Safety, immunogenicity and duration of protection of a candidate malaria vaccine in Mozambique

Seguridad, inmunogenicidad y duración de protección del candidato a vacuna contra la malaria en Mozambique

Pedro Carlos Paulino Aide

TESIS DOCTORAL

Safety, immunogenicity and duration of protection of a candidate malaria vaccine in Mozambique

Seguridad, inmunogenicidad y duración de protección del candidato a vacuna contra la malaria en Mozambique

Pedro Carlos Paulino Aide

CRESIB BARCELONA

cism centro de investigação em saúde de manhiça

PROGRAMA DE DOCTORADO DE MEDICINA

FACULTAD DE MEDICINA

Seguridad, inmunogenicidad y duración de protección del candidato a vacuna contra la malaria en Mozambique

Safety, immunogenicity and duration of protection of a candidate malaria vaccine in Mozambique

Tesis presentada por **Pedro Carlos Paulino Aide**

para optar al grado de Doctor en Medicina

Director de tesis: Professor Dr. **Pedro L. Alonso Fernández**

Línea de investigación: Agresión biológica y mecanismos de la respuesta

Centre de Recerca en Salut Internacional de Barcelona (CRESIB)

Junio 2011

To

My wife

My parents

My brothers & sisters

ACKNOWLEDGMENTS

At the beginning of an end I owe many thanks to so many people…forgive me those who I might have forgotten to mention.

Special thanks to my mentor, boss and thesis director, Dr. Pedro Alonso, for the continuous support and guidance since the very early stages of my (short) research career. There are not enough words to express my gratitude. Sharing the same first name with you got me the sense of being 'respected' and easily recognized but, in some circumstances, I had to let clear that I was the 'other' Pedro as being *you* could potentially put me in troubles.

Another big thank goes to my colleagues and friends Jahit Sacarlal and Diana Quelhas with whom I have shared all the good and some of *those not so good* moments during the trials. Together we have learnt how strong and committed we need to be when the storm is coming… just to be always ready!

I am also very grateful to all the malaria vaccine team at CISM, especially Tonecas, Fanuel, Sofia, Sidónio, Esselina, Mr. Filipe and Mr. Lumbelane, and all other fieldworkers and supervisors. Thanks also to all personal who supported us during all these years, among others, C. Sacoor, I. Mandomando, E. Macete, B. Sigaúque, J.Chilengue, S. Noguera, D. Nhalungo, A. Langa, A. Nhabanga, L. Morais, S. Machevo and K. Munguambe.

To all staff at CRESIB, thanks a lot for letting me to be one of you. A special recognition goes to John Aponte, Montse Renom, Caterina Guinovart and Quique Bassat. Your advice and support has been greatly appreciated.

Great thanks to *all my day* friends Tacilta and Nélia… Barcelona would not be such a nice place without you around. The same goes to Vanda, Tufária, Sónia, Paulo, Tytas, Inácio, Cláudia and Dércio. With Pep Figueras and Marc I have enjoyed some of the best football games at Camp Nou. I felt at home with you guys!

Assante sana to my mother (Marta), my father (God have you), my brothers and sisters (Bony, Tina, Belinha, Titos, George, Judy & Iony).

Finally I would like to thank my dear wife Ercília, source of my inspiration… thanks for being so supportive and patient during all these years!

My stay in Barcelona was supported by a scholarship from the *Agencia Española de Cooperación Internacional para el Desarrollo* (AECID). Gracias!

PRESENTATION

The current doctoral thesis is presented following the University of Barcelona recommendations on presentation of doctoral thesis by compendium of publications.

The thesis is presented as a collection of four articles published in peer-reviewed international journals on work conducted at the *Centro de Investigação em Saúde da Manhiça* (CISM) in Mozambique, and at the Barcelona Centre for International Health Research (CRESIB), in Spain.

The first manuscript is an introductory review article on the challenges towards the development on an effective malaria vaccine. The other three articles provide original data from the RTS,S/AS candidate malaria vaccine clinical trials conducted in Mozambican children and infants.

LIST OF ARTICLES

This thesis is based on the following articles:

I. **Aide P,** Bassat Q, Alonso PL.; Towards an effective malaria vaccine**. Arch. Dis. Child. 2007**;92;476-479.; doi:10.1136/adc.2005.092551, PMID: 17515617; Impact Factor: 2.834

II. **Aide P,** Aponte JJ, Renom M, Nhampossa T, Sacarlal J, et al. (2010) Safety, immunogenicity and duration of protection of the RTS,S/AS02$_D$ malaria vaccine: one year follow-up of a randomized controlled phase I/IIb trial. **PLoS ONE 5(11): e13838**. doi:10.1371/journal.pone.0013838, PMID: 21079803; Impact Factor: 4.351

III. Barbosa A, Naniche D, Aponte JJ, Manaca M, Mandomando I, **Aide P**, e al. (2009) *Plasmodium falciparum*-specific cellular immune responses after immunization with the RTS,S/AS02$_D$ candidate malaria vaccine in infants living in an area of high endemicity in Mozambique. **Infection and Immunity 77(10);** 4502–4509.; doi:10.1128/IAI.00442-09, PMID: 19651872; Impact Factor: 3.933

IV. **Aide P**, Dobaño C, Sacarlal J, Aponte JJ, et al. Four year immunogenicity of the RTS,S/AS02$_A$ malaria vaccine in Mozambican children during a phase IIb trial. **Vaccine** (2011), doi:10.1016/j.vaccine.2011.03.041, PMID: 21443960; Impact Factor: 3.616

LIST OF ACRONYMS

ACT	Artemisin Based Therapy
ADI	Active Detection of Infection
Anti-CS	Antibody to the *P. falciparum* circumsporozoite (CS) repeat protein
Anti-HBsAg	Antibody to the hepatitis B surface antigen
AMA	Apical Membrane Antigen
AS	Adjuvant System
ATP	According to Protocol
CISM	Centro de Investigação em Saúde de Manhiça
CMI	Cell Mediated Immunity
CRESIB	Centre de Recerca en Salud Internacional de Barcelona
CSP	Circumsporozoite Protein
DDT	Dichlorodiphenyltrichoroethane
DSMB	Data and Safety Monitoring Board
DTPwHib	Diphtheria, Tetanus, whole-Pertussis and Haemophilus b vaccine
EBA-175	Erythrocyte-binding antigen 175
ELISA	Enzyme Linked Immunosorbent Assay
EPI	Expanded Programme on Immunisation
GMT	Geometric Mean Titer
G6DP	Glucose-6-phosphate dehydrogenase
GLURP	Glutamate-rich Protein
GSK	GlaxoSmithKline
HBsAg	Hepatitis B surface antigen
ICS	Intracellular cytokine staining
IFAT	Indirect fluorescent antibody test
IFN-γ	Interferon gamma
IL-2	Interleukin 2

IL-4	Interleukin 4
IPTi	Intemitent Preventive Treatment in infants
IPTp	Intemitent Preventive Treatment in pregnant women
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
ITT	Intention to Treat
LLITN	Long Lasting Insecticide Treated Net
MPL	Monophosphoryl lipid
MSP	Merozoite Surface Protein
MVI	Malaria Vaccine Initiative
NAI	Naturally Acquired Immunity
NANP	Tetrapeptide repeat motif
NMCP	National Malaria Control Program
OPD	Outpatient Department
PATH	Programme for Appropriate Technology in Health
PCD	Passive Case Detection of Infection
QS21	*Quilaja saponaria* 21: a triterpene glycoside purified from the bark of Quillaja saponaria
RTS,S	Particulate antigen, containing both RTS and S (hepatitis B surface antigen) proteins
SI	Stimulation index
SERA	Serine repeated antigen
VE	Vaccine Efficacy
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research

Table of contents

1. SUMMARY

1.1. Resumen (Castellano)

La malaria, causada por el parásito *Plasmodium falciparum* sigue siendo un gran problema de salud pública y una causa importante de mortalidad y morbilidad en el África Sub-Sahariana, especialmente entre los niños y lactantes. El parásito y su vector mosquito *Anofeles spp.* tienen una tremenda capacidad de adaptación, incluyendo la capacidad de adquirir resistencia a los fármacos antipalúdicos e insecticidas. Es por tanto prioritario desarrollar nuevas herramientas preventivas, entre las cuales una vacuna segura y eficaz, un elemento clave para contrarrestar esta tendencia.

La vacuna candidata contra la malaria más avanzada, denominada RTS,S/AS, ha progresado hasta un ensayo de Fase III siguiendo un plan de investigación y desarrollo clínico resultado de una colaboración sin precedentes entre centros de investigación Africanos, Europeos y Americanos junto con la GSK Biologicals y PATH Malaria Vaccine Initiative (MVI).

Esta tesis describe algunas de las fases críticas de estos logros, reportando los ensayos clínicos de la vacuna candidata contra la malaria RTS,S/AS llevados a cabo en niños y lactantes de Mozambique.

Esta tesis analiza en detalle la evaluación de la seguridad de esta estrategia, la respuesta inmunológica (tanto humoral como celular) que esta vacuna confiere, y la duración de la protección durante un período de seguimiento de un año en los lactantes. Presentamos también los datos completos de 4 años de seguimiento de la inmunogenicidad de la RTS,S/AS02$_A$ administrada a niños de 1 a 4 años de edad en el momento de su primera vacunación.

Estos estudios demuestran que la vacuna es segura, bien tolerada y altamente inmunogénica, produciendo respuestas tanto humorales como celulares. La vacuna también protege a los niños y lactantes contra la malaria clínica. Además se describe por primera vez una asociación entre el riesgo de malaria clínica y los niveles de anticuerpos contra la proteína de circumsporozoito del *Plasmodium falciparum*.

Los resultados aquí presentados apoyan la tesis de que el desarrollo de una vacuna eficaz, inmunogénica y segura contra la malaria es posible, y deberá ser la base, junto con otros estudios, del proceso de registro de la que podrá ser la primera generación de vacunas contra la malaria.

1.2. Summary (English)

Malaria, caused by *Plasmodium falciparum* parasites remains a huge public health problem and a major cause of morbidity and mortality in sub-Saharan Africa, especially among children and infants. The parasite and its vector – the *Anopheles spp* mosquito - have tremendous adaptability capacities, including the acquisition of resistance to anti-malarial drugs and insecticides, making the development of new preventive tools, such as a safe and effective vaccine, a key element to counter balance this tendency.

The most advanced malaria vaccine candidate, RTS,S/AS has progressed to a Phase III trial through a research and development plan as a result of an unforeseen partnership between African, European and American research institutions together with GSK Biologicals and the PATH Malaria Vaccine Initiative (MVI).

This thesis describes some critical stages of the clinical development plan of this vaccine, reporting clinical trials of the RTS/AS candidate malaria vaccine conducted in Mozambican children and infants. Here we illustrates the assessment of the RTS,S/AS02$_D$ safety, humoral and cellular mediated immune responses and the duration of protection over a one year period in infants. We also provide a detailed immunogenicity data of 4 years of follow-up of children aged 1 to 4 years by the time of immunization with RTS,S/AS02$_A$.

These studies principally show that the vaccine is safe, well tolerated and highly immunogenic, eliciting both humoral and cell-mediated antibodies. The vaccine also protects children and infants against clinical malaria. Importantly, they also describe for the first time an association between the risk of clinical malaria and *Plasmodium falciparum* anti-circumsporozoite antibody titters.

The presented results support the hypothesis that developing a safe, immunogenic and efficacious malaria vaccine is feasible and, together with other studies, they should be the basis for the registry process of what should be the first generation of vaccines against malaria.

2. INTRODUCTION

2.1. Brief history of malaria

The name **malaria** – from ***mala*** and ***aria*** meaning bad air - was first used by Italians in 1740 associated to the cause of intermittent fevers (also known as jungle fever, marsh fever, paludal fever, or swamp fever) after exposure to marsh air or miasma.

Early references from malaria can be traced back to 2700 BC in China, Mesopotamia (2000 BC), Egyptian papyri (1570 BC), Ancient Greece (500 BC) and Hindu texts (VI century BC). Hippocrates (400 BC) distinguished the intermittent nature of malaria fever from the continuous fever associated to other infectious diseases affecting humans (1). Descriptions of severe periodic tertian and quartan fevers and spleen enlargements can be found in some of these historical documents (2).

The evidence that the causal agent of malaria were parasites was first presented in 1880 by the French army doctor Alphonse Laveran (1845 - 1922), who described microorganisms in the blood of patients with malaria. While examining blood slides from a group of malaria patients (using a crude light microscope), Laveran recognized four distinct forms of the malaria parasite in different stages of its life cycle (later proved to correspond to both male and female gametocytes, schizont and trophozoites). In 1907, Laveran received the Nobel Prize for such discovery.

In 1897, Ronald Ross described for the first time the malaria parasite in wild-caught mosquitoes. He identified sporozoites in the salivary glands of mosquitoes that had been previously fed on malaria infected birds (2, 3). Subsequently, he infected 21 of 28 fresh sparrows with these mosquitoes further demonstrating the causal relationship (4). In 1902 he was awarded the Nobel Prize for this discovery. However, Ross was not the only researcher who confirmed the malaria life cycle in the mosquito. Between 1898 and 1900, the causal agent of human malaria and its vector was described in parallel by a group of Italian scientists (Grassi, Bignami, Bastianelli, Celli, Golgi and Marchiafava), who contributed not without controversy and animated scientific debate to the detailed description of the parasite life cycle and its mode of transmission.

2.2. The first attempt of malaria eradication

A few decades later, the scientific community embarked in an ambitious plan to eradicate malaria. Feeling that the complexity of the malaria parasite's life cycle had been untangled, and in possession of effective antimalarial therapeutic and preventive arsenal, the WHO launched in 1955 the Global Malaria Eradication Programme. The main premise was the combination of massive deployment of vector control strategies using the powerful insecticide dichlorodiphenyl-trichloroethane (DDT) and the use of chloroquine (then extremely effective and cheap) for the treatment of clinical cases. DDT was available since 1874, but its insecticidal proprieties were only discovered in 1939 by Paul Muller (5), and chloroquine was a by-

product of the second world war, developed to protect soldiers fighting in endemic areas.

By 1978, 37 out of 143 malaria endemic countries in 1950 were freed from malaria, with only 10 of these countries located outside Europe and the Americas (6). Success of the activities in Africa, the continent most severely affected by the disease, was limited, and for this reason WHO's policy was switched in that same year from eradication and elimination to malaria control (focusing on prevention and treatment).

The great success obtained by the eradication programme in the temperate climate areas of Europe, some countries in the Americas, northern of Africa and Middle East (where malaria could be eradicated) was overcome by a failure in most of sub-Saharan Africa. One of the biggest obstacles was the widespread distribution of *Anopheles gambiae*, a long-lived and aggressive malaria vector, associated with several logistic, administrative, financial, political, infrastructural and social challenges. With the exception of a few pilot programs, no sustained malaria control efforts were ever set up in sub-Saharan Africa (7).

By the end of 1996, campaigns using DDT, elimination of mosquito breeding sites and mass treatment had freed more than 500 million people from the threat of disease (8), but significant endemic areas remained scattered around the globe, highlighting the terrible burden that this disease caused.

2.3. Worldwide burden and distribution of malaria

Malaria is the most important parasitic infection affecting humans and remains endemic in 106 countries (Figure 1). Sub-Saharan Africa accounts for a significant piece of the malaria burden and the vast majority of malaria deaths (9, 10).

Between 2005 and 2009, global estimates of malaria cases decreased by 19 million (from 244 to 225 million annual cases respectively). The total number of deaths also decreased from 985.000 to 781.000 during the same period and Africa accounted for the largest absolute decline of cases and fatalities (11).

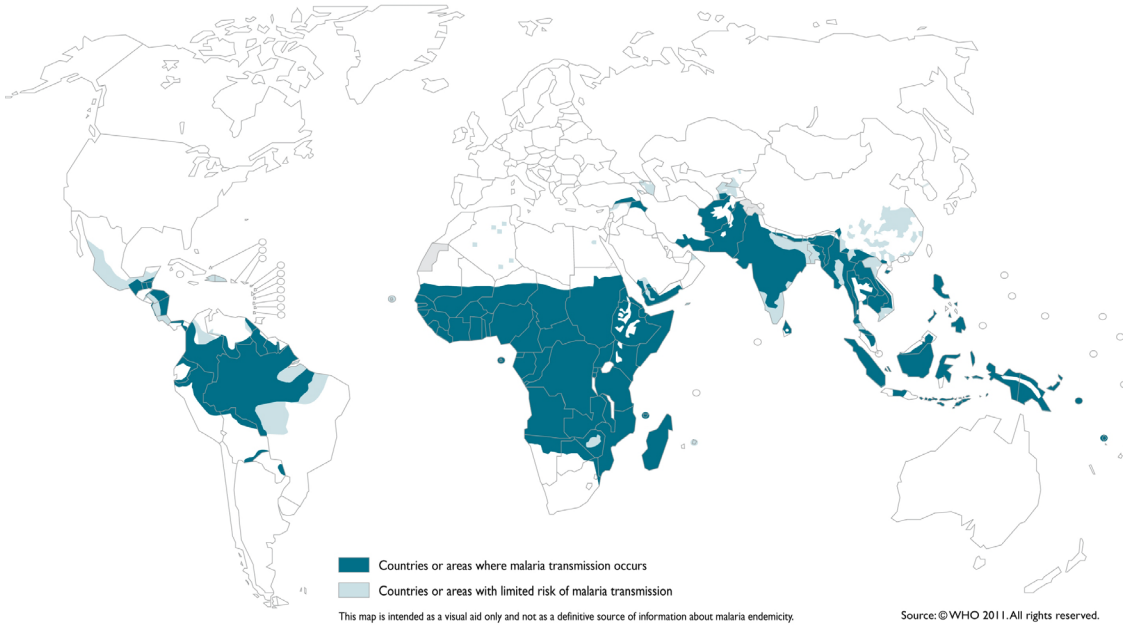


Fig. 1: Distribution of malaria endemic countries (Source: WHO World Malaria Report 2010).

The malaria burden estimation, especially in Africa, relies primarily on mortality and morbidity data collected by the health information systems. In most endemic affected areas, this information are based on history of reported febrile illness and clinical signs, thus over or underestimating the true burden of the disease. Additionally, the health system network in areas where malaria is endemic is usually fragile and scarce, further hindering the management of this disease.

In recent years, increased interest and funding has substantially supported the scale-up of malaria control interventions such us the use of long lasting insecticide treated bed-nets (LLITNs), campaigns of indoor-residual spraying (IRS) and the adoption of effective artemisinin-based combination therapy (ACT) as first drug of choice for malaria treatment that are leading to a reduction in malaria morbidity and mortality in certain areas (11).

The WHO reports that eleven countries in Africa (Algeria, Cape Verde, Botswana, Madagascar, Namibia, Sao Tome and Principe, South Africa, Swaziland, Eritrea, Rwanda and Zambia) experienced around 50% reduction in malaria cases or malaria admissions and deaths although Rwanda, Sao Tome and Principe, and Zambia noticed malaria resurgence in 2009 (after a previous decreasing tendency) (11).

Malaria is not uniformly distributed. Various factors account for the larger variation of the malaria transmission intensity including, among others, the distribution and behavior of the human host, the vector and parasite

species, the geophysical and climatic features, the environment and the socio-economic status (12).

A current categorization of malaria endemicity divides it into three types (Table 1) (13). Stable endemic malaria refers to the continuous exposure of a population to a fairly constant rate of malaria inoculation. On the other hand, in an unstable endemic malaria situation, a population is subjected to more or less permanent malaria transmission but under circumstances in which there are large fluctuations in the rates at which malarial inoculations are delivered to individuals within the population. Epidemic malaria occurs when a population, or even a small group of individuals, is exposed to an increase in malaria transmission rates above that previously or normally experienced. If *P. falciparum* is implicated, malaria epidemics can be accompanied by significant fatality rates.

Table 1: Characteristics of the malaria transmission (13)

Type of Malaria	Distribution	Inoculation rates	Protective immunity	Transmission characteristics
Stable	Sub-Saharan Africa	Regular, low to very high	High in older age groups; low in children <5 years	Perennial or seasonal; regular contact between vectors and human hosts
Unstable	Mediterranean, Asia and Western Pacific, Central and South America and Caribbean	Irregular, low to medium	Unreliable in older age groups; absent in children < 5 year old	Perennial or seasonal; irregular contact between vectors and human hosts
Endemic	Highlands areas of tropical Africa; Central Asia and Caucuses; Asia and Latin America	Rising suddenly, low to medium	Low or absent in all age groups	Very variable, subject to sudden and rapid change

Recently, a new malaria eradication strategy has been developed based in three main premises (14):

- Aggressive control in highly endemic countries, to achieve low transmission and mortality in countries with the highest disease and mortality burdens.
- Progressive elimination of malaria from the endemic margins, to shrink the malaria map.
- Research into vaccines and improved drugs, diagnostics, insecticides and other interventions, and into delivery methods that reach all at risk populations.

Applied to malaria, eradication is defined as the permanent reduction to zero of the worldwide incidence of malaria as a result of deliberate efforts while elimination refers to the cessation of malaria transmission (no incidence of locally contracted cases) in a defined geographic area or an entire country. The WHO defines malaria control as the reduction of the disease burden to a level at which it is no longer a public health problem (6). By 2010, 109 countries were categorized as malaria free, 67 controlling endemic malaria and 32 as malaria-eliminating countries (Figure 2) (14).

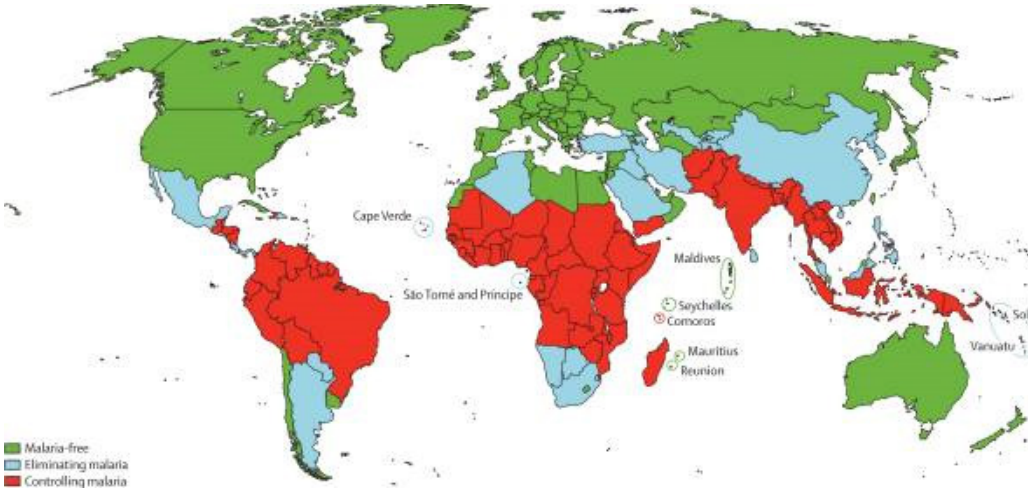


Figure 2: Categorization of countries as malaria free, eliminating or controlling malaria, 2010 (Source: Feachem et al, Lancet 2010)

There is no doubt that *P. falciparum* still remains a challenging enemy to defeat and that is fighting back to survive, either using immune system evasion mechanisms or acquiring resistance to anti-malarial drugs, now including the artemisinin derivates as seen in Asia (15). Additionally, the development of insecticide resistance from the mosquito vector further complicates control efforts (16). Albeit the development of an effective malaria vaccine has been hampered by the parasite's extreme complexity and poorly understood immune mechanisms, there is an overall consensus that, together with other preventive measures, it will constitute a key tool for the control, elimination, or even eradication of the disease (17, 18).

2.4. Malaria in Mozambique

Mozambique is situated in a region which presents favorable conditions for malaria occurrence. The climatic and environmental conditions (high temperature and humidity, precipitation pattern, abundance of natural breeding sites), geography (low altitude, several rivers and lakes), type of predominant vector species (*Anopheles gambiae*), population distribution (majority of population live in high-risk areas), poor socio-economic status conditions and limited access to health services are in favor of the high persistence of the disease.

Malaria is characterized to be endemic throughout Mozambique, varying between mesoendemic and hyperendemic areas. The transmission is constant, with highest peaks during and following the rainy seasons (January to April). Ninety percent of malaria prevalence is attributable to *Plasmodium falciparum,* while only 9.1 and 0.9% of all infections are caused respectively by *P. malariae* and *P. ovale* (19).

Malaria is also responsible for almost 44% of the total outpatient visits, 57% of admissions in the pediatric population and about one third of all inpatient deaths (20). Most of the deaths are related to severe malaria cases that often presents as cerebral malaria, respiratory distress or as malaria associated with severe anemia which invariably requires a blood transfusion to save the patient, especially in children under 5 years.

Malaria during pregnancy is also a major public health issue in Mozambique. According to the malaria national control program,

approximately 34% of pregnant women are infected with *P. falciparum,* with the highest parasite prevalence linked to primigravidae (21).

The association of maternal anemia and malaria infection is one of the most common co-morbidities presented at the health facilities (22). At least 68% of pregnant women have hematocrit levels below 33% (21), situation that also contributes to the elevated maternal mortality rates observed in rural areas (400 per 100,000 births).

The Manhiça Health Research Center (CISM) established in 1996 in Manhiça district, southern Mozambique runs a continuous demographic and morbidity surveillance system. Using a reputable platform for epidemiological and intervention studies, this center helps in filling the gap in accuracy of national health statistics and contributes with evidence-based policy changes to the entire country and internationally.

An initial study to establish the malariometric indexes in the Manhiça region was done from 1996 to 1999 as part of a larger epidemiological study in Mozambique (20). About 2057 children bellow 10 years of age were enrolled in the study. The authors estimated a 90% prevalence of *Plasmodium falciparum* among all malaria infections. The prevalence of asexual *P. falciparum* ranged from 13.7-21.7% at the end of the dry season to 30.5-34.0% at the end of rainy season. A 36% crude malaria attributable fraction (MAF) of fever was depicted from a separate case-control study using 1021 hospital based cases of fever and their corresponding

community non-febrile age-matched controls. To estimate the incidence of clinical malaria episodes, weekly home-based active case detection was done in two separate cohorts. Every child had between 0 to 6 clinical malaria episodes. Importantly, no episodes occurred in very young children (newborns and children less than 2 months) and in up to 71% of the children evaluated, proving that malaria clusters significantly in certain population groups. The highest incidence ranging from 0.65 to 0.74 episodes per 100 person-weeks at risk was found in children aged 6 to 48 months, highlighting that infants and young children were at highest risk. Saúte and colleagues also noted significant differences in the spatial incidence of malaria episodes in regions just a few miles apart with a higher incidence in children living near the river or in swampy areas.

Malaria has been estimated as the leading cause of death in the district, according to a study based on verbal autopsies reports (23).

Treatment and prevention

As in most of African countries, the WHO global malaria eradication program previously described failed to succeed in Mozambique. In the early 70's the attention of the program was changed to treatment rather than prevention. In 1982, the newly created Mozambican national malaria control program adopted a strategy focused on the prompt malaria diagnosis and treatment, vector control and health promotion. A number of reasons, including an inadequate coverage of the national health system

(almost absent from rural areas), insufficient infrastructures, the civil conflict, the concentration of residual spraying campaigns only in the major urban areas, and the advent of resistance to chloroquine rendered this strategy to be ineffective for the adequate control of malaria.

Two trials done in Manhiça supported the malaria first line treatment policy changes in Mozambique. These studies demonstrated a 69% parasitological resistance of *P. falciparum* to chloroquine and 100% clinical efficacy of three different drug combinations, namely amodiaquine plus sulphadoxine-pyrimethamine (AQ+SP), amodiaquine plus artesunate (AQ+AS) and sulfadoxine-pyrimethamine plus artesunate (SP+AS) (24). In line with these results, the Mozambican national malaria control program changed the first line treatment for uncomplicated malaria from chloroquine to the combination AQ+SP in 2002. Following WHO recommendations to use artemisinin-based combination therapy (ACTs) to treat malaria in *P. falciparum* endemic areas (25), the first line treatment was then successively changed to AS+SP in 2004 and finally arthemether plus lumefantrine (AL) in 2009. Quinine is the drug of choice for the treatment of severe malaria or for cases where other treatment alternatives are not indicated or unavailable (26).

Other preventive measures currently used in Mozambique include the free distribution of ITNs (preferably LLITNs), indoor residual spraying (IRS) campaigns and the intermittent preventive treatment in pregnant women (IPTp).

3. Malaria: the parasites, life-cycle and vectors

3.1. The *Plasmodium* species and distribution

Malaria transmission is granted through the bites of an infected female *Anopheles* mosquito. There are around 400 *Anopheles* species in the world but only 30 are of major importance. Five species of *Plasmodium* can infect humans under natural conditions: *P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi.* Each of those species has particularities in terms of their morphology, immunology, geographical distribution, clinical patterns and drug susceptibility. *Plasmodium falciparum* is responsible of the most severe forms of the malaria and its associated death toll, especially in Sub-Saharan Africa. *P. ovale* is confined to West Africa and is the rarest parasite. *P. malariae* can be found worldwide with relatively low frequency while *P. vivax*, the most widespread malaria specie, rarely causes fatal outcomes, but is now known to be less benign than previously considered. *P. ovale* and *P. vivax* life cycles have some particularities. Regardless of producing normal hepatic schizonts, these species can develop some forms of dormant liver stage known as hypnozoites. These dormant forms can remain inactive for long periods (weeks, months and sometimes years) before being re-activated and causing malaria relapses. *Plasmodium falciparum* and *P. malariae* do not produce hypnozoites, thus not being able to relapse. Thus, the recurrence of malaria in patients infected by these

species reflects the proliferation of surviving blood-stage parasites from an earlier infection (malaria recrudescence), or just a new infection.

Recently, *P. knowlesi*, a malaria parasite usually affecting monkeys and easily misdiagnosed as *P. malariae*, has been implicated in severe or even life threatening forms of human malaria particularly on the island of Borneo (27-29).

Although rare, malaria transmission not always involves mosquitoes. Some infections can be acquired following transfusion of malaria parasitized blood products, exposure to tissues with contaminated erythrocytes, organ transplantation or through the placental barrier.

3.2. Life cycle

The malaria parasite infects both human and mosquitoes spending its lifecycle partly in the mosquito and partly in the human host (Figure 3).

When an infected female *Anopheles* mosquito takes a blood meal on human, sporozoites contained in their salivary gland are injected into the skin reaching the blood stream. New evidences indicate that a proportion of sporozoites are captured by the lymph nodes and degraded (30). In less than an hour they reach the hepatocytes (liver cells) probably intermediated by Kupfer cells. Inside the hepatocytes, the sporozoites replicate and develop into schizonts, at this stage called pre-erytrocytic schizonts, which contain thousands of merozoites. When the liver cell ruptures, the

merozoites are released into the bloodstream (starting the asexual erytrocytic stage). Every merozoite try to invade an erythrocyte and multiply inside them forming what is known as erytrocytic schizonts. After 48 hour-long waves for all plasmodia species (with the exception of *P. malariae* which lasts 72 hours), these schizonts burst, killing the erythrocyte, and releasing new merozoites to the blood stream, a phenomenon that perpetuates the erythrocytic stage of the cycle. The release of the parasites from blood schizonts triggers the classical malarial paroxysm, i.e. the occurrence of fever, sweats and chills. The periodicity of these intermittent fevers is determined by the duration of these waves. The 48–hour paroxysms caused by *P. falciparum* are less predictable, especially in non-immune patients, because of the occurrence of non-synchronized waves of blood schizonts rupture, and today's use of antipyretic medication. *Plasmodium knowlesi* has a daily (quotidian) cycle and, if unchecked, can rapidly reach potentially lethal densities (27).

After several erythrocytic cycles, some of merozoites invade erythrocytes and instead of differentiating into schizonts, may follow a different pathway and become gametocytes, the sexual stages of the life cycle.

To perpetuate the lifecycle, the gametocytes need to be ingested by another female *Anopheles* mosquito. In the mosquito gut, the gametocytes quickly divide into a number of microgametocytes (male gametes) each with a flagellum (exflagelation). They become free and following contact with a female gamete (macrogametocyte), fertilization occurs. The zygote

develops into a motile ookinete, which traverses the stomach wall of the mosquito, forming an oocyst. Inside it, thousands of sporozoites are formed. Once mature, the oocysts bursts spreading the sporozoites to all parts of the mosquito, particularly to the salivary glands and are injected into the human body during the next mosquito meal.

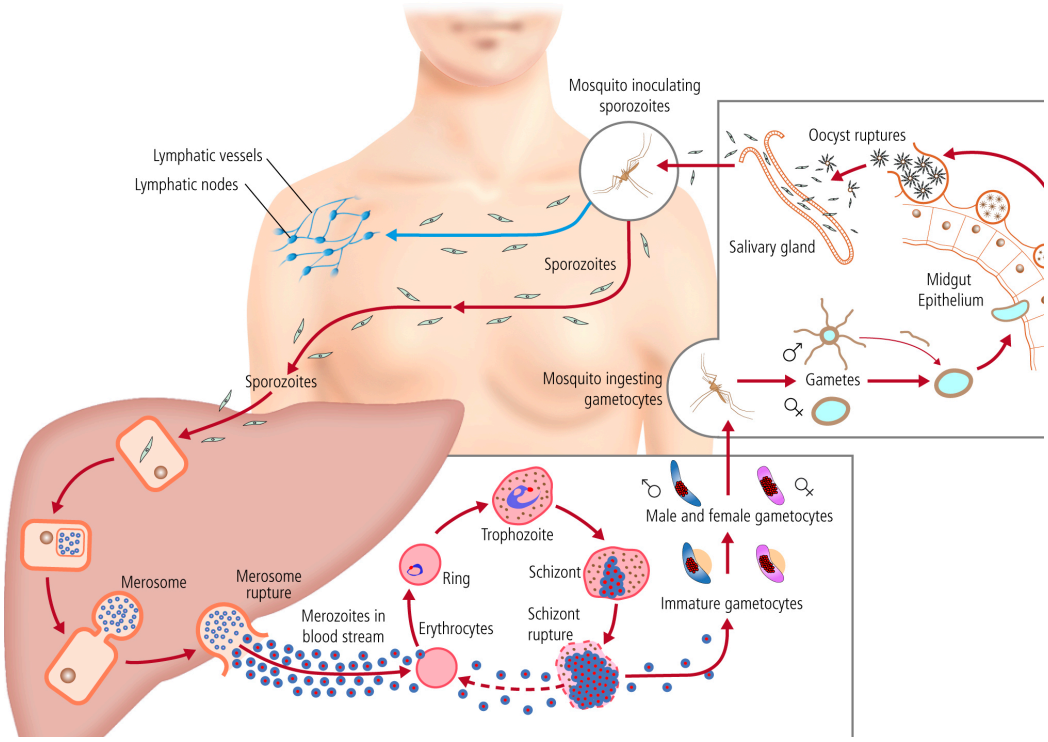


Figure 3: *Plasmodium falciparum* life-cycle

3.3. The *Anopheline* vector

There are around 400 Anopheles species in the world, of which 60 species are malaria vectors, within them only 30 species are of major importance. They are distributed mostly in tropical and subtropical regions, although some species can survive in temperate climates. In general, mosquitoes are not found in regions with altitudes above 2000-2500 meters.

The *Anopheles gambiae* complex and *Anopheles funestus* group, closely linked to the malaria infection transmission in several regions across Africa, include the most efficient mosquito vectors of malaria affecting humans (31-33). These vectors have a strong preference for feeding on humans and a long lifespan compared with other *anopheline* species. An adult female *anopheline* lives an average of 3 weeks or more under optimal conditions while their parent males live only a few days. In conditions where the ambient temperature exceeds 35^{O}C, or humidity falls below 50 percent, the longevity of the anophelines is significantly reduced, directly influencing the transmission of malaria (34).

The terms *anthropophagic* or *anthropophilic* and *zoophagic* or *zoophilic* are used to describe the mosquitoes preference for humans or animals (cattle) respectively. Most of the anopheline feed between dusk and dawn. *Anopheles gambiae,* can feed over a period starting at 10.00 pm to 06.00 am. The resting habits of the mosquitoes are important for targeting preventive measures. For instance, some female mosquitoes rest during

daylight in the walls within house interiors where the environment is cool and humid. The use of indoor residual spraying is suitable for these species (34).

