Immune responses to *Plasmodium falciparum* in Mozambican infants receiving Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine

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A Doctoral Thesis submitted by **Diana Iris Silveira Quelhas**

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Thesis directors: **Dr. Carlota Dobaño** *and* **Dr. Clara Menéndez**
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DOCTORAL THESIS

Immune responses to *Plasmodium falciparum* in Mozambican infants receiving Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine

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This work is dedicated to my family
and to all children vulnerable to malaria.

PRESENTATION

Thesis submitted by **Diana Iris Silveira Quelhas** to aspire to the degree of Doctor in Medicine by the Universitat de Barcelona, under direction of **Dr. Carlota Dobaño** and **Dr. Clara Menéndez**, and tutorship of **Dr. Pedro Alonso**.

Línia de recerca: Agressió biològica i mecanismes de resposta
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The present Doctoral Thesis is presented following the University's recommendations on presentation of Doctoral Theses by compendium of publications, as certified by the directors of this Thesis.

Dr. Carlota Dobaño

Dr. Clara Menéndez

Barcelona, May 2011

The following original articles make the body of this thesis. The authors declare that the results obtained in these studies provide novel information on this line of research.

1. **Impact of Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine on Antibody Responses to Erythrocytic Stage Antigens in Mozambican Infants**
   Quelhas D, Puyol L, Quintó L, Serra-Casas E, Nhampossa T, Macete E, Aide P, Mayor A, Mandomando I, Sanz S, Aponte JJ, Chitnis C, Alonso PL, Menéndez C, Dobaño C.
   Clin Vaccine Immunol. 2008 Aug; 15(8): 1282-1291
   Impact factor (2011): 2.373

2. **Age-dependent IgG subclass responses to Plasmodium falciparum EBA-175 are differentially associated with incidence of malaria in Mozambican children**
   Dobaño C, Quelhas D, Puyol L, Quintó L, Serra-Casas E, Mayor A, Nhampossa T, Macete E, Aide P, Mandomando I, Sanz S, Aponte JJ, Puniya SK, Singh B, Gupta P, Bhattacharya A, Chauhan VS, Chitnis C, Alonso PL, Menéndez C.
   ***Submitted to Malaria Journal*.**
   Impact factor (2011): 3.000

3. **IgG against *Plasmodium falciparum* Variant Surface Antigens and Growth Inhibitory Antibodies in Mozambican Children receiving Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine**
   Quelhas D, Jiménez A, Quintó L, Serra-Casas E, Mayor A, Cisteró P, Puyol L, Wilson DW, Richards JS, Nhampossa T, Macete E, Aide P, Mandomando I, Sanz S, Aponte JJ, Alonso PL, Beeson JG, Menéndez C, Dobaño C.
   Immunobiology. 2011 Jul; 216(7): 793-802
   Impact factor (2011): 3.586

4. **Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine does not modify plasma cytokines and chemokines or intracellular cytokine responses to *Plasmodium falciparum* in Mozambican Children**
   Quelhas D, Puyol L, Quintó L, Nhampossa T, Serra-Casas E, Macete E, Aide P, Sanz S, Aponte JJ, Doolan DL, Alonso PL, Menéndez C, Dobaño C.
   ***Submitted to Immunobiology*.**
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ACRONYMS and ABBREVIATIONS

ACT - Artemesinin-based combination treatment
AECID – Agencia Española de Cooperación Internacional para el Desarrollo (Spanish Agency for International Cooperation and Development)
AIDS – acquired inmuno-deficiency syndrome
AL –Artemether-Lumefantrine
AMA-1 – apical membrane antigen 1
APC – allophycocyanin
AQ – Amodiaquine
AS – Artesunate
BHC – benzene hexachloride
BMGF – Bill and Melinda Gates Foundation
BSA – bovine serum albumin
CD – cluster of differentiation
CI – confidence intervals
CISM – Centro de Investigação em Saúde da Manhiça (Manhiça Health Research Centre)
CRESIB – Centre de Recerca en Salut Internacional de Barcelona (Barcelona Centre for International Health Research)
CQ – Chloroquine
CV – coefficient of variation
DDT – Dichlorodiphenyltrichoroetane
DNA – deoxyribonucleic acid
DP – Dihydroartemisinin-Piperaquine
DTP/OPV/Hep B – diphteria + tetanus + pertussis/oral polio vaccine/hepatitis B
EBA-175 – erythrocyte binding antigen 175
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme-linked immunosorbent assay
EPI – expanded program of immunization
FITC – fluorescein isothiocyanate
FSC – forward scatter
G-CSF – granulocyte colony stimulating factor
GFP – green fluorescence protein
GIA – growth inhibition assay
GM – geometric mean
GM-CSF – granulocyte macrophage colony stimulating factor
HIV – human immunodeficiency virus
ICS – intracellular cytokine staining
IE – infected erythrocytes
IFAT – immunofluorescence antibody test
IFN – interferon
Ig – immunoglobulin
IL – interleukin
INS – Instituto Nacional de Saúde (National Institute of Health)
IPTc – Intermittent Preventive Treatment in children
IPTi – Intermittent Preventive Treatment in infants

IPTp – Intermittent Preventive Treatment in pregnancy
IRR – incidence rate ratio
IRS – indoor residual spraying
ITC – insecticide-treated curtains
ITN – insecticide-treated bed nets
LLIN – long lasting insecticidal nets
MCAF – monocyte chemotactic and activating factor
MCP – monocyte chemotactic protein
MFI – mean fluorescence intensity
MIM – Multilateral Initiative on Malaria
MIP – macrophage inflammatory protein
MISAU – Ministério da Saúde (Ministry of Health)
MMV – Medicines for Malaria Venture
MQ – Mefloquine
MSP-1 – merozoite surface protein 1
MVI – Malaria Vaccine Initiative
NAI – naturally acquired immunity
NIE – non-infected erythrocytes
OMS – Organización Mundial de la Salud/ Organização Mundial da Saúde
P. falciparum – *Plasmodium falciparum*
PA – Pyronaridine-Artesunate
PARPA – Poverty Reduction Paper
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PE – phycoerythrin
PerCP – peridinin chlorophyll protein
PIESA – parasite-induced erythrocyte surface antigen
pLDH – *Plasmodium* lactate dehydrogenase
PMI – President's Malaria Initiative
PNCM – Programa Nacional de Controlo da Malária (National Malaria Control Program)
PYAR – person years at risk
QN – Quinine
RBM – Roll Back Malaria
RDT – rapid diagnostic test
SMP – Sulphamethoxypyrazine-Pyrimethamine
SP – Sulfadoxine-Pyrimethamine
SSC – side scatter
TB – tuberculosis
TGF – transforming growth factor
Th – T helper
TNF – tumor necrosis factor
UNICEF – United Nations Children's Fund
USAID – United States Agency for International Development
VSA – variant surface antigens
WBA – whole blood assay
WHO – World Health Organization

SUMMARY
(English)

Malaria has a high toll on the lives of infants and children under the age of five in endemic areas. Control, and more recently, elimination agendas consist on implementing several measures simultaneously, including vector control, vaccines and drugs. The current Millennium Development Goals aim at reducing malaria mortality and morbidity in the two most vulnerable groups: pregnant women and children.

Intermittent Preventive Treatment in infants with Sulphadoxine-Pyrimethamine (IPTi-SP) delivered through routine EPI vaccination programs, has shown to be efficacious in different transmission settings and the WHO has recently recommended its implementation as a malaria control tool. However, one of the main concerns has been the potential impairment of acquisition of naturally acquired immunity, and this could be a concern regarding IPTi performance.

This thesis reports on a series of studies conducted in Manhiça, a malaria endemic area of Mozambique, to assess the impact of IPTi-SP on the development of immune responses to the *Plasmodium falciparum* parasite. In this work, we measured antibody and cellular immune parameters considered to be markers of protective immunity against malaria identified to date.

The most consistent finding was that IPTi-SP does not modify the magnitude of the immune responses acquired over the first two years of age in Mozambican children. In addition, we described the factors that affect the antibody and cellular immune responses measured, and reported those that correlated with prospective malaria incidence. Based on the studies presented herein, we can conclude that IPTi-SP does not interfere with immune responses that are considered major contributors to the acquisition of protective immunity to malaria in early infancy.

In summary, this thesis contributes to a more complete assessment of IPTi as a control measure that will possibly be implemented in the near future in malaria endemic areas of Africa and elsewhere. In addition, it aims to contribute to advance the knowlegde on the acquisition of natural immunity in infancy, which is poorly understood. Furthermore, this information will help to understand the factors that should be taken into account when designing and deploying other control measures for this age group.

RESUMEN
(Castellano)

La malaria causa una elevada mortalidad en niños menores de 5 años de edad en zonas endémicas. Estrategias de control, y más recientemente de eliminación, consisten en implementar varias medidas simultáneamente, como el control del vector, las vacunas y los fármacos. Los actuales Objetivos de Desarrollo del Milenio pretenden reducir la mortalidad y la morbilidad debidas a la malaria en los dos grupos más vulnerables de la población: las mujeres embarazadas y los niños.

El Tratamiento Preventivo Intermitente en niños con Sulfadoxina-Pirimetamina (IPTi-SP) administrado através de los programas nacionales de vacunación ha demostrado ser eficaz en zonas con diferentes patrones de trasmisión. La OMS ha recomendado recientemente su implementación como herramienta de control contra la malaria. Sin embargo, una de las principales preocupaciones ha sido la posible interferencia en la adquisición de la inmunidad natural frente a la malaria, que podria comprometer el desempeño del IPTi.

Esta tesis presenta una serie de estudios realizados en Manhiça, una zona endémica de malaria en Mozambique, en los que se ha evaluado el impacto del IPTi-SP en el desarrollo de las respuestas inmunes contra el parásito *Plasmodium falciparum*. En este trabajo se han medido respuestas de anticuerpos y celulares, consideradas importantes marcadores de protección frente a la malaria identificados hasta ahora.

La observación más consistente ha sido que el IPTi-SP no modifica las respuestas inmunes adquiridas en los dos primeros años de vida en niños mozambiqueños. Además, se describen los factores que afectan a las respuestas humorales y celulares, y los que se correlacionan con la incidencia de malaria. En base a estos estudios, concluimos que el IPTi-SP no interfiere con respuestas inmunes consideradas relevantes para la adquisición de inmunidad protectora contra la malaria en la infancia.

En resumen, esta tesis contribuye a una evaluación más completa del IPTi como medida de control que posiblemente será implementada en un futuro próximo en zonas endémicas de malaria en África y otras partes del mundo. Además, pretende contribuir al avance del conocimiento sobre la adquisición de la inmunidad natural en la infancia, de la cual tenemos datos limitados. Finalmente, esta información ayudará a entender mejor los factores que deben ser tomados en cuenta a la hora de diseñar e implementar otras medidas de control de la malaria en este grupo de edad.

SÍNTESE
(Português)

A malária causa uma elevada taxa de mortalidade em crianças menores de 5 anos de idade em zonas endémicas. Estratégias de control, e mais recentemente de eliminação, consistem em implementar várias medidas simultaneamente, como o control do vector, as vacinas e os fármacos. Os actuais Objectivos de Desenvolvimento do Milénio pretendem reduzir a mortalidade e a morbilidade devidas à malária nos dois grupos mais vulneráveis da população: as mulheres grávidas e as crianças.

O Tratamento Preventivo Intermitente em crianças com Sulfadoxina-Pirimetamina (IPTi-SP) administrado através dos programas nacionais de vacinação demonstrou ser eficaz em zonas com diferentes padrões de transmissão. A OMS recomendou recentemente a sua implementação como ferramenta de control contra a málaria.

Porém, uma das principais preocupações tem sido a possível interferência na aquisição da imunidade natural frente à malária, que poderia comprometer o desempenho do IPTi.

Esta tese apresenta uma série de estudos realizados em Manhiça, uma zona endémica de malária em Moçambique, nos quais se avaliou o impacto do IPTi-SP no desenvolvimento das respostas imunes contra o parasita Plasmodium falciparum. Neste trabalho mediram-se diversos parâmetros imunológicos, tanto respostas de anticorpos como respostas celulares, considerados os mais importantes marcadores de proteção contra a malária identificados actualmente.

A observação mais consistente foi que o IPTi-SP não modifica a magnitude das respostas imunes adquiridas durante os dois primeiros anos de vida em crianças moçambicanas. Descrevem-se também os factores que afectam as respostas humorais e celulares, e os factores que correlacionam com a posterior incidência de malária. Com base nos estudos aqui apresentados, podemos concluir que o IPTi-SP não interfere com as respostas imunitárias que se consideram relevantes para a aquisição de imunidade protectora contra a malária na infância.

Em resumo, esta tese contribui para uma avaliação mais completa do IPTi como medida de control que possivelmente será implementada num futuro próximo em zonas endémicas de malária em África e outras partes do mundo. Adicionalmente, pretende contribuir ao avanço do conhecimento sobre a aquisição da imunidade natural na infância, da qual temos dados limitados. Finalmente, esta informação ajudará a entender melhor os factores que devem ser tomados em conta ao desenhar e implementar outras medidas de control da malária neste grupo de idade.

LHOKO MHAKA
(Changana)

Mavabji ya dzedzedze ma vanga rifo le rikulu ka swivanana switsongo ka n´tanga ya 5 wa malembe ka tindhau leti tingani matshamelo manenen ka wone. Mitirho ya ku vonelela, na kungari khale ku zama ku mahelisa, hiku yentxa mitirho yinnyingi yaku hambana hambana hi n´karhi wunwe, inga ku lavisela a switsotso swima hangalsako, ku lavisela ti nyedzana ni mimirhi. Mikongometo ya xiswoswi ya ku yampswisa ka malembe ya swoswi yi zama ku pumba a rifo ni tindlela ta rona hi kola kaku vangiwa hi dzedzedze ka mitlhawa mimbirhi leyi yinga ka mhangu a ka xitsungu: vavasati va ti nyimba ni swivanana.

Wudjahu ra wu voneleli ra n´kama ni n´kama ka swivanana hi murhi lowu wu txhuliwaka Sulfadoxina-Pirimetamina (IPTi-SP) wu nyikiwaka hi tindlela ta tixaxameto ta tiko ta ku txavelela ka tinyedzana wu kombisiwile swa swinene ka ti ndhau leti tinga ni matshamelo yaku hambana hambana ka makumelo ya wone. A n´tlhangano wa OMS wu nyikile yangulo ka n´kama wa swoswi ku wu tirhisiwa kota wunwe ka ntirho waku vonelela ka mavabji ya dzedzedze.

Hi xileswo, xinwe ka swinyingi swivangelo mayelano ni IPTi hiku lavisela niku vona a ntamu wa swivikelo swa ntumbuluko phambeni ka mavabji ya dzedzedze.

Mavonela lawa ma kombisa a tidjondzo tinyingi tinga yentxiwa ka Manhiça, Musambiki, katona ku yentxiwile a kulavisela kaku tirhisa IPTi-SP ku voneleleni ni tin´lhamulo ta wuvikeleli kulua ni xipunguana *Plasmodium falciparum*. Ka ntirho lowu ku pimiwile wu vikeleli ro hambana hambana ku tiyela ka rona, ka tin´lhamulo ta swi tsongua-tsonguana swa wu vikeli ni ka swinwanyane switsongua-tsonguana a nyameni, hi swikombisa ku swone swi lulameke kuva swikombiso ka wuvikeli kulua ni mavabji ya dzedzedze swi kombisiweke ka nkama lowu.

Leswi swi vonekeke ka n´tssima n´tshima hile swaku a IPTi-SP a yi txintxi n´txumu ka n´talo wa tin´lhamulo ta wuvikelo leri ri kumiweke ndzeni ka malembe mambirhi yo rhanga ka wutomi ra vatsonguana ka tiko ra Musambiki. Swa voneka kambe swinwanyane swivangelo swi yentxaka ku hambanisanyana ka matshamela ya wutomi ni ka switsongua-tsonguana, swinwanyane swivangelo swaku lava ku fanana ni makumela ya dzedzedze a wuganwini. Hikola ka tidjondzo leti ti kombisiweke laha, ha swikota ku nyika n´seketelo yaku IPTi-SP ayina wungenelo kumbe nkatsano ka tin´lhamulo ta wuvikeli leti ti tshembiweke hi n´tshima n´tshima aku kumeni ka matshamelo ya wuvikeli kulua ni mavabji ya dzedzedze ka vatsonguana.

Hiku katsakanya, a yangulo leyi yinyika ku lavisisa ka kuringana ka IPTi kota a m´pimo lowu wunene wutaka kota ku tirhisiwa min´kama leyi yitaka ka ti ndhau leti ti mavabji ya dzedzedze ma nga yandza phakati ka matiko ya África ni ka manwanyane matiko. Hiku patsana, hin´kwaswo leswo swikongomisa ku tiva a wukumelo ra ku vikela ka n´tumbuluko kumbe ra matsalua a wutsonguanine, ka leswo hi kumeka na hiri ni swikombiso swa ripimo ritsongo. Hiku gamesa, a tlhamuxelo lowu wuta pfunisa ku twisiseka lo kunene swivangelo leswi swi fanelaka ku tirhisiwa kota tindlela taku vonelela ka mavabji ya dzedzedze ka n´tlhawa lowu wa ntanga.

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I. INTRODUCTION

1. Historical perspective

Malaria or a disease resembling malaria has been noted for more than 4,000 years and has probably influenced to a great extent human populations and human history (Carter 2002).

Ancient history (2700 b.c. - 340 a.d.)

The symptoms of malaria were described in ancient Chinese medical writings. In 2700 b.c., several characteristic symptoms of what would later be named malaria were described in the *Nei Ching*, The Canon of Medicine. *Nei Ching* was edited by Emperor Huang Ti. Malaria became widely recognized in Greece by the 4th century b.c., and it was responsible for the decline of many of the city-state populations. Hippocrates noted the principal symptoms. By the age of Pericles, there were extensive references to malaria in the literature and depopulation of rural areas was recorded. In the *Susruta*, a Sanskrit medical treatise from India, the symptoms of malarial fever were described and attributed to the bites of certain insects and were associated with enlarged spleens. A number of Roman writers attributed malarial diseases to the swamps.

In China, during the 2nd century b.c., the Qinghao plant (*Artemisia annua*) was described in the medical treatise, *52 Remedies*, found in the Mawangdui Tomb. In 340 a.d., the anti-fever properties of Qinghao were first described by Ge Hong of the East Yin Dynasty. The active ingredient of Qinghao, known as artemisinin, was isolated by Chinese scientists in 1971. Derivatives of this extract, known collectively as artemisinins, are today very potent and effective antimalarial drugs, especially in combination with other medicines.

By the beginning of the Christian era malaria was widespread in all the Mediterranean area, the Middle East and in Asia and South East Asia, spreading

then to northern Europe during the Middle Ages. From central and south America it probably expanded to the rest of the Americas during the 18th century (Carter 2002).

Quinine (early 17th century)

Following their arrival in the New World, Spanish Jesuit missionaries learned from indigenous Indian tribes of a medicinal bark used for the treatment of fevers. With this bark, the Countess of Chinchón, the wife of the Viceroy of Peru, was cured of her fever. The bark from the tree was then called *Peruvian bark* and the tree was named Cinchona after the countess (Lee 2002a). The medicine from the bark is now known as the antimalarial Quinine. Along with artemisinins, Quinine is one of the most effective antimalarial drugs available today.

"Malaria" or "paludisme"

In the 18th century the term "mal'aria", derived from the Italian word for "bad air", was adopted to name the disease, as it was believed to be caused by gases emanating from swamps and stagnant waters. Later the term "paludisme" was introduced from the French "palud" (marshes).

Discovery of the malaria parasite (1880)

Charles Louis Alphonse Laveran, a French army surgeon stationed in Constantine, Algeria, was the first to notice parasites in the blood of a patient suffering from malaria. This occurred on the 6th of November 1880.

Differentiation of species of malaria (1886)

Camillo Golgi, an Italian neurophysiologist, established that there were at least two forms of the disease, one with tertian periodicity (fever every other day) and one with quartan periodicity (fever every third day). He also observed that the forms produced differing numbers of merozoites (new parasites) upon maturity and that fever coincided with the rupture and release of merozoites into the blood stream.

Naming of human malaria parasites (1890, 1897)

The Italian investigators Giovanni Batista Grassi and Raimondo Filetti first introduced the names *Plasmodium vivax* and *P. malariae* for two of the malaria parasites that affect humans in 1890. Laveran had believed that there was only one species, *Oscillaria malariae*. An American, William H. Welch, reviewed the subject and, in 1897, he named the malignant tertian malaria parasite *P. falciparum*. There were many arguments against the use of this name; however, the use was so extensive in the literature that a change back to the name given by Laveran was no longer thought possible. In 1922, John William Watson Stephens described the fourth human malaria parasite, *P. ovale*. *P. knowlesi* was first described by Robert Knowles and Biraj Mohan Das Gupta in 1931 in a long-tailed macaque. The first documented human infection with *P. knowlesi* was in 1965.

Discovery that mosquitoes transmit malaria parasites (1897-1898)

On August 20th, 1897, Ronald Ross, a British officer in the Indian Medical Service, was the first to demonstrate that malaria parasites could be transmitted from infected patients to mosquitoes. In further work with bird malaria, Ross showed that mosquitoes could transmit malaria parasites from bird to bird. This necessitated a sporogonic cycle (the time interval during which the parasite developed in the mosquito). Thus, the problem of malaria transmission was solved.

Discovery of the transmission of the human malaria parasites *Plasmodium* (1898-1899)

Led by Giovanni Batista Grassi, a team of Italian investigators, which included Amico Bignami and Giuseppe Bastianelli, collected *Anopheles claviger* mosquitoes and fed them on malarial patients. The complete sporogonic cycle of *P. falciparum, P. vivax*, and *P. malariae* was demonstrated. In 1899, mosquitoes infected by feeding on a patient in Rome were sent to London where they fed on two volunteers, both of whom developed malaria.

The U.S. public health service and malaria (USPHS) (1914-1942)

During the U.S. military occupation of Cuba and the construction of the Panama Canal at the turn of the 20th century, U.S. officials made great strides in the control of malaria and yellow fever. In 1914 Henry Rose Carter and Rudolph H. von Ezdorf of the USPHS requested and received funds from the U.S. Congress to control malaria in the country. Various activities to investigate and combat malaria in the U.S. followed from this initial request.

Chloroquine (Resochin®) (1934, 1946)

Chloroquine was discovered by a German, Hans Andersag, in 1934 at Bayer I.G. Farbenindustrie A.G. laboratories in Eberfeld, Germany. He named his compound Resochin. Through a series of lapses and confusion brought about during the war, Chloroquine was finally recognized and established as an effective and safe antimalarial in 1946 by British and U.S. scientists.

Dichloro-diphenyl-trichloroethane (DDT) (1939)

A German chemistry student, Othmer Zeidler, synthesized DDT in 1874, for his thesis. The insecticidal property of DDT was not discovered until 1939 by Paul Müller in Switzerland. Various militaries in World War II utilized the new insecticide initially for control of louse-borne typhus. DDT was used for malaria control at the end of World War II after it had proven effective against malaria-carrying mosquitoes by British, Italian, and American scientists.

North America malaria control in war areas (MCWA) (1942-1945)

MCWA was established to control malaria around military training bases in the southern U.S. and its territories, where malaria was still problematic. Many of the bases were established in areas where mosquitoes were abundant, and more person-days were lost among military personnel due to malaria than to bullets in malaria endemic regions in the 20th century. MCWA aimed to prevent reintroduction of malaria into the civilian population by mosquitoes that would

have fed on malaria-infected soldiers, in training or returning from endemic areas. During these activities, MCWA also trained state and local health department officials in malaria control techniques and strategies. From these efforts, stemmed various malaria control and research programs based in North America, such as the CDC (Communicable Disease Center now known as Centers for Disease Control and Prevention), WRAIR (Walter Reed Army Institute of Research), and the NMRC (Naval Medical Research Centre).

With the successful reduction of malaria in the U.S., these institutions switched their malaria focus from elimination efforts to prevention, surveillance, and technical support both domestically and internationally. These are still some of the main institutions investing in malaria research and control today.

Synthetic Quinine and other antimalarial drugs (1944)

In 1944 Quinine was synthesized, but the process was slow and expensive, and extraction from the bark continues to be used (Lee 2002b). Quinine supplies were not ensured in Europe during the beginning of the 20th century, and that led to the development after the First World War of the first synthetic antimalarial drugs: Chloroquine, Proguanil, Amodiaquine, Primaquine and Pyrimethamine. Chloroquine, an effective, cheap and safe antimalarial, has played a major role in the control of malaria. It was widely used for decades in all malaria-endemic areas and was key to decrease malaria mortality in the second half of the 20th century. However, in the 1960s the first resistances of *P. falciparum* to Chloroquine appeared in South East Asia and South America and expanded with time to other parts of the world. In 1978 the first resistances appeared in East Africa and within the following ten years resistances were reported in all countries of sub-Saharan Africa. Despite high levels of resistance, Chloroquine was used as first line treatment in most countries until recently, and some authors claim that this was the cause of a rise in malaria mortality in Africa during the last decades (Trape 2001). Nowadays the only area with no resistance of *P. falciparum* to Chloroquine is Central America. *P. vivax* remains sensitive to Chloroquine in most areas, except

for Indonesia, Papua New Guinea and other parts of Oceania (*WHO 2005a*).

First eradication efforts worldwide: success and failure (1955-1978)

With the success of DDT, the advent of less toxic, more effective synthetic antimalarials, and the enthusiastic and urgent belief that time and money were of the essence, the World Health Organization (WHO) submitted at the World Health Assembly in 1955 an ambitious proposal for the eradication of malaria worldwide. The Global Malaria Eradication Program (GMEP, 1955-1969) efforts began and focused on house spraying with residual insecticides, antimalarial drug treatment, and surveillance, and would be carried out in 4 successive steps: preparation, attack, consolidation, and maintenance. Successes included elimination in nations with temperate climates and seasonal malaria transmission. Some countries such as India and Sri Lanka had sharp reductions in the number of cases, followed by increases to substantial levels after efforts ceased. Other nations had negligible progress (such as Indonesia, Afghanistan, Haiti, and Nicaragua). Some nations were excluded completely from the eradication campaign (most of sub-Saharan Africa). The emergence of drug resistance, widespread resistance to available insecticides, wars, massive population movements, difficulties in obtaining sustained funding from donor countries, and lack of community participation, made the long-term maintenance of the effort untenable. Completion of the eradication campaign was eventually abandoned fourteen years later in 1969.

