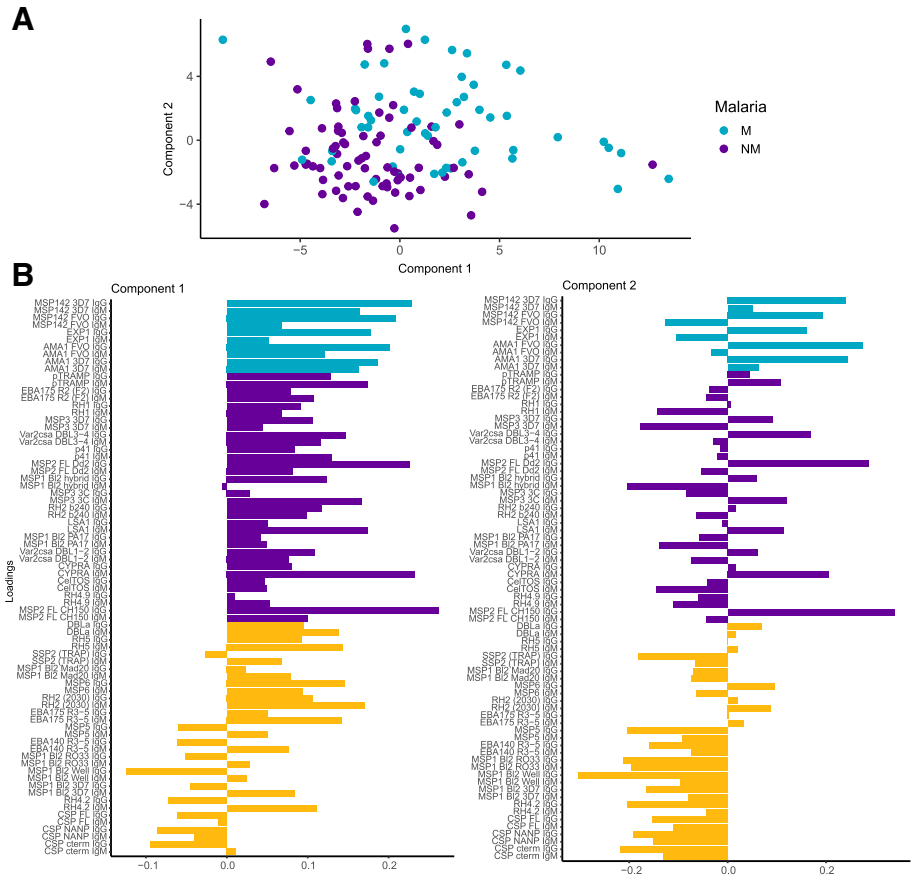

Fig. 6 Multimarker analysis of antibody responses to RTS,S and non-RTS,S antigens after vaccination and malaria protection. **a** Partial least squares discriminant analysis (PLS-DA) scatter-plot with the two PLS-DA components that best enable discrimination between malaria cases (blue) and non-malaria controls (pink). **b** Loadings of antibody response for each PLS-DA component colored by antigen group: group i (light blue), group ii (dark purple), group iii plus vaccine antigens (yellow)

antibodies at birth are also a reflection of MTI in the community, and higher levels at baseline are associated with prospective malaria risk. IgG to some antigens like AMA1 and VAR2CSA DBL1–2 and DBL3–4 appear to be longer-lived maternal antibodies, as they remain higher in infants than in children also at M3.

On the other hand, our data also show that vaccination with RTS,S can result in an increase in antibody levels to other antigens (group iii) with respect to comparators and baseline. Stratified by age, the effect of increased antibody levels at M3 by RTS,S (not comparator) vaccination is manifested in both infants and children but appears more pronounced in children. This could be because children have more exposure cumulatively and no maternal IgG remaining at M3 that could interfere with the build-up of their own antibodies. It could also be that children are able to produce stronger responses just because of intrinsic age-related particularities of the immune system, such as maturation of T-dependent B cell responses [25]. Interestingly, upon RTS,S vaccination, IgG levels to group iii antigens increased in both sites, Manhiça and Kintampo; however, Manhiça had a greater increase post-vaccination than Kintampo, perhaps representing complex interactions between host immune response and levels of parasite exposure. Remarkably, the M0 to M3 antibody kinetics and the analysis of factors affecting the Ig levels suggest that group iii antigens are weaker markers of exposure than group i and ii antigens and that infants are not born with high IgG to them. It could be that antibodies to these antigens might be transferred less transplacentally (due to antigen-driven polarization of subclasses or glycoprofiles affecting binding to FcRn) or that mothers have lower levels. Thus, adults could have lower IgG levels to group iii antigens than children, i.e., an age pattern such that antibodies increase in early infancy and childhood but at some point decrease and are lower in adults, contrary to group i antigens. For some antigens like EBA140, this is consistent with prior analyses of RNA expression in parasites that decreased with age [26]. The different age pattern of group iii antibodies (e.g., EBA140) points to

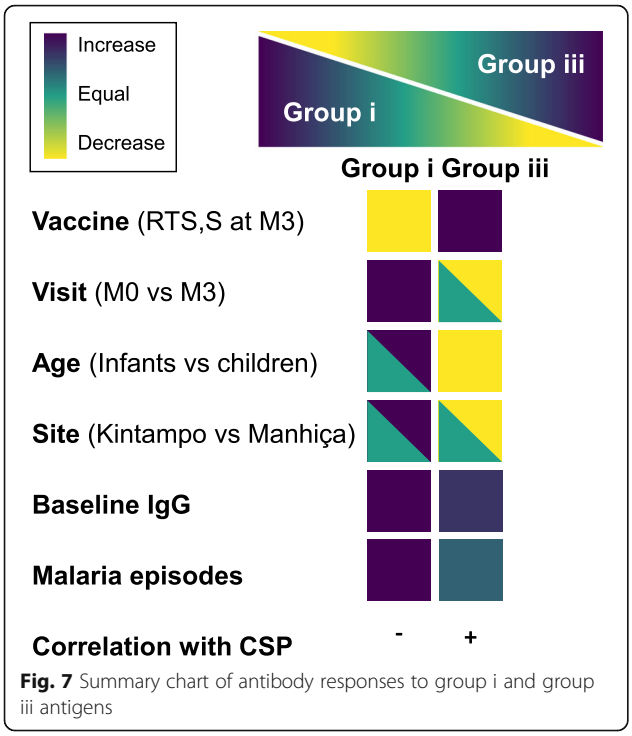

Fig. 7 Summary chart of antibody responses to group i and group iii antigens

different roles in anti-disease vs anti-parasite immunity, which may be acting at different phases of NAI acquisition. Therefore, it could be that the antibody "enhancement" seen with RTS,S for group iii antigens may be related mostly to the absence/low levels of maternally derived IgGs to these antigens such that host responses are not interfered with, in contrast to group i and ii antigens. Overall, there seems to be an inverse relationship between group i + ii ("exposure") vs group iii antigens (see opposite patterns in Fig. 7 and Additional file 1: Table S2 and Figure S2). Interestingly, antibody levels are heterogeneous within antigen groups, as different behaviors are observed upon close examination that imply that the determinants and patterns of antibody responses to each individual antigen are more complex and diverse than these three categories. For example, when IgG to group i and ii antigens are stratified by age, IgG responses to EXP1 and MSP2 in RTS,S-vaccinated children (who do not have maternal antibodies at M3) resemble those of group iii antigens.

How could a vaccine that reduces exposure to the parasite be associated with an increase in antibody levels? This could be related to the fact that RTS,S may not result in sterile immunity, particularly in the longer term. Instead, it appears to be a partially effective or "leaky" PE vaccine. To explain the differences in duration of vaccine efficacy between two cohorts subject to diverse MTI in the Mozambican phase 2b trial, we hypothesized that partial protection afforded by RTS,S/AS0 may stimulate protective antibodies to certain asexual BS target antigens, through a reduction in merozoite release from the liver, leading to attenuated BS parasitemia. As CSP-specific highly protective immunity decays in the short term [27–30], incoming infections would be partially controlled resulting in subpatent low antigen doses that elicit enhanced antibody production to certain antigens. This would be reflected in accelerated acquisition of BS protective immunity [10, 12, 29]. As this is an exposure-dependent mechanism, the outcome will differ depending on the force of infection to which subjects are exposed, i.e., MTI- and age-dependence. Therefore, a certain MTI window (enough but not too much) may maximize benefits of RTS,S vaccination and baseline factors (e.g., frequency/intensity of parasite exposure, presence of maternal antibodies) may modulate non-RTS,S antigen responses affected by the vaccine. This would imply that the potential positive impact of immunization with RTS,S or other leaky vaccines may not be apparent in sites with too low MTI and that it might be lost in sites with too high MTI, which may overwhelm the initial required protective effect in the liver stage. It is remarkable that this antibody enhancement phenomenon can occur in the short 3-month interval of our study (during vaccination and 1 month post-vaccination). The concept of immunologically favorable impact of low-level infections is not new, as a similar explanation was proposed for the prolonged protective effect of intermittent preventive treatment in infants with sulfadoxine-pyrimethamine (IPTi-SP) in Tanzania upon interruption of treatment [31]. Thus, induction of effective and sustained immunity against malaria by IPTi (or RTS,S) could be due to the generation of low-dose blood-stage inocula and attenuated infections [32], and this could translate into higher antibody responses [33]. Additionally, insecticide-treated bednet use can also enhance antibodies [34] and ultra-low dose of BS infection can protect [11], although residual antimalarial concentration before challenge compromised these results, and protection was not thought to be antibody-mediated. Furthermore, not only BS but also PE antibodies like anti-SSP2 IgG and IgM increased. Conceivable, this could be an independent mechanism whereby RTS,S-induced CSP antibodies [7] recognizing parasites may increase phagocytocis of sporozoites which leads to presentation of other PE antigens to the immune system and consequent antibody responses. The fact that RTS,S affects non-vaccine antibody responses would suggest an association between antibody levels to CSP and group iii antigens, as shown in the PCA and correlation analyses here. At least at the level of primary structure, there is little evidence that RTS,S-induced CSP antibodies cross-react with group iii antigens with the alignment strategy used here. This does not

Table 2 Summary table of associations between IgG responses and malaria protection. Dark purple represents association with protection and light blue with risk

		IgG							
		t-test				Multivariable logistic regression model		PLS-DA	
		M3		M3-M0		M3	M3-M0	M3	
		Comparator	RTS,S	Comparator	RTS,S			Component 1	Component 2
Group i	$MSP1_{42}$ 3D7								
	$MSP1_{42}$ FVO								
	EXP1								
	AMA1 FVO								
	AMA1 3D7								
Group ii	pTRAMP								
	EBA175 R2(F2)								
	PfRH1								
	MSP3 3D7								
	Var2csa DBL3-4								
	p41								
	MSP2 FL Dd2								
	MSP2 FL CH150								
	MSP1 Bl2 hybrid								
	MSP3 3C								
	RH2 b240								
	LSA1								
	MSP1 Bl2 PA17								
	Var2csa DBL1-2								
	CyRPA								
	CelTOS								
	RH4.9								
Group iii	DBL-α								
	RH5								
	SSP2 (TRAP)								
	MSP1 Bl2 Mad20								
	MSP6								
	RH2 (2030)								
	EBA175 R3-5								
	MSP5								
	EBA140 R3-5								
	MSP1 Bl2 RO33								
	MSP1 Bl2 Well								
	MSP1 Bl2 3D7								
	RH4.2								

preclude the possibility of tertiary structure or other undetected similarities between any of the antigens tested with CSP, but does suggest that the group iii antigens identified in this analysis represent targets of specific antibodies affected by RTS,S vaccination.

What is the immunological relevance of RTS,S-mediated changes in non-vaccine antibodies on malaria protective immunity? Antibodies to group i and ii antigens were generally associated with increased malaria risk, consistent with their role as markers for identifying individuals under the highest attack rates. There is little dispute that antibodies to exposure antigens such as AMA1 can protect against malaria, particularly allele-specific infection [35], but these effects are often confounded in immunoepidemiological studies by the strong effect of prior exposure on both antibodies and future risk of repeated infection [36]. In contrast, antibodies to group iii antigens seem to have a clearer association with NAI in our study. Among group iii antigens, the most consistent associations between M3 antibody levels and immunity were for MSP1 Bl2 [RO33, Well, 3D7], MSP5, EBA140, RH4.2, and SSP2 antigens. Antibody responses to MSP1 Bl2 [37], MSP5 [38, 39], EBA140 [40], and RH4.2 [41, 42] BS antigens have been previously associated with protection against malaria, and SSP2 (TRAP) is the target of other leading subunit PE vaccine candidates [43]. Functionally, antibodies against erythrocyte binding antigens, including EBA140 [44], and against the reticulocyte binding protein-like homolog family, particularly RH4 and RH5 [45], have shown in vitro inhibition of erythrocyte invasion. These parasite proteins play critical roles as ligands for host erythrocytes and the process of merozoite invasion. It follows that RTS,S enhancement of such antibodies could provide an additional layer of protective immunity on the back end of an infection. The functional relevance of MSP5 antibodies is less clear, although antibodies to MSP5 orthologues have been

protective in animal models [46, 47]. Interestingly, MSP5 has high levels of expression in *P. falciparum* sporozoites (PlasmoDB.org; [48]), and MSP5 antibodies correlate with the PE sterile protection observed in humans immunized with irradiated sporozoites (Campo et al., in preparation), perhaps hinting at a multistage function of MSP5 antibodies. Under certain circumstances (e.g., in children with low baseline Ig), antibody responses to EXP1 and MSP2 and other antigens may also have a role in protection.

What is the net impact of the interaction between RTS,S immunization and NAI on overall protection against malaria? On one hand, there is a potential negative effect whereby RTS,S vaccination, by providing protection against malaria infection, might reduce NAI that would normally be induced by repeated infections. This could render vaccinated children more susceptible when the vaccine effect wanes [49] if such antibodies were mediators of NAI, and potentially lead to malaria "rebound" [50]. However, it appears that antibodies that decrease following RTS,S vaccination are mostly markers of exposure that reflect vaccine efficacy [12, 13, 24]; thus, this may not be a concern. On the other hand, there is a potential positive impact whereby NAI could improve overall vaccine efficacy in the capacity of RTS,S to protect against malaria, combining the effect of experimentally elicited and NAI-dependent immunity, and this may be age- and/or exposure-dependent. Malaria prevention in early life when the infant is at highest risk of the negative effects of infection is thought to contribute to an overall lower risk of severe morbidity and thus mortality in childhood [51]. Varying endemicity may differentially influence the long-term morbidity and mortality risk so that the benefits of RTS,S vaccination may be maximized in moderate MTI settings. In the pediatric field trials of RTS,S, there was evidence of different vaccine efficacies depending on malaria surveillance system and MTI [29], although in the phase 3 trial differences due to MTI were only significant in children when administered with a vaccine booster dose [49]. The long-term impact of the interaction between RTS,S vaccination and NAI on duration of vaccine efficacy and overall protection against malaria will be investigated in follow-up studies including the subsequent trial visits at M20, M21, and M32 till study end. We speculate that antibodies altered by RTS,S vaccination will affect the longevity of protective immunity in an age- and MTI-dependent manner.

Importantly, our study provides evidence for a positive effect of RTS,S on antibody responses to certain antigens that are associated with protection. Since multivariable logistic regression models in which IgGs to MSP1 Bl2, MSP5, and SSP2 (that are increased by vaccination) were associated with protection were adjusted by RTS,S vaccination, there is an additional protective effect of these antibodies on top of the protection afforded by the RTS, S vaccine. We postulate that these responses are contributing to the RTS,S-induced malaria protection and that these antigens could improve efficacy in a future multivalent next-generation vaccine. Such formulations raise the possibility of saturation of the immune system, having to react simultaneously to multiple antigens. However, there are vaccines given through the expanded program of immunization (EPI) that contain more than 3 antigens and are able to induce adequate immune responses. Moreover, it is envisaged that in the pilot implementation starting 2019 in three African countries, RTS,S will not be administered to infants as part of the EPI but to children age 5–17 months; thus, immune saturation may not represent a significant limitation.

The study design has some limitations that could be addressed in follow-up studies including more sites and larger sample size. In Kintampo, all children who fulfilled the inclusion criteria, most of whom had malaria, were analyzed. Whereas in Manhiça, a case-control design was needed because there were fewer cases; a cohort design would have required increasing the number of samples substantially to have power to detect association with protection. In Manhiça, there was also an age imbalance, as most cases were in infants. In addition, the phase 3 trial design did not allow for collection of genetics data from the volunteers. Therefore, host genetics-based differences in the immune responses to vaccination between the two African populations, including hemoglobinopathies such as the sickle cell trait, could not be assessed here. Despite this and having many antigens and multiple comparisons, patterns emerged that were consistent and biologically plausible.

Conclusion

Baseline characteristics related to age and exposure appear to modulate the nature and magnitude of antibody responses to distinct sets of *P. falciparum* antigens induced by the interaction between vaccination and NAI. Importantly, antibodies to certain antigens which may be targets of protective NAI may be enhanced by RTS, S/AS01E vaccination, contributing to vaccine efficacy. These antigens could potentially be candidates of multivalent next-generation RTS,S formulations to improve vaccine efficacy as additive (or synergistic) responses with CSP may occur. We postulate that antibodies affected by RTS,S/AS01E vaccination at peak response will contribute to the duration of protection. We plan to test this hypothesis in the extended follow-up of the phase 3 trial.

Additional file

Additional file 1: Supplementary information including figures, tables, methods, results, and references. **Figure S1.** Antibody responses to non-RTS,S *Plasmodium falciparum* antigens per visit and vaccination group. **Figure S2.** Antibody responses to non-RTS,S *Plasmodium falciparum* antigens after RTS,S/AS01E vaccination stratified by age and site. **Figure S3.** Association between IgG antibody levels and malaria risk stratified by age and site. **Table S1.** Antigens included in the multiplex quantitative suspension array panel. **Table S2.** Effect of RTS,S vaccination and other variables on antibody levels at each study visit by univariable linear regression models. **Table S4.** Multivariable analysis of the effect of RTS,S/AS01E vaccination and demographic, clinical, and epidemiological variables on antibody levels to *Plasmodium falciparum* pre-erythrocytic and blood-stage antigens. (DOCX 1736 kb)

Abbreviations

a.a.: Amino acid; AIC: Akaike information criterion; AUC: Area under the curve; BI: Block; BS: Blood stage; BSA: Bovine serum albumin; CI: Confidence interval; CSP: Circumsporozoite protein; FC: Fold change; GAM: Generalized additive models; GST: Glutathione *S*-transferase; HAZ: Height-for-age *Z*-score; IPTi-SP: Intermittent preventive treatment in infants with sulfadoxine-pyrimethamine; IQR: Interquartile ranges; MFI: Median fluorescence intensity; MTI: Malaria transmission intensity; NAI: Naturally acquired immunity; OR: Odds ratio; PCA: Principal component analysis; PCD: Passive case detection; PE: Pre-erythrocytic; PLS-DA: Partial least squares discriminant analysis; qSAT: Quantitative suspension array technology; WAZ: Weight-for-age *Z*-score

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Authors' contributions

CD, JJC, and GM contributed to the conceptualization. CD contributed to the writing and the original draft preparation. CD, GM, RS, JJC, IU, CJ, BG, MV, AJ, DD, AJN, AA, RA, NAW, NDP, DL, VC, CC, SD, DG, EA, KPA, SO, CV, BGa, RLC, DC, and JGB contributed to the writing and the review and editing of the manuscript. AA, IU, RS, CV, and GM contributed to data curation. AA, IU, RS, CV, and GM contributed to the formal analysis. AJN, CJ, JJC, GM, CD, DD, SO, MV, AJ, IU, BG, NAW, NDP, DL, VC, CC, SD, DG, EA, KPA, BGa, RLC, DC, and JGB contributed to the investigation. NAW, NDP, and CD contributed to the project administration. IU, CD, JJC, GM, RA, CV, and BG contributed to the methodology. DL, VC, CC, SD, DG, EA, BGa, RLC, DC, and JGB contributed to the resources. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files or are available from the authors on request.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committees from Spain, Mozambique, and Ghana, and written informed consent was obtained from parents or guardians before starting study procedures.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Supplementary materials, methods and results from Article 3

Dobaño C, Ubillos I, **Jairoce C,** Gyan B, Vidal M, Jiménez A, Santano R, Dosoo D, Nhabomba AJ, Ayestaran A, Aguilar R, Williams NA, Díez-Padrisa N, Lanar D, Chauhan V, Chitnis C, Dutta S, Gaur D, Angov E, Asante KP, Moncunill G. **RTS, S/AS01E immunization increases antibody responses to vaccine-unrelated Plasmodium falciparum antigens associated with protection against clinical malaria in African children: a case-control study.** BMC medicine. 2019 Dec;17:1-9.