4. Pathogenesis and clinical features

The infection with malaria parasites may result in a wide diversity of clinical features virtually ranging from the complete absence of symptoms to a mild, moderate, severe or even life-threatening disease (35), depending on the immune status of the infected host. In children living in endemic areas, the most common clinical manifestation of *P. falciparum* malaria is a common nonspecific febrile illness that requires laboratory confirmation. Most of the clinical symptoms associated with malaria are caused by the inflammatory cascade prompted by erythrocyte lyses at the end of a cycle of asexual parasite development.

Malaria disease is normally classified as uncomplicated (the most common and widespread presentation) or complicated (severe malaria, mostly associated to *P. falciparum* infection, believed to occur in only 1-2% of all cases). Malaria complications can happen extremely fast in children and fatal outcomes may occur in the very early stages of the disease. Uncomplicated malaria does not pose an immediate danger but requires prompt treatment, especially if *P. falciparum* is present, to avoid it developing into severe disease. The classical symptoms are the

occurrence of recurrent fever, dizziness, arthromyalgias, anorexia, vomiting, malaise and headaches. Spleen enlargement can be often found in children.

Severe (complicated) malaria cases can present with coma, hypoglycemia, seizures, severe anemia, acute renal failure, jaundice, respiratory distress, pulmonary edema, shock and acidosis.

In general, high levels of peripheral parasitaemia are associated to poorer prognoses although as a result of the parasite's capacity to sequester in the deep microvasculature, low peripheral parasitemias may not represent a high parasite mass and can also be associated to severe cases. Furthermore, two children with similar levels of parasitemia can present a complete different pattern of disease, from cerebral malaria or severe anemia to a virtual absence of the disease.

There are some differences in the clinical patterns of the severe malaria cases among non-immune adults and semi-immune African children with organ failure being frequently found in adults rather than in children (36). Children in Africa frequently present severe anemia and cerebral malaria but, when occurring, respiratory distress is also a dangerous form of severe malaria, particularly if associated to other syndromes (36-38).

In endemic areas, cerebral malaria typically occurs in children under 5 years of age, and is rarely seen above the age of 10 in children who have

been exposed to *P. falciparum* from birth. The definition of cerebral malaria is not straightforward, but a simplified way of typifying it is based on the presence of *P. falciparum* asexual parasitemia and altered consciousness (Blantyre coma scale ≤2), in the absence of other obvious causes of impaired consciousness. The Blantyre coma scale is useful for assessing children not yet able to speak (39). It gives a total score from 0 (worst) to 5 (best) based on motor and verbal responses, and eye movements. Children with cerebral malaria usually develop a coma after 1–3 days history of fever. This coma may be accompanied by one or more seizures, an arched back, posturing, an altered respiratory pattern and/or gas abnormalities (39). Other acute neurologic features include a generalized decrease in muscle tone, cranial nerve palsies and retinal abnormalities including hemorrhage (40). The resulting neurological sequelae may include ataxia, hemiplegia, speech disorders, blindness, paralysis, epilepsy, and cognitive and behavioral deficits.

Signs of respiratory distress include deep (acidotic) breathing, low chest wall indrawing, increased respiratory rate and nasal flaring. It is believed that the malaria associated respiratory distress is caused by a metabolic disturbance rather than by a pathological process at the lung level, as documented by the findings of normal oxyhemoglobin concentrations and normal chest X-rays in most of affected patients (41, 42).

The deep sequestration of mature blood schizonts within the venous microvasculature of vital organs is the core pathological process in severe

malaria *P. falciparum* malaria. The organ distribution of sequestration determines the resulting clinical syndrome (43). If sufficient numbers of *P. falciparum* schizonts are attached to blood vessels in the brain, cerebral malaria can result (44); in the placenta, premature delivery, low birth weight and increased neonatal mortality can result as consequence of the reduced fetal blood supply (45, 46).

Severe malaria-related anemia (hemoglobin concentration less than or equal to 5 g/dL) also consists of a group of conditions with multifactorial causes, including the direct and indirect immune mediated destruction of both parasitized and non-parasitized erythrocytes, and bone-marrow suppression associated with imbalances in cytokine concentrations (47). Blood transfusions and iron supplementation remain the cornerstone of anemia treatment in malaria endemic areas (48). Children with severe anemia tend to be younger than those with cerebral malaria, but the two conditions often overlap.

Prompt access, diagnosis and treatment of malaria is critical to save lives, especially in Africa where more than three quarters of deaths are estimated to occur in the first 24 hours after hospital admission, and within the first 2 days of onset of symptoms (49).

5. Immunity and host genetics

A complete understanding of the host immune response to malaria parasites is of paramount importance to achieve the ambition of an effective and safe malaria vaccine (50).

The exact immune mechanisms against malaria are complex and still not fully elucidated. Uncertainty also regards which of the 5300 antigens encoded by the *P. falciparum* parasite produces the key protective immune responses in humans, although some evidence implicates about 20 of these (51). This is due to the complex life cycle of the malaria parasites that consists of a sexual phase in the mosquito vector and a pre-erythrocytic or liver-stage in the human host. For people living in endemic zones, continuously exposed to infective bites throughout their life, natural acquired immunity (NAI) to malaria begins to develop early in childhood. This slowly build up of immunity is age dependent, stage-specific and most importantly, quickly wanes in the absence of a continuous exposure to the *plasmodium* species.

The initial protection acquired is against the life threatening forms of the disease (cerebral malaria, severe malaria anemia, malaria with respiratory distress and death). Gradually it encompasses a protection against more milder clinical disease (anti-disease immunity) and eventually is capable of suppressing the circulating parasites (anti-parasite immunity) (52). Anti-disease immunity is reflected by the age evolution of fever and is believed

to quickly develop, with a reduction in the frequency of clinical presentation of malaria. The second type, anti-parasite immunity is slowly acquired and leads to a progressive decrease in parasite prevalence and multiplicity of infection (53). On the contrary to other viral and bacterial infections, there is no sterilizing immunity under natural circumstances. As a result, as an individual living in an endemic area grows up, the number and severity of clinical malaria episodes decreases, although infection continues to occur.

The acquisition of immunity is also mediated by the intensity of transmission. People living in areas where transmission is perennial and high tend to quickly develop NAI, thus concentrating the burden of severe disease and death in children less than 5 years of age and pregnant women. Generally this immunity persists as long as the individual remains in the area of stable transmission. Conversely, in low transmission areas, this acquisition takes longer to occur and all age groups show similar levels of clinical disease, with adults being also vulnerable to severe disease episodes. A similar situation is characteristic of areas were transmission of malaria is epidemic or unstable, not allowing the acquisition of NAI as transmission is extremely variable and during short time periods.

Individuals not previously exposed to *Plasmodium* infective bites, and therefore ‘non-immune’ or ‘malaria-naïve’, are at great risk of rapidly developing severe disease and death when not promptly treated.

Both humoral and cellular immune responses are thought to be involved in the protective response against malaria. Paradoxically, part of the immune responses may play an important role in the malaria pathogenesis (54, 55).

The duration of this antimalarial immunity can vary from person to person. As the immunity wanes, the risk of potential life-threatening complications increases. A shift for older-age cases of malaria is generally the first indicator that immunity has been lost in an entire community (13). For this reason preventive malaria control programs must be sustained for long and continuous periods even after proved success, as the whole populations becomes vulnerable to epidemic malaria. A good example occurred in the highlands of Madagascar between 1949 and 1960. After almost a complete malaria transmission interruption, using a combination of IRS and mass chloroquine treatment (56), malaria reemerged and a severe epidemic caused by *P. falciparum* in 1986 ended with high fatality rates in all age groups during the following two years (57).

Some conditions alter the human susceptibility to malaria. Most of these human genetic polymorphisms, especially those affecting red blood cells have been selected to high frequencies because they have protected against the effects of malarial infections (13). There is strong epidemiological evidence that thalassemias, sickle cell hemoglobin and glucose-6-phosphate dehydrogenase (G6PD) deficiency protect against

severe *falciparum* malaria. Some authors describe a reduction of the risk of *P. falciparum* death of about 50% and 90% in patients with alpha thalassemias/G6PD or in West African child with sickle cell trait respectively (58-61). The current burden of some genetic diseases seems to be a result of our past contact with malaria. About one-third of a million to half a million babies are born each year with severe forms of these inherited disorders (62).

How exactly the immune system acts in preventing malaria is still not yet clearly understood (63). Despite recent advances in the biotechnology field of research, no clear surrogate markers of immune response fully predictive of protection against malaria have been yet identified.

6. Malaria control strategies

In the last decade, several international initiatives were created to tackle malaria, among others, the WHO's Roll Back Malaria program, the Medicines for Malaria Venture, the PATH-Malaria Vaccine Initiative, the Multilateral Initiative in Malaria and the Global Fund to Fight AIDS, TB and Malaria, aimed to support and implement prevention and treatment programs as well as to support research in innovative therapies and prevention tools.

There are four main strategies to deal with malaria: 1) Effective drugs for malaria treatment and prevention, 2) Vector control, 3) Mechanisms to reduce contact between mosquitoes and humans and 4) Vaccines to prevent malaria.

6.1. Effective drugs for malaria treatment and prevention

The first widely used effective malaria treatment, Quinine, used since the 17th century for the treatment of fevers, was finally isolated in 1820 from extracts from the Andean *Cinchona* tree and is still being used currently as the first line therapy for severe malaria in most endemic countries. Chloroquine was the basic treatment for the erytrohrocytic stages of the malaria parasites, although the dormant hepatic stages of *P. vivax* and *P. ovale* also require further treatment with primaquine, the only drug that currently is available and effective against hypnozoites. The advent of resistance of *P. falciparum* to chloroquine (first reported in Thailand and

then globally widespread) in most endemic countries has limited its use, despite its low price, wide availability and efficacy. Other antimalarial drugs such as proguanil, amodiaquine, primaquine, sulfadoxine-pyrimethamine, halofantrine, mefloquine and lumenfatrine were developed to overcome the parasite resistance to chloroquine and the antifolates. In recent years, a new class of drugs, artemisinins (extracted from the millenarian Chinese plant *Artemisia annua*) has become the base of the antimalarial treatment. In fact, artemisinin based combination therapy are the current recommended WHO malaria first line treatment.

The rationale for the use of ACTs strategy is that the synergistic result of the combined therapy is more effective compared to the monotherapy, and also provides a way in which resistance can be defeated (64-66). The probability in which resistance is developed simultaneously to two therapeutic agents with independent mechanisms of action is extremely low, in the order of 1: 10^{12} treatments (this frequency being the product of the probabilities of acquiring a resistant mutation to each therapeutic agent multiplied by the total number of parasites in a typical infection) (67). Furthermore, in account of their gametocydal action, artemisinin derivatives may also reduce malaria transmission.

Intermittent preventive treatment (IPT) in pregnant women and children has also emerged as a novel strategy to tackle malaria. It implies the administration of a full course of antimalarial treatment to a population at risk at a specified time points, benefiting from the contacts of this

population with the health systems, independently of the infection status (68). Antenatal visits are used to deliver the drug to pregnant women, and vaccination visits part of the EPI schemes are used to deliver them to infants. Different regimens for this preventive therapy are used (and are being explored), depending on the patterns of susceptibility and resistance (69). Sulfadoxine-pyrimethamine is the most widely used drug for IPT in children and pregnant women.

6.2. Vector control

As previously said, vector control has been the main principal strategy of antimalarial control programs. This strategy is based on the use of insecticides, larvicides and other environmental procedures. Indoor residual spraying (IRS) with DDT or a pyrethroid has been shown to be effective in reducing the vectorial capacity and thus malarial disease in a wide variety of settings, and is particularly effective in locations where mosquitoes are indoor-resting and malaria is seasonally transmitted.

The efficacy of IRS depends on a variety of factors such as the chemical proprieties of the product, the susceptibility of the vector, the quality of indoor spraying, the residual efficacy and the cooperation of communities to get complete coverage of their houses (70). Despite some controversy and increased scientific evidence supporting the use of DDT, WHO has re-allowed its use in areas where the malaria vectors remains susceptible (71).

6.3. Mechanisms to reduce contact between mosquitoes and humans

Insecticide-treated mosquito nets (ITNs) prevent malaria transmission by not allowing a contact between the infected mosquitoes and the human host. The addition of insecticide further enhances the physical barrier that the net provides between the vector and the host.

Randomized clinical studies have demonstrated that the use of ITNs reduced both malaria specific and all-cause pediatric deaths (72, 73), proving that ITNs are among the most cost effective public health tools. Also a series of community based randomized controlled trials in a range of malaria transmission settings in sub-Saharan African have shown a reduction by a third in all-cause under 5 child mortality in association to the use of ITNs (74).

Despite its proven benefits, current ITN use in sub-Saharan Africa also is low and needs to be scaled-up. Also the rapid loss of efficacy of ITNs because of washing and associated low retreatment rates limits the effectiveness of ITNs programs. The adoption of long lasting insecticide treated nets can render these programs more cost-effective.

6.4. Vaccines to prevent malaria

6.4.1. The beginning

The need of new and innovative tools to be integrated in the fight against malaria has guided the search for malaria vaccine for years. In line with some technological advances during the last century, the first successful human immunization against mosquito-transmitted malaria was reported in 1973. It consisted of several bites of X-ray irradiated *Plasmodium* infected mosquitoes which delivered viable attenuated sporozoites (75), conferring an immune response without being capable of causing clinical disease. This strategy was, however, clearly unpractical to be considered in terms of mass immunization. Irradiated sporozoites of *P. falciparum* and *P. vivax* protected one naïve volunteer against challenge by infective mosquitoes for at least 3 and 6 months respectively as reflected by a positive species-specific circumsporozoite reaction (76).

During the 90's several trials of the SPf66, the first synthetic malaria vaccine, contributed to generate a mix of hope and controversy, but further development of this candidate was halted after consistent demonstration of low or no efficacy results (77-81). Since then, many potential vaccine candidates entered pre-clinical development with only a few reaching ulterior phases. However, a vaccine against malaria remains the missing tool in the malaria preventive arsenal.

Several progresses occurred during the last years, accompanying an unforeseen investment in this area of research and development (82, 83). The ideal vaccine should be one capable of inducing a lifelong sterilizing immunity, provide cross-species protection, protect children and infants and be compatible with routine immunization schedules. The objective of most vaccines is to induce antibody and T-cell responses to one or a few antigens, but for effective vaccination these need to be of greater magnitude, duration, and strain-transcedence than in naturally acquired immunity (51). Almost all of the vaccines under development are directed *at P. falciparum*, responsible for the vast majority of severe malaria disease and deaths. However, several challenges face the development of an effective malaria vaccine since the very beginning, among others, the lack of immune correlates of protection, the lack of reliable and predictive animal models and the multiple stages and antigenic diversity and variability of the parasites.

6.4.2. Goals and target for malaria vaccines

Classically, the development of malaria vaccines has been directed to target one of the different stages of the *Plamodium* life cycle in the human (pre-erythrocitic and erythrocytic stages) or mosquito hosts (sexual stage)(Figure 4).

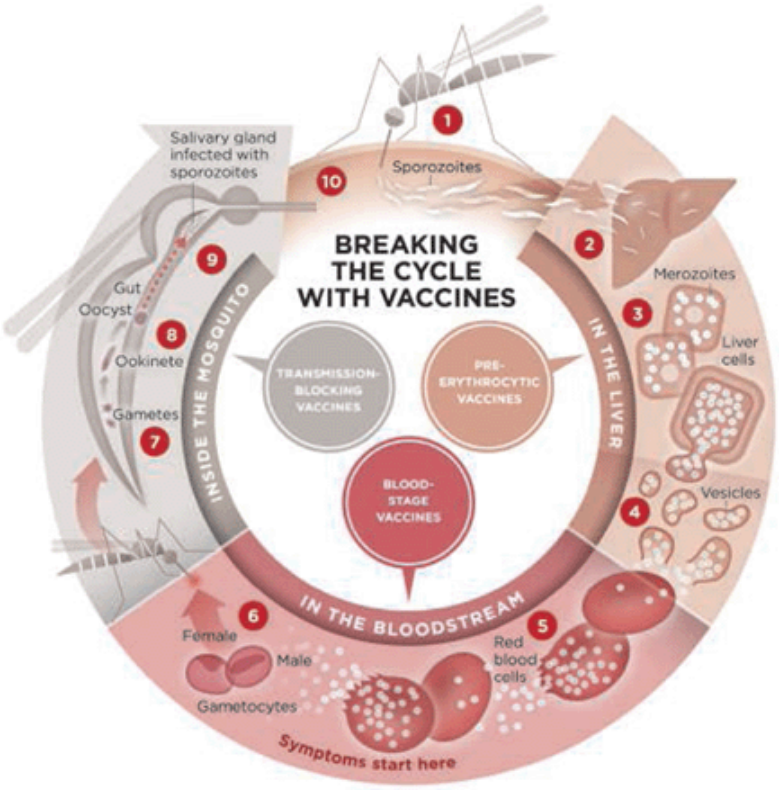


Figure 4: Targets for malaria vaccines (84).

6.4.3. Pre-erythrocytic vaccines

Pre-erythrocytic vaccines strategies are designed to elicit an antibody response to neutralize sporozoites and prevent them from invading the hepatocyte, as well as to elicit a cell-mediated immune response that will inhibit intra-hepatic parasite development. Therefore this type of vaccine would prevent infection and the advent of clinical disease. The observation that immunization of mice with irradiated sporozoites conferred protection (85), and that this protection could be obtained vaccinating with the CS protein alone (86) are the hallmark of the pre-erythrocytic vaccines. The RTS,S/AS is the most advanced malaria

vaccine in development and targets the *Plasmodium falciparum* circumporozoite protein (CSP) in its pre-erythrocytic stage. This vaccine will be further described below as part of the current thesis.

6.4.4. Erythrocytic (blood) stage vaccines

Erythrocytic stage vaccines aim to elicit antibodies to inactivate merozoite antigens and/or antigens expressed on RBC surface through antibody-dependent cellular cytotoxicity and/or complement lysis, as well as T-cell responses able to inhibit the development of the parasite in erythrocytes. By controlling the parasite density limiting RBC invasion, these vaccines would reduce the morbidity, although not preventing the infection.

Some blood stages vaccines in clinical development include apical membrane antigen 1 (AMA-1), erythrocyte-binding antigen-175 (EBA-175), glutamate-rich protein (GLURP), merozoite surface protein 1 (MSP-1), MSP-2, MSP-3 and serine-repeat antigen 5 (SERA5), all of which are highly expressed on the surface of the merozoite (17, 87-96). A recent phase II trials of the most advanced blood-stage candidates, AMA1 and MSP-1 showed no efficacy in African children (17, 93, 97). New strategies are being implemented to enhance the efficacy of some of these vaccine candidates.

6.4.5. Sexual stage vaccines (transmission blocking)

Sexual stage vaccines aim to prevent the transmission of the parasite from the vector to new hosts, not to prevent infection or disease. The rationale is that vaccinated humans elicit anti-gametocyte antibodies that could be transferred to the female *anophelines* during their blood meal, thus blocking the parasite development inside the vector (98). These antibodies, inhibiting the parasite development in the mosquito's midgut, would block further transmission. Efficacious transmission blocking vaccines are thought to be highly desirable in pre elimination settings where interruption of transmission becomes a key aim of an immunization programme. The main antigens assessed as vaccine candidate are the *Plasmodium falciparum* surface proteins *Pfs*25, Pfs28, Pfs48/45 and Pfs230.

7. Malaria vaccine development

7.1. Clinical development pathway

For testing new malaria vaccines candidates in a logical and most economical approach, the following pathway has been proposed (Figure 5) (99):

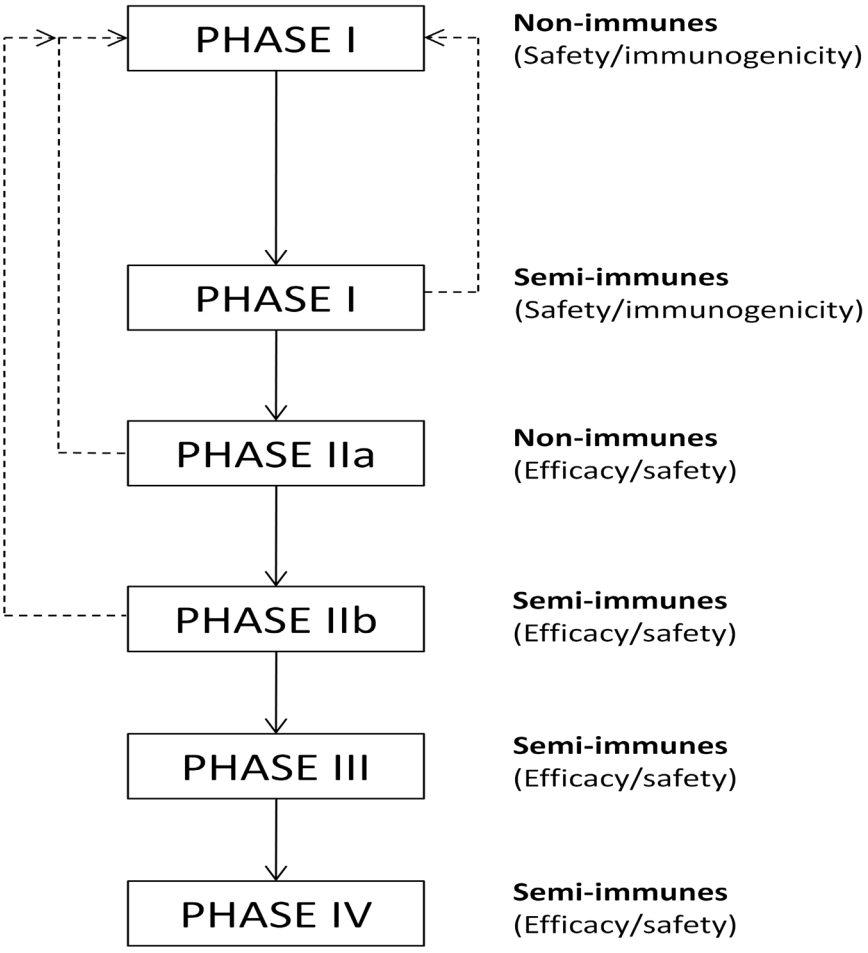


Figure 5: The pathway of malaria vaccine development

Phase 1 trials are designed to primarily evaluate the safety profile and immune response of an experimental vaccine candidate in malaria-naïve and malaria exposed volunteers. Only a small number of volunteers is recruited and both local and systemic side effects following vaccination are evaluated.

Phase 2 studies are sub-divided in two categories: phase 2a, in which tens of non-immune volunteers are artificially challenged with malaria and phase 2b in which natural exposure to infected mosquitoes is allowed to occur. These studies evaluate safety and potential side effects, immune responses, efficacy against infection and clinical disease and determine optimal dosage and schedule.

A phase 3 clinical trial continues evaluating safety and potential side effects as well as efficacy on a large scale. They are conducted with the final vaccine formulation and in the target population in which it will be used if licensed.

Phase 4 trials are designed to monitor post-marketing safety, duration of protection and assess vaccine compliance. They are also important for surveillance of rare adverse effects not detected during the previous phases.

8. RTS,S/AS vaccine clinical research and development plan

Early clinical development of RTS,S starts with the alliance between SmithKline (currently GlaxoSmithKline) Biologicals and the Walter Reed Army Institute of Research (WRAIR) in 1984. This initial development was based under the hypothesis that a subunit vaccine based on the *P. falciparum* circumsporozoite (CS) protein would protect humans from malaria infection (100). In a subunit vaccine, like the current hepatitis B vaccine, parts or the complete antigen that elicits protective immunity to the whole organism are identified and engineered from the proteomic complement of the pathogen (51).

A distinguishing characteristic of RTS,S compared with other previous CS-based vaccine candidates, was the inclusion of both the CS repeat antibody targets as well as portions of the C-terminal nonrepeat regions that constitute targets for cell-mediated immunity (101). The result was a recombinant protein vaccine, composed by the Hepatitis B surface antigen DNA that was fused to DNA encoding a large part of the *P. falciparum* circumsporozoite protein (CSP). Once expressed in yeast, the fusion product (RTS) binds hepatitis B surface antigen (S) forming RTS,S particles that are mixed with a new family of adjuvant system (AS) designed to stimulate strong immune responses in humans (51)

The formulation RTS,S associated to the proprietary adjuvant $AS02_A$ has been demonstrated to be more immunogenic and confer higher efficacy in experimental sporozoite challenges than RTS,S formulated with other

adjuvants (102-104). $AS02_A$ is an oil-in-water emulsion with monophosphoryl lipid MPL immunostimulants and the saponin derivative QS21 (a triterpene glycoside purified from the bark of *Quillaja saponaria*). A new recent developed adjuvant, AS01, in which a liposome replaces the oil-in-water emulsion, in the formulation with MPL and QS21 immunostimulants, has been shown to be less reactogenic, more immunogenic and to be more protective than RTS,S/$AS02_A$ (105, 106).

8.1. RTS,S/AS clinical trials

8.1.1. Adult trials

The RTS,S/AS02 vaccine entered clinical trials in malaria non-endemic and endemic countries. Studies with malaria-exposed adults in The Gambia and Kenya (107, 108) and with malaria-naïve adults in Belgium and the USA (103) have shown the vaccine to be safe and immunogenic. The vaccine, given according to a 2 or 3 dose immunization schedule, protected between 42% and 86% of healthy non-immune volunteers against infection in homologous sporozoite challenge studies (109). In addition a prolongation of the pre-patent period was observed in the majority of non-protected volunteers (109).

In a double-blind, randomized, controlled Phase IIb study in an endemic region of The Gambia, the vaccine efficacy against infection was 34%

(95% CI: 8% to 53%; p = 0.014) over one malaria transmission season, but quickly waned over time: during the first 9 weeks of surveillance efficacy was 71% (95% CI: 46% to 85%; p < 0.0005), decreasing to 0% (95% CI: -52% to 34%) over the subsequent 6 weeks (108). After a single booster dose given prior to the next malaria season, the estimated vaccine efficacy against infection was 47% (95% CI 4% to 71%; p=0.037).

8.1.2. Pediatric trials

The final target of the vaccine is the administration to a pediatric population, and desirably its inclusion in the Expanded Program on Immunization (EPI). This premise has determined the next steps on the RTS,S/AS vaccine development plan: the selection of the optimal dose and formulation to be used in children and infants. Two sequential phase I double-blind, randomized controlled, staggered, age and dose de-escalation trials to evaluate the safety and immunogenicity of a 0.1 mL, 0.25 mL and 0.5 mL doses of RTS,S/AS02$_A$ were conducted in Gambian children aged 6 to 11 years and 1 to 5 years respectively in a 0, 1 and 3-month vaccination schedule (110). The control arms of these studies received rabies vaccine. The 0.25 mL dose RTS,S/AS02$_A$ (25 µg RTS,S antigen in 0.25 mL AS02) was selected for future pediatric development, because it exhibited comparable immunogenicity to the 0.5 mL dose, with a tendency to a lower reactogenicity profile (110). Another expected observation was that the antibody response to the

vaccine components was better at the youngest age group than in adults.

Prior to the launch of a larger phase IIb proof-of-concept efficacy study, a small phase I, double-blind randomized controlled trial in 60 Mozambican children aged 1 to 4 years further confirmed the 0.25 mL dose of RTS,S/AS02$_A$ as having a good safety and immunogenicity profile (104).

In the larger phase IIb trial carried out in Mozambique, 2022 children aged 1 to 4 years of age at the time of enrollment were randomized to receive RTS,S/AS02$_A$ or the control vaccines. The study was designed to evaluate efficacy against *P. falciparum* clinical malaria and efficacy against malaria infection in two separate cohorts. The vaccine efficacy against the first clinical malaria episode was 29.9% (95% CI: 11 – 44.8; p=0.004) and led to a 57.7% (95% CI: 16.2 – 80.6; p=0.019) reduction in admissions for severe malaria during the six-month follow-up period; the study also showed a 45% reduction in rate of infection in the RTS,S vaccinated children compared to controls (111). In an extended surveillance period of the same group of children, the vaccine efficacy against clinical malaria was estimated to be 35.5% (95% CI: 21.6 – 46.6; p<0.0001) and against severe malaria was 48.6% (95% CI: 12.3 – 71.0; p=0.02) at 18 months follow-up (112). Clinical benefit persisted for 3.5 years after vaccination (VE against clinical malaria of 30.5% over 42

months), with no rebound of malaria cases in the RTS,S/AS02 vaccinated group (113).

In preparation for the infant and phase III trials, it was necessary to adequate the formulation to the standard self-disposable syringes used to deliver vaccines through the EPI. A bridging phase I/II randomized double-blind study to evaluate the safety and immunogenicity of 3 doses of RTS,S/AS02$_D$ (0.5 mL dose) in comparison to 3 doses of the existing formulation RTS,S/AS02$_A$ (0.25 mL dose) was then carried out in Mozambique. Both formulations contain the same constituents but the final volume was adjusted to be compatible with existing EPI practices. In other words, the terminology RTS,S/AS02$_A$ stands for the adult formulation while RTS,S/AS02$_D$ for the pediatric one. This study showed that the two presentations had similar safety and immunogenicity profile and demonstrated that the response of the vaccine to the HBsAg component was not inferior when compared to the licensed control vaccine Engerix-B™ (GSK Biologicals, Belgium) (114).

The first administration of the RTS,S/AS02$_D$ vaccine to infants was done in Mozambique between 2005 and 2007. In this study, 214 infants were randomly assigned to receive three doses either of RTS,S/AS02$_D$ at or the hepatitis B vaccine Engerix-B at 10, 14 and 18 weeks of age, as well as routine EPI vaccines (DTPwHib, Hepatitis B and Polio vaccines) given at 8, 12, and 16 weeks of age. The vaccine

was exceptionally well tolerated and reduced the risk of infection by 65.9% (CI: 42.6 – 79.8; p<0.0001) during the initial three month follow-up period after the final immunization (115). Furthermore, when the same vaccine was co-administered with the EPI vaccines to Tanzanian infants, it showed no interference with the immunological responses and similar degree of reduction in malaria infection incidence (116).

Another trial in children 5 to 17 months old, this time with the adjuvant AS01, in Tanzania and Kenya showed that the vaccine was well tolerated and antibody responses against CSP were consistently high (GMT 540 EU/mL, 95% CI: 501 -582). The time to first episode of clinical malaria was reduced by 53% (95% CI 28 – 69, p<0.001) over an 8 month follow-up period after the third dose (117).

At this stage it was considered that enough evidence to lead to a phase III trial had been accumulated under different transmission settings, different ethnic populations and different researchers (101).

9. HYPOTHESIS AND OBJECTIVES

9.1. Hypothesis

9.1.1. The RTS,S/AS02$_D$ is a candidate malaria vaccine with an acceptable reactogenicity and safety profile in children and infants.

9.1.2. The RTS,S/AS02$_D$ vaccine confers partial efficacy against clinical malaria to infants living in an endemic country that lasts at least 12 months after immunization.

9.1.3. The RTS,S/AS02$_D$ vaccine elicits detectable cellular immune responses to both CSP and HbS antigens after infant immunization.

9.1.4. The RTS,S/AS02$_A$ vaccine is immunogenic and induces long-lasting anti-circumsporozoite antibodies.

9.2. Objectives

9.2.1. General objective

To generate information on the safety, immunogenicity and duration of protection conferred by the RTS,S candidate malaria vaccine in young children and infants in a malaria endemic area.

9.2.2. Specific objectives

9.2.2.1. To summarize and describe the developments towards an effective malaria vaccine

9.2.2.2. To describe the safety, immunogenicity and duration of protection of the RTS,S/AS02$_D$ malaria candidate vaccine in infants less than 1 year during 14 months of follow-up of a randomized phase IIb trial.

9.2.2.3. To evaluate the cellular immune responses in infants after immunization with the RTS,S/AS02$_D$ malaria vaccine candidate.

9.2.2.4. To evaluate the humoral immune responses to the *P. falciparum* circumsporozoite protein (CSP) of the RTS,S/AS02$_A$ malaria vaccine during a four year period after immunization.

10. MATERIALS & METHODS

10.1. The study site and population

All studies were conducted at the Manhiça Health Research Centre (CISM), located in the Manhiça district, southern Mozambique. CISM runs a demographic surveillance system in the area since 1996 (118). There are two distinct seasons, a warm and rainy (from November to April) and a cool and dry period throughout the rest of the year. The average temperature is 23^{o}C

The total population of district is around 150.000 inhabitants, approximately half of which being covered by the demographic surveillance system. Every person living for at least 3 months in the surveillance area has a unique permanent identifier number. A full description of the geographical and socio-demographic characteristics of the study community can be found elsewhere (118).

10.2. Morbidity surveillance

A morbidity surveillance system, using a passive case detection approach, was established in 1996 in the main health facility (Manhiça District Hospital) and has integrated progressively other five peripheral health centers (111, 119). All outpatient and inpatient visits from children under 15 years of age are recorded using standardized questionnaires. Information collected includes personal and demographic data (linked to the demographic surveillance system through the unique permanent identification number), clinical data

(signs and symptoms, physical examination, duration of signs/symptoms), auxiliary diagnostic results data (blood parasites reading, packed cell volume), final diagnoses and treatment received as well as the outcome. Additional information, such as more detailed and comprehensive physical examination and further auxiliary laboratory including blood cultures (routinely collected from all children under 2 years of age and under specific criteria to all children), chest X-rays, blood sugar, total blood counts among others are collected from admitted children.

The participants of the clinical trials reported in the current thesis were living in the catchment area of the Manhiça District Hospital and the health posts of Maragra, Ilha Josina Machel and Taninga (figure 6).

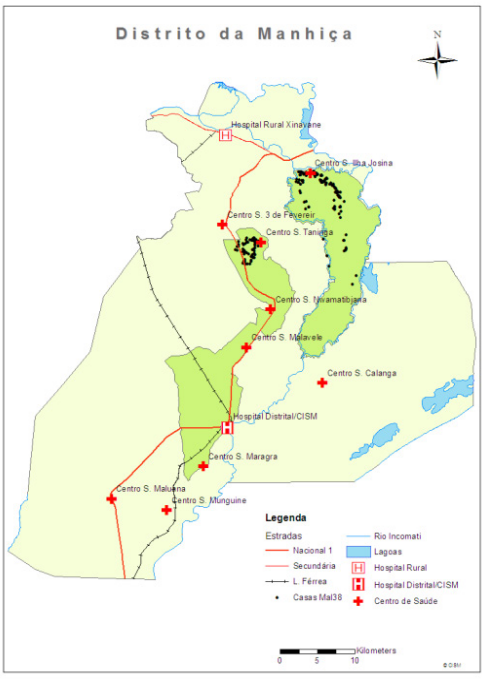


Figure 6: Map showing the Manhiça district, the study area (in green) and the local health network.

10.3. Methodology of studies

10.3.1. First article

The first manuscript is a review article which presents an overview of the need of a safe and efficacious malaria vaccine, and was based on a non-systematic review of the scientific literature. It included a brief description of the challenges towards the development of a malaria vaccine.

10.3.2. Second and third articles

These two manuscripts come from the first RTS,S/AS02$_D$ candidate malaria vaccine trial in infants living in a malaria endemic area. This study was a phase IIb, randomized controlled trial to assess the safety, immunogenicity and efficacy of the RTS,S/AS02$_D$ candidate malaria vaccine administered to 214 infants aged 10, 14 and 18 weeks of age staggered with the administration of of DTPw, Hib, and Polio vaccines at 8, 12 and 14 weeks of age. The control arm received Hepatitis B vaccine (Engerix-B^{TM}) at the times RTS,S/AS02$_D$ was given. The participants, from the villages of Taninga and Ilha Josina Machel (Manhiça district, southern Mozambique), were followed up for 12 months post administration of the third dose. Study activities were completed on December 27th, 2007, when the last recruited child completed 14 months of follow-up. Figure 7 represents the study design.