During the 1970s and 1980s, because of economic and financial crises, international support for malaria control declined rapidly. Since then, the goal of most current National Malaria Prevention and Control Programs and most malaria activities conducted in endemic countries has been to reduce malaria transmission to a level where it is no longer a public health problem.

However, in the past decade, following increasing demands from endemic countries and promising results from scaling up of control activities, a significant increase in resources has been devoted to the development of new control tools,

including drugs and vaccines, and an increased international commitment has led to the creation of several multilateral initiatives and partnerships between the public and the private sector to tackle malaria worldwide.

Renewed eradication agenda: malERA (2011)

In October 2007 the Bill & Melinda Gates Foundation (BMGF) called for a global commitment to eradicating malaria. As a result of this commitment, an unprecedented consultative process to define a research and development agenda for malaria eradication (malERA) was established in 2008. The consultative process lasted two years and involved more than 250 of the leading scientists in the malaria field and beyond.

In January 2011 a white paper comprising a collection of 12 reviews was published as part of a sponsored supplement of *PLoS Medicine* (Alonso 2011). The nine research and development agendas defined the priority lines of research for different thematic areas including basic science and enabling technologies; drugs; vaccines; vector control; health systems and operational research; modelling; diagnoses and diagnostics; and monitoring, evaluation and surveillance. An additional paper identified research priorities that are common to several of the thematic areas. The collection included an analysis of the last Global Malaria Eradication Program and outlined lessons for future eradication programs (Najera 2011). Finally, it also includes an analysis examining the role that research has played in eradication or elimination initiatives for smallpox, poliomyelitis, and measles, and from this analysis derived nine cross-cutting lessons for malaria eradication (Levine 2011).

This effort was put in place in the hope that by publishing this collection, better transparency in defining research priorities will be achieved for malaria researchers and policymakers around the world.

2. Malaria transmission: the parasite, the vector, the human host, the environment

Intensity of transmission varies significantly with the species of the parasite, the species of Anopheline vectors present, the biological factors of the human host, environmental and climatic conditions as well as behavioural, social and economic factors. Understanding and monitoring each of these factors has been the key to designing specific malaria control measures to reduce or eliminate transmission.

The parasite

The malaria parasites are protozoa that belong to the *Plasmodium* genus, *Plasmodiidae* family. There are four species of *Plasmodium* that affect humans, *P. falciparum*, *P. vivax*, *P. malariae, P. ovale*; in addition, *P.knowlesi* has recently been described to affect humans in certain forest areas.

P. falciparum is the most virulent of the four and is responsible for most of the severe morbidity and mortality worldwide. This is the predominant species in sub-Saharan Africa and thus the main focus of this thesis. *P. vivax* is the species with the wider geographical distribution (predominant in Central and parts of South America, North Africa, the Middle East and the Indian subcontinent), but the clinical presentation is usually milder and rarely results in death. *P. malariae* and *P. ovale* account for a small percentage of the cases and are found predominantly in sub-Saharian Africa (Boutin 2005) as well as in Papua New Guinea (Mehlotra 2000) and some areas of South East Asia (Win 2002). *P. knowlesi*, in addition to infecting the long-tailed macaque monkeys, it was also detected for the first time in humans in Malaysia in the 1960's and can now be found in other areas of South East Asia (Singh 2004, Cox-Singh 2008); the infection is commonly misidentified as *P. malariae.*

At a molecular level, natural populations of *P. falciparum* are genetically diverse, consisting of parasite genotypes or "strains" (Walliker, 1991). These strains differ

in their composition of a number of polymorphic molecules, including antigens. Because of this, it has been suggested that immunity to *P. falciparum* may be essentially strain-specific (Wilson & Phillips, 1976).

The vector

Malaria is transmitted by female Anopheline mosquitoes. Although more than 400 species of anopheles have been identified, only approximately 60 are considered to be potential vectors of malaria parasites (Gilles 1988, Molineaux 1988), the most relevant ones being *Anopheles gambiae* (Africa), *A. farauti* (Papua New Guinea), *A. minimus* (Thailand), and *A. darlingi* (South America).

The *A. gambiae* complex is mainly responsible for 80% of malaria morbidity and mortality that occurs in sub-Saharan Africa (Breman 2001). Differences in malaria vector competence among members of the complex have been recognized and are attributed primarily to preferences for feeding on humans versus animals, tendency to enter houses, and ability to recover in number after dry seasons (White 1974).

The human host

A part of the parasite's life cycle occurs in the bloodstream of the human host, where the process of differentiation and development of gametocytes guarantees the maintenance and transmission of the infection. However, once an infected mosquito bites a human host, transmitting the malaria parasites, the severity and progression of this infection will depend on multiple factors. From the human host's standpoint, these include the genetic characteristics, innate natural defences, and the degree of naturally acquired immunity (NAI).

It is presumed that as an evolutionary mechanism, in highly endemic areas, the high mortality from *P. falciparum* has modified the human genome by selection of genetic variants that reduce the risk of death. For example, in Africans and Melanesians many otherwise deleterious genes are thought to confer a survival

advantage against malaria. Merozoites of *P. vivax* and *P. knowlesi* use a particular blood group on the red blood cell (RBC) surface, the Duffy antigen, as a receptor to enter into the RBC. The near absence of this antigen in West African populations renders them resistant to infection by *P. vivax* (reviewed in Miller, 1996). Other RBC defects which offer some protection against *P. falciparum* are haemoglobinopathies: sickle cell anaemia, α and β-thalassaemias, foetal haemoglobin syndrome, and glucose-6-phosphate deficiency. Distributions of these traits correlate geographically with areas of *P. falciparum* endemicity.

In addition, polymorphisms in genes encoding elements of the immune system have also been associated with apparent protection against malaria in humans. The possession of the human major histocompatibility complex (HLA) Bw53 allele, widespread in West Africans but rare in Caucasians, appeared to correlate with protection against severe malaria (Hill 1991), although this has not been reproduced in other studies.

The environment

The natural environment and climatic conditions have a significant effect on parasite and vector development and proliferation. Temperatures ranging between 20°C and 30°C offer an optimal window for development of both aquatic stages and geotropic maturation in different vector species. Within that range, higher temperatures tend to increment the growth rate, by shortening the minimum generation time of vector populations. A high relative humidity, preferably at least over 60%, lengthens the life span of adult vectors, thus making it possible to transmit the infection to as many as possible human hosts (Molineaux 1988).

The malaria vectors differ on their breeding habits. In general each vector species breed specifically in a certain type of water surface, although some have adapted perfectly to changes in the type of water surface available. Rain pools are favored by certain species (*A. arabiensis, A. gambiae*) while other species breed in salt-

water or in swamps. In addition, some species of vectors are affected by sunlight or by shade or emerging vegetation around the breeding sites (Molineaux 1988).

Similarly, altitude (due to its relation to temperature) also determines mosquito survival, thus malaria transmission is believed to be difficult above 2,000 meters. However, with ongoing climatic changes, there have been some deviations from this paradigm.

3. Malaria infection: the parasite's life cycle

P. falciparum has a complex life cycle that is divided between 2 phases: one that takes place in the human host (which includes the asexual stage) and one that develops in the anopheline mosquito, the vector (which includes the sexual stage). The interaction between the two reservoirs occurs when the female anopheline bites a human and feeds on its blood, which she requires in order to reproduce.

The human phase of the cycle

When an infected female mosquito bites a human, it injects around 100 sporozoites from its salivary glands, and into the human skin. From here, a proportion is drained by lymphatics to lymph nodes, where most sporozoites are degraded (Amino 2006), and the rest enter blood capillaries and reach the liver. After an initial replication in the liver (exo-erythrocytic schizogony) which may take one to two weeks, the merosome ruptures releasing thousands of merozoites into the blood stream. What is known as the pre-erythrocytic stage of the life cycle, ends here. Merozoites then invade erythrocytes and undergo asexual multiplication inside them (erythrocytic schizogony). The ring stage matures into trophozoites and then into schizonts, which rupture triggering the clinical symptoms and releasing merozoites (an average of 8 to 16 each) that will invade new erythrocytes.

After erythrocytic invasion, some of the merozoites differentiate into sexual erythrocytic stages (male and female gametocytes), which are then ingested by another female mosquito during a blood meal.

The mosquito phase of the cycle

Again when a female mosquito bites a human, the gametocytes that circulate in the bloodstream are ingested. Here, parasites undergo a sporogonic cycle of multiplication. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes) and invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

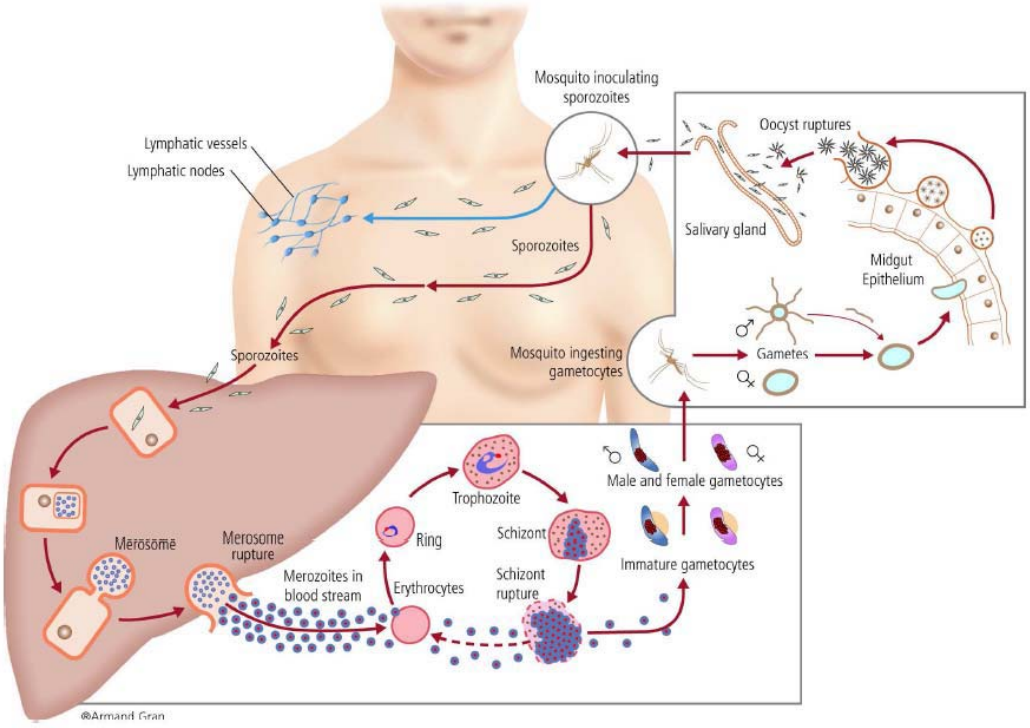

FIGURE 1. The life cycle of *Plasmodium falciparum.*

The overall life cycle of the four human species is similar. However *P. vivax* and *P. ovale* have a peculiarity, as some sporozoites differentiate into hypnozoites in the liver. These are dormant forms of the parasite that can reactivate after months and cause a new asexual blood-stage infection with clinical symptoms.

A particularity of *P. falciparum* is that, unlike other species which specifically invade different stages of maturation of the erythrocyte, this species can invade RBC of any age, thus accounting for a greater virulence and potential to multiply faster reaching higher peripheral parasitaemias.

4. Clinical features of malaria and diagnosis

The presentation of malaria can vary from an asymptomatic infection to mild clinical malaria to severe malaria to death. The anti-malarial immunity level of the host is one of the key determinants of the infection outcome and will also determine the parasitaemia threshold at which an infection is symptomatic. The other critical factors are the rapid diagnosis and the availability of an effective antimalarial treatment.

Clinical presentation

The commonest clinical presentation of all human malarias is an acutely febrile illness that can be accompanied by chills. Other frequent symptoms are headache, vomiting, diarrhoea, nausea, myalgia, arthralgia and general prostration, and typical signs are splenomegaly and hepatomegaly (White & Pukrittayakamee 1993). These are features of mild or uncomplicated malaria (UM).

The manifestations of severe or complicated malaria (SM), as defined by WHO criteria, are highly variable but are associated almost exclusively with *P. falciparum* infections (reviewed in Warrell 1990). The case fatality rate of severe malaria cases ranges from 2 to 10% (Taylor 2006, Bassat 2008). The spectrum of severe *P.*

falciparum disease includes severe malarial anaemia (SMA), cerebral malaria (CM), acidosis, respiratory distress, shock, apparent disseminated intravascular coagulation (DIC), renal failure and pulmonary oedema. Some manifestations are more common in particular age groups and/or geographical areas. Cerebral malaria is widespread in sub-Saharan Africa and South East Asia (and can occur in South America) (reviewed in Mendis & Carter 1995). In Africa, about 2% of malaria cases between 6 months and 5 years of age are classified as severe, mainly involving CM (Greenwood 1991). A few CM cases also occur in the 5-10 year age group, and are only occasionally reported in adults. In contrast, in South East Asia CM typically occurs in adults as well as children. Anemia is common mainly in African children and pregnant women. Organ failure mainly affects adults in Asia and South America.

Malaria mortality in African children, where the heaviest burden of disease falls, is attributed mostly to CM and SMA. However, life threatening pulmonary and kidney complications are increasing in areas of multidrug resistant malaria such as in South East Asia. Liver pathology may also be a more significant feature of malarial infection than has been realized. In addition, malaria-induced metabolic acidosis in children seems to be an important cause of mortality in East Africa (Marsh & Snow 1997).

Cerebral malaria is defined in clinical terms as the presence of coma associated with the presence of malaria parasites (Warrell 1990). In Africa, the mortality rate for CM is between 15-30%. In addition, a consistent minority of survivors (9-12%) are discharged with neurological sequelae, though half of these recover fully within 4-6 weeks (Newton 1998).

Severe malarial anaemia is defined as a haemoglobin concentration < 5g/dl, or a haematocrit (or packed RBC volume, PCV) < 16%, in a patient with a parasitaemia in excess of 10,000 trophozoites/μl of blood, although SMA can develop at lower parasitaemias. In most cases, blood transfusions improve the clinical findings. Overall, mortality rates for children hospitalized with SMA range from 4.7% to 14-

16% (Newton 1998). In addition, SMA contributes significantly to morbidity during pregnancy, and may indirectly increase the fatality rate of post-partum haemorrhage (Menendez 1995). In areas of high malaria transmission, SMA mainly occurs in primigravidae (Bardaji 2008). In the foetus maternal malaria can cause preterm birth or low birth weight, contributing to increased perinatal mortality, and, less frequently, congenital malaria (Menendez 2006, Desai 2007).

Pathogenesis of severe malaria

The main pathological feature of severe *P. falciparum* malaria is the sequestration of parasitized erythrocytes (mostly mature forms of the parasite) in the microvasculature (post-capillary venules and capillaries) of vital organs. Parasite antigens expressed on the infected erythrocytes' surface bind to human endothelial receptors (leading to decreased blood flow and local release of cytokines and other inflammatory mediators), to non-infected erythrocytes (rosetting), to platelets (platelet clumping) and to syncytiotrophoblasts (placental malaria). The most important parasite ligand is the PfEMP-1 protein (*P. falciparum* erythrocyte membrane protein 1) family, encoded by the *var* gene family, and the human receptors include ICAM-1, CD36, VCAM-1, hyaluronic acid, chondroitin sulphate A (CSA), P-selectin, E-selectin, CD35 and CD31.

Diagnosis

Malaria is often misdiagnosed, as its clinical presentation overlaps with other diseases, mainly pneumonia (English 1996). In fact, there is no standard definition for clinical malaria. In most places malaria is defined as fever or history of fever with asexual parasitaemia.

The gold standard for the diagnosis of malaria parasitaemia is to observe by microscopy asexual forms of the parasite on Giemsa-stained blood smears. Unfortunately, clinical symptoms of malaria often commence at about the same time or just before parasites are readily diagnosed in the peripheral blood by microscopy. More sensitive detection by polymerase chain reaction (PCR) can now

detect parasites in the blood for up to 1 week before they can be detected in slides (Cheng 1997). Alternatively, rapid diagnostic tests (RDTs), based on an immunochromatographic assay directed against parasite antigens, can be used. The sensitivity and specificity of the newest RDTs is >85% for *P. falciparum*, but lower for non-*P. falciparum* infections (Wongsrichanalai 2007). However, microscopy or RDTs are often not available in health centres and thus malaria is frequently diagnosed on a clinical basis, which leads to the administration of antimalarial treatment to all children who have fever or a history of fever.

5. Malaria in the world

5.1. Spatial distribution and burden of disease

Malaria is presently found throughout the tropics and subtropics. In the past it was established in other areas such as Europe, Northern Asia and North America, but it has long been eradicated from those areas.

Cases: In 2009 there were an estimated 225 million cases of malaria (5^{th}–95^{th} centiles, 169–294 million) worldwide (Table 1) down from an estimated 244 million cases in 2005. The global number of cases was estimated to have increased between 2000 and 2005 in line with population growth and decreased subsequently possibly due to the impact of malaria control (*WHO World Malaria Report 2010*).

Deaths: The global number of malaria deaths is estimated to have decreased from 985,000 in 2000 to 781,000 in 2009. The largest percentage decreases were seen in the region of the Americas (48%); the largest absolute decline was observed in the African region. It is estimated that 91% of deaths in 2009 were in the African region, followed by the South East Asia (6%) and Eastern Mediterranean regions (2%). About 85% of deaths globally were in children under 5 years of age. The estimated numbers of deaths for prior years are consistent with those reported in

the *World Malaria Report 2009* but are lower in the African region probably because the effects of increased intervention are taken into account (*WHO World Malaria Report 2010*).

ESTIMATES OF MALARIA CASES AND DEATHS BY WHO REGION, 2000–2009

CASES (in thousands)	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	Uncertainty bounds	
											lower	*upper*
African	173 000	178 000	181 000	185 000	187 000	188 000	187 000	186 000	181 000	176 000	117 000	241 000
Americas	2 800	2 300	2 200	2 100	1 900	1 900	1 700	1 500	1 100	1 100	1 000	1 300
Eastern Mediterranean	15 000	15 000	17 000	16 000	15 000	12 000	12 000	12 000	13 000	12 000	14 000	16 000
European	47	34	27	22	13	7	4	2	1	1	1	1
South-East Asia	38 000	37 000	35 000	34 000	37 000	39 000	34 000	33 000	34 000	34 000	28 000	41 000
Western Pacific	2 800	2 400	2 200	2 500	2 800	2 300	2 500	2 100	1 900	2 300	2 000	2 500
World	233 000	235 000	237 000	240 000	243 000	244 000	238 000	234 000	231 000	225 000		
lower bound	181 000	181 000	182 000	184 000	185 000	185 000	179 000	175 000	171 000	169 000		
upper bound	302 000	304 000	308 000	313 000	314 000	317 000	310 000	304 000	298 000	294 000		

DEATHS	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	Uncertainty bounds	
											lower	*upper*
African	900 000	893 000	885 000	880 000	870 000	853 000	832 000	802 000	756 000	709 000	554 000	892 000
Americas	2 400	2 300	1 400	1 400	1 500	1 600	1 600	1 400	1 100	1 300	900	1 700
Eastern Mediterranean	18 000	18 000	21 000	19 000	17 000	17 000	16 000	15 000	16 000	16 000	12 000	892 000
European	0	0	0	0	0	0	0	0	0	0	0	1
South-East Asia	58 000	55 000	51 000	50 000	52 000	50 000	48 000	43 000	48 000	49 000	37 000	892 000
Western Pacific	6 800	5 800	5 200	5 900	6 500	4 900	5 400	4 700	4 200	5 300	3 400	7 300
World	985 000	974 000	963 000	957 000	947 000	927 000	904 000	867 000	826 000	781 000		
lower bound	797 000	785 000	775 000	769 000	765 000	744 000	725 000	694 000	662 000	628 000		
upper bound	1 228 000	1 214 000	1 199 000	1 191 000	1 174 000	1 153 000	1 120 000	1 075 000	1 024 000	968 000		

TABLE 1. Estimates of malaria cases and deaths by WHO region between 2000-2009 (WHO World Malaria Report 2010)

The need for an updated and dynamic estimate of malaria endemic areas for the malaria control community at a global scale has been addressed by the Malaria Atlas Project (MAP) founded in 2005. The MAP team assembled a unique spatial database of linked information based on medical intelligence and satellite-derived climate data to constrain the limits of malaria transmission and the largest ever archive of community-based estimates of parasite prevalence. The initial focus of MAP has been centred on *P. falciparum* due to its global epidemiological significance and its better prospects for elimination and control. To date they have collated 24,492 parasite rate surveys (*P.f.* 24,178; *P.v.* 8,866) from an aggregated sample of 4,373,066 slides in 85 countries. As a result, a study was recently published that estimated the global burden of malaria in 2007 and the most

accurate maps available to date (Hay 2009).

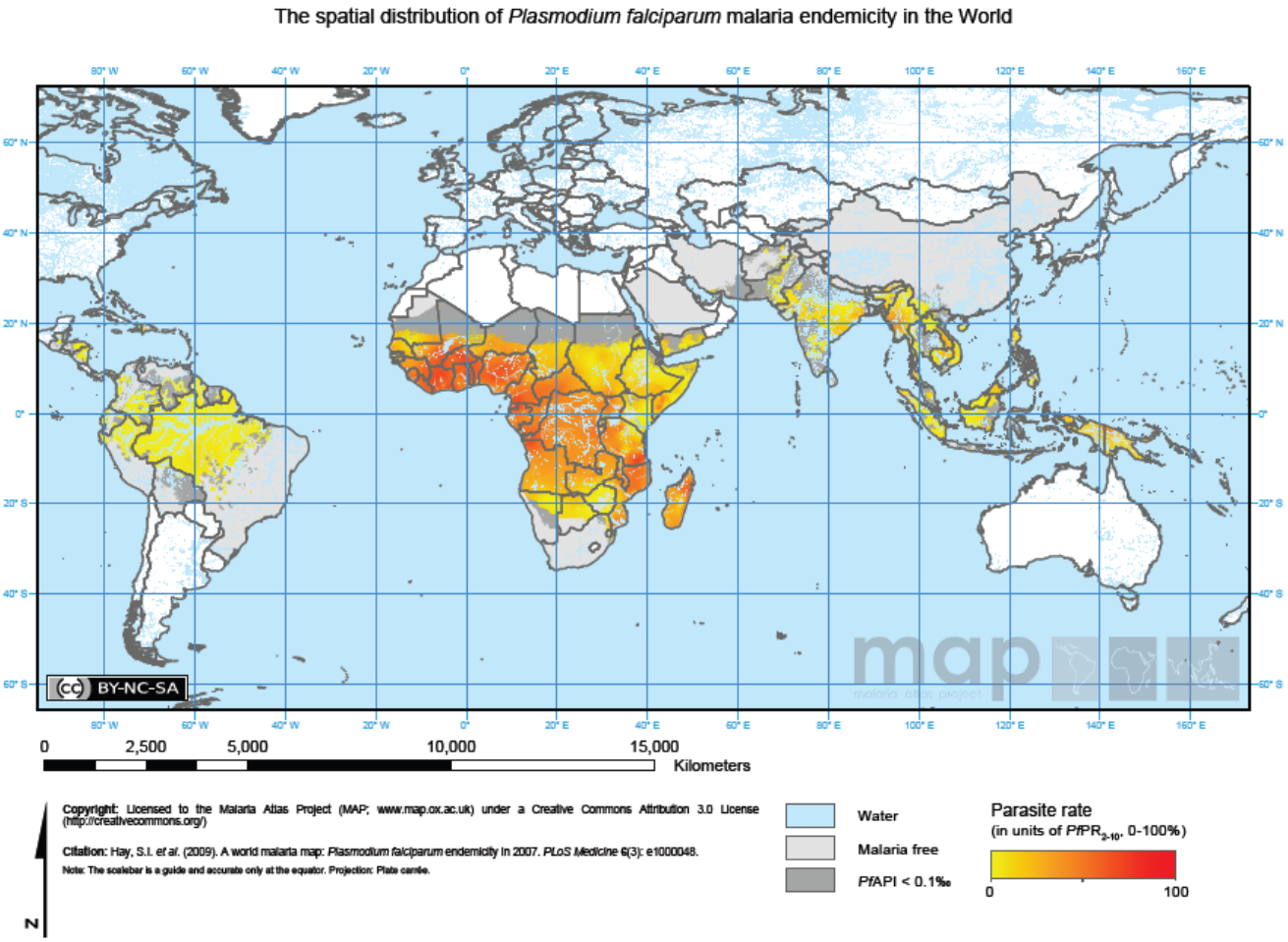

FIGURE 2. Spatial distribution of *P. falciparum* malaria endemicity in the World (Hay 2009)

African region

In 2009 there were an estimated 68,925,435 cases of malaria in the African region (78% of worldwide estimates) and 111,885 malaria deaths (91% of worldwide estimates) (*WHO Malaria World Report 2010*).

To compound the human toll of this disease, recent macroeconomic analysis by the WHO Commission on Macroeconomics and Health has found that malaria reduces economic growth in sub-Saharan Africa by > 1% per year, with major long term consequences for the gross national product of the afflicted countries (Sachs & Malaney 2002).

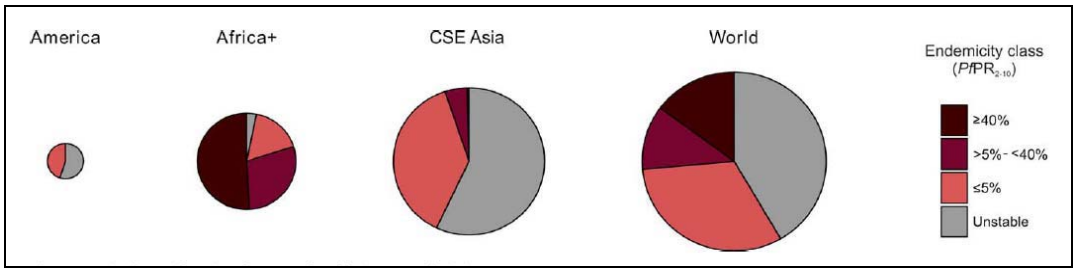

FIGURE 3. Pie Charts showing the population at risk of *P. falciparum* malaria in 2007. The charts show the proportion of the population living in each predicted *P.f.* parasite rate endemicity classes for the America, Africa, Central and South East Asia regions, and worldwide. The charts are scaled proportionally to the total population at risk in each region. (Hay 2009)

5.2. Global control measures

Malaria control is at present mostly based on three main strategies: (i) reducing human-vector contact (ii), vector control and (iii) drugs for treatment of cases and prophylaxis (including vaccines).