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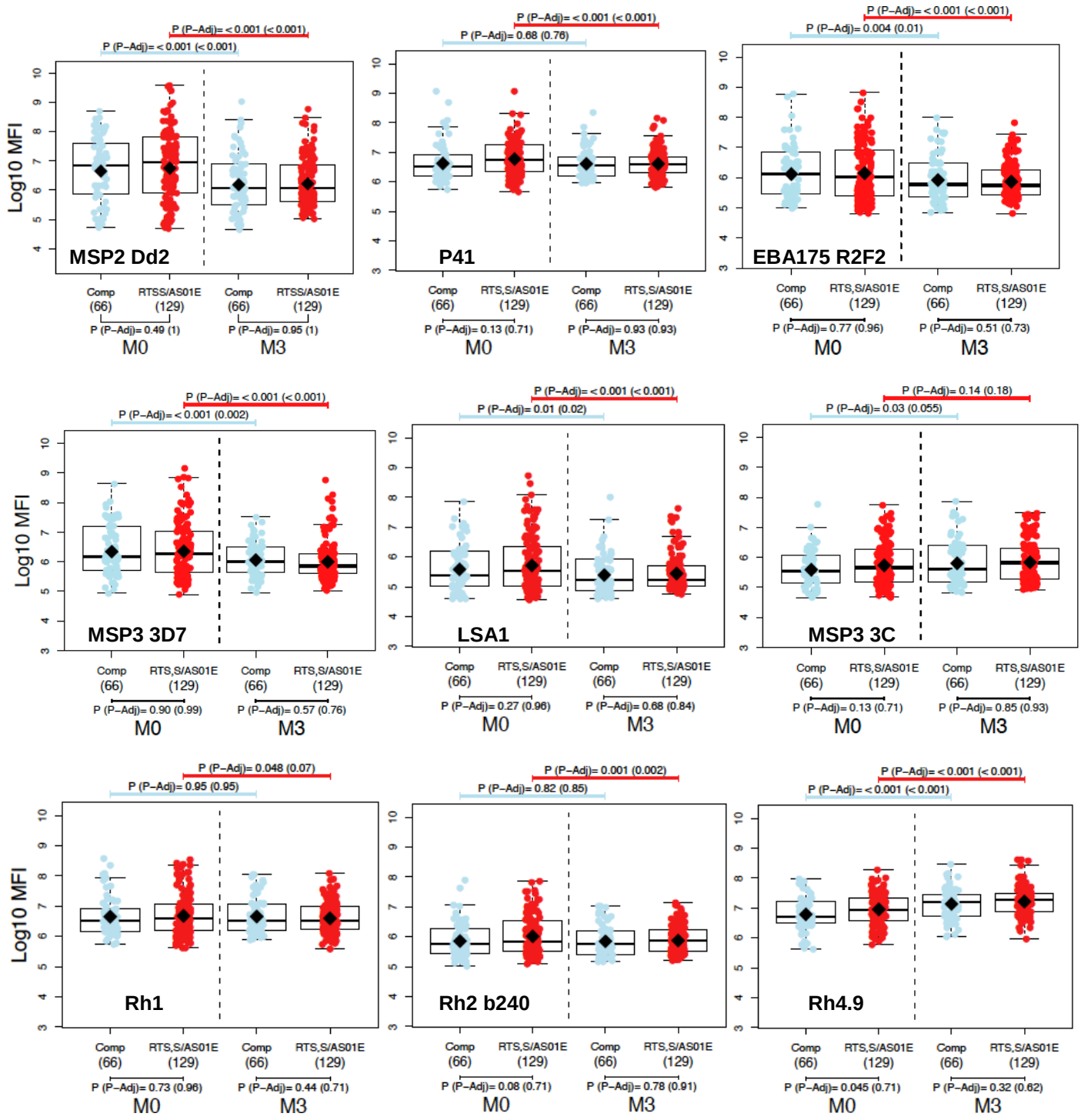
SUPPLEMENTARY INFORMATION

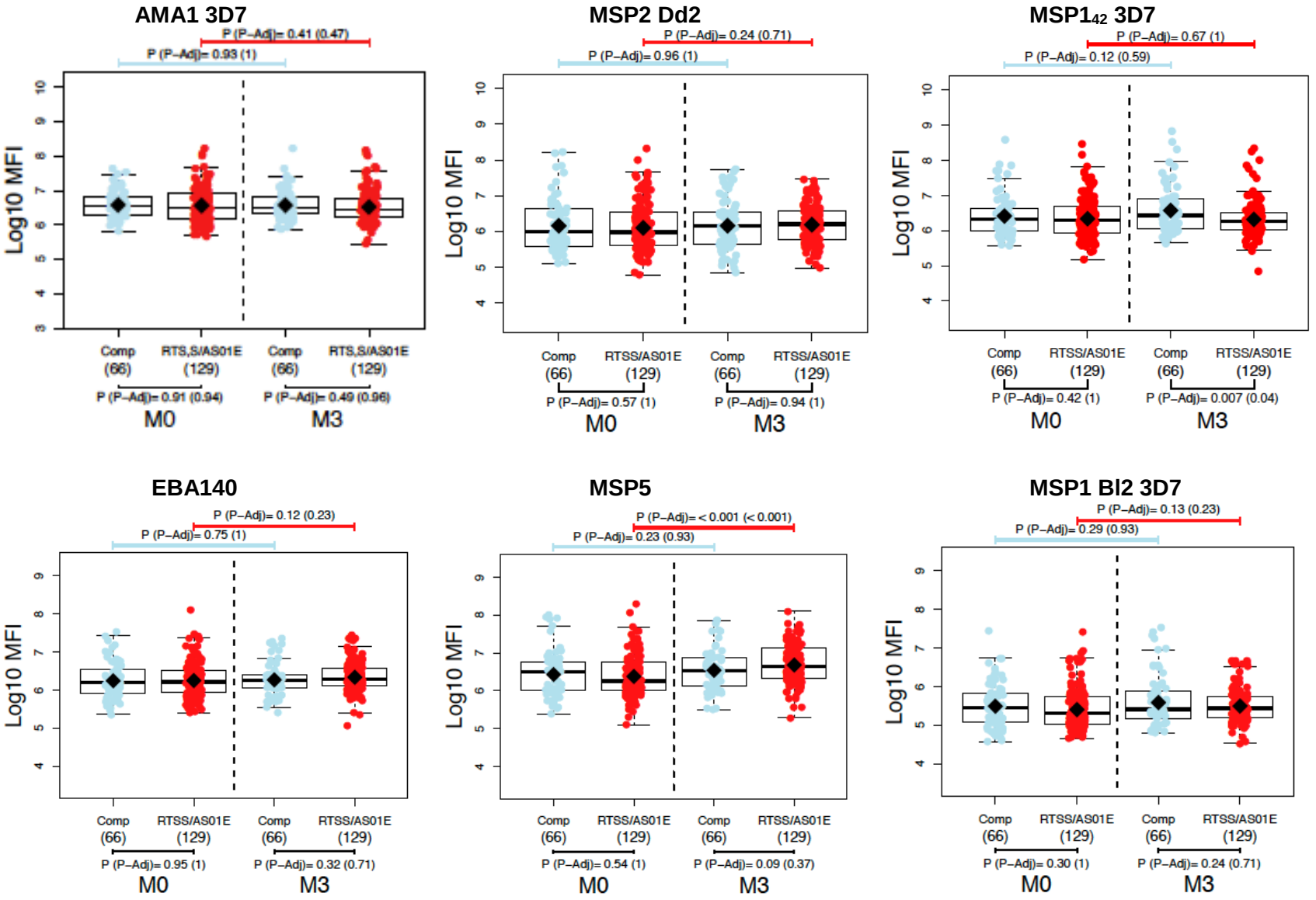
SUPPLEMENTARY TABLES AND FIGURES

Supplementary Fig 1. Antibody responses to non-RTS,S *Plasmodium falciparum* antigens per visit and vaccination group. Representative examples.

a) IgG: group ii antigens that do not change upon RTS,S vaccination



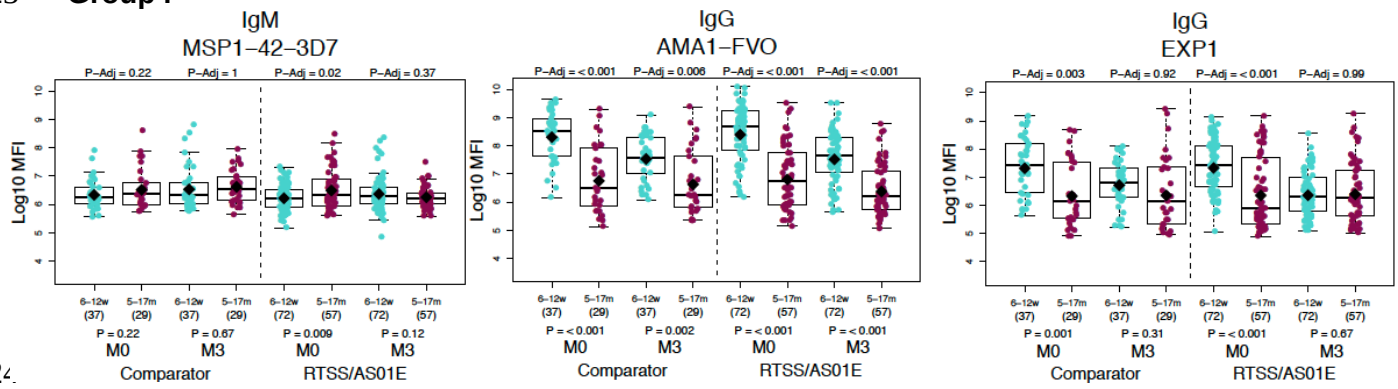
b) IgM: antibody responses mostly do not change upon RTS,S vaccination except $MSP1_{42}$ 3D7 that follows the pattern of group i antigens (decrease)



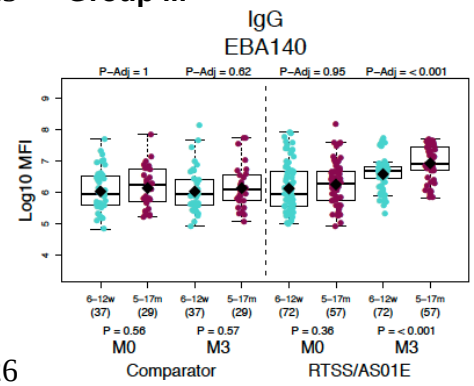
Supplementary Fig 2. Antibody responses to non-RTS,S *Plasmodium falciparum* antigens after RTS,S/AS01E vaccination stratified by age and site. Crude Ig levels (log_{10} MFI).

a) By age

Group i

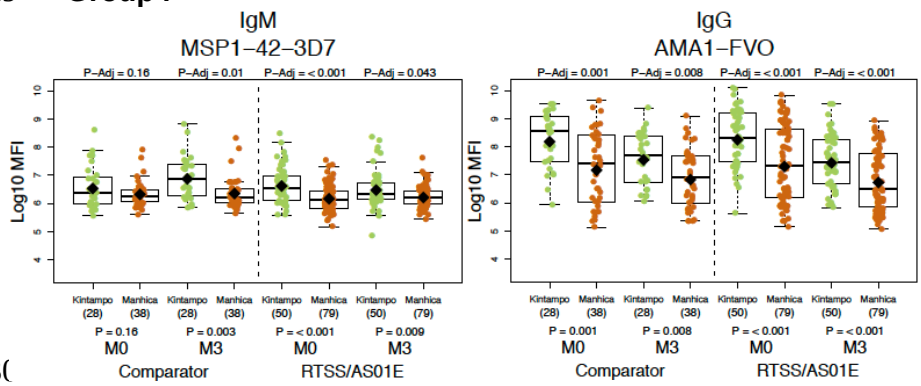


Group iii

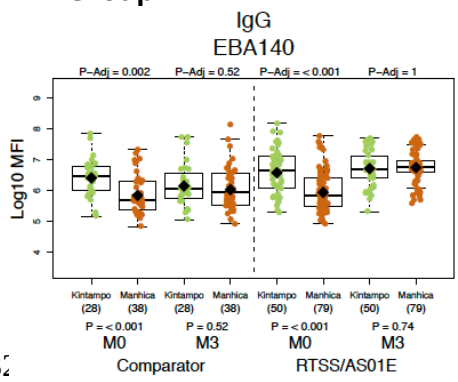


b) By site

Group i

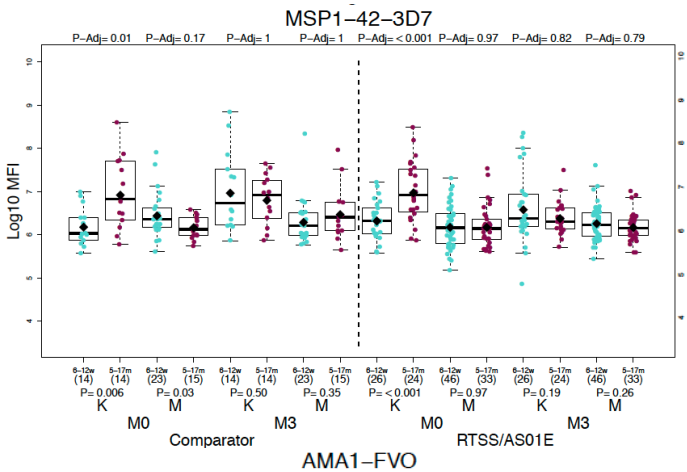


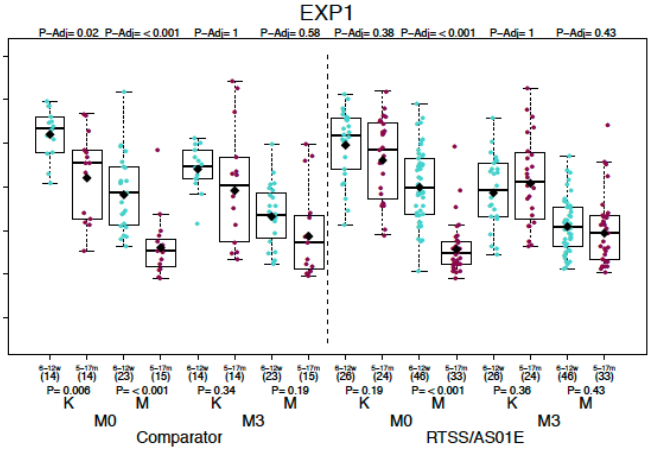
Group iii

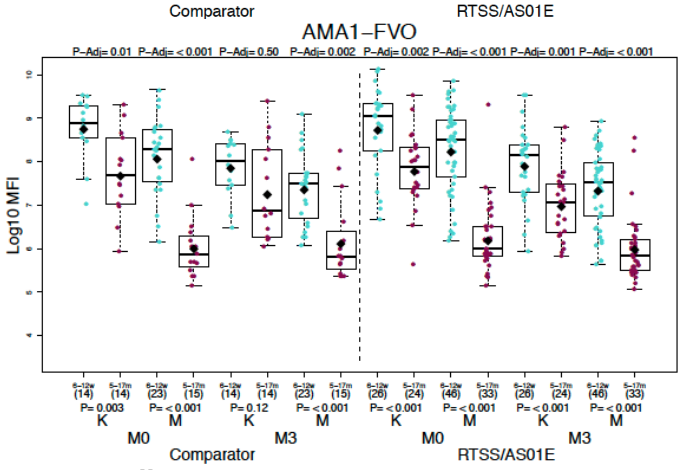


c) By age and by site. All are IgG except $MSP1_{42}$ that is IgM. K = Kintampo, M = Manhiça.

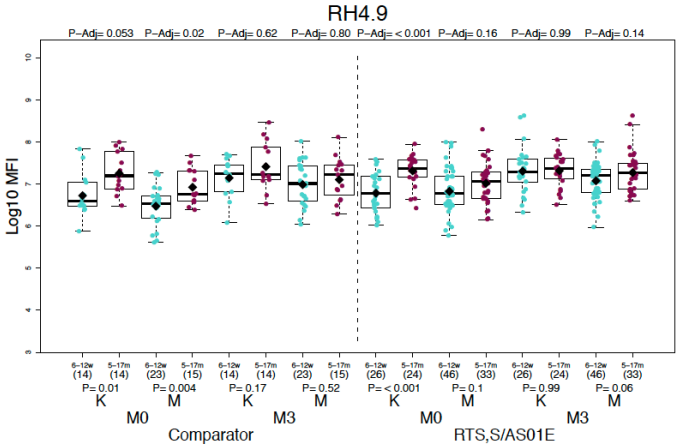
Group i



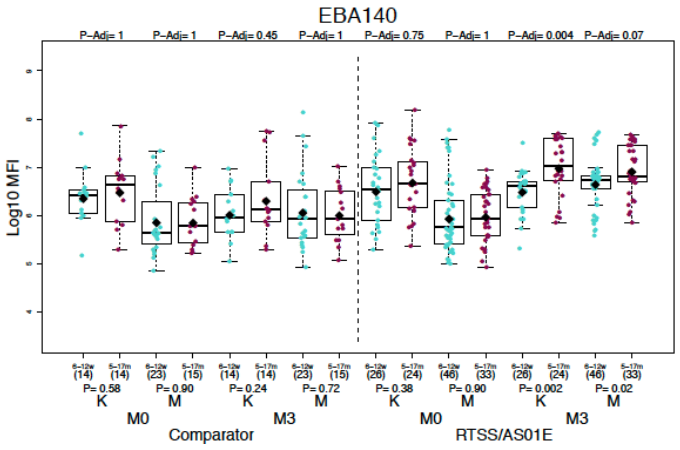


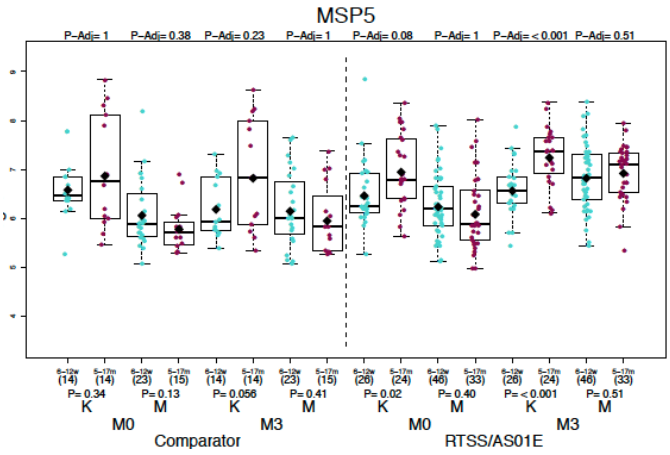


Group ii



Group iii



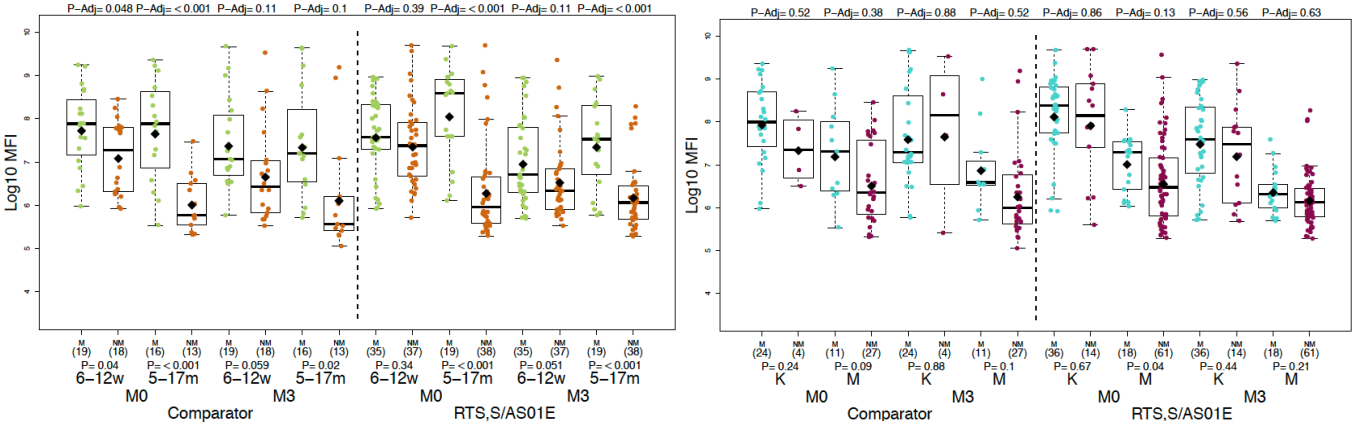


Supplementary Fig 3. Association between IgG antibody levels and malaria risk stratified by age and site. M = Malaria *vs* NM = No malaria. K = Kintampo *vs* M = Manhiça.