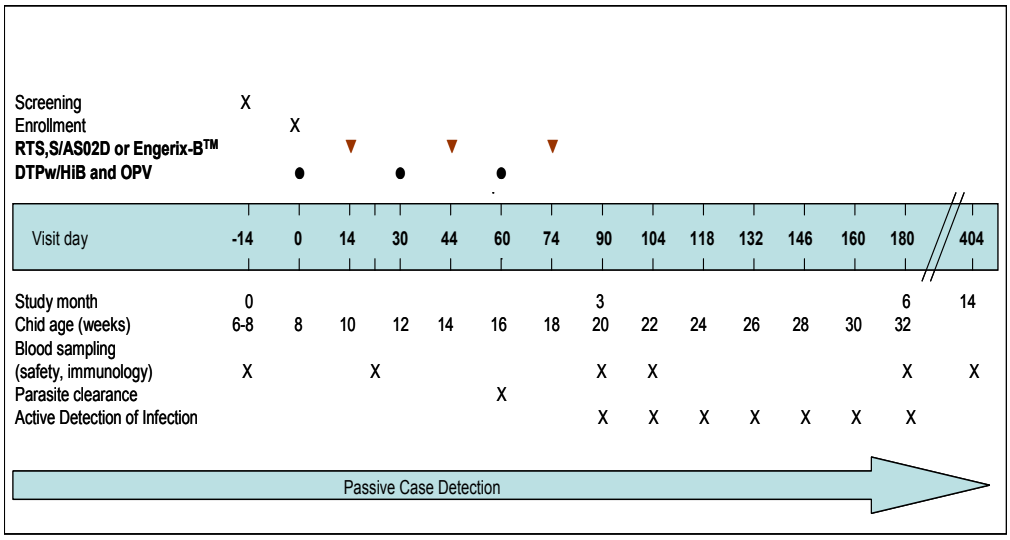


Figure 7: Trial design (second and third articles)

Safety endpoints included the occurrence of solicited and unsolicited symptoms within 7 and 30 days after each vaccination respectively and the occurrence of serious adverse events (SAEs) during the entire 14 month-long period. All SAEs were reported within 24 hours after detection.

Antibody titres were measured against hepatitis B surface antigen (anti-HBs) and *P. falciparum* circumsporozoite protein (anti-CS) at screening and 1, 3½ and 12 months post dose 3. Cellular mediated immune responses (secreted IFN-γ, IL-2 and IL-4) were also measured at the same time points, except for the last visit.

Cases of clinical malaria and malaria infection by *P. falciparum* were ascertained by a combination of passive case detection (PCD) and active detection of infection (ADI). Two weeks prior to dose 3, all

children received a combination of amodiaquine and sulfadoxine-pyrimethamine to clear any parasitemia. Two weeks after dose 3, children with negative slides started ADI (performed bi-weekly for 12 weeks). At each ADI visit, axillary temperature was recorded and parasitemia determined. Children with positive results received antimalarial treatment regardless of the presence or absence of symptoms and were withdrawn from further ADI evaluation. PCD was performed at Manhiça District Hospital and Ilha Josina and Taninga Health posts.

The primary case definition of clinical malaria was the presence of fever (axillary temperature ≥ 37.5ºC) with a *P. falciparum* asexual parasitemia > 500/µL. This definition has a sensitivity and specificity > 90% in this age group. The secondary case definition was fever or history of fever in the previous 24 hours plus any asexual *P. falciparum* parasitemia.

Exploratory efficacy endpoints were first or only clinical episode of *P. falciparum* malaria as well as multiple episodes of clinical malaria detected by PCD during 14 months after dose 1. An additional endpoint was first or only clinical episode of *P. falciparum* malaria detected by a combination of ADI and PCD.

Analyses were done for intention to treat (ITT) and according to protocol (ATP) cohorts, following a predefined analytical plan. The ITT cohort included all children who received at least one dose of the study

vaccine. All safety analyses were based on the ITT population. The ATP cohort included participants that met all eligibility criteria, completed the vaccination course and contributed to follow-up time during the evaluation period. For exploratory analyses, the ATP cohort was split into two follow-up periods: follow-up over study months 3-9 (ATP_{3-9}) and study months 3-14 (ATP_{3-14}). Vaccine efficacy (VE) explored both first or only episode and multiple episodes of clinical malaria detected during the two study periods.

Analysis of immunogenicity was based on the ATP cohort, excluding children that received any blood product, immunosuppressants or immune-modifying therapies. Measurements of anti-CS and anti-HBs antibodies were summarized by Geometric Mean Titres (GMTs) with 95% confidence intervals (95% CI). Titres below the cut-off were assigned an arbitrary value of half the cut-off of the assay for the purpose of GMT calculation.

Reverse cumulative distribution plots of cytokine concentration were used for rapid visual assessment of the distributions. Differences between both vaccine groups in intracellular median stimulation indexes and cytokine median concentrations in the supernatant were assessed by using the Wilcoxon rank-sum test and comparison of proportions was performed by using the Fisher exact test. McNemar chi-square and Wilcoxon ranksum tests were used to compare pre-immune and post-immune proportions and the distribution of positive responses in

supernatant responders.

Person years at risk (PYAR) accounted for absences from the study area and use of antimalarial drugs as previously described (111). VE was defined as 1 minus the hazard ratio multiplied by 100 [(1 – HR)*100] and was adjusted for distance to health facility and community of residence (111). The adjusted VE was assessed using Cox regression models (for the first or only episode) and Poisson regression (for multiple episodes).

A test based on the Schoenfeld residuals was performed to assess whether the hazard was constant over the surveillance period, and alternative approaches were applied if the assumption of proportional hazards was not supported.

The risk of clinical malaria as a function of immune response was evaluated by comparing post-vaccination anti-CS titters for RTS,S/AS02$_D$ recipients who either did or did not experience at least one episode of clinical malaria meeting the primary case definition over ATP$_{3\text{-}14}$ follow-up, using the Wilcoxon Rank Sum test. The hazard rate per 2-fold increase in post-vaccination anti-CS response was calculated for both ATP$_{3\text{-}9}$ and ATP$_{3\text{-}14}$ follow-up, along with their 95% confidence intervals.

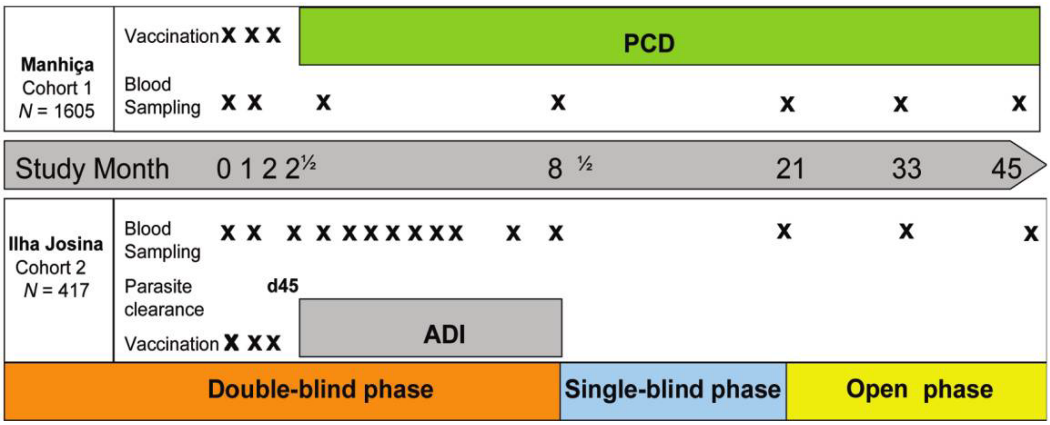
10.3.3. Fourth article

This manuscript derives from largest phase IIb trial of the safety, immunogenicity and efficacy of the RTS,S/AS02$_A$ candidate malaria vaccine study. 2022 healthy children aged 1 to 4 years were enrolled to receive either the RTS,S/AS02$_A$ or a control vaccine administered intramuscularly in the deltoid following a 0, 1, 2 month schedule, after written or thumb printed informed consent provided by their parents or guardians.

In the control group, children aged 24 months and older received three paediatric doses of hepatitis B vaccine (*Engerix-B*™). Because children under 24 months in this group had already received hepatitis B immunization by the time they were enrolled in the trial (as part of their previous EPI immunization), they were vaccinated with 2 paediatric doses of a 7-valent pneumoccocal conjugate vaccine (*Prevnar*™) administered at the first and third vaccinations and one dose of *Haemophilus influenzae type b* vaccine (*Hiberix*™) at the second vaccination. Vaccines were administered at the Manhiça and Ilha Josina health centres.

Children were enrolled into two cohorts to measure the vaccine efficacy against either clinical malaria or malaria infection. In cohort 1, based in Manhiça and Maragra, 1605 participants were followed-up using passive surveillance to detect clinical episodes of malaria. In cohort 2,

based in Ilha Josina, 417 participants were followed-up using active surveillance to detect malaria infection, through visits that started 14 days after the third vaccine dose and were done every 2 weeks for 2.5 months and then monthly for 2 additional months. In children from cohort 2, asymptomatic parasitaemia was presumptively cleared with a combination of amodiaquine and sulfadoxine-pyrimethamine 14 days prior to dose 3. Figure 8 illustrates the trial design.



PCD: Passive case detection; ADI: Active detection of infection

Figure 8: Trial design (fourth article)

Blood samples for determining anti-CSP antibody concentrations in both cohorts and anti-HBsAg antibodies (only cohort 2) were obtained at study months 0 (pre-vaccination), 3 (1 month after the third and final vaccine dose), 8½, 21, 33 and 45. RF1-like antibodies were measured prior to vaccination and at study month 3, only in cohort 2. Serum was separated for antibody determinations. Indirect fluorescent antibody tests (IFAT) for blood stage anti-parasite antibodies were performed

prior to vaccination. Primary analysis of immunogenicity was performed on the ATP cohort (primary analysis).

The levels of IgG antibodies to the NANP repeat region of CSP (B cell epitope) were measured by a standard, validated enzyme-linked immunosorbent assay (ELISA) using plates adsorbed with the R32LR antigen at a GSK validated laboratory (CEVAC, University of Ghent, Belgium). Anti-HBsAg antibody levels were measured only in samples from Cohort 2 at GSK laboratories by ELISA with a commercial kit (AUSAB EIA, Abbott Laboratories, Abbott Park, IL) for the first 5 samplings, and with an in-house developed HBsAg ELISA for the last month 45 sample. RF1-like antibodies levels were determined using an in-house developed ELISA based competition assay with plate adsorbed HBs antigen, performed at CEVAC, University of Ghent, Belgium.

HBsAg levels were determined in both cohorts by ELISA with a commercial kit (ETI-MAK-4® DiaSorin®, Saluggia, Italy) at the Microbiology Service of Hospital Clinic, Universitat de Barcelona, Spain, according to the manufacturer's instructions.

To determine the level of naturally-acquired *P. falciparum*-specific antibodies prior to vaccination, IFAT in baseline serum samples from children in the two study cohorts was conducted at the Barcelona

Center for International Health Research (CRESIB, Hospital Clinic, Universitat de Barcelona, Spain).

For each treatment group, the seropositivity rate for anti-CSP antibodies (proportion of subjects with anti-CSP antibody concentration of ≥ 0.5 EU/mL) and their 95% Confidence Intervals (CI) was tabulated for each time point. Reverse cumulative distribution curves were plotted stratified by age at day 0 (<24 months, ≥24 months) for serum antibody titers measured prior to immunization and at months $8^{1/2}$, 21 and 45.

For each treatment group in Cohort 2, the seroprotection rate for anti-HBsAg antibodies (proportion of subjects with anti-HBsAg antibody titers of ≥ 10 mIU/mL) and their 95% CI were tabulated for each time point. GMTs for anti-HBsAg antibodies measured in mIU/mL with 95% CI were calculated for each group at each time point when a serology sample was taken.

The seroconversion rate for anti-RF1 antibodies (proportion of subjects with anti-RF1 antibody titers of ≥ 33 mIU/mL) were tabulated with 95% CI for all time points at which anti-RF1 antibodies were measured.

GMT calculations were performed by taking the anti-log of the mean of the log titer transformations (log base 10). Titres below the cut-off were assigned an arbitrary value of half the cut-off of the assay for the purpose of GMT calculation.

The relationship between blood stage IFAT titers and anti-CSP antibodies in children vaccinated with RTS,S/AS02$_A$ was assessed by multiple regression methods. Age at vaccination was categorized in four groups, each one corresponding to a one year interval.

The relation between anti-CSP antibody concentrations as measured 30 days post dose 3 and the risk of infection and clinical malaria was assessed in RTS,S/AS02$_A$ recipients. The hazard ratio of participants with anti-CSP antibody titres in the highest tertile against those in the lowest tertile was estimated, as well as the hazard ratio per ten-fold increase in the value of anti-CSP antibodies, using Cox regression models.

11. ETHICAL ISSUES

The protocols involved in the two clinical trials presented in the second, third and for the fourth articles were approved by the Mozambican National Ethics Review Committee, the Hospital clinic of Barcelona (University of Barcelona) Ethics Review Committee and the PATH Human Subjects Protection Committee. The trials were conducted according to the International Conference on Harmonization of Good Clinical Practice Guidelines, and were monitored by GSK Biologicals. A local safety monitor and a Data and Safety Monitoring Board (DSMB) closely reviewed the conduct and the safety data of the studies. A written (or thumbprint) informed consent was obtained prior to start of any trial related procedures. The trials are registered at the ClinicalTrials.gov with the identifiers numbers NCT00197028, NCT00197041 and NCT00323622.

The first article was a review article, therefore not needing a specific ethical clearance.

12. FULL ARTICLES

1st Article: Towards an effective malaria vaccine**. Arch. Dis. Child. 2007**

2nd Article: Safety, immunogenicity and duration of protection of the RTS,S/AS02$_D$ malaria vaccine: one year follow-up of a randomized controlled phase I/IIb trial. **PLoS ONE 2010**

3rd Article: *Plasmodium falciparum*-specific cellular immune responses after immunization with the RTS,S/AS02$_D$ candidate malaria vaccine in infants living in an area of high endemicity in Mozambique. **Infection and Immunity. 2009**

4th Article: Four year immunogenicity of the RTS,S/AS02$_A$ malaria vaccine in Mozambican children during a phase IIb trial. **Vaccine. 2011**

Article 1: Towards an effective malaria vaccine

Pedro Aide, Quique Bassat and Pedro L. Alonso

Arch. Dis. Child. 2007; 92;476-479

Malaria vaccine

Towards an effective malaria vaccine

Pedro Aide, Quique Bassat, Pedro L Alonso

An effective malaria vaccine may be developed in the near future

When in 1955 the malariologist Paul Russell predicted without hesitation the imminent end of malaria,[1] little could he have imagined that half a century later malaria would still be one of the most important public health challenges in the world. At the beginning of the 21st century, 3000 million people (almost half the world's population) living in malaria endemic areas in 100 countries are at risk, with the biggest burden of both disease and death concentrated in African countries. Between 300 and 500 million clinical cases and up to 2.7 million deaths are believed to occur annually.[2 3]

Although there are four species of *Plasmodium* that infect humans, only two (*P vivax* and *P falciparum*) cause significant disease, with nearly all deaths being caused by *P falciparum*.

IS A MALARIA VACCINE NECESSARY?

Over the last century, malaria has disappeared from significant areas of the world, and in some places this has been due to the use of control measures. Nevertheless, in areas where this infection still occurs, we are witnessing an increase in the total number of malaria cases due to population growth, which implies that today more people die from this disease than 40 years ago.[4]

The causes of this resurgence are many. The parasite's extended and increasing resistance to the most common antimalarial drugs, the mosquito's resistance to the widely used insecticides, the hitherto insufficient interest of the pharmaceutical industry in developing new drugs, the shortcomings in the implementation of available control measures, the collapse of national malaria control programmes and the increase in tourism and the migration of non-immune populations to malaria endemic areas, have all contributed to the general rise in malaria cases.[5]

Despite the increasing availability of effective malaria control tools, which should have a combined positive effect on the dynamics of the pandemic, a better and definitive approach to deal with this disease is clearly needed. Vaccines, traditionally considered first-class public health tools, are relatively cheap, easy to administer and deployable through existing universal schemes. A malaria vaccine could therefore become the key element to boost malaria control.

WHY IS A MALARIA VACCINE NOT ALREADY AVAILABLE?

The development of a malaria vaccine is an old jigsaw puzzle which has not yet been solved and presents a formidable scientific challenge. Several factors may explain this historic failure to produce an effective vaccine.

From the immunological point of view, the parasite shows great complexity. The *Plasmodium* genus presents a myriad of antigens which vary throughout the different stages of its life cycle, and against which sequential consecutive immune responses are required. Moreover, many parasitic proteins exhibit high polymorphism, and a single parasitic clone may have up to 50 different copies of the gene coding for an essential protein, expressing a different version of such protein in each successive wave of parasitaemia. This particular antigenic variability appears critical for the parasite's survival, and clearly is a disadvantage not only for the infected individual but also for the scientists aiming to design a vaccine.

Our knowledge about the acquired immunity developed against the disease is limited and incomplete. So far, no surrogate of immunity has been found and there is no certainty about which specific antigens play a key role in the development of immunity.

Moreover, no appropriate animal model exists and the only way of testing the efficacy of a vaccine depends on logistically complex clinical trials being carried out in malaria endemic areas. The high calculated mean cost of developing a malaria candidate vaccine (around $500 million) and the length of the process before it can be marketed (up to 10–12 years),[6] has discouraged pharmaceutical companies from investing in vaccines destined for a market eager for solutions but too poor in resources to pay for them.

IS A MALARIA VACCINE FEASIBLE?

There are four lines of argument supporting the idea that malaria vaccines are feasible.

The first argument is based on the naturally acquired immunity that individuals living permanently in endemic areas develop. Partial immunity against the most severe forms of disease[7] (death and severe disease) is progressively acquired, followed by immunity against clinical episodes and finally suppression of the parasitaemia to low or undetectable levels.[8] Such protection requires a continued booster effect which, however, does not confer sterilising[9] immunity, as individuals may become infected although they do not develop clinical symptoms. If such a model could be reproduced by a vaccine, we would be able to confer solid protection against the disease.

The second model implies evidence of potential passive immunity against malaria. The administration of purified immunoglobulins from ''immune'' malaria patients has been shown to protect patients exposed to the infection.[10 11] Moreover, in endemic areas, newborn infants seem to be protected against clinical forms of the disease, a possible consequence of the passive transfer of maternal antimalarial antibodies during pregnancy.[12]

The third line of argument is supported by experiments carried in the 1970s, during which non-immune volunteers were intensively exposed to UV irradiation-weakened sporozoites. When the volunteers were re-challenged by normally infecting sporozoites, they had acquired, in up to 90% of cases,[13] complete (sterilising) although short-lived immunity. This supports the viability of a vaccine, and should be, despite obvious practical limitations, another model to imitate.[14] Recent research using genetically modified *Plasmodium* parasites (UIS3-deficient) has also shown that this model can be replicated successfully in rodents.[15]

Finally, several studies[16 17] have shown the efficacy of experimental malaria candidate vaccines in humans (adults and children). Nevertheless, despite different candidate vaccines successfully protecting individuals in clinical phase II trials and despite extensive immunological analysis, we still do not know on what immunological basis these individuals are protected, as no clear surrogate measures of immunity have been found.

STRATEGIES FOR VACCINE DESIGN

The ideal malaria vaccine would probably be one that was safe and induced sterilising life-long immunity against infection from childhood. However, this is unlikely in the short term. Given the lack of surrogate markers of protection and our incomplete understanding of

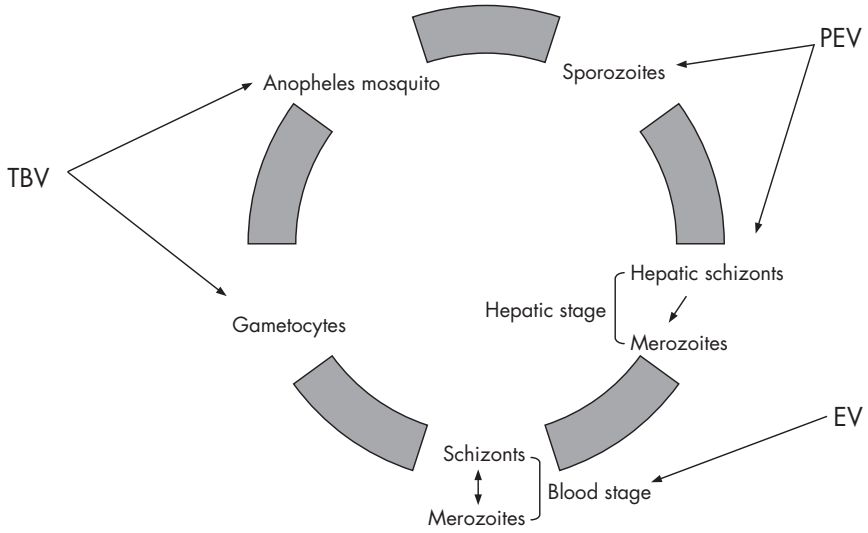


Figure 1 *Plasmodium* life cycle and theoretical activity points of the different malaria vaccines.

malaria immunity, the choice of adequate antigens becomes particularly difficult. It would be reasonable to suppose that antigens should be as conserved as possible, play a vital role in the parasite's life cycle and be amenable to immune challenge. Moreover, immune responses to that antigen should ideally correlate with a reduced risk of malaria. In the past, there has been a greater emphasis on trying to induce cellular responses together with antibody responses, particularly when targeting the pre-erythrocytic stages.[18–20]

The last few years have highlighted the key role that improved and more potent adjuvants may play. Identifying new powerful adjuvants that remain safe, effective and not too reactogenic will surely enhance the possibilities of the existing candidate antigens.

Malaria vaccines can be designed according to the target population or the life cycle stage targeted.

Vaccines designed according to the target population

Different vaccines are needed for different populations; a vaccine aimed at protecting children living in a malaria endemic area is not necessarily similar in its concept to a vaccine aimed at protecting non-immune individuals. In the first case, the vaccine does not need to be 100% effective, as its effect will add to the naturally acquired immunity. This vaccine would need to be directed against the asexual stages and imitate naturally acquired immunity. However, a vaccine aimed at protecting the non-immune individual (for instance, a tourist) requires 100% efficacy, as it would need to neutralise the parasite before it can reach the bloodstream and cause clinical symptoms. The model to follow in this case would be that of immunisation with irradiated sporozoites.

Vaccines designed according to the life cycle stage targeted

The complexity of the *Plasmodium's* life cycle suggests the possibility of establishing different antigenic targets for each stage. Figure 1 summarises the *Plasmodium* life cycle and the respective targets of the different types of stage-specific vaccines.

- Pre-erythrocytic vaccines (PEV) are directed against sporozoites or intrahepatic parasitic stages, and are designed to stop the parasite from reaching its erythrocytic stage so as to prevent any clinical manifestation.
- Blood stage or erythrocytic vaccines (EV) are directed against the blood stage antigens of the life cycle. They should therefore prevent the invasion of red blood cells by post-hepatic merozoites, speed the parasitised erythrocytes' clearance and therefore avoid their sequestration in the microvasculature. The vaccine would not interfere with infection but it would decrease the severity of symptoms.
- Transmission blocking or "altruistic" vaccines (TBV) would not benefit the individual but the community where vaccinated individuals live, by blocking human to human transmission. By targeting the parasite's sexual stages (using antigens expressed in the mosquito stages rather than in humans), this vaccine could prevent the appearance of mutant strains. Since the mosquito does not have an adaptive immune response, the *Plasmodium* genes coding for the mosquito-stage life cycle are remarkably conserved, and thus easier to identify and target.

The combination of a vaccine of this kind with a PEV or an EV could then avert the appearance of potentially dangerous immune selection.[21 22]

In reality, the predicted effects of such types of vaccines are generally wider than expected and may intertwine. Partially effective PEVs have shown protection against severe disease,[16] a characteristic traditionally believed to be typical of EVs.[23] It is believed that by decreasing the initial parasite inoculum, and subsequently causing a delay in the rupture of hepatic schizonts, a more benign illness may occur,[22] an identical mechanism to that proposed for bed nets.[24]

A possible strategy is to combine antigens from different stages (multistage vaccines) in order to trigger an intense and sequential immune response, or different antigens from the same phase (multivalent vaccines), so as to increase the efficacy and reduce the risk of emergent resistance. However, the inclusion of unnecessary components may increase both the cost and any undesired effects.

VACCINES IN CLINICAL TRIALS

The development of a malaria vaccine takes a long time and is expensive, and several phases must occur before a candidate vaccine can be tried in children.

Currently, several candidate vaccines are being developed, most of which are still in the preclinical phases. More than 50% of the approximately 75 candidate vaccines in active development today are based on just three antigens cloned two decades ago: the circumsporozoite protein (CSP), the merozoite surface protein (MSP) and the apical membrane antigen 1 (AMA-1).[18] The *Plasmodium falciparum* genome project has identified hundreds of parasite proteins that could form the basis for new vaccines.[25]

The most advanced candidate vaccine, the RTS,S/AS02A, has been developed and jointly financed by GlaxoSmithKline and the Malaria Vaccine Initiative (MVI).[26] This pre-erythrocytic subunit vaccine is based on the fusion of the surface antigen from the circumsporozoite (CS) with the hepatitis B surface antigen (HBsAg), formulated with the AS02A adjuvant. In a phase IIb clinical trial carried out in 2003 in children from 1 to 4 years of age in Mozambique, this vaccine was shown to be safe, immunogenic and efficacious, reducing *P falciparum* clinical malaria cases by 30% and episodes of severe disease by up to 58%.[16] Moreover, this efficacy did not seem to wane[22] after an 18 month follow-up period, when the protection was maintained.[27] These promising results need now to be confirmed in the ideal target population, which is children less than

Table 1 Major malaria candidate vaccines in clinical development[12 18 28]

Antigen	Name	Adjuvant	Clinical phase	Producer/group
Pre-erythrocytic (PEV)				
CSP	RTS,S[16 27 29]	AS02A	1a, 1b, 2a, 2b	MVI/GSK
CSP	RTS,S	AS01B	1a, 2a	WRAIR/GSK
CSP	RTS,S	AS01E	1a, 1b, 2a	MVI/GSK/WRAIR
Fowl pox 9 CSP+LSA-1 epitope/ MVA CSP+LSA-1 epitope		None	1a, 1b, 2a	Oxford
Fowl pox 9MVA polyprotein		None	1a, 2a	Oxford/EMVI
LSA-1 *E coli* expressed	LSA-NRC	AS02A	1a, 2a	GSK/WRAIR
LSA-1 *E coli* expressed	LSA-NRC	AS01B E	1a, 2a	GSK/WRAIR
Erythrocytic (EV)				
MSP-1 42 3D7 (FMP-1) *E coli* expressed	FMP1[30]		1a, 1b, 2a, 2b	WRAIR
MSP-1-C1 42	(FVO+ 3D7)	ALOH *P pastoris* expressed	1a	MVDU/NIH
MSP-1-C1 42	(FVO+ 3D7)	ALOH+CPG *P pastoris* expressed	1a	MVDU/NIH
AMA-1 3DT[31]	FMP2.1	AS02 *E coli* expressed	1a, 1b	WRAIR
AMA-1 C1	(FVO+ 3D7)	ALOH *E coli* expressed	1a, 1b	MVDU/NIH
AMA-1 C1	(FVO+ 3D7)	ALOH + CPG *E coli* expressed	1a	MVDU/NIH
AMA-1 C1	PfCP-2.9	ALOH/MontanideISA720/AS02 *P pastoris* expressed	1a	BPRC
SE 36	SERA	ALOH *E coli* expressed	1a	Osaka University, BIKEN Foundation
MSP3/GLURP	GMZ 2	ALOH *L lactis* expressed recombinant	1a	
MSP-1 19/AMA-1 chimera	PfCP2.9	*P pastoris* expressed	1a	SMMHS/Wanxing/MVI/WHO
Combination multi-stage vaccines				
Recombinant FMP-1 plus RTS,S, MSP-1 3DT+CSP			1a, 2a	WRAIR
Mimetopes delivered on virosome CSP, AMA-1			1a, 2a	Pevion
Transmission blocking vaccines (TBV)				
Pvs25 Saccharomyces expressed[32]		ALOH	1a	MVDU
Other approaches and targets				
PfEMP1	Malaria in pregnancy] vaccines[33		Pre-clinical	
Attenuated parasite (sporozoite)	Attenuated sporozoite vaccine[14 15]		Pre-clinical	Sanaria
GPI	Anti-toxic] vaccines[34		Pre-clinical	

ALOH, aluminium hydroxide; AMA, apical membrane antigen; CSP, circumsporozoite protein; EV, erythrocytic vaccines; GLURP, glutamate-rich protein; GPI, glycosylphosphatidylinositol; GSK, GlaxoSmithKline Biological; LAS, liver-stage antigen; MDVU, Malaria Vaccine Development Unit; MSP, merozoite surface protein; MVA, modified vaccine Ankara; NIH, National Institute of Health; PEV, pre-erythrocytic vaccines; WRAIR, Walter Reed Army Institute of Research.

1 year of age. Should this vaccine be similarly effective in this age group, the vaccine could be included in the Expanded Programme of Immunization (EPI), one of the few existing effective mechanisms for the universal distribution of health measures in poor countries.

Other candidate malaria vaccines in different stages of clinical development and further vaccine development strategies (including prime boost, virosomes, virus-like particles and peptides based on the important parasite antigens) are summarised in table 1. In the past 5 years, the number of groups working with malaria vaccines has grown from three to 11[21] and in the next few years we should have a clearer picture of the efficacy of these candidate vaccines.

CONCLUSIONS

The promising advances that the beginning of the 21st century is witnessing in the field of malaria vaccine research are framed in an atmosphere of optimism and research impetus that cannot and must not be wasted. Different private initiatives have worked together with the public sector in order to finance the research needed to obtain a vaccine that once seemed too far away. It is essential that this momentum is maintained to guarantee the development of an effective vaccine. We face the possibility of solving a formidable scientific challenge and must not undermine it. Vaccination of children from malaria endemic areas with an effective and safe vaccine, combined with the use of other proven effective control measures, could contribute decisively to decreasing the intolerable malaria toll. It may now be the appropriate moment to reflect upon the strategies that will be needed in the future to distribute this control tool at an affordable cost among those who need it most, an equal or even bigger challenge.[35]

Arch Dis Child 2007;**92**:476–479.
doi: 10.1136/adc.2005.092551

Authors' affiliations
Pedro Aide, National Institute of Health, Ministry of Health, Mozambique
Pedro Aide, Quique Bassat, Pedro L Alonso, Manhica Health Research Center, Maputo, Mozambique
Quique Bassat, Pedro L Alonso, Barcelona Center for International Health, Hospital Clinic, University of Barcelona, Barcelona, Spain

Correspondence to: Pedro L Alonso, Centre de Salut Internacional, Hospital Clínic de Barcelona, Villarroel 170, 08036 Barcelona, Spain; palonso@clinic.ub.es

Accepted 7 February 2007

Competing interests: None.

REFERENCES

1 **Russell PF**. *Man's mastery of malaria*. Oxford: Oxford University Press, 1955.

2 **Hay SI**, Guerra CA, Tatem AJ, *et al.* The global distribution and population at risk of malaria: past, present, and future. *Lancet Infect Dis* 2004;**4**(6):327–36.
3 **WHO**. *WHO expert committee on malaria*, WHO technical report series 892. Geneva: World Health Organization, 2000.
4 **Guerin PJ**, Olliaro P, Nosten F, *et al.* Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. *Lancet Infect Dis* 2002;**2**(9):564–73.
5 **Hoffman SL**, Miller LH. Perspectives on malaria vaccine development. In: Hoffman SL, ed. *Malaria vaccine development:a multi-immune response approach*. Washington: American Society for Microbiology, 1996:1–13.
6 **Bonn D**. Filling the vaccine gap. *Lancet Infect Dis* 2005;**5**(1):7.
7 **Gupta S**, Snow RW, Donnelly CA, *et al.* Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nat Med* 1999;**5**(3):340–3.
8 **Webster D**, Hill AV. Progress with new malaria vaccines. *Bull World Health Organ* 2003;**81**(12):902–9.
9 **Raghunath D**. Malaria vaccine: are we anywhere close? *J Postgrad Med* 2004;**50**(1):51–4.
10 **Cohen S**, McGregor IA, Carrington S. Gamma-globulin and acquired immunity to human malaria. *Nature* 1961;**192**:733–7.
11 **Sabchareon A**, Burnouf T, Ouattara D, *et al.* Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. *Am J Trop Med Hyg* 1991;**45**(3):297–308.
12 **Ballou WR**, Arevalo-Herrera M, Carucci D, *et al.* Update on the clinical development of candidate malaria vaccines. *Am J Trop Med Hyg* 2004;**71**(Suppl 2):239–47.
13 **Rieckmann KH**, Beaudoin RL, Cassells JS, *et al.* Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. *Bull World Health Organ* 1979;**57**(Suppl 1):261–5.
14 **Hoffman SL**, Goh LM, Luke TC, *et al.* Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *J Infect Dis* 2002;**185**(8):1155–64.
15 **Mueller AK**, Labaied M, Kappe SH, *et al.* Genetically modified Plasmodium parasites as a protective experimental malaria vaccine. *Nature* 2005;**433**(7022):164–7.
16 **Alonso PL**, Sacarlal J, Aponte JJ, *et al.* Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. *Lancet* 2004;**364**(9443):1411–20.
17 **Alonso PL**, Smith T, Schellenberg JR, *et al.* Randomised trial of efficacy of SPf66 vaccine against Plasmodium falciparum malaria in children in southern Tanzania. *Lancet* 1994;**344**(8931):1175–81.
18 **Girard MP**, Reed ZH, Friede M, *et al.* A review of human vaccine research and development: malaria. *Vaccine* 2007;**25**(9):1567–80.
19 **Doolan DL**, Martinez-Alier N. Immune response to pre-erythrocytic stages of malaria parasites. *Curr Mol Med* 2006;**6**(2):169–85.
20 **Yazdani SS**, Mukherjee P, Chauhan VS, *et al.* Immune responses to asexual blood-stages of malaria parasites. *Curr Mol Med* 2006;**6**(2):187–203.
21 **Moorthy VS**, Good MF, Hill AV. Malaria vaccine developments. *Lancet* 2004;**363**(9403):150–6.
22 **Van de Perre P**, Dedet JP. Vaccine efficacy: winning a battle (not war) against malaria. *Lancet* 2004;**364**(9443):1380–3.
23 **Greenwood BM**. What can be expected from malaria vaccines? *Ann Trop Med Parasitol* 1997;**91**(Suppl 1):S9–S13.
24 **Greenwood B**, Marsh K, Snow R. Why do some African children develop severe malaria? *Parasitol Today* 1991;**7**(10):277–81.
25 **Gardner MJ**, Hall N, Fung E, *et al.* Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* 2002;**419**(6906):498–511.
26 **Graves P**, Gelband H. Vaccines for preventing malaria. *Cochrane Database Syst Rev* 2003;(1):CD000129.
27 **Alonso PL**, Sacarlal J, Aponte JJ, *et al.* Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet* 2005;**366**(9502):2012–8.
28 **Dubovsky F**, Rabinovich R. Malaria vaccines. In: Plotkin SA, Orenstein WA, eds. *Vaccines*. Philadelphia: Saunders, 2004:1282–9.
29 **Stoute JA**, Slaoui M, Heppner DG, *et al.* A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. *N Engl J Med* 1997;**336**(2):86–91.
30 **Stoute JA**, Gombe J, Withers MR, *et al.* Phase 1 randomized double-blind safety and immunogenicity trial of Plasmodium falciparum malaria merozoite surface protein FMP1 vaccine, adjuvanted with AS02A, in adults in western Kenya. *Vaccine* 2007;**25**(1):176–84.
31 **Malkin EM**, Diemert DJ, McArthur JH, *et al.* Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for Plasmodium falciparum malaria. *Infect Immun* 2005;**73**(6):3677–85.
32 **Malkin EM**, Durbin AP, Diemert DJ, *et al.* Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. *Vaccine* 2005;**23**(24):3131–8.
33 **Smith JD**, Deitsch KW. Pregnancy-associated malaria and the prospects for syndrome-specific antimalaria vaccines. *J Exp Med* 2004;**200**(9):1093–7.
34 **Schofield L**, Hewitt MC, Evans K, *et al.* Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* 2002;**418**(6899):785–9.
35 **Moree M**, Ewart S. Policy challenges in malaria vaccine introduction. *Am J Trop Med Hyg* 2004;**71**(Suppl 2):248–52.

Article 2: Safety, immunogenicity and duration of protection of the RTS,S/AS02$_D$ malaria vaccine: one year follow-up of a randomized controlled phase I/IIb trial.

Pedro Aide, John J. Aponte, Montse Renom, Tacilta Nhampossa, Jahit Sacarlal, Inacio Mandomando, Quique Bassat, Maria Nélia Manaca, Amanda Leach, Marc Lievens, Johan Vekemans, Marie-Claude Dubois, Christian Loucq, W. Ripley Ballou, Joe Cohen and Pedro L. Alonso

PLoS ONE 2010 5(11): e13838.