Reduction of human-vector contact

The main tool to reduce human-vector contact is the use of insecticide-treated nets (ITNs). The use of ITNs to reduce the impact of malaria on endemic populations is one of the public health recommendations for which there is most evidence of its efficacy. WHO recommends the use of ITNs for all children under the age of five and pregnant women (*Malaria Control Today. Current WHO Recommendations. Working document. 2005*). Nevertheless, the cost and operational problems with the distribution and re-impregnation of the nets are responsible for a low coverage, as only 8% of children younger than 5 years are sleeping under an ITN in Africa (*UNICEF & Roll Back Malaria 2007*).

Research on ITNs can be divided in two periods. From 1982 to 1991 strong evidence was developed of the impact of ITNs on the number of infective bites (Charlwood 1987, Lines 1987, Carnevale 1988, Li 1989), but there was contradictory evidence of the impact on clinical malaria (Graves 1987, Snow 1987, Snow 1988a, Snow 1988b, Carnevale 1991). In 1991 a trial showed a large impact on infection, clinical malaria, all-cause and malaria-specific mortality (Alonso

1991). At that time WHO withheld taking a policy decision until other large scale mortality trials in different epidemiological and geographical settings had been performed, under the coordination of WHO. Four more trials to evaluate the efficacy of ITNs against malaria-related morbidity and mortality in young children were conducted in several African settings, with different transmission intensities and age patterns (D'Alessandro 1995b, Binka 1996, Nevill 1996, Habluetzel 1997, Phillips-Howard 2003). All trials showed that ITNs were efficacious in reducing child mortality. A Cochrane review estimated that ITNs reduced overall mortality in children under 5 years by 17% compared to no nets, and in areas of stable transmission reduced the incidence of clinical malaria by 50% (Lengeler 2004). The effect of the intervention is sustained over time and has no deleterious effect on child mortality, as have shown the studies of the long-term follow-up of ITN use (Binka 2002, Diallo 2004, Lindblade 2004).

Several trials have evaluated the impact of ITNs on the consequences of malaria during pregnancy, showing that in Africa ITNs compared to no nets reduced low birth weight by 23% and miscarriages/stillbirths by 33% in the first pregnancies, while the reduction in placental parasitaemia was 23% for all gravidae (Gamble 2006, Gamble 2007).

New mechanisms for the funding and deployment of this tool, including the distribution of long-lasting insecticide treated nets that do not need re-impregnation, are needed.

Vector control

Indoor residual spraying (IRS), elimination of mosquito larvae with *gambusia* fish or larvicides and reduction of mosquito breeding sites are the main vector control measures. However, environmental management activities are expensive, require technical engineering staff and several years to be implemented, and can only be used in areas where it is logistically and economically possible. They are difficult to implement in sub-Saharan Africa outside urban areas and in fact, most of the

previously successful environmental management studies were conducted outside this region, mainly in Europe, the Americas and South East Asia (Keiser 2005). An example of successful environmental management to control malaria is the activities conducted during the building of the Panama Canal at the end of the 19th century. The area was covered with jungle and swamps and there was no water sanitation, which led to the death of more than 22,000 employees of the Canal Company. Drainage and vegetation management was then introduced and malaria incidence decreased from 821 per 1,000 in 1906 to 14 per 1,000 in 1917 (Keiser 2005).

IRS played a key role in the malaria eradication campaign and has been extensively used for the control of malaria in areas of low or epidemic transmission, especially in urban areas and in Latin America and Asia. However, it is debatable whether it is a useful control tool in moderate or high transmission settings in sub-Saharan Africa. Its implementation requires expensive logistics, large human resources, high coverage levels and sustainability in time, making it difficult for African countries to implement it. Moreover, there are problems with resistance of the mosquitoes to the insecticides and acceptability of the population to having their houses sprayed.

Different insecticides can be used for IRS (organochlorine, pyrethroids, organophosphates and carbamates), the most widely used and cheapest one being DDT (*WHO. Indoor residual spraying. Position paper. 2006*). DDT was largely used from 1945 to 1970, both for IRS to control malaria and other vector-borne diseases and for agriculture to control insects. In the 1970's and 80's several countries banned its use based on harmful ecological effects, due to persistence of DDT in the environment and bioaccumulation that interferes with reproduction of some animals. At the Stockholm Convention on Persistent Organic Pollutants in 2001 it was proposed to ban DDT and 11 other persistent organic pollutants globally. Most countries signed the Convention, which entered into force in 2004, but some countries, mainly African, asked for an exemption for DDT for public health

purposes (*Stockholm Convention on Persistent Organic Pollutants*). In 2006 WHO endorsed the re-introduction of DDT for IRS for malaria control where it is indicated in their guidelines (*WHO 2006*). This decision has been controversial and has generated a lot of debate, mainly because of the potential toxic effects of DDT on human health. The use of IRS has increased substantially in many African countries during the last years and in many areas the insecticide DDT is being used again (*WHO 2005*).

Although exposure to DDT at amounts that would be needed for malaria control have not generally shown any toxic effects in humans, studies conducted in North America showed DDT to cause preterm birth and early weaning. If these effects were the same in sub-Saharan Africa this could lead to an increase in infant mortality, that could reduce or eliminate the benefit achieved through the reduction of malaria (Rogan 2005). Although DDT can be useful for malaria control if applied correctly, the risks for human health should be taken into account and, if possible, other malaria control measures or other insecticides should be used.

Drugs for treatment of cases

The cornerstone of malaria control continues to be early diagnosis and prompt treatment of malaria cases with an effective drug. Chloroquine has been used for decades for first line treatment and prophylaxis of malaria in endemic countries. After the appearance of the first resistances in the 60's in South East Asia and their later spread to most endemic areas, Chloroquine had to be substituted. Some countries changed to Sulphadoxine-Pyrimethamine (SP), but resistance to SP also appeared first in South East Asia and then in Africa. Development of new antimalarial drugs received very little attention until recently, and very few alternatives to Chloroquine and SP were available for first line treatment.

In the last few years more research and funds have been devoted to the development of new antimalarials. Initiatives such as the Medicines for Malaria Venture (MMV) have greatly contributed to the field with funding and building of

public-private partnerships, putting together a pipeline of new drugs or combination of drugs.

Artemisinin derivatives of Qinghaosu (*Artemisia annua*), were shown to be highly efficacious against malaria (Lee 2002b). However, it was not until a few years ago that artemisinins were produced under good manufacturing practices (GMP) and tested and registered in western regulatory agencies. Artemisinins have a short half-life, rapidly decreasing parasite load, and a gametocyticidal effect. Artemisinin-combination treatments (ACTs) have been shown to be effective, safe and well tolerated in multiple clinical trials (Ogbonna 2008).

Currently the WHO recommendation is to use ACTs as first line antimalarials for the treatment of mild malaria in sub-Saharan Africa. The recommended ACTs are Artemether-Lumefantrine, Artesunate + Amodiaquine, Artesunate + Mefloquine or Artesunate + Sulfadoxine–Pyrimethamine (WHO. *Malaria Control Today. Current WHO Recommendations. Working document. 2005*). The rationale is that the combination of two drugs with different modes of action increases the therapeutic efficacy and decreases the development of resistance to the individual drugs of the combination.

Most countries in sub-Saharan Africa have now adopted ACTs as the first line treatment. Nevertheless the coverage and uptake within the countries is still low. Several factors hamper the large-scale deployment of ACTs, including their high cost, the poor delivery systems and the low quality of the drugs, as the GMP production does not yet cover the demand (Ogbonna 2008). Also, co-formulations of ACTs would improve compliance of treatment courses.

Quinine continues to be used worldwide for severe and complicated malaria and is in many places the only alternative to multi-resistant antimalarials. However, there might be a shift from this policy in the near future. WHO has recommended Artesunate for SM in adults since 2006, but very recently revised its guidelines to

include children, based on findings from a nine-country trial in Africa in 2010, which found that for every 41 children treated with Artesunate instead of Quinine, one life could be saved (Dondorp 2010). It is thus suggested that parenteral Artesunate should replace Quinine as the treatment of choice for severe falciparum malaria worldwide.

Treatment of malaria in pregnancy is nowadays a matter of concern, as Chloroquine and SP were the only drugs widely used for case management in pregnant women. Little is known about the pharmacokinetics, efficacy or safety of ACTs in pregnancy, as pregnant women have not been included in previous clinical trials. Research on alternative drugs for case management in pregnancy and on the safety of ACTs, especially in the first trimester of pregnancy, is urgently needed (Greenwood 2007, Nosten 2007).

New combinations of drugs are in development, the most advanced ones being Dihydroartemisinin-Piperaquine (DP) and Pyronaridine-Artesunate (PA). In a non-inferiority study carried out in five African countries (Burkina-Faso, Kenya, Mozambique, Uganda and Zambia), DP showed to be as efficacious as Artemether-Lumefrantine (AL) for treating UM in children 6-59 months. Furthermore it showed a statistically superior efficacy in preventing new infections (Bassat 2009). This new ACT will potentially be recommended in the near future.

Drugs for prophylaxis

In the past, weekly chemoprophylaxis with Chloroquine was recommended for all infants, young children and pregnant women in endemic areas. This strategy was very effective in reducing morbidity and mortality, but had several setbacks, including drug toxicity and safety concerns, risk of developing Chloroquine resistance, rebound increase in malaria once chemoprophylaxis ended and logistical challenges in implementing such an intervention at the population level (Geerligs 2003, Menendez 2007, Greenwood 2004).

Continuous chemoprophylaxis for children was abandoned many years ago, and chemoprophylaxis for pregnant women is no longer recommended in Sub-Saharan Africa, but is still used in India and several Latin American countries.

Some years ago, a new approach to prophylaxis was explored attempting to maximize the benefits and minimize the problems of continuous chemoprophylaxis: intermittent preventive treatment. It consists of administering an antimalarial at therapeutic doses to infants or pregnant women, independently of their parasitaemic status, at pre-defined time points.

Intermittent Preventive Treatment in pregnancy (IPTp)

Intermittent preventive treatment in pregnancy is the administration under direct observation of two or three doses of an antimalarial to pregnant women during the antenatal clinic visits.

Evidence on the safety and efficacy of IPTp initially came from four studies (Schultz 1994, Parise 1998, Verhoeff 1998) of which only one was a randomized-controlled trial (Shulman 1999). Overall they showed that 2 doses of SP decreased the prevalence of maternal anemia, placental parasitaemia and low birth weight. Despite the limited evidence and the gaps in knowledge on the mechanism of action of IPTp it is now a recommendation of WHO to administer at least 2 doses of IPTp with SP to all pregnant women in the second and/or third trimesters at least one month apart in stable transmission areas, and many sub-Saharan African countries have already implemented it (*WHO 2004, WHO 2005a*). In HIV-positive women the efficacy of 2 doses of IPTp is lower, and three doses are recommended (Parise 1998, Filler 2006, ter Kuile 2007, *WHO 2004*).

Although IPTp and ITNs had already shown separately to be efficacious in reducing the harmful effects of malaria during pregnancy, the safety and efficacy of both interventions together were later also assessed in different settings (Njagi 2003, Mbaye 2006, Menendez 2008). In the latter double-blind randomized trial, carried

out in Mozambique, two-dose SP was associated with a 40% reduction in the incidence of clinical malaria during pregnancy and reductions in the prevalence of peripheral and placental parasitaemia (Menendez 2008). It was also reported that SP administration did not translate into significant improvement in other maternal or birth outcomes, which suggested that the use of ITNs during pregnancy may reduce the need to administer IPTp or at least the number of doses (Menendez 2008). However, in a follow-up study of the newborns up to 12 months of age, it was suggested that IPTp-SP could reduce neonatal mortality (Menendez 2010).

SP is the only drug that has been used so far for IPTp, but increasing resistance to SP calls for the evaluation of alternative drugs. A comparison between the safety and efficacy of SP versus Mefloquine (MQ) is currently being carried out in a multi-site trial, within the Malaria in Pregnancy Preventive Alternative Drugs (MiPPAD) consortium.

Intermittent Preventive Treatment in infants (IPTi)

WHO is now recommending a new intervention against *P. falciparum* malaria, targeted at the most vulnerable age group: Intermittent Preventive Treatment for infants (IPTi).

IPTi-SP is the administration of a full therapeutic course of SP delivered through the Expanded Program on Immunization (EPI) at defined intervals corresponding to routine vaccination schedules to all infants irrespective of infection status. Further details on this intervention are described in **section 7** of this thesis' introduction.

Malaria vaccines

Anti-malarial vaccines would be a major tool to contribute to malaria control and eradication. The development of a safe and efficacious malaria vaccine has remained an elusive goal for the last eighty years. However, the last decade has witnessed a new impetus in the development of malaria vaccines and it is likely that registration of the first one will occur in the next decade. This first generation

vaccine is unlikely to provide complete protection, but it may become a useful tool to be added to the available control measures against malaria.

Developing a malaria vaccine remains an enormous scientific, technical, financial and political challenge. The *Plasmodium* is a highly complex parasite, with a multi-stage life cycle, during which the parasite presents multiple antigens that show significant variability. The main hindrance to developing a vaccine is the choice of the antigen(s) to be included, as, despite numerous studies that have tried to correlate specific immune responses against the *Plasmodium* with the risk of infection or disease, still no surrogate of protection for malaria has been found. In order to choose an antigen we would ideally need to know that immune responses induced by that antigen are protective.

Association between specific immune responses and the risk of infection or disease has been found for different antigens in studies of naturally acquired immune responses in humans. However, association does not mean protection, as exposure to the parasite is an important confounding factor in these studies. Adequate study designs to evaluate whether antigen-specific immune responses are protective (i.e. causative pathways) include randomized controlled trials and longitudinal prospective studies in malaria endemic areas.

Types of malaria vaccines

Classically, there are different types of malaria vaccines targeting different stages of the parasite life cycle: pre-erythrocytic, blood-stage, and transmission-blocking vaccines, as well as so-called anti-toxic vaccines, targeting putative malaria toxins. Vaccines for malaria eradication need to have an impact on transmission rather than focusing on mortality and morbidity reduction alone, therefore MalERA has proposed a new concept called “Vaccines that interrupt malaria transmission” (VIMT). This includes not only classical transmission-blocking vaccines that target the sexual and mosquito stages but also pre-erythrocytic and asexual stage vaccines that have an effect on transmission.

At present there are around 100 candidate malaria vaccines in development (*WHO 2006a, WHO 2006b*), however, most of them are based on a small number of *P. falciparum* antigens (half of them are based on MSP-1, CSP and AMA-1), and only a few have entered clinical trials. A high percentage of the candidates are discarded along the malaria vaccine development pipeline and only a few have reached advanced clinical trials in humans.

Future challenges and opportunities in the control of malaria

Although the availability of a malaria vaccine is currently the single most promising tool to prevent the disease, when vaccines do become available these will have to be administered together with other control strategies, as integration of different strategies is needed if we are to have an impact on the burden of disease. Thus, evaluating the impact of vaccines when used in combination with other malaria control tools (ITNs, IPTp, IPTi, etc.) will be required in future studies.

The last decade has witnessed a renewed international interest for malaria, with the creation of several multilateral initiatives and public-private partnerships to tackle malaria, such as the Multilateral Initiative on Malaria (MIM), the Global Fund to fight AIDS, Tuberculosis and Malaria, MMV, the Malaria Vaccine Initiative (MVI) or the President's Malaria Initiative (PMI). These initiatives have led to a great increase in the funds and research to advance in the control of malaria. Increased political commitment has been reflected in several international development targets, like the objectives set at the African Summit on Roll Back Malaria in Abuja and the Millennium Development Goals (MDG) set by the United Nations.

The Abuja summit held in Nigeria in 2000 set the objective to increase by the year 2005 to at least 60% access to appropriate treatment for malaria cases, coverage of ITNs for children < 5 years and pregnant women and chemoprophylaxis or IPTp for pregnant women (*WHO 2000a*) and the MDG 6 is to reduce morbidity and

mortality due to malaria and other diseases by 2015.

The increased funding and commitment has accelerated the implementation of malaria control tools and the renewal of the national malaria control plans. However, the coverage of these interventions remains ominously low in most countries (*UNICEF & Roll Back Malaria 2007*) and operational and logistic difficulties, weak health services and lack of human resources continue to be great barriers for the control of malaria.

In this renewed effort to control malaria, a combination of tools such as ITNs, ACTs, RDTs and IRS are, and will increasingly be, deployed in many countries. All these strategies have been evaluated individually in efficacy trials but their effectiveness when combined and used in "real life" conditions is unknown. There is a need to bridge the gap between basic research and efficacy studies and the evaluation of the combined implementation of different tools through national malaria control programs.

A wide scale deployment of the currently available tools could massively decrease the malaria burden in most parts of the world. Nevertheless, research on new drugs, vaccines, improved diagnostic tools, new vector control strategies and new delivery mechanisms and operational research are required. Equally important are a coordinated management and rigorous monitoring of the progress of such programs.

6. Malaria in Mozambique

6.1. Spatial distribution and burden of disease

The first attempt to characterize the ailments afflicting Mozambican inhabitants dates from early 1846, in a work entitled "Draft on the illnesses of the African

eastern coast", described by Jacques Salis, a Swiss physician working for the king of Portugal in Ilha de Moçambique, the first colonial capital city of the Portuguese province of Mozambique (Santos Reis 1982). The pathogenesis of various infectious diseases was not yet well understood at that time; however, the author remarkably stressed the relationship between the illnesses, the environment conditions and type of soil. When describing the region's climate, the author emphasized the alteration between dry and rainy seasons, hot temperatures, predominant southern winds and the lower altitude that are the typical characteristics of the Mozambican costal line. Fevers were the most important ailments, always referred to as endemic fevers and believed to emanate from the swamps. The treatment of those fevers consisted of infusions including lemon syrups, cooked barley and other seasonings containing sulphured Quinine. Other ailments described were dysenteries, hepatitis, tick bite fever, flegmasiasis, elephantiasis, ulcers, tetanus, etc. The author had described the region as "unhealthy and flooded" (Reis 1982). At the time, no specific antimalarial activities were carried out with the exception of the symptomatic treatment of fever cases.

Today malaria continues to be one of the major public health problems in Mozambique due to a multitude of factors such as climatological/ environmental (favourable temperatures and rain patterns, abundant breeding sites) and socio-economical (poverty related improper housing/shelter, unaffordable preventive means). It is considered that 100% of the Mozambican population lives in malaria high-risk areas since malaria is endemic throughout the country, varying between mesoendemic and hyperendemic areas (*WHO World Malaria Report 2009*; Table 1). Transmission is perennial, with peaks during and after rainy seasons (January to April). However the intensities of transmission may vary depending on the amount of rain and air temperatures observed each year and depending on the specific local conditions.

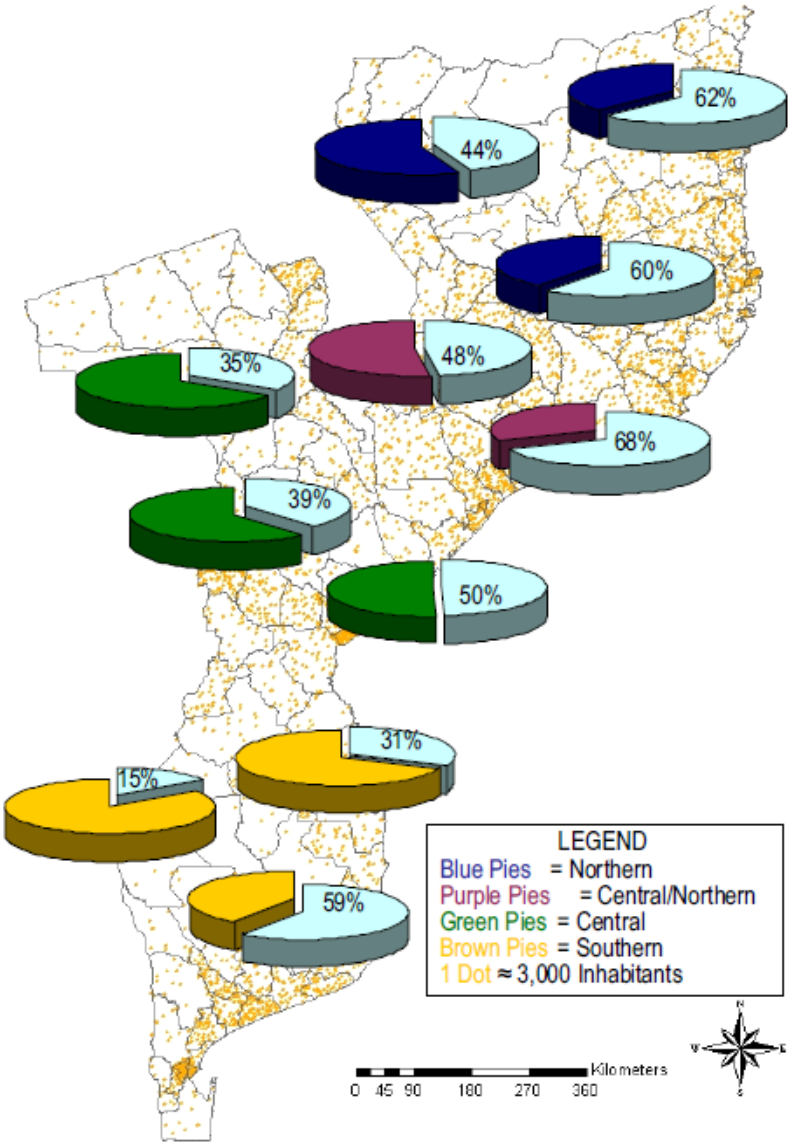

FIGURE 4. Mean estimation point prevalence of *P. falciparum* infection among children aged < 10 years old across different epidemiological settings in Mozambique (Mabunda 2008).

At present there is a gap in the available data of detailed endemicity levels in the country, and the data presented in this section are mostly from 2002, even though most recently published data (*WHO Malaria World Report 2010*) reports that malaria has decreased in Mozambique since 2007. This is consistent with the preliminary results from a study carried out in Manhiça recently (Guinovart *et al*, in preparation).

Population (in thousands)*	**2009**	%
All ages	22 894	
< 5 years	3 842	17
Rural	14 276	62
Population by malaria endemicity (in thousands)	**2009**	%
High transmission (≥1 case per 1000 population)	22 894	100
Low transmission (0–1 cases per 1000 population)	0	0
Malaria-free (0 cases)	0	0
Vector and parasite species		
Major *Anopheles* species	*gambiae, arabiensis, funestus*	
Major *Plasmodium* species	*falciparum*	

** UN Population Division estimates*

TABLE 2. Mozambican population and malaria endemicity (WHO World Malaria Report 2009)

Nevertheless, malaria is still a very common presentation to health facilities. It is also the most common cause of admissions to wards. Severe malaria presents as CM or as SMA, often requiring life saving blood transfusion. In 2002, malaria accounted for an estimated 44% of all outpatient attendance, 57% of paediatric admissions and 29% of all hospital deaths in rural and provincial hospitals. The case fatality rate is thought to vary between 1.8% and 9.9%, depending on the category of the health facility. *P. falciparum* is responsible for > 90% of all malaria cases (*PNCM 2002*).

Incidence of clinical malaria established through weekly active case detection suggested that the risk of clinical malaria is highest between the ages of 1 and 3 years when children experience an average of > 2 episodes per year. Overall, malaria is the main cause of morbidity in children < 5 years accounting for over 30% of reported deaths in this age group. The risk of malaria drops sharply after the age of 6 years, based on a continuous demographic surveillance system (DSS) and verbal autopsies carried out at the Centro de Investigação em Saúde da Manhiça (CISM).

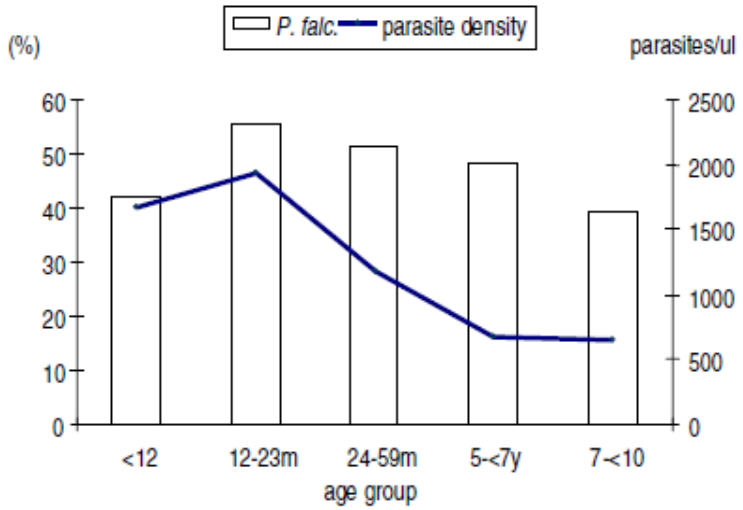

FIGURE 5. Overall *P. falciparum* prevalence and mean parasite density among children < 10 years of age in Mozambique (Mabunda 2008).

Malaria is also a major problem in pregnant women in rural areas. Approximately 20% of women are parasitaemic, and among them primigravids show the highest prevalence (31%). Anaemia is often associated with malaria, and a study in rural southern Mozambique revealed a prevalence of maternal anaemia associated with microscopic parasitaemia to be 59% (Saúte 2002).

The real burden of malaria and its impact on the society or economy are not known due to lack of detailed studies. However, general references such as episodes of illness due to malaria contribute to a loss of agricultural productivity, industrial labour and increased school absenteeism (Wernsdorfer 1988).

Finally, the emergence of the HIV/ AIDS epidemic in Mozambique poses a particularly difficult challenge. Although the direct effects of HIV infection on malaria associated morbidity and mortality are not clearly established, by placing an unprecedented demand on the already meagre health resources, HIV is bound to have a major negative impact on malaria control.