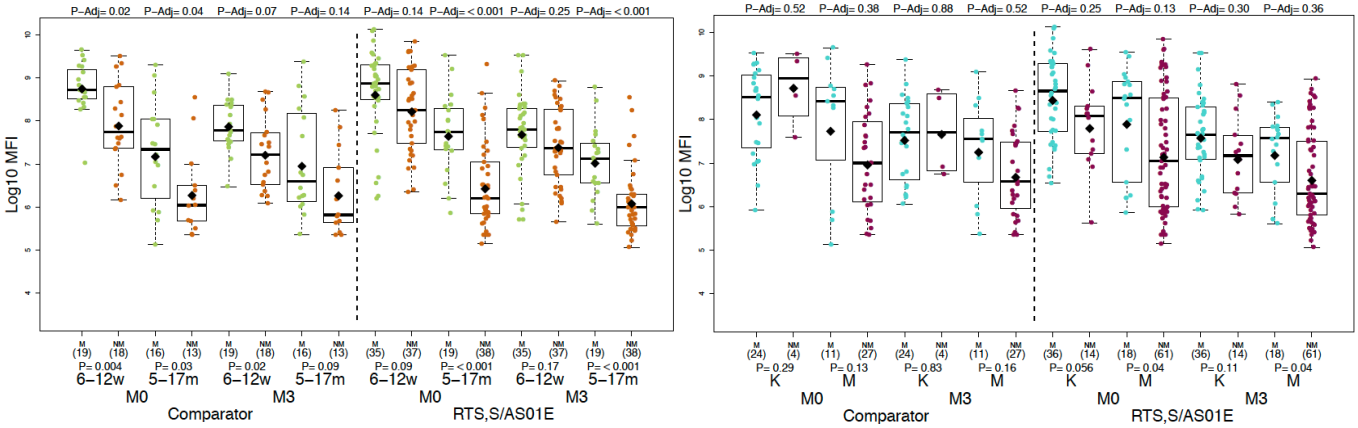
AGE **SITE**

a) Group i antigens

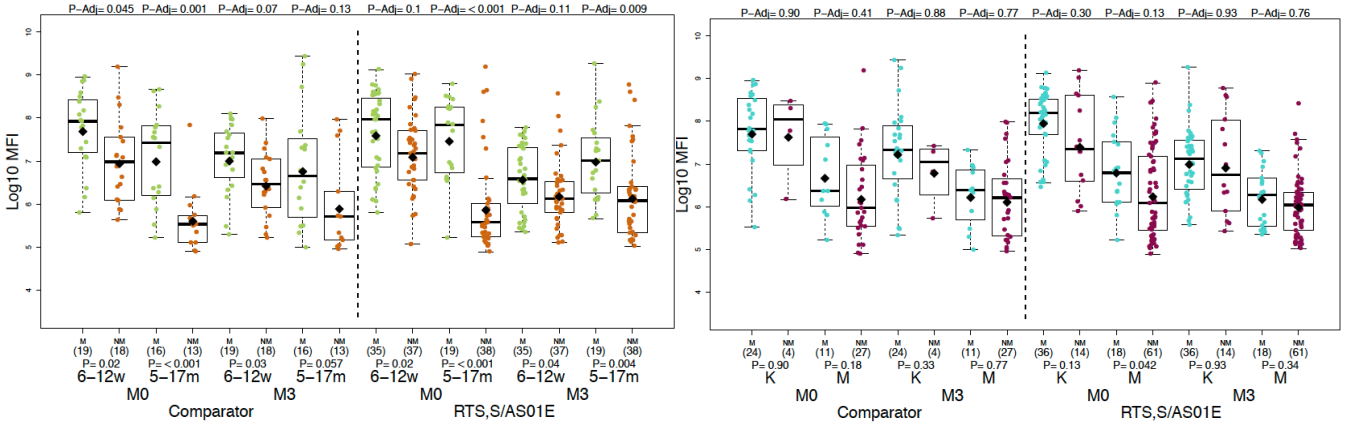
MSP1$_{42}$



AMA1



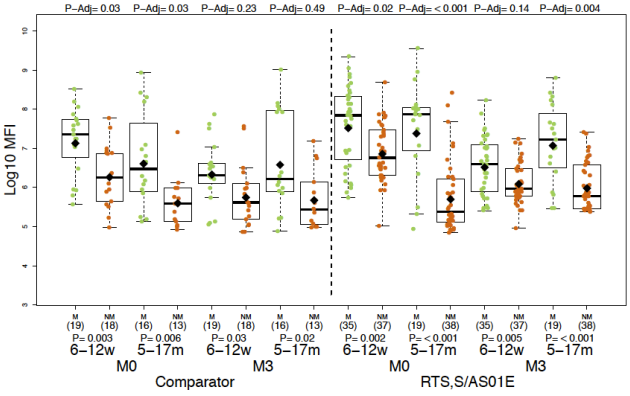
EXP1

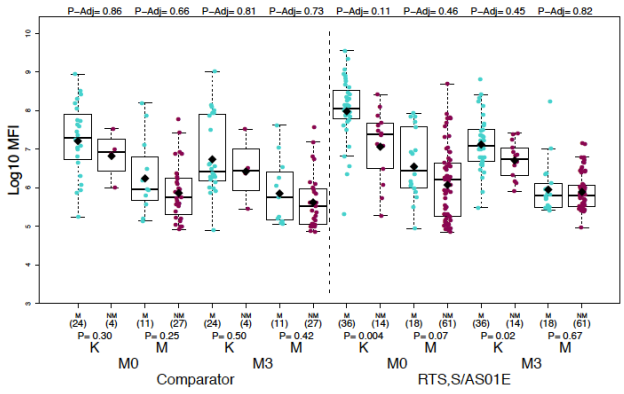


AGE **SITE**

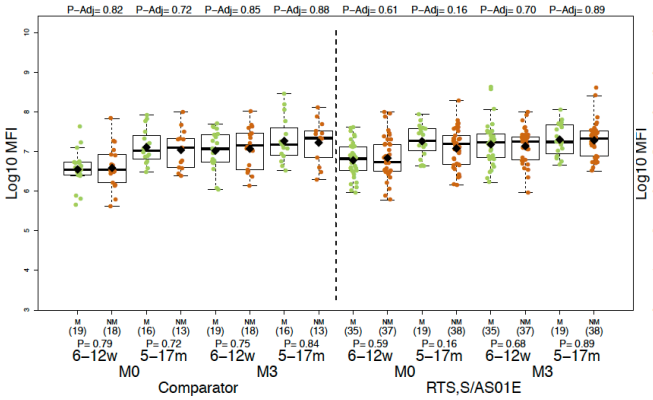
b) Group ii antigens

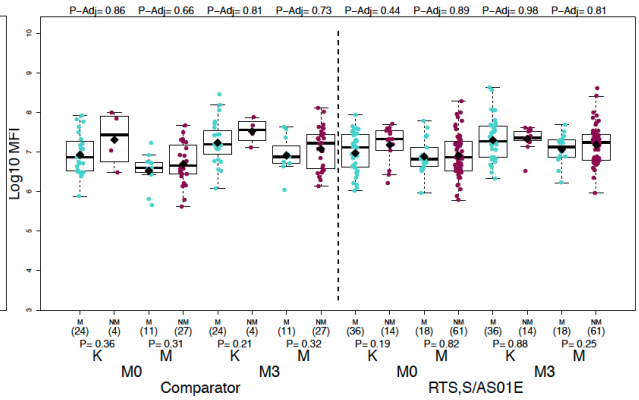
MSP2 CH150





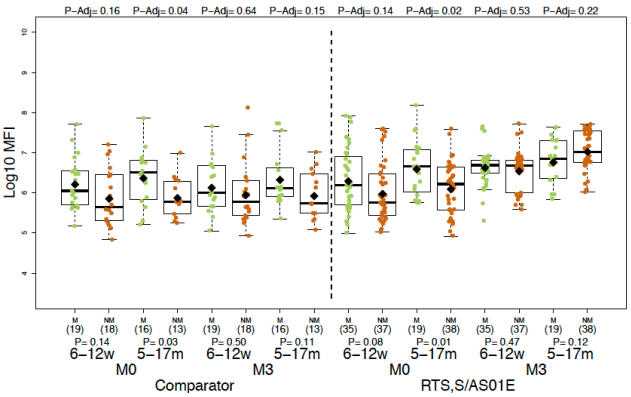
Rh4.9

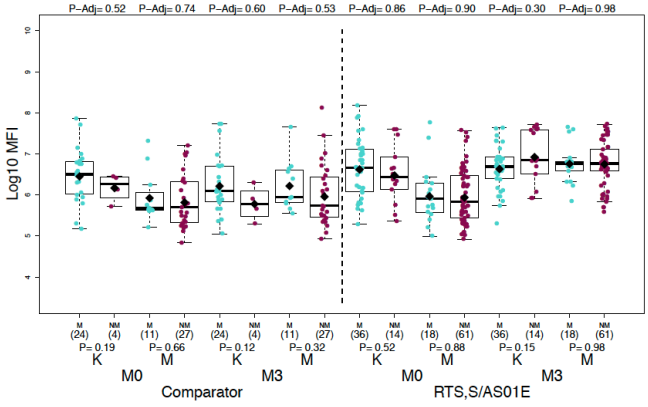




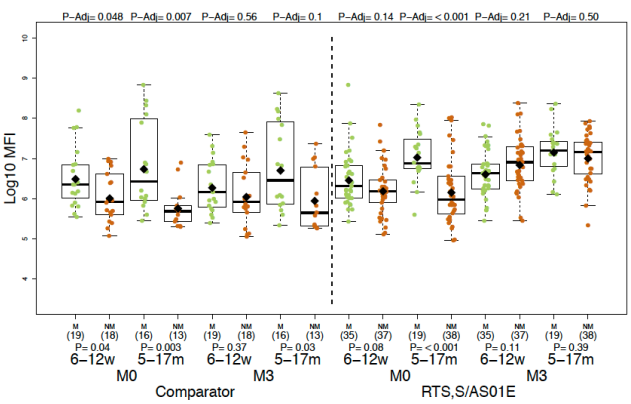
c) Group iii antigens

EBA140





MSP5



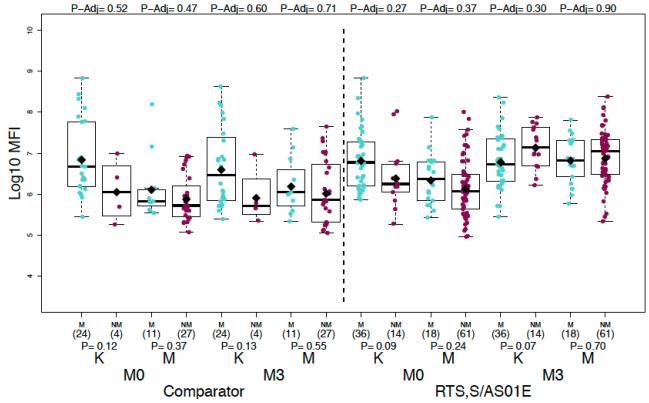


Table S1. Antigens included in the multiplex quantitative suspension array panel

Antigen	Rationale	Malaria life stage	Expression	Reference
CSP full length	Component of RTS,S vaccine	Sporozoite		[1,2]
CSP NANP repeat			GST fusion	[3]
CSP C-terminus			GST fusion	[4]
SSP2 or TRAP	Exposure to sporozoite infection			[5,6]
CelTOS				[7,8]
LSA1		Liver		[9,10]
EXP1	Exposure to asexual blood stage infection			[11]
AMA1 3D7 (FMP2.1)		Merozoite		[12,13]
AMA1 FVO (FMP009)				[12]
CyRPA full length				[14]
EBA140 region 3-5			GST fusion	[15]
EBA175 region 2 PfF2				[16]
EBA175 region 3-5			GST fusion	[15]
$MSP1_{42}$ 3D7				[12,17]
$MSP1_{42}$ FVO				[12,17]
MSP1 Block 2 PA17			GST fusion	[18]
MSP1 Block 2 3D7			GST fusion	[18]
MSP1 Block 2 MAD20			GST fusion	[18]
MSP1 Block 2 RO33			GST fusion	[18]
MSP1 Block 2 Wellcome			GST fusion	[18]
MSP1 Block 2 hybrid			GST fusion	[19]
MSP2 CH150 full length (5/6) Type A			GST fusion	[20]
MSP2 Dd2 full length (13/14) Type B			GST fusion	[20]
MSP3 3C				[20]
MSP3 3D7				[21]
MSP5				[22,23]
MSP6			GST fusion	[24]
PTRAMP				[25]
P41				[26]
RH1				[27]
RH2 b240				[28]
RH2 (2030)			GST fusion	[29]
RH4.2			GST fusion	[30,31]
RH4.9				[30,31]
RH5				[14,32]
DBLα		Trophozoite		[33]
VAR2CSA DBL1-2	Representative of maternally-transferred antibodies			[34]
VAR2CSA DBL3-4				[35]

74 **Table S2. Effect of different variables on month 3 IgG levels to *Plasmodium falciparum* antigens.**

Antibody	Vaccine (RTS,S)		Age (5-17 months)		Site (Manhiça)		Baseline IgG levels		Malaria episodes	
Antigen	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens										
$MSP1_{42}$ 3D7	**0.58 (0.37;0.91)**	**0.02**			**0.56 (0.33;0.96)**	**0.03**	**3.42 (2.74;4.27)**	**< 0.001**	**15.66 (7.51;32.65)**	**< 0.001**
$MSP1_{42}$ FVO	0.62 (0.36;1.05)	0.08			**0.38 (0.2;0.72)**	**0.003**	**2.76 (2.17;3.51)**	**< 0.001**	**19.4 (8.09;46.48)**	**< 0.001**
EXP1	0.65 (0.42;1.01)	0.054	**3.5 (2.17;5.64)**	**< 0.001**			**4.16 (3.4;5.09)**	**< 0.001**	**3.69 (1.82;7.47)**	**< 0.001**
AMA1 FVO	0.7 (0.48;1.02)	0.06			1.44 (0.95;2.18)	0.08	**4.79 (4.15;5.54)**	**< 0.001**	**6.37 (3.45;11.75)**	**< 0.001**
AMA1 3D7	**0.65 (0.45;0.93)**	**0.02**			1.33 (0.89;1.99)	0.16	**4.87 (4.25;5.58)**	**< 0.001**	**4.69 (2.63;8.36)**	**< 0.001**
Group ii antigens										
pTRAMP							**2.78 (2.22;3.48)**	**< 0.001**	**3.02 (1.84;4.97)**	**< 0.001**
EBA175 R2(F2)			**1.49 (1.12;1.99)**	**0.006**	1.28 (0.93;1.77)	0.13	**3.29 (2.79;3.88)**	**< 0.001**	**3.64 (2.28;5.82)**	**< 0.001**
PfRH1			**1.6 (1.2;2.13)**	**0.001**			**2.72 (2.19;3.38)**	**< 0.001**	**2.06 (1.3;3.26)**	**0.002**
MSP3 3D7			**2.45 (1.76;3.42)**	**< 0.001**	**1.45 (1.02;2.05)**	**0.04**	**4.19 (3.44;5.1)**	**< 0.001**	**2.62 (1.61;4.26)**	**< 0.001**
Var2csa DBL3-4			0.83 (0.65;1.07)	0.14			**3.01 (2.66;3.41)**	**< 0.001**	**1.57 (1.11;2.22)**	**0.01**
p41			**1.34 (1.06;1.68)**	**0.01**			**2.79 (2.32;3.37)**	**< 0.001**	**1.88 (1.31;2.71)**	**< 0.001**
MSP2 FL Dd2			**3.89 (2.56;5.9)**	**< 0.001**	1.48 (0.91;2.41)	0.12	**4.72 (3.79;5.88)**	**< 0.001**	**4.52 (2.56;7.98)**	**< 0.001**
MSP2 FL CH150			**2.95 (2.06;4.22)**	**< 0.001**			**3.55 (3.04;4.15)**	**< 0.001**	**6.37 (3.68;11.02)**	**< 0.001**
MSP1 Bl2 hybrid			**1.58 (1.14;2.19)**	**0.006**	1.33 (0.91;1.96)	0.14	**2.98 (2.51;3.54)**	**< 0.001**	**4.67 (2.71;8.06)**	**< 0.001**
MSP3 3C			0.65 (0.41;1.03)	0.07	**2.06 (1.25;3.41)**	**0.005**	**2.87 (2.04;4.04)**	**< 0.001**	**2.05 (0.97;4.34)**	0.059
PfRH2 b240			**1.5 (1.18;1.9)**	**0.001**			**2.41 (2;2.92)**	**< 0.001**	**3.16 (2.13;4.68)**	**< 0.001**
LSA1			**1.6 (1.15;2.23)**	**0.006**	**1.48 (1;2.19)**	**0.05**	**2.99 (2.43;3.67)**	**< 0.001**	**2.29 (1.31;4)**	**0.004**
MSP1 Bl2 PA17							**2.27 (1.88;2.74)**	**< 0.001**	**2.54 (1.52;4.25)**	**< 0.001**
Var2csa DBL1-2							**2.64 (2.18;3.2)**	**< 0.001**		
CyRPA							**3.43 (2.69;4.39)**	**< 0.001**		
CelTOS							**2.41 (1.85;3.14)**	**< 0.001**	**1.72 (1.07;2.78)**	**0.03**
RH4.9							**3.31 (2.58;4.24)**	**< 0.001**	**1.81 (1.19;2.74)**	**0.006**

Group iii antigens										
DBL-α	1.29 (0.95;1.75)	0.1	**1.77 (1.33;2.37)**	**< 0.001**			**2.74 (2.1;3.58)**	**< 0.001**		
RH5	**1.52 (1.08;2.14)**	**0.02**	**1.61 (1.16;2.23)**	**0.005**			**1.97 (1.5;2.58)**	**< 0.001**	**1.93 (1.13;3.29)**	**0.02**
SSP2 (TRAP)	**1.65 (1.2;2.28)**	**0.003**	**3.51 (2.55;4.83)**	**< 0.001**	**1.65 (1.16;2.34)**	**0.006**	**3.79 (2.87;5)**	**< 0.001**	**2.65 (1.56;4.5)**	**< 0.001**
MSP1 Bl2 Mad20	**1.9 (1.64;2.2)**	**<0.001**	**1.3 (1.13;1.49)**	**< 0.001**	**1.19 (1.01;1.4)**	**0.03**	**2 (1.64;2.42)**	**< 0.001**	**1.44 (1.15;1.81)**	**0.002**
MSP6	**1.91 (1.39;2.62)**	**<0.001**	**1.82 (1.35;2.45)**	**< 0.001**			**2.51 (2.06;3.06)**	**< 0.001**	**2.39 (1.46;3.92)**	**< 0.001**
RH2 (2030)	**2.18 (1.63;2.92)**	**<0.001**	**1.61 (1.14;2.27)**	**0.007**	**1.47 (1.04;2.06)**	**0.03**	**2.51 (2.01;3.13)**	**< 0.001**	**3.27 (2.01;5.33)**	**< 0.001**
EBA175 R3-5	**3.67 (2.41;5.61)**	**<0.001**	**2.06 (1.35;3.15)**	**< 0.001**			**2.14 (1.69;2.7)**	**< 0.001**	1.61 (0.83;3.1)	0.16
MSP5	**3.52 (2.18;5.68)**	**<0.001**	1.5 (0.95;2.38)	0.08	1.6 (0.95;2.67)	0.08	**1.89 (1.4;2.54)**	**< 0.001**	**3.68 (1.74;7.8)**	**< 0.001**
EBA140 R3-5	**4.01 (2.71;5.93)**	**<0.001**	**1.6 (1.09;2.34)**	**0.02**	**1.61 (1.03;2.52)**	**0.04**	**1.8 (1.36;2.38)**	**< 0.001**	1.66 (0.87;3.16)	0.12
MSP1 Bl2 RO33	**5.16 (3.66;7.28)**	**<0.001**	**1.51 (1.07;2.11)**	**0.02**			**1.72 (1.34;2.2)**	**< 0.001**	**2.45 (1.43;4.21)**	**0.001**
MSP1 Bl2 Well	**5.83 (4.34;7.82)**	**<0.001**	**1.78 (1.32;2.4)**	**< 0.001**	**1.53 (1.12;2.09)**	**0.008**	**2.58 (1.93;3.46)**	**< 0.001**	1.51 (0.93;2.44)	0.09
MSP1 Bl2 3D7	**6.84 (4.58;10.2)**	**<0.001**	**1.53 (1.04;2.26)**	**0.03**			**1.78 (1.38;2.29)**	**< 0.001**	1.8 (0.96;3.36)	0.07
RH4.2	**6.55 (4.51;9.51)**	**<0.001**	**2.15 (1.5;3.1)**	**< 0.001**	1.49 (0.99;2.24)	0.054	**1.96 (1.45;2.66)**	**< 0.001**	**2.05 (1.11;3.78)**	**0.02**

Each line shows multivariable linear regressions results with antibody levels for each antigen (log_{10} Median Fluorescence Intensity measured by quantitative suspension array technology) as outcome and vaccine, age, site, baseline (month 0, M0) IgG levels and malaria episodes from M0 to M3 as predictor covariables (in parenthesis the category that is compared to the reference). Coefficients (10^Beta) with 95% confidence intervals and p-values are shown for variables retained in the models according to the minimum akaike information criterion. The order of the antigens is based on coefficients and p-values of the predictor variable Vaccine. Month 0 data are shown in Table S4. Associations for the following variables were only significant for a few antigens and are not included in the table: season (pTRAMP, 2.65, p=0.004; MSP1 Bl2 Mad20, 1.54, p=0.007), hemoglobin (MSP1 Bl2 Well, 0.87, p=0.01), weight-for-age Z-score (MSP2 FL [full-length] CH150, 0.86, p=0.046) height-for-age Z-score (AMA1 3D7, 0.83, p=0.01; P41, 1.17, p=0.01; CyRPA, 1.13, p=0.01; RH2 2030, 0.82, p=0.02). Sex was retained in the models for MSP3 3C, CyRPA, MSP1 Bl2 PA17 and SSP2 but it was not significantly associated with antibody levels. Bl2: block 2.