OPEN ACCESS Freely available online

PLoS one

Safety, Immunogenicity and Duration of Protection of the RTS,S/AS02$_D$ Malaria Vaccine: One Year Follow-Up of a Randomized Controlled Phase I/IIb Trial

Pedro Aide[1,3]*, John J. Aponte[1,2], Montse Renom[1,2], Tacilta Nhampossa[1,3], Jahit Sacarlal[1,4], Inacio Mandomando[1,3], Quique Bassat[1,2], Maria Nélia Manaca[1], Amanda Leach[5], Marc Lievens[5], Johan Vekemans[5], Marie-Claude Dubois[5], Christian Loucq[6], W. Ripley Ballou[5], Joe Cohen[5], Pedro L. Alonso[1,2]

1 Centro de Investigação em Saúde da Manhiça (CISM), Maputo, Mozambique, **2** Barcelona Centre for International Health Research, Hospital Clinic, University of Barcelona, Barcelona, Spain, **3** Instituto Nacional de Saúde, Ministério de Saúde, Maputo, Mozambique, **4** Faculdade de Medicina, Universidade Eduardo Mondlane, Maputo, Mozambique, **5** Glaxo-SmithKline Biologicals, Rixensart, Belgium, **6** PATH Malaria Vaccine Initiative, Bethesda, Maryland, United States of America

Abstract

Background: The RTS,S/AS02$_D$ vaccine has been shown to have a promising safety profile, to be immunogenic and to confer protection against malaria in children and infants.

Methods and Findings: We did a randomized, controlled, phase I/IIb trial of RTS,S/AS02$_D$ given at 10, 14 and 18 weeks of age staggered with routine immunization vaccines in 214 Mozambican infants. The study was double-blind until the young child completed 6 months of follow-up over which period vaccine efficacy against new *Plasmodium falciparum* infections was estimated at 65.9% (95% CI 42.6–79.8, $p<0.0001$). We now report safety, immunogenicity and estimated efficacy against clinical malaria up to 14 months after study start. Vaccine efficacy was assessed using Cox regression models. The frequency of serious adverse events was 32.7% in the RTS,S/AS02$_D$ and 31.8% in the control group. The geometric mean titers of anti-circumsporozoite antibodies declined from 199.9 to 7.3 EU/mL from one to 12 months post dose three of RTS,S/AS02$_D$, remaining 15-fold higher than in the control group. Vaccine efficacy against clinical malaria was 33% (95% CI: −4.3–56.9, $p = 0.076$) over 14 months of follow-up. The hazard rate of disease per 2-fold increase in anti-CS titters was reduced by 84% (95% CI 35.1–88.2, $p = 0.003$).

Conclusion: The RTS,S/AS02$_D$ malaria vaccine administered to young infants has a good safety profile and remains efficacious over 14 months. A strong association between anti-CS antibodies and risk of clinical malaria has been described for the first time. The results also suggest a decrease of both anti-CS antibodies and vaccine efficacy over time.

Trial Registration: ClinicalTrials.gov NCT00197028

Citation: Aide P, Aponte JJ, Renom M, Nhampossa T, Sacarlal J, et al. (2010) Safety, Immunogenicity and Duration of Protection of the RTS,S/AS02$_D$ Malaria Vaccine: One Year Follow-Up of a Randomized Controlled Phase I/IIb Trial. PLoS ONE 5(11): e13838. doi:10.1371/journal.pone.0013838

Editor: Jennifer Keiser, Swiss Tropical Institute, Switzerland

Received June 12, 2010; **Accepted** October 15, 2010; **Published** November 4, 2010

Funding: GlaxoSmithKline and Centro de Investigação em Saúde de Manhiça both received financial support from the PATH Malaria Vaccine Initiative (MVI), which was involved in all aspects of the study design and interpretation. MVI funded this work through a grant from the Bill & Melinda Gates Foundation. Core funding for CISM is provided by the Spanish Agency for International Cooperation (AECI). The funders had no role in data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: MVI supports the development and testing of a number of malaria vaccines that can be seen as competitors. Amanda Leach, Marc Lievens, Johan Vekemans, Marie-Claude Dubois, W. Ripley Ballou, and Joe Cohen are current or previous employees of GlaxoSmithKline Biologicals. Amanda Leach, W. Ripley Ballou, Marie-Claude Dubois and Joe Cohen own shares in GlaxoSmithKline. Both Joe Cohen and W. Ripley Ballou are listed as the 'Inventors' of patented malaria vaccines. However, neither individual holds a patent for a malaria vaccine. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials. None of the other authors in this paper have declared a conflict of interest.

* E-mail: pedro.aide@manhica.net

Introduction

Plasmodium falciparum malaria is one of the most serious public health problems worldwide[1]. The need for improved prevention tools cannot be overemphasized. A safe and effective malaria vaccine to be used in malaria-endemic areas, particularly during early stages of life, could greatly contribute to reducing the enormous burden of malaria, and perhaps contribute to future eradication efforts.

The last decade has witnessed important progresses in the development of a first generation malaria vaccine. GlaxoSmithKline's (GSK) RTS,S, formulated with the Adjuvant System AS02 or AS01, is currently the world's most clinically-advanced malaria vaccine candidate. This vaccine has been shown to be safe and efficacious against malaria infection and disease in adult naïve and semi-immune volunteers [2,3]. In 2004, we reported the first proof-of-concept study in African children aged 1 to 4 years showing that the vaccine was safe, immunogenic and reduced the risk of *P. falciparum* infection, uncomplicated malaria and severe disease, and that protection lasted for at least 45 months [4,5,6].

Recognizing that malaria control strategies must prioritize protection in infants [7,8,9] led us to a I/IIb proof-of-concept trial

to assess the safety, immunogenicity and efficacy of RTS,S/AS02$_D$ in children less than 12 months of age. Vaccine efficacy (VE) against malaria infection was 65.9% (95% CI 42.6–79.8, $p<0.0001$) at the end of 6 months of follow-up [10].

A subsequent trial of the RTS,S/AS02$_D$ in Tanzanian infants has recently shown very similar results [vaccine efficacy of 65.2% (95% CI 20.7–84.7, $p = 0.01$)] [11]. Furthermore, another trial in children 5–17 months old with RTS,S/AS01$_E$ in Tanzania and Kenya yielded a 53% (95% CI 28–69, $p<0.001$) reduction of clinical malaria episodes over an 8 month follow up period [12].

This paper reports the safety, reactogenicity, immunogenicity and efficacy of the complete 14 months follow-up period of the Mozambican phase I/IIb proof-of-concept trial in infants, with particular emphasis on safety and reactogenicity, given that it was the first time that RTS,S formulated with AS02 was administered to infants.

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Study site

The study was carried out by the Centro de Investigação em Saúde de Manhiça (CISM) in the rural areas of Taninga and Ilha Josina Machel, 50 Km north of Manhiça village, Mozambique, from June 2005 to December 2007. Detailed description of the area can be found elsewhere [10,13].

Study Design

This study was a phase I/IIb, randomized controlled trial to assess the safety, immunogenicity and efficacy of the RTS,S/AS02$_D$ vaccine administered to infants at 10, 14 and 18 weeks of age, staggered with EPI vaccines (DTPw/Hib [TETRActHib^{TM} Aventis Pasteur]) at 8, 12 and 16 weeks of age. The study was double-blind until the youngest child completed 6 months of follow-up. After the unblinding, the study was considered single blinded although both participants and field investigators remained blinded. Only a senior statistician had access to the treatment codes allocated to the subjects, and he was not involved in the children follow-up. Data provided to the field investigators did not include information of the allocated treatment per subject during the entire duration of the trial.

A total of 214 children were enrolled and randomized to receive either RTS,S/AS02$_D$ or the control hepatitis B vaccine, *Engerix-B*^{TM}. Details of the malaria and control vaccines as well as the trial profile for the double-blind phase have been presented elsewhere [10]. Briefly, all women who considered enrolling their infant in the study were screened for hepatitis B surface antigen (HBsAg) and human immunodeficiency virus (HIV) in their third trimester of pregnancy. Written informed consent was obtained before any blood was taken for testing.

Infants were screened between 6 and 12 weeks of age and a second written informed consent was obtained from parents/guardians of all participants. Infants were enrolled if they were born after a normal gestational period and in the absence of obvious medical abnormalities. Children born to Hepatitis B and HIV positive mothers were not included in the trial. Children were excluded as well from participation if BCG vaccine had not been given at least one week before the first study vaccination or if any other vaccinations, other than the first dose of oral polio vaccine (OPV) given at birth with BCG, had been given prior to enrolment. Identification cards were provided soon after recruitment. Study activities were completed on December 27th, 2007, when the last recruited child completed 14 months of follow-up.

The protocol (NCT00197028) was approved by the Mozambican National Bioethics Committee, the Hospital Clínic of Barcelona Ethics Review Committee and the PATH Human Subjects Protection Committee and implemented according to the International Conference of Harmonization and Good Clinical Practices guidelines. GSK monitored the study. A Local Safety Monitor and a Data and Safety Monitoring Board oversaw the design, conduct and results of the trial.

The sample size for the original study was based on an evaluation of vaccine safety [10]. A trial with 100 subjects in each group had 80% power to detect a 2.6-fold increase in SAE rates if the rate in controls was at least 10%. The trial also had 90% power to detect an efficacy against malaria infection of 45% or more assuming an attack rate of at least 75% in the control group over the surveillance period. Efficacy against clinical malaria was an exploratory endpoint.

Evaluation of safety

Safety endpoints included the occurrence of solicited and unsolicited symptoms within 7 and 30 days after each vaccination respectively and the occurrence of serious adverse events (SAEs) during the entire 14 month follow-up period. All SAEs were reported within 24 hours after detection.

Vaccine safety was evaluated using active and passive follow-up. All study participants were observed for at least one hour after each vaccine dose by a physician equipped with an emergency kit. Children were visited daily in their homes for 6 days after vaccination where any adverse events (AEs), local or general, were registered on diary cards. Study physicians evaluated all suspected grade 3 AEs and guided clinical management.

Passive follow-up was done through a health facility based morbidity surveillance system [5,14]. All AEs irrespective of their severity or relationship to vaccination were recorded during the 30 day period after each dose. SAEs were similarly detected and reported throughout the study. Detailed definitions for solicited and unsolicited AEs and SAEs as well as the classification of the intensity can be found elsewhere [15].

Participants with SAEs were followed-up until events resolved. Deaths occurring at home were investigated by a review of available medical records and by verbal autopsy, as described elsewhere [16].

Safety monitoring of hematological parameters [hemoglobin, hematocrit, whole blood cell (WBC) and platelets] and biochemical parameters [alanine amino transferase (ALT), total bilirubin and creatinine] were measured one week after dose 1 and 1, 3½, and 12 months after dose 3. Normality values considered were: hemoglobin $\geq$80 g/L, hematocrit $\geq$25%, WBC 5-17$\times10^9$/L, platelets $\geq100\times10^9$/L, ALT $\leq$60 µmol/L, creatinine $\leq$45 µmol/L and bilirubin $\leq$34 µmol/L.

Biochemical, hematological and packed cell volume (PCV) tests were determined as described elsewhere [10].

Evaluation of immunogenicity

Antibody titres were measured against hepatitis B surface antigen (anti-HBs) and *P. falciparum* circumsporozoite protein (anti-CS) at screening and 1, 3½ and 12 months post dose 3.

Anti-CS antibodies were measured by a standardized ELISA, using plates absorbed with recombinant R32LR with an assay cut-off of 0.5 EU/mL. Anti-HBs antibodies were quantified using the EIA kit from Abbott Laboratories and a GSK validated sandwich ELISA described elsewhere [17]. The cut-off for the anti-HBs ELISA was set at 10 mIU/mL.

Evaluation of vaccine efficacy

Cases of clinical malaria and malaria infection by *P. falciparum* were ascertained by a combination of passive case detection (PCD) and active detection of infection (ADI) as described elsewhere [10]. Briefly, two weeks prior to dose 3, all children received a combination of amodiaquine and sulfadoxine-pyrimethamine to clear any parasitemia. Two weeks after dose 3, children with negative slides started ADI (performed bi-weekly for 12 weeks). At each ADI visit, axillary temperature was recorded and parasitemia determined. Children with positive results received antimalarial treatment regardless of the presence or absence of symptoms and were withdrawn from further ADI evaluation. PCD was performed at Manhica District Hospital and Ilha Josina and Taninga Health posts as described elsewhere [5,14].

The primary case definition of clinical malaria was the presence of fever (axillary temperature $\geq 37.5^{\circ}C$) with a *P. falciparum* asexual parasitemia $>500/\mu L$. This definition has a sensitivity and specificity $>90\%$ in this age group [18]. The secondary case definition was fever or history of fever in the previous 24 hours plus any asexual *P. falciparum* parasitemia.

Exploratory efficacy endpoints were first or only clinical episode of *P. falciparum* malaria as well as multiple episodes of clinical malaria detected by PCD during 14 months after dose 1. An additional endpoint was first or only clinical episode of *P. falciparum* malaria detected by a combination of ADI and PCD.

Statistical methods

Analyses were done for intention to treat (ITT) and according to protocol (ATP) cohorts, following a predefined analytical plan. The ITT cohort included all children who received at least one dose of the study vaccine. All safety analyses were based on ITT. The ATP cohort included participants that met all eligibility criteria, completed the vaccination course and contributed to follow-up time during the evaluation period. For exploratory analyses, the ATP cohort was split into two follow-up periods: follow-up over study months 3–9 (ATP_{3-9}) and study months 3–14 (ATP_{3-14}). VE explored both first or only episode and multiple episodes of clinical malaria detected during the two study periods.

Analysis of immunogenicity was based on the ATP cohort, excluding children that received any blood product, immunosuppressant or immune-modifying therapy. Measurements of anti-CS and anti-HBs antibodies were summarized by Geometric Mean Titres (GMTs) with 95% confidence intervals (95% CI). Titres below the cut-off were assigned an arbitrary value of half the cut-off of the assay for the purpose of GMT calculation.

Person years at risk (PYAR) accounted for absences from the study area and use of antimalarial drugs as previously described [5].

VE was defined as 1 minus the hazard ratio multiplied by 100 [(1 – HR)*100] and was adjusted for distance to health facility [5] and community of residence. The adjusted VE was assessed using Cox regression models (for the first or only episode) and Poisson regression (for multiple episodes).

A test based on the Schoenfeld residuals was performed to assess whether the hazard was constant over the surveillance period, and alternative approaches were applied if the assumption of proportional hazards was not supported.

The risk of clinical malaria as a function of immune response was evaluated by comparing post-vaccination anti-CS titters for $RTS,S/AS02_D$ recipients who either did or did not experience at least one episode of clinical malaria meeting the primary case definition over ATP_{3-14} follow-up, using the Wilcoxon Rank Sum test. The hazard rate per 2-fold increase in post-vaccination anti-CS response was calculated for both ATP_{3-9} and ATP_{3-14} follow-up, along with their 95% confidence intervals.

Analyses were performed using SAS version 9.1 (Cary, NC, USA) and STATA version 10 (College Station, TX, USA).

Results

Of the 251 infants aged 6 to 12 weeks screened for eligibility, 214 were recruited and randomized to the $RTS,S/AS02_D$ group (107) or the control group (107). A total of 177 children completed the 14 months follow-up period: 91 in the $RTS,S/AS02_D$ group and 86 in the *Engerix-B*™ group (Fig. 1). Results of the initial 3.5 months of follow-up were reported elsewhere [10].

Vaccine safety

Safety data was available for 214 children. 107 received 301 doses of $RTS,S/AS02_D$ and 309 doses of *TETRActHib*™ and 107 received 303 doses of *Engerix-B*™ and 311 doses of *TETRActHib*™. Compliance for completion of symptoms questionnaires was 100%.

Solicited AEs after $RTS,S/AS02_D$ or Engerix-B™ vaccinations. Three recipients of the *Engerix-B*™ vaccine reported grade 3 solicited general symptoms, all of them considered to be related to the vaccine but resolving within the 7 day follow-up period (Table 1). None of the $RTS,S/AS02_D$ group participants reported grade 3 solicited general events. None of the solicited local symptoms reported in either group were of grade 3 intensity.

In both groups the most common solicited local symptom was pain at the injection site. There was no apparent trend in incidence of either pain or swelling with subsequent doses of $RTS,S/AS02_D$ or *Engerix-B*™.

Solicited AEs after TETRActHib™ vaccinations. Five children (4 in the *Engerix-B*™ and 1 in the RTS,S group) experienced grade 3 solicited general symptoms following either the first or the second *TETRActHib*™ dose (Table 2). All of these events were considered to be related to vaccination and the children fully recovered. None of the solicited local symptoms were reported to be of grade 3 intensity.

Pain at the injection site was the most frequently reported solicited local symptom. The incidence of pain and swelling was similar in both vaccine groups. There was no apparent trend in incidence of either pain or swelling with subsequent doses of *TETRActHib*™.

Unsolicited adverse events. Unsolicited AEs occurring within 30 days following vaccination were reported by 86.9% of participants in both vaccine groups. In both groups, the most frequently reported diagnosis was upper respiratory tract infection (49.5% of subjects in the $RTS,S/AS02_D$ and 45.8% of subjects in the *Engerix-B*™ group) (data not shown).

No unsolicited AE was considered to be causally related to the study vaccines.

Grade 3 unsolicited events were rare, occurring with similar frequency in both comparison groups. Five subjects (4.7%) reported ten grade 3 unsolicited AEs in the $RTS,S/AS02_D$ group [anemia (4), bronchopneumonia (1), *P. falciparum* infection (4), pneumonia (1)] and seven subjects (6.5%) reported twelve grade 3 unsolicited AEs in the *Engerix-B*™ group [anaemia (1), conjunctivitis (1), pyrexia (1), bronchitis (1), bronchopneumonia (1), skin furuncle (1), gastroenteritis (3), pneumonia (2), bronchospasm (1)] (data not shown).

SAEs. There were 69 children with at least one SAE (35 in the $RTS,S/AS02_D$ and 34 in the *Engerix-B*™ group) as shown in Table 3. The proportion of subjects reporting an SAE was similar

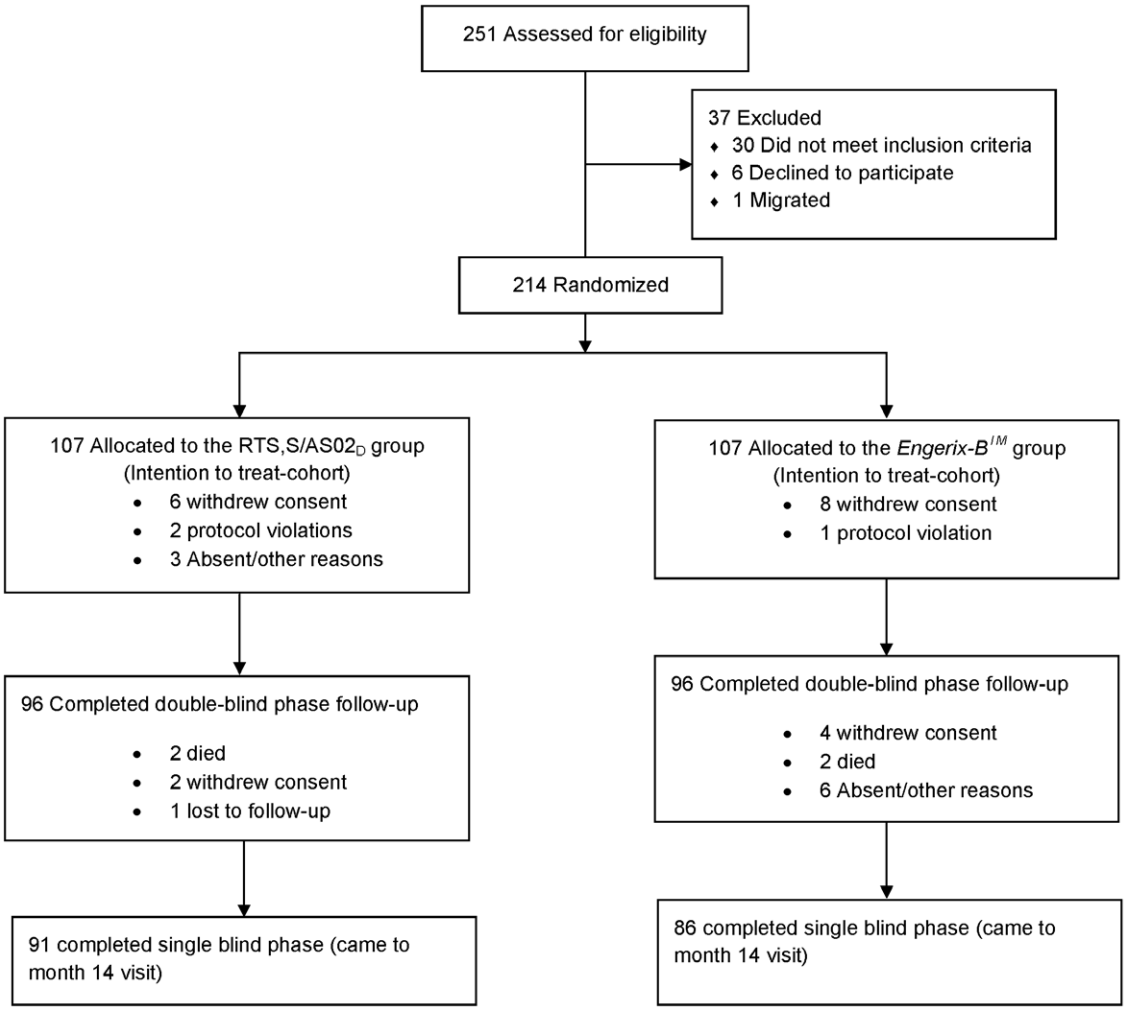


Figure 1. Trial Profile.
doi:10.1371/journal.pone.0013838.g001

in the RTS,S/AS02$_D$ (32.7%, 95% CI 24.0–42.5) and the control group (31.8%, 95% CI 23.1–41.5). None of them were considered to be related to vaccination. The total number of SAEs classified according to the Medical Dictionary for Regulatory Activities (MedDRA)[19] preferred terms was 157 (75 in the RTS,S/AS02$_D$ and 82 in the control group).

During the entire follow-up period, 15 participants in the RTS,S/AS02$_D$ group reported *P. falciparum* as an SAE requiring hospitalization, corresponding to 14.0% (95% CI 8.1–22.1). In the control group, there were 13 participants hospitalized with malaria, corresponding to 12.1% (95% CI 6.6–19.9). All cases fully recovered. The other main diagnoses of SAEs requiring admission were anaemia (15.9% vs 12.1%) gastroenteritis (12.1% vs 16.1%) and pneumonia (8.4% vs 7.5%) in the RTS,S/AS02$_D$ and *Engerix-B*^{TM} groups, respectively (data not shown).

Four deaths occurred during follow-up (two in each group). None of the deaths was judged to be related to vaccination. In the RTS,S/AS02$_D$ group, an eight month old girl died at home four months after having received a study vaccine. The presumptive diagnosis based on the verbal autopsy obtained from the mother was staphylococcal septic shock (recorded history of fever, generalised vesicular eruption, skin peeling and face swelling prior to death).

The second death in this group also occurred at home nine months after the child had received the last study vaccination. The 15 month HIV negative old boy had previously been admitted for a *Streptococcus pneumoniae* pneumonia (confirmed by a positive blood culture) and anaemia. According to the verbal autopsy, the death occurred after about 3 weeks of fever, vomiting, diarrhoea, abdominal pain and pallor. The parents did not seek treatment at any health facility. The final diagnosis following verbal autopsy review was chronic gastroenteritis with severe dehydration.

In the *Engerix-B*^{TM} group, a 10 month old boy died at home six months after receiving the third dose of the vaccine. The child had been seen by a field worker 3 days before he died and he appeared to be in good health. The verbal autopsy performed to the mother revealed that 24 hours prior to the fatal event the child abruptly started with intense vomiting and diarrhoea. The mother took him to a traditional healer who administered him ''traditional medication''. The child died shortly after. The probable cause of death was severe dehydration from gastroenteritis. The possibility of an adverse effect secondary to traditional medicine ingestion could not be excluded.

The second death was of an 11 month old girl, who died at home 7 months after the last vaccination with *Engerix-B*^{TM}.

Table 1. Incidence of solicited general symptoms by dose within the 7-day follow-up after RTS,S/AS02$_D$ or *Engerix-B*^{TM}.

	After dose 1				After dose 2				After dose 3			
	RTS,S/AS02$_D$		*Engerix -B*		RTS,S/AS02$_D$		*Engerix -B*		RTS,S/AS02$_D$		*Engerix-B*	
	(N = 105)		(N = 106)		(N = 99)		(N = 100)		(N = 97)		(N = 97)	
	n	%	n	%	N	%	n	%	n	%	n	%
General symptoms												
Drowsiness												
Any	27	25.7	37	34.9	32	32.3	26	26.0	28	28.9	32	33.0
Related	10	9.5	11	10.4	8	8.1	3	3.0	3	3.1	5	5.2
Fever												
Any	11	10.5	5	4.7	10	10.1	10	10.0	8	8.2	9	9.3
Related	11	10.5	5	4.7	10	10.1	9	9.0	8	8.2	9	9.3
Grade 3	0	-	0	-	0	-	1	1.0	0	-	1	1.0
Grade 3 related	0	-	0	-	0	-	1	1.0	0	-	1	1.0
Irritability												
Any	43	41.0	39	36.8	49	49.5	39	39.0	42	42.3	46	47.4
Related	27	25.7	13	12.3	25	25.3	16	16.0	17	17.5	20	20.6
Grade 3	0	-	0	-	0	-	0	-	0	-	1	1.0
Grade 3 related	0	-	0	-	0	-	0	-	0	-	1	1.0
Loss of appetite												
Any	19	18.1	27	25.5	25	25.3	24	24.0	28	28.9	29	29.9
Related	2	1.9	1	0.9	1	1.0	1	1.0	3	3.1	3	3.1
Local symptoms												
Pain												
Any	103	98.1	95	89.6	92	92.9	82	82.0	80	82.5	81	83.5
Grade 3	0	-	0	-	0	-	0	-	0	-	0	-
Swelling												
Any	10	9.5	12	11.3	11	11.1	8	8.0	8	8.2	8	8.2
Grade 3	0	-	0	-	0	-	0	-	0	-	0	-

N = Number of subjects with at least one symptom sheet completed; n/% = number and percentage of subjects reporting a specified symptom.

doi:10.1371/journal.pone.0013838.t001

According to the child's father, the child had diarrhoea and fever for 4 days, stopped eating, and progressively developed sunken eyes and pallor. The child was not brought to the health centre. Clinician's review of the verbal autopsy report concluded that the most probable cause of death was severe dehydration secondary to gastroenteritis.

Monitoring of hematological and biochemical parameters. Hematological values outside the normal range were infrequent. The majority of abnormal values of hemoglobin, white blood cells and platelets were of grade 1 intensity and occurred with similar incidence in the two groups. One child with concomitant malaria in the RTS,S/AS02$_D$ group had a low platelet count (44×10^9/L) of grade 2 intensity one month after the last vaccination. This value was within the normal range (167×10^9/L) at month 6.

Biochemistry values outside the normal range were also infrequent. The majority of out of range ALT and bilirubin values were of grade 1 intensity, occurring with a similar incidence in the two groups. One participant in the RTS,S/AS02$_D$ group had a grade 2 ALT value one week after the first dose (162 µmol/L) which dropped to 38 µmol/L one month after the third dose and to 39 µmol/L by study month 6. No creatinine values were outside the normal ranges.

Vaccine immunogenicity

ATP analysis of vaccine immunogenicity at month 14 included 151 children (73 in the RTS,S/AS02$_D$ and 78 in the control group). The anti-CS antibody GMTs declined from 199.9 EU/mL one month post dose 3 to 58.8 EU/mL and 7.3 EU/mL by 3.5 and 12 months post dose 3 respectively in the RTS,S/AS02$_D$ group. In the control group, anti-CS antibody GMTs were below the assay cut off (0.5 EU/mL) at all post vaccination time points.

In the RTS,S/AS02$_D$ group, the anti-HBs antibody GMTs declined from 10082 mIU/mL one month after dose 3 to 2751 mIU/mL by 12 months post dose 3. In the *Engerix-B*^{TM} group, the anti-HBs GMTs were 392.4 mIU/mL and 263.9 mIU/mL at the same time points. All children of both RTS,S/AS02$_D$ and control groups were seroprotected for Hepatitis B at 12 months post dose 3.

Vaccine efficacy

Results of VE analyzed over three different time periods are summarized in Table 4.

It should be noted that the trial was not powered for VE against clinical malaria and all analyses herein are exploratory.

VE analysis between months 3 to 9 of follow-up (ATP_{3-9}) was 48.8% (95% CI 11.3–70.4, p = 0.017) against first or only clinical

Table 2. Incidence of solicited general symptoms by dose within the 7-days follow-up after *TETRActHib*^{TM} according to randomization group.

	After dose 1				After dose 2				After dose 3			
	RTS,S/AS02$_D$		*Engerix -B*		RTS,S/AS02$_D$		*Engerix -B*		RTS,S/AS02$_D$		*Engerix -B*	
	(N = 107)		(N = 107)		(N = 102)		(N = 104)		(N = 100)		(N = 100)	
	n	%	n	%	N	%	n	%	n	%	n	%
General symptoms												
Drowsiness												
Any	31	29.0	25	23.4	29	28.4	27	26.0	28	28.0	30	30.0
Related	12	11.2	12	11.2	3	2.9	8	7.7	5	5.0	3	3.0
Grade 3	0	-	1	0.9	0	-	0	-	0	-	0	-
Grade 3 related	0	-	1	0.9	0	-	0	-	0	-	0	-
Fever												
Any	10	9.3	13	12.1	6	5.9	12	11.5	8	8.0	5	30.0
Related	10	9.3	13	12.1	6	5.9	12	11.5	8	8.0	5	3.0
Grade 3	0	-	1	0.9	1	1.0	1	1.0	0	-	0	-
Grade 3 related	0	-	1	0.9	1	1.0	1	1.0	0	-	0	-
Irritability												
Any	59	55.1	59	55.1	44	43.1	48	46.2	49	49.0	50	50.0
Related	40	37.4	47	43.9	18	17.6	29	27.9	26	26.0	23	23.0
Grade 3	0	-	1	0.9	0	-	0	-	0	-	0	-
Grade 3 related	0	-	1	0.9	0	-	0	-	0	-	0	-
Loss of appetite												
Any	22	20.6	15	14.0	26	25.5	20	19.2	27	27.0	30	30.0
Related	3	2.8	2	1.9	0	-	1	1.0	4	4.0	3	3.0
Local symptoms												
Pain												
Any	105	98.1	103	96.3	98	96.1	102	98.1	92	92.0	93	93.0
Grade 3	0	-	0	-	0	-	0	-	0	-	0	-
Swelling												
Any	22	20.6	18	16.8	14	13.7	22	21.2	16	16.0	22	22.0
Grade 3	0	-	0	-	0	-	0	-	0	-	0	-

N = Number of subjects with at least one symptom sheet completed; n/% = number and percentage of subjects reporting a specified symptom.
doi:10.1371/journal.pone.0013838.t002

episodes and 53.7% (95% CI 21.4–72.7, p = 0.004) against multiple episodes.

VE against first or only episodes of clinical malaria over the entire follow-up period up to month 14 (ATP_{3-14}) was 33.0% (95% CI -4.3–56.9, p = 0.076) and VE against multiple malaria episodes was 25.9% (95% CI -15.7–52.6, p = 0.167). Figure 2 shows Kaplan-Meier curves of the cumulative incidence of first or only episodes of clinical malaria in both groups. A test based on the

Table 3. Percentage of participants reporting SAEs classified by MedDRA primary organ class and preferred term over 14 months follow-up.

	Engerix-B (N = 107)			RTS,S/AS02$_D$ (N = 107)		
	n	%	95% CI	n	%	95% CI
Number of subjects with at least one SAE reported	34	31.8	23.1–41.5	35	32.7	24.0–42.5
Number of SAEs reported classified by MedDRA preferred term*	82	76.6	67.5–84.3	75	70.1	60.5–78.6

N = number of subjects with at least one administered dose and included in ITT cohort.
n/% = number/percentage of subject reporting at least once the symptom.
*Symptoms reported by a subject after a given dose and classified by the same Preferred Term are counted once.
doi:10.1371/journal.pone.0013838.t003

Table 4. Vaccine efficacy evaluated for different follow-up periods.

	Engerix B (n = 92)			RTS,S/AS02$_D$ (n = 93)			Vaccine Efficacy		
	Events	PYAR	Rate	Events	PYAR	Rate		95% CI	p
ATP$_{(3–9)}$									
First or only (FO) episode of fever and parasitemia >500/µl	34	31.5	1.08	21	38.2	0.55	48.8%	11.3–70.4	0.017
FO episode of fever or history of fever* and parasitemia >0/µl	48	27.7	1.74	29	36.4	0.80	54.5%	27.3–71.5	0.001
Multiple episodes of fever and parasitemia>500/µl	45	36.2	1.24	23	40.2	0.57	53.7%	21.4–72.7	0.004
Multiple episodes of fever or history of fever* and parasitemia >0/µl	72	36.0	2.00	34	40.0	0.85	58.9%	35.8–73.6	<0.001
ATP$_{(3–14)}$									
First or only (FO) episode of fever and parasitemia >500/µl	45	51.3	0.88	36	61.7	0.58	33.0%	−4.3–56.9	0.076
FO episode of fever or history of fever* and parasitemia >0/µl	57	41.4	1.38	45	57.1	0.79	41.9%	13.7–60.9	0.007
Multiple episodes of fever and parasitemia>500/µl	74	68.9	1.07	58	72.5	0.80	25.9%	−15.7–52.6	0.187
Multiple episodes of fever or history of fever* and parasitemia >0/µl	120	68.4	1.75	85	72.3	1.18	35.1%	2.2–57.0	0.039
ITT**$_{(0–14)}$									
First or only (FO) episode of fever and parasitemia >500/µl	54	79.4	0.68	46	90.2	0.51	25.9%	−9.9–50.0	0.136
Multiple episodes of fever and parasitemia>500/µl	105	111	0.94	82	113	0.72	24.3%	−12.9–49.2	0.173
Multiple episodes of fever and parasitemia>500/µl (PCD only)	95	112	0.85	80	113	0.71	17.6%	−24.2–45.3	0.355

*History of fever in previous 24 hours.
**ITT: n = 107 for each group.
PYAR = Persons-years at risk. Vaccine efficacy adjusted estimates for area and distance from health center (km).
ATP = According to the Protocol; ITT = Intention to Treat; PCD = Passive Case Detection.
doi:10.1371/journal.pone.0013838.t004

Schoenfeld residuals (p = 0.049) suggested that the hazard was not proportional over the follow-up period, consistent with the notion that VE waned over the course of the study.

Analysis of the relationship between anti-CS antibody levels and VE against clinical malaria suggested that within RTS,S recipients, the hazard rates of disease per 2 fold increase anti-CS titres at one month post dose 3 were significantly reduced by 84.1% (95% CI 43.5–95.5, p = 0.004) and 72.4% (95% CI 35.1–88.2, p = 0.003) for the two follow-up periods (ATP$_{3–9}$ and ATP$_{3–14}$), respectively.

Discussion

This is the first comprehensive safety and reactogenicity report of RTS,S/AS malaria vaccine in infants. We previously reported that VE against new *P. falciparum* infections was 65.9% during the

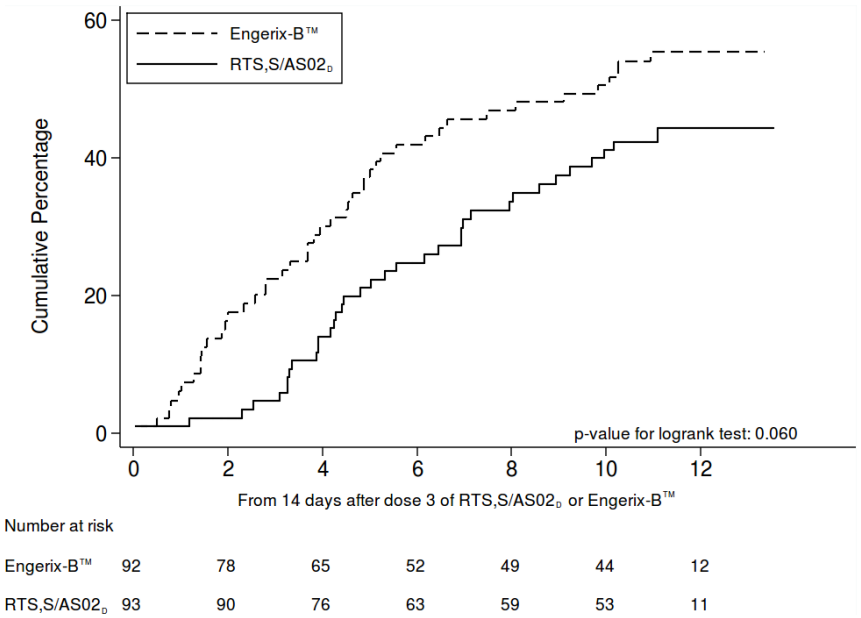


Figure 2. Kaplan-Meier curves for the cumulative proportion of children with at least one episode of clinical malaria between study months 3 to14 (ATP $_{3–14}$).
doi:10.1371/journal.pone.0013838.g002

initial three months after dose 3 in infants immunized in a staggered schedule with routine EPI vaccines [10]. This report goes further to include an exploratory analysis of VE against clinical malaria observed during the study.