6.2. Local control measures

In Mozambique as in many other places around the world, the history of malaria control began during the early 1900's with the discovery of synthetic antimalarial drugs and the implementation of anti-larval activities. Later, the discovery of the insecticidal effect of DDT, benzene hexachloride (BHC) and dieldrin increased malaria control measures worldwide (Molineaux & Gramiccia 1980).

The scarce information available of malaria reflects the weakness of the surveillance system and the absence of a national plan for malaria control during the colonial period. However, some statistics compiled in the main hospital in Lourenço Marques (LM) city (now Maputo) during the period between 1900 and 1909 showed an increased number of fever cases admitted to the hospital. The second most important disease was anemia. Clearly malaria and malaria-related anemia were the main causes of admission at the LM Hospital between that period (Sant'Anna 1910). Not surprisingly, the case fatality rates were very high given that the treatment of fever cases was palliative.

The first antimalarial activities were initiated in May 1907 in LM city and consisted of environment management (elimination of breeding sites and application of larvicides such as residual oils) (Serrão de Azevedo 1910).

Lack of both human and financial resources was the most important obstacle for expansion to a larger scale implementation of the anti-malarial activities to the rest of the territory. In addition, in those areas where these activities were carried out, local communities were refractory to comply with health authorities' recommendations concerning anti-malarial actions (Serrão de Azevedo 1910).
Between 1935 and 1939 a first large-scale control of rural malaria using pyrethrum spraying was successfully implemented in South Africa.

The positive results achieved within neighbouring territories of the Republic of South Africa influenced the sanitary entities of the bordering regions, particularly

in the southern part of Mozambique, and consequently, during subsequent years entomologists from Transvaal, South Africa, jointly with Mozambican authorities, initiated various studies towards malaria vectors identification, behaviour and habit characterization to provide elements to plan antimalarial interventions in the Maputo region (Paiva Martins 1941).

The first large-scale antimalarial interventions started in LM city and surroundings in 1942 and consisted in the application of pesticides, using kerosene and Pyrethrum and larviciding, and applying oils in all identified permanent breeding sites. In semi-urban areas, breeding sites were treated using residual oils. These activities were later expanded to Beira city in 1946. A complementary measure included spatial fogging with a Tifa® machine and was carried out weekly in LM city and suburbs to decrease the vector density. In addition, anti-larval brigades were responsible for house-to-house treatment and control campaigns for example to avoid in-house breeding sites like domestic water containers (Soeiro 1959).

Use of DDT was introduced in 1946, again in LM and surroundings, then expanded to João Belo, Inhambane and the Limpopo valley, in the southern region of the country. In Ressano Garcia, a small town bordering South Africa and with an important railway station, activities were successfully implemented with the support of the Mozambican railway company CFM (Soeiro 1959). Later, Beira, Quelimane, António Enes, Nampula and Porto Amélia in the northern provinces, were included in the DDT program.

However, the lack of information about vector distribution, parasite prevalence or epidemiology of the disease meant that malaria transmission was very poorly monitored. The first countrywide comprehensive study was carried out between 1949-1951 (Soeiro 1952).

FIGURE 6. Stamps highlighting malaria eradication efforts by vector control in former Portuguese colonies in Africa.

The National Malaria Control Program (PNCM – *Programa Nacional de Controlo da Malária*) was established in 1982 after the worldwide change from malaria eradication campaigns to malaria prevention and control strategies.

In 1991 the program adopted three main strategies: (i) to provide early diagnosis, and prompt and effective treatment through health care services; (ii) implementation of selective preventive measures to reduce man-vector contact ie. IRS and ITNs; (iii) health promotion and community mobilization. In 1999 a joint international consultancy mission concluded that these strategies had not been effective towards malaria control in Mozambique, for the following reasons:

1. The national health system lacked the capacity to reach the majority of the rural population;
2. Health infrastructures were insufficient after 16 years of civil war and destruction, and linkages between health services and the community were weak;
3. Chloroquine resistance and the limited availability of drugs at community level. Despite an abundance of data suggesting that Chloroquine began to loose efficacy in East Africa in the 1980s, most East African countries were reticent to change their malaria drug policies;
4. IRS campaigns against mosquitoes were concentrated in urban areas;
5. Health promotion, information and communication often failed to reach the target population, and had been ineffective;
6. The population had limited capacity to recognize important malaria signs and

symptoms, and certain cultural practices prevented people from seeking health care.

In response to this situation, the Mozambican Ministry of Health adopted a new approach for its malaria control program, in line with the Roll Back Malaria initiative for the African region. The strategy aimed to promote civil society involvement in health, focusing on the capacity at family level to prevent, recognize and, when necessary, manage malaria appropriately or go to a health facility. This strategy targets children < 5 and pregnant women. Malaria is also regarded as a priority, both in the PARPA (Poverty Reduction Paper) and the Health Sector Strategic Plan.

Current national malaria control strategies (past decade to date)

The current global impact goal is to reduce the malaria burden (malaria parasite prevalence and case fatality rate) by half by 2015 as compared to levels found in 2001 (40% - 80%), thereby achieving the MDG for malaria control. Amongst other specific impact targets, it is proposed to reduce SM incidence rate in children < 5, from 55 per 10,000 found in 2000 to 41 per 10,000 in 2010 and 22.5 per 10,000 in 2015.

The Strategic Plan defined targets and indicators to monitor implementation of activities and measure the impact of malaria control interventions. The key indicators are now monitored annually, as defined in annual plans of action and reports.

The current backbone of the National Malaria Control Program consists of:

(i) Diagnosis, case management and drug supply

(ii) Integrated vector management and personal protection

(iii) Health promotion and community mobilization

(iv) Emergency response

(v) Program management and systems development

(vi) Monitoring and evaluation (surveillance, information and research)

Treatment policies:

Normally two standard regimens are recommended for treating uncomplicated *P. falciparum* malaria, a first-line treatment for routine use and a second-line alternative that should be used if the first treatment has failed, is contraindicated or is not available. The third-line is recommended for treating SM.

Before 2002, the following was the treatment policy:

1st line: Chloroquine (CQ)

2nd line: Amodiaquine (AQ) + Sulfadoxine-Pyrimethamine (SP)

3rd line: Quinine (QN)

On approval of the change to the Mozambican malaria treatment policy in 2002, AQ-SP was introduced as an interim first-line treatment:

1st line: Amodiaquine (AQ) + Sulfadoxine-Pyrimethamine (SP)

2nd line: Artemether-Lumefantrine (AL)

3rd line: Quinine (QN)

Given the fact that AQ had been banned by WHO due to alleged severe side effects, together with its similarities to CQ and potential cross-resistance between the two drugs (Chloroquine was still being used at community level), in late 2004 Artesunate (AS) replaced AQ (combined with SP) as the first-line malaria treatment:

1st line: Artesunate (AS) + Sulfadoxine-Pyrimethamine (SP)

2nd line: Artemether-Lumefantrine (AL)

3rd line: Quinine (QN)

Because of concerns about resistance to SP, national malaria treatment guidelines were updated in 2007, replacing AS–SP with AL as first-line treatment of UM. AQ was re-introduced as 2nd line treatment after the WHO reconsidered its efficacy and safety, since the previously alleged serious adverse reactions to it had not been confirmed. These changes were implemented in 2009:

1st line: Artemether-Lumefantrine (AL)

2nd line: Artesunate (AS) + Amodiaquine (AQ)

3rd line: Quinine (QN)

Although not included in the written guidelines, the PNCM has stated that Artesunate rectal suppositories can be used for the emergency treatment of SM in children in settings in which intramuscular or intravenous Quinine cannot be administered, as recommended by the WHO. The treatment guidelines also state that AS-SP should not be used in children under six months of age but no alternative is offered (*USAID report 2007*).

Malaria prevention in pregnant women: IPTp

While the use of IPTp for all pregnant women is national policy in Mozambique since 2006, its uptake has been limited (33% coverage in 2009) particularly in the Northern provinces. This is probably due to a combination of factors, including poorly coordinated training of staff, lack of supervision together with poor reporting practices (*PMI Malaria Operational Plan for Mozambique –financial year 2011*).

Vector control and reduction of human-vector contact

Vector management has been done mainly by two means, IRS and ITNs (Morel 2005), and it has been concluded that both interventions are effective across a large range of settings and that costs are similar (Lengeler & Sharp 2003). A comparison by Curtis & Mnzava (2000) had previously concluded that ITNs were at least as efficacious as IRS.

However, vector control often requires bordering countries to adopt a coordinated strategy. In this sense, a study recently carried out in the Southern African region (Cliff 2010) reported that a disparate mix of interests and ideas in the region slowed the uptake of ITNs in Mozambique and Zimbabwe and prevented its uptake in South Africa. In all three countries, national policy makers favoured IRS, and only in Mozambique did national researchers support ITNs. Outside interests in favour of IRS included manufacturers who supplied the insecticides and groups opposing

environmental regulation. International research networks, multilateral organizations, bilateral donors and international NGOs supported ITNs. Other factors influenced in the decision to invest in one or the other approaches: the end of apartheid permitted a strongly pro-IRS South Africa to influence the region, however in Mozambique and Zimbabwe, floods in the early 2000's provided conditions conducive to ITN distribution.

The Lubombo Spatial Development Initiative (LSDI), a trilateral initiative between the governments of Mozambique, South Africa and Swaziland, was created to develop the area bounded by the Lubombo mountains into a globally competitive economic zone, mainly in agriculture and tourism, and with the objective of creating a strong employment hub in the region. They soon realized that these were highly endemic malaria areas in the three countries, and that no development could occur in the region if the burden of malaria was not reduced. Thus since 1999 the South Africa's Medical Research Council led the LSDI Malaria Control Program which aimed at reducing malaria transmission in South Africa's KwaZulu Natal province, Mozambique's Maputo and Gaza provinces and Swaziland.

In 2003, the Global Fund to fight AIDS, Tuberculosis and Malaria approved a grant of US$32 million for the initiative. IRS was implemented as the main intervention for vector control, complemented by provision of RDTs for timely diagnosis and the provision of ACTs for treatment of malaria cases.

Despite the positive contribution of this initiative to the reduction in the prevalence of malaria vectors and parasitaemia in the past years in southern Mozambique, there has always been a concern regarding this approach: its sustainability in the long-term and its geographic limits. In fact, LSDI's activities have been practically brought to a halt due to the Global Fund's recent cut down on funding.

The new National Malaria Control Strategic Plan for 2010 to 2014 outlines the new national ITN distribution policy, which focuses on universal coverage (approximately one LLIN for every two persons) for the entire population at risk of malaria in areas that do not have IRS activities. This strategy foresees that the distribution to the high risk groups (children < 5 years and pregnant women) as a platform to reach the universal coverage in Mozambique. The policy also states that LLINs should continue to be distributed free-of-charge.

National and international collaborations

In 2005, the National Malaria Control Commission (CNLM) was established, as a decision-making board led by the MISAU. Besides the CNLM, there is a partners' forum directly involved in malaria control (Malaria Control Technical Coordination Committee), which supports policy design and strategy development, including relevant operational aspects of the PNCM. Members include the WHO, UNICEF, USAID, Malaria Consortium, PSI, PMI, CISM, LSDI and the INS.

Current challenges and opportunities

- Although infant, child, and maternal mortality rates in Mozambique have been decreasing in recent years, they are still among the highest in Africa and the world. While the Government of Mozambique is committed to building an equitable health system that is affordable and sustainable, the health infrastructure, provision of services, and networks are not sufficiently developed to meet the health needs of a highly dispersed population, resulting in poor quality healthcare.
- At this stage, malaria diagnostic capacity is still limited. This is due both to a limited laboratory network and to a shortage of laboratory staff (technicians and lab assistants) to respond to the demand of those who use these services.
- More than half of Mozambicans are estimated to live more than 20 Km from the nearest health facility. In this context, prompt treatment upon fever detection is a challenge.

- Prevention is an increasingly strong focus of malaria control programs, however the available tools (IRS, ITN, IPTp) are dependent on effective delivery systems.
- National programs should take advantage of their health systems' strengths in order to deploy preventive measures in a sustainable way. For example in Mozambique the EPI coverage rates are considered high (approximately 80% in 2005, according to Mozambique's MoH "EPI comprehensive multi-year plan 2007-2009"), and is generally well accepted in the communities; this is believed to be a strength and an opportunity to reach the infant population for additional measures.

7. Intermittent Preventive Treatment in Infants (IPTi)

Intermittent preventive treatment in infants consists of administering three doses of an antimalarial during infancy through the EPI at defined intervals corresponding to routine vaccination schedules to all infants irrespective of infection status.

7.1. IPTi with SP

Initial evidence on the safety and efficacy of IPTi came from studies conducted in Tanzania. The first randomized double-blind placebo-controlled trial of IPTi administered SP or placebo to infants attending for routine vaccination at 2, 3, and 9 months of age. IPTi-SP showed a 59% reduction in clinical malaria and a 50% reduction in anaemia during the first year of life. The treatment was safe and well tolerated and no interaction with the EPI vaccines was found (Schellenberg 2001). In contrast, in a previous trial in which continuous chemoprophylaxis was administered during the first year of life there was a rebound effect during the second year of life, with a higher incidence of clinical malaria and severe anaemia in children who had received chemoprophylaxis compared to controls (Menendez

1997). IPTi not only presented no rebound effect but showed a sustained protective efficacy against clinical malaria of 36% during the second year, suggesting that IPTi could facilitate development of naturally acquired immunity to *P. falciparum* (Schellenberg 2005).

A subsequent study was a randomized placebo-controlled trial in which Amodiaquine was administered to infants three times with intervals of 60 days. The treatment was safe, protective efficacy for malarial fever and anaemia was 65% and 67% respectively and no rebound morbidity was detected during a 4 month follow-up period (Massaga 2003).

These results pointed to a possible large scale implementation of IPTi as a malaria control tool, however it was decided that further evaluation of clinical, immunological and molecular aspects was warranted before a policy recommendation could be made. The IPTi Consortium was founded as an international consortium of research organizations, the WHO and UNICEF and funding from the BMGF to define the research questions that needed to be answered in a coordinated way in order to inform policy and implement the intervention as a public health strategy (Schellenberg 2006). The objectives of the consortium included the following key elements: estimate the efficacy against clinical malaria and anaemia under different epidemiological settings, estimate the efficacy of different antimalarial drugs, evaluate the interactions with the EPI vaccines, analyse the cost-effectiveness of the IPTi and establish a policy platform.

Several IPTi trials with different antimalarials were conducted in different countries with moderate to high transmission of malaria. A pooled analysis of the six trials that used SP provided data for 7930 infants (IPTi, n=3958; placebo, n=3972). IPTi had a combined protective efficacy of 30.3% (95% CI 19.8-39.4, $p<0.0001$) against clinical malaria, 21.3% (8.2-32.5, $p=0.002$) against the risk of anaemia, 38.1% (12.5-56.2, $p=0.007$) against hospital admissions associated with malaria parasitaemia, and 22.9% (10.0-34.0, $p=0.001$) against all-cause hospital

admissions. There was no evidence of a rebound effect during the second year (Aponte 2009). It was reported that IPTi-SP offers a personal protection against clinical malaria for a period of approximately 35 days following the administration of each dose (*IoM Report, 2008).*

The Institute of Medicine of the U.S. was commissioned an assessment of the role of IPTi with SP for malaria in infants, and concluded that IPTi is a tool in which it is worth investing further (Committee on the Perspectives on the Role of Intermittent Preventive Treatment for Malaria in Infants 2008).

Based on the assessments carried out, the WHO announced in March 2010 that it recommends the implementation of IPTi with SP as a malaria control tool. WHO policy recommendation was made at the fourth consultative meeting of the WHO Technical Expert Group (TEG) of Preventive Chemotherapy in April 2009 (*WHO 2009a*) then reviewed and endorsed by WHO's Strategic Advisory Group of Experts on Immunization (SAGE) in October 2009 (*WHO 2009b*).

Sulfadoxine–Pyrimethamine

Fansidar®, the commercial name by which SP has been known, was developed by Roche but it is now off-patent and therefore publicly available for local production (Roche position on access to medicines and diagnostics).

Typically, each tablet of Fansidar® contains 500 mg N^1-(5,6-dimethoxy-4-pyrimidinyl) sulfanilamide (Sulfadoxine) and 25 mg 2,4-diamino-5-(p-chlorophenyl)-6-ethylpyrimidine (Pyrimethamine).

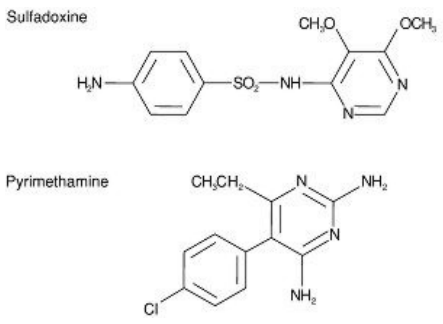

FIGURE 7. Molecular structure of Sulfadoxine and of Pyrimethamine

Mechanism of action: Sulfadoxine and Pyrimethamine are folic acid antagonists. Sulfadoxine inhibits the activity of dihydropteroate synthase whereas Pyrimethamine inhibits dihydrofolate reductase. These are active against the asexual erythrocytic stages of *P. falciparum* and may also be effective against strains resistant to Chloroquine. Thus, the mechanism of action of IPTi-SP appears to be predominantly one of chemoprophylaxis and its protective efficacy is related to the half-life of the medicine and the susceptibility of the malaria parasite to SP.

Drug resistance: Strains of *P. falciparum* with decreased susceptibility to Sulfadoxine and/or Pyrimethamine can be selected *in vitro* or *in vivo*. *P. falciparum* malaria that is clinically resistant to SP occurs frequently in parts of South East Asia and South America, and is also prevalent in East and Central Africa. Therefore, parasite resistance to SP in the area should serve as a guide to adoption of a policy on SP-IPTi. The recommendation of a threshold of a parasite genetic marker of SP-resistance (50% prevalence of the *pfdhps* 540) above which IPTi-SP is not recommended for use is based on the following:

• The presence of mutations at codons 437 and 540 of *pfdhps* together with the triple mutation of *pfdhfr* (quintuple mutation) is a significant predictor of SP treatment failure. The *pfdhps* 540 mutant is a useful epidemiological marker of the quintuple mutation in Africa.

• In areas with up to 50% prevalence of the *pfdhps* 540 parasite mutant in infants and children, clinical trials showed a 30% protective efficacy of IPTi-SP against clinical malaria over one year.

• One trial conducted in an area where the prevalence of the *pfdhps* 540 parasite mutant was approximately 90% found no demonstrable protective efficacy for SP-IPTi.

Despite the above recommendation of a 50% prevalence threshold, in the context of the same IPTi trial in which this thesis is based (Macete 2006), a molecular study was carried out to compare the frequency of clinical episodes of malaria caused by *P. falciparum* parasites with mutations in *pfdhfr* and *pfdhps* among sick children

who received SP or placebo. It was reported that half of the children who received placebo harbored quintuple-pure mutant parasites. Nevertheless, the protective efficacy of IPTi within the 35 days after the third dose was 70.8% (95% CI, 40.7%–85.6%) (Mayor 2008).

In vitro studies: It has been shown that both drugs have complex effects on the biology of gametocytogenesis (Kone 2010). SP treatment is known to select *pfdhfr* and *pfdhps* mutant asexual parasites (Tekete 2009) and gametocytes (Beavogui 2010) and the prolonged parasite clearance time conferred by such mutations is believed to be responsible for increased gametocyte prevalence in SP treated individuals (Kone 2010).

However, using a direct feeding assay in a study in Mali, Kone and colleagues showed that gametocytes present in peripheral venous blood post-SP treatment had an impaired development and thus reduced infectivity for *A. gambiae* mosquitoes. On the other hand, in their experiment, day 3 concentrations of SP decreased mosquito survival by up to 65% ($P < 0.001$) demonstrating that SP is deleterious in vitro for gametocyte infectivity as well as mosquito survival (Kone 2010).

To some extent this confirmed what earlier studies had reported. Analogous experiments performed in the 1950s with Pyrimetamine, suggested that the low rate of infectivity of post-treatment gametocytes could be due to a sporontocidal effect of the drug that is ingested together with gametocytes during the mosquitoes' blood meals (Jeffery 1958). The effect of drugs on infectivity appeared to be dose-related (Shute & Maryon 1954, Young & Burgess 1957). Furthermore, the sporontocidal effect of Pyrimetamine appears to be dependent on the rate of efficacy of the drug (Jeffery 1958, Young & Burgess 1957). Hogh and colleagues (1998) found that SP decreased infectivity. By contrast the effect of Sulfadoxine on gametocyte infectivity has been much less studied (Laing 1965). This may be due to the fact that Sulfadoxine and sulfa-drugs were almost never widely used alone for the treatment of malaria. Therefore, this study by Kone and colleagues provided one of the first indications that Sulfadoxine could also have a

sporontocidal effect.

Box 1. Summary of WHO recommendations and considerations on IPTi-SP (adapted from the Institute of Medicine report)

WHO recommends

The co-administration of IPTi-SP with DTP2, DTP3 and measles immunization to infants, through routine EPI in countries in sub-Saharan Africa, in areas:

• with moderate-to-high malaria transmission (Annual Entomological Inoculation Rates 10), and

• where parasite resistance to SP is not high – defined as a prevalence of the *pfdhps* 540 mutation of ≤ 50%.

Contra-indications

IPTi-SP should not be given to infants receiving a sulfa-based medication for treatment or prophylaxis, including co-trimoxazole (trimethoprim-sulfamethoxazole), which is widely used as prophylaxis against opportunistic infections in HIV infected infants.

Considerations and caveats for implementation

• In situations where a national-scale implementation may not be feasible due to varying levels of the *pfdhps* 540 mutation, IPTi may be implemented at a provincial or district scale, targeting areas with *pfdhps* 540 mutation prevalence ≤ 50%.

• Programmes implementing the IPTi-SP strategy should regularly monitor and evaluate the impact on immunization services and performance.

• Pharmacovigilance systems to monitor potentially serious adverse reactions to SP should be strengthened.

• Surveillance of parasite resistance to SP should accompany the implementation of IPTi-SP as a surrogate measure of its efficacy.

Intermittent preventive treatment in children (IPTc)

In areas of markedly seasonal malaria transmission, the main burden of malaria is in older children rather than infants, and the risk of clinical malaria is restricted largely to a few months each year (Etard 2004, Jaffar 1997). In such areas, administration of IPT to children several times during the seasonal peak in malaria transmission (IPTc) has been investigated as a method of preventing malaria. A systematic review and meta-analysis was carried out based on existing data on the safety and efficacy of IPTc administered seasonally to children under five years of age in different settings and using several different drug regimens (Wilson 2011). All of the controlled studies identified demonstrated a protective effect of IPTc against clinical episodes of malaria during the malaria transmission season ranging from 31% to 93%; the overall protective efficacy (PE) of IPTc administered monthly was 82%. Analysis of the efficacy trials allowed some conclusions to be made about the efficacy of individual drugs and dosing regimens in preventing episodes of clinical malaria. The highest PE was observed using two drugs with a medium to long half-life in combination, SP+AQ or SP+PQ. Long acting drugs used alone or in combination with short acting drugs, such as AS or DHA showed lower PEs. No advantage was seen from the use of artemisinin combination therapies, which should probably be reserved for treatment of clinical malaria when the rapid action of artemisinins is of particular benefit. As would be expected, bimonthly administration of SP or AS+AQ demonstrated a lower PE than monthly IPTc administration. The results add weight to the growing body of evidence which suggests that IPT works largely by providing a period of post-treatment prophylaxis and that the length of this period of protection is determined by the pharmacodynamics of the drugs used (Cairns 2008).

It is known that extensive use of anti-malarials for prevention (as used in chemoprophylaxis) adds to drug pressure and may facilitate the emergence and spread of drug resistant parasites. Drug pressure will be higher when a drug is used for IPTc, covering up to 20% of the population, than when used for IPT in infants or pregnant women (2–5% of the population). Some evidence supporting this view

was obtained from a study in Senegal where the proportion of parasites carrying SP resistance markers was higher at the end of the malaria transmission season in children who had received IPTc (Sokhna 2008).

As for any other successful malaria control measure, an effective IPTc regimen will reduce exposure to malaria parasites and thus has the potential to impair the development of naturally acquired immunity. Meta-analysis indicated a small increase in the incidence of clinical malaria episodes during the malaria transmission season in the year following IPTc administration among children who received IPTc compared to control children. A limitation of these efficacy studies is that IPTc was only administered for one transmission season. It is likely that if children are given IPTc each year for their first five years of life this will have a greater impact on the development of naturally acquired immunity to malaria, as observed in The Gambia when fortnightly chemoprophylaxis during the rainy season was given each year for many years (Greenwood 1995). None of the above mentioned studies included an assessment of specific markers of acquired immunity to malaria.

From an implementation point of view, IPTc has a major limitation: unlike IPTi, for older children there is no health system infrastructure in which this intervention could be delivered systematically, and this would compromise its sustainability.

7.2. IPTi with other drugs

Some of the trials of IPTi carried out previous or during the Consortium period were not included in the meta-analysis by Aponte and colleagues (Aponte 2009) because they used different drugs and did not deliver IPT at the same time as EPI vaccines (Massaga 2003), used IPT as a treatment for anaemia in children (Desai 2003, Verhoef 2002), gave IPT to schoolchildren (Clarke 2008), or gave IPT in monthly intervals to children up to 5 or 10 years of age in settings with highly seasonal transmission of malaria (Cisse 2006, Sokma 2008, Dicko 2008).

More recent IPTi studies with alternative drugs were carried out after the Consortium ended.

- In Kenya IPTi consisted of SP plus 3 days of Artesunate, 3 days of Amodiaquine-Artesunate, or 3 days of short-acting Chlorproguanil-Dapsone (CD) (Odhiambo 2009).
- In Tanzania the protective efficacy and safety (Gosling 2009) and the duration of protection against clinical malaria (Cairns 2010) were assessed in a trial with three regimens of IPTi: SP, CD and Mefloquine (MQ).
- In Papua New Guinea IPTi consisted of a combination of SP and Amodiaquine or Artesunate (*unpublished*).

Other potential efficacious drugs

Jansen and colleagues (2011) are in favour of Artesunate and Sulphamethoxypyrazine-Pyrimethamine (SMP) combination and they recommended that this combination should be tested in future field studies of IPTi. This drug combination has shown to be efficacious and safe (Sagara 2006, Adam 2006, Rulisa 2007, Penali 2008, Sagara 2009) and the full curative dosing can be done over 24 h (dose interval 12h) or 48 h (dose interval 24 h).