Table S3. Effect of RTS,S vaccination and other variables (columns) on antibody levels (log_{10}MFI) at each study visit by univariable linear regression models (in parenthesis the category that is compared to the reference). Coefficients (10^Beta), 95% confidence intervals and p values (significant values in bold) are shown. Antigens are ordered according to the RTS,S vaccination coefficient values (lower to higher) of their IgG responses, and this is used to define the three pattern groups (i, ii, iii) presented in this study.

a) Antibody levels at month 3 for IgG and IgM.

IgG												
Antigen	**Vaccine (RTS,S)**		**Age (5-17 month)**		**Site (Manhiça)**		**Season (Low)**		**Baseline IgG levels**		**Previous malaria**	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens												
$MSP1_{42}$ 3D7	0.51 (0.24;1.08)	0.21	0.65 (0.32;1.34)	0.37	**0.06 (0.03;0.11)**	**<0.001**	0.7 (0.15;3.34)	0.72	**4.87 (3.93;6.03)**	**< 0.001**	**89.36 (33.92;235.44)**	**< 0.001**
$MSP1_{42}$ FVO	0.53 (0.24;1.18)	0.3	0.97 (0.45;2.09)	0.93	**0.06 (0.03;0.11)**	**<0.001**	0.55 (0.1;2.88)	0.69	**4.15 (3.27;5.26)**	**< 0.001**	**104.33 (36.57;297.66)**	**< 0.001**
EXP1	0.63 (0.33;1.24)	0.4	0.89 (0.47;1.69)	0.82	**0.1 (0.06;0.18)**	**<0.001**	0.4 (0.1;1.57)	0.41	**3.72 (3.07;4.51)**	**< 0.001**	**20.67 (8.14;52.48)**	**< 0.001**
AMA1 FVO	0.74 (0.36;1.55)	0.62	**0.09 (0.05;0.17)**	**< 0.001**	**0.21 (0.1;0.4)**	**<0.001**	1.61 (0.35;7.27)	0.71	**4.92 (4.25;5.69)**	**< 0.001**	**10.83 (3.7;31.67)**	**< 0.001**
AMA1 3D7	0.77 (0.37;1.59)	0.64	**0.1 (0.05;0.18)**	**< 0.001**	**0.2 (0.1;0.39)**	**<0.001**	1.37 (0.31;6.08)	0.72	**4.89 (4.27;5.61)**	**< 0.001**	**7.51 (2.57;21.96)**	**< 0.001**
Group ii antigens												
pTRAMP	0.78 (0.52;1.17)	0.46	1.22 (0.83;1.8)	0.43	**0.53 (0.36;0.79)**	**0.003**	1.86 (0.81;4.3)	0.36	**3.05 (2.44;3.82)**	**< 0.001**	**4.52 (2.51;8.14)**	**< 0.001**
EBA175 R2 F2	0.85 (0.54;1.34)	0.64	0.94 (0.61;1.45)	0.82	**0.38 (0.25;0.58)**	**<0.001**	0.58 (0.23;1.45)	0.45	**3.24 (2.77;3.79)**	**< 0.001**	**6.69 (3.54;12.63)**	**< 0.001**
RH1	0.86 (0.59;1.25)	0.62	**2.11 (1.5;2.98)**	**< 0.001**	**0.39 (0.28;0.55)**	**<0.001**	0.8 (0.37;1.73)	0.71	**3.16 (2.57;3.89)**	**< 0.001**	**4.11 (2.39;7.06)**	**< 0.001**
MSP3 3D7	0.88 (0.56;1.39)	0.74	0.79 (0.51;1.21)	0.4	**0.4 (0.26;0.61)**	**<0.001**	0.84 (0.33;2.13)	0.73	**3.2 (2.69;3.8)**	**< 0.001**	**3.26 (1.67;6.37)**	**0.001**
VAR2CSA DBL3-4	0.96 (0.63;1.46)	0.94	**0.27 (0.19;0.38)**	**< 0.001**	**0.34 (0.24;0.5)**	**<0.001**	0.73 (0.31;1.71)	0.69	**3.23 (2.92;3.59)**	**< 0.001**	**3.01 (1.62;5.59)**	**< 0.001**
p41	0.99 (0.72;1.35)	0.95	**1.53 (1.14;2.06)**	**0.01**	**0.59 (0.44;0.8)**	**0.001**	0.67 (0.35;1.29)	0.45	**2.97 (2.46;3.58)**	**< 0.001**	**3.29 (2.09;5.17)**	**< 0.001**
MSP2 FL Dd2	1.02 (0.55;1.89)	0.95	0.91 (0.51;1.65)	0.82	**0.1 (0.06;0.16)**	**<0.001**	0.39 (0.11;1.37)	0.36	**3.8 (3.22;4.48)**	**< 0.001**	**22.32 (9.71;51.3)**	**< 0.001**
MSP1 Bl2 hybrid	1.03 (0.63;1.69)	0.95	1.08 (0.67;1.73)	0.82	**0.33 (0.21;0.52)**	**<0.001**	0.45 (0.16;1.23)	0.36	**3.02 (2.56;3.55)**	**< 0.001**	**7.71 (3.84;15.49)**	**< 0.001**
MSP3 3C	1.05 (0.64;1.73)	0.94	1.06 (0.66;1.7)	0.84	1.06 (0.65;1.71)	0.85	0.76 (0.28;2.12)	0.71	**2.4 (1.74;3.3)**	**< 0.001**	1.97 (0.93;4.19)	0.08
RH2 b240	1.05 (0.75;1.48)	0.89	**1.51 (1.1;2.08)**	**0.02**	**0.46 (0.34;0.63)**	**<0.001**	0.62 (0.31;1.24)	0.41	**2.76 (2.26;3.38)**	**< 0.001**	**5.23 (3.3;8.27)**	**< 0.001**
LSA1	1.1 (0.7;1.73)	0.81	1.2 (0.78;1.84)	0.53	**0.46 (0.3;0.71)**	**<0.001**	0.65 (0.26;1.63)	0.57	**2.83 (2.35;3.4)**	**< 0.001**	**3.65 (1.89;7.08)**	**< 0.001**
MSP1 Bl2 PA17	1.13 (0.75;1.7)	0.71	1.11 (0.75;1.64)	0.72	**0.61 (0.41;0.9)**	**0.02**	0.64 (0.28;1.47)	0.5	**2.44 (2.02;2.96)**	**< 0.001**	**4.07 (2.26;7.35)**	**< 0.001**
VAR2CSA DBL1-2	1.15 (0.84;1.58)	0.58	**0.59 (0.44;0.78)**	**0.001**	**0.61 (0.45;0.82)**	**0.002**	0.87 (0.46;1.66)	0.72	**2.57 (2.13;3.1)**	**< 0.001**	1.61 (1.01;2.59)	0.055
CYRPA	1.16 (0.88;1.55)	0.53	**1.71 (1.32;2.21)**	**< 0.001**	0.85 (0.65;1.12)	0.28	1.01 (0.56;1.8)	0.99	**3.38 (2.64;4.33)**	**< 0.001**	1.56 (1.02;2.4)	0.05
CelTOS	1.18 (0.83;1.67)	0.58	**1.64 (1.19;2.27)**	**0.008**	**0.69 (0.5;0.96)**	**0.045**	0.51 (0.25;1.03)	0.26	**2.56 (1.97;3.32)**	**< 0.001**	**2.35 (1.4;3.94)**	**0.002**
RH4.9	1.19 (0.85;1.67)	0.53	1.43 (1.04;1.96)	0.051	**0.66 (0.48;0.9)**	**0.02**	0.81 (0.41;1.62)	0.71	**3.46 (2.7;4.43)**	**< 0.001**	**2.38 (1.44;3.91)**	**0.001**
Group iii antigens												
DBL-α	1.23 (0.86;1.75)	0.48	**1.85 (1.34;2.56)**	**< 0.001**	0.73 (0.52;1.02)	0.09	0.79 (0.39;1.64)	0.71	**2.77 (2.11;3.64)**	**< 0.001**	**1.79 (1.05;3.05)**	**0.04**
MSP2 FL CH150	1.55 (0.85;2.82)	0.35	1.24 (0.7;2.21)	0.57	**0.08 (0.05;0.14)**	**<0.001**	0.29 (0.09;1)	0.25	**3.44 (2.89;4.08)**	**< 0.001**	**20.66 (9.17;46.53)**	**< 0.001**
RH5	1.57 (1.08;2.29)	0.057	**1.92 (1.35;2.73)**	**0.001**	**0.56 (0.39;0.81)**	**0.003**	0.4 (0.18;0.86)	0.17	**2.37 (1.81;3.09)**	**< 0.001**	**2.76 (1.57;4.86)**	**< 0.001**

SSP2 (TRAP)	1.68 (1.09;2.59)	0.057	**3.11 (2.12;4.57)**	**< 0.001**	0.77 (0.5;1.17)	0.26	0.57 (0.23;1.39)	0.44	**3.49 (2.55;4.76)**	**< 0.001**	**3.14 (1.64;6)**	**0.001**
MSP1 Bl2 MAD20	**1.75 (1.49;2.06)**	**< 0.001**	1.17 (0.98;1.38)	0.14	0.91 (0.76;1.08)	0.31	0.91 (0.63;1.31)	0.71	**1.75 (1.42;2.16)**	**< 0.001**	**1.44 (1.1;1.88)**	**0.01**
MSP6	**2.01 (1.35;3.01)**	**0.003**	**1.7 (1.15;2.51)**	**0.02**	**0.43 (0.3;0.64)**	**<0.001**	0.48 (0.2;1.11)	0.3	**2.78 (2.26;3.42)**	**< 0.001**	**4.2 (2.3;7.66)**	**< 0.001**
EBA175 R3-5	**2.14 (1.47;3.1)**	**<0.001**	0.82 (0.57;1.18)	0.4	**0.55 (0.39;0.8)**	**0.003**	0.49 (0.22;1.08)	0.29	**2.33 (1.92;2.82)**	**< 0.001**	**4.15 (2.39;7.23)**	**< 0.001**
RH2 2030	**3.85 (2.41;6.15)**	**< 0.001**	1.38 (0.85;2.23)	0.31	0.66 (0.41;1.08)	0.14	0.45 (0.16;1.25)	0.36	**2.01 (1.58;2.56)**	**< 0.001**	**2.38 (1.11;5.1)**	**0.03**
MSP5	**3.95 (2.4;6.5)**	**<0.001**	**1.96 (1.19;3.24)**	**0.02**	0.71 (0.43;1.19)	0.25	**0.17 (0.06;0.5)**	**0.047**	**2.04 (1.51;2.76)**	**< 0.001**	**3.4 (1.53;7.54)**	**0.004**
EBA140 R3-5	**4.32 (2.86;6.54)**	**< 0.001**	**1.87 (1.21;2.87)**	**0.01**	1.01 (0.65;1.58)	0.97	0.61 (0.23;1.56)	0.5	**1.84 (1.38;2.46)**	**< 0.001**	1.64 (0.81;3.31)	0.17
MSP1 Bl2 RO33	**5.17 (3.54;7.56)**	**< 0.001**	**1.72 (1.13;2.61)**	**0.02**	0.7 (0.46;1.08)	0.14	0.39 (0.16;0.96)	0.23	**2.12 (1.59;2.82)**	**< 0.001**	**2.9 (1.49;5.63)**	**0.003**
MSP1 Bl2 Well	**5.71 (4.03;8.09)**	**< 0.001**	**2.52 (1.71;3.7)**	**< 0.001**	1.1 (0.73;1.66)	0.69	0.29 (0.12;0.68)	0.08	**2.94 (2.02;4.26)**	**< 0.001**	1.12 (0.58;2.15)	0.74
MSP1 Bl2 3D7	**6.78 (4.45;10.34)**	**< 0.001**	1.34 (0.83;2.15)	0.37	0.68 (0.42;1.1)	0.15	0.31 (0.11;0.85)	0.17	**1.82 (1.36;2.45)**	**< 0.001**	2.14 (1;4.57)	0.056
RH4.2	**7.19 (4.74;10.9)**	**<0.001**	**2.55 (1.61;4.03)**	**< 0.001**	0.89 (0.55;1.45)	0.69	0.27 (0.1;0.74)	0.13	**2.34 (1.64;3.33)**	**< 0.001**	1.98 (0.93;4.24)	0.08

No statistical significant differences were detected for sex, weight for age Z-score, height for age Z-score or hemoglobin (data not shown).

IgM												
Antigen	**Age (5-17 month)**		**Site (Manhiça)**		**HAZ**		**Season (Low)**		**Baseline IgM levels**		**Previous malaria**	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens												
$MSP1_{42}$ 3D7	0.86 (0.58;1.28)	0.56	**0.42 (0.29;0.61)**	**<0.001**	0.87 (0.73;1.03)	0.19	0.54 (0.23;1.26)	0.22	1.3 (0.94;1.8)	0.11	**4.74 (2.62;8.61)**	**<0.001**
$MSP1_{42}$ FVO	1.53 (1.03;2.28)	0.09	**0.42 (0.29;0.62)**	**< 0.001**	0.83 (0.7;0.98)	0.1	**0.35 (0.15;0.81)**	**0.041**	**2.02 (1.46;2.81)**	**<0.001**	**4.36 (2.37;8.01)**	**<0.001**
EXP1	**2.76 (1.68;4.51)**	**<0.001**	**0.39 (0.24;0.65)**	**< 0.001**	0.96 (0.77;1.2)	0.71	**0.26 (0.09;0.77)**	**0.041**	**2.31 (1.64;3.24)**	**<0.001**	**9.91 (4.66;21.1)**	**<0.001**
AMA1 FVO	0.85 (0.64;1.14)	0.4	**0.62 (0.46;0.82)**	**0.002**	0.89 (0.78;1)	0.13	0.69 (0.37;1.28)	0.32	**1.73 (1.27;2.34)**	**<0.001**	**2.2 (1.4;3.46)**	**0.001**
AMA1 3D7	0.88 (0.65;1.19)	0.52	**0.55 (0.41;0.74)**	**< 0.001**	0.91 (0.8;1.04)	0.24	0.89 (0.46;1.72)	0.73	**1.68 (1.23;2.29)**	**0.001**	**2.59 (1.62;4.14)**	**<0.001**
Group ii antigens												
pTRAMP	0.9 (0.71;1.14)	0.51	**0.64 (0.5;0.81)**	**<0.001**	**0.82 (0.74;0.91)**	**0.006**	0.87 (0.52;1.46)	0.62	**2.11 (1.51;2.93)**	**< 0.001**	**1.79 (1.23;2.62)**	**0.004**
EBA175 R2 F2	1.37 (0.98;1.9)	0.14	**0.5 (0.36;0.69)**	**< 0.001**	0.9 (0.78;1.03)	0.21	0.52 (0.25;1.05)	0.12	**2.07 (1.51;2.84)**	**<0.001**	**2.52 (1.51;4.22)**	**0.001**
RH1	1.21 (0.93;1.56)	0.28	**0.64 (0.5;0.82)**	**0.001**	0.92 (0.83;1.03)	0.24	0.62 (0.35;1.07)	0.14	**2.38 (1.81;3.15)**	**<0.001**	**2.14 (1.43;3.19)**	**<0.001**
MSP3 3D7	1.06 (0.78;1.46)	0.77	**0.7 (0.51;0.96)**	**0.03**	0.89 (0.78;1.02)	0.19	0.55 (0.28;1.08)	0.14	**1.9 (1.43;2.51)**	**<0.001**	1.51 (0.91;2.49)	0.11
VAR2CSA DBL3-4	0.98 (0.76;1.25)	0.87	**0.63 (0.5;0.81)**	**< 0.001**	**0.84 (0.76;0.94)**	**0.02**	0.74 (0.43;1.26)	0.35	**1.98 (1.46;2.67)**	**<0.001**	**1.7 (1.14;2.52)**	**0.01**
p41	0.99 (0.75;1.31)	0.93	**0.63 (0.48;0.83)**	**0.002**	0.88 (0.78;1)	0.12	0.86 (0.47;1.57)	0.63	**2.1 (1.54;2.87)**	**<0.001**	**2.09 (1.35;3.23)**	**0.002**
MSP2 FL Dd2	**2.1 (1.43;3.09)**	**0.001**	**0.46 (0.31;0.69)**	**< 0.001**	0.83 (0.7;0.99)	0.1	**0.18 (0.08;0.41)**	**< 0.001**	**2.04 (1.57;2.65)**	**<0.001**	**3.54 (1.91;6.56)**	**<0.001**
MSP1 Bl2 hybrid	**1.77 (1.2;2.63)**	**0.02**	0.74 (0.49;1.11)	0.14	0.93 (0.78;1.1)	0.44	**0.33 (0.14;0.77)**	**0.04**	**1.8 (1.36;2.39)**	**<0.001**	**4.36 (2.37;8.01)**	**<0.001**
MSP3 3C	0.91 (0.61;1.37)	0.75	**0.64 (0.42;0.96)**	**0.03**	0.89 (0.74;1.06)	0.24	0.66 (0.28;1.59)	0.41	**2.22 (1.68;2.95)**	**<0.001**	**2.48 (1.31;4.71)**	**0.008**
RH2 b240	1.36 (0.99;1.86)	0.14	**0.61 (0.44;0.83)**	**0.004**	**0.83 (0.72;0.95)**	**0.042**	**0.41 (0.21;0.79)**	**0.04**	**2.15 (1.61;2.89)**	**<0.001**	**2.28 (1.39;3.74)**	**0.002**
LSA1	1.66 (1.06;2.6)	0.08	**0.27 (0.17;0.4)**	**< 0.001**	0.89 (0.73;1.08)	0.26	0.61 (0.23;1.61)	0.38	**2.05 (1.55;2.7)**	**<0.001**	**4.5 (2.25;8.99)**	**<0.001**
MSP1 Bl2 PA17	1.5 (0.96;2.33)	0.15	**0.53 (0.34;0.82)**	**0.007**	0.88 (0.73;1.07)	0.24	0.35 (0.14;0.91)	0.07	**1.82 (1.3;2.53)**	**<0.001**	**5.73 (2.92;11.23)**	**<0.001**
VAR2CSA DBL1-2	1.09 (0.77;1.53)	0.73	**0.53 (0.38;0.75)**	**< 0.001**	0.84 (0.73;0.97)	0.08	0.44 (0.21;0.91)	0.06	**1.76 (1.27;2.42)**	**<0.001**	1.62 (0.94;2.8)	0.08
CYRPA	0.8 (0.56;1.14)	0.33	**0.39 (0.28;0.55)**	**< 0.001**	**0.77 (0.66;0.89)**	**0.009**	0.76 (0.35;1.63)	0.52	**2.58 (1.9;3.51)**	**<0.001**	**2.32 (1.33;4.05)**	**0.005**
CelTOS	**1.57 (1.15;2.15)**	**0.02**	**0.63 (0.46;0.87)**	**0.007**	0.92 (0.8;1.05)	0.26	0.64 (0.32;1.27)	0.28	**2.49 (1.82;3.42)**	**<0.001**	**1.95 (1.18;3.23)**	**0.01**
RH4.9	**2.15 (1.6;2.89)**	**<0.001**	**0.65 (0.48;0.9)**	**0.01**	0.88 (0.77;1.01)	0.13	**0.36 (0.19;0.7)**	**0.02**	**3.06 (2.26;4.14)**	**<0.001**	**1.93 (1.18;3.18)**	**0.01**
Group iii antigens												