The RTS,S/AS02$_D$ reactogenicity profile was similar to that recorded in previous trials in older age groups [2,5]. The safety profile in infants remained promising during the extended follow-up, with no significant differences between groups in the frequency of SAEs. No safety signals were found in the monitoring of hematologic and biochemistry data.

We observed no strong evidence of significant differences in the immunological responses to vaccination with RTS,S/AS02$_D$ in infants in this trial compared to older children. Anti-CS antibody titters decayed over time. However, there is no evidence that they did so more precipitously in this trial compared to other pediatric trials, where antibody decay profiles are consistent with a half-life of 6 to 8 weeks. In this trial, anti-CS responses at 12 months post-dose 3 remain 15 fold higher in the RTS,S/AS02$_D$ group than in the control group. Where longer follow-up data are available, low but persistently elevated anti-CS responses have been reported (10–30 times higher than in controls) also [6]. This is consistent with the induction of long term T cell and B cell memory responses by RTS,S/AS02. Such persistence of antibody responses is likely to be seen in this infant population but this can only be confirmed by longer follow-up as planned in the ongoing Phase 3 trials.

Anti-HBs responses were higher throughout the follow-up in recipients of RTS,S/AS02$_D$ than of the licensed Hepatitis B control vaccine probably reflecting the use of a different adjuvant system. HBs antibody titters also decayed over time. However, all children vaccinated with both RTS,S/AS02$_D$ and *Engerix-B*^{TM} vaccines reached seroprotection levels for anti-HBs 12 months post Dose 3.

Vaccine efficacy against clinical malaria over the 12 months follow-up period after dose three was 33% (95% CI -4.3–56.9, $p = 0.076$), whereas during the initial 3.5 months of double-blind follow-up the efficacy was 65.8% (95% CI 25.3–84.4, $p = 0.007$). This difference could be due to chance as the confidence intervals of the two estimates overlap, and the study is underpowered for such analyses. Nevertheless, together with the data showing that the hazard was not proportional over the follow-up periods, the results suggest that VE against clinical malaria may have waned over the 14 months follow-up period.

Caution is needed when attempting to compare the results of this study with data reported from a previous phase IIb trial conducted in this same area among children aged 1 to 4 years [5]. Cohort 2 of that trial had a very similar design to the infant study that we are reporting, including the administration of presumptive treatment with effective antimalarials between dose 2 and 3 and an initial follow-up through intense active detection of infections (ADI). In both studies, VE against clinical malaria appeared to wane over time [20]. This is in sharp contrast to cohort 1 of the phase IIb trial where children were only followed-up by passive case detection and did not have presumptive treatment. Among these children VE persisted at 30% for 45 months [6]. Reasons for this apparent differences in the duration of protection are discussed elsewhere [20].

While several previous trials have shown a relationship between anti-CS antibody responses and risk of malaria infection, this study provides the first evidence of a similar relationship between anti-CS antibodies and protection against clinical malaria. It is probable that in trials in older populations similar analyses have been confounded by the superimposed naturally acquired immunity.

In summary, these results confirm the good safety and immunogenicity profile of RTS,S/AS02$_D$ malaria vaccine in African infants, as well as confirm protection against clinical malaria for at least one year. Together they support the rationale for the ongoing Phase III trial.

Supporting Information

Protocol S1 Trial Protocol
Found at: doi:10.1371/journal.pone.0013838.s001 (0.92 MB PDF)

Checklist S1 CONSORT Checklist
Found at: doi:10.1371/journal.pone.0013838.s002 (7.60 MB RTF)

Acknowledgments

Special thanks to the participating children, mothers and fathers, and the entire Ilha Josina Machel and Taninga communities. We would also like to specially thank all the investigator assistants and field workers closely involved with the trial as well as all the CISM staff and the District, Provincial and National Health Authorities for their continuous support. Special thanks to Joe Campo and Caterina Guinovart for critically reviewing the manuscript.

Author Contributions

Conceived and designed the experiments: PA JJA JS AL ML JV MCD CL WRB JC PLA. Performed the experiments: PA MR TN JS IM QB PLA. Analyzed the data: JJA ML. Contributed reagents/materials/analysis tools: JJA IM MM. Wrote the paper: PA JJA MR TN JS IM QB MM AL ML JV MCD CL WRB JC PLA.

References

1. WHO (2008) World Malaria Report, 2008. Genever: World Health Organization.
2. Bojang KA, Milligan PJ, Pinder M, Vigneron L, Allouecche A, et al. (2001) Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet 358: 1927–1934.
3. Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, et al. (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med 336: 86–91.
4. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, et al. (2005) Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. Lancet 366: 2012–2018.
5. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, et al. (2004) Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. Lancet 364: 1411–1420.
6. Sacarlal J, Aide P, Aponte JJ, Renom M, Leach A, et al. (2009) Long-Term Safety and Efficacy of the RTS,S/AS02A Malaria Vaccine in Mozambican Children. J Infect Dis 200: 329–336.
7. Hutton G, Tediosi F (2006) The costs of introducing a malaria vaccine through the expanded program on immunization in Tanzania. Am J Trop Med Hyg 75: 119–130.
8. Schellenberg D, Menendez C, Kahigwa E, Aponte J, Vidal J, et al. (2001) Intermittent treatment for malaria and anaemia control at time of routine vaccinations in Tanzanian infants: a randomised, placebo-controlled trial. Lancet 357: 1471–1477.
9. Aponte JJ, Schellenberg D, Egan A, Breckenridge A, Carneiro I, et al. (2009) Efficacy and safety of intermittent preventive treatment with sulfadoxine-pyrimethamine for malaria in African infants: a pooled analysis of six randomised, placebo-controlled trials. Lancet.
10. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, et al. (2007) Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet 370: 1543–1551.

11. Abdulla S, Oberholzer R, Juma O, Kubhoja S, Machera F, et al. (2008) Safety and Immunogenicity of RTS,S/AS02D Malaria Vaccine in Infants. N Engl J Med.
12. Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, et al. (2008) Efficacy of RTS,S/AS01E Vaccine against Malaria in Children 5 to 17 Months of Age. N Engl J Med.
13. Nhacolo AQ, Nhalungo DA, Sacoor CN, Aponte JJ, Thompson R, et al. (2006) Levels and trends of demographic indices in southern rural Mozambique: evidence from demographic surveillance in Manhica district. BMC Public Health 6: 291.
14. Guinovart C, Bassat Q, Sigauque B, Aide P, Sacarlal J, et al. (2008) Malaria in rural Mozambique. Part I: children attending the outpatient clinic. Malar J 7: 36.
15. Macete E, Aponte JJ, Guinovart C, Sacarlal J, Ofori-Anyinam O, et al. (2007) Safety and immunogenicity of the RTS,S/AS02A candidate malaria vaccine in children aged 1-4 in Mozambique. Trop Med Int Health 12: 37–46.
16. Sacarlal J, Nhacolo AQ, Sigauque B, Nhalungo DA, Abacassamo F, et al. (2009) A 10 year study of the cause of death in children under 15 years in Manhica, Mozambique. BMC Public Health 9: 67.
17. Cambron P, Jacquet JM, Hoet B, Lievens M (2009) Development and technical and clinical validation of a quantitative enzyme-linked immunosorbent assay for the detection of human antibodies to hepatitis B surface antigen in recipients of recombinant hepatitis B virus vaccine. Clin Vaccine Immunol 16: 1236–1246.
18. Saute F, Aponte J, Almeda J, Ascaso C, Abellana R, et al. (2003) Malaria in southern Mozambique: malariometric indicators and malaria case definition in Manhica district. Trans R Soc Trop Med Hyg 97: 661–666.
19. (2008) MEDRA, Version 11.1 Geneva: Intenational Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). .
20. Guinovart C, Aponte JJ, Sacarlal J, Aide P, Leach A, et al. (2009) Insights into long-lasting protection induced by RTS,S/AS02A malaria vaccine: further results from a phase IIb trial in Mozambican children. PLoS ONE 4: e5165.

Article 3: *Plasmodium falciparum*-specific cellular immune responses after immunization with the RTS,S/AS02$_D$ candidate malaria vaccine in infants living in an area of high endemicity in Mozambique.

Arnoldo Barbosa, Denise Naniche, John J. Aponte, M. Nelia Manaca, Inacio Mandomando, Pedro Aide, Jahit Sacarlal, Montse Renom, Sarah Lafuente, W. Ripley Ballou and Pedro L. Alonso

Infection and Immunity 2009, 77(10); 4502–4509

INFECTION AND IMMUNITY, Oct. 2009, p. 4502–4509 Vol. 77, No. 10
0019-9567/09/$08.00+0 doi:10.1128/IAI.00442-09

Plasmodium falciparum-Specific Cellular Immune Responses after Immunization with the RTS,S/AS02D Candidate Malaria Vaccine in Infants Living in an Area of High Endemicity in Mozambique▽

Arnoldo Barbosa,[1,2] Denise Naniche,[2] John J. Aponte,[1,2] M. Nelia Manaca,[1] Inacio Mandomando,[1,4] Pedro Aide,[1,4] Jahit Sacarlal,[3] Montse Renom,[1,2] Sarah Lafuente,[1,2] W. Ripley Ballou,[5] and Pedro L. Alonso[1,2]*

Centro de Investigação en Saúde de Manhiça, Manhiça, Mozambique[1]; Barcelona Centre for International Health Research, Hospital Clinic/Institut d'Investigacions Biomediques August Pi i Sunyer, Universitat de Barcelona, Barcelona, Spain[2]; Facultade de Medicina, Universidade Eduardo Mondlane, Maputo, Mozambique[3]; National Institute of Health, Ministry of Health, Maputo, Mozambique[4]; and GlaxoSmithKline Biologicals, Rixensart, Belgium[5]

Received 20 April 2009/Returned for modification 6 June 2009/Accepted 23 July 2009

Results from clinical trials in areas where malaria is endemic have shown that immunization with RTS,S/AS02A malaria vaccine candidate induces partial protection in adults and children and cellular effector and memory responses in adults. For the first time in a malaria vaccine trial, we sought to assess the cell-mediated immune responses to RTS,S antigen components in infants under 1 year of age participating in a clinical phase I/IIb trial of RTS,S/AS02D in Mozambique. Circumsporozoite protein (CSP)-specific responses were detected in approximately half of RTS,S-immunized infants and included gamma interferon (IFN-γ), interleukin-2 (IL-2), and combined IL-2/IL-4 responses. The median stimulation indices of cytokine-producing $CD4^+$ and $CD8^+$ cells were very low but significantly higher in RTS,S-immunized infants than in infants that received the comparator vaccine. Protection against subsequent malarial infection tended to be associated with a higher percentage of individuals with CSP-specific IL-2 in the supernatant ($P = 0.053$) and with higher CSP-specific IFN-γ-producing $CD8^+$ T-cell responses ($P = 0.07$). These results report for the first time the detection of malaria-specific cellular immune responses after vaccination of infants less than 1 year of age and pave the way for future field studies of cellular immunity to malaria vaccine candidates.

Malaria remains one of the major world heath problems affecting between 200 and 400 million people annually and causing 2 to 3 million deaths, mostly children and pregnant women living in sub-Saharan Africa (37). Infections by *Plasmodium falciparum*, one of the four species of plasmodia that affect humans, cause 80 to 90% of the malaria cases and are responsible for 95% of all malaria-associated deaths (14). Since most of the worldwide malaria burden is due to *P. falciparum*, efforts for prevention and eradication of malaria have focused on this parasite, and a *P. falciparum*-customized malaria vaccine is one of most promising tools (12, 25, 26).

The most abundant and immunogenic antigen on the surface of *Plasmodium* sporozoites is the circumsporozoite protein (CSP), which is a target for vaccine development (9, 10, 17, 27). In vaccines based on irradiated sporozoites and CSP in human and mouse models, antibodies to circulating sporozoites, followed by cell-mediated responses to the protein after invasion of hepatocytes, have been described as crucial for the generation of protective responses (7, 11, 13, 28, 29).

RTS,S is a subunit malaria vaccine candidate based on the CSP of *P. falciparum* that has been under study for many years. The chimeric vaccine contains a portion of the NANP-repeats, all four NVDP-repeats, and the complete carboxyl-terminal region of CSP suggested to be targets for humoral and cellular immunity, along with the amino-terminal region of HbsAg (HBS) (16). The malaria vaccine candidate RTS,S (GlaxoSmithKline, Rixensart, Belgium) formulated with the adjuvant system AS01 or AS02 has proven to confer partial protective immunity against malaria infection in malaria-naive adults (20, 21, 41), as well as in adults and infants in areas where malaria is endemic (2–6). Clinical safety, immunogenicity, and efficacy trials in infants and children have shown RTS,S/AS02 to be safe and protective and to induce high antibody titers (2, 4, 6, 34).

Although the induction of a CSP-specific humoral response after RTS,S vaccination has been well described, the generation of cellular immune responses has not yet been addressed in infants or young children immunized with the RTS,S vaccine candidate. In adults, protection conferred by the RTS,S vaccine has been associated with acquisition of strong antibody and cellular responses to the CSP fragment of RTS,S (20, 22). Malaria naive volunteers immunized with RTS,S/AS02 frequently develop strong proliferative and IFN-γ-producing T-cell responses to peptides representing T-cell epitopes (Th2R and Th3R) present in the vaccine (22). A correlation between protection against experimental challenge and the CSP-specific production of IFN-γ by $CD4^+$ and $CD8^+$ T cells has been described in a limited number of individuals (42).

* Corresponding author. Mailing address: Barcelona Centre for International Health Research (CRESIB), Hospital Clinic/IDIBAPS, C. Rossello 132, 4°, 2≫, Barcelona 08036, Spain. Phone: (34) 93 227 5706. Fax: (34) 93 227 9853. E-mail: palonso@clinic.ub.es.

▽ Published ahead of print on 3 August 2009.

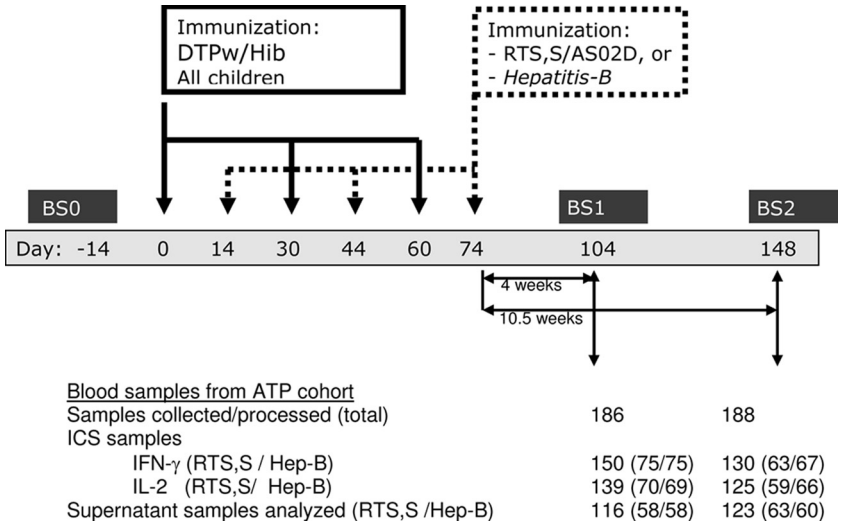


FIG. 1. Timeline of immunizations and blood sample collection. Timing for the blood samples used for cellular immunity studies are indicated: BS0, preimmune; BS1, 4 weeks postimmunization; and BS2, 10.5 weeks postimmunization.

Current efforts are under way to proceed to phase III clinical trials with the RTS,S vaccine, despite no currently identified immune correlates of protection for vaccination with RTS,S in infants or young children. The present study was integrated into a phaseI/IIb clinical trial of the RTS,S/AS02D candidate vaccine in infants in a rural area of malaria endemicity in Mozambique (4). We sought here to examine the cellular responses in infants vaccinated with RTS,S/AS02D and further the development of assays for use in malaria vaccine trials in infants and young children, the population most vulnerable to severe malaria.

MATERIALS AND METHODS

Study population. This study was integrated into a randomized placebo-controlled phase I/IIb clinical trial of the RTS,S/AS02D malaria candidate vaccine in infants living in a rural area of Mozambique (4) (registry URL, clinicaltrials.gov; trial registration no., NCT00197028). Briefly, mothers in their last trimester of pregnancy living in the Ilha Josina and Taninga communities of Manhiça district in Maputo, Mozambique, were invited to enroll their newborns after delivery in the clinical trial. After informed consent was obtained, the mothers were counseled regarding sexually transmitted diseases and tested for human immunodeficiency virus and hepatitis B virus infection (UniGold HIV [Trinity Biotech]; Determine HIV1-2 and HBsAg [Abbott Laboratories]). Mothers with a positive result for either serological test were excluded from the study and referred to the Manhiça District Hospital for clinical management according to national guidelines.

Vaccines. The pediatric version of RTS,S/AS02A contains 25 μg of RTS,S antigen and 0.125 ml of AS02D adjuvant as described by Macete et al. in 2007 (23, 24). The control vaccine Engerix-B is from GlaxoSmithKline, Belgium. TETRActHib (Adventis Pasteur, France), the vaccine used in the Expanded Programme on Immunization (EPI) program, is a lyophilized vaccine combining diphtheria and tetanus toxoid-pertussis vaccine (DTP) with *Haemophilus influenzae* type b conjugate vaccine (polyribosyl ribitol phosphate conjugated to tetanus protein).

Immunizations. All enrolled children received the standard polio (Chiron/Novartis, San Francisco, CA) and DTPw/Hib (DTP plus *H. influenzae* type b) vaccines as their EPI vaccinations at 8, 12, and 16 weeks of age at their local community health post. At the first EPI vaccination visit, they were randomly distributed into two immunization groups to receive either the RTS,S/AS02D or the hepatitis B Engerix-B vaccines. These intramuscular immunizations were given in a staggered manner blindly 2 weeks after the EPI vaccines at weeks 10, 14, and 18. A diagram of the immunization schedule is shown in the Fig. 1.

Blood samples. Approximately 2.0 ml of blood was drawn from infants prior to study vaccination (blood sample BS0) and then 4 and 10.5 weeks (blood samples BS1 and BS2) after the third immunization with RTS,S/AS02D or hepatitis B vaccine by heel prick, finger prick, or venipuncture for later visits. Blood was collected into heparin tubes at an ambient temperature and processed within 3 h for cellular immunology and biochemistry assays.

Malaria case detection. Malaria infections caused by *P. falciparum* were assessed by active detection and by passive case detection at health facilities in the study area as described previously (4). Active detection occurred at predefined intervals in which a blood slide for parasitemia determination was collected, and the axillary temperature was recorded irrespective of symptoms. Passive case detection was done through monitoring of all attendances at health facilities and ascertainment of episodes of clinical malaria, including blood smear for infants with documented fever (37.5°C or higher) or history of fever in the preceding 24 h, as described in detail elsewhere (24). Infants with any parasitemia > 0 were considered cases and used in the current analysis. After vaccination, parasitemia-positive infants were not included in the risk period for malaria for 28 days after receiving treatment. Clinical management was provided according to standard national guidelines.

Whole-blood cultures and peptide stimulation. To stimulate and culture whole blood, 250 μl of heparinized blood was dispensed into three aliquots supplemented with 150 μl of RPMI 1640 (incomplete medium; Invitrogen/Gibco-BRL), containing 20 mM HEPES, 100 U of penicillin ml^{-1}, 100 μg of streptomycin ml^{-1}, 2 mM L-glutamine, and mouse anti-human CD28 and anti-human CD49d antibodies (BD Biosciences, San Jose, CA) at final concentrations of 1.2 μg ml^{-1} each. The cultures were stimulated as follows with (i) CSP peptides encompassing sequences present in RTS,S; (ii) HBS peptides present in RTS,S; and (iii), as a negative control, the peptide diluent alone, dimethyl sulfoxide at a 1/1,000 dilution. CSP and HBS peptides were 15-mer long, overlapping peptides by 11 amino acids used at 1.25 μg ml^{-1} as described previously (39). Due to small volumes of blood, positive control stimulation with mitogen (phytohemagglutinin) was not possible for most of the donors and was performed in ca. 10% of the samples in parallel with acridine orange-ethidium bromide staining to ensure viability of >95%. Peptide stimulation incubation was performed at 37°C in 5% CO_2 for ~42 h, after which 100 μl of the supernatant was collected and stored at −80°C until use in cytokine detection assays. An approximate 42- to 48-h stimulation yielded the best signal-to-noise ratio compared to 24- and 72-h incubations (data not shown). Brefeldin A was added to the culture (Golgi Plug; BD Pharmingen, San Diego, CA), and cells were kept in incubation for another 6 h prior to flow cytometry.

Intracellular cytokine staining (ICS). After an approximately 42-h peptide stimulation, erythrocytes were lysed by adding fluorescence-activated cell sorting lysing solution (BD Biosciences). Cells were then stained with fluorochrome-conjugated antibodies to the cell surface markers anti-CD8/APC, anti-CD4/fluorescein isothiocyanate, and anti-CD3/PerCP (BD Pharmingen); fixed and

permeabilized with a solution containing paraformaldehyde and saponin (Cytofix/Cytoperm; BD Biosciences), and stained with phycoerythrin-conjugated anti-IFN-γ or anti-IL-2 antibodies (FastImmune; BD Biosciences). A minimum of 50,000 gated lymphocyte events were collected on a BD FACSCalibur cytometer, and the unlabeled clones for anti-CD3, anti-CD4, anti-CD8, anti-IFN-γ, and anti-IL-2 immunoglobulin G antibodies and Calibrite beads (BD Biosciences) were used for compensation settings. To reduce interassay variations, we used the control stimulated cells as a reference for specific stimulation. Plots of CD3-CD4 and CD3-CD8 versus each cytokine were generated for control stimulated cells, and a threshold gating was placed to objectively exclude at least 99.5% cytokine-phycoerythrin-negative control cells from the cytokine-positive quadrant. The same threshold was placed on the cells stimulated with CSP and HBS peptides. Thus, antigen-specific cells were defined as the $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ cells in the quadrant above the threshold. Stimulation indexes (SIs) were calculated as ratios between the proportions of peptide-specific cells over the proportion of control stimulated cells for each individual in order to account for donor variation in background signal.

Determination of concentration of cytokines in supernatant. Detection of human IFN-γ, IL-2, and IL-4 in 25 μl of each supernatant, diluted 1:2, was performed by using a four-bead CBA-Flex customized kit and analyzed using the FCAP array software (v.1.0.1; BD Biosciences). To assess the antigen-specific production of cytokines, concentration values from the control-stimulated culture supernatants were subtracted from the concentration values from peptide-stimulated supernatants for the same blood sample. Threshold values for evaluating positive responses in supernatant were placed above the 98th percentile for all preimmune cytokine levels using reverse cumulative distribution plots. The cytokine cutoffs were therefore set as follows: IFN-γ at 20 pg ml^{-1}, IL-2 at 100 pg ml^{-1}, and IL-4 at 5 pg ml^{-1}.

Statistical methods. The according-to-protocol (ATP) cohort included subject samples that met all eligibility criteria and complying with all procedures defined in protocol. Criteria for analysis of cellular immunity results included children who correctly completed the protocol of three immunizations that were followed up for 10 weeks and had an available sample (referred to as the cell-mediated immunity [CMI] cohort) (Fig. 1).

Reverse cumulative distribution plots were used for rapid visual assessment of the distributions (32). Differences between both vaccine groups in intracellular median SIs and cytokine median concentrations in the supernatant were assessed by using the Wilcoxon rank-sum test and comparison of proportions was performed by using the Fisher exact test. McNemar chi-square and Wilcoxon rank-sum tests were used to compare preimmune and postimmune proportions and the distribution of positive responses in supernatant responders. Analyses were performed using Stata 9 software (StataCorp LP).

RESULTS

Study population and samples included for analysis of cellular immunity. From August 2005 to September 2006, 214 infants were enrolled in the phase I/IIb double-blind randomized placebo-controlled trial of RTS,S/AS02D in Mozambique as described previously (4). Whole-blood cell cultures for cell immunogenicity measures were performed from 206 blood samples taken before immunization at BS0, from 186 samples taken 4 weeks after the third immunization (BS1), and from 188 samples taken 10.5 weeks after the third immunization (BS2) (Fig. 1). Detection of secreted (supernatant) and nonsecreted (intracellular) cytokines were measured in the same whole-blood cell cultures. The data were available for the assessment of secreted cytokines at BS1 and BS2 for 62 and 65% of the samples, respectively, whereas for ICS from BS1 and BS2 the data were available from 80 and 70% of the samples, respectively. Missing data were equally distributed between the immunization groups.

Basal levels of secreted cytokines. Prior to assessing postimmunization cytokine secretion, basal preimmune cytokine production was analyzed. Preimmune specific levels of IFN-γ, IL-2, and IL-4 were low (apart from one outlier) ranging from mostly undetectable up to 45.2, 51.6, and 5.3 pg ml^{-1}, respectively. Median white blood cell counts were similar between the RTS,S/ASO2D and hepatitis B immunization groups at BS0 (9.7, with an interquartile range [IQR] of 8.3 to 11.8, and 9.9, with an IQR of 8.7 to 11.6), as well as at other time points (data not shown).

CSP-specific cytokine responses in supernatants after immunization. CSP-specific cytokine concentrations were assessed after immunization at 4 weeks (BS1) and 10.5 weeks (BS2) postimmunization. The median concentration of CSP-specific IL-2 was significantly higher in the RTS,S/AS02D vaccine group compared to the hepatitis B vaccine group both at BS1 and BS2 (Fig. 2). The median CSP-specific IL-2 concentrations at BS1 for the RTS,S/AS02D group and for the hepatitis B group were 24.5 (IQR = 1.0 to 43.6) and 0.0 (IQR = 1.0 to 22.6), respectively, and at BS2 the medians were 25.3 (IQR = 1.0 to 95.5) and 0.0 (IQR = 1.0 to 20.8), respectively. A higher proportion of infants produced IFN-γ, IL-2, and IL-4 after immunization with RTS,S/AS02D compared to the preimmune group (Fig. 3). In particular, the proportion of infants with an IL-2 response was significantly higher in the RTS,S/AS02D group than in the hepatitis B group at both 4 weeks (BS1, $P < 0.01$ [Fisher exact test]) and 10.5 weeks (BS2, $P < 0.001$ [Fisher exact test]) after immunization (Fig. 3B). Interestingly, as shown in Fig. 3, a significantly higher proportion of positive responders was more often observed at BS2 after a longer (10.5-week) postimmunization interval.

HBS-specific production of cytokines in supernatant after immunization. Since HBsAg was present in both the RTS,S/AS02D and the control hepatitis B vaccines, HBS stimulation was expected to generate cytokine production in both immunized groups. Children immunized with the RTS,S formulation showed slightly higher HBS-specific IFN-γ and IL-2 responses compared to the hepatitis B vaccine group (data not shown). Compared to preimmune cytokine production, the proportion of children with a HBS-specific IFN-γ and IL-2 production increased significantly for both vaccine groups at both 4 weeks (BS1) and 10.5 weeks (BS2) (Fig. 3C and D). However, there was a greater proportion of infants producing HBS-specific IL-2 in the RTS,S group than in the hepatitis B vaccine group (Fig. 3, $P_{IL\text{-}2} = 0.05$ [Fisher exact test]).

Assessment of CSP- and HBS-specific intracellular cytokine production. To describe the role of $CD4^+$ and $CD8^+$ T cells in IFN-γ and IL-2 responses to RTS,S, intracellular IFN-γ and IL-2 cytokine staining was performed in the same cell culture after supernatant collection. Averages of 21,155 ± 9,763 $CD4^+$ T cells and 8,532 ± 4,521 $CD8^+$ T cells were analyzed for IFN-γ intracellular staining, and averages of 17,100 ± 7,542 $CD4^+$ T cells and 6,917 ± 3,773 $CD8^+$ T cells were analyzed for IL-2 intracellular staining. The percentage of antigen-specific cytokine-expressing cells was generally very low and, after subtraction of control-stimulated values, the maximum ranged from 3.5% to 6% depending on the stimulating antigen. Median SIs for both $CD4^+$ and $CD8^+$ T cells ranged from 0.81 to 1.34 in both immunization groups (Table 1). When CSP-specific responses were assessed, it was observed that children immunized with RTS,S had a higher median SI of CSP-specific IFN-γ-producing $CD8^+$ T cells (Table 1, $P_{IFN\text{-}\gamma} = 0.029$ [Wilcoxon rank-sum test]) and CSP-specific IL-2-producing $CD4^+$ T cells than did the hepatitis B vaccination group (Table 1, $P_{IL\text{-}2} = 0.043$ [Wilcoxon rank-sum test]). Evaluation of HBS-

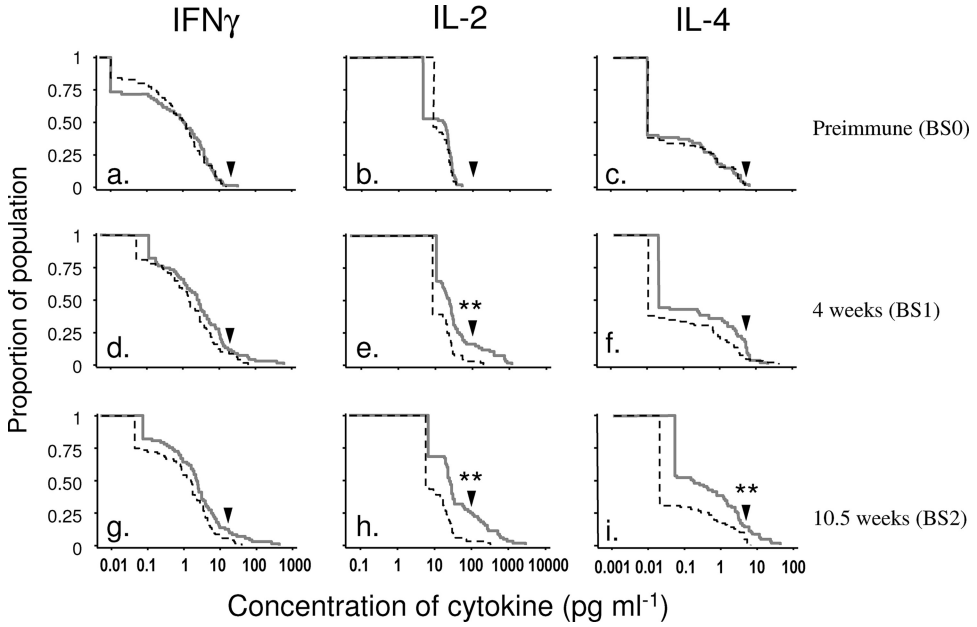


FIG. 2. Reverse cumulative distribution of cytokine concentration in supernatants from CSP-stimulated cell cultures. Plots for preimmune (a to c) and for 4-week (d to f) and 10.5-week (g to i) postimmunization samples are shown in the top, middle, and bottom rows, respectively. Immunization groups are represented by boldface (RTS,S/AS02D) and dotted (hepatitis B) lines. Arrowheads show the concentration cutoffs for positive responses: 20 pg ml-1 for IFN-γ, 100 pg ml-1 for IL-2, and 5 pg/ml-1 for IL-4. The Wilcoxon rank-sum test was used to compare cytokine concentrations between both immunization groups. Significant differences ($P < 0.01$) are indicated by asterisks (**).

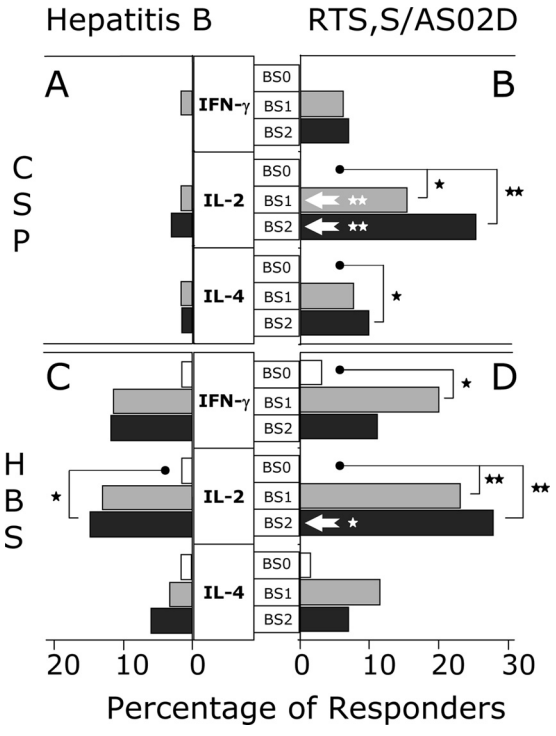


FIG. 3. Frequency of CSP- and HBS-specific cytokine responses in supernatants of children immunized with RTS,S/AS02D and hepatitis B (Engerix-B) vaccines before immunization (BS0, □), 4 weeks postimmunization (BS1, ▨), and 10.5 weeks postimmunization (BS2, ■). Differences in the proportions of positive responders before and after immunization (brackets) and between immunization groups (arrows) were assessed by using the McNemar chi-square and Fisher exact tests, respectively. Significant differences: *, $P < 0.05$; **, $P < 0.001$.

specific responses showed that the median SI of HBS-specific IL-2 and IFN-γ-producing $CD8^+$ T cells was also higher in the RTS,S group than in the hepatitis B vaccine group (Table 1, $P_{IFN-\gamma} = 0.015$ and $P_{IL-2} = 0.030$ [Wilcoxon rank-sum test]).

Evaluation of association between CSP-specific cytokine responses and malaria infection. As described by Aponte et al. (4), "first or only" (FO) cases of *P. falciparum* infection postimmunization, as assessed by both active and passive detection of parasitemia, were documented during the follow-up period in 22 infants from the RTS.S/AS02D group and in 46 infants from the hepatitis B control group, with a resulting adjusted vaccine efficacy of 65.9% for malaria infection. The percentages of infants having had a FO malaria case were similar between the efficacy ATP cohort and the CMI cohort (24.4 and 24.2%, respectively). We sought to assess whether there was any difference in CSP-specific cytokine responses in the RTS,S/AS02D group between infants with reported malaria infection and those without. Table 2 shows the median CSP-specific intracellular $CD4^+$ and $CD8^+$ SIs for IL-2 and IFN-γ responses and the proportion of infants with positive IL-2 supernatant responses, according to FO cases of malaria parasitemia.