Other new ACT combinations of drugs are under development, such as Dihydroartemisinin-Piperaquine (DP) and Pyronaridine-Artesunate (PA). In a non-inferiority study carried out in five African countries (Burkina-Faso, Kenya, Mozambique, Uganda and Zambia), DP showed to be as efficacious as Artemether-Lumefrantine (AL) for treating UM in children 6-59 months. Furthermore it showed a statistically superior efficacy in preventing new infections (Bassat 2009). These could be potential candidates for administration as IPTi.

8. Development of immunity during infancy and early childhood

Riley and colleagues have suggested a model for the development of antimalarial immunity in early life (Artavanis-Tsakonas 2003) that seems to fit both the immunological and epidemiological data. The hypothesis is that, in endemic populations, primary malaria infections in infants induce low levels of IFN-γ and TNF via an innate pathway and, at the same time, antigen-specific T cells are primed. The infection induces minimal clinical symptoms and parasites are cleared, either immunologically (via opsonization by maternal antibody or cytokine-mediated parasite killing) or because parasites fail to establish themselves in the face of physiological barriers (e.g. foetal haemoglobin and dietary deficiencies). Upon re-infection, the malaria primed T cells produce greatly increased amounts of IFN-γ which synergise with malarial glycosyl phosphatidyl inositol (GPI) to up-regulate the production of TNF (and/or lymphotoxin) leading to an increased risk of CM or systemic shock. Further infections induce effective antiparasitic immunity which reduces the parasite load and thus the concomitant level of antigenic stimulation, thereby dampening the pro-inflammatory cytokine cascade. Falling antigen concentrations or other factors lead to a switch in the predominant T cell phenotype from Th1 (IFN-γ producing) to a regulatory T (Treg) cell phenotype (IL-10 and TGF-β producing). The clinically immune individual can then clear the infection without running the risk of overproducing dangerous inflammatory mediators. The risk of severe disease may thus depend on the relative speed with which the different components of the antimalarial immune response develop.

By contrast, non immune adults who contract malaria during travel to an endemic area, or during an epidemic in a previously malaria-free region, have no protective immunity and are unable to control their infections. Innate responses may provide a degree of protection, as has been described for some individuals with experimental malaria infections, but cross-reactively primed T cells appear to contribute to development of severe disease. Non-immune children on the other

hand may have fewer cross-reactively primed cells (due to lower levels of exposure to cross-reacting microbes) and are, subsequently, at lower risk of CM or SM.

This model of antimalarial immunity has obvious implications for vaccine development; Th1-like cellular responses are clearly required for parasite clearance, but need to be induced in a controlled, site- or organ-specific manner in order to avoid systemic disease.

Immune system development in infants

The developing immune system is influenced by many factors, including intrinsic genetic differences (e.g. ethnicity), disease status (e.g. immunodeficiency) and environmental exposures (e.g. birth weight, undernutrition).

Infants have limited exposure to antigens *in utero* to induce adaptive immunity. Therefore, they are thought to be heavily dependent on their innate immune system for protection against infections (PrabhuDas 2011). Toll-like receptor (TLR)-mediated cytokine production by mononuclear cells differ among blood samples from neonates, toddlers and adults (Kollmann 2009). Thus, infant blood monocytes produce less IFN-γ and IL-12 than cells obtained from adults. However, production of these cytokines rapidly increased between birth and 1 or 2 years of age. In contrast, infant cells showed a greater capacity to produce IL-10 and strong IL-17-producing helper T cell (Th17 cell)–promoting function in response to TLR stimulation by producing IL-6 and IL-23. Furthermore, individual infant cells are less able than adult cells to produce multiple cytokines simultaneously in response to TLR agonists (i.e. less polyfunctional). The predominance of a Th17-like pattern combined with considerable IL-10 production may contribute to diminished Th1 responses, resulting in greater susceptibility to intracellular infections and diminished vaccine responses during infancy.

Understanding the molecular mechanisms that regulate the pattern of cytokine

expression by innate cells in infants will be necessary for the development of protective measures, such as vaccines, for this population. The development of adaptive immune cells in early life (cell-mediated and antibody-mediated responses) is another understudied area of research. Studies of cell-mediated responses suggest that infants are able to mount T cell responses in most circumstances. However, the quantity and quality of the response may differ from that in adults. For example, CD4+ T cell responses, but not CD8+ T cell responses, develop more slowly in infants than in adults after primary infection with cytomegalovirus or herpes simplex virus (Wilson 2008). In addition, responses to some vaccines, such as hepatitis B virus and oral poliovirus vaccine, are diminished in Th1 activity and biased toward Th2 function (Adkins 2007). Although there is evidence for the presence of many cytokines in response to vaccines, good correlates of protective cellular immunity that are informative have not yet been defined. The mechanisms underlying the functionally distinct responses of infant T cells are not well understood but are probably multifactorial. First, infants have a dominant anti-inflammatory cytokine profile that seems to be induced during foetal life (Adkins 2007). For example, in the in utero environment, Treg cells dominate the foetal circulation, suppressing reactivity to non-inherited maternal antigens (Mold 2008) and possibly promoting a generally suppressive environment. Second, there is a propensity for Th2-polarizing cytokine responses in infants, related to the degree of methylation of cytokine loci regulating the expression of cytokine genes. Antigen presenting cells from neonatal human cord blood appear to have impaired Th1 responses to many stimuli, including most TLR agonists.

The B cell arm of adaptive immunity also commonly seems to be compromised in early life. Infant antibody responses are typically of shorter duration, have a delayed onset, differ in the distribution of IgG isotypes (lower titers of IgG2) and are of lower affinity than are adult responses (Siegrist 2009). Furthermore, although primary IgG responses to vaccines can be elicited within 2 months after birth, the persistence of protective antibody titers is poor. It is likely that young

children mostly have short-lived plasma cells and thus experience rapid decline in antibody levels, while older children have long-lived plasma cells producing longer lasting antibody responses (Onome 2008). Because antibodies are key components of protective vaccine responses, greater understanding of B cell function during infancy will be important for the development of effective infant vaccines. Limited infant B cell responses to vaccines may be due in part to diminished T cell help, immature antigen presentation, the presence of maternal antibodies, lower IgG responses to protein and particularly to polysaccharide antigens, and less antibody persistence. For example, passively acquired maternal antibodies impede the effectiveness of antibody-mediated protection elicited by measles vaccines (Gans 1998).

Infant immunity in the developing world

The administration of vaccines in early infancy is a cost-effective strategy for protecting infants from infectious disease in the developing world. However, many factors may limit vaccine efficacy in resource-limited settings. There is a high prevalence of co-infections in developing countries (HIV, malaria, helminths or CMV), which may hinder vaccine efficacy. Malaria can restrict fetal growth, result in preterm delivery and low birth weight in newborns (Bardaji 2011), diminish the transfer of maternal immunity, and dampen responses to other infectious diseases such as *Streptococcus pneumoniae*, tetanus and measles.

In addition, prenatal exposure to *P. falciparum* antigens stimulates an immune response in the foetus that can be strongly immunomodulatory and persist into childhood. The effects of foetal exposure to parasites or their products on the development of immune responses in the face of multiple co-infections, and the degree to which pathogen-specific immune responses acquired *in utero* persist into early life, remain poorly understood.

9. Immune responses to *P. falciparum*

Host genetic resistance to parasites and innate immune mechanisms can protect non-immune individuals from infection to some extent. In addition, people living in malaria endemic areas who are exposed to repeated *P. falciparum* infections from infancy develop NAI to the disease later in life (Doolan 2009). Adults in such endemic areas, though often harbouring low but detectable numbers of parasites in the blood, rarely develop clinical symptoms or die as a result of the infection. This form of acquired immunity to the disease, also referred to as "premunition", "clinical tolerance" or "antitoxic / antidisease immunity", develops slowly over the years and is never complete. There is, generally, also a substantial decrease in the parasite load in infected adults, indicating that there is also an "anti-parasite immunity" component in malaria protection which develops with age. Thus, the long period required to achieve protection is probably dependent on age and exposure, but it has not been conclusively explained which component prevails. On one hand, the malaria parasite may be poorly immunogenic at inducing protective responses. On the other hand, if immunity was essentially strain-specific, a long period could be required to be exposed to a large repertoire of diverse strains. Another hypothesis suggests that immunity to malaria develops differently in adults and children; a more mature immune system would allow an adult to acquire immunity more rapidly than a child under the same exposure (Baird 1995).

Despite the fragmented knowledge about host immune responses to plasmodial infection in humans, there is an increasing awareness of the roles played by the various arms of the immune system against the invading parasite, which involve both humoral and cellular immune responses.

9.1. Erythrocytic stage antigens: AMA-1, MSP-1, EBA-175, PIESA

Most of the symptoms and complications in human infections are associated with asexual blood stages of the parasite, actively multiplying within host erythrocytes. Because of their contribution to the pathologic changes associated with malaria, asexual blood stage parasites are being included as targets of immunization with the hope of inducing anti-disease immunity rather than preventing infection (Jones & Hoffman 1994). Many merozoite antigens have been identified by monoclonal antibodies that block invasion, or by protection studies in animal models immunised with affinity-purified parasite proteins (Anders & Saul 1994).

In our studies, in addition to detecting a general response to whole parasite antigens, we have chosen to evaluate immune responses to three specific merozoite surface proteins all of which are involved in erythrocyte invasion; these are the merozoite surface protein 1 (MSP-1), the apical membrane antigen 1 (AMA-1) and the erythrocyte binding antigen 175 KDa (EBA-175). Furthermore, we evaluated responses to parasite-induced erythrocyte surface antigens (PIESA) which are expressed once the erythrocyte has been invaded; these include the so called variant surface antigens (VSA).

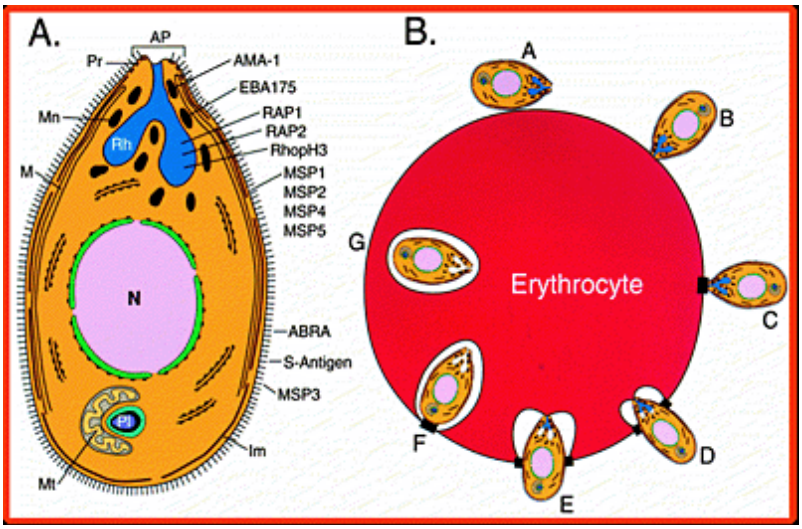

FIGURE 8. A) Schematic of a *P. falciparum* merozoite highlighting the major merozoite proteins that are potential vaccine candidates; B) Stepwise schematic of the invasion of a *P. falciparum* merozoite into an uninfected erythrocyte (Cowman 2000).

MSP-1 is one of the best characterized malaria proteins. It was initially discovered in *P. yoelii* with a mAb against Py230 (Freeman 1980), the homologue of *P. falciparum* MSP-1. This protein is the precursor of major antigens found on the surface of the merozoite (Holder & Freeman 1984, McBride & Heidrich 1987). It is encoded by a single copy gene and is synthesised as a protein of 185-220 kDa at the schizont stage. MSP-1 is anchored into the merozoite membrane through the attachment of a GPI moiety to the C-terminus of the polypeptide (Schofield & Hackett 1993). Most of the protein is shed around the time of RBC invasion and the only fragment that remains associated with the parasite after invasion is a 19 kDa fragment, MSP-1_{19}, a result of a secondary processing of the 42 kDa C-terminal fragment MSP-1_{42} (Blackman 1991, 1993). MSP-1_{19} consists of two domains, each with six highly conserved cysteine residues that are characteristic of epidermal growth factor (EGF)-like motifs, which are targets of inhibitory antibodies (Chappel & Holder 1993). Major epitopes recognised by human antibodies depend on a conformation created by disulphide bonds in the EGF-like motifs (Egan 1995).

MSP-1_{19} is one of the promising vaccine candidates. Evidence supporting the importance of MSP-1_{19} includes inhibitory activities of anti-MSP-1_{19} mAbs, and numerous animal vaccine trials using purified parasite MSP-1_{19}, synthetic peptides, or recombinant proteins. In *P. falciparum*, mAbs specific for either conserved epitopes of MSP-1_{19} (Blackman 1990) or polymorphic epitopes in the N-terminal block 2 region of MSP-1 (Locher 1996) inhibit merozoite invasion in vitro. No robust *in vitro* correlates of protection were established in these animal models. In mice, some though not all studies suggested that antibodies were the means of protection following immunisation. In contrast, in monkeys there was no clear association between antibody titres or between the ability of sera to inhibit merozoite invasion *in vitro* and protection.

SPf66, a synthetic polypeptide antigen constituted the first malaria vaccine to undergo extensive field trials. SPf66 combined three synthetic peptides (35.1, 55.1 and 83.1), together with the peptide $(NANP)_2$ from CSP. Peptide 83.1 was derived

from the N-terminus of MSP-1 whereas the other two peptides were derived from poorly characterised proteins. Initial results from vaccine trials in both *Aotus* monkeys and in humans in South America were very encouraging (Moreno & Patarroyo 1989). SPf66 was safe, immunogenic and protective against *P. falciparum* in semi-immune Colombians (Amador 1993). However, the nature of the protective immune mechanisms remained unclear; no correlation was established between antibody titres and the incidence of malaria. The first randomised double-blind placebo-controlled trial of SPf66 in a highly endemic area of Tanzania reported a reduced risk of clinical malaria among children, with an estimated efficacy of 31%, but confidence limit ranged from 0-52% (Alonso 1994). These results were not reproduced in further phase III trials in The Gambia (D'Alessandro 1995) and Thailand (Nosten 1996) or in infants.

AMA-1 is an 83-kDa precursor protein localized in the micronemes at the apical end of the merozoite, the erythrocyte-invading stage of the parasite (Peterson 1989, Bannister 2003, Healer 2002, Healer 2004). This precursor is proteolytically cleaved to form a 66-kDa protein which then translocates from micronemes to the surface of the merozoite (Narum 1994, Howell 2001) and as such is thought to mediate merozoite reorientation to the erythrocyte (Mitchell 2004). AMA-1 has also been found on the surface of sporozoites and on hepatic merozoites (Silvie 2004); thus, this “blood stage” antigen may be a target for protective immune responses against both the invading sporozoite and liver stage of the parasite.
Several studies have shown that anti-AMA-1 antibodies may play a role in protective immunity in adults living in malaria-endemic areas, and while these studies did demonstrate cross-reactivity to heterologous alleles, the degree to which these antibodies reacted varied (Hodder 2001, Polley 2004).

Other studies with T-cells from naturally-exposed subjects have reported proliferation in response to peptides derived from AMA-1 (Udhayakumar 2001).
Preclinical studies in mice have demonstrated protection (survival with reduced parasitaemia) against the rodent parasite *P. chabaudi* by active immunization with

homologous recombinant AMA-1 protein formulated with potent adjuvants, as well as by passive transfer of immunoglobulin from vaccinated rabbits (Anders 1988). Additionally, active immunization of rhesus monkeys with *P. knowlesi* AMA-1 adjuvanted in saponin resulted in some animals demonstrating a delayed prepatent period when challenged with *P. knowlesi* schizonts (Deans 1988). In *Aotus* monkeys immunized with *P. falciparum* recombinant AMA-1 in complete Freund's adjuvant, significant delays in parasitaemia after homologous blood stage challenge were seen as compared to monkeys immunized with a similarly adjuvanted control malarial antigen (Stowers 2002). T-cell responses to AMA-1 were detected in naïve adult volunteers immunized with irradiated *P. falciparum* sporozoites (Krzych 1995), suggesting that AMA-1 may be able to elicit cellular host immune responses to act against pre-erythrocytic stages of *P. falciparum* infection.

Two Phase 1 dose-escalation adult vaccine trials have been completed, one at WRAIR and one in Mali, evaluating FMP2.1, an AMA-1 recombinant protein vaccine based on the 3D7 allele, formulated with the GlaxoSmithKline (GSK) proprietary Adjuvant System, AS02A (Polhemus 2007, Thera 2008). Both studies demonstrated the vaccine to be well-tolerated and immunogenic. Recent preclinical data suggests another GSK Adjuvant System, AS01B, may be more potent than AS02A, which may translate into improved efficacy of vaccines adjuvanted with this System (Mettens 2008, Stewart 2006).

A phase 1/2a study was the first to compare the safety and immunogenicity of an AMA-1-based vaccine in both AS01B and AS02A adjuvant systems, and the first to assess the efficacy of such a vaccine in malaria-naïve adults using a homologous primary sporozoite challenge model (Spring 2009). In addition, ongoing phase 1b and 2b FMP2.1/AS02A pediatric vaccine studies in Mali, with a single adjuvant and single AMA-1 allele, will possibly provide valuable information regarding the mechanisms and cross-reactivity of the immune response to *P. falciparum* in an endemic pediatric population (*unpublished*).

Early studies suggested that some regions of AMA-1 were sufficiently homologous

between different strains of plasmodia to give cross-protection in animals and, perhaps, in people (Good 1997). However, with more recent studies on the genetic diversity of the gene encoding AMA-1 in natural populations of *P. falciparum* from Kenya, Thailand, India, and Venezuela it has been shown that it is in fact a considerably polymorphic protein (Escalante 2001). This has risen the concern and debate about whether AMA polymorphisms would elicit strain-specific immunity and perhaps less protection thus constituting a major challenge for vaccine development (Cortes 2003, Healer 2004 Cortes 2005a,b).
It has been suggested that it is possible to circumvent this issue by combining multiple AMA genotypes when designing these vaccines.

EBA-175 is a 175-kDa protein that is expressed in the micronemes of merozoites. Its potential as a malaria vaccine antigen is based on the fact that the majority of *P. falciparum* isolates use EBA-175 as a ligand for the invasion of erythrocytes (Camus 1985, Okoyeh 1999). EBA-175 binds to sialic acid-dependent epitopes on erythrocyte glycophorin A (Klotz 1992, Sim 1994) and it is thought to be involved in the formation of a junction between the erythrocyte and the apical portion of the merozoite just before invagination (Hadley 1986). This step is a key part of the erythrocyte invasion process and provides a logical target for vaccine-mediated immunity. EBA-175 is structurally divided into seven regions (Adams 1992), and the cysteine-rich region II, consisting of the F1 and F2 domains, functions as the erythrocyte-binding ligand domain (Narum 2000, Pandey 2002, Sim 1994). Antibodies to the F2 domain of EBA-175 can partially inhibit invasion of *P. falciparum* merozoites into human erythrocytes (Pandey 2003).

Genes of other merozoite surface proteins, e.g. MSP-3 and MSP-4, have been identified. MSP-3 is a target of cytophilic Abs that promote *P. falciparum* killing by co-operation with blood monocytes in antibody-dependent cellular inhibition assay (ADCI) (Oeuvray 1994). MSP-4 is a 40 kDa protein containing a single EGF-

like domain at the C-terminus, and anchored to the merozoite membrane by a GPI moiety (Marshall 1997).

PIESA. Malaria parasites spend a considerable part of their life cycle inside erythrocytes, in which they grow and multiply. The surface of parasite-infected erythrocytes becomes modified by the insertion of parasite molecules (parasite-induced erythrocyte surface antigens, PIESA), which dramatically alter the behaviour of infected cells (Craig 2001). Infected erythrocytes are highly immunogenic and can cytoadhere to other host cells. Such interactions appear to play a role in modulating the host immune response (Urban 1999) and in sequestering the parasites within tissues, potentially contributing to the particular virulence of *P. falciparum*.

Both the antigenic and functional (cytoadherent) properties of PIESA can be largely attributed to the family of diverse parasite molecules PfEMP-1. These molecules are inserted into the surface of infected erythrocytes by parasites during the asexual stage of growth. PfEMP-1 molecules are encoded by a multifamily of about 60 highly diverse *var* genes (Gardner 2002) that undergo rapid switching in vitro and are thought to be largely responsible for the phenomenon of clonal antigenic variation (Roberts 1992, Su 1995, Kyes 2001). In addition, they appear to be central to changes in cytoadherence properties that lead to the sequestration of infected erythrocytes in capillary beds, potentially a key step in the pathology of severe disease (Beeson 2001). The molecules are made up of combinations of different domains, each mediating a specific range of interactions with molecules on host endothelial cells (Baruch 1996, Smith 2000), platelets (Pain 2001), uninfected erythrocytes (Rowe 1997) and dendritic cells (Urban 1999).

9.2. Antibody responses against malaria

A protective role for anti- *P. falciparum* antibodies was identified and demonstrated by classical studies of passive transfer of immune IgG (Cohen 1961, McGregor & Carrington 1963) and reproduced later (Bouharoun-Tayoun 1990).

Transfer of pooled γ-globulin from West African immune adults into East African or Thai children acutely infected with *P. falciparum* caused a sharp drop in parasitaemia. However, in experimental animal models protective immunity to malaria does not correlate simply with antibody levels and can be even induced in their absence (Good 1997). It is generally considered that antibodies would target extracellular parasites and block their capacity to invade new cells, whereas cell-mediated responses would prevent the development of intracellular forms.

9.2.1. Antibodies against merozoite antigens

During the asexual blood phase, antibodies are thought to be the major effector mechanisms. In principle, many putative effector mechanisms involving antibodies can be envisaged. Firstly, these can cause direct damage to free parasites, either by themselves or by activating the complement system. Secondly, they can neutralise a function of the parasite e.g. block its attachment and/or subsequent invasion to a host cell. They can agglutinate the merozoite at rupture of mature schizonts, and block merozoite invasion of erythrocytes. Thirdly, antibodies can enhance phagocytosis by macrophages, which could be increased even more by the presence of complement. Finally, antibodies engulfed with the merozoite at invasion could inactivate the intraerythrocytic parasite, either directly or via antibody-dependent cell-mediated cytotoxicity (ADCC), as macrophages and neutrophils bearing Fc receptors may become killer cells in ADCC (Good 1997). An in vitro ADCC (or antibody-dependent cellular inhibition, ADCI, as defined by the authors) assay correlated with the efficacy of the pooled serum that markedly reduced parasitaemia *in vivo*. It was thought that if blood monocytes were required for biological function of the antibodies, then the isotypes of the antibodies might be a relevant variable (Bouharoun-Tayoun 1992). IgG1 and IgG3, both cytophilic isotypes, predominated in protected subjects, whereas subjects experiencing a primary attack of malaria had IgG2 or IgM isotypes (Oeuvray 1994). It was suggested that non-protective antibody isotypes during childhood may block the activity of protective isotypes, and thus explaining the age-related acquisition of immunity. Cytophilic classes co-operated with monocytes in vitro via Fc receptor

to produce TNF. Competition assays suggested that Ig from non-protected individuals could block the activity of those from immune individuals.

Although other points in the biological cycle of *Plasmodium* also offer attractive sites for prophylactic intervention, all clinical symptoms are provoked by the parasite's blood stage.

9.2.2. Antibodies against parasite-induced erythrocyte surface antigens

In many areas where malaria is endemic, the main load of malarial disease is experienced by children and adolescents, and a degree of immunity to severe, life-threatening malaria is apparent after only a few disease episodes (Gupta 1999) and is essentially complete before the age of five years. By contrast, the prevalence of asymptomatic parasitaemia in the community often continues to rise well after the age at which immunity to SM has developed (Smith 1999).

These epidemiological features, together with the fact that episodes of malaria can occur on a background of preexisting infection, have often been used as evidence that some important targets of immunity are polymorphic (Molineaux 1996). Thus, a component of immunity to malaria might develop through the piecemeal acquisition of a repertoire of antibodies to all the different parasite variants circulating in the human population (Lines 1992, Marsh 1992).

In addition, the immunological properties of PIESA appear to reflect some of the epidemiological features of NAI (Bull 2002). Naturally induced antibodies to PIESA are extremely diverse and this could contribute to the apparent strain-specific component of immunity. Nonetheless, the diversity of PIESA appears to be finite as immune sera from African adults can recognize parasite-infected erythrocytes from different continents (Aguiar 1992).

Although there is no formal typing system for studying the epidemiology of specific

surface antigen variants, isolates vary markedly in the frequency with which they are recognized by the antibodies carried by children from the same community. Frequently recognized isolates are associated with infections of children with SM, raising the possibility that restricted subsets of prevalent (perhaps functionally 'fit') PfEMP-1 variants are associated with SM (Bull 2000). If this is the case, a repertoire of protective antibodies against SM might accumulate relatively quickly, explaining the fact that immunity to SM develops more rapidly than immunity to UM (Nielsen 2002).

Other studies have demonstrated an association between *P. falciparum* infection and enhanced anti-erythrocyte surface antibody responses to a range of isolates (Iqbal 1993, Giha 1999a,b, Ofori 2002). In support of this, more recent data demonstrated that the proportion of isolates recognised was substancially higher amongst children with a microscopically detectable parasitaemia at the time of assay compared with those without, and that this association was not just due to cumulative exposure (Mackintosh 2008). Rather, it suggests that the presence of parasites reveals short-lived, more cross-reactive responses (Bull 2002, Kinyanjui 2004b). The presence of parasites at the time of serum collection not only leads to increased antibody recognition but also modifies the likelihood that this measured response will be associated with protection from both SM and UM (Bull 2002, Kinyanjui 2004b, Osier 2007). The precise target on the infected erythrocyte surface for these short-lived responses is currently unknown.

In a longitudinal study, Mackintosh and colleagues (2008) showed that failure to mount an antibody response to the surface of the erythrocyte infected with any isolate tested predicts subsequent susceptibility to malaria amongst asymptomatically parasitized children. They also observed a strong correlation in individual antibody responses to each parasite tested, suggesting a more conserved target on the infected erythrocyte surface for these responses.