DBL-α	0.82 (0.61;1.1)	0.3	**0.67 (0.5;0.9)**	**0.01**	**0.83 (0.74;0.94)**	**0.03**	0.79 (0.42;1.49)	0.52	**1.5 (1.1;2.04)**	**0.01**	**1.66 (1.04;2.63)**	**0.03**
MSP2 FL CH150	**1.98 (1.34;2.93)**	**0.004**	**0.4 (0.27;0.59)**	**<0.001**	0.88 (0.74;1.04)	0.22	**0.23 (0.1;0.53)**	**0.005**	**1.85 (1.36;2.51)**	**<0.001**	**3.34 (1.79;6.23)**	**<0.001**
RH5	1.13 (0.81;1.56)	0.56	**0.54 (0.39;0.74)**	**<0.001**	0.84 (0.73;0.97)	0.06	0.56 (0.28;1.12)	0.16	**2.02 (1.5;2.73)**	**<0.001**	**2.58 (1.56;4.27)**	**<0.001**
SSP2 (TRAP)	**2.03 (1.46;2.84)**	**<0.001**	**0.62 (0.44;0.88)**	**0.01**	0.85 (0.73;0.98)	0.1	**0.41 (0.2;0.86)**	**0.045**	**2.15 (1.58;2.93)**	**<0.001**	**1.83 (1.06;3.18)**	**0.03**
MSP1 Bl2 MAD20	**2.03 (1.56;2.64)**	**<0.001**	**0.6 (0.46;0.79)**	**<0.001**	0.93 (0.82;1.05)	0.26	**0.44 (0.24;0.79)**	**0.03**	**2.4 (1.87;3.07)**	**< 0.001**	**2.14 (1.38;3.32)**	**0.002**
MSP6	1.32 (0.89;1.96)	0.3	**0.49 (0.33;0.72)**	**<0.001**	0.81 (0.68;0.95)	0.06	0.53 (0.22;1.24)	0.22	**1.92 (1.41;2.6)**	**<0.001**	**3.9 (2.12;7.17)**	**< 0.001**
EBA175 R3-5	1.2 (0.85;1.68)	0.41	**0.54 (0.39;0.75)**	**< 0.001**	0.87 (0.76;1.01)	0.14	0.46 (0.22;0.95)	0.07	**1.39 (1.03;1.88)**	**0.03**	**2.3 (1.35;3.92)**	**0.004**
RH2 2030	1.04 (0.8;1.33)	0.83	**0.69 (0.54;0.89)**	**0.007**	0.87 (0.78;0.97)	0.06	0.59 (0.34;1.01)	0.1	**1.82 (1.35;2.45)**	**<0.001**	**1.89 (1.27;2.8)**	**0.003**
MSP5	1.47 (1.04;2.07)	0.08	**0.68 (0.48;0.97)**	**0.04**	0.86 (0.74;1)	0.13	**0.24 (0.11;0.49)**	**0.001**	**1.63 (1.22;2.18)**	**0.001**	**1.83 (1.05;3.19)**	**0.03**
EBA140 R3-5	**1.43 (1.09;1.89)**	**0.04**	0.78 (0.59;1.04)	0.09	0.9 (0.8;1.02)	0.19	**0.45 (0.25;0.82)**	**0.04**	**1.55 (1.17;2.05)**	**0.002**	**1.85 (1.19;2.87)**	**0.009**
MSP1 Bl2 RO33	1.26 (0.89;1.78)	0.3	**0.63 (0.44;0.88)**	**0.01**	0.89 (0.77;1.04)	0.22	0.67 (0.32;1.4)	0.35	**1.59 (1.19;2.13)**	**0.002**	**1.86 (1.08;3.22)**	**0.03**
MSP1 Bl2 Well	**2.87 (2.11;3.89)**	**<0.001**	0.77 (0.54;1.08)	0.13	0.95 (0.82;1.1)	0.54	**0.24 (0.12;0.48)**	**< 0.001**	**2.33 (1.82;2.98)**	**<0.001**	**2.1 (1.23;3.58)**	**0.009**
MSP1 Bl2 3D7	1.52 (1.07;2.16)	0.06	**0.58 (0.41;0.83)**	**0.005**	0.97 (0.83;1.13)	0.71	**0.39 (0.18;0.82)**	**0.041**	**1.85 (1.37;2.5)**	**<0.001**	**3.75 (2.18;6.43)**	**<0.001**
RH4.2	1.32 (0.97;1.8)	0.15	**0.62 (0.45;0.85)**	**0.004**	0.91 (0.8;1.05)	0.24	**0.25 (0.13;0.48)**	**< 0.001**	**1.65 (1.26;2.17)**	**<0.001**	**2.44 (1.5;3.96)**	**<0.001**

HAZ: height for age Z-score. No statistical significant differences were detected for vaccination, sex, weight for age Z-score or hemoglobin (data not shown).

b) Antibody levels at month 0 for IgG and IgM.

IgG						
Antigen	**Age (5-17 month)**		**Site (Manhiça)**		**Hemoglobin**	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens						
$MSP1_{42}$ 3D7	**0.29 (0.14;0.61)**	**0.002**	**0.05 (0.03;0.09)**	**< 0.001**	0.94 (0.72;1.23)	0.8
$MSP1_{42}$ FVO	0.55 (0.24;1.22)	0.2	**0.05 (0.03;0.11)**	**< 0.001**	0.86 (0.64;1.15)	0.53
EXP1	**0.12 (0.06;0.24)**	**< 0.001**	**0.04 (0.02;0.08)**	**< 0.001**	0.99 (0.75;1.3)	0.93
AMA1 FVO	**0.03 (0.01;0.06)**	**< 0.001**	**0.12 (0.05;0.28)**	**< 0.001**	1.08 (0.8;1.47)	0.79
AMA1 3D7	**0.03 (0.02;0.06)**	**< 0.001**	**0.13 (0.06;0.3)**	**< 0.001**	1.05 (0.77;1.42)	0.83
Group ii antigens						
pTRAMP	1.55 (0.98;2.46)	0.09	**0.26 (0.17;0.4)**	**< 0.001**	0.79 (0.67;0.93)	0.051
EBA175 R2 F2	**0.38 (0.21;0.69)**	**0.003**	**0.18 (0.1;0.31)**	**< 0.001**	0.99 (0.79;1.23)	0.93
RH1	**2.06 (1.33;3.18)**	**0.003**	**0.14 (0.1;0.2)**	**< 0.001**	**0.76 (0.65;0.89)**	**0.02**
MSP3 3D7	**0.16 (0.09;0.27)**	**< 0.001**	**0.2 (0.11;0.35)**	**< 0.001**	1.16 (0.94;1.43)	0.4
VAR2CSA DBL3-4	**0.09 (0.05;0.16)**	**< 0.001**	**0.12 (0.06;0.21)**	**< 0.001**	1.19 (0.94;1.5)	0.4
p41	1.12 (0.75;1.68)	0.6	**0.31 (0.21;0.45)**	**< 0.001**	0.85 (0.73;0.98)	0.16
MSP2 FL Dd2	**0.12 (0.06;0.24)**	**< 0.001**	**0.03 (0.02;0.06)**	**< 0.001**	0.91 (0.69;1.19)	0.69
MSP1 Bl2 hybrid	**0.44 (0.22;0.86)**	**0.03**	**0.14 (0.07;0.25)**	**< 0.001**	0.88 (0.69;1.12)	0.53
MSP3 3C	**2.18 (1.4;3.38)**	**0.001**	**0.32 (0.21;0.49)**	**< 0.001**	0.89 (0.76;1.05)	0.4
RH2 b240	0.93 (0.61;1.43)	0.75	**0.26 (0.17;0.38)**	**< 0.001**	0.88 (0.76;1.03)	0.33
LSA1	0.56 (0.31;1.01)	0.09	**0.15 (0.09;0.25)**	**< 0.001**	0.83 (0.67;1.02)	0.33
MSP1 Bl2 PA17	0.74 (0.43;1.29)	0.38	**0.3 (0.17;0.51)**	**< 0.001**	0.9 (0.74;1.1)	0.53
VAR2CSA DBL1-2	**0.34 (0.23;0.51)**	**< 0.001**	**0.25 (0.17;0.36)**	**< 0.001**	1.07 (0.92;1.24)	0.63

CyRPA	**2.5 (1.93;3.26)**	**< 0.001**	**0.66 (0.5;0.89)**	**0.007**	0.94 (0.85;1.05)	0.53
CelTOS	**2.07 (1.45;2.96)**	**< 0.001**	**0.37 (0.26;0.53)**	**< 0.001**	0.9 (0.78;1.02)	0.33
RH4.9	**2.45 (1.78;3.37)**	**< 0.001**	**0.64 (0.45;0.9)**	**0.01**	0.97 (0.86;1.1)	0.8
Group iii antigens						
DBL-α	1.11 (0.78;1.58)	0.6	**0.36 (0.26;0.49)**	**< 0.001**	0.95 (0.84;1.08)	0.64
MSP2 FL CH150	**0.17 (0.08;0.35)**	**< 0.001**	**0.04 (0.02;0.07)**	**< 0.001**	0.83 (0.63;1.09)	0.4
RH5	**1.71 (1.14;2.55)**	**0.02**	**0.23 (0.16;0.32)**	**< 0.001**	0.88 (0.77;1.02)	0.33
SSP2 (TRAP)	0.9 (0.61;1.31)	0.6	**0.46 (0.32;0.66)**	**< 0.001**	0.88 (0.77;1.01)	0.33
MSP1 Bl2 MAD20	0.81 (0.63;1.04)	0.14	**0.47 (0.37;0.59)**	**< 0.001**	1.02 (0.93;1.11)	0.83
MSP6	0.8 (0.48;1.33)	0.43	**0.16 (0.1;0.25)**	**< 0.001**	0.82 (0.68;0.98)	0.17
EBA175 R3-5	**0.27 (0.15;0.48)**	**< 0.001**	**0.2 (0.11;0.36)**	**< 0.001**	1.06 (0.85;1.31)	0.79
RH2 2030	**0.19 (0.12;0.3)**	**< 0.001**	**0.21 (0.13;0.35)**	**< 0.001**	1.03 (0.85;1.24)	0.83
MSP5	1.3 (0.77;2.19)	0.39	**0.24 (0.15;0.4)**	**< 0.001**	0.88 (0.73;1.06)	0.4
EBA140 R3-5	1.3 (0.81;2.09)	0.38	**0.25 (0.16;0.39)**	**< 0.001**	0.98 (0.82;1.16)	0.83
MSP1 Bl2 RO33	1.27 (0.8;2)	0.38	**0.25 (0.16;0.37)**	**< 0.001**	0.8 (0.68;0.93)	0.051
MSP1 Bl2 Well	**2 (1.46;2.74)**	**< 0.001**	**0.65 (0.47;0.91)**	**0.01**	0.96 (0.85;1.08)	0.69
MSP1 Bl2 3D7	**0.52 (0.32;0.87)**	**0.02**	**0.26 (0.16;0.42)**	**< 0.001**	0.93 (0.78;1.11)	0.65
RH4.2	1.21 (0.8;1.83)	0.43	**0.32 (0.22;0.48)**	**< 0.001**	0.81 (0.7;0.94)	0.051

No statistically significant differences were detected for vaccination group, sex, weight-for-age Z score, height-for-age Z score or season (data not shown).

IgM								
Antigen	**Age (5-17 month)**		**Site (Manhiça)**		**WAZ**		**Hemoglobin**	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens								
$MSP1_{42}$ 3D7	**1.81 (1.23;2.66)**	**0.004**	**0.41 (0.28;0.6)**	**< 0.001**	**0.72 (0.61;0.86)**	**< 0.001**	**0.77 (0.67;0.88)**	**< 0.001**
$MSP1_{42}$ FVO	**2.36 (1.64;3.4)**	**< 0.001**	**0.4 (0.27;0.57)**	**< 0.001**	**0.73 (0.62;0.86)**	**< 0.001**	**0.73 (0.65;0.84)**	**< 0.001**
EXP1	**3.23 (2.09;4.98)**	**< 0.001**	**0.22 (0.15;0.34)**	**< 0.001**	**0.78 (0.63;0.96)**	**0.02**	**0.73 (0.62;0.85)**	**< 0.001**
AMA1 FVO	1.22 (0.91;1.65)	0.2	**0.53 (0.4;0.71)**	**< 0.001**	**0.76 (0.67;0.87)**	**< 0.001**	**0.88 (0.79;0.98)**	**0.02**
AMA1 3D7	1.16 (0.85;1.58)	0.35	**0.52 (0.38;0.7)**	**< 0.001**	**0.82 (0.71;0.94)**	**0.007**	0.94 (0.84;1.05)	0.29
Group ii antigens								
pTRAMP	**1.28 (1.02;1.61)**	**0.03**	**0.69 (0.55;0.87)**	**0.002**	**0.81 (0.73;0.89)**	**< 0.001**	**0.89 (0.82;0.96)**	**0.003**
EBA175 R2 F2	**1.75 (1.27;2.4)**	**0.001**	**0.49 (0.36;0.67)**	**< 0.001**	**0.82 (0.71;0.94)**	**0.009**	**0.79 (0.7;0.88)**	**< 0.001**
RH1	**1.84 (1.41;2.4)**	**< 0.001**	**0.66 (0.5;0.87)**	**0.004**	**0.82 (0.72;0.92)**	**0.002**	**0.84 (0.76;0.93)**	**< 0.001**
MSP3 3D7	1.24 (0.88;1.76)	0.23	**0.61 (0.43;0.87)**	**0.008**	**0.77 (0.66;0.89)**	**0.002**	**0.77 (0.68;0.87)**	**< 0.001**
VAR2CSA DBL3-4	**1.65 (1.28;2.11)**	**< 0.001**	**0.61 (0.48;0.79)**	**< 0.001**	**0.81 (0.72;0.9)**	**< 0.001**	**0.87 (0.79;0.95)**	**0.003**
p41	**1.54 (1.17;2.02)**	**0.003**	**0.57 (0.44;0.75)**	**< 0.001**	**0.82 (0.73;0.93)**	**0.003**	**0.8 (0.73;0.88)**	**< 0.001**
MSP2 FL Dd2	**3.52 (2.29;5.43)**	**< 0.001**	**0.32 (0.2;0.5)**	**< 0.001**	**0.76 (0.62;0.93)**	**0.01**	**0.7 (0.59;0.82)**	**< 0.001**
MSP1 Bl2 hybrid	**2.48 (1.62;3.81)**	**< 0.001**	**0.57 (0.37;0.89)**	**0.02**	**0.74 (0.61;0.9)**	**0.004**	**0.8 (0.68;0.93)**	**0.006**
MSP3 3C	**2.09 (1.36;3.19)**	**0.001**	0.69 (0.45;1.08)	0.11	**0.83 (0.69;1.02)**	**0.07**	**0.81 (0.69;0.94)**	**0.006**
RH2 b240	**2.21 (1.61;3.01)**	**< 0.001**	**0.57 (0.41;0.79)**	**0.002**	**0.77 (0.66;0.88)**	**< 0.001**	**0.8 (0.71;0.9)**	**< 0.001**

LSA1	**4.31 (2.72;6.81)**	**< 0.001**	**0.21 (0.13;0.32)**	**< 0.001**	**0.81 (0.65;1.02)**	**0.07**	**0.63 (0.53;0.75)**	**< 0.001**
MSP1 Bl2 PA17	**2.17 (1.43;3.28)**	**< 0.001**	**0.48 (0.31;0.73)**	**0.001**	**0.7 (0.58;0.84)**	**< 0.001**	**0.81 (0.7;0.94)**	**0.006**
Var2csa DBL1-2	**2.31 (1.68;3.18)**	**< 0.001**	**0.56 (0.4;0.78)**	**0.001**	**0.76 (0.66;0.88)**	**< 0.001**	**0.79 (0.7;0.88)**	**< 0.001**
CYRPA	**1.47 (1.05;2.07)**	**0.03**	**0.42 (0.3;0.59)**	**< 0.001**	**0.83 (0.71;0.97)**	**0.02**	**0.77 (0.68;0.87)**	**< 0.001**
CelTOS	**2.16 (1.63;2.87)**	**< 0.001**	0.81 (0.59;1.1)	0.17	**0.81 (0.7;0.92)**	**0.003**	**0.82 (0.74;0.91)**	**< 0.001**
RH4.9	**3.06 (2.37;3.96)**	**< 0.001**	**0.67 (0.49;0.9)**	**0.01**	**0.79 (0.69;0.9)**	**0.001**	**0.85 (0.76;0.94)**	**0.003**
Group iii antigens								
DBL-α	**1.46 (1.08;1.97)**	**0.02**	**0.6 (0.45;0.81)**	**0.002**	**0.81 (0.71;0.92)**	**0.003**	**0.82 (0.74;0.91)**	**< 0.001**
MSP2 FL CH150	**3.14 (2.15;4.59)**	**< 0.001**	**0.32 (0.22;0.47)**	**< 0.001**	**0.73 (0.61;0.87)**	**0.001**	**0.78 (0.67;0.9)**	**0.001**
RH5	**1.79 (1.29;2.49)**	**0.001**	**0.43 (0.31;0.59)**	**< 0.001**	**0.83 (0.72;0.97)**	**0.02**	**0.82 (0.73;0.92)**	**0.001**
SSP2 (TRAP)	**2.63 (1.91;3.62)**	**< 0.001**	**0.68 (0.48;0.96)**	**0.03**	**0.85 (0.73;0.99)**	**0.04**	**0.8 (0.71;0.9)**	**< 0.001**
MSP1 Bl2 MAD20	**2.56 (1.89;3.48)**	**< 0.001**	**0.51 (0.37;0.71)**	**< 0.001**	**0.73 (0.63;0.84)**	**< 0.001**	**0.82 (0.73;0.92)**	**0.001**
MSP6	**2.49 (1.69;3.67)**	**< 0.001**	**0.43 (0.29;0.64)**	**< 0.001**	**0.72 (0.6;0.86)**	**< 0.001**	**0.76 (0.66;0.87)**	**< 0.001**
EBA175 R3-5	**1.82 (1.27;2.6)**	**0.002**	**0.52 (0.36;0.74)**	**< 0.001**	**0.78 (0.66;0.91)**	**0.004**	**0.81 (0.71;0.92)**	**0.002**
RH2 2030	**1.39 (1.07;1.82)**	**0.02**	0.77 (0.59;1.01)	0.06	**0.87 (0.77;0.98)**	**0.02**	**0.89 (0.81;0.98)**	**0.02**
MSP5	**2.19 (1.52;3.17)**	**< 0.001**	0.69 (0.47;1.02)	0.07	**0.79 (0.67;0.94)**	**0.01**	**0.76 (0.67;0.87)**	**< 0.001**
EBA140 R3-5	**1.72 (1.26;2.35)**	**0.001**	**0.62 (0.45;0.85)**	**0.004**	**0.75 (0.65;0.86)**	**< 0.001**	**0.77 (0.69;0.86)**	**< 0.001**
MSP1 Bl2 RO33	**1.75 (1.21;2.54)**	**0.004**	**0.43 (0.3;0.62)**	**< 0.001**	**0.84 (0.71;0.99)**	**0.04**	**0.77 (0.67;0.87)**	**< 0.001**
MSP1 Bl2 Well	**3.02 (2.08;4.39)**	**< 0.001**	**0.62 (0.42;0.93)**	**0.03**	**0.71 (0.59;0.84)**	**< 0.001**	**0.82 (0.71;0.94)**	**0.006**
MSP1 Bl2 3D7	**2.18 (1.52;3.11)**	**< 0.001**	**0.6 (0.41;0.86)**	**0.008**	**0.74 (0.63;0.87)**	**< 0.001**	**0.81 (0.71;0.93)**	**0.003**
RH4.2	**2.71 (1.94;3.79)**	**< 0.001**	**0.55 (0.38;0.79)**	**0.002**	**0.74 (0.63;0.87)**	**< 0.001**	**0.75 (0.67;0.85)**	**< 0.001**

WAZ: weight-for-age Z score. No statistically significant differences were detected for vaccination group, sex, height-for-age Z score or season (data not shown).