Among infants immunized with RTS,S, there was a trend toward a higher proportion of individuals who did not have an episode of malaria infection with a positive CSP-specific IL-2 response in supernatants (>100 pg ml^{-1}) compared to infants who suffered an episode of malarial infection ($P = 0.053$ [Fisher exact chi-square test], Table 2). Likewise, the median SI for IFN-γ-producing $CD8^+$ T cells was higher in infants with no FO malarial infection than in those with FO malaria, although this difference did not reach significance ($P = 0.07$, Table 2). There was no difference in cytokine production be-

TABLE 1. Comparison of CSP- and HBS-specific IFN-γ and IL-2 $CD4^+$ and $CD8^+$ responses between RTS,S/AS02D and control hepatitis B immunization groups[a]

T-cell stimulus	Time (wk) after third dose	Immunization group						P^b
		Hepatitis B			RTS,S/AS02D			
		n	SI		*n*	SI		
			Median	IQR		Median	IQR	
IFN-γ								
CSP $CD4^+$	4	75	1.20	0.77–1.61	75	1.16	0.85–1.75	0.611
	10.5	69	1.09	0.85–1.47	63	1.18	0.90–1.59	0.213
CSP $CD8^+$	4	75	1.04	0.67–1.57	75	1.04	0.66–1.57	0.891
	10.5	69	1.05	0.67–1.41	63	1.25	0.84–1.85	0.029*
HBS $CD4^+$	4	75	1.17	0.71–1.75	75	1.24	0.76–1.67	0.533
	10.5	69	1.03	0.74–1.47	63	1.08	0.77–1.59	0.769
HBS $CD8^+$	4	75	1.08	0.63–1.71	75	1.06	0.74–1.70	0.423
	10.5	69	0.81	0.58–1.21	63	1.08	0.68–1.49	0.015*
IL-2								
CSP $CD4^+$	4	69	1.05	0.71–1.65	70	1.32	0.95–2.00	0.052
	10.5	68	1.00	0.66–1.44	59	1.25	0.92–1.74	0.043*
CSP $CD8^+$	4	69	1.04	0.66–1.86	70	1.14	0.78–1.86	0.626
	10.5	68	0.86	0.54–1.38	59	1.04	0.76–1.61	0.086
HBS $CD4^+$	4	69	1.19	0.69–1.79	70	1.34	0.95–1.79	0.185
	10.5	68	1.04	0.72–1.37	59	1.18	0.81–1.88	0.065
HBS $CD8^+$	4	69	1.14	0.62–1.67	70	1.16	0.83–1.65	0.451
	10.5	68	0.89	0.52–1.26	59	1.28	0.63–1.65	0.030*

[a] Results are from ICS assays and are expressed as SIs as described in Materials and Methods. IQR = quartile 25−75. *n*, Number of subjects.
[b] As determined by the Wilcoxon rank-sum test. *, $P < 0.05$ (significance).

tween infants who suffered malaria infection in the hepatitis B vaccine group ($P = 1.00$ [data not shown]).

DISCUSSION

This study is the first description of cellular immune responses induced by a candidate malaria vaccine (RTS,S/AS02D) in infants less than 1 year of age. Evaluation of cell-mediated responses to both the CSP and HBsAg components of RTS,S was performed in infants participating in a phase I/IIb clinical trial (4). Our data show that the RTS,S/AS02D vaccine was immunogenic in infants, eliciting detectable cellular immune responses to both CSP and HBS antigens after immunization.

Secreted IL-2 was the strongest and most frequent CSP-specific response observed and was detected in about a quarter (25.3%) of the RTS,S/AS02D-immunized infants at 10.5 weeks after immunization. In addition, HBS-specific IFN-γ and IL-2 responses were more frequently induced by the RTS,S/AS02D vaccine than by the control hepatitis B vaccine, possibly due to a stronger Th1 adjuvant effect of AS02D compared to alum present in the Engerix-B hepatitis B vaccine.

Although RTS,S/AS02D immunization induced statistically significant CSP-specific cellular immune responses detectable by ICS compared to the hepatitis B group or preimmune responses, the percentage of positive cells was very low, as shown by the SIs close to 1.0, and the biological significance of this is

TABLE 2. Associations between CSP-specific cytokine responses and malaria infection in RTS,S/AS02D-immunized infants

Variable[a]	Time (wk) after third dose	Malaria cases[b]						P^c
		No			Yes			
		n	Median SI (IQR)	% Responders (no.)	*n*	Median SI (IQR)	% Responders (no.)	
SI								
IFN-γ ($CD8^+$ T cells)	4	57	1.030 (0.650–1.571)		18	1.119 (0.852–1.455)		0.669
	10.5	46	1.398 (0.960–2.061)		17	1.121 (0.636–1.484)		0.074
IL-2 ($CD4^+$ T cells)	4	56	1.355 (0.915–2.000)		14	1.294 (1.045–1.667)		0.860
	10.5	44	1.271 (0.943–1.794)		15	1.222 (0.903–1.594)		0.741
Cytokines in supernatant								
IFN-γ	10.5	54		9.3 (5)	17		0 (0)	0.328
IL-2	10.5	54		31.5 (17)	17		5.9 (1)	0.053
IL-4	10.5	54		13.0 (7)	17		0 (0)	0.188

[a] CSP-specific cytokine responses are expressed as either SI values from ICS assays or as a percentage of infants with cytokines in supernatant (lower panel). *n*, Number of subjects.
[b] ATP cohort, for first or only episode of malaria infection from 2.5 to 6 months of follow-up.
[c] As determined by the Wilcoxon rank-sum test for the ICS SI values and as determined by the Fisher exact chi-square test for the proportion with supernatant cytokine response.

unknown. When significant differences were observed between immunization groups, median SIs for CSP-induced responses were ~0.25 higher in the RTS,S/AS02D group than those in the hepatitis B vaccine group. This translates into approximate 0.08 and 0.09% increments, respectively, in the CSP-specific $CD4^+$ and $CD8^+$ T-cell populations in children immunized with RTS,S. When comparing ICS to supernatant cytokines, no correlation was observed between secreted and intracellular cytokine levels (data not shown). This has often been described for different assays and may be due to the duration of in vitro stimulation and assay conditions favoring secretion and not having reached the peak of intracellular cytokine accumulation (1).

A limitation of our ICS assay was that, due to the small volumes of blood available, whole blood was used instead of peripheral blood mononuclear cells (PBMC). ICS assays have been extensively optimized in PBMC, particularly from adults living in North America or Europe. Whole-blood assays may give more background in ICS than PBMC, potentially weakening the signal-to-noise ratio for antigen-specific responses and complicating compensation settings. The true differences between the immunization groups may thus be greater than detected in the present study. Furthermore, several studies have observed that the background levels in cells from African subjects may be higher due to more chronic activation and inflammation (18, 33, 43). The whole-blood ICS assay will thus require further optimization for use in African infants for malaria trials, similar to what has been done in the context of tuberculosis studies (15).

There are concerns about the adverse effect of maternal immunity and the immaturity of infants' immune systems on the induction of adequate antibody and effector T-cell IFN-γ responses (Th1) by malaria vaccines in neonates and infants living in areas of endemicity (35, 36). In this safety/efficacy trial in infants, RTS,S/AS02D immunization induced high titers of anti-CSP antibodies, suggesting that the presence of maternally transferred antibodies at a young immunization age did not significantly modify the antibody immune response (4). It has been suggested that IFN-γ T-cell responses to natural malaria exposure are infrequent in children (8, 38), and it is hypothesized that repeated exposure and a mature immune system may be required. In the present study, although weak, RTS,S immunization clearly induced CSP-specific T-cell responses, thus demonstrating that CSP-specific T-cell immunity can be elicited in infants less than 1 year of age.

In the main efficacy study, the RTS,S/AS02D immunization, along with EPI vaccines, was shown to be safe, to induce a strong antibody response to both CSP and HBS components, and to have a calculated efficacy of 65.9% against malaria infection (4). Since it is known that humoral immunity is not sufficient for protection against malaria infection, the role of cytokine-producing T cells has been under intense study in the effort to identify correlates of malaria vaccine efficacy. In past studies in adults, the RTS,S vaccine candidate has been shown by various techniques to elicit cellular immune responses (5, 20, 22, 30, 40–42).The use of different assays to measure antigen-specific T-cell activity, including both ex vivo and cultured lymphoproliferation, IFN-γ enzyme-linked immunospot, and ICS methods, complicates interpretations. In malaria-naive adults, a trend has been observed toward a higher proportion of CSP-specific IFN-γ $CD4^+$ and $CD8^+$ responses in a small number of protected individuals (19, 42). In adults in an area of malaria endemicity, cultured enzyme-linked immunospot assay revealed an association between IFN-γ-producing $CD4^+$ cells and protection after RTS,S vaccination (31). To date, no trials of RTS,S vaccination in areas of malaria endemicity have reported detectable $CD8^+$ responses, and published data in infants and young children are limited to antibody immunogenicity.

We found a suggestive association between not suffering an infection episode and higher levels of CSP-specific IL-2 in supernatant at 10.5 weeks postimmunization. In addition, there was a trend toward an association between not having a malaria infection episode and CSP-specific $CD8^+$ IFN-γ responses, although these associations did not reach statistical significance, possibly due to the lack of power to make these associations with the given sample size. Surprisingly, stronger cytokine responses were found after longer postimmunization periods. The parallel increase of HBS-specific cytokine responses allows speculation that natural boosting by *P. falciparum* during the 6-week interval between BS1 and BS2 was not solely responsible for the increase. This suggests that a certain time period may be important to allow the development of RTS,S-induced protective T-cell immunity, as suggested in a previous RTS,S trial in adults (19). This interval may be more relevant in the development of infant immunity.

As an initial description of CSP-specific T-cell immune responses elicited in infants immunized with the RTS,S vaccine, the present study provides crucial background data for conducting further malaria immunology studies in infants and in identifying targets for immune correlates of protection. The methods and results reported here pave the way for future investigation both in optimizing assays adapted for detection of cell-mediated immunity in young African infants and in incorporating other parameters of T-cell function and phenotype in order to identify correlates of RTS,S vaccine efficacy.

ACKNOWLEDGMENTS

We are grateful to the participating children, their families, and the communities of Ilha Josina and Taninga in Mozambique and to the clinical and laboratory staff at the Centro de Investigação en Saúde de Manhiça, Diana Quelhas, and Lázaro Mussacate for assistance in processing the samples and acquiring the fluorescence-activated cell sorting data. We thank Carlota Dobaño and Joe Campo for critically reviewing the manuscript and Laura Puyol for assistance in the purchasing and shipping of reagents. We thank Phillipe Moris, Marc Lievens, Marie-Ange Demoitié, and Joe Cohen for helpful discussions and input during the study. We also thank Ann Stewart and Shannon McGrath at the Walter Reed Army Institute of Research for their valuable comments on the ICS technique.

This study was funded by MVI and The Bill and Melinda Gates Foundation.

MVI supports the development and testing of several malaria vaccines. W.R.B. was employed by GlaxoSmithKline Biologicals (GSK) at the time of the study. W.R.B. own shares of GSK. W.R.B. and A.B. are listed as the inventors of a different subunit malaria vaccine; however, neither of them individually holds a patent for a malaria vaccine. None of the other authors declares any conflict of interest.

REFERENCES

1. **Alheim, M., U. Lazdina, D. R. Milich, and M. Sallberg.** 2001. Flow cytometric determination of cytokine production and proliferation in hepatitis B core antigen specific murine CD4 cells: lack of correlation between number of cytokine producing cells and cytokine levels in supernatant. J. Immunol. Methods **258:**157–167.
2. **Alonso, P. L., J. Sacarlal, J. J. Aponte, A. Leach, E. Macete, P. Aide, B.**

Sigauque, J. Milman, I. Mandomando, Q. Bassat, C. Guinovart, M. Espasa, S. Corachan, M. Lievens, M. M. Navia, M. C. Dubois, C. Menendez, F. Dubovsky, J. Cohen, R. Thompson, and W. R. Ballou. 2005. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. Lancet **366:**2012–2018.

3. **Alonso, P. L., J. Sacarlal, J. J. Aponte, A. Leach, E. Macete, J. Milman, I. Mandomando, B. Spiessens, C. Guinovart, M. Espasa, Q. Bassat, P. Aide, O. Ofori-Anyinam, M. M. Navia, S. Corachan, M. Ceuppens, M. C. Dubois, M. A. Demoitie, F. Dubovsky, C. Menendez, N. Tornieporth, W. R. Ballou, R. Thompson, and J. Cohen.** 2004. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet **364:**1411–1420.
4. **Aponte, J. J., P. Aide, M. Renom, I. Mandomando, Q. Bassat, J. Sacarlal, M. N. Manaca, S. Lafuente, A. Barbosa, A. Leach, M. Lievens, J. Vekemans, B. Sigauque, M. C. Dubois, M. A. Demoitie, M. Sillman, B. Savarese, J. G. McNeil, E. Macete, W. R. Ballou, J. Cohen, and P. L. Alonso.** 2007. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet **370:**1543–1551.
5. **Bojang, K. A., P. J. Milligan, M. Pinder, L. Vigneron, A. Allouecheb, K. E. Kester, W. R. Ballou, D. J. Conway, W. H. Reece, P. Gothard, L. Yamuah, M. Delchambre, G. Voss, B. M. Greenwood, A. Hill, K. P. McAdam, N. Tornieporth, J. D. Cohen, and T. Doherty.** 2001. Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium falciparum* infection in semi-immune adult men in The Gambia: a randomised trial. Lancet **358:**1927–1934.
6. **Bojang, K. A., F. Olodude, M. Pinder, O. Ofori-Anyinam, L. Vigneron, S. Fitzpatrick, F. Njie, A. Kassanga, A. Leach, J. Milman, R. Rabinovich, K. P. McAdam, K. E. Kester, D. G. Heppner, J. D. Cohen, N. Tornieporth, and P. J. Milligan.** 2005. Safety and immunogenicity of RTS,S/AS02A candidate malaria vaccine in Gambian children. Vaccine **23:**4148–4157.
7. **Chatterjee, S., M. Wery, P. Sharma, and V. S. Chauhan.** 1995. A conserved peptide sequence of the *Plasmodium falciparum* circumsporozoite protein and antipeptide antibodies inhibit *Plasmodium berghei* sporozoite invasion of Hep-G2 cells and protect immunized mice against *P. berghei* sporozoite challenge. Infect. Immun. **63:**4375–4381.
8. **Chelimo, K., P. O. Sumba, J. W. Kazura, A. V. Ofula, and C. C. John.** 2003. Interferon-gamma responses to *Plasmodium falciparum* liver-stage antigen-1 and merozoite-surface protein-1 increase with age in children in a malaria holoendemic area of western Kenya. Malaria J. **2:**37.
9. **Clyde, D. F., V. C. McCarthy, R. M. Miller, and R. B. Hornick.** 1973. Specificity of protection of man immunized against sporozoite-induced falciparum malaria. Am. J. Med. Sci. **266:**398–403.
10. **Dame, J. B., J. L. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Schneider, D. Roberts, et al.** 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science **225:**593–599.
11. **Doolan, D. L., R. A. Houghten, and M. F. Good.** 1991. Location of human cytotoxic T-cell epitopes within a polymorphic domain of the *Plasmodium falciparum* circumsporozoite protein. Int. Immunol. **3:**511–516.
12. **Engers, H. D., and T. Godal.** 1998. Malaria vaccine development: current status. Parasitol. Today **14:**56–64.
13. **Good, M. F., L. H. Miller, S. Kumar, I. A. Quakyi, D. Keister, J. H. Adams, B. Moss, J. A. Berzofsky, and R. Carter.** 1988. Limited immunological recognition of critical malaria vaccine candidate antigens. Science **242:**574–577.
14. **Greenwood, B., and T. Mutabingwa.** 2002. Malaria in 2002. Nature **415:**670–672.
15. **Hanekom, W. A., J. Hughes, M. Mavinkurve, M. Mendillo, M. Watkins, H. Gamieldien, S. J. Gelderbloem, M. Sidibana, N. Mansoor, V. Davids, R. A. Murray, A. Hawkridge, P. A. Haslett, S. Ress, G. D. Hussey, and G. Kaplan.** 2004. Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency in field studies. J. Immunol. Methods **291:**185–195.
16. **Heppner, D. G., Jr., K. E. Kester, C. F. Ockenhouse, N. Tornieporth, O. Ofori, J. A. Lyon, V. A. Stewart, P. Dubois, D. E. Lanar, U. Krzych, P. Moris, E. Angov, J. F. Cummings, A. Leach, B. T. Hall, S. Dutta, R. Schwenk, C. Hillier, A. Barbosa, L. A. Ware, L. Nair, C. A. Darko, M. R. Withers, B. Ogutu, M. E. Polhemus, M. Fukuda, S. Pichyangkul, M. Gettyacamin, C. Diggs, L. Soisson, J. Milman, M. C. Dubois, N. Garcon, K. Tucker, J. Wittes, C. V. Plowe, M. A. Thera, O. K. Duombo, M. G. Pau, J. Goudsmit, W. R. Ballou, and J. Cohen.** 2005. Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. Vaccine **23:**2243–2250.
17. **Hoffman, S. L., L. M. Goh, T. C. Luke, I. Schneider, T. P. Le, D. L. Doolan, J. Sacci, P. de la Vega, M. Dowler, C. Paul, D. M. Gordon, J. A. Stoute, L. W. Church, M. Sedegah, D. G. Heppner, W. R. Ballou, and T. L. Richie.** 2002. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J. Infect. Dis. **185:**1155–1164.
18. **Kassu, A., A. Tsegaye, B. Petros, D. Wolday, E. Hailu, T. Tilahun, B. Hailu, M. T. Roos, A. L. Fontanet, D. Hamann, and T. F. De Wit.** 2001. Distribution of lymphocyte subsets in healthy human immunodeficiency virus-negative adult Ethiopians from two geographic locales. Clin. Diagn. Lab. Immunol. **8:**1171–1176.
19. **Kester, K. E., J. F. Cummings, C. F. Ockenhouse, R. Nielsen, B. T. Hall, D. M. Gordon, R. J. Schwenk, U. Krzych, C. A. Holland, G. Richmond, M. G. Dowler, J. Williams, R. A. Wirtz, N. Tornieporth, L. Vigneron, M. Delchambre, M. A. Demoitie, W. R. Ballou, J. Cohen, and D. G. Heppner, Jr.** 2008. Phase 2a trial of 0, 1, and 3 month and 0, 7, and 28 day immunization schedules of malaria vaccine RTS,S/AS02 in malaria-naive adults at the Walter Reed Army Institute of Research. Vaccine **26:**2191–2202.
20. **Kester, K. E., D. A. McKinney, N. Tornieporth, C. F. Ockenhouse, D. G. Heppner, T. Hall, U. Krzych, M. Delchambre, G. Voss, M. G. Dowler, J. Palensky, J. Wittes, J. Cohen, and W. R. Ballou.** 2001. Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental *Plasmodium falciparum* malaria. J. Infect. Dis. **183:**640–647.
21. **Kester, K. E., D. A. McKinney, N. Tornieporth, C. F. Ockenhouse, D. G. Heppner, Jr., T. Hall, B. T. Wellde, K. White, P. Sun, R. Schwenk, U. Krzych, M. Delchambre, G. Voss, M. C. Dubois, R. A. Gasser, Jr., M. G. Dowler, M. O'Brien, J. Wittes, R. Wirtz, J. Cohen, and W. R. Ballou.** 2007. A phase I/IIa safety, immunogenicity, and efficacy bridging randomized study of a two-dose regimen of liquid and lyophilized formulations of the candidate malaria vaccine RTS,S/AS02A in malaria-naive adults. Vaccine **25:**5359–5366.
22. **Lalvani, A., P. Moris, G. Voss, A. A. Pathan, K. E. Kester, R. Brookes, E. Lee, M. Koutsoukos, M. Plebanski, M. Delchambre, K. L. Flanagan, C. Carton, M. Slaoui, C. Van Hoecke, W. R. Ballou, A. V. Hill, and J. Cohen.** 1999. Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant *Plasmodium falciparum* malaria vaccine. J. Infect. Dis. **180:**1656–1664.
23. **Macete, E., J. J. Aponte, C. Guinovart, J. Sacarlal, O. Ofori-Anyinam, I. Mandomando, M. Espasa, C. Bevilacqua, A. Leach, M. C. Dubois, D. G. Heppner, L. Tello, J. Milman, J. Cohen, F. Dubovsky, N. Tornieporth, R. Thompson, and P. L. Alonso.** 2007. Safety and immunogenicity of the RTS,S/AS02A candidate malaria vaccine in children aged 1–4 in Mozambique. Trop. Med. Int. Health **12:**37–46.
24. **Macete, E. V., J. Sacarlal, J. J. Aponte, A. Leach, M. M. Navia, J. Milman, C. Guinovart, I. Mandomando, Y. Lopez-Pua, M. Lievens, A. Owusu-Ofori, M. C. Dubois, C. P. Cahill, M. Koutsoukos, M. Sillman, R. Thompson, F. Dubovsky, W. R. Ballou, J. Cohen, and P. L. Alonso.** 2007. Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase I/IIb randomized double-blind bridging trial. Trials **8:**11.
25. **Malkin, E., F. Dubovsky, and M. Moree.** 2006. Progress toward the development of malaria vaccines. Trends Parasitol. **22:**292–295.
26. **Matuschewski, K., and A. K. Mueller.** 2007. Vaccines against malaria: an update. FEBS J. **274:**4680–4687.
27. **Moorthy, V. S., M. F. Good, and A. V. Hill.** 2004. Malaria vaccine developments. Lancet **363:**150–156.
28. **Nardin, E., Y. D. Munesinghe, A. Moreno, P. Clavijo, M. C. Calle, R. Edelman, J. Davis, D. Herrington, and R. S. Nussenzweig.** 1992. T-cell responses to repeat and non-repeat regions of the circumsporozoite protein detected in volunteers immunized with *Plasmodium falciparum* sporozoites. Mem. Inst. Oswaldo Cruz **87**(Suppl. 3)**:**223–227.
29. **Nussenzweig, R. S., and F. Zavala.** 1997. A malaria vaccine based on a sporozoite antigen. N. Engl. J. Med. **336:**128–130.
30. **Pinder, M., W. H. Reece, M. Plebanski, P. Akinwunmi, K. L. Flanagan, E. A. Lee, T. Doherty, P. Milligan, A. Jaye, N. Tornieporth, R. Ballou, K. P. McAdam, J. Cohen, and A. V. Hill.** 2004. Cellular immunity induced by the recombinant *Plasmodium falciparum* malaria vaccine, RTS,S/AS02, in semi-immune adults in The Gambia. Clin. Exp. Immunol. **135:**286–293.
31. **Reece, W. H., M. Pinder, P. K. Gothard, P. Milligan, K. Bojang, T. Doherty, M. Plebanski, P. Akinwunmi, S. Everaere, K. R. Watkins, G. Voss, N. Tornieporth, A. Allouecheb, B. M. Greenwood, K. E. Kester, K. P. McAdam, J. Cohen, and A. V. Hill.** 2004. A $CD4^+$ T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease. Nat. Med. **10:**406–410.
32. **Reed, G. F., B. D. Meade, and M. C. Steinhoff.** 1995. The reverse cumulative distribution plot: a graphic method for exploratory analysis of antibody data. Pediatrics **96:**600–603.
33. **Saathoff, E., P. Schneider, V. Kleinfeldt, S. Geis, D. Haule, L. Maboko, E. Samky, M. de Souza, M. Robb, and M. Hoelscher.** 2008. Laboratory reference values for healthy adults from southern Tanzania. Trop. Med. Int. Health **13:**612–625.
34. **Sacarlal, J., J. J. Aponte, P. Aide, I. Mandomando, Q. Bassat, C. Guinovart, A. Leach, J. Milman, E. Macete, M. Espasa, O. Ofori-Anyinam, J. Thonnard, S. Corachan, M. C. Dubois, M. Lievens, F. Dubovsky, W. R. Ballou, J. Cohen, and P. L. Alonso.** 2008. Safety of the RTS,S/AS02A malaria vaccine in Mozambican children during a Phase IIb trial. Vaccine **26:**174–184.
35. **Sedegah, M., M. Belmonte, J. E. Epstein, C. A. Siegrist, W. R. Weiss, T. R. Jones, M. Lu, D. J. Carucci, and S. L. Hoffman.** 2003. Successful induction of CD8 T cell-dependent protection against malaria by sequential immunization with DNA and recombinant poxvirus of neonatal mice born to immune mothers. J. Immunol. **171:**3148–3153.

36. **Siegrist, C. A.** 2000. Vaccination in the neonatal period and early infancy. Int. Rev. Immunol. **19:**195–219.
37. **Snow, R. W., C. A. Guerra, A. M. Noor, H. Y. Myint, and S. I. Hay.** 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature **434:**214–217.
38. **Ssewanyana, I., C. Pietras, C. A. Baker, F. Nghania, N. G. Jones, P. J. Rosenthal, G. Dorsey, and H. Cao.** 2008. Pattern of malaria-specific T-cell responses in a cohort of Ugandan children. J. Trop. Pediatr. **54:**6–13.
39. **Stewart, V. A., S. M. McGrath, P. M. Dubois, M. G. Pau, P. Mettens, J. Shott, M. Cobb, J. R. Burge, D. Larson, L. A. Ware, M. A. Demoitie, G. J. Weverling, B. Bayat, J. H. Custers, M. C. Dubois, J. Cohen, J. Goudsmit, and D. G. Heppner, Jr.** 2007. Priming with an adenovirus 35-circumsporozoite protein (CS) vaccine followed by RTS,S/AS01B boosting significantly improves immunogenicity to *Plasmodium falciparum* CS compared to that with either malaria vaccine alone. Infect. Immun. **75:**2283–2290.
40. **Stoute, J. A., K. E. Kester, U. Krzych, B. T. Wellde, T. Hall, K. White, G. Glenn, C. F. Ockenhouse, N. Garcon, R. Schwenk, D. E. Lanar, P. Sun, P. Momin, R. A. Wirtz, C. Golenda, M. Slaoui, G. Wortmann, C. Holland, M. Dowler, J. Cohen, and W. R. Ballou.** 1998. Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. J. Infect. Dis. **178:**1139–1144.
41. **Stoute, J. A., M. Slaoui, D. G. Heppner, P. Momin, K. E. Kester, P. Desmons, B. T. Wellde, N. Garcon, U. Krzych, M. Marchand, et al.** 1997. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. N. Engl. J. Med. **336:**86–91.
42. **Sun, P., R. Schwenk, K. White, J. A. Stoute, J. Cohen, W. R. Ballou, G. Voss, K. E. Kester, D. G. Heppner, and U. Krzych.** 2003. Protective immunity induced with malaria vaccine, RTS,S, is linked to *Plasmodium falciparum* circumsporozoite protein-specific $CD4^+$ and $CD8^+$ T cells producing IFN-gamma. J. Immunol. **171:**6961–6967.
43. **Tsegaye, A., D. Wolday, S. Otto, B. Petros, T. Assefa, T. Alebachew, E. Hailu, F. Adugna, W. Measho, W. Dorigo, A. L. Fontanet, D. van Baarle, and F. Miedema.** 2003. Immunophenotyping of blood lymphocytes at birth, during childhood, and during adulthood in HIV-1-uninfected Ethiopians. Clin. Immunol. **109:**338–346.

Editor: W. A. Petri, Jr.

Article 4: Four year immunogenicity of the RTS,S/AS02$_A$ malaria vaccine in Mozambican children during a phase IIb trial.

Pedro Aide, Carlota Dobaño, Jahit Sacarlal, John J. Aponte, Inácio Mandomando, Caterina Guinovart, Quique Bassat, Montse Renom, Laura Puyol, Eusebio Macete, Esperanza Herreros, Amanda Leach, Marie-Claude Dubois, Marie-Ange Demoitie, Marc Lievens, Johan Vekemans, Christian Loucq, W. Ripley Ballou, Joe Cohen and Pedro L. Alonso

Vaccine (2011), doi:10.1016/j.vaccine.2011.03.041

Vaccine 29 (2011) 6059–6067

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Four year immunogenicity of the RTS,S/AS02$_A$ malaria vaccine in Mozambican children during a phase IIb trial

Pedro Aide[a,b,*], Carlota Dobaño[a,c], Jahit Sacarlal[a,d], John J. Aponte[a,c], Inácio Mandomando[a,b], Caterina Guinovart[a,c], Quique Bassat[a,c], Montse Renom[a,c], Laura Puyol[c], Eusebio Macete[a,b], Esperanza Herreros[g], Amanda Leach[e], Marie-Claude Dubois[e], Marie-Ange Demoitie[e], Marc Lievens[e], Johan Vekemans[e], Christian Loucq[f], W. Ripley Ballou[e], Joe Cohen[e], Pedro L. Alonso[a,c]

[a] *Centro de Investigação em Saúde de Manhiça (CISM), Manhiça, Mozambique*
[b] *Instituto Nacional de Saúde, Ministério de Saúde, Maputo, Mozambique*
[c] *Barcelona Centre for International Health Research/Hospital Clinic/University of Barcelona, Spain*
[d] *Faculdade de Medicina, Universidade Eduardo Mondlane, Maputo, Mozambique*
[e] *GlaxoSmithKline Biologicals, Rixensart, Belgium*
[f] *PATH Malaria Vaccine Initiative, Bethesda, MD, USA*
[g] *GlaxoSmithKline, Tres Cantos, Madrid, Spain*

ARTICLE INFO

Article history:
Received 25 September 2010
Received in revised form 9 March 2011
Accepted 12 March 2011
Available online 7 April 2011

Keywords:
RTS
S/AS02$_A$
Malaria
Vaccine
Immunogenicity
Children

ABSTRACT

Previous studies with the malaria vaccine RTS,S/AS02$_A$ in young children in a malaria endemic area of Mozambique have shown it to have a promising safety profile and to reduce the risk of *Plasmodium falciparum* infection and disease.

In this study, we assessed the antibody responses to the *P. falciparum* and hepatitis B components of the RTS,S/AS02$_A$ vaccine over a 45 months surveillance period in a large phase IIb trial which included 2022 children aged 1–4 years at recruitment.

The RTS,S/AS02$_A$ vaccine induced high anti-circumsporozoite antibody levels with at least 96% of children remaining seropositive during the entire follow-up period. IgG titers decayed over the first 6 months of follow-up to about 25% of the initial level, but still remained 30-fold higher until month 45 compared to controls. Children with higher levels of naturally acquired immunity at baseline, assessed by blood stage indirect fluorescent antibody test, had slightly higher anti-circumsporozoite levels, after adjusting for the effect of age.

The RTS,S/AS02$_A$ vaccine also induced high levels of anti-hepatitis B surface antigen antibodies (seroprotection >97%).

RTS,S/AS02$_A$ vaccine is immunogenic and induces long-lasting anti-circumsporozoite antibodies, persisting at least 42 months after immunization. These antibodies may play a role in protection against malaria.

1. Introduction

Plasmodium falciparum is responsible for the high malaria morbidity and mortality in malaria endemic countries, accounting for around 250 million clinical malaria cases and 863,000 deaths every year [1]. The GlaxoSmithKline (GSK) Biologicals pre-erythrocytic RTS,S malaria vaccine antigen is a virus-like particle containing a mixture of RTS, a chimeric recombinant protein combining polypeptide regions of *P. falciparum* circumsporozoite protein (CSP) and the hepatitis B virus surface antigen (HBsAg), and S, the recombinant HBsAg alone. It is formulated in the AS02 adjuvant system [2,3]. Developments of this vaccine has included sequential steps of phase I and phase IIa studies in adults in the USA [3], phase I/IIb studies in adults in The Gambia [4], and finally children and infant studies in Mozambique [5–7] and Tanzania [8]. We have shown that the vaccine is immunogenic, inducing immunoglobulin G (IgG) humoral antibodies and CD4$^+$ T cell and cytokine responses to *P. falciparum* CSP [9]. Some previous trials have shown an association between anti-CSP IgG levels in serum (measured by a standardized ELISA) and vaccine efficacy against malaria infection, but not against clinical disease [7,10,11]. The protective immune mechanism of RTS,S/AS remains poorly understood, and

* Corresponding author at: Centro de Investigação em Saúde da Manhiça (CISM), Instituto Nacional de Saúde, Ministério de Saúde, Rua 12, P.O. Box 1929, Maputo, Mozambique. Tel.: +258 21 810181; fax: +258 21 810002.
E-mail addresses: pedro.aide@manhica.net, pcpaid@gmail.com (P. Aide).

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.03.041

is thought to involve humoral as well as cell-mediated immunity [9].

The hepatitis B virus (HBV) HBsAg portion of RTS,S is also highly immunogenic. The quality of the immune response to vaccination with HBsAg (whether in *Engerix-B*™ or RTS,S/AS02$_A$) cannot be evaluated directly by a neutralisation test, since the hepatitis B virus does not replicate *in vitro*. Nevertheless, the generation of monoclonal antibodies against various epitopes of HBsAg has allowed the identification of a protective epitope in non human primates. The RF1 monoclonal antibody (RF1 mAb) recognises this conformational epitope on peptide 124–137 of the S protein and is able to protect chimpanzees against infection with the virus [12]. In the absence of a neutralisation assay for HBV, evaluation of the presence of RF1-like antibodies in serum from vaccinated subjects can be used as a surrogate marker of protective capacity and thus provide a qualitative evaluation of the immune response to vaccination [12].

Previous reports of the pivotal proof-of-concept trial of RTS,S/AS02$_A$ in Mozambique have presented limited immunogenicity data [5,6,13]. In this article we report a more detailed analysis of the anti-CSP, and anti-HBsAg antibody responses during the entire 45 month follow-up period of the largest phase IIb trial of RTS,S/AS conducted.

2. Materials and methods

2.1. Study site

This study was conducted by the Centro de Investigação em Saúde de Manhiça, located in the Manhiça District, Southern Mozambique, from April 2003 to May 2007. The characteristics of the area have been described in detail elsewhere [14–16]. Hepatitis B immunization (given at 2, 3 and 4 months of age together with DTPw and oral Polio vaccines) was introduced in the Expanded Program on Immunization (EPI) in Mozambique in July 2001.

2.2. Study design

This study was a phase IIb double-blind, randomised controlled trial to assess the efficacy, safety and immunogenicity of the candidate RTS,S/AS02$_A$ malaria vaccine RTS,S/AS02$_A$. Details of the candidate malaria vaccine, the control vaccines, the trial design and efficacy and safety analyses for the double-blind (study months 0–8.5), single-blind (study months 8.5–21) and open phases (study months 21–45) have been presented elsewhere [5,6,13,17]. Briefly, 2022 healthy children aged 1–4 years were enrolled to receive either the candidate malaria vaccine or a comparator vaccine after written or thumb printed informed consent provided by their parents/guardians. HBsAg status was assessed at baseline, but positivity was not an exclusion criterion for the trial.

The RTS,S/AS02$_A$ and control vaccines were administered intramuscularly in the deltoid following a 0, 1, 2-month schedule. Children in the control group aged 24 months and older received three paediatric doses (0.5 ml) of hepatitis B vaccine (*Engerix-B*™, GSK Biologicals, Rixensart, Belgium). Children under 24 months in the control group had already received hepatitis B immunization by the time they were enrolled in the trial as part of their previous EPI immunization, and were therefore vaccinated with 2 paediatric doses of a 7-valent pneumoccocal conjugate vaccine (*Prevnar*™, Wyeth Lederle Vaccines, USA) administered at the first and third vaccinations and one dose of *Haemophilus influenzae type b* vaccine (*Hiberix*™, GSK Biologicals, Belgium) at the second vaccination. Vaccines were administered at the Manhiça and Ilha Josina health centres.

Children were enrolled into two cohorts to measure the vaccine efficacy against either clinical malaria or malaria infection. In Cohort 1, based in Manhiça and Maragra, 1605 participants were followed-up using passive surveillance to detect clinical episodes of malaria. In Cohort 2, based in Ilha Josina, where malaria transmission intensity was 10 times higher [17], 417 participants were followed-up using active surveillance to detect malaria infection, through visits that started 14 days after the third vaccine dose and were done every 2 weeks for 2.5 months and then monthly for 2 additional months. In children from Cohort 2, asymptomatic parasitaemia was presumptively cleared with a combination of amodiaquine and sulfadoxine-pyrimethamine 14 days prior to dose 3.

Blood samples for determining anti-CSP antibody concentrations in both cohorts and anti-HBsAg antibodies (only Cohort 2) were obtained at study months 0 (prevaccination), 3 (1 month after the third vaccine dose), 8½, 21, 33 and 45. RF1-like antibodies were measured prior to vaccination and at study month 3, only in Cohort 2. Serum was separated for antibody determinations. Indirect fluorescent antibody tests (IFAT) for blood stage anti-parasite antibodies were performed prior to vaccination. Primary analysis of immunogenicity was performed on the ATP cohort (primary analysis).

2.3. Antibody responses to the RTS,S/AS02$_A$ vaccine

The levels of IgG antibodies to the NANP repeat region of CSP (B cell epitope) were measured by a standard, validated enzyme-linked immunosorbent assay (ELISA) using plates adsorbed with the R32LR antigen at a GSK validated laboratory (CEVAC, University of Ghent, Belgium). Antibody concentrations were calculated using a reference standard curve with a 4 parameter logistic fitting algorithm and expressed in EU/mL, with cut-off set at 0.5 EU/mL [18].