The identification of specific host immune responses and molecular targets that explain these characteristics of immunity would clearly contribute greatly to both an understanding of the biology of the parasite–host interaction and to the development of vaccines. For this reason, there has been a long-standing interest in parasite proteins that are expressed either on the parasite surface or on the surface of the cells that they invade (Bolad 2000, Snounou 2000).

9.2.3. Antibodies that mediate inhibition of parasite growth

Functional antibodies that inhibit parasite invasion of erythrocytes (Egan 1999, Hodder 2001) or act in conjunction with monocytes to inhibit parasite growth (Bouharoun-Tayoun 1995) are thought to play a role in immunity.

The mechanisms by which the antibodies neutralize parasites in vitro differ greatly depending on the target antigen. Modalities include merozoite opsonization, targeting them toward phagocytic cells of the host (Groux 1990), prevention of invasion (Perkins 1981), inhibition of parasite development within the erythrocyte (Woehlbier 2006), and interference with merozoite dispersal by agglutination (Green 1981, Chulay 1981).

Most antibody assays used in studies until recently, were only able to detect and quantify antibody levels but did not detect or quantify their functional capacity. More recent methods have been developed for analysing functional antibodies against blood stage parasites in vitro and these include microscopic evaluation of blood smears, detection of DNA in erythrocytes or the measurement of enzymatic activity of the parasite-derived lactate dehydrogenase (pLDH).

Some methods are able to distinguish between erythrocyte invasion and parasite growth by assessing parasite viability, other methods asume these two mechanisms are representative of the same capacity of the parasite.

Other recent advances using transfection approaches and transgenic parasite lines have facilitated the development of assays to measure functional inhibitory

antibodies to specific antigens, such as MSP-1 (O'Donnell 2001, Saul 2001) and AMA-1 (Healer 2004), suitable for application in large population studies.

Several studies have demonstrated that antibodies against MSP-1_{19} and AMA-1 are major components of the invasion-inhibitory response in individuals immune to malaria. MSP-1_{19} is composed almost entirely of two Cys-rich EGF-like domains that form a tightly packed, disc-like structure (Chitarra 1999). The MSP-1_{19} EGF domains form reduction-sensitive epitopes that are recognized by invasion-inhibitory monoclonal and polyclonal antibodies (O'Donnell 2000, Blackman 1990, Chappel 1993, Chang 1992). MSP-1_{19}-specific inhibitory antibodies are also present in the sera of individuals naturally exposed to *P. falciparum* (Egan 1999). These antibodies recognize epitopes formed by the double EGF domain and by the second EGF domain alone (Egan 1999). The mechanism of inhibition by MSP-1_{19} antibodies is not fully understood, however, those that prevent the secondary processing of a precursor molecule and hence the formation of MSP-1_{19} also effectively inhibit merozoite invasion of RBCs (Blackman 1994).

O'Donnell and colleagues (2001) showed that MSP- 1_{19}-specific antibodies comprise a large component of the total invasion–inhibitory response of sera from many *P. falciparum*–immune adults from Papua New Guinea. These results are consistent with epidemiological evidence linking the presence of MSP-1_{19}-specific antibodies to protection from clinical malaria (Egan 1996, Conway 2000, Branch 1998). It has also been suggested that MSP-1_{19}-specific antibodies in human immune sera include a mix of inhibitory and noninhibitory antibodies as well as "blocking" antibodies that interfere with the inhibitory effect of MSP-1_{19} antibodies (Blackman 1994, Patino 1997, O'Donell 2001, Miura 2008).

Another well studied target of inhibitory activity is AMA-1 (Peterson 1989). AMA-1 appears to play a pivotal role in erythrocyte invasion (Triglia 2000), participating in the attachment and reorientation of the merozoite to the host red cell surface (Mitchell 2004). In this context, the AMA-1 region closest to the merozoite

membrane has been reported to bind to the Kx erythrocyte surface protein (Kato 2005). Its critical role in merozoite invasion has been supported by its presence in all plasmodial species examined as well as other apicomplexan parasites (Donahue 2000, Waters 1990) and by the failure to obtain parasites lacking the AMA-1 gene in knockout experiments (Triglia 2000).

It has also been suggested that some AMA-1-specific antibodies may interfere with this proteolytic cleavage, thereby inhibiting invasion (Dutta 2003).

9.3. Cytokine and chemokine responses against malaria

Cytokines and chemokines upon *P. falciparum* infection: a complex interplay between pathology and immunity

Cellular immunity mediates the production of cytokines and chemokines by activated lymphocytes, monocytes, macrophages, and, for some cytokines, also fibroblasts, neutrophils, endothelial cells, or mast cells. In malaria, both immunity and immunopathology are associated with T cell activation, but the precise mechanisms contributing to one or the other remain obscure.

Cytokines and pathology

Many field studies have described an association of specific cytokines with severity of malarial disease, in particular TNF (Kern 1989, Grau 1989, Kwiatkowski 1989, Kwiatkowski 1990, Shaffer 1991, Molyneux 1991, Othoro 1999, Brown 1999, Ramharter 2003, Körner 2010, Scuderi 1986), IFN-γ (Day 1999, Ramharter 2003), IL-1β (Brown 1999), IL-6 (Kern 1989, ME Molyneux 1991), IL-10 (Winkler 1998, Kurtzhals 1998, Day 1999, Othoro 1999, Ramharter 2003), IL-2 (Ramharter 2003), IL-12 (Luty 2000, Perkins, Weinberg, and Kremsner 2000), IL-4 (Kumaratilake and Ferrante 1992, Troye-Blomberg 1994, Eisenhut 2010), MIP-1β (Ochiel 2005), and TGF-β (Perkins, Kremsner, and Weinberg 2001). All of these studies have investigated individual or a few cytokines but it is more likely that a network of cytokine and chemokines determines protection or susceptibility from *P. falciparum* infections. Prakash and colleagues (2006) analysed the association between plasma levels of a range of cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-

10, IL-12, TNF, and TGF-β) in clinically well-defined groups of *P. falciparum*–infected patients with UM, SM, or CM by cluster and principal component analysis. They found that cytokines such as IL-1β, IL-12, and IFN-γ discriminated CM from SM, that high IL-1β levels were associated with CM, and that high IL-12 and IFN-γ levels were associated with non-cerebral SM (Prakash 2006).

Proinflammatory cytokines like TNF are thought to play an important role in malaria pathogenesis, particularly in CM, by increasing the surface expression of adhesion molecules on cerebral endothelial cells that enhance parasite attachment (Grau 1994, Beremdt 1989, Hommel 1990, Miller 1989). Excessive production of TNF in patients with CM seems to be a consequence of genetic variation in the host's propensity to produce this cytokine (McGuire 1994, McGuire 1999, Knight 1999, Ubalee 2001). Furthermore, increased plasma levels of TNF, together with increased production of IFN-γ, IL–1β, and IL-6 and reduced production of IL-4 and TGF-β, have been reported in patients with SM (Peyron 1994, De Kossodo 1993).

Evidence that GPI is involved in inducing the pro-inflammatory response has come from an assay measuring the release of TNF by macrophages *in vitro*. The isolation of subcellular components from the parasite coupled with this *in vitro* assay led to the identification of the GPI anchor from the parasite proteins MSP-1 and MSP-2 as an inducer of pro-inflammatory cytokines. Antibodies to the GPI anchor are associated with a lack of disease in adults (Naik 2000), but there is no proof that this is causally related.

The role of anti-inflammatory cytokines in malaria severity remains controversial, because different studies have reported high concentrations of IL-10 as being associated either with SM or with protection against SM in humans (Peyron 1994, Ho 1998, Kurtis 1999, Nussenblatt 2001). In addition, it has been shown that the balance between IL-10 and TNF concentrations determines the severity of anaemia in infected children (Othoro 1999). Along the same line of evidence, plasma levels

of TGF-β, a cytokine that acts as an anti-inflammatory cytokine at high concentrations, are inversely correlated with malaria severity in murine models as well as in humans. In Kenya, lower levels of TGF-β in serum and cerebrospinal fluid from children with CM were found to be associated with higher levels of TNF (Esamai 2003). Indeed, the outcome of *P. falciparum* infection may depend on a fine balance between appropriate and inappropriate induction of these immune regulatory factors.

Early life priming and innate responses

Interestingly, in malaria-endemic populations, CM is uncommon in very young children experiencing their first malaria infection, but is common in slightly older children undergoing second or subsequent malaria infections. These children have already acquired a degree of antimalarial immunity as demonstrated by high levels of circulating antimalarial antibodies (Erunkulu 1992). It has been hypothesized that priming of malaria-specific αβ T cells during early life leads to excessive IFN-γ production on reinfection (Artavanis-Tsakonas 2003), predisposing an individual to the over-production of TNF and subsequent onset of severe pathology (Riley 1999).

Whilst acquired immune responses eventually confer significant protection against malarial pathology, studies in mice undergoing a primary malaria infection have shown that the profile of cytokines, including IFN-γ, released in the first few hours of malaria infection predicts the course of infection and the final outcome (De Souza 1997, Choudhury 2000). Such rapid production of IFN-γ in naïve animals implies production either from pre-existing, cross-reactively primed, effector memory T cells or from cells of the innate immune system, e.g. phagocytic granulocytes, macrophages, NK cells or γδ T cells.

Cell-mediated immune effector mechanisms

There is less field data available on the relevance of individual cytokines in NAI to malaria, e.g. IL-12 (Sedegah, Finkelman, and Hoffman 1994, Dodoo 2002, Torre

2002), IFN-γ (Chizzolini 1990, Riley 1992, Harpaz 1992, Luty 1999, Torre 2002, Dodoo 2002), TNF (Harpaz 1992, Bouharoun-Tayoun 1995, Dodoo 2002), or IL-10 (John 2000).

IFN-γ and TNF have been involved as effector mechanisms conferring protection against *Plasmodium* infection (Taylor-Robinson 1993, Sedegah 1994, Winkler 1999). IFN-γ derived from NK, γδT or Th1 cells activates macrophages for enhanced phagocytosis and killing of parasitized erythrocytes (Fritsche 2001) and inhibition of parasite growth and development inside hepatocytes by CD8+ cytotoxic and IFN-γ-producing T cells (Tsuji 2003). In addition, nitric oxide (NO), produced by macrophages in response to parasitic components and T cell IFN-γ production, can have antiparasitic effects (Brunet 2001). TNF has also been associated with the development of resistance against malaria. Children having suffered from a malarial attack kept low plasma TNF levels compared to adults and asymptomatic children during a longitudinal survey in an endemic area (Peyron 1990). In another longitudinal study measuring plasma concentration of various cytokines in individuals continuously exposed to malaria, levels of TNF and IFN-γ were higher in parasitaemic than aparasitaemic individuals and donors who had clinical malaria had higher levels of TNF, IFN-γ and IL-6 than asymptomatic parasitaemic donors (Mshana 1991). There was a negative correlation between age of the individual and the concentration of plasma TNF and IFN-γ suggesting that the production of these cytokines could be modulated by repeated malarial infections. Asymptomatic parasitaemic children 5-7 years of age had higher levels of plasma TNF than clinically similar children below or above this age group, suggesting that refractoriness to the clinical effects of TNF may be an important factor in the ability of these children to resist clinical malaria.

Cellular assays in clinical studies

Cytokines have taken centre stage in many clinical and basic research studies because they play such a primary role in transmitting regulatory signals between various cell types. This intercellular communication system is comprised of multiple stimulatory and inhibitory cytokines, and its complex and dynamic

makeup largely determines the type and intensity of a particular immune or inflammatory response. Thus, to study the role of a particular cytokine, a multiparameter approach is often required since the role of each cytokine may be influenced by other cytokines that are present in the experimental system.

Although cytokines function on a microenvironmental level, human cytokines are most commonly assessed at the macro level, by measuring serum or plasma concentrations or levels in the supernatant of *in vitro* stimulated blood mononuclear cells. Reasons for this approach are largely practical. Serum and plasma levels can be readily assessed by using commercially available enzyme immunoassays, while cell-specific cytokine assessment has traditionally been laborious, involving *in situ* hybridization, cell separation followed by PCR measurement of mRNA, limiting dilution assays, plaque and enzyme-linked immunospot (ELIspot) assays, or T-cell cloning. In recent years, improved reagents have permitted flow cytometric cell specific cytokine assessments in which *ex vivo* peripheral blood cell stimulation or nonstimulation is followed by cell permeabilization, fixation, fluorescent staining, and cytokine detection (Jason 1997, Jung 1993). With multiparameter flow cytometry, very specific cell populations can be identified by surface antigen staining, without cell separation or cloning, and the production of multiple cytokines by individual cells can be assessed. In addition, the use of whole blood assays (WBA) instead of isolated peripheral blood mononuclear cells (PBMC) for measuring antigen-specific responses by intracellular cytokine staining (ICS) is an approach that provides an environment more similar to that existing *in vivo*, and it is faster and more economical (Struik 2004, Malhotra 2005, Walther 2006, Barbosa 2009).

Finally, with the development of luminex-based microsphere suspension array methods it is now possible to measure multiple cytokines and chemokines free in small volumes of plasma (Jason 2001, Metenou 2007).

Gaps in knowledge

Most studies of cytokine responses have been cross-sectionals done after the onset of symptoms and at the initial stages of clinical illness (Kremsner 1990, Deloron, Lepers, and Coulanges 1989, Nguyen-Dinh and Greenberg 1988, Rhodes-Feuillette 1985). Only a few studies have assessed longitudinally the evolution of cellular responses to infection, and those have been mostly in adult populations (Peyron 1990, Riley 1993, Dodoo 2002). Furthermore, while a number of studies have evaluated newborn cytokines responses in cord blood (Fievet 1996, King 2002, Bouyou-Akotet 2004, Malhotra 2005, Brustoski 2005a, Brustoski 2005b, Brustoski 2006, Breitling 2006, Metenou 2007), very few have measured prospectively cellular responses to *P. falciparum* in infants (Hesran 2006) or young children (Peyron 1990, Mshana 1991, Dodoo 2002).

Hesran and collegues (2006) conducted a cohort study to identify naturally acquired immune responses to *P. falciparum*. Cellular responses of Cameroonian neonates from birth to 36 months of age were evaluated every 6 months by cell proliferation and cytokines (IFN-γ, IL-2 and IL-4) production after *in vitro* culture in the presence of schizont extract and Pf155/RESA peptides. Their results suggested that *P. falciparum* specific immune responses are first oriented towards a Th2-type of response, and later switch to Th1-type of response.

As pointed out by Riley and Greenwood (1990) measurements of in vitro cellular immune responses to malaria antigens are influenced by a variety of external factors. For example, malaria-related immunosuppression may be both generalised and antigen specific. Although in vitro responses to malaria antigens are suppressed in acutely infected individuals, there is evidence that lymphocyte activation does occur in vivo.

There is still a major gap in the knowledge on how immune effector mechanisms determine protection or susceptibility to malaria. More longitudinal studies, especially

in children and infants, correlating specific immune responses with subsequent malaria morbidity, and using modern approaches, are required to identify potentially protective antigens and appropriate effector mechanisms.

10. Impact of control measures on the development of acquired immunity

As pointed out by Schofield and Mueller (2006), current thinking concerning rebound vs. sustained protection of IPTi hinges on concepts of an immunological nature, yet very few studies have evaluated the impact of IPTi or other malaria control interventions on the development of immune responses.

Insecticide-treated bed nets

ITNs reduce contact between the mosquito vector and the human host and have been documented to reduce malaria morbidity and mortality (Lengeler 2004). When used widely in a community, ITNs reduce the population of sporozoite-positive mosquitoes (Curtis 1998), which is reflected in a reduction in the plasma antibody levels to the circumsporozoite protein among bed net users (Marsh 1986). Even though the short- and medium-term beneficial effect of ITN are well documented, concerns have been raised about the long-term effect, since the reduction in infectious inoculations might affect both the development and the maintenance of malaria immunity (Snow and Marsh 1995).

Several studies have reported that there was no evidence for a difference in all-cause mortality during follow-up of young children being protected compared to those not being protected with ITNs (Binka 2002, Diallo 2004, Lindblade 2004, Muller 2006). These trials were not, however, specifically designed to address the issue of possible delayed or rebound mortality.

Very few studies have assessed the impact of ITNs on immune responses. A study

has reported that children using ITNs had lower VSA antibody levels and recognized a smaller proportion of VSA types (Askjaer 2001). Another study in Kenya found significantly higher prevalence of IgG to MSP-1_{19} in children from ITNs areas, concluding that in areas of stable malaria transmission, interventions that reduce the number of asexual parasitemic episodes do not delay the development of antibody responses to blood-stage malarial antigens (Kariuki 2003).

Finally, permethrin-treated bednets showed a significant impact on percent lean body mass in Kenyan school children, thought to be due to decreased production of pro-inflammatory cytokines TNF, IL-1 and IL-6 in this group (Friedman 2003).

Insecticide-treated curtains (ITC)

In a study that measured efficacy of ITC (Modiano 1998), it was suggested that the impact of the intervention on infection rates was positively correlated with the levels of anti-malaria immunity. Authors concluded that since decreased transmission entails a reduction of immunity, the efficacy of the intervention in the long term cannot be taken for granted.

Another study assessing growth inhibition capacity of antibodies showed that the use of ITC in children did not translate into a reduction of GIA activity (Bolad 2004). However it has been described that malaria transmission intensity does influence the level of growth-inhibitory antibodies (McCallum 2008) so there is a need to investigate this further in more studies of interventions that reduce transmission intensity.

Continuous chemoprophylaxis

Early studies of continuous malaria chemoprophylaxis raised concerns regarding the loss of or delay in the acquisition of protective immunity (Geerligs 2003, Laing 1984, Pringle 1966). Weekly chemoprophylaxis between 2 and 12 months of age in infants in Tanzania significantly reduced the incidence of malaria and anaemia during the first year of life, but the risk increased in the second year after stopping

the intervention (Menendez 1997), suggesting that protection against *P. falciparum* infection during infancy had delayed the development of immunity to malaria. However, continuous chemoprophylaxis for 3 years in Gambian children resulted in higher lymphoproliferative responses and IFN-γ production (Otoo 1989), and there was no clinical rebound of malaria one year after termination of prophylaxis (despite a decrease in anti-malarial antibody levels).

IPTp and malaria in pregnancy

Although concerning a different target group, this is the most similar intervention to the one assessed in this thesis (IPTi). A comprehensive study was carried out to assess the effect of IPTp on malarial antibodies and delivery outcomes (Serra-Casas 2010). The results showed that a reduction in maternal and cord humoral immune responses to malaria was observed after administration of 2 IPTp-SP doses to HIV-positive pregnant women, but not to HIV-negative mothers, probably reflecting the combined effect of HIV immunosuppression and the reduction in exposure to malaria antigens resulting from the intervention. This reduction in *P. falciparum*-specific antibodies (both against pregnancy-specific antigens [(VSAs from a CSA-binding line] and for non–pregnancy-associated antigens [AMA-1 and VSAs from a paediatric isolate]) did not translate into an enhanced risk of malaria-associated morbidity in mothers and infants.

Another study reported that pregnant women receiving IPTp had reduced levels of anti-VSA IgG (Staalsoe 2004a) that have been associated with reduced prevalence of low birth weight and maternal anaemia (Staalsoe 2004b). However, it remains to be established whether other immunologic mechanisms, such as cellular immunity, could be affected by IPTp regimens with SP or with alternative antimalarial drugs currently under evaluation.

Reduced exposure by subtherapeutic drugs

Low exposure related to subtherapeutic drug concentrations of antimalarials due to moderate resistance has also shown to have important effects on immune

responses (Mayxay 2001). Higher titers of IgG against MSP-1_{19} enhanced the likelihood of parasitological clearance in individuals treated with suboptimal drug regimens, and it was suggested that recovery from uncomplicated malaria in patients carrying drug-resistant *P. falciparum* is a measure (phenotypic marker) of acquired functional immunity (Djimdé 2003, Pinder 2006).

The importance of low doses of parasite antigen in the induction of protective immunity is supported by studies in animal models and humans. Subpatent infections with blood stage *P. chabaudi* or *P. yoelii* rodent malarias stimulate good level of immunity, which differ from that induced by patent infection (Taylor-Robinson 1998, Favila-Castillo 1999, Belnoue 2004, Elliott 2005). In humans, subpatent infections with very low inoculum of *P. falciparum* induce protective immunity (Pombo 2002). A study in Kenya also showed that children with low intensity of malaria exposure during the first 2 years of life had higher subsequent levels of IgG to MSP-1_{19} (Singer 2003).

Regarding **IPTi-SP,** no previous studies had assessed the potential impairment that this malaria control tool could exert on the acquisition of malaria immunity. Therefore, this thesis set out to assess the impact of IPTi-SP on the development of immune responses to the *P. falciparum* parasite in young children in Manhiça, a malaria endemic area of Mozambique. To address this, we measured multiple antibody and cellular immune mediators considered to be among the most promising markers of protective immunity to malaria identified to date.

II. JUSTIFICATION AND OBJECTIVES

One of the main concerns regarding the use of malaria control interventions such as continuous chemoprophylaxis has been the potential impairment of naturally acquired immunity. An important objective of the IPTi Consortium was to assess the possibility of a rebound increase in clinical malaria after IPTi due to an interference with the development of anti-malarial immunity.

Primary aim of this PhD thesis

To investigate whether IPTi with SP administered within the EPI scheme (3, 4 and 9 months) interferes with the development of immune responses to falciparum malaria at ages 5, 9, 12 and 24 months in Manhiça, Mozambique.

Secondary aims

To describe the natural history of immune responses to *P. falciparum* in children up to 2 years of age living in a malaria endemic area of sub-Saharan Africa.

- To determine what factors affect the magnitude of antibody and cytokine immune responses
- To determine whether the magnitude of immune responses is associated with subsequent incidence of clinical malaria.

The HYPOTHESIS of this research work is that IPTi does not interfere with the acquisition of *P. falciparum*-specific humoral or cellular immunity.

In order to assess the development of NAI in young children, we measured those immunological parameters that have been shown to be the most appropriate markers of protective immunity against malaria identified to date. Given the complexity of the parasite and the multitude of immune responses which may be involved, it is unlikely that a single immune response directed against a single antigen expressed during one stage of the parasite's life cycle is responsible for protection.

III. STUDY AREA, POPULATION, AND ETHICAL CONSIDERATIONS

Study area and population

The randomised double-blind placebo-controlled IPTi trial was conducted at the Centro de Investigação em Saúde da Manhiça (CISM), Manhiça District (Maputo Province), in southern Mozambique. The characteristics of the area have been described in detail elsewhere (*INDEPTH Network. 2002*). The most recent data on the efficacy of SP in children in this area showed a combined (early and late) therapeutic efficacy rate of 83%, with an in vivo parasitological sensitivity of 83.6% at day 14 (Macete 2006).

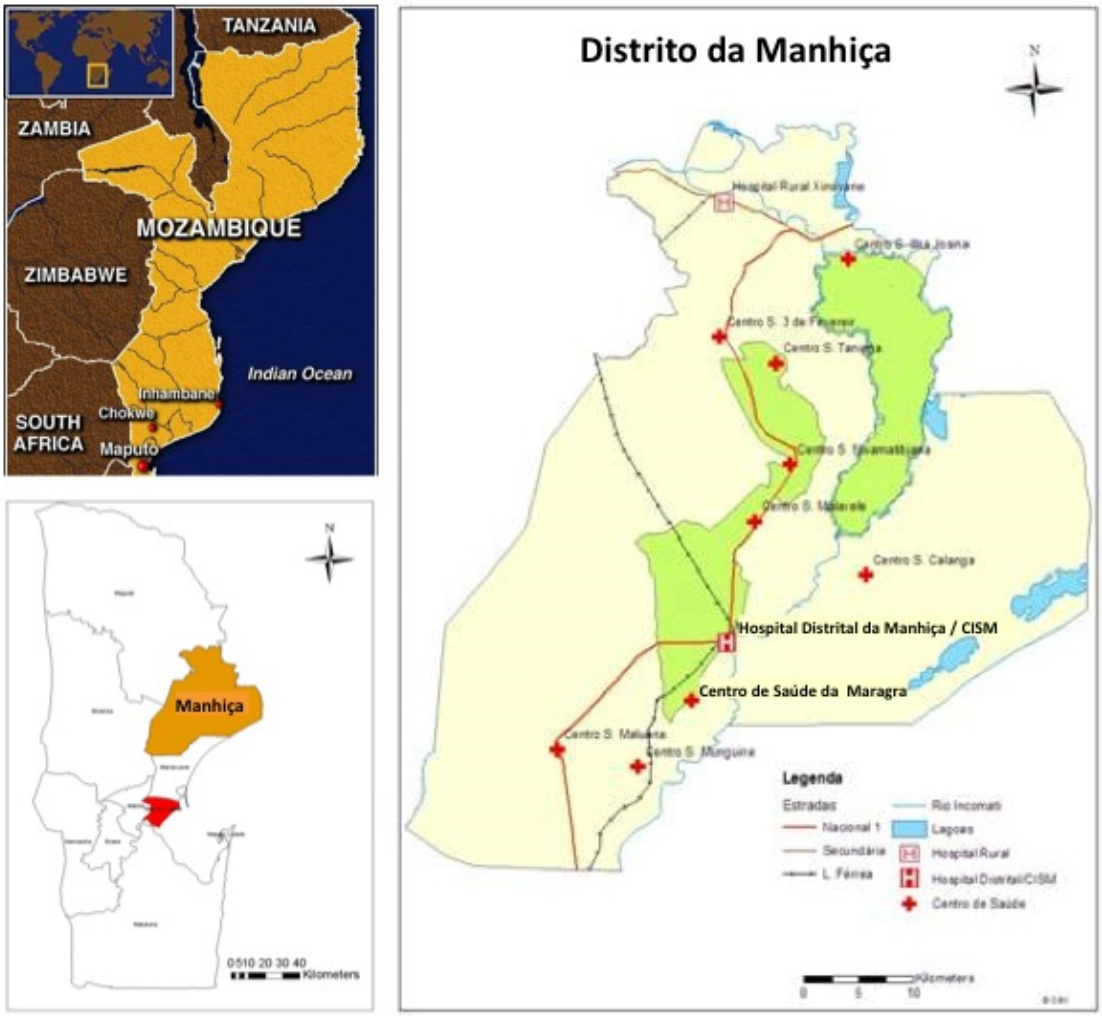

FIGURE 9. Study area: Manhiça District

The recruitment of patients and collection of samples for the safety and efficacy IPTi trial was done at the EPI clinics of the Manhiça Health Centre and the Maragra Health Post immediately after they received dose 2 of diphtheria-tetanus toxoid–pertussis (DTP)/oral polio (OPV) vaccine between September 2002 and February 2004. Treatment with Sulfadoxine-Pyrimethamine (SP) or placebo was administered at 3, 4, 9 months of age alongside routine EPI vaccinations. Further details have been described elsewhere (Macete 2006).