Table S4. Multivariable analysis of the effect of RTS,S/AS01E vaccination and demographic, clinical and epidemiological variables on antibody levels to *Plasmodium falciparum* pre-erythrocytic and blood stage antigens. Linear regression with antibody levels (log_{10}MFI) as outcome and other covariates adjusted (in parenthesis the category that is compared to the reference). Coefficients (10^Beta) with 95% confidence intervals and p values are shown (significant in bold).

a) Month 3 IgM

Antigen	**Vaccine (RTS,S)**		**Age (5-17 month)**		**Site (Manhiça)**		**Baseline IgM levels**		**Malaria episodes**		**Season (Low)**		**HAZ**		**Sex (Male)**	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens																
$MSP1_{42}$ 3D7	**0.49 (0.33;0.73)**	**<0.001**	0.73 (0.51;1.05)	0.09	**0.57 (0.38;0.84)**	**0.005**			**3.23 (1.75;5.95)**	**< 0.001**	0.47 (0.21;1.07)	0.07			1.39 (0.97;1.98)	0.07

$MSP1_{42}$ FVO	0.75 (0.5;1.12)	0.15			0.7 (0.45;1.07)	0.1	**1.49 (1.06;2.09)**	**0.02**	**2.75 (1.44;5.23)**	**0.002**	0.44 (0.19;1.02)	0.055	0.87 (0.74;1.03)	0.1		
EXP1			**1.93 (1.17;3.16)**	**0.01**			**1.45 (1.01;2.08)**	**0.042**	**6.66 (3.1;14.31)**	**<0.001**	0.48 (0.18;1.3)	0.15				
AMA1 FVO			0.79 (0.6;1.04)	0.09	0.75 (0.55;1.01)	0.06	**1.49 (1.08;2.04)**	**0.01**	**1.68 (1.05;2.71)**	**0.03**					**1.34 (1.02;1.76)**	**0.04**
AMA1 3D7			0.81 (0.61;1.09)	0.16	**0.68 (0.49;0.94)**	**0.02**	1.36 (0.99;1.87)	0.057	**1.89 (1.15;3.11)**	**0.01**						
Group ii antigens																
pTRAMP			0.8 (0.64;1.01)	0.06	0.79 (0.62;1.02)	0.07	**1.73 (1.22;2.44)**	**0.002**	1.42 (0.96;2.12)	0.08			**0.88 (0.79;0.97)**	**0.01**	1.21 (0.97;1.52)	0.09
EBA175 R2(F2)					**0.63 (0.44;0.89)**	**0.009**	**1.66 (1.19;2.31)**	**0.003**	1.53 (0.89;2.61)	0.12						
PfRH1					**0.77 (0.6;1)**	**0.05**	**2.07 (1.55;2.76)**	**<0.001**	1.4 (0.93;2.11)	0.11						
MSP3 3D7							**1.87 (1.41;2.47)**	**<0.001**			0.57 (0.3;1.08)	0.09				
VAR2CSA DBL3-4			0.84 (0.65;1.07)	0.15	**0.73 (0.57;0.93)**	**0.01**	**1.82 (1.32;2.51)**	**<0.001**					0.9 (0.81;1)	0.054		
p41					0.78 (0.59;1.05)	0.1	**1.77 (1.28;2.45)**	**<0.001**	1.53 (0.97;2.41)	0.07					**1.3 (1;1.69)**	**0.048**
MSP2 FL Dd2			1.4 (0.94;2.07)	0.1			**1.58 (1.19;2.09)**	**0.002**	**2.48 (1.38;4.47)**	**0.003**	**0.27 (0.12;0.6)**	**0.001**	0.86 (0.74;1)	0.057		
MSP1 Bl2 hybrid			1.34 (0.9;1.99)	0.15			**1.43 (1.07;1.93)**	**0.02**	**3.15 (1.7;5.86)**	**< 0.001**	**0.44 (0.19;0.99)**	**0.047**				
MSP3 3C			**0.64 (0.44;0.94)**	**0.02**			**2.2 (1.65;2.93)**	**<0.001**	**2.02 (1.11;3.67)**	**0.02**					0.71 (0.49;1.03)	0.07
PfRH2 b240							**1.96 (1.46;2.62)**	**<0.001**	**1.85 (1.15;2.97)**	**0.01**	**0.49 (0.27;0.92)**	**0.03**	**0.87 (0.77;0.99)**	**0.03**	1.29 (0.97;1.72)	0.08
LSA1	0.73 (0.48;1.11)	0.14			**0.38 (0.24;0.61)**	**< 0.001**	**1.43 (1.07;1.92)**	**0.02**	1.95 (0.97;3.9)	0.06						
MSP1 Bl2 PA17							**1.37 (0.98;1.92)**	**0.07**	**4.66 (2.34;9.27)**	**< 0.001**	0.48 (0.2;1.19)	0.11				
VAR2CSA DBL1-2			0.76 (0.54;1.08)	0.12	**0.59 (0.42;0.83)**	**0.002**	**1.74 (1.24;2.43)**	**0.001**			0.53 (0.26;1.07)	0.08				
CYRPA			**0.68 (0.5;0.93)**	**0.02**	**0.52 (0.37;0.73)**	**< 0.001**	**2.14 (1.57;2.92)**	**<0.001**					**0.87 (0.76;0.99)**	**0.042**	1.31 (0.97;1.78)	0.08
CelTOS					**0.67 (0.5;0.9)**	**0.009**	**2.37 (1.73;3.26)**	**< 0.001**								
RH4.9			1.35 (0.97;1.88)	0.07			**2.38 (1.67;3.39)**	**< 0.001**	1.51 (0.97;2.36)	0.07	0.57 (0.31;1.04)	0.07	0.91 (0.81;1.03)	0.13	**1.38 (1.05;1.81)**	**0.02**
Group iii antigens																
DBL-α			0.77 (0.58;1.02)	0.07	0.79 (0.58;1.09)	0.15	**1.37 (1;1.89)**	**0.05**	1.43 (0.88;2.35)	0.15			**0.87 (0.77;0.99)**	**0.04**	1.24 (0.93;1.64)	0.14
MSP2 FL CH150			**1.64 (1.12;2.39)**	**0.01**	**0.51 (0.34;0.77)**	**0.001**			**2.04 (1.09;3.82)**	**0.03**	**0.41 (0.18;0.93)**	**0.03**				
PfRH5					0.75 (0.53;1.07)	0.11	**1.64 (1.2;2.24)**	**0.002**	**1.79 (1.05;3.04)**	**0.03**			0.88 (0.77;1)	0.054		

SSP2 (TRAP)	**1.41 (1.01;1.98)**	**0.044**	**1.63 (1.15;2.31)**	**0.006**	0.72 (0.52;1.01)	0.058	**1.63 (1.17;2.27)**	**0.004**					0.89 (0.77;1.02)	0.1		
MSP1 BI2 MAD20			**1.47 (1.12;1.92)**	**0.006**			**1.92 (1.47;2.51)**	**<0.001**	**1.55 (1.04;2.32)**	**0.03**	0.64 (0.37;1.09)	0.1				
MSP6							**1.57 (1.15;2.13)**	**0.005**	**3.26 (1.77;6)**	**< 0.001**			**0.81 (0.69;0.95)**	**0.01**		
RH2 (2030)							**1.65 (1.22;2.22)**	**0.001**	**1.67 (1.13;2.47)**	**0.01**	0.63 (0.37;1.05)	0.08	**0.89 (0.8;0.98)**	**0.02**		
EBA175 R3-5					**0.62 (0.43;0.89)**	**0.01**			**1.8 (1.03;3.15)**	**0.04**					1.26 (0.91;1.74)	0.16
MSP5							**1.45 (1.09;1.93)**	**0.01**	1.51 (0.88;2.59)	0.13	**0.28 (0.14;0.58)**	**< 0.001**	0.9 (0.78;1.04)	0.14		
EBA140 R3-5			1.25 (0.94;1.66)	0.13			1.28 (0.95;1.73)	0.11	**1.59 (1.02;2.5)**	**0.042**	0.56 (0.31;1.02)	0.058	0.92 (0.81;1.03)	0.15		
MSP1 BI2 RO33					0.71 (0.5;1.03)	0.07	**1.45 (1.07;1.98)**	**0.02**								
MSP1 BI2 Well			**1.94 (1.42;2.65)**	**< 0.001**			**1.74 (1.36;2.24)**	**<0.001**	1.5 (0.95;2.39)	0.08	**0.39 (0.21;0.72)**	**0.003**				
MSP1 BI2 3D7	0.72 (0.5;1.04)	0.08					**1.53 (1.14;2.06)**	**0.005**	**2.79 (1.62;4.79)**	**< 0.001**	**0.39 (0.19;0.81)**	**0.01**				
RH4.2							**1.44 (1.1;1.88)**	**0.008**	**1.89 (1.18;3.03)**	**0.009**	**0.29 (0.16;0.54)**	**< 0.001**				

HAZ: height-for-age Z score. Hemoglobin and weight-for-age Z score were retained in some antigen models but were not statistically significant (data not shown).

b) Month 0

IgM														
Antigen	**Age (5-17 month)**		**Site (Manhiça)**		**Season (Low)**		**Hemoglobin**		**WAZ**		**HAZ**		**Sex (Male)**	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens														
$MSP1_{42}$ 3D7	1.44 (0.99;2.1)	0.058	**0.52 (0.35;0.78)**	**0.001**	**3.32 (1.53;7.19)**	**0.003**	**0.82 (0.72;0.94)**	**0.004**	**0.72 (0.58;0.89)**	**0.002**	1.17 (0.96;1.43)	0.12		
$MSP1_{42}$ FVO	**1.8 (1.28;2.53)**	**<0.001**	**0.59 (0.41;0.85)**	**0.005**			**0.81 (0.71;0.91)**	**<0.001**	**0.83 (0.71;0.97)**	**0.02**				
EXP1	**2.56 (1.73;3.79)**	**<0.001**	**0.34 (0.22;0.53)**	**<0.001**			**0.84 (0.73;0.96)**	**0.01**			0.87 (0.73;1.03)	0.1		
AMA1 FVO			**0.71 (0.52;0.97)**	**0.03**			0.92 (0.83;1.02)	0.1	**0.8 (0.71;0.91)**	**0.001**				
AMA1 3D7			**0.65 (0.46;0.9)**	**0.01**							**0.86 (0.75;0.98)**	**0.02**		
Group ii antigens														
pTRAMP					1.4 (0.9;2.17)	0.13	**0.89 (0.83;0.96)**	**0.003**	**0.82 (0.75;0.9)**	**<0.001**			0.86 (0.7;1.05)	0.14
EBA175 R2(F2)	**1.54 (1.13;2.1)**	**0.007**	**0.65 (0.47;0.91)**	**0.01**	1.62 (0.84;3.12)	0.15	**0.84 (0.75;0.93)**	**0.001**			0.89 (0.78;1.02)	0.09		
PfRH1	**1.56 (1.2;2.02)**	**<0.001**					**0.89 (0.81;0.98)**	**0.01**	**0.86 (0.76;0.96)**	**0.008**				

MSP3 3D7			**0.7 (0.5;0.99)**	**0.043**			**0.8 (0.71;0.91)**	**<0.001**	**0.84 (0.72;0.97)**	**0.02**				
VAR2CSA DBL3-4	**1.44 (1.13;1.83)**	**0.004**	0.81 (0.62;1.05)	0.12			0.92 (0.84;1.01)	0.07	**0.85 (0.76;0.95)**	**0.004**				
p41	1.24 (0.96;1.61)	0.1	0.77 (0.58;1.01)	0.06			**0.83 (0.76;0.91)**	**<0.001**	**0.88 (0.78;0.99)**	**0.03**				
MSP2 FL Dd2	**2.65 (1.76;4)**	**0.001**	**0.44 (0.29;0.69)**	**< 0.001**			**0.79 (0.68;0.91)**	**0.002**						
MSP1 Bl2 hybrid	**2.23 (1.46;3.39)**	**<0.001**					0.87 (0.75;1.02)	0.08			**0.78 (0.65;0.93)**	**0.006**		
MSP3 3C	**1.8 (1.16;2.79)**	**0.009**					**0.85 (0.72;0.99)**	**0.04**						
PfRH2 b240	**1.94 (1.42;2.65)**	**<0.001**	0.77 (0.55;1.08)	0.13	1.63 (0.84;3.13)	0.14	**0.87 (0.78;0.97)**	**0.01**	**0.84 (0.73;0.96)**	**0.01**				
LSA1	**3 (2;4.5)**	**<0.001**	**0.29 (0.19;0.44)**	**< 0.001**			**0.74 (0.64;0.85)**	**<0.001**						
MSP1 Bl2 PA17	**1.74 (1.16;2.61)**	**0.008**					0.88 (0.76;1.01)	0.08	**0.74 (0.61;0.88)**	**<0.001**				
VAR2CSA DBL1-2	**1.92 (1.41;2.62)**	**<0.001**					**0.85 (0.76;0.95)**	**0.004**	**0.83 (0.72;0.95)**	**0.007**				
CyRPA			**0.55 (0.39;0.78)**	**< 0.001**			**0.78 (0.7;0.88)**	**<0.001**						
CelTOS	**1.98 (1.48;2.64)**	**<0.001**			**1.9 (1.04;3.48)**	**0.04**	**0.88 (0.8;0.98)**	**0.02**	**0.86 (0.76;0.98)**	**0.02**				
RH4.9	**2.86 (2.22;3.68)**	**<0.001**							**0.83 (0.74;0.93)**	**0.002**				
Group iii antigens														
DBL-α							**0.83 (0.75;0.92)**	**<0.001**	**0.84 (0.73;0.96)**	**0.008**				
MSP2 FL CH150	**2.94 (2.04;4.24)**	**<0.001**	**0.45 (0.3;0.66)**	**<0.001**	2 (0.9;4.43)	0.09	0.9 (0.79;1.02)	0.1			**0.83 (0.71;0.97)**	**0.02**		
PfRH5	**1.68 (1.21;2.32)**	**0.002**	**0.56 (0.4;0.79)**	**0.001**	**2.04 (1.03;4.01)**	**0.04**	**0.87 (0.78;0.98)**	**0.02**			0.9 (0.78;1.03)	0.12		
SSP2 (TRAP)	**2.36 (1.71;3.25)**	**<0.001**					**0.88 (0.78;0.99)**	**0.03**			**0.87 (0.76;0.99)**	**0.04**		
MSP1 Bl2 Mad20	**2.11 (1.57;2.83)**	**<0.001**	**0.7 (0.51;0.96)**	**0.03**			0.92 (0.83;1.02)	0.12	**0.8 (0.7;0.91)**	**<0.001**				
MSP6	**1.92 (1.32;2.79)**	**<0.001**	**0.64 (0.43;0.97)**	**0.03**			**0.84 (0.73;0.96)**	**0.01**	**0.81 (0.68;0.96)**	**0.02**				
RH2 (2030)	**1.32 (1;1.73)**	**0.048**			**1.93 (1.1;3.41)**	**0.02**	0.92 (0.84;1.01)	0.09	**0.88 (0.78;0.99)**	**0.04**				
EBA175 R3-5	**1.59 (1.11;2.27)**	**0.01**	0.71 (0.48;1.04)	0.08	1.81 (0.85;3.84)	0.12	**0.86 (0.76;0.98)**	**0.02**	**0.84 (0.72;0.99)**	**0.04**				
MSP5	**1.76 (1.21;2.55)**	**0.003**					**0.82 (0.72;0.93)**	**0.003**	0.86 (0.73;1.01)	0.07				
EBA140 R3-5	1.32 (0.99;1.77)	0.06					**0.81 (0.73;0.9)**	**<0.001**	**0.8 (0.7;0.91)**	**<0.001**				
MSP1 Bl2 RO33	1.36 (0.96;1.93)	0.08	**0.6 (0.41;0.87)**	**0.008**			**0.8 (0.7;0.91)**	**<0.001**						
MSP1 Bl2 Well	**2.65 (1.84;3.82)**	**<0.001**							**0.74 (0.63;0.87)**	**<0.001**				
MSP1 Bl2 3D7	**1.77 (1.25;2.52)**	**0.002**					**0.88 (0.78;1)**	**0.051**	**0.79 (0.67;0.92)**	**0.003**				
RH4.2	**2.13 (1.54;2.94)**	**<0.001**					**0.83 (0.74;0.93)**	**0.002**	**0.81 (0.7;0.94)**	**0.004**				

IgG														
Antigen	**Age (5-17 month)**		**Site (Manhiça)**		**Season (Low)**		**Hemoglobin**		**WAZ**		**HAZ**		**Sex (Male)**	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Group i antigens														
$MSP1_{42}$ 3D7	**0.21 (0.12;0.38)**	**<0.001**	**0.05 (0.03;0.09)**	**<0.001**										
$MSP1_{42}$ FVO	**0.4 (0.2;0.8)**	**0.01**	**0.04 (0.02;0.09)**	**<0.001**										
EXP1	**0.09 (0.05;0.15)**	**<0.001**	**0.04 (0.02;0.07)**	**<0.001**										
AMA1 FVO	**0.02 (0.01;0.04)**	**<0.001**	**0.1 (0.05;0.18)**	**<0.001**			0.84 (0.67;1.04)	0.11						
AMA1 3D7	**0.02 (0.01;0.04)**	**<0.001**	**0.1 (0.05;0.2)**	**<0.001**			0.81 (0.64;1.02)	0.07						
Group ii antigens														
pTRAMP			**0.35 (0.22;0.55)**	**<0.001**			**0.81 (0.7;0.94)**	**0.005**						
EBA175 R2(F2)	**0.32 (0.18;0.55)**	**<0.001**	**0.16 (0.09;0.27)**	**<0.001**										
PfRH1	**1.56 (1.09;2.22)**	**0.01**	**0.18 (0.12;0.26)**	**<0.001**			**0.84 (0.74;0.96)**	**0.009**						
MSP3 3D7	**0.14 (0.09;0.22)**	**<0.001**	**0.17 (0.11;0.28)**	**<0.001**										
VAR2CSA DBL3-4	**0.07 (0.04;0.11)**	**<0.001**	**0.11 (0.07;0.17)**	**<0.001**	**0.25 (0.09;0.7**)	**0.008**								
p41			**0.38 (0.25;0.57)**	**<0.001**			**0.88 (0.77;1)**	**0.054**						
MSP2 FL Dd2	**0.08 (0.05;0.12)**	**<0.001**	**0.03 (0.02;0.06)**	**<0.001**			**0.82 (0.68;0.98)**	**0.03**						
MSP1 BI2 hybrid	**0.35 (0.19;0.63)**	**<0.001**	**0.11 (0.06;0.21)**	**<0.001**										
MSP3 3C	**2.01 (1.32;3.05)**	**0.001**	**0.33 (0.22;0.51)**	**<0.001**										
PfRH2 b240			**0.28 (0.18;0.42)**	**<0.001**										
LSA1	**0.47 (0.27;0.79)**	**0.005**	**0.14 (0.08;0.24)**	**<0.001**			**0.8 (0.66;0.97)**	**0.02**	1.32 (1.04;1.68)	0.02				
MSP1 BI2 PA17	0.65 (0.38;1.1)	0.11	**0.27 (0.16;0.46)**	**<0.001**										
VAR2CSA DBL1-2	**0.29 (0.21;0.41)**	**<0.001**	**0.24 (0.17;0.33)**	**<0.001**	0.52 (0.25;1.1)	0.09							1.27 (0.91;1.77)	0.15
CyRPA	**2.41 (1.86;3.14)**	**<0.001**	**0.7 (0.54;0.92)**	**0.009**										
CelTOS	**1.87 (1.34;2.61)**	**<0.001**	**0.38 (0.27;0.53)**	**<0.001**										
RH4.9	**2.34 (1.71;3.2)**	**<0.001**	**0.64 (0.46;0.88)**	**0.007**							1.17 (1.02;1.34)	**0.02**		
Group iii antigens														

DBL-α			**0.42 (0.29;0.6)**	**<0.001**							0.9 (0.78;1.03)	0.12		
MSP2 FL CH150	**0.11 (0.06;0.19)**	**<0.001**	**0.04 (0.02;0.06)**	**<0.001**			**0.78 (0.64;0.95)**	**0.01**						
PfRH5	**1.5 (1.06;2.13)**	**0.02**	**0.25 (0.17;0.37)**	**<0.001**										
SSP2 (TRAP)			**0.47 (0.32;0.69)**	**<0.001**							0.88 (0.75;1.03)	0.1		
MSP1 Bl2 Mad20	**0.75 (0.6;0.94)**	**0.01**	**0.46 (0.37;0.59)**	**<0.001**							0.92 (0.83;1.02)	0.11	0.82 (0.65;1.03)	0.08
MSP6	**0.63 (0.4;0.99)**	**0.045**	**0.19 (0.12;0.31)**	**<0.001**			**0.84 (0.71;0.99)**	**0.03**					1.47 (0.95;2.27)	0.09
RH2 (2030)	**0.17 (0.11;0.26)**	**<0.001**	**0.24 (0.15;0.38)**	**<0.001**							**0.83 (0.69;1)**	**0.045**		
EBA175 R3-5	**0.24 (0.14;0.4)**	**<0.001**	**0.16 (0.09;0.27)**	**<0.001**					1.23 (0.96;1.56)	0.1				
MSP5			**0.27 (0.16;0.45)**	**< 0.001**							0.84 (0.68;1.04)	0.11		
EBA140 R3-5			**0.23 (0.15;0.37)**	**< 0.001**										
MSP1 Bl2 RO33			**0.3 (0.19;0.47)**	**< 0.001**			**0.82 (0.71;0.95)**	**0.009**					0.74 (0.49;1.11)	0.15
MSP1 Bl2 Well	**1.92 (1.4;2.63)**	**< 0.001**	**0.68 (0.49;0.93)**	**0.02**										
MSP1 Bl2 3D7	**0.45 (0.28;0.71)**	**< 0.001**	**0.27 (0.16;0.44)**	**< 0.001**										
RH4.2			**0.39 (0.26;0.6)**	**< 0.001**			**0.84 (0.73;0.96)**	**0.01**						

HAZ: height-for-age Z score; WAZ: weight-for-age Z score.