Anti-HBsAg antibody levels were measured only in samples from Cohort 2 at GSK laboratories by ELISA with a commercial kit (AUSAB EIA, Abbott Laboratories, Abbott Park, IL) for the first 5 samplings, and with an in-house developed HBsAg ELISA for the last month 45 sample (described elsewhere) [19]. RF1-like antibodies levels were determined using an in-house developed ELISA based competition assay with plate adsorbed HBs antigen, performed at CEVAC, University of Ghent, Belgium. Dilutions of the test samples and the reference serum were mixed with a fixed amount of RF1 mAb that was revealed through a colorimetric reaction. The signal obtained was inversely proportional to the amount of anti-RF1 like antibodies present in the samples. The amount of antibody competing with RF1 mAb for binding to the coated HBsAg was quantified by comparison to a reference serum using a 4 parameters equation (Softmax Pro Software), with an assay cut-off of 33 EU/ml.

2.4. Hepatitis B virus surface antigen

HBsAg levels were determined in both cohorts by ELISA with a commercial kit (ETI-MAK-4® DiaSorin®, Saluggia, Italy) at the Microbiology Service of Hospital Clinic, Universitat de Barcelona, Spain, according to the manufacturer's instructions.

2.5. Antibodies to blood-stage P. falciparum antigens by IFAT

To determine the level of naturally acquired *P. falciparum*-specific antibodies prior to vaccination, IFAT in baseline serum samples from children in the two study cohorts was conducted at the Barcelona Center for International Health Research (CRESIB, Hospital Clinic, Universitat de Barcelona, Spain). *In vitro* cultures containing mostly mature asexual blood stages of *P. falciparum* strains were grown at GSK Tres Cantos, Madrid, Spain. A pool was

prepared with a mixture of 3D7, K1, FCR3 and HB3 cultures, and parasitized erythrocytes were harvested and washed twice in PBS. Cells were resuspended to 3–5% hematocrit in PBS and 20–25 μl aliquots were placed onto 12-well multispot slides (Cell-Line Associates, Newfield, NJ, USA), dried, packed and stored at −20 °C in self-sealed plastic bags containing silica gel as desiccant.

Two-fold serial dilutions of the test sera were prepared (highest dilution tested was 1/81,920), and 25 μl of each serum dilution together with positive and negative control sera were placed onto acetone-fixed IFAT slides containing whole *P. falciparum* parasites and incubated in a wet chamber for 1 h at room temperature. After washing the slides 3 times with PBS-Tween 0.05%, 15 μl of FITC-labelled secondary antibody diluted in Evans Blue (1/100) were added and incubated for 30 min at 37 °C. Slides were washed 3 times with PBS-Tween 0.5%, mounted with buffered glycerine containing DAPI (1:100,000), and examined with a NIKON fluorescence microscope. The highest dilution giving positive fluorescence above the negative control levels was scored under the UV light. For each reading and each antibody, the end-point titer corresponded to the reciprocal of the greatest serum dilution that yielded a positive fluorescence.

The protocols (NCT00197041 and NCT00323622) were approved by the Mozambican National Bioethics Committee, the Hospital Clinic of Barcelona Ethics Review Committee and the PATH Human Subjects Protection Committee and implemented according to the International Conference of Harmonization and Good Clinical Practices guidelines. A Local Safety Monitor and a Data and Safety Monitoring Board oversaw the design, conduct and results of the trial.

2.6. Statistical analysis

For each treatment group, the seropositivity (S+) rate for anti-CSP antibodies (proportion of subjects with anti-CSP antibody concentration of ≥0.5 EU/mL) and their 95% Confidence Intervals (CI) were tabulated for each time point. Reverse cumulative distribution curves [20] were plotted stratified by age at day 0 (<24 months, ≥24 months) for serum antibody titers measured prior to immunization and at months $8^{1/2}$, 21 and 45.

For each treatment group in Cohort 2, the seroprotection (SP) rate for anti-HBsAg antibodies (proportion of subjects with anti-HBsAg antibody titers of ≥10 mIU/mL) and their 95% CI were tabulated for each time point. GMTs for anti-HBsAg antibodies measured in mIU/mL with 95% CI were calculated for each group at each time point when a serology sample was taken.

The seroconversion rate for anti-RF1 antibodies (proportion of subjects with anti-RF1 antibody titers of ≥33 mIU/mL) were tabulated with 95% CI for all time points at which anti-RF1 antibodies were measured.

GMT calculations were performed by taking the anti-log of the mean of the log titer transformations (log base 10). Titers below the cut-off were assigned an arbitrary value of half the cut-off of the assay for the purpose of GMT calculation.

The relationship between blood stage IFAT titers and anti-CSP antibodies in children vaccinated with RTS,S/AS02$_A$ was assessed by multiple regression methods. Age at vaccination was categorized in four groups, each one corresponding to a one year interval.

The relation between anti-CSP antibody concentrations as measured 30 days post dose 3 and the risk of infection and clinical malaria was assessed in RTS,S/AS02$_A$ recipients. The hazard ratio of participants with anti-CSP antibody titers in the highest tertile against those in the lowest tertile was estimated, as well as the hazard ratio per ten-fold increase in the value of anti-CSP antibodies, using Cox regression models.

Table 1
Association between anti-CSP antibody responses and baseline IFAT titers, age and cohort in vaccinated children.

Variable	Relative change[a]	95% CI	*p* value
IFAT at baseline[b]	1.07	1.014–1.117	<0.0001
Cohort 1	1		0.04
Cohort 2	1.23	1.000–2.404	
1 year at D1	1	1	<0.0001
2 years at D1	0.64	0.0661–1.632	
3 years at D1	0.41	0.378–0.963	
4 years at D1	0.46	0.510–1.303	

Cohorts: 1 = Manhica, 2 = Ilha Josina; D1 = Dose 1; Global $p < 0.0001$.

[a] Relative change in anti-CSP antibody geometric mean concentration at 1 month post dose 3.

[b] Per doubling the value of IFAT.

Analyses were done with SAS version 8 (Cary, NC, USA) and STATA version 9.0 (College Station, TX, USA).

3. Results

A total of 2022 children aged 1–4 years were randomised and received at least one vaccine dose of RTS,S/AS02$_A$ or control vaccine. Of these, 1565 were included in the immunogenicity analysis: 795 in the RTS,S/AS02$_A$ group and 770 in the control group. Fig. 1 shows the trial profile for the study.

3.1. Anti-CSP antibody responses

The magnitude and longevity of anti-CSP antibodies in the two groups receiving RTS,S/AS02$_A$ (cohorts 1 and 2) as well as in the corresponding control groups is shown in Fig. 2. Among RTS,S/AS02$_A$ recipients, a robust response in the development of anti-CSP IgG is followed by a decay in antibody concentrations over the first 6 months of follow-up to about 25% of the initial level. However 42 months after dose 3, 96% of participants remained seropositive (30-fold higher compared to controls). Also worth noting is the apparent lack of increase in anti-CSP antibodies or the proportion of seropositives among the control group while being exposed to high *P. falciparum* transmission.

The effect of age at time of vaccination on antibody responses has been explored by plotting the reverse cumulative distribution curves for anti-CSP antibody GMTs by age group (<24 months, ≥24 months) in both cohorts at different follow up periods (Fig. 3). There is some evidence of higher immunogenicity 30 days after the third dose among the younger age group, but this difference disappears over the subsequent follow-up period.

We also explored the relationship between blood stage IFAT titers, as a reflection of intensity of *P. falciparum* transmission, and anti-CSP immunogenicity. IFAT baseline values were significantly higher in Cohort 2 (GMT [95% CI], 25,623 [21,360–30,737] in controls and 27,496 [22,520–33,571] in vaccinated) compared to Cohort 1 (2490 [2084–2976] in controls and 2449 [2107–2964] in vaccinated) reflecting the higher malaria transmission intensity in Ilha Josina. When doubling the IFAT titers at baseline, the vaccine-induced anti-CSP antibodies modestly increased by 1.07 (95% CI: 1.04–1.09, $p < 0.0001$) having adjusted for the effect of age and cohort. However, children in Cohort 2 had 1.23 (95% CI: 1.01–1.50, $p = 0.04$) times higher anti-CSP antibody titers compared to those in Cohort 1, having adjusted for both IFAT and age (Table 1).

We also looked at whether anti-CSP antibody responses induced by the RTS,S/AS02$_A$ were influenced by pre-vaccination HBsAg status (Table 2). In the RTS,S/AS02$_A$ group, 16 subjects in Cohort 1 and 9 in Cohort 2 were HBsAg positive at pre-vaccination. Responses to anti-CSP were slightly lower in HBsAg positive children in comparison to HBsAg negative participants. However, in both cohorts,

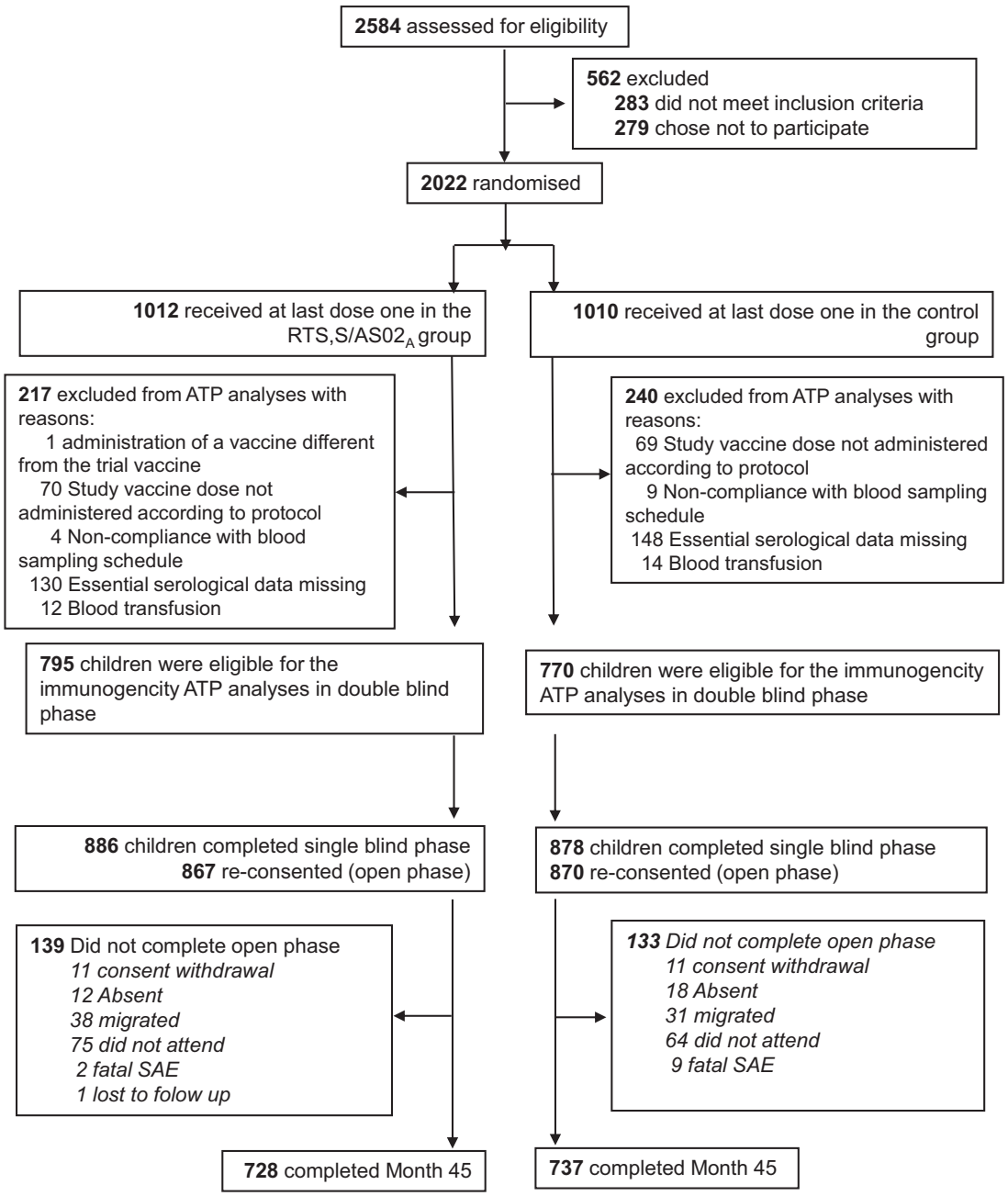


Fig. 1. Trial profile.

almost 100% of the subjects were seropositive for anti-CSP antibodies 1 month post dose 3.

Finally, we examined the relation between anti-CSP pre-vaccination antibodies and anti-HBsAg IgG titers. Subgroup analysis for Cohort 2 children showed no association between baseline anti-HBsAg antibody titers and anti-CSP IgG titers 1 month post dose 3. Even when adjusting by baseline IFAT and age, there was no evidence of increased RTS,S immunogenicity with higher anti-HBsAg titers (doubling the anti-HBsAg titers was associated with an increase of 1.01 (95% CI: 0.94–1.08) in the anti-CSP titers ($p = 0.854$)).

3.2. Anti-HBsAg antibody responses

Table 3 shows anti-HBsAg seroprotection rates and antibody GMTs, measured in samples from children in Cohort 2. We sub-divided age groups in two categories: a first group of children aged 24 months or older and that had not been previously immunised with hepatitis B vaccine, and a second group of participants younger than 2 years that had received hepatitis B vaccine through the routine EPI program. In children aged ≥24 months the seroprotective levels of anti-HBsAg antibodies at day 0 were approximately 20%, reflecting natural exposure. Immunisation with RTS,S/AS02$_A$ resulted in an increase of anti-HBsAg antibody titers from a GMT of 9.1 mIU/mL at baseline to 11368.6 mIU/mL 1 month post dose 3, and subsequently decreasing by 60% at month 8½ (4556 mIU/mL) and by 86% at month 45 (1557 mIU/mL). However, approximately 98% of subjects had seroprotective levels of anti-HBsAg antibodies at all time points. Among the control group that received *Engerix-B*™ approximately 90% of recipients were seroprotected following vaccination and remained so throughout the follow up. However, anti-HBsAg antibody titers were lower than among RTS,S recipients.

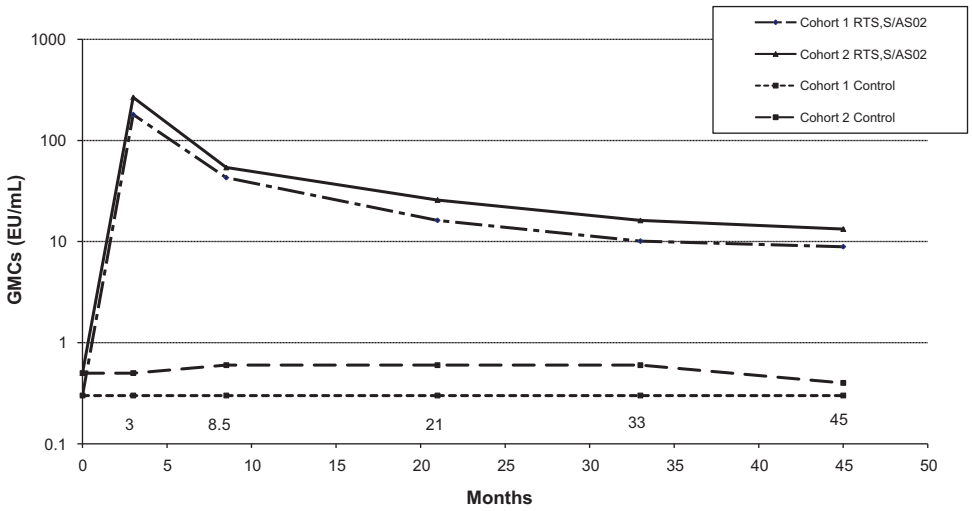


Fig. 2. The anti-CSP Antibody responses in children aged 1–4 years during the 45 months follow-up period. The figure represents the geometric mean concentration in the RTS,S/AS02$_A$ and control groups in both cohorts.

In children <24 months of age, the pre-vaccination seroprotective levels of anti-HBsAg antibodies were high in both the RTS,S/AS02$_A$ and control groups (>77%), reflecting the prior HBV immunisation. Following administration of RTS,S, seroprotection rates increased to 97% and remained so throughout the entire follow-up. GMT values for anti-HBsAg antibodies in this group increased from 62.9 mIU/mL at baseline to 51,035 mIU/mL 1 month post dose 3. This value decreased by 75% (to 13,642 mIU/mL) at month 8½ and by 93% (to 3324 mIU/mL) at month 45. Among the control group that received *Prevnar*™ and *Hiberix*™, seroprotection levels declined from an average of 79% pre vaccination to 56% at month 45. GMT of anti-HB antibodies also declined to 26.6 (95% CI 13.0–54.1) at the end of follow-up, reflecting the natural decay in antibodies and protection afforded by hepatitis B immunisation during the first year of life.

3.3. RF1-like antibody responses

Table 4 shows anti-RF1 antibody GMTs and seropositivity rates in Cohort 2 children at day 0 and 1 month after dose 3. No increase

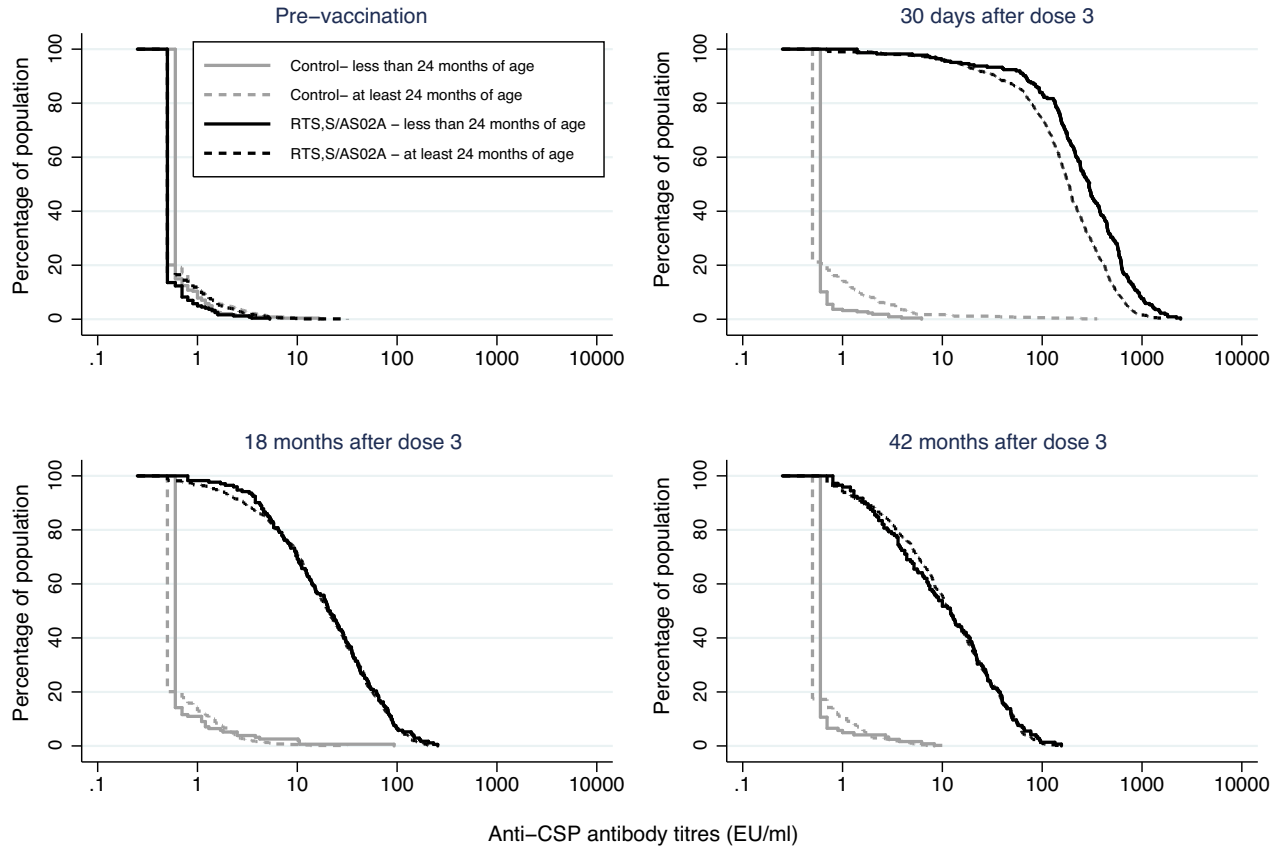


Fig. 3. Reverse cumulative distribution curves for the anti-CSP antibody titers by group, stratified by age at first dose in Cohort 1 and 2.

Table 2
Anti-CSP seropositivity rates and antibody GMCs by pre-vaccination HBsAg serostatus and study cohort in the RTS,S/AS02$_A$ vaccine group.

Cohort	Pre-vaccination status	Timing	*N*	Seropositivity for anti-CSP *n* (%)	Anti-CSP GMCs (95% CI)
	HBsAg	Baseline	584	69 (11.8)	0.3 (0.3–0.3)
	Negative	M3	584	579 (99.1)	181.4 (164.7–199.8)
		M8½	584	583 (99.8)	43.0 (38.8–47.7)
		M21	544	534 (98.2)	16.2 (14.4–18.1)
		M33	476	457 (96.0)	10.0 (8.8–11.4)
Cohort 1		M45	440	422 (95.9)	8.9 (7.8–10.1)
	HBsAg	Baseline	16	1 (6.3)	0.3 (0.2–0.3)
	Positive	M3	16	16 (100)	154.2 (89.4–265.9)
		M8½	16	16 (100)	42.0 (23.5–75.1)
		M21	14	14 (100)	17.7 (9.3–33.6)
		M33	13	13 (100)	11.4 (5.4–24.1)
		M45	11	11 (100)	9.5 (4.1–22.0)
	HBsAg	Baseline	176	62 (35.2)	0.5 (0.4–0.5)
	Negative	M3	169	169 (100)	267.9 (229.4–312.9)
		M8½	143	143 (100)	55.2 (45.5–67.0)
		M21	153	153 (100)	26.3 (22.0–31.5)
		M33	141	139 (98.6)	16.9 (13.7–20.9)
Cohort 2		M45	141	141 (100)	15.8 (13.0–19.2)
	HBsAg	Baseline	9	2 (22.2)	0.3 (0.2–0.5)
	Positive	M3	9	9 (100)	197.2 (88.7–438.3)
		M8½	9	9 (100)	30.1 (10.2–89.1)
		M21	9	9 (100)	18.2 (7.3–45.2)
		M33	8	7 (87.5)	7.7 (1.7–35.7)
		M45	9	9 (100)	10.0 (3.5–28.3)

N = number of subjects with available results.
N/% = number/percentage of subjects with titer within the specified range.
95% CI = 95% confidence interval.
M3, M8, M21, M33, M45 = months 3, 8, 21, 33 and 45 post dose 3
GMC = geometric mean concentrations.

in anti-RF1 seropositivity was observed in the *Prevnar*™ and *Hiberix*™ control group. Among participants receiving *Engerix-B*™, there was a 2.5 fold increase in anti-RF1 GMT and about 47% were seroprotected. However, among RTS,S/AS02$_A$ recipients seropositivity rates were greater than 98% in both age groups. There was also a marked increase in anti-RF1 GMT, being highest in children less than 24 months than in the older age group (a 55 and 22-fold increase in anti-RF1 antibody GMTs, respectively).

4. Discussion

Previous studies with RTS,S/AS02$_A$ in a endemic area of Mozambique showed that vaccination of children aged 1–4 years induces partial protection against infection and clinical malaria including severe disease [5,6,13], and the clinical benefit conferred by the vaccine is sustained over at least 45 months [13]. Here we report the immunogenicity of RTS,S/AS02$_A$ with respect to both the

Table 3
Anti-HBsAg seroprotection rates and antibody GMTs by age category and vaccine group in Cohort 2.

Age	Group	Timing	*N*	Seroprotection *n* (%)	Anti-HBs GMTs (95% CI)
	RTS,S/AS02$_A$	Baseline	44	34 (77.3)	62.9 (37.5–105.4)
		M3	41	40 (97.6)	51,035.4 (27,918.9–93,291.8)
		M 81/2	33	32 (97.0)	13,642.0 (7342.2–25,347.1)
		M21	37	36 (97.3)	5935.3 (3218.6–10,945.1)
		M33	33	32 (97.0)	4008.6 (2266.7–7089.1)
Less than 24 months at		M45	35	34 (97.1)	3323.8 (1908.4–5788.8)
day 0	*Prevnar*™ and	Baseline	42	33(78.6)	92.4 (47.1–181.1)
	Hiberix™	M3	33	26 (78.6)	67.7 (33.9–135.4)
		M81/2	31	23 (74.2)	40.1 (21.1–76.4)
		M21	37	23 (62.2)	35.5 (17.7–71.2)
		M33	33	16 (47.1)	20.3 (10.9–54.1)
		M45	35	18 (56.3)	26.6 (13.0–54.1)
	RTS,S/AS02$_A$	Baseline	148	28 (18.9)	9.1 (7.3–11.4)
		M3	134	132 (98.5)	11,368.6 (8518.9–15,171.6)
		M81/2	121	118 (97.5)	4556.4 (3499.8–5932.1)
		M21	125	123(98.4)	2877.0 (2241.5–3692.8)
At least 24 months old		M33	116	114 (98.3)	1842.5 (1413.7–2401.3)
at day 0		M45	115	113 (98.3)	1557.0 (1187.6–2041.4)
	Engerix-B™	Baseline	142	29 (20.4)	9.0 (7.2–11.2)
		M3	118	108 (91.5)	349.9 (236.7–517.0)
		M81/2	115	103 (89.6)	153.5 (110.6–213.0)
		M21	127	109 (85.8)	103.8 (74.8–144.1)
		M33	115	90 (78.3)	67.4 (47.5–95.7)
		M45	113	102 (90.3)	99.4 (73.1–135.1)

N = number of subjects with available results.
N/% = number/percentage of subjects with titer within the specified range.
95% CI = 95% confidence interval.
M3, M8, M21, M33, M45 = months 3, 8, 21, 33 and 45 post dose 3
GMT = geometric mean titer.

Table 4
Anti-RF1 seropositivity rates and antibody geometric mean titers (GMT) by age category in Cohort 2.

Age	Group	Timing	*N*	Seroprotection *n* (%)	Anti-RF1 GMT (95% CI)
Less than 24 months old at day 0	RTS,S/AS02$_A$	Baseline	43	2 (4.7)	20.2 (14.6–28.0)
		M3	39	39 (100)	1113.6 (810.2–1530.7)
	Prevnar™ and *Hiberix*™	Baseline	40	1 (2.5)	17.0 (16.0–18.1)
		M3	34	1 (2.9)	17.5 (15.5–19.7)
At least 24 months old at day 0	RTS,S/AS02$_A$	Baseline	146	4 (2.7)	19.0 (16.3–22.0)
		M3	132	130 (98.5)	421.7 (346.1–513.8)
	Engerix-B™	Baseline	143	2 (1.4)	17.9 (15.9–20.1)
		M3	107	50 (46.7)	43.2 (33.0–56.5)

N = number of subjects with available results.
N/% = number/percentage of subjects with titer within the specified range.
95% CI = 95% confidence interval.
M3 = month 3 post dose 3.
GMT = geometric mean titers.

P. falciparum and HBV components of the vaccine in the largest phase IIb trial of RTS,S/AS02$_A$ conducted and over a total surveillance period of 45 months. The RTS,S/AS02$_A$ candidate vaccine was shown to be immunogenic in young African children, inducing high anti-CSP antibody levels after three doses.

Recent Phase IIb trials of RTS,S/AS01$_E$ (a slightly modified Adjuvant System improving immunogenicity) in children have indicated that higher titers of anti-CSP IgG antibodies may be induced in children who have received previous immunization with HBV vaccine [21,22]. Several possible mechanisms have been proposed to explain this observation, including both B and/or T cell priming by prior HBV vaccination [21]. However, in our study we found no evidence that pre-vaccination anti-HBsAg antibody titers had an influence on the levels of anti-CSP antibodies induced by RTS,S/AS02$_A$.

High levels of antibodies to asexual blood-stage antigens acquired by natural exposure to *P. falciparum* infection were seen by IFAT, especially in Ilha Josina. IFAT served as an indirect measure to compare intensity of malaria transmission in the two trial sites [6]. RTS,S/AS02$_A$ recipients in Ilha Josina (Cohort 2) had higher anti-CSP antibodies throughout the follow-up period than those in Manhiça (Cohort 1). Together with the observed higher level of anti-CSP antibodies among the control group in Cohort 2 versus Cohort 1, and the higher production of anti-CSP antibodies following immunization in Cohort 2 compared to Cohort 1, it would appear that in areas of higher transmission, immunogenicity is higher and this may be a reflection of limited natural boosting.

Natural exposure induces a poor anti-CSP antibody response, including that achieved at high transmission. As such, pre-vaccination anti-CSP antibody levels were low in all study children and remained so in the control group of Cohort 2 throughout follow-up, therefore suggesting that parasite exposure induced poor anti-CSP responses (Fig. 2) that did not increase during the four year surveillance period despite a very high incidence of infection and disease; consequently we did not observe evidence of natural boosting of anti-CSP antibodies.

In the RTS,S/AS02$_A$ group, anti-CSP antibody levels peaked 30 days post dose 3, declining over the next 6 months to about ¼ of the peak level, but remaining 30-fold higher than the cut-off level until month 45 compared to pre-vaccination GMT or control individuals. These results are consistent with those observed in a 5 year follow-up study conducted in Gambian semi-immune adults vaccinated with RTS,S/AS02$_A$ [23] It is not clear if the sustained IgG levels, presumably by long-lived plasma cells in the bone marrow, are sufficient for an antibody-mediated contribution in protection. It would be of interest to study the kinetics of memory B-cell response to CSP stimulus and the magnitude of expansion and up-regulation of antibody production following subsequent exposures to *P. falciparum* sporozoite infections. Alternatively, vaccine-specific antibodies could last for decades in the absence of antigenic re-exposure, as has been shown with smallpox vaccination [26] and recently with other malaria antigens upon natural infection [24,25]. Other human vaccines (e.g. live measles, mumps, and poliovirus) also induce serum antibody responses that persist for years, however it is unclear how durable antibody responses may be affected by intermittent re-exposure to the antigen, and this question remains open also for RTS,S/AS02$_A$ vaccination.

Another interesting observation is the effect of age on the antibody titers induced by the vaccine. One month after dose 3 of RTS,S/AS02$_A$, anti-CSP IgG levels were higher in the younger children (<2 years) compared to the older children (≥2 years), further demonstrating that the vaccine is highly immunogenic also in young children.

We previously assessed the role of anti-CSP antibody responses induced by RTS,S/AS02$_A$ in protection against malaria and found that higher levels of anti-CSP IgG were associated with a lower risk of infection in Cohort 2 [26]. The hazard ratio for *P. falciparum* infection of children per ten-fold increase in the value of anti-CSP antibodies was 0.41 (95% CI 0.28–0.60, $p < 0.001$). This is consistent with results from subsequent infant phase I/IIb trials of RTS,S/AS02$_D$ conducted in the same area and in Tanzania, in which high antibody titers were also associated with protection against infection [7,8]. However, no association was found between peak CSP antibody responses and protection against clinical malaria in any of the cohorts.

This indicates that anti-CSP antibodies, probably together with other cellular immune responses, as shown in other RTS,S/AS studies in naïve adults [11] and as measured in Mozambican infants [9], are involved in initial protection against infection. However, higher anti-CSP antibody titers did not predict greater protection against clinical malaria [13]. One hypothesis is that the longevity of protection found in Cohort 1 may be explained by the fact that as a "leaky" vaccine, RTS,S/AS vaccination in the context of ongoing malaria exposure has allowed the acquisition of natural immunity to liver or blood-stage antigens in vaccine recipients that are similar to those that develop in unvaccinated controls [26]. Additional studies will be required to determine the validity of this hypothesis. In addition to the magnitude of the antibodies, the quality and type of the IgGs may be relevant to their anti-parasite effector role. Therefore, for a more complete understanding of the mechanism of action of RTS,S/AS, future studies should include evaluation of IgG isotypes, avidity and affinity and the biological function, e.g. the neutralizing effect of antibodies to inhibit the migration, invasion or development of sporozoites or an opsonizing capacity [27] that may facilitate the phagocytosis and digestion of sporozoites by antigen presenting cells. Likewise, the mechanism of induction and maintenance of memory B cell responses to RTS,S/AS needs to be investigated more thoroughly to better understand the pattern of affinity maturation and kinetics of vaccine-specific anti-CSP

antibody responses. Similarly, the contribution of cellular immunity to RTS,S/AS-induced protection needs further investigation. It remains to be established whether a fourth vaccine dose will boost the antibody responses and help sustain their durability. This will be tested in the ongoing phase III trial of RTS,S/AS01$_E$, the primary objective of which is to gather the safety, efficacy and immunogenicity data necessary for vaccine registration. The study will also provide a unique opportunity to try to understand the vaccine mechanisms of action and investigate immune correlates of vaccine-induced protection. Possibilities for improving on the partial protection against malaria observed in previous RTS,S/AS0 trials would include the combination of this vaccine with other *P. falciparum* antigens and/or delivery systems in heterologous prime-boost immunization schemes.

Finally, we evaluated the antibody responses induced by the HBV portion of RTS,S. This trial gave us the unique opportunity of having a head to head comparison of RTS,S and a licensed hepatitis B vaccine (*Engerix-B*™) in children older than 24 months. RTS,S induced higher antibody levels and achieved greater levels of seroprotection than did the commercially available hepatitis B vaccine.

5. Conclusions

RTS,S/AS02A has been found to be safe and to induce moderate levels of protection against different malaria endpoints. Further work is required to understand the exact immune mechanisms of action. Detectable anti-CSP GMCs antibodies persisted up to month 45 (42 months after dose 3) in RTS,S/AS02$_A$ recipients, and at least 97% of the vaccine recipients had seroprotective levels of anti-HBsAg antibodies 45 months after immunization. The anti-HBsAg antibody response was significantly higher with RTS,S/AS02$_A$ compared to *Engerix-B*™. The candidate malaria vaccine was highly immunogenic for anti-CSP and anti-HBsAg antibodies, especially in children younger than 24 months.

This vaccine, currently undergoing phase III trials will require further improvements to expand its protection. Understanding the immune mechanisms of action will increase our chance of improving its performance.

Engerix-B™ and *Hiberix*™ are trademarks of the GlaxoSmithKline group of companies. *Prevnar*™ is a trademark of Lederle.

Acknowledgements

A complex trial like this one would not have been possible without the contribution of a large number of people. We would first like to thank the children and parents participating in the study, as well as the entire Manhiça community. We would also like to thank all the CISM staff and the District, Provincial and National Health Authorities for their support.

We thank Dr Domingo Gargallo-Viola, María Roncalés, Pedro Torres, Sonia Lozano and Mª Jesús Almela at GSK, Tres Cantos, Madrid, and Dr Denise Doolan, formerly at the Naval Medical Research Center, Maryland, for their invaluable contributions to the IFAT determinations. We thank Joe Campo for critically reviewing the manuscript.

Dr Esperança Sevene was the Local Safety Monitor for this trial. She provided invaluable and continuous support and guidance. Professor Malcolm Molyneux was the Chairman of the Data and Safety Monitoring Board. His advice and support were greatly appreciated by the investigators.

We thank Dr Julia Donnelly for her expert editorial help and Dr Lode Schuerman for critically reviewing the manuscript.

Contributors: Each of the authors participated in the design, implementation, analysis and interpretation of the study. Pedro Aide, Jahit Sacarlal, Pedro Alonso and John J. Aponte were involved in all phases of the study. John J. Aponte and Marc Lievens led the data analysis. Pedro Aide, Jahit Sacarlal, Caterina Guinovart, Quique Bassat, Montse Renom and Eusebio Macete were responsible for the field and hospital activities as well as safety surveillance. Inacio Mandomando coordinated all laboratory work at CISM. Carlota Dobaño planned and conducted the IFAT analyses and Laura Puyol executed the IFAT measurements at CRESIB. Esperanza Herreros prepared the *P. falciparum* cultures for IFAT slides at GSK Tres Cantos. Marie-Ange Demoitie was coordinator for the anti-CSP, anti-HBsAg and RF-1 serologies. Amanda Leach led the clinical team at GSK Biologicals. Marie-Claude Dubois was the malaria vaccine project manager at GSK Biologicals. Joe Cohen and W. Ripley Ballou headed the malaria vaccine research and development at GSK Biologicals (WRB at the time of this trial). Christian Loucq serves as the Director of the PATH Malaria Vaccine Initiative (MVI). Pedro Aide, Carlota Dobaño and Jahit Sacarlal led the manuscript preparation with inputs from all other investigators. *Conflict of interests*: MVI supports the development and testing of a number of malaria vaccines that can be seen as competitors. Amanda Leach, Esperanza Herreros, Marc Lievens, Johan Vekemans, Marie-Ange Demoitie, Marie-Claude Dubois, W Ripley Ballou and Joe Cohen are current or previous employees of GlaxoSmithKline Biologicals. Amanda Leach, W. Ripley Ballou, Marie-Claude Dubois and Joe Cohen own shares in GlaxoSmithKline. Both Joe Cohen and W. Ripley Ballou are listed as the 'Inventors' of patented malaria vaccines. However neither individual holds a patent for a malaria vaccine. None of the other authors in this paper have declared conflicts of interest. *Financial disclosure*: GSK and CISM both received financial support to conduct the work described in this paper from PATH Malaria Vaccine Initiative (MVI). The initial support to CISM by MVI was passed through GSK for administrative purposes. GSK and MVI (sponsors and funders) participated in the design of the trial and interpretation of the data, review and approval of the analysis presented in this article. GSK also participated in the implementation of the trial. Core funding for CISM is provided by the Spanish Agency for International Cooperation (AECI).