Patients and samples

For the immunological study, 501 children were recruited at age 5 months, as we expected that a significant number of children would not attend the subsequent cross-sectional hospital visits during the follow up. All participants were recruited at the end of the main IPTi clinical trial between November 2003 and May 2004.

The study involved four bleedings at 5, 9, 12 and 24 months of age. At each cross sectional visit, 1 ml of blood was collected from each child by fingerprick into EDTA microtainers, to obtain plasma and erythrocyte pellet, and 0.5 ml of blood into heparin microtainers for whole blood cellular stimulation assays.

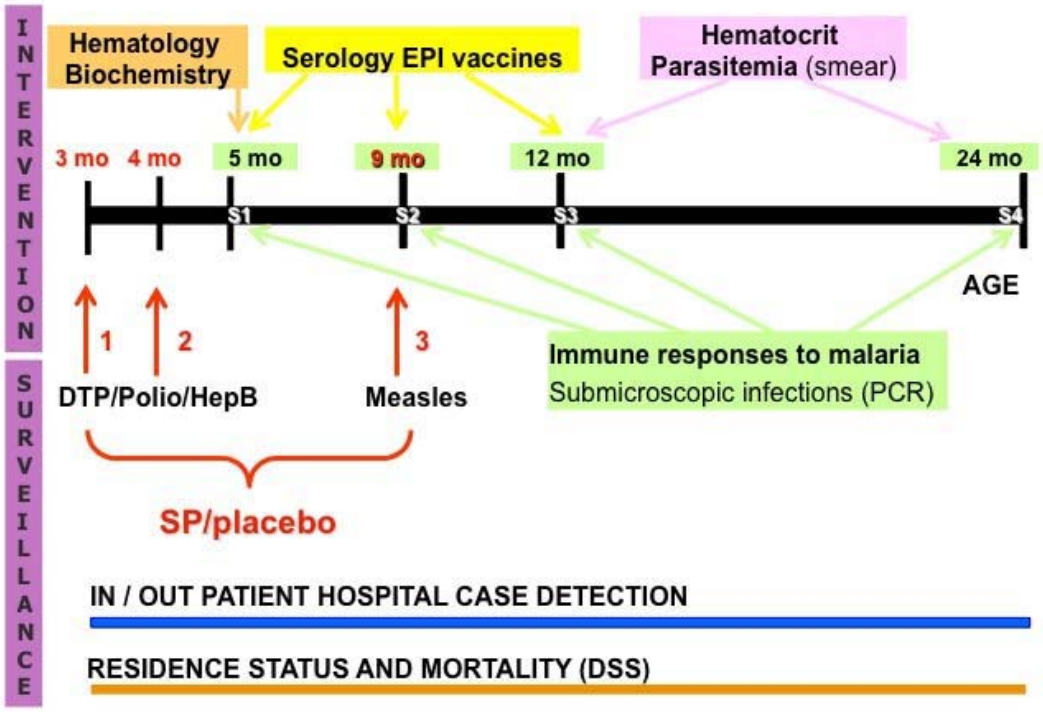

FIGURE 10. Study design diagram

Antibody and cytokine responses were analysed in a subgroup of 306 children who met the following criteria: (i) full SP or placebo treatment had been administered, (ii) blood samples were collected preferably at all four cross sectionals, (iii) there was enough volume of sample available and (iv) there was an equal distribution of SP- and placebo-recipients.

Immune markers measured

- **IgG** responses to whole *P. falciparum* blood stage parasites, measured by indirect immunofluorescence antibody test (**IFAT)**
- **IgM, IgG, IgG1, IgG2, IgG3, IgG4** to asexual blood stage proteins of merozoites involved in invasion of erythrocytes measured by **ELISA**

  MSP-1_{19}, C-terminal fragment 19 kD, 3D7 strain

  AMA-1, ectodomain, 3D7 strain

  EBA-175, region II, fragment II, CAMP strain
- **IgG** to variant antigens on the surface of infected erythrocytes (**VSA**) by **FACS**
- Growth inhibitory antibodies (**GIA**), measured by **FACS**
- Antigen-specific T cell cytokines in blood, measured by intracellular cytokine staining (**ICS-FACS**)
- **Cytokines** in plasma by **luminex**: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ, TNF, MIP-1β, MCP-1, RANTES, GM-CSF, G-CSF

In addition, submicroscopic *P. falciparum* infections were detected by nested **PCR**

Ethical considerations

Written informed consent was obtained from all parents or guardians and ethical approval for the protocol was obtained from the ethics review committees of the Ministry of Health of Mozambique (Comitê Nacional de Bioética em Saúde - CNBS) and the Hospital Clinic, Barcelona, Spain (Comité Etico de Investigación Clinica - CEIC).

IV. PUBLISHED ARTICLES

ARTICLE 1

Impact of Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine on Antibody Responses to Erythrocytic-Stage *Plasmodium falciparum* Antigens in Infants in Mozambique

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We evaluated the impact of intermittent preventive treatment in infants (IPTi) with sulfadoxine-pyrimethamine (SP), which was given at ages 3, 4, and 9 months through the Expanded Program on Immunization (EPI), on the development of antibody responses to *Plasmodium falciparum* in Mozambique. Immunoglobulin M (IgM) and IgG subclass antibodies specific to whole asexual parasites and to recombinant MSP-1_{19}, AMA-1, and EBA-175 were measured at ages 5, 9, 12, and 24 months for 302 children by immunofluorescence antibody tests and by enzyme-linked immunosorbent assays. Antibody responses did not significantly differ between children receiving IPTi with SP and those receiving a placebo at any time point measured, with the exception of the responses of IgG and IgG1 to AMA-1 and/or MSP-1_{19}, which were significantly higher in the SP-treated group than in the placebo group at ages 5, 9, and/or 24 months. IPTi with SP given through the EPI reduces the frequency of malarial illness while allowing the development of naturally acquired antibody responses to *P. falciparum* antigens.

Malaria remains one of the major infectious diseases globally, causing up to 3 million deaths and close to 5 billion episodes of clinical illness per year (7). In areas characterized by hyperendemic transmission, the greatest burden of malaria occurs in children less than 12 months of age (38); consequently, infants in sub-Saharan Africa are the main target population for any malaria control tool.

Intermittent preventive treatment in infants (IPTi) that consists of the administration of a full dose of an antimalarial within the Expanded Program on Immunization (EPI) has proven to reduce the risk of malaria in this vulnerable group (37). This strategy has gained increasing interest, and several intervention trials evaluating the efficacy of IPTi in the reduction of malaria morbidity have been and are still being carried out in several sub-Saharan countries (Tanzania, Ghana, Senegal, Mozambique, Gabon, and Kenya) as part of an international consortium (www.ipti-malaria.org). However, before setting any policy recommendation for the large-scale implementation of IPTi for malaria control, it is necessary to fully evaluate the consequences that this early preventive intervention may have later in life.

An important issue that needs to be considered is the impact that IPTi may have on the development of naturally acquired immunity to malaria. Early studies of continuous malaria chemoprophylaxis raised concerns regarding the loss of or delay in the acquisition of protective immunity (16, 23, 34). Weekly chemoprophylaxis between 2 and 11 months of age in infants in Tanzania significantly reduced the incidence of malaria and anemia during the first year of life, but the risk increased in the second year after stopping the intervention (26), suggesting that protection against *Plasmodium falciparum* infection during infancy had delayed the development of immunity to malaria. However, subsequent studies of IPTi in Tanzania (37) and Mozambique (24) showed that, as opposed to continuous chemoprophylaxis, intermittent prevention reduced the risk of malaria without being followed by a clinical rebound once the intervention was stopped. Furthermore, IPTi resulted in a sustained protective effect during the second year of life after the cessation of treatment (36), suggesting that the intervention had unanticipated beneficial effects in the acquisition of immunity.

Nevertheless, studies completed so far have been limited to the evaluation of the safety and efficacy of IPTi without the parallel assessment of immune responses to *P. falciparum*. Therefore, in the context of a randomized, placebo-controlled trial of IPTi in Mozambique that resulted in a 22.2% (95% confidence interval [CI], 3.7 to 37.0%; $P = 0.020$) reduction in the incidence of clinical malaria in the first year of life (24), we evaluated whether IPTi with sulfadoxine-pyrimethamine (SP), administered alongside the EPI system, could affect the qualitative and/or quantitative immune responses to malaria antigens. As surrogates of protective immunity, we measured the type and quality of antibodies to the blood-stage antigens mero-

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zoite surface protein (MSP-1) (27), apical membrane antigen (AMA-1) (32), and erythrocyte binding antigen (EBA-175) (8, 40). These antigens play critical roles in erythrocyte invasion and are leading vaccine candidates (9). Antibodies raised against these parasite proteins inhibit the invasion of erythrocytes in vitro (11, 27, 30). Immunoglobulin G (IgG) antibodies are important in protection against blood-stage malaria infection, as demonstrated by the classical Ig passive-transfer studies (5, 10), and their protective effect has been attributed to the cytophilic (IgG1 and IgG3) rather than the noncytophilic (IgG2 and IgG4) subclasses (28). We hypothesized that the levels of *P. falciparum*-specific antibodies during the first 2 years of life would not significantly differ between children who received IPTi with SP and those who received a placebo.

MATERIALS AND METHODS

Study area and design. The study was conducted at the Centro de Investigação em Saúde da Manhiça, Manhiça District, southern Mozambique. The characteristics of the area have been described in detail elsewhere (21). The most recent data on the efficacy of SP in children in this area showed a combined (early and late) therapeutic efficacy rate of 83%, with an in vivo parasitological sensitivity of 78.6% at day 14 (1). The efficacy study was an individually randomized, placebo-controlled trial (24). Infants were recruited from those attending the EPI clinic to receive dose 2 of the diphtheria/oral polio/hepatitis B vaccine between September 2002 and February 2004. Treatment with SP or a placebo was administered at 3, 4, and 9 months of age alongside the routine vaccinations. Cross-sectional visits were scheduled at 5, 9, 12, and 24 months of age. For the immunological studies of IPTi, we included the last 501 children recruited in the main trial. Among those, 302 were selected for the analysis of antibody responses, because they fulfilled the following criteria: (i) having received all three doses of SP or placebo, (ii) having plasma available to conduct all the serological determinations, and (iii) having an equal representation of SP and placebo recipients. Those children who came for all four visits were prioritized. At each cross-sectional visit, 1 ml of blood was collected by finger prick into EDTA microtainers to obtain plasma and an erythrocyte pellet. All immunological assays were performed by personnel in a blind manner. Clinical surveillance for malaria morbidity was done through passive case detection. Ethical approval for the protocol was obtained from the ethics review committees of Mozambique and the Hospital Clinic, Barcelona, Spain. The trial registration number is NCT00209795 (http://clinicaltrials.gov).

IFAT. In vitro cultures of *P. falciparum* (strain 3D7) were used at 3.5% parasitemia and 3 to 5% hematocrit to prepare immunofluorescence antibody test (IFAT) slides. Infected erythrocytes were resuspended in phosphate-buffered saline (PBS), and droplets of 25-μl cell suspensions were loaded onto 12-well microscopy slides (Cell-Line Associates, Newfield, NJ), dried, and fixed with 100% acetone. Twenty-five microliters of test plasma (twofold serial dilutions from 1/20 to 1/163,840) was placed in each well and incubated with the parasites for 30 min. Positive and negative control plasma pools were used in each slide. After the samples were washed, 15 μl of fluorescein isothiocyanate-labeled anti-human IgG antibody (1:120) in Evans Blue solution (0.01% [wt/vol] in PBS) (Sigma, St. Louis, MO) was loaded in each well for 30 min. To stain parasite DNA, slides were incubated with a drop of 4′,6′-diamidino-2-phenylindole–PBS (300 nM) for 1 to 5 min and rinsed with PBS before being mounted with glycerol and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy. The highest dilution giving positive green fluorescence was scored. Data are presented as endpoint IgG titers, i.e., the reciprocal of the last plasma dilution causing positive fluorescence above-the negative-control levels.

ELISA. For enzyme-linked immunosorbent assays (ELISA), all samples were assayed for IgG and IgM to the recombinant proteins MSP-1_{19} (19-kDa C-terminal fragment, 3D7 strain), AMA-1 (3D7), and EBA-175 (region II, fragment II, CAMP strain) from the ICGEB (New Delhi, India). High-binding 96-well microplates (Nunc Maxisorp, Denmark) were coated with 200 ng per well of antigen diluted in 0.05 M carbonate-bicarbonate buffer and incubated overnight at 4°C. Plates were washed with 0.05% Tween 20 in PBS (PBS-Tween), blocked with 2% bovine serum albumin in PBS-Tween for 8 h at 4°C, and washed with PBS-Tween, and then plasma samples (1:200) were added in duplicate along with positive (a pool from eight adults with lifelong exposure to malaria)- and negative (nine nonexposed adults)-control plasma samples. Plates were incubated overnight at 4°C and washed, and peroxidase-conjugated goat anti-human IgG or IgM secondary antibodies (Sigma, St. Louis, MO) were added at 1:30,000 and 1:2,000 dilutions, respectively. After 1 h of incubation and washing, 100 μl of a phosphate solution with 0.012% H_2O_2 substrate and *o*-phenylendiamine chromogen was added per well for 5 min, and the colorimetric reaction was stopped with 25 μl of 3 M H_2SO_4. The specific reactivities of plasma samples were obtained as optical density (OD) values (absorbance measured at 492 nm using a Multiskan EX; Labsystems, Finland), normalized against a positive control (1:200) run in the same experiment, and used as continuous variables (in arbitrary units and percentages) or converted to a categorical variable (positive versus negative) using a cutoff OD value (the arithmetic mean of negative controls plus three standard deviations) for statistical analyses.

IgG isotypes were analyzed in the samples in which a positive IgG response was detected for the corresponding antigen. Identical sets of antigen-coated plates were prepared for the determination of IgG, IgG1, IgG2, IgG3, or IgG4 in assays performed with any one plasma sample (1:200, duplicates) in parallel on the same day. Wells were incubated for 3 h with peroxidase-conjugated sheep anti-human IgG1 (1:6,000), IgG2 (1:3,000), IgG3 (1:6,500), or IgG4 (1:5,000) (Binding Site, Birmingham, United Kingdom) or peroxidase-conjugated rabbit anti-human IgG specific for gamma chains (1:6,000; DAKO, Glostrup, Denmark). In parallel, purified human myeloma proteins IgG1, IgG2, IgG3, and IgG4 (Binding Site) were coated on plates at twofold dilutions from 2 to 0.001 μg/ml. Peroxidase-conjugated antibodies to each IgG subclass (Binding Site), at the same dilutions as those used for plasma samples, were reacted with the myeloma proteins and used as positive controls. All plates were developed and read as described above. IgG isotype data was reported as the OD at 492 nm (OD_{492}).

Malaria parasitemia. *P. falciparum* infections were detected by microscopy at 12 and 24 months of age. Thick and thin blood films were stained and read according to quality control procedures (2). Submicroscopic infections were assessed by PCR from erythrocyte pellets collected at all visits (5, 9, 12, and 24 months). Parasite DNA was extracted using the QIAamp 96 DNA blood kit (Qiagen, Venlo, The Netherlands). The amplification of genus- and species-specific *P. falciparum* DNA was done as described previously (42) in an MJ Research DYAD 96-well thermocycler, and PCR products were visualized on a 1.5% agarose gel in 1× Tris-borate-EDTA buffer.

Definitions and statistical methods. Malaria infection was defined as the presence of asexual *P. falciparum* parasites of any density in a blood smear. A clinical malaria episode was defined as the latter plus an axillary temperature of ≥37.5°C. Children were not considered to be at risk for 28 days after the start of each episode of clinical malaria.

Antibody values (IFAT IgG endpoint titers, ELISA-normalized OD values [given as percentages] for total IgM and IgG, and raw OD values for IgG isotypes) were logarithmically transformed, and averages within groups are presented as geometric means (GM) plus 95% CIs. Differences in antibody levels between children receiving SP and the placebo were estimated with the *t* test, and differences in the frequencies of positive responses were estimated with Fisher′s exact test. The distributions of antibody responses to each antigen at each time point in SP and placebo recipients were presented as (i) weighted scattered plots, with significance tests performed using linear regression models and adjusted for previous malaria episodes and present infections, and (ii) reverse cumulative distribution plots (35), with significance tests performed using the Kruskal-Wallis test corrected for continuity.

To correct for the multiple comparisons performed, we used the Monte Carlo permutations test (17), applying 1,000 random permutations; the cases in which Monte Carlo correction altered the significance of *P* values are indicated in the text.

Multivariate regression models using a stepwise procedure were estimated to identify variables independently associated with antibody measures. In a first model that included all children, the variables were intervention group (SP or placebo), age at visit, and the occurrence of previous clinical episodes (yes/no). In a second model, including only children with at least one clinical malaria episode, the variables were intervention group, age at visit, number of previous malaria episodes, age at first episode, parasite density at first and last episode, maximum parasite density before visit, time from first and last episode, and time from the episode of maximum density.

In all of these analyses, intraindividual and interindividual variabilities were taken into account and adjusted for. Data analysis was performed using Stata 9.2 (Stata Corporation, College Station, TX). Statistical significance was defined as a P of <0.05.

TABLE 1. IgG responses to erythrocytic-stage antigens after IPTi with SP

Age[a]	Antigen	SP group				Placebo group				*P* value[d]	*P* value[e]
		GM[b]	95% CI	*n*[c]	% Pos[c]	GM	95% CI	*n*	% Pos		
5[f]	MSP-1_{19}	11.19	9.70–12.90	30	20	12.13	10.18–14.45	43	30	0.479	0.079
	AMA-1	32.49	27.89–37.86	93	63	26.12	22.32–30.55	86	61	0.050	0.809
	EBA-175	19.07	17.16–21.19	35	24	17.57	15.53–19.89	36	25	0.321	0.892
9[g]	MSP-1_{19}	13.74	11.45–16.50	43	29	12.41	10.38–14.83	45	31	0.429	0.703
	AMA-1	18.87	16.34–21.80	62	42	17.52	15.07–20.35	54	38	0.480	0.473
	EBA-175	17.05	15.29–19.01	26	18	17.21	15.27–19.40	31	22	0.907	0.460
12[h]	MSP-1_{19}	12.26	10.44–14.40	35	24	12.66	10.65–15.04	42	29	0.789	0.355
	AMA-1	15.32	13.36–17.56	43	29	15.42	13.29–17.88	51	35	0.950	0.318
	EBA-175	17.61	15.88–19.52	29	20	17.42	15.54–19.53	29	20	0.891	1.000
24[i]	MSP-1_{19}	16.93	14.04–20.41	25	21	13.84	11.71–16.36	27	24	0.114	0.753
	AMA-1	21.39	18.76–24.39	27	23	23.90	20.88–27.35	37	32	0.244	0.109
	EBA-175	18.33	16.48–20.38	20	17	18.67	16.94–20.58	20	18	0.798	1.000

[a] Age in months.
[b] GM of ELISA antibody levels, expressed as arbitrary units of OD normalized against results for the positive control (in percentages).
[c] Number (*n*) and frequency (Pos) of positive responders. Average GM values for negative controls were the following: AMA-1, 15.96%; MSP-1_{19}, 15.17%; and EBA-175, 21.75%.
[d] Determined by *t* test for the comparison of the magnitude of antibody responses.
[e] Determined by Fisher's exact test for the comparison of the frequency of positive antibody responses.
[f] Number of samples at 5 months: SP ($n = 148$) and placebo ($n = 145$).
[g] Number of samples at 9 months: SP ($n = 147$) and placebo ($n = 143$).
[h] Number of samples at 12 months: SP ($n = 146$) and placebo ($n = 145$).
[i] Number of samples at 24 months: SP ($n = 118$) and placebo ($n = 114$).

RESULTS

Crude analysis of antibody responses to *P. falciparum* blood-stage antigens in relation to IPTi with SP. We found no significant differences in IgG IFAT titers (given in parentheses as GM [95% CI]) between the SP and the placebo groups at any of the following time points: 5 months, SP (11,192 [8,946 to 14,000]) and placebo (11,880 [9,608 to 14,689]), $P = 0.702$; 9 months, SP (10,364 [8,101 to 13,259]) and placebo (10,240 [8,153 to 12,861]), $P = 0.943$; 12 months, SP (9,132 [6,922 to 12,048]) and placebo (10,053 [7,817 to 12,930]), $P = 0.612$; and 24 months, SP (445 [286 to 692]) and placebo (736 [450 to 1,204]), $P = 0.131$.

Similarly, crude IgG responses to *P. falciparum* merozoite antigens MSP-1_{19}, AMA-1, and EBA-175, measured by ELISA, did not significantly differ overall between children receiving IPTi with SP or those receiving a placebo at any of the cross-sectional visits, when analyzed either as continuous or as discontinuous variables (Table 1). There was one exception: IgG responses to AMA-1 at age 5 months (after two IPTi doses at 3 and 4 months), which were significantly higher in children who received SP than those who received the placebo, as determined by reverse distribution cumulative plots ($P = 0.032$; data not shown) and by *t* test of the GM ($P = 0.050$ in Table 1; the corrected *P* value by Monte Carlo permutations test was 0.056). Correction for multiple tests slightly altered the significance of only five comparisons, in which *P* values already were borderline significant; these are indicated in the text. For all other cases, permutations did not significantly change the *P* values and, thus, the correction is not reported. IgM responses to the same antigens did not significantly differ between the two treatment groups (data not shown).

Likewise, IgG subclass responses to MSP-1_{19}, AMA-1, or EBA-175 did not significantly differ between SP and placebo recipients for most of the cross-sectional visits when analyzed either as continuous (Table 2) or as categorical (data not shown) variables. There were, however, three exceptions in which the levels of cytophilic IgG1 antibodies were significantly higher in the SP than in the placebo group, as determined by reverse distribution cumulative plots (data not shown): at 5 months the *P* value for AMA-1 was 0.035 and the *P* value for EBA-175 was 0.050 (corrected $P = 0.060$), and at 9 months the *P* value for MSP-1_{19} was 0.033.

Factors affecting IgG antibody responses after IPTi with SP-adjusted analysis. The occurrence of previous clinical malaria episodes was strongly associated with IgG levels. The number of children who had previous clinical episodes was 18 at 5 months (3 SP, 15 placebo), 54 at 9 months (25 SP, 29 placebo), 60 at 12 months (26 SP, 34 placebo), and 82 at 24 months (34 SP, 48 placebo). Children with a previous clinical malaria episode had, on average, 2.01 times higher IgG responses to MSP-1_{19} than children without a previous episode (CI, 1.72 to 2.34; $P < 0.001$). Comparable associations were found for AMA-1 and EBA-175. Similarly, children with current parasitemia (36 children at 5 months, 62 at 9 months, 37 at 12 months, and 78 at 24 months), detected by microscopy and PCR, had 1.34 times higher IgG responses to MSP-1_{19} than did children without current parasitemia (CI, 1.17 to 1.54; $P < 0.001$). Present infections also were significantly associated with IgM levels ($P = 0.001$ for MSP-1_{19}, $P < 0.001$ for AMA-1, and $P = 0.055$ for EBA-175).

Adjusted IgG responses to whole parasites (data not shown), MSP-1_{19}, AMA-1, and EBA-175 (Fig. 1 and 2) generally did not differ between SP and placebo groups, except for a few cases in which IgG levels were significantly higher in the SP group than the placebo group, namely, with the (i) IgG response to MSP-1_{19} at 24 months, adjusting for previous clinical malaria episodes ($P = 0.041$) and present malaria parasitemia ($P = 0.033$); (ii) IgG and IgG1 responses to AMA-1 at 5 months, adjusting for previous clinical episodes ($P = 0.012$ and $P = 0.004$, respectively) and present parasitemia ($P = 0.049$

TABLE 2. IgG subclass responses to erythrocytic-stage antigens after IPTi with SP

Age[a]	Antigen	IgG subclass	Response by intervention group				*P* value[c]
			SP		Placebo		
			GM[b]	95% CI	GM	95% CI	
5[d]	MSP-1_{19}	IgG1	0.21	0.14–0.32	0.25	0.18–0.36	0.526
		IgG2	0.06	0.05–0.06	0.06	0.06–0.07	0.151
		IgG3	0.11	0.08–0.16	0.14	0.10–0.18	0.384
		IgG4	0.05	0.05–0.06	0.05	0.05–0.06	0.237
	AMA-1	IgG1	0.53	0.46–0.61	0.43	0.37–0.50	0.035
		IgG2	0.06	0.06–0.06	0.06	0.06–0.07	0.142
		IgG3	0.11	0.09–0.12	0.12	0.11–0.14	0.128
		IgG4	0.05	0.05–0.06	0.05	0.05–0.06	0.676
	EBA-175	IgG1	0.35	0.29–0.42	0.26	0.21–0.33	0.058
		IgG2	0.05	0.05–0.06	0.05	0.05–0.06	0.952
		IgG3	0.13	0.10–0.16	0.11	0.09–0.14	0.447
		IgG4	0.06	0.05–0.08	0.07	0.05–0.09	0.676
9[e]	MSP-1_{19}	IgG1	0.43	0.29–0.64	0.25	0.19–0.34	0.029
		IgG2	0.07	0.06–0.08	0.07	0.06–0.07	0.571
		IgG3	0.16	0.12–0.21	0.13	0.09–0.17	0.347
		IgG4	0.06	0.05–0.06	0.05	0.05–0.06	0.381
	AMA-1	IgG1	0.34	0.28–0.41	0.35	0.27–0.44	0.863
		IgG2	0.07	0.06–0.07	0.07	0.06–0.07	0.678
		IgG3	0.14	0.11–0.19	0.15	0.12–0.19	0.937
		IgG4	0.06	0.05–0.06	0.06	0.05–0.06	0.424
	EBA-175	IgG1	0.29	0.23–0.36	0.26	0.20–0.33	0.520
		IgG2	0.06	0.06–0.06	0.06	0.06–0.07	0.226
		IgG3	0.13	0.10–0.16	0.14	0.11–0.19	0.489
		IgG4	0.05	0.05–0.05	0.06	0.05–0.08	0.064
12[f]	MSP-1_{19}	IgG1	0.27	0.20–0.35	0.28	0.20–0.40	0.814
		IgG2	0.07	0.06–0.08	0.07	0.06–0.08	0.689
		IgG3	0.09	0.07–0.11	0.10	0.08–0.13	0.368
		IgG4	0.05	0.05–0.06	0.06	0.05–0.06	0.455
	AMA-1	IgG1	0.32	0.25–0.41	0.29	0.23–0.37	0.576
		IgG2	0.07	0.07–0.08	0.07	0.06–0.07	0.655
		IgG3	0.14	0.10–0.18	0.13	0.10–0.16	0.761
		IgG4	0.05	0.05–0.06	0.06	0.05–0.06	0.958
	EBA-175	IgG1	0.24	0.19–0.31	0.22	0.18–0.29	0.617
		IgG2	0.06	0.06–0.06	0.06	0.06–0.07	0.581
		IgG3	0.11	0.09–0.14	0.14	0.10–0.18	0.235
		IgG4	0.05	0.05–0.06	0.07	0.05–0.10	0.077
24[g]	MSP-1_{19}	IgG1	0.40	0.29–0.56	0.48	0.36–0.64	0.400
		IgG2	0.07	0.06–0.07	0.07	0.06–0.08	0.272
		IgG3	0.15	0.10–0.21	0.21	0.14–0.31	0.212
		IgG4	0.05	0.05–0.06	0.06	0.05–0.07	0.280
	AMA-1	IgG1	0.56	0.43–0.73	0.60	0.45–0.79	0.753
		IgG2	0.08	0.07–0.09	0.09	0.08–0.10	0.145
		IgG3	0.22	0.15–0.30	0.22	0.17–0.30	0.890
		IgG4	0.06	0.05–0.06	0.06	0.06–0.07	0.270
	EBA-175	IgG1	0.32	0.27–0.39	0.41	0.32–0.51	0.104
		IgG2	0.08	0.07–0.10	0.08	0.07–0.09	0.370
		IgG3	0.13	0.10–0.16	0.13	0.10–0.16	0.911
		IgG4	0.21	0.12–0.37	0.22	0.12–0.41	0.866

[a] Age in months.
[b] GM of ELISA antibody levels, expressed as OD values.
[c] Determined by *t* test for the comparison of the magnitude of antibody responses.
[d] Number of samples at 5 months: MSP-1_{19}, SP ($n = 29$) and placebo ($n = 43$); AMA-1, SP ($n = 92$) and placebo ($n = 84$); EBA-175, SP ($n = 35$) and placebo ($n = 34$).
[e] Number of samples at 9 months: MSP-1_{19}, SP ($n = 43$) and placebo ($n = 44$); AMA-1, SP ($n = 58$) and placebo ($n = 52$); EBA-175, SP ($n = 25$) and placebo ($n = 30$).
[f] Number of samples at 12 months: MSP-1_{19}, SP ($n = 34$) and placebo ($n = 40$); AMA-1, SP ($n = 42$) and placebo ($n = 51$); EBA-175, SP ($n = 27$) and placebo ($n = 28$).
[g] Number of samples at 24 months: MSP-1_{19}, SP ($n = 24$) and placebo ($n = 26$); AMA-1, SP ($n = 25$) and placebo ($n = 36$); EBA-175, SP ($n = 18$) and placebo ($n = 19$).

[corrected $P = 0.056$], and $P = 0.045$ [corrected $P = 0.054$], respectively) (data not shown for IgG1), consistently with the initial crude analysis; (iii) IgG1 response to MSP-1_{19} at 9 months, adjusting for the presence of parasitemia ($P = 0.013$); and (iv) IgG1 response to EBA-175 at 5 months, adjusted for previous clinical malaria episodes ($P = 0.038$).

We next stratified children into those with and those without previous malaria/present infection and compared their IgG levels by a reverse cumulative distribution function. In this subgroup analysis, children with malaria exposure had significantly higher antibody responses after IPTi with SP for some antigens and at some time points. In particular, in children who

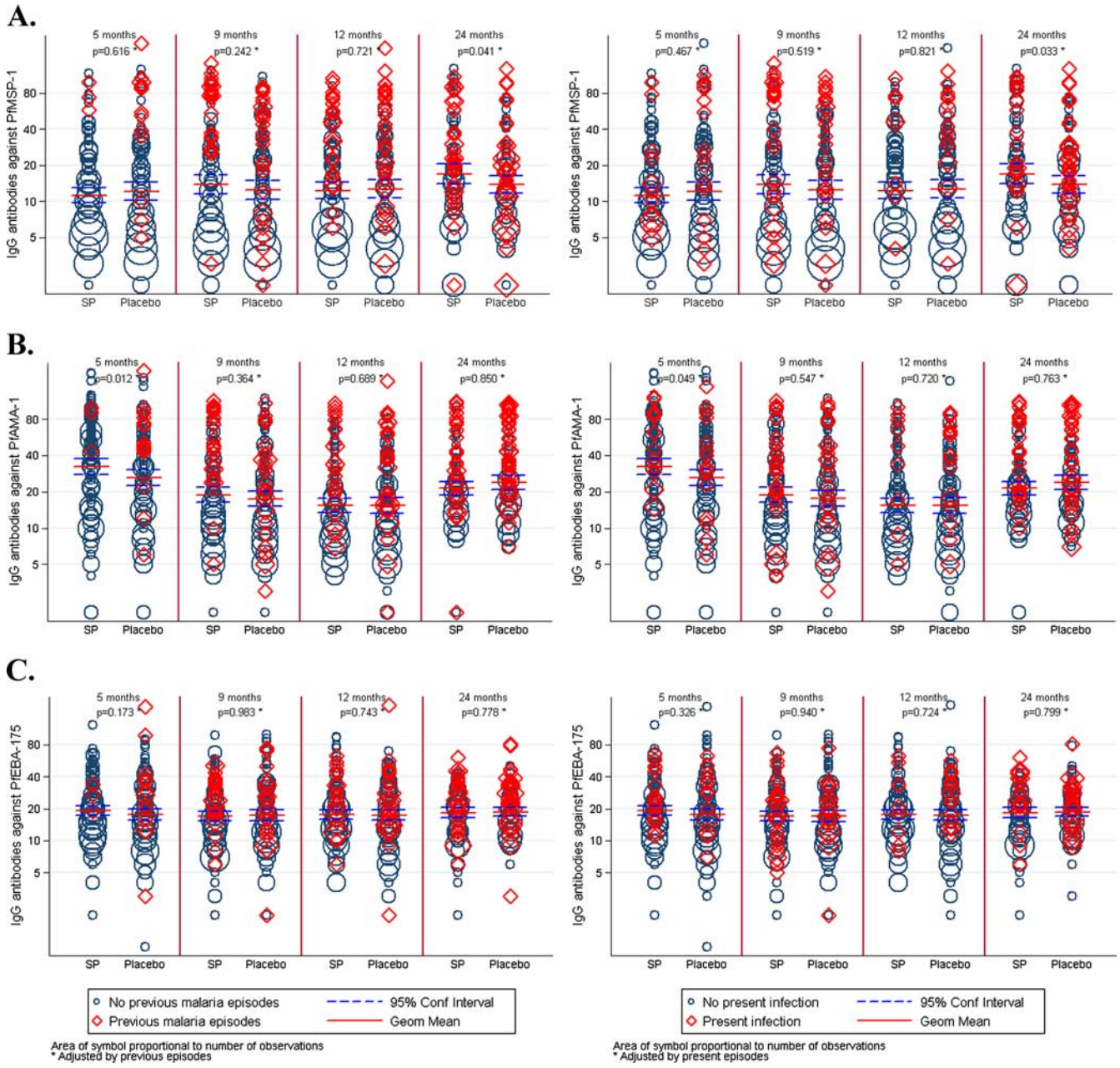


FIG. 1. IgG responses to MSP-1_{19} (PfMSP-1) (A), AMA-1 (PfAMA-1) (B), and EBA-175 (PfEBA-175) (C) in Mozambican infants receiving IPTi with SP or the placebo, adjusted for previous clinical malaria episodes (left) or present malaria infection (right) by linear regression analysis. IgG levels (*y* axes) are expressed as normalized OD values (as percentages). In the weighted scatter plots, the area of the symbol is proportional to the number of observations. Geometric (Geom) mean IgG levels and 95% confidence (Conf) intervals are indicated by horizontal red and blue lines. Red symbols correspond to IgG levels in children with previous/present infection.

have had previous clinical malaria episodes, IgG IFAT titers were significantly higher in the SP than in the placebo group at the age of 9 months ($P = 0.018$; data not shown), and ELISA antibody levels also were significantly higher in the SP than in the placebo group for MSP-1_{19} at 9 (IgG, $P = 0.001$ [Fig. 3A]; IgG1, $P = 0.024$ [data not shown]) and 24 (IgG, $P = 0.022$; Fig. 3A) months of age and for AMA-1 at 9 months (IgG, $P = 0.010$; Fig. 3B). In children with present infection, the levels of IgG1 response to MSP-1_{19} were significantly higher in the SP than in the placebo group at 9 months ($P = 0.011$; data not shown). For children with no documented previous clinical episodes, significant differences were observed only at 5 months, when only a few malaria episodes had occurred due to their young age, particularly in the SP group. At that time, the levels of the IgG and IgG1 responses to AMA-1 were higher in the SP than in the placebo group ($P = 0.010$ [Fig. 3B]; $P = 0.006$ [data not shown]), as found in the initial crude analysis. Similarly, the levels of the IgG1 response to EBA-175 were higher in the SP than in the placebo group at age 5 months ($P = 0.014$; corrected $P = 0.056$; Fig. 3C), also consistent with the above analyses.

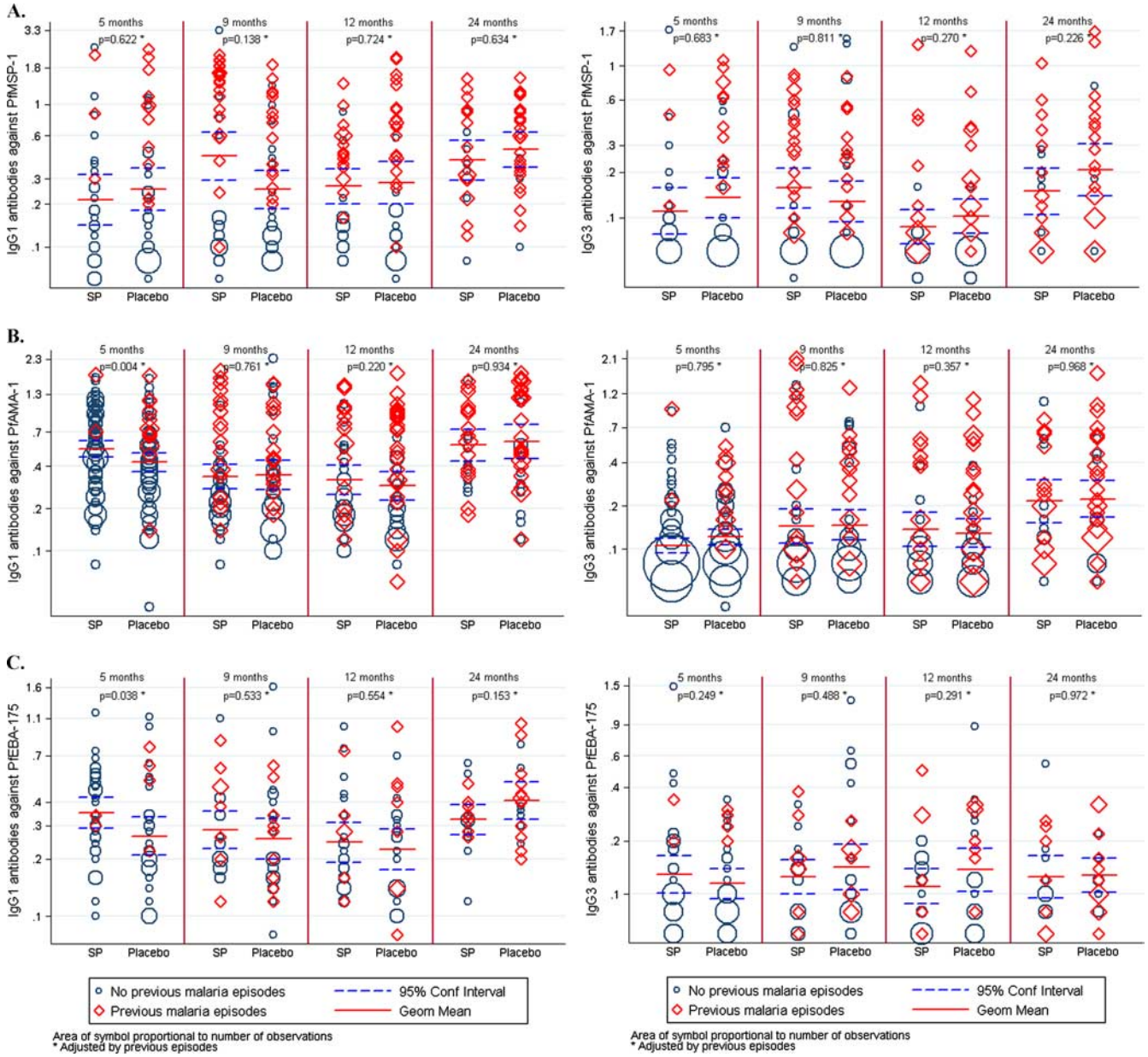
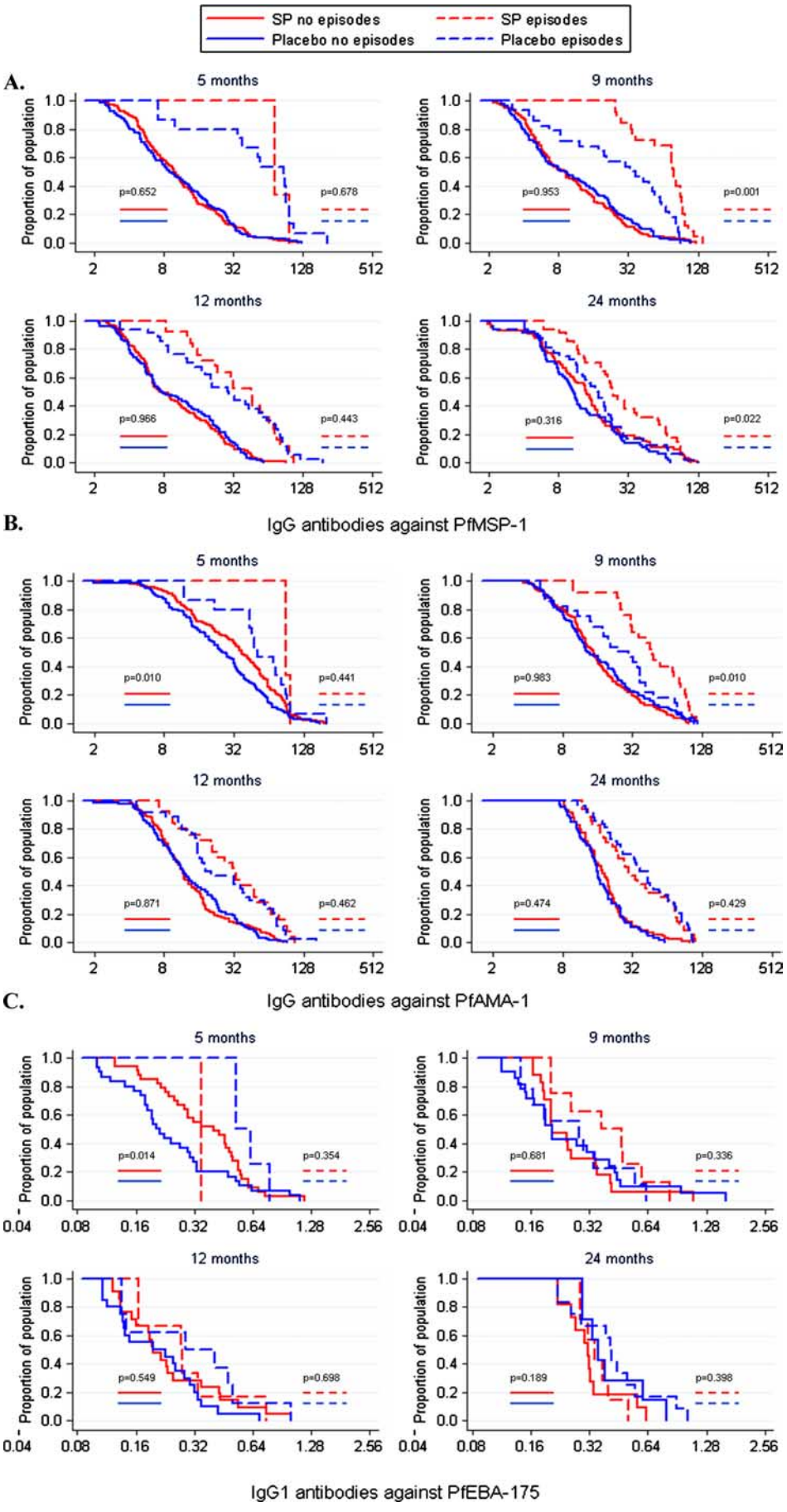


FIG. 2. IgG1 and IgG3 cytophilic isotype responses to MSP-1_{19} (PfMSP-1) (A), AMA-1 (PfAMA-1) (B), and EBA-175 (PfEBA-175) (C) in Mozambican infants receiving IPTi with SP or a placebo, adjusted for previous clinical malaria episodes by linear regression analysis. Antibody data adjusted for present malaria infections are not shown, but statistically significant differences found for IgG1 are indicated in Results. IgG2 and IgG4 levels generally were very low and are not shown. IgG levels (*y* axes) are expressed as OD values. In the weighted scatter plots, the area of the symbol is proportional to the number of observations. Geometric (Geom) mean IgG levels and 95% confidence (Conf) intervals are indicated by horizontal red and blue lines. Red symbols correspond to IgG levels in children with previous clinical episodes.

To investigate why IPTi with SP would result in enhanced antibody levels to some antigens in children with previous *P. falciparum* exposure, we used a multilevel regression model that included only children with at least one clinical episode of malaria and looked at whether intervention group status, age, number of previous episodes, time from past episodes, parasite density, and infection status were independently associated with IgG levels. On average, the levels of IgG in response to MSP-1_{19} were significantly higher in the SP than in the placebo group: according to the estimated models, they were 0.55 times higher (CI, 0.40 to 0.74; $P < 0.001$) after being adjusted for all of the other variables and taking into account repetitive measures. In addition, in these same children, the levels of IgG in response to MSP-1_{19} in parasitemic children were 1.65 times higher than in those who were aparasitemic (CI, 1.27 to 2.13; $P < 0.001$). Figure 3A illustrates that children who received IPTi with SP and who had had previous exposure to malaria have higher anti-MSP-1_{19} IgG levels than those who received placebo, particularly during the first year. Different ages at first episode or different parasite densities did not seem to explain



the higher IgG levels after IPTi with SP. No other significant associations in relation to SP treatment were found with the other antigens and antibodies studied.

DISCUSSION

This study shows that IPTi with SP administered at 3, 4, and 9 months of age does not modify the levels of antibodies to *P. falciparum* erythrocytic-stage antigens in the first 2 years of life, indicating that this intervention does not negatively affect the development of naturally acquired antibody responses to malaria. These results contrast with those of previous studies of continuous chemoprophylaxis, in which a significant reduction of IFAT malaria antibody responses was observed (4, 6, 18–20, 29, 45). In some cases, the decrease in antibody titers was accompanied by a rebound in clinical and/or parasite prevalence (18, 29). Moreover, we found some evidence indicating that IPTi with SP is associated with higher IgG responses to certain antigens at certain time points. This was the case for IgG and/or IgG1 responses to AMA-1 and EBA-175 at 5 months and for IgG and/or IgG1 to MSP-1 and AMA-1 at 9 and/or 24 months. Furthermore, it appeared that the pattern of responses in the SP and placebo groups was different according to past/present malaria history. These analyses took into account the confounding effect of having had previous clinical episodes of malaria and being parasitemic at the visits; both variables were independently associated with high levels of antibodies to all antigens. Despite the multiple comparisons performed, results were always internally consistent, and in no case was the antibody level superior in placebo recipients than in SP recipients. When analyses were corrected for multiple comparisons, the overall outcome did not vary significantly.

Previous studies by Schellenberg et al. (36) show that subjecting Tanzanian infants to IPTi with SP may result in a sustained protection from malaria during the second year of life after the therapeutic effect of the drug had ceased. This indicates that IPTi facilitates the development of protective immunity to malaria. It has recently been hypothesized that the induction of effective and sustained immunity against malaria by IPTi could be due to the generation of low-dose blood-stage inocula and attenuated infections because of the long serum half-life of SP and its activity against developing hepatic parasite stages (43). It is possible that higher IgG and cytophilic IgG1 responses in SP recipients, particularly to highly immunogenic antigens, are associated with the enhanced acquisition of clinical immunity.

As pointed out by Schofield and Mueller (39), current thinking concerning rebound versus sustained protection hinges on concepts of an immunological nature, but to date there have been no direct measurements of the immunological impact of IPTi. It is possible that the consistently lower levels of IgG in response to AMA-1 at 5 months in placebo recipients is explained by the higher clearance of antibodies, probably of maternal origin, due to the higher incidence of infection. In addition, the significant differences in IgG levels between SP and placebo recipients at 5, 9, and 24 months of age may be related to immunological processes resulting from SP administration. We speculate that subtherapeutic drug concentrations due to partial SP resistance could have attenuated parasites in vivo, resulting in subpatent infections of very low densities between 3 and 5 months. Low-antigenic-dose stimulation in SP recipients may have more adequately primed the immune system, resulting in the induction of cytophilic IgG1 antibodies at higher titers later on. Conversely, high-density parasitemia and clinical malaria episodes between 3 and 5 months in placebo recipients may result in immune suppression and a less efficient mounting of antibody responses. This interpretation is consistent with a study from Kenya showing that children with a low intensity of malaria exposure during the first 2 years of life had higher subsequent IgG responses to MSP-1_{19} (41). A trial of insecticide-treated bed nets in the same area also found a significantly higher prevalence of IgG responses to MSP-1_{19} in children from bed net areas, concluding that in areas of intense malaria transmission, interventions that reduce the number of asexual parasitemic episodes do not delay the development of antibody responses to blood-stage malarial antigens (22).

The importance of low doses of parasite antigen in the induction of protective immunity also is supported by other studies of animal models and humans. Subpatent infections with blood-stage *Plasmodium chabaudi* or *Plasmodium yoelii* rodent malarias stimulate a good level of immunity, which differed from that induced by patent infection (3, 14, 15, 44). In humans, subpatent infections with a very low inoculum of *P. falciparum* induce protective immunity (33). Low-level exposure related to subtherapeutic drug concentrations of antimalarials due to moderate resistance also has been shown to have important effects on immune responses (25). Higher titers of IgG responses to MSP-1_{19} enhanced the likelihood of parasitological clearance in individuals treated with a suboptimal drug regimen, and it was suggested that recovery from uncomplicated malaria in patients carrying drug-resistant *P. falciparum* is a phenotypic marker of acquired functional immunity (12, 13, 31).

In addition to parasite density, the age at which the infant's immune system first encounters an infection also may play an important role in determining the magnitude and quality of subsequent immune responses. It is probable that infants who receive SP have their first clinical episode 2 months later, on average, than those who receive the placebo due to the protection granted by the treatment. However, our study was not

FIG. 3. IgG responses to MSP-1_{19} (PfMSP-1) (A) and AMA-1 (PfAMA-1) (B) and IgG1 responses to EBA-175 (PfEBA-175) (C) in Mozambican infants receiving IPTi with SP (red) or the placebo (blue), stratified by those with previous clinical malaria episodes (discontinuous lines) and those without (continuous lines) and illustrated as reverse cumulative distribution functions. IgG levels (*x* axes) are expressed as normalized OD values (as percentages) (A and B), and IgG1 levels (*x* axis) are expressed as OD values (C). The *y* axes represent the proportion of children from each group that has a given OD value or higher. Statistical significance between the two treatment groups was analyzed by a Kruskal-Wallis test adjusted for ties. *P* values shown correspond to the comparison between SP and placebo groups of children with previous episodes (right) and without previous episodes (left).

designed or powered to test this hypothesis, and further studies are now under way in Manhiça that will help clarify this possibility.

Finally, currently we are investigating whether IPTi with SP also has an effect on other types of antibody responses that are thought to be involved in the acquisition of protective immunity to malaria, such as IgG to *P. falciparum* variant surface antigens and functional growth-inhibitory antibodies.

In conclusion, IPTi with SP is safe and protects children against clinical malaria without a rebound and without negatively affecting the development of *P. falciparum*-specific antibody responses to blood-stage antigens considered targets of immunity to malaria. Furthermore, IPTi with a partially effective drug such as SP may have unanticipated benefits by allowing immune priming with lower parasite densities at earlier ages, which may result in higher levels of cytophilic IgG subclass responses to some antigens, which may contribute to the acquisition of protective immunity. It remains to be established whether IPTi with more efficacious drug combinations will have a similar impact on the development of naturally acquired immune responses.

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ARTICLE 2

Age-Dependent IgG Subclass Responses to *Plasmodium falciparum* EBA-175 are Differentially Associated with Incidence of Malaria in Mozambican Children

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Abstract

Background

Plasmodium falciparum blood stage antigens such as the 19 KDa fragment of the merozoite surface protein (MSP)-1, the apical membrane antigen (AMA)-1, and the 175 kDa erythrocyte binding antigen (EBA-175) are considered important targets of naturally acquired immunity to malaria. However, it is not clear whether antibodies to these antigens are effectors in protection against clinical disease or mere markers of exposure.

Methods

In the context of a randomized, placebo-controlled trial of intermittent preventive treatment in infants conducted between 2002 and 2004, antibody responses to *Plasmodium falciparum* blood stage antigens were evaluated by immunofluorescence antibody test and enzyme-linked immunosorbent assay at 5, 9, 12 and 24 months of age in a cohort of 302 Mozambican children recruited at age 3 months and followed up till age 2 years.

Results

Past and present exposure to *P. falciparum* resulted in