SUPPLEMENTARY MATERIALS AND METHODS

Antibody measurements. Antigen-coupled beads were added to a 96-well µClear® flat bottom plate (Greiner Bio-One) in multiplex (1,000 microspheres/analyte/well) resuspended in 50µL of PBS, 1% BSA, 0.05% Azide pH 7.4 (PBS-BN). Fifty µL of sample, negative or positive control were added to multiplex wells and incubated overnight at 4ºC in a shaker protected from light. Plates were washed three times with 200µL/well of wash buffer (PBS-Tween 20 0.05%) using a manual magnetic washer. Then, 100µL of biotinylated secondary antibody were added diluted in PBS-BN: anti-human IgG (Sigma) and anti-human IgM (Sigma) were added. All antibody incubations were performed for 45 min, at room temperature, with agitation and protected from light. Next, streptavidin-R-phycoerythrin (Sigma) in PBS-BN was added to all wells and incubated 30 min, at room temperature, with agitation and protected from light. Plates were washed as before and resuspended in 100 µL/well of PBS-BN. Plates were stored at 4°C overnight protected from light and read the next day using the Luminex xMAP® 100/200 analyser; at least 50 microspheres per analyte were acquired per sample.

Test samples were assayed at 4 dilutions for IgG (500, 5000, 50,000 and 500,000) and IgM (100, 1000, 10,000 and 50,000) to ensure that at least one dilution lie in the linear range of the respective standard curve, i.e. close to the highest slope between two dilution points. For IgG assays, 18 to 22 serial dilutions 1:2 of a positive control were used to perform antigen-isotype/subclass specific standard curves. The positive control consisted of a WHO Reference Reagent for anti-malaria *P. falciparum* human serum (NIBSC code: 10/198) at 1:50 plus a pool of plasmas from RTS,S/AS02 vaccinated children with high IgG titers against CSP at 1:100. For the IgM assay, 18 serial dilutions 1:2 of a pool of samples from ISGlobal repository with high IgM levels against *P. falciparum* antigens were used. A total of 69 different negative control samples from malaria-naïve adult donors were assayed along the study to calculate the cutoffs of seropositivity (mean + 3 standard deviations [SD]). Blanks were added to each plate in triplicates for quality control purposes. Sample distribution across plates was designed to ensure a balanced distribution of vaccination groups, age cohorts and time-points. Data were captured using xPonent software.

Statistical analysis

Data pre-processing. The assay quality control for each antigen and plate was based on the estimation of the % coefficient of variation (CV) of the 3 blank controls and the performance of the standard curves. The standard curve for each antigen-isotype/subclass-plate was estimated using the *drLumi* R package flow [36], fitted in a 5-parameter logistic (5-PL) regression model, and data points logarithmically transformed. If the model did not converge, 4-PL or exponential regressions were fitted. The standard curves were visually inspected, and the percentage of plates within an analyte-isotype/subclass with CV Emax SE/Beta <15%, CV Emin SE/Beta <15%, R^2 >0.95 and Model fit p value >0.05, were calculated.

To select the sample dilution in the linear part of the sigmoidal curve (antigen, isotype/subclass and plate specific), an algorithm that detects the two points with the highest slope between them was used. The slope was computed as: $m = (\log_{10} MFI_i - \log_{10} MFI_{i+1}) / (dilution\_factor_i - dilution\_factor_{i+1})$. The mean $\log_{10}$ MFI value of the two points was computed, and the nearest $\log_{10}$MFI of the test sample and the corresponding dilution was selected. The MFI measurement of the selected dilution was corrected multiplying by its corresponding dilution factor and transformed to $\log_{10}$ scale to stabilize the variance.

Blank and GST background signals were not subtracted. GST subtraction distorted and increased the variability of the data due to the lack of correlation between GST-fusion proteins and GST alone[37].

SUPPLEMENTARY RESULTS

Additional factors affecting antibody responses. In adjusted multivariable models, season was not generally associated with antibody levels except for significantly lower IgM levels to about half of the antigens if the post-vaccination visit (M3) occurred during the low compared to the high transmission season (**Table S4**). Sex was not significantly associated with antibody levels at baseline (M0) or M3, except for AMA1 FVO, P41 and RH4.9 for which M3 IgM levels were higher in males than females (**Table S4**).

Local alignment of antigens to CSP. The default BLAST E-value threshold of 10 returned hits for two full length sequences in addition to SSP2 (TRAP): MSP2 (a.a. 173-210, % identity = 34.2, E-value = 1.1) and VAR2CSA (a.a. 2003-2022, % identity = 45, E-value = 9.9). These areas of local alignment mapped to CSP a.a. 247-283 and a.a. 349-367, respectively. The region of VAR2CSA, a duffy-binding-like region, maps to the thrombospondin type 1 domain of CSP (PlasmoDB.org). At the peptide level, four 25mer sequences aligned to CSP in addition to the two SSP2 sequences reported in the main text: 1) VAR2CSA a.a. 1999-2023 (% identity = 45, E-value = 2.6) mapped to CSP a.a. 349-367; 2) MSP1 a.a. 379-403 (% identity = 45.5, E-value = 5.3) mapped to CSP a.a. 51-61; 3) VAR2CSA a.a. 361-385 (% identity = 35.3, E-value = 6.2) mapped to CSP a.a. 39-55; and 4) LSA1 a.a. 1099-1123 (% identity = 46.7, E-value = 7.2) mapped to CSP a.a. 307-321.

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Discussion

Summary of the key findings

This PhD project was developed to characterize the immunogenicity of RTS,S/AS0 immunization after primary and booster dose schedules, and the kinetics of induced immune response, recognizing the suboptimal efficacy of this first generation product and the need for improved vaccines that induce longer-lasting immunity to malaria in African children. The work of this thesis shows that the immunogenicity varies according to primary or booster vaccination, including the magnitude of antibodies and preferential patterns of IgG class switching, and impacts NAI, decreasing IgG to some blood stage immunogenic antigens but also increasing IgG against some other malaria antigens not included in the vaccine. These findings have significant implications for our understanding of malaria protective immunity and the design of future vaccines.

Firstly, contrary to our hypothesis, we have shown that after primary schedule vaccination, the RTS,S-induced IgG recognize similarly the native-like fucosylated and the vaccine-like non-fucosylated CSP-TSR antigens, suggesting that an O-fucosylated CSP-based vaccine would probably not improve RTS,S immunogenicity and VE.

Secondly, for the first time, we have reported that levels of IgG1, IgG3 and IgG4 subclasses, but not IgG2 nor IgM, against vaccine antigens, are increased one month after the 4th dose. Overall, antibody responses to the booster were lower than the initial peak response to the primary immunization, infants had lower IgG and IgG1 levels than children, and the RTS,S booster induced antibody response was different from that induced following primary vaccination, highlighting a temporal change in subclass patterns, with age and under malaria exposure.

Moreover, after the booster dose, we detected higher anti-Rh5 IgG and IgG1-4 levels, suggesting that RTS,S afforded partial protection could affect the acquisition of adaptive immunity to malaria by increasing some blood stage antibody responses. Related to this, we have shown a complex interplay between RTS,S vaccination and naturally acquired immunity to malaria after the primary vaccination schedule. We found that immunization with RTS,S affects the acquisition of IgG and IgM to *P. falciparum* antigens that are not part of the vaccine formulation, and such responses impact the overall protection against malaria. Of note, one month after post-vaccination, RTS,S decreased antibody responses to some parasite antigens ($MSP1_{42}$,

AMA1) that are considered markers of exposure and their levels correlated with clinical malaria risk over 12-month follow-up. On the contrary, we showed for the first time that RTS,S vaccination increased IgG levels to a specific group of pre-erythrocytic and blood-stage antigens, which levels correlated with protection against clinical malaria, including MSP5, MSP1 block 2, RH4.2, EBA140, and SSP2/TRAP antigens.

Considerations on native post-translational modifications on vaccine design and protective immunity

The evidence that the CSP is O-fucosylated in their TSR domains (316) has raised questions about how these post-translation modifications could impact the selection of vaccine antigens, immunogen design and efficacy. Therefore, in Article 1, we hypothesized that the RTS,S vaccine-induced antibodies could bind less to a native-like fucosylated CSP, which could compromise the overall protective efficacy. We primarily aimed to characterize the vaccine IgG reactivity to native-like fucosylated CSP after primary vaccination with RTS,S and postulated their significance in improving the immunogenicity and overall protection in a potential O-fucosylated CSP-based vaccine. Surprisingly, we found that IgG levels against fucosylated CSP full-length and TSR constructs were similar to those of their vaccine-related non-fucosylated counterpart. This same phenomenon was also seen in pre- and post-vaccination comparator samples containing IgG induced against fuco-CSP naturally found in the parasites. When examining how age, sex, and clinical malaria status could affect IgG levels, we found no significant differences. Moreover, the intensity of malaria transmissions did not seem to affect the IgG levels. Altogether, these findings suggest that the RTS,S vaccine-induced antibodies can recognize both CSP full-length and TSR constructs independently of their fucosylation status; the contrary is also true as we found that IgG generated by natural exposure, probably to native-like fucosylated CSP on sporozoites, also recognize equally well vaccine-related antigens. These findings could have some considerations. Firstly, the recombinant fucosylated CSP full-length was expressed in *Pichia pastoris,* and the recombinant TSR C-terminal domain of CSP was expressed in *E. coli* and not derived from *P. falciparum* sporozoites. It could be that the construct used here did not sufficiently resemble the sporozoites in their native form. Mixed results were reported in a trial where a glycosylated $MSP1_{42}$ purified from the milk of transgenic mice was found to be associated with malaria risk in *Aotus nancymai* monkeys, whereas the baculovirus-expressed form of $MSP1_{42}$ conferred protection (372), highlighting the relevant role of antigen expression system and glycosylation status. Secondly, we could have missed minor population antibodies against fucosylated CSP full-length and TSR relevant for protection by measuring total IgG due to the polyclonal antibody nature of the immunoassays used in this experiment. However, these results are not yet

conclusive. The characterization of the *Plasmodium* glycome has just begun, and their potential therapeutic significance and relevance for vaccine design are not mature enough. In fact, to the best of our knowledge, this is the first work linking the RTS,S and naturally induced antibodies reactivity to native-like fucosylated CSP, serving as a baseline study exploring the role of fucosylation in the magnitude of immune response using samples from a phase 2b clinical trial. The importance of glycoproteins has been emphasized recently in the HIV vaccine field, where researchers have advanced in generating broadly neutralizing antibodies analogous to those that develop naturally in a subset of infected individuals by mimicking the glycan shield (373). In malaria, the incomplete protection elicited by recombinant *P. falciparum* MSP1 antigens has been linked to a lack of protein glycosylation (315). These advances highlight the potential of carbohydrate modifications to induce an immune response, as structural studies suggest that fucosylation contributes to antigenic determinants by changing their shape and how they are recognized by the immune system (316). In addition, several studies demonstrated that glycosylation modulates T-cell immune responses by participating in the TCR epitopes or by T-cell recognition and interaction between glycan-containing antigens and T cells (374); the implications of these interactions could be studied. Although glycosylation plays a critical role in modulating immune responses, it is a double-edged sword that can also be a tool for immune evasion by pathogens by shielding specific protein epitopes from antibody neutralization (375,376). Therefore, in-depth characterization, understanding of the correct glycosylation and suitable secretion systems that lead to good yields, stability, and correct immunogen presentation and, thus, a high-quality immune response are crucial to inform vaccine design strategies and development. Different approaches will be needed to determine how much glycosylation potentially impacts the immunogenicity of the antigen and VE.

Despite not seeing differences in RTS,S-induced IgG recognition to CSP O-fucosylated vs. non-O-fucosylated, it is possible that a vaccine with an O-fucosylated CSP could lead to a broader immune response by engaging T-cells and that the IgG raised could better recognize native sporozoites. Vaccination models testing both humoral and cellular responses and VE of glycosylated versus nonglycosylated vaccines head-to-head could shed more light on to the impact of glycosylation on immunogenicity and induction of protective responses.

Characterisation of the magnitude, nature, and duration of the immune response to the booster dose

Due to the growing evidence that immunity to malaria involves multiple antibody effector functions, we characterized the immune responses beyond the measurement of IgG titers to only one B cell epitope of the CSP (the NANP-repeat region). In article 2, we measured levels of total IgM, IgG, and IgG1-4 subclasses against three CSP constructs (full-length, the C-term, and the NANP-repeat region) and HBsAg, all vaccine-related antigens after the booster dose.

Different patterns than primary vaccination emerged one month after the booster dose. We found increased IgG, IgG1, IgG3, and IgG4 against vaccine constructs, and the levels remained above those of the non-vaccinated group throughout the entire follow-up period. Contrary to the primary three-dose schedule, which increased IgG2 that was associated with a malaria risk (347), the booster dose did not increase IgG2 or IgM. The temporal changes in subclass antibody patterns upon the booster vaccine highlight differential patterns of IgG classes with primary vaccination and the booster dose seems to induce class-switched memory B cell response.

After the booster dose, we found that the highest levels were against full-length CSP, followed by the CSP NANP region, the CSP C-term and HBsAg similar to primary vaccination. The predominant subclass was IgG1, followed by IgG3, then lower levels of IgG2 and the least for IgG4.

The inclusion of full-length CSP in our experiments has an additional advantage in detecting responses of lower magnitude, possibly due to the additive response to multiple B cell epitopes. The RTS,S has a truncated protein that does not contain any portion of the N-terminal domain (393), junction region (394), or the NVDP repeats (395), that compose the whole CSP and some of the antigens not included in RTS,S have been associated with induction of protective antibodies. Notably, it was suggested that antibodies that target epitopes in both regions, the NANP-repeat region of CSP and its N terminus simultaneously, were more potent than antibodies that exclusively recognize each site (377). The relatively conserved R1-NANP junction has also been associated with eliciting protective antibody responses (378). A better understanding of the role of these specific epitopes in protective humoral responses could inform next-generation CSP-based vaccine development.

We also characterized the IgG anti-HBsAg post-booster response and found that the mean increase in antibody levels against HBsAg was proportionally lower than that of CSP constructs. Although our study was not designed to draw an association between HBsAg post-booster response and protection, we previously

reported the association of anti-HBsAg antibody levels with protection against malaria in RTS,S vaccinees one month after the primary vaccination dose schedule (347). How the induced anti-HBsAg antibodies are related to protection against malaria remains unclear. We found a correlation between anti-CSP and anti-HBsAg IgG titers (347), which could indicate children's better immune status and capacity to respond to malaria and non-malaria antigens epitopes after vaccination. Moreover, since the CSP and HBsAg antigens are presented to effector cells at the same time as a component of the RTS,S vaccine, it could be the reflection of the direct immune mechanism whereby the HBsAg-specific T cells might provide help to B cells to produce high affinity anti-CSP antibodies (379).

We found that the booster dose increased the level of antibodies, albeit below the initial peak response to primary immunization, as previously reported (341,380). The reasons for this still need to be fully understood. Different hypotheses have been put forward to explain this phenomenon. One is that the pre-exposure to *P. falciparum*, whether through repeated malaria infection or vaccination, leads to hypo-responsiveness of B cell lymphocytes (393) due to the recruitment of fewer antigen-specific B cells into successive responses, with the B cell reservoir being exhausted by repeat or high-dose antigen exposure (381). Another possibility is that pre-existing antibodies due to natural infection or primary vaccination course could accelerate clearance of vaccine antigen after booster dose and limit B cell priming after vaccination (382), suggesting that increasing the time to some extent between primary and booster vaccinations could benefit the immune response. In the long term, we saw that IgG and IgG1 levels continued to wane from 1 month after booster dose to the end of the study 11 months later. It also seems that the pattern of antibody waning in the booster dose responses was similar to that of the primary vaccination response. It was proposed that the waining pattern was due to the presence of the short-lived component of the antibody response, which wanes rapidly, followed by the long-lived component that wanes gradually over time (341), suggesting that the vaccine induces short-lived plasmablast responses with limited durable antibody immunity. The long-lived component, on the other hand, has been associated with GC reaction, MBC and LLPCs, suggesting that the ideal vaccine strategy for both primary and booster doses would be the one that is capable of improving the generation of LLPCs and MBC, and this would be achieved via modifying the immunogen and adjuvant or altering the vaccine regimen (383,384).

When we assessed the interaction between the booster dose vaccine and NAI, we did not find consistent differences in antibody levels between pre- and post-booster doses in most of the blood-stage antigens we tested. However, some changes were seen after the booster, where higher levels of Rh5 IgG and all IgG subclasses were higher in the RTS,S booster group than in the comparator vaccine groups. Unfortunately,

the study was not powered to assess the association with protection. The RH5.1/Matrix-M has recently progressed to phase 2b trial after showing a favorable safety profile and immunogenicity in healthy Tanzanian 5–17-month-old children, and if successful, it could used as a second line of defense to complement partially effective RTS,S, and R21 approved vaccines (385).

Several other unexamined aspects prevent us from making conclusive remarks about the booster doses and the mechanism by which the vaccine mediates protection based on Article 2. Other antibody properties and many antigens must be assessed, as the magnitude of antibody responses alone was shown not to be enough.

Factors affecting the vaccine immunogenicity and vaccine efficacy

Our research has provided valuable insights into the factors affecting vaccine response, but it also highlights the need for further investigation.

Effect of age - It has been previously reported that the RTS,S vaccination induces a substantial increase of anti-CSP and HBsAg IgG responses in children compared to infants, which aids in protecting against clinical malaria (341,347). These lower levels of antibodies in the infants group have mainly been attributed to the interference of anti-CSP maternally transferred antibodies or the immature immune system in neonates and babies.

We have previously demonstrated a negative association between pre-vaccination levels and post-vaccination total IgG and IgG1 responses to anti-NANP CSP, particularly in infants (347). Additionally, because of maternally acquired antibodies, infants tend to have a lower increase in anti-CSP antibodies from pre-vaccination and one month after primary vaccination. Maternally transferred antibodies inhibit active infant immunization, hampering the immune response to the vaccines. The exact mechanisms by which the maternally transferred antibodies affect the outcome of RTS,S vaccine response are unknown. However, different mechanisms have been proposed as responsible for inhibition by the maternal antibody on infant vaccine responses, including 1) epitope masking by maternal antibody, thus impeding antigen presentation to infant B cells and limiting their priming (347,386), 2) inhibition of infant B cell activation by Fcγ-receptor mediated signaling, and (3) Fc-dependent phagocytosis leading to elimination of maternal antibody-coated vaccine antigens (386).

In Article 1, we observed IgG antibody levels reacting to fucosylated or non-fucosylated CSP antigens were not affected by age group, contrary to what was reported previously in the same trial where younger children

(<2 years) were found to have higher levels of anti-CSP IgG (381). In that case, the maternally transferred antibodies do not play a significant role, as the younger children were vaccinated at over 1 year in the phase 2b trial and maternal antibodies are detected till 6-9 months of age. However, we found a lower increase of IgG to fucosylated TSR in older children (2–4 years) compared to younger children (1–2 years) after primary RTS,S vaccination. These findings underscore the complexity of vaccine response and the need for further research to fully understand and optimize vaccine efficacy.