References

[1] WHO. World Malaria Report 2009, 2009 [cited 2010 12 July]; Available from: http://whqlibdoc.who.int/publications/2009/9789241563901_eng.pdf.
[2] Gordon DM, McGovern TW, Krzych U, Cohen JC, Schneider I, LaChance R, et al. Safety, immunogenicity, and efficacy of a recombinantly produced *Plasmodium falciparum* circumsporozoite protein-hepatitis B surface antigen subunit vaccine. J Infect Dis 1995;171(January (6)):1576–85.
[3] Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med 1997;336(January (2)):86–91.
[4] Bojang KA, Milligan PJ, Pinder M, Vigneron L, Alloueche A, Kester KE, et al. Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium falciparum* infection in semi-immune adult men in The Gambia: a randomised trial. Lancet 2001;358(December (9297)):1927–34.
[5] Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Aide P, et al. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. Lancet 2005;366(December (9502)): 2012–8.
[6] Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. Lancet 2004;364(October (9443)):1411–20.
[7] Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet 2007;370(November (9598)): 1543–51.
[8] Abdulla S, Oberholzer R, Juma O, Kubhoja S, Machera F, Membi C, et al. Safety and Immunogenicity of RTS,S/AS02D Malaria Vaccine in Infants. N Engl J Med 2008;(December).
[9] Barbosa A, Naniche D, Aponte JJ, Manaca MN, Mandomando I, Aide P, et al. *Plasmodium falciparum*-specific cellular immune responses after immunization

with the RTS,S/AS02D candidate malaria vaccine in infants living in an area of high endemicity in Mozambique. Infect Immun 2009;77(October (10)):4502–9.
[10] Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, Vekemans J, et al. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med 2008;(December).
[11] Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis 2009;200(August (3)):337–46.
[12] Waters JA, Pignatelli M, Brown D, O'Rourke S, Lever A, Thomas HC. The immune response to hepatitis B virus. Postgrad Med J 1987;63(Suppl. (2)):51–6.
[13] Sacarlal J, Aide P, Aponte JJ, Renom M, Leach A, Mandomando I, et al. Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children. J Infect Dis 2009;200(August (3)):329–36.
[14] Alonso PL, Saúte, F., Aponte, J.J., Gómez-Olivé, F.X., Nhacolo, A., Thompson, R., Macete, E., Abacassamo, F., Ventura, P.J., Bosch, X., Menéndez, C., Dgedge, M. (2002) Manhiça DSS, Mozambique. In population and health in developing countries. 1st ed. Ottawa: International Development Research Centre (IDRC).
[15] Aranda C, Aponte JJ, Saute F, Casimiro S, Pinto J, Sousa C, et al. Entomological characteristics of malaria transmission in Manhica, a rural area in southern Mozambique. J Med Entomol 2005;42(March (2)):180–6.
[16] Nhacolo AQ, Nhalungo DA, Sacoor CN, Aponte JJ, Thompson R, Alonso P. Levels and trends of demographic indices in southern rural Mozambique: evidence from demographic surveillance in Manhica district. BMC Public Health 2006;6:291.
[17] Sacarlal J, Aponte JJ, Aide P, Mandomando I, Bassat Q, Guinovart C, et al. Safety of the RTS,S/AS02A malaria vaccine in Mozambican children during a Phase IIb trial. Vaccine 2008;26(January (2)):174–84.
[18] Macete E, Aponte JJ, Guinovart C, Sacarlal J, Ofori-Anyinam O, Mandomando I, et al. Safety and immunogenicity of the RTS,S/AS02A candidate malaria vaccine in children aged 1-4 in Mozambique. Trop Med Int Health 2007;12(January (1)):37–46.
[19] Cambron P, Jacquet JM, Hoet B, Lievens M. Development and technical and clinical validation of a quantitative enzyme-linked immunosorbent assay for the detection of human antibodies to hepatitis B surface antigen in recipients of recombinant hepatitis B virus vaccine. Clin Vaccine Immunol 2009;16(August (8)):1236–46.
[20] Reed GF, Meade BD, Steinhoff MC. The reverse cumulative distribution plot: a graphic method for exploratory analysis of antibody data. Pediatrics 1995;96(September (3)):600–3.
[21] Lell B, Agnandji S, von Glasenapp I, Haertle S, Oyakhiromen S, Issifou S, et al. A randomized trial assessing the safety and immunogenicity of AS01 and AS02 adjuvanted RTS,S malaria vaccine candidates in children in Gabon. PLoS One 2009;4(10):e7611.
[22] Owusu-Agyei S, Ansong D, Asante K, Kwarteng Owusu S, Owusu R, Wireko Brobby NA, et al. Randomized controlled trial of RTS,S/AS02D and RTS,S/AS01E malaria candidate vaccines given according to different schedules in Ghanaian children. PLoS One 2009;4(10):e7302.
[23] Bojang K, Milligan P, Pinder M, Doherty T, Leach A, Ofori-Anyinam O, et al. Five-year safety and immunogenicity of GlaxoSmithKline's candidate malaria vaccine RTS,S/AS02 following administration to semi-immune adult men living in a malaria-endemic region of The Gambia. Hum Vaccin 2009;5(April (4)):242–7.
[24] Amanna IJ, Slifka MK, Crotty S. Immunity and immunological memory following smallpox vaccination. Immunol Rev 2006;211(June):320–37.
[25] Wipasa J, Suphavilai C, Okell LC, Cook J, Corran PH, Thaikla K, et al. Long-lived antibody and B Cell memory responses to the human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*. PLoS Pathog 2010;6(2):e1000770.
[26] Guinovart C, Aponte JJ, Sacarlal J, Aide P, Leach A, Bassat Q, et al. Insights into long-lasting protection induced by RTS S/AS02A malaria vaccine: further results from a phase IIb trial in Mozambican children. PLoS One 2009;4(4):e5165.
[27] Schwenk R, Asher LV, Chalom I, Lanar D, Sun P, White K, et al. Opsonization by antigen-specific antibodies as a mechanism of protective immunity induced by *Plasmodium falciparum* circumsporozoite protein-based vaccine. Parasite Immunol 2003;25(January (1)):17–25.

13. SUMMARY OF RESULTS

Article 1: Towards an effective malaria vaccine. Arch. Dis. Child. 2007

Malaria still affects almost half of the world's population, with a very significant burden of related morbidity and mortality. An effective malaria vaccine, used in combination with the existing control tools, would act as a key element to boost malaria control.

Several factors explain why to date this has not yet been achieved, among others, the great complexity of the parasite, our limited knowledge about how immunity against malaria is acquired and the lack of appropriate animal models.

Despite that, there is currently enough evidence to support the development of an effective malaria vaccine. Ideally, the perfect malaria candidate vaccine should be safe, 100% effective not only in children but also in infants, cheap, easy to administer and capable of conferring long lasting immunity, an unrealistic proposal in the short term.

Malaria vaccines are being designed according to the target population or according to the parasite life cycle (pre-erythrocytic, erythrocytic or transmission blocking vaccines). Strategies to combine antigens from different stages, aiming to trigger an intense and sequential immune response, or different antigens from the same phase (multivalent

vaccines), so as to increase the efficacy and reduce the risk of emergent resistances, are also under consideration.

Currently, several candidate vaccines have reached different development stages, although most of them are still in the preclinical phases. The most advanced candidate vaccine, the RTS,S/AS02$_A$, has been developed and jointly financed by GlaxoSmithKline and the Malaria Vaccine Initiative (MVI). Two other candidate vaccines, the MVA-ME TRAP (a pre-erythrocytic vaccine) and the MSP1/AS02$_A$ (an erythrocytic vaccine using the same adjuvant as the RTS,S/AS02$_A$) have also undergone clinical trials in children from malaria endemic areas.

The significant, although partial, efficacy results established by recent trials of the RTS,S candidate malaria vaccine have granted a reasonable optimism with regards to the development of an effective malaria vaccine in the near future.

Article 2: Safety, immunogenicity and duration of protection of the RTS,S/AS02$_D$ malaria vaccine: one year follow-up of a randomized controlled phase I/IIb trial.

Safety data was available for 214 children. 107 received 301 doses of RTS,S/AS02$_D$ and 309 doses of *TETRActHib*^{TM} and 107 received 303 doses of *Engerix-B*^{TM} and 311 doses of *TETRActHib*^{TM}. The frequency of serious adverse events was 32.7% (95% CI: 24.0 – 42.5) in the RTS,S/AS02$_D$ and 31.8% (95% CI: 23.1–41.5) in the control group.

The geometric mean titers of anti-circumsporozoite antibodies declined from 199.9 to 7.3 EU/mL from one to 12 months post dose three of RTS,S/AS02$_D$, remaining 15-fold higher than in the control group.

Vaccine efficacy between months 3 to 9 of follow-up was 48.8% (95% CI: 11.3–70.4, p=0.017) against first or only clinical episodes and 53.7% (95% CI: 21.4–72.7, p=0.004) against multiple episodes. VE over the entire follow-up period (months 3 to 14) was 33.0% (95% CI -4.3–56.9, p=0.076) and 25.9% (95% CI -15.7–52.6, p=0.167) against first or only and multiple clinical malaria episodes respectively.

The hazard rate of disease per 2-fold increase in anti-CS titters at one month post dose 3 was reduced by 84% (95% CI 35.1–88.2, p=0.003) and 72.4% (95% CI: 35.1 – 88.2, p=0.003) for the two follow-up periods ($ATP_{3–9}$ and ATP_{3-14}), respectively.

Article 3: *Plasmodium falciparum*-specific cellular immune responses after immunization with the RTS,S/AS02$_D$ candidate malaria vaccine in infants living in an area of high endemicity in Mozambique.

Whole-blood cell cultures for cell immunogenicity measures were performed from 206 blood samples taken before immunization, and from 186 and 188 samples taken at 4 and 10.5 weeks after the third dose. Preimmune specific levels of IFN-γ, IL-2, and IL-4 were low ranging from mostly undetectable up to 45.2, 51.6, and 5.3 pg ml^{-1}, respectively.

The median stimulation index of cytokine- producing CD4$^+$ and CD8$^+$ cells intracellular cytokine production by CD4$^+$ and CD8$^+$ cells was very low but significantly higher in RTS,S immunized infants compared to those infants having received the comparator vaccine.

Children immunized with the RTS,S formulation showed slightly higher HBS-specific IFN-γ and IL-2 responses as compared to the Hepatitis-B vaccine group.

Medians of stimulation indices for both CD4$^+$ and CD8$^+$ T cells ranged from 0.81 to 1.34 in both immunization groups.

Protection against subsequent malaria infection tended to associate with a higher percentage of individuals with CSP-specific IL-2 in supernatant (p=0.053) and with higher CSP-specific IFN-γ CD8$^+$ T responses (p = 0.07) observed 10.5 weeks after immunization.

Article 4: Four year immunogenicity of the RTS,S/AS02$_A$ malaria vaccine in Mozambican children during a phase IIb trial.

A total of 1565 children were included in the immunogenicity analysis: 795 in the RTS,S/AS02$_A$ and 770 in the control groups.

The RTS,S/AS02$_A$ vaccine induced high anti-circumsporozoite antibody levels with at least 96% of children remaining seropositive during the entire follow-up period. IgG titers decayed over the first 6 months of follow-up to about 25% of the initial level, but still remained 30-fold higher until month 45 compared to controls.

When doubling the IFAT titers at baseline, the vaccine-induced anti-CSP antibodies modestly increased by 1.07 (95% CI: 1.04 – 1.09, p<0.0001) after adjusting for the effect of age and cohort. Children in Cohort 2 had 1.23 (95% CI: 1.01– 1.50, p=0.04) times higher anti-CSP antibody titers compared to those in Cohort 1.

The RTS,S/AS02$_A$ vaccine also induced high levels of anti-hepatitis B surface antigen antibodies (seroprotection >97%).

In children < 24 months of age, the pre-vaccination seroprotective levels of anti-HBsAg antibodies were high in both the RTS,S/AS02$_A$ and control groups (>77%), reflecting the prior HBV immunisation. Following administration of RTS,S, seroprotection rates increased to 97% and remained so throughout the entire follow-up. GMT values for anti-HBsAg antibodies in this group increased from 62.9 mIU/mL at baseline to 51035

mIU/mL 1 month post dose 3. This value decreased by 75% (to 13642 mIU/mL) at month 8½ and by 93% (to 3324 mIU/mL) at month 45.

14. CONCLUSIONS

i. The development of a safe and effective malaria vaccine is feasible.

ii. The RTS,S/AS02$_D$ malaria vaccine administered to young infants has a good safety and reactogenicity profile.

iii. In infants, anti-CS antibodies levels decreased over time but remained 15 fold higher in the RTS,S/AS02$_D$ group than in the control group at 12 months post third immunization.

iv. A strong association between anti-CS antibodies and risk of clinical malaria has been described for the first time in infants vaccinated with RTS,S/AS02$_D$.

v. In infants, both anti-CS antibodies and vaccine efficacy appear to decrease over time.

vi. The RTS,S/AS02$_D$ vaccine elicits detectable cellular mediated immune responses to both CS and HBs antigens following immunization of infants less than 1 year of age.

vii. In children, the RTS,S/AS02$_A$ vaccine induces anti-circumsporozoite antibodies that persist for at least 42 months after immunization.

15. REFERENCES

1. Cox FE. History of the discovery of the malaria parasites and their vectors. Parasit Vectors 2010;3(1):5.

2. Bruce-Chwatt L. History of malaria from prehistory to eradication. In: Wernsdorfer WH, Mac Gregor I, editors. Malaria: principles and practice of malariology. Edinburgh. Churchill Livingstone; 1988.

3. Gilles HM, Lucas AO. Tropical medicine: 100 years of progress. Br Med Bull1998;54(2):269-80.

4. Sherman IW. Malaria: parasite biology, pathogenesis, and protection. Washington: ASM Press; 1998.

5. Smith AG. Chlorinated hydrocarbon insecticides. In: Hayes WJ, Laws ER, editors. Handbook of pesticide toxicology / Vol 3, Classes of pesticides. San Diego/New York: Academic Press, San Diego; 1991. p. 731 - 915.

6. WHO. Global malaria control and elimination: report of a technical review. Geneva 2008; Available from: http://whqlibdoc.who.int/publications/2008/9789241596756_eng.pdf.

7. Greenwood B, Mutabingwa T. Malaria in 2002. Nature2002 Feb 7;415(6872):670-2.

8. Shiff C. Integrated approach to malaria control. Clin Microbiol Rev2002 Apr;15(2):278-93.

9. Greenwood BM, Bradley AK, Greenwood AM, Byass P, Jammeh K, Marsh K, et al. Mortality and morbidity from malaria among children in a rural area of The Gambia, West Africa. Trans R Soc Trop Med Hyg1987;81(3):478-86.

10. Snow RW, Craig M, Deichmann U, Marsh K. Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. Bull World Health Organ1999;77(8):624-40.

11. WHO. World Malaria Report 2010. Geneva 2010 [cited 2010 12 March]; Available from: http://whqlibdoc.who.int/publications/2010/9789241564106_eng_Chapter6.pdf.

12. Molineaux L. The epidemiology of human malaria as an explanation of its distribution, including some implications to its control. In: Wernsdorfer WH, Mac Gregor I, editors. Malaria : principles and practice of malariology. Edinburgh Churchill Livingstone; 1988. p. 913-98.

13. Carter R, Mendis KN. Evolutionary and historical aspects of the burden of malaria. Clin Microbiol Rev2002 Oct;15(4):564-94.

14. Feachem RG, Phillips AA, Hwang J, Cotter C, Wielgosz B, Greenwood BM, et al. Shrinking the malaria map: progress and prospects. Lancet2010 Nov 6;376(9752):1566-78.

15. Dondorp AM, Yeung S, White L, Nguon C, Day NP, Socheat D, et al. Artemisinin resistance: current status and scenarios for containment. Nat Rev Microbiol2010 Apr;8(4):272-80.

16. N'Guessan R, Corbel V, Akogbeto M, Rowland M. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. Emerg Infect Dis2007 Feb;13(2):199-206.

17. Crompton PD, Pierce SK, Miller LH. Advances and challenges in malaria vaccine development. J Clin Invest2010 Dec 1;120(12):4168-78.

18. Alonso PL, Ballou R, Brown G, Chitnis C, Loucq C, Moorthy V, et al. A research agenda for malaria eradication: vaccines. PLoS Med;8(1):e1000398.

19. PNCM. Plano estratégico para o controlo de malária em Moçambique 2006-2009. MISAU, Maputo.

20. Saute F, Aponte J, Almeda J, Ascaso C, Abellana R, Vaz N, et al. Malaria in southern Mozambique: malariometric indicators and malaria case definition in Manhica district. Trans R Soc Trop Med Hyg2003 Nov-Dec;97(6):661-6.

21. Saute F, Menendez C, Mayor A, Aponte J, Gomez-Olive X, Dgedge M, et al. Malaria in pregnancy in rural Mozambique: the role of parity, submicroscopic and multiple Plasmodium falciparum infections. Trop Med Int Health2002 Jan;7(1):19-28.

22. Menendez C. Malaria during pregnancy: a priority area of malaria research and control. Parasitol Today1995 May;11(5):178-83.

23. Sacarlal J, Nhacolo AQ, Sigauque B, Nhalungo DA, Abacassamo F, Sacoor CN, et al. A 10 year study of the cause of death in children under 15 years in Manhica, Mozambique. BMC Public Health2009;9:67.

24. Abacassamo F, Enosse S, Aponte JJ, Gomez-Olive FX, Quinto L, Mabunda S, et al. Efficacy of chloroquine, amodiaquine, sulphadoxine-pyrimethamine and combination therapy with artesunate in Mozambican children with non-complicated malaria. Trop Med Int Health2004 Feb;9(2):200-8.

25. WHO. Antimalaria drug combination therapy: Report of a WHO Technical Consultation Geneva: World Health Organization; 2001; Available from: http://whqlibdoc.who.int/hq/2001/WHO_CDS_RBM_2001.35.pdf.

26. Programa Nacional de Controlo de Malária. Normas de tratamento de malária. Maputo: MISAU; 2005.

27. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, et al. Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis2008 Jan 15;46(2):165-71.

28. White NJ. Plasmodium knowlesi: the fifth human malaria parasite. Clin Infect Dis2008 Jan 15;46(2):172-3.

29. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. Lancet2004 Mar 27;363(9414):1017-24.

30. Amino R, Thiberge S, Shorte S, Frischknecht F, Menard R. Quantitative imaging of Plasmodium sporozoites in the mammalian host. C R Biol2006 Nov;329(11):858-62.

31. Hunt RH, Coetzee M, Fettene M. The Anopheles gambiae complex: a new species from Ethiopia. Trans R Soc Trop Med Hyg1998 Mar-Apr;92(2):231-5.

32. Coetzee M. Distribution of the African malaria vectors of the Anopheles gambiae complex. Am J Trop Med Hyg2004 Feb;70(2):103-4.

33. Levine RS, Peterson AT, Benedict MQ. Geographic and ecologic distributions of the Anopheles gambiae complex predicted using a genetic algorithm. Am J Trop Med Hyg2004 Feb;70(2):105-9.

34. Arrow KJ, Gelband H, Panosian C, ebrary Inc. Saving lives, buying time[Recurso electrónico] :] economics of malaria drugs in an age of resistance. Washington, D.C.: National Academies Press; 2004.

35. Breman JG. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am J Trop Med Hyg2001 Jan-Feb;64(1-2 Suppl):1-11.

36. Greenwood BM, Bojang K, Whitty CJ, Targett GA. Malaria. Lancet2005 Apr 23-29;365(9469):1487-98.

37. Schellenberg D, Menendez C, Kahigwa E, Font F, Galindo C, Acosta C, et al. African children with malaria in an area of intense Plasmodium falciparum transmission: features on admission to the hospital and risk factors for death. Am J Trop Med Hyg1999 Sep;61(3):431-8.

38. Newton CR, Marsh K, Peshu N, Mwangi I. Blood transfusions for severe anaemia in African children. Lancet1992 Oct 10;340(8824):917-8.

39. Molyneux ME, Taylor TE, Wirima JJ, Borgstein A. Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children. Q J Med1989 May;71(265):441-59.

40. Lewallen S, Harding SP, Ajewole J, Schulenburg WE, Molyneux ME, Marsh K, et al. A review of the spectrum of clinical ocular fundus findings in P. falciparum malaria in African children with a proposed classification and grading system. Trans R Soc Trop Med Hyg1999 Nov-Dec;93(6):619-22.

41. English M, Waruiru C, Amukoye E, Murphy S, Crawley J, Mwangi I, et al. Deep breathing in children with severe malaria: indicator of metabolic acidosis and poor outcome. Am J Trop Med Hyg1996 Nov;55(5):521-4.

42. Taylor TE, Borgstein A, Molyneux ME. Acid-base status in paediatric Plasmodium falciparum malaria. Q J Med1993 Feb;86(2):99-109.

43. Pongponratn E, Turner GD, Day NP, Phu NH, Simpson JA, Stepniewska K, et al. An ultrastructural study of the brain in fatal Plasmodium falciparum malaria. Am J Trop Med Hyg2003 Oct;69(4):345-59.

44. Adams S, Brown H, Turner G. Breaking down the blood-brain barrier: signaling a path to cerebral malaria? Trends Parasitol2002 Aug;18(8):360-6.

45. Beeson JG, Reeder JC, Rogerson SJ, Brown GV. Parasite adhesion and immune evasion in placental malaria. Trends Parasitol2001 Jul;17(7):331-7.

46. Scherf A, Pouvelle B, Buffet PA, Gysin J. Molecular mechanisms of Plasmodium falciparum placental adhesion. Cell Microbiol2001 Mar;3(3):125-31.

47. Ekvall H. Malaria and anemia. Curr Opin Hematol2003 Mar;10(2):108-14.

48. Menendez C, Schellenberg D, Quinto L, Kahigwa E, Alvarez L, Aponte JJ, et al. The effects of short-term iron supplementation on iron status in infants in malaria-endemic areas. Am J Trop Med Hyg2004 Oct;71(4):434-40.

49. Marsh K, Forster D, Waruiru C, Mwangi I, Winstanley M, Marsh V, et al. Indicators of life-threatening malaria in African children. N Engl J Med1995 May 25;332(21):1399-404.

50. Stevenson MM, Zavala F. Immunology of malaria infections. Parasite Immunol2006 Jan-Feb;28(1-2):1-4.

51. Moorthy VS, Good MF, Hill AV. Malaria vaccine developments. Lancet2004 Jan 10;363(9403):150-6.

52. Snow RW, Nahlen B, Palmer A, Donnelly CA, Gupta S, Marsh K. Risk of severe malaria among African infants: direct evidence of clinical protection during early infancy. J Infect Dis1998 Mar;177(3):819-22.

53. Mayor A, Aponte JJ, Fogg C, Saute F, Greenwood B, Dgedge M, et al. The epidemiology of malaria in adults in a rural area of southern Mozambique. Malar J2007;6:3.

54. Marsh K, Snow RW. Host-parasite interaction and morbidity in malaria endemic areas. Philos Trans R Soc Lond B Biol Sci1997 Sep 29;352(1359):1385-94.

55. Perlmann P, Troye-Blomberg M. Malaria blood-stage infection and its control by the immune system. Folia Biol (Praha)2000;46(6):210-8.

56. Hamon J, Mouchet J, Chauvet G, Lumaret R. [Review of 14 Years of Malaria Control in the French-Speaking Countries of Tropical Africa and in Madagascar. Considerations on the Persistence of Transmission and Future Prospects]. Bull Soc Pathol Exot Filiales1963 Sep-Oct;56:933-71.

57. Mouchet J, Laventure S, Blanchy S, Fioramonti R, Rakotonjanabelo A, Rabarison P, et al. [The reconquest of the Madagascar highlands by malaria]. Bull Soc Pathol Exot1997;90(3):162-8.

58. Allen SJ, O'Donnell A, Alexander ND, Alpers MP, Peto TE, Clegg JB, et al. alpha+-Thalassemia protects children against disease caused by other infections as well as malaria. Proc Natl Acad Sci U S A1997 Dec 23;94(26):14736-41.

59. Gilles HM, Fletcher KA, Hendrickse RG, Lindner R, Reddy S, Allan N. Glucose-6-phosphate-dehydrogenase deficiency, sickling, and malaria in African children in South Western Nigeria. Lancet1967 Jan 21;1(7482):138-40.

60. Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, et al. Common west African HLA antigens are associated with protection from severe malaria. Nature1991 Aug 15;352(6336):595-600.

61. Ruwende C, Khoo SC, Snow RW, Yates SN, Kwiatkowski D, Gupta S, et al. Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. Nature1995 Jul 20;376(6537):246-9.

62. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ2001;79(8):704-12.

63. Stevenson MM, Riley EM. Innate immunity to malaria. Nat Rev Immunol2004 Mar;4(3):169-80.

64. Kremsner PG, Krishna S. Antimalarial combinations. Lancet2004 Jul 17-23;364(9430):285-94.

65. Adjuik M, Babiker A, Garner P, Olliaro P, Taylor W, White N. Artesunate combinations for treatment of malaria: meta-analysis. Lancet2004 Jan 3;363(9402):9-17.

66. Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T, et al. Effects of artemisinin derivatives on malaria transmissibility. Lancet1996 Jun 15;347(9016):1654-8.

67. White NJ. Preventing antimalarial drug resistance through combinations. Drug Resist Updat1998 Mar;1(1):3-9.

68. Vallely A, Vallely L, Changalucha J, Greenwood B, Chandramohan D. Intermittent preventive treatment for malaria in pregnancy in Africa: what's new, what's needed? Malar J2007;6:16.

69. Greenwood B. Review: Intermittent preventive treatment--a new approach to the prevention of malaria in children in areas with seasonal malaria transmission. Trop Med Int Health2006 Jul;11(7):983-91.

70. Raghavendra K, Barik TK, Reddy BP, Sharma P, Dash AP. Malaria vector control: from past to future. Parasitol Res Apr;108(4):757-79.

71. WHO. Use of indoor residual spraying for scaling up global malaria control and elimination Geneva: 2006. WHO/HTM/MAL/2006.1112.

72. Alonso PL, Lindsay SW, Armstrong JR, Conteh M, Hill AG, David PH, et al. The effect of insecticide-treated bed nets on mortality of Gambian children. Lancet1991 Jun 22;337(8756):1499-502.

73. Lengeler C. Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database Syst Rev2004(2):CD000363.

74. Schellenberg D, Menendez C, Kahigwa E, Aponte J, Vidal J, Tanner M, et al. Intermittent treatment for malaria and anaemia control at time of routine vaccinations in Tanzanian infants: a randomised, placebo-controlled trial. Lancet2001 May 12;357(9267):1471-7.

75. Clyde DF, Most H, McCarthy VC, Vanderberg JP. Immunization of man against sporozite-induced falciparum malaria. Am J Med Sci1973 Sep;266(3):169-77.

76. Clyde DF. Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. Am J Trop Med Hyg1975 May;24(3):397-401.

77. Alonso PL, Tanner M, Smith T, Hayes RJ, Schellenberg JA, Lopez MC, et al. A trial of the synthetic malaria vaccine SPf66 in Tanzania: rationale and design. Vaccine1994 Feb;12(2):181-6.

78. D'Alessandro U, Leach A, Drakeley CJ, Bennett S, Olaleye BO, Fegan GW, et al. Efficacy trial of malaria vaccine SPf66 in Gambian infants. Lancet1995 Aug 19;346(8973):462-7.

79. Acosta CJ, Galindo CM, Schellenberg D, Aponte JJ, Kahigwa E, Urassa H, et al. Evaluation of the SPf66 vaccine for malaria control when delivered through the EPI scheme in Tanzania. Trop Med Int Health1999 May;4(5):368-76.

80. Nosten F, Luxemburger C, Kyle DE, Ballou WR, Wittes J, Wah E, et al. Randomised double-blind placebo-controlled trial of SPf66 malaria vaccine in children in northwestern Thailand. Shoklo SPf66 Malaria Vaccine Trial Group. Lancet1996 Sep 14;348(9029):701-7.

81. Graves P, Gelband H. Vaccines for preventing malaria (SPf66). Cochrane Database Syst Rev2006(2):CD005966.

82. Malkin E, Dubovsky F, Moree M. Progress towards the development of malaria vaccines. Trends Parasitol2006 Jul;22(7):292-5.

83. Nardin EH, Nussenzweig V, Nussenzweig RS, Collins WE, Harinasuta KT, Tapchaisri P, et al. Circumsporozoite proteins of human malaria parasites Plasmodium falciparum and Plasmodium vivax. J Exp Med1982 Jul 1;156(1):20-30.

84. Vogel G. The 'do unto others' malaria vaccine. Science2010 May 14;328(5980):847-8.

85. Nussenzweig RS, Vanderberg J, Most H, Orton C. Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. Nature1967 Oct 14;216(5111):160-2.

86. Nussenzweig V, Nussenzweig RS. Development of a sporozoite malaria vaccine. Am J Trop Med Hyg1986 Jul;35(4):678-88.

87. Sagara I, Ellis RD, Dicko A, Niambele MB, Kamate B, Guindo O, et al. A randomized and controlled Phase 1 study of the safety and immunogenicity of the AMA1-C1/Alhydrogel + CPG 7909 vaccine for Plasmodium falciparum malaria in semi-immune Malian adults. Vaccine2009 Dec 9;27(52):7292-8.

88. El Sahly HM, Patel SM, Atmar RL, Lanford TA, Dube T, Thompson D, et al. Safety and immunogenicity of a recombinant nonglycosylated erythrocyte binding antigen 175 Region II malaria vaccine in healthy adults living in an area where malaria is not endemic. Clin Vaccine Immunol2010 Oct;17(10):1552-9.

89. Esen M, Kremsner PG, Schleucher R, Gassler M, Imoukhuede EB, Imbault N, et al. Safety and immunogenicity of GMZ2 - a MSP3-GLURP fusion protein malaria vaccine candidate. Vaccine2009 Nov 16;27(49):6862-8.

90. Hermsen CC, Verhage DF, Telgt DS, Teelen K, Bousema JT, Roestenberg M, et al. Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of Plasmodium falciparum in a phase 1 malaria vaccine trial. Vaccine2007 Apr 12;25(15):2930-40.

91. Audran R, Cachat M, Lurati F, Soe S, Leroy O, Corradin G, et al. Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. Infect Immun2005 Dec;73(12):8017-26.

92. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, et al. A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. J Infect Dis2002 Mar 15;185(6):820-7.

93. Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, et al. Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. PLoS One2009;4(3):e4708.

94. Druilhe P, Spertini F, Soesoe D, Corradin G, Mejia P, Singh S, et al. A malaria vaccine that elicits in humans antibodies able to kill Plasmodium falciparum. PLoS Med2005 Nov;2(11):e344.

95. Sirima SB, Tiono AB, Ouedraogo A, Diarra A, Ouedraogo AL, Yaro JB, et al. Safety and immunogenicity of the malaria vaccine candidate MSP3 long synthetic peptide in 12-24 months-old Burkinabe children. PLoS One2009;4(10):e7549.

96. Horii T, Shirai H, Jie L, Ishii KJ, Palacpac NQ, Tougan T, et al. Evidences of protection against blood-stage infection of Plasmodium falciparum by the novel protein vaccine SE36. Parasitol Int2010 Sep;59(3):380-6.

97. Sagara I, Dicko A, Ellis RD, Fay MP, Diawara SI, Assadou MH, et al. A randomized controlled phase 2 trial of the blood stage AMA1-C1/Alhydrogel malaria vaccine in children in Mali. Vaccine2009 May 18;27(23):3090-8.

98. Carter R, Mendis KN, Miller LH, Molineaux L, Saul A. Malaria transmission-blocking vaccines--how can their development be supported? Nat Med2000 Mar;6(3):241-4.

99. Greenwood B, Alonso P. Malaria vaccine trials. Chem Immunol2002;80:366-95.

100. Ballou WR, Cahill CP. Two decades of commitment to malaria vaccine development: GlaxoSmithKline Biologicals. Am J Trop Med Hyg2007 Dec;77(6 Suppl):289-95.

101. Ballou WR. The development of the RTS,S malaria vaccine candidate: challenges and lessons. Parasite Immunol2009 Sep;31(9):492-500.

102. Ballou WR, Arevalo-Herrera M, Carucci D, Richie TL, Corradin G, Diggs C, et al. Update on the clinical development of candidate malaria vaccines. Am J Trop Med Hyg2004 Aug;71(2 Suppl):239-47.

103. Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med1997 Jan 9;336(2):86-91.

104. Macete E, Aponte JJ, Guinovart C, Sacarlal J, Ofori-Anyinam O, Mandomando I, et al. Safety and immunogenicity of the RTS,S/AS02A candidate malaria vaccine in children aged 1-4 in Mozambique. Trop Med Int Health2007 Jan;12(1):37-46.

105. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis2009 Aug 1;200(3):337-46.

106. Mettens P, Dubois PM, Demoitie MA, Bayat B, Donner MN, Bourguignon P, et al. Improved T cell responses to Plasmodium falciparum circumsporozoite protein in mice and monkeys induced by a novel formulation of RTS,S vaccine antigen. Vaccine2008 Feb 20;26(8):1072-82.

107. Doherty JF, Pinder M, Tornieporth N, Carton C, Vigneron L, Milligan P, et al. A phase I safety and immunogenicity trial with the candidate malaria vaccine RTS,S/SBAS2 in semi-immune adults in The Gambia. Am J Trop Med Hyg1999 Dec;61(6):865-8.

108. Bojang KA, Milligan PJ, Pinder M, Vigneron L, Alloueche A, Kester KE, et al. Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet2001 Dec 8;358(9297):1927-34.

109. Kester KE, McKinney DA, Tornieporth N, Ockenhouse CF, Heppner DG, Hall T, et al. Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental Plasmodium falciparum malaria. J Infect Dis2001 Feb 15;183(4):640-7.

110. Bojang KA, Olodude F, Pinder M, Ofori-Anyinam O, Vigneron L, Fitzpatrick S, et al. Safety and immunogenicty of RTS,S/AS02A candidate malaria vaccine in Gambian children. Vaccine2005 Jul 14;23(32):4148-57.

111. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. Lancet2004 Oct 16-22;364(9443):1411-20.

112. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Aide P, et al. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. Lancet2005 Dec 10;366(9502):2012-8.

113. Sacarlal J, Aide P, Aponte JJ, Renom M, Leach A, Mandomando I, et al. Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children. J Infect Dis2009 Aug 1;200(3):329-36.

114. Macete EV, Sacarlal J, Aponte JJ, Leach A, Navia MM, Milman J, et al. Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase I/IIb randomized double-blind bridging trial. Trials2007;8:11.

115. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet2007 Nov 3;370(9598):1543-51.

116. Abdulla S, Oberholzer R, Juma O, Kubhoja S, Machera F, Membi C, et al. Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. N Engl J Med2008 Dec 11;359(24):2533-44.

117. Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, Vekemans J, et al. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med2008 Dec 11;359(24):2521-32.

118. Alonso PL, Saúte, F., Aponte, J.J., Gómez-Olivé, F.X., Nhacolo, A., Thompson, R., Macete, E., Abacassamo, F., Ventura, P.J., Bosch, X., Menéndez, C., Dgedge, M. Manhiça DSS, Mozambique. In Population and Health in Developing Countries. 1st ed. INDEPTH, editor. Ottawa: International Development Research Centre (IDRC); 2002.

119. Guinovart C, Bassat Q, Sigauque B, Aide P, Sacarlal J, Nhampossa T, et al. Malaria in rural Mozambique. Part I: children attending the outpatient clinic. Malar J2008;7:36.