We further aimed to understand how age could affect the booster dose responses following primary vaccination in the phase 3 trial. In article 2, we saw that children have only higher total IgG and IgG1 antibody levels against CSP antigens at the time of the booster than infants. The booster dose was also more immunogenic in children than infants one month after the booster dose, and this tendency continued throughout the whole study follow-up. The results align with the previously reported (341), where anti-CSP antibody titer after primary vaccination significantly predicted anti-CSP antibody titer after the booster dose. It was proposed that since the children received the primary immunization with a relatively more mature immune system, they could mount a relatively more effective memory response that led to effective boosting since no maternal antibodies were circulating in both age groups at the moment of booster dose vaccination.

In article 3, our assessment of how age can affect the acquisition of NAI after primary vaccination revealed intriguing patterns. We observed mixed patterns of antibody levels one month after primary vaccination, with both infants (6-12 weeks) and children (5-17 months) age groups showing an increase in antibody levels in an antigen-dependent manner. However, children tend to show a more pronounced increase in antibody levels one month after vaccination than infants, a finding that sheds light on the age-related development of effective adaptive immunity.

Apart from the level of residual maternal antibodies at the time of immunization, other factors might also interfere with the build-up of adequate vaccine response in babies, including the nature and dose of antigen, the number of vaccine doses, and their underdeveloped immune systems, pre-existing immunity due to primary vaccination or infections. Appropriate immunization strategies using conventional or novel vaccines for early priming are necessary to achieve effective vaccine responses earlier in the presence of maternal, vaccine or infection-related antibodies. This is particularly important for infants due to their still immature immune systems.

The effect of malaria intensity transmission - Our prior study (347) found higher levels of anti-CSP antibodies at pre-vaccination in a high malaria transmission setting than at a low, but found a negligible difference one month after the primary vaccination. However, the baseline difference significantly affected the antibody level change from pre-vaccination to one month after primary immunization. Mixed patterns were reported depending on age group, where infants had higher pre-vaccination IgG levels than children, presumably representing passively transferred maternal antibodies. However, the decayed rate from pre-vaccination to one month post-primary vaccination was generally higher in high malaria intensity transmission (MTI) settings than in low due to higher malaria exposure in the mothers. On the other hand, children showed higher pre-vaccination IgG and IgM levels in high MTI settings than in low MTI settings, representing responses acquired upon infection.

We found no evidence that the MTI affects the binding of vaccine or naturally induced IgG to fucosylated or non-fucosylated CSP antigens when comparing a high MTI transmission setting and a low MTI area on Article 1, suggesting that the binding properties are not affected by the level of malaria exposure.

In article 3, our close examination of antibody levels to individual antigens revealed a complex and diverse effect of MTI on antibody responses. For instance, the antibody levels against the two groups of antigens found to be decreased or unchanged upon RTS,S vaccination were significantly higher in Kintampo (higher MTI) than in Manhiça (lower MTI) upon one month post-vaccination, and most of these antigens behaved as markers of exposure. On the other hand, IgG levels to the third group of antigens found to be increased by vaccination were showed a more significant increase post-vaccination in the lower MTI setting. Our results corroborate the notion that high levels of previous malaria exposure could have a detrimental impact on RTS,S clinical efficay. Multiple mechanisms have been postulated to explain the altered vaccine response in highly exposed children. It was shown that *P. falciparum* could drive the expansion of atypical MBCs (387,388) and phenotypically exhausted $CD4^+$ T cells (357), and loss of $CD4^+$ T cell function may deny normal costimulation to B cells, resulting in poor antibodies induction and impaired memory responses. Additionally, malaria exposure was found to be associated with a poorer induction and maintenance of functional antibody responses to RTS,S (359). Strategies that combat the negative effect of malaria exposure may improve RTS,S vaccine efficacy and longevity. However, recent evidence points towards the opposite direction as it was recently reported an improved VE against first new infections in children who were malaria infected at first vaccination with RTS,S and also with children experiencing greater force of infection (389,390). This improved VE could possibly be a result of the acquisition of NAI afforded by previous malaria

infections. These findings merit further investigation and validation as they could impact current malaria development and implementation strategies.

Prexisting immunity- The presence of antibodies at vaccination with RTS,S due to prior natural exposure or the mother through maternally transferred antibodies is known to impact the induction of vaccine-related immunity and protective efficacy. The impact depends on age and levels of malaria exposure.
After the booster dose in Article 2, no significant differences were detected when comparing antibody levels at booster or post-booster visits in individuals who had either presented or not with clinical malaria before. However, we may not have detected the difference due to the small sample size, and the study also reported a few malaria cases. As explained before, we found that the peak response after the booster dose is lower than at the primary scheduled vaccination and also different patterns in IgG subclasses exist between the booster and the primary vaccinations. It remains unclear if the lower response to the booster dose compared to primary vaccination or the different IgG subclass pattern observed in Article 2 is modulated by pre or post-vaccination exposure.
In the phase 3 trial (Article 3), some covariates affected vaccine-unrelated antigens response one month after RTS,S primary vaccination in three main ways: decreasing antibody levels, not affecting antibody levels, and increasing antibody levels. As mentioned previously, antigen groups where antibody levels decreased or remained unchanged seem to indicate a predominantly maternal origin, as IgG levels for many of those antigens are elevated at baseline, significantly decay from pre-vaccination to one month post-vaccination and were higher in infants than in children. Higher maternally transferred antibodies are also an indication of MTI in the community and are associated with prospective malaria risk.

The interaction between RTS,S and NAI and its relevance in malaria protective immunity

RTS,S was approved to be used in children living in malaria-endemic areas, prioritizing moderate and high transmission areas. In these areas, children are constantly exposed to *P. falciparum* and build NAI. However, how a partially protective vaccine could impact the acquisition of NAI to *P. falciparum* upon subsequent parasite infection remains to be unequivocally defined. It was proposed that the long-term protection against clinical disease afforded by RTS,S was due to the partially protective vaccine-induced pre-erythrocytic response that lasts several months and partially inhibits hepatocyte invasion and the subsequent liver stage

development, allowing the “leakage” of low-dose merozoites emerging from the liver into the bloodstream and stimulating a long-lasting asexual-stage immune response (391). This hypothesis was consistent with notions and observations from other antimalarial interventions, including insecticide-impregnated bed nets or curtains and intermittent preventive therapy leading to low parasitaemias that induce or maintain protective immune responses (392,393). The alternative hypothesis was that RTS,S vaccination would reduce naturally acquired immune responses to blood-stage parasites by reducing high-level exposure to blood-stage parasites in the long term, raising fear of the "rebound malaria" phenomenon, particularly in high-transmission areas (380,394). In the phase 2b (391), designed with two cohorts, cohort 1 (low MTI) to asses the efficacy against clinical malaria and cohort 2 (high MTI) to asses the efficacy against infection, the researchers found that that the RTS,S VE decreased quickly with time in children who received the vaccine in cohort 2, contrary to the observation in cohort 1 where the vaccine conferred protection against clinical malaria for at least 21 months. Due to the protocol design, the cohort 2 children were followed by both passive case detection and active case detection, and with antimalarial treatment, it was hypothesized that this reduced duration of protection was a result of the early diagnosis and treatment of infections, limiting sufficient exposure to asexual-stage antigens.

On the contrary, the long-term protection against clinical disease observed in cohort 1, where children were followed by passive case detection, might have been a consequence of prolonged exposure to low-dose blood-stage asexual parasitemia (391). However, mixed results were reported when using samples from the same trial to decipher the exact mechanism by which this pre-erythrocytic vaccine, targeting the asymptomatic sporozoite and liver stages, protects against disease caused by blood-stage parasites. After screening for antibodies to limited asexual blood stage antigens AMA-1, MSP-$1_{42,}$ EBA-175, DBL-α, and VSA_{R29}, prior work from our group did not find any difference in antibody levels between treatment groups, meaning that RTS,S/AS02 neither enhances nor impairs antibodies naturally acquired against blood stage antigens (380). The “leaky vaccine” hypothesis could not explain the difference in duration of protection between the two cohorts (low and high MTI) (395). Another study using the same phase 2b trial serum samples reported decreased exposure to *P. falciparum* in both liver and blood-stage parasites in RTS,S vaccinated (396). The findings above did not support the "leaky vaccine" hypothesis; however, they were insufficient to reject it due to the limited panel of antigens, sample selection regimens, study design, and epidemiological confounding factors of exposure.

In article 2, we explored the impact of RTS,S booster dose vaccination on naturally acquired immunity by measuring antibodies to vaccine-unrelated *P. falciparum* antigens. In this exploratory analysis, we included only seven *P. falciparum* blood stage antigens (MSP1 [block 2 and $MSP1_{42}$ fragments, 3D7 strain], MSP5, EBA140, EBA175 region 3-5, Rh4.2 and Rh5) that were shown to be affected by vaccination in our previous studies or because they are leading vaccine candidates. After the booster dose, we found higher anti-Rh5 IgG and IgG_{1-4}, suggesting that RTS,S partial protection could increase some blood stage antibody responses after a booster dose.

In Article 3, we evaluated IgM and IgG responses to an expanded panel of 38 pre-erythrocytic and blood-stage antigens presumably involved in NAI using serum samples from the phase 3 clinical trial. As mentioned above, three major patterns of IgG responses to vaccine-unrelated antigens after vaccination with RTS,S emerged. The first group of antigens showed decreasing antibody levels; in this group, we found some antigens such as AMA-1 and $MSP1_{42}$; a similar pattern was previously seen in the phase 2b trial (380). The second group of antigens included antigens whose antibody levels did not change after vaccination, which also accords with the previous finding (395).

In a nutshell, the above two groups of antigens do not support the "leaky vaccine" hypothesis, and antigens in these major groups behave as markers of exposure. However, our data have shown a third group of antigens, including (DBL-α, MSP1 Block2 (3D7, Well, RO33 and Mad20 strains), EBA140 R3-5, EBA175 R3-5, MSP5, MSP6, RH2 2030, RH4.2, RH5, and SSP2 (TRAP), the antibody levels of which were increased in vaccinated children than in comparators. More importantly, levels of antibodies to some of these pre-erythrocytic and blood-stage antigens, mainly (MSP5, MSP1 block 2, RH4.2, EBA140, and SSP2/TRAP) were found to be correlated with protection against clinical malaria in multivariable logistic regression analyses, after 12 months follow-up period. To the best of our knowledge, this was the first study showing that the RTS,S vaccination can favor NAI by increasing levels of IgG against some vaccine-unrelated blood stage antigens.

Interestingly, we found that the RTS,S increased IgG and IgM to pre-erythrocytic and leading vaccine candidate TRAP/SSP2 (397), which levels were associated with protection. It was previously demonstrated that radiation-attenuated sporozoites immunization could induce the acquisition of sterile immunity, independently of the CSP-specific cellular and humoral responses induced by vaccination, suggesting that potential other multiple sporozoites or liver stage antigens are likely involved (398,399). Conflicting evidence exists regarding whether or not the CSP can be combined with TRAP antigens in vaccine formulations. The

co-immunization of RTS,S/AS02 and TRAP/AS02 was detrimental to the protection afforded by RTS,S/AS02 alone (400). On the other hand, active immunizations in mouse challenge using a combination of CSP and TRAP consistently resulted in enhanced sterile protection compared to CSP-alone immunizations (401). Moreover, experiments in mice TRAP-CSP fusion protein antigen were found to be immunogenic and protective in mice (396), suggesting that co-immunization and protein fusion strategies have the potential to enable synergy between epitopes and still hold promise. However, issues possibly related to immune interference should be addressed.

It remains unclear if the RTS,S partial protection could lead to an increase of antibody responses to vaccine-unrelated antigens after primary or booster dose or if the increase in antibodies is due to cross-reactivity, even with no clear sequence similarities, at least at the primary structure. To unravel this complex interplay of vaccine-induced and naturally acquired immunity, our team further investigated the effects between RTS,S vaccination and antibody responses to vaccine-unrelated antigens (402). We used a protein array to measure levels of IgG antibodies to 1000 *P. falciparum* antigens in infants and children from six African sites of phase 3 clinical trial at pre- and post-primary and booster doses vaccination. One month after vaccination, a subset of vaccine-unrelated probed antigens showed differences in IgG responses between vaccine groups, whereas no pre-vaccination differences were found consistent with the results of this thesis. The IgG levels to a subset of these antigens were strongly correlated with CSP IgG levels, with a similar waning pattern over time and re-increasing after booster dose, suggesting that vaccine-induced IgG recognizes other malaria antigens (off-target). Notably, the strong off-target responses were associated with greater vaccine protection against clinical malaria. The structure-function relationships and whether the induced IgG could functionally limit malaria infection and disease still need to be elucidated.

Contribution of the current thesis

Firstly, this PhD thesis highlights the pressing need to characterize and deeply understand the interaction between glycan-containing antigens and the immune system and the implications of these interactions in vaccine development since glycosylation significantly alters recognition epitopes. Few studies have rigorously examined the antibody response to fucosylated constructs and their correlation with protection. Recently, the WHO recommended the R21/Matrix-M vaccine for malaria prevention, the second malaria vaccine after the RTS,S vaccine. To date, no evidence shows that one vaccine performs better than the other since the two WHO-recommended vaccines have not been tested in a head-to-head trial (403). Both vaccines

include HBsAg fused with the glycoprotein CSP, which self-assembles into VLPs in yeast (404). An important area for further research and enhancement of VE could be the incorporation of glycans into current vaccine antigens. This is particularly significant because CSP is fucosylated in *P. falciparum* but not in yeast, where CSP from both vaccines is recombinantly expressed.

Secondly, we have elucidated the RTS,S booster dose responses, which differ from primary dose vaccinations, particularly the differing IgG subclasses not previously studied.

Finally, our study has shown that the RTS,S/AS01 vaccine significantly increases antibody responses against some pre-erythrocytic and blood-stage antigens, namely (MSP5, MSP1 block 2, RH4.2, EBA140, and SSP2/TRAP) after the primary vaccination schedule. These levels were correlated with protection against malaria, a promising discovery that instills hope for developing more effective vaccines. Moreover, anti-RH5 antibody levels increased upon natural infections after a booster dose, further bolstering our optimism. The results have paved the way for a new line of research in our group, leading to an additional published article exploring the vaccine off-target IgG responses and their correlation with protection, which confirmed the results of this thesis. We hypothesize that the off-target response could be caused by cross-reactivity, a potential avenue for further research. These antigens are attractive vaccine candidates, and their inclusion in next-generation multivalent or multistage vaccines has the potential to generate more efficacious malaria vaccines. This could be achieved through co-immunization with a CSP-based vaccine or vaccine formulations where CSP is fused to these antigens, or they can be used as booster vaccines to primary CSP-based primary vaccination.

Limitations and Future Perspectives

While this PhD thesis shows biologically plausible findings, it also has some shortcomings that might affect the generalizability of our results, which should be addressed in follow-up studies. The main limitation is related to the study design. Firstly, the studies had a limited sample size (Articles 2 and 3), affecting the ability to draw important associations. Secondly, the studies were limited to Manhiça-Mozambique (Articles 1 and 2) and Manhiça-Mozambique and Kintampo-Ghana (Article 3), possibly not reflecting the spectrum of malaria intensity transmission settings. Finally, the imbalance between age groups, malaria and non-malaria cases, might also have impacted our results in article 3. About 11 research centers in seven sub-Saharan African countries participated in the phase 3 efficacy and safety trial in areas with different malaria transmission intensities and seasonalities. At least 7 of these centers participated in the MAL067 immunology ancillary

study that was part of the main clinical trial, namely Manhiça-Mozambique; Nanoro-Burkina Faso, Siaya-Kenya, Bagamoyo- Tanzania; Kombewa-Kenya; Kintampo-Ghana and Lambaréne-Gabon. That being stated, the primary focus of the follow-up studies should be to perform more balanced longitudinal studies with larger sample sizes, balanced groups, and multiple sites representing diverse malaria intensity transmission settings and seasonalities. Moreover, a more detailed characterization of the type (subclass), magnitude, breadth, functions, and quality (avidity) of antibody responses against multiple RTS,S/AS01$_E$ vaccine-related, and vaccine-unrelated antigens is needed in children at baseline and study months 3, 20, 21 and 32 in a phase 3 trial.

WHO's currently recommended CSP-based vaccines offer only marginal protection, underscoring the urgency for innovative vaccine design and development. Understanding the lack of durable protection in endemic areas is crucial. The most promising path to accelerating future vaccine development lies in a deeper understanding of the host-pathogen interaction and the nature of naturally acquired immunity, including its immune-evasion mechanisms. These studies will provide the information to guide the design of future malaria vaccines and other malaria control tools.

A comprehensive characterization of *P. falciparum* glycoprotein structures, function, and immunological properties is crucial and may help to make educated decisions on vaccine design. Although we do not have evidence that a glycosylated CSP-based vaccine could perform better than current CSP-based formulations, head-to-head clinical trials involving glycosylated and non-glycosylated vaccines would be ideal for properly controlling variables affecting vaccine immunogenicity and protective efficacy. Moreover, it will help to understand the strength of immune responses they trigger and how well each vaccine protects against infection and disease. Furthermore, to obtain a bona fide *Plasmodium* glycosylation, an alternative expression system might be considered for malaria vaccine design.

It is crucial to understand the interaction between vaccine responses induced by RTS,S and NAI to malaria and its relevance for the duration of protective immunity, as we have shown that the RTS,S-induced protective mechanism reaches beyond the CSP antibody response. Our recent findings on off-target antibody reactivity occurring after primary and booster dose vaccination with similar anti-CSP antibody kinetics also open new doors for research, as we postulate that the antibodies to strongly reacting off-target antigens could cross-react anti-CSP repeat region antibodies, possibly due short identical sequences, and merit further investigations.

Moreover, epitopes from various *P. falciparum* proteins encompassing several parasite life stages or several different alleles of a single antigen into a single platform could appropriately address the significant antigenic diversity of target epitopes and the capacity of the *P. falciparum* for immune invasion and eventually enhance the scope of immunity. Although combining multiple protein-in adjuvants formulations has proven challenging (397), this may be more easily achieved by using mRNA-based vaccines, as they can code multiple antigens (405).

Since the growing evidence base has shown the multiple ways in which antibodies function to protect against malaria infection and disease or reduce transmission, future research should also be focused on quantifying multiple functional activities and other properties of antibodies to understand better their role in mediating immunity and how these functional activities can be harnessed to maximize malaria vaccine efficacy. Multi-functional antibody assays should include but not be limited to those that quantify direct antibody inhibition, complement fixation and activation, and assays that quantify Fcγ-receptor mediated functional activity. In addition, the integrated analysis of cellular populations and cytokine secretion and antibody data may provide insights into the lack of protection afforded by RTS,S.

Finally, accounting for other biasing factors that can significantly impact the immune response to vaccines, such as prior malaria episodes, maternally-transferred IgGs, baseline immune status, nutritional status, HIV, sickle cell disease, other co-infections, and the composition of the gut microbiota will be useful in generating new hypotheses, as these factors may potentially lead to impaired quality and duration of protective immune response.

The potential improvement of the vaccine could be achieved by various means, including the development of novel adjuvants, rational antigen selection, rational immunogen design, improved vaccine schedules, improving the induction and maintenance of antibodies that promote phagocytosis and cellular functions, and combating the possible negative effect of malaria exposure on vaccine responses.

Conclusions

1. The post-translational modifications by O-fucosylation in the circumsporozoite protein present in *Plasmodium falciparum* do not affect the antigen binding of IgG antibodies induced by non-fucosylated circumsporozoite protein included in the RTS,S vaccine. In addition, IgG generated by naturally acquired immunity, presumably to fucosylated CSP on sporozoites, recognizes equally non-fucosylated antigen epitopes. Nevertheless, we cannot rule out any potential benefit of an alternative fucosylated RTS,S vaccine.

2. The RTS,S/AS01$_E$ booster dose induces responses that differ from the primary vaccine immune response. Although the booster increased IgG, IgG1 and IgG3 antibody levels for all vaccine antigen constructs, which waned substantially over time, the peak was lower than the primary vaccination peak response. Moreover, the booster dose did not increase the levels of IgG2, as seen after the primary vaccination, highlighting the temporal changes in antibody subclasses after a booster dose.

3. RTS,S vaccination alters naturally acquired immunity to malaria, decreasing IgG levels against immunogenic malaria antigens, which are markers of malaria exposure, while increasing IgG levels against malaria antigens not present in the vaccine. The increase of IgG levels by RTS,S vaccination is associated with increased protection and may be a new correlate of RTS,S-induced protection.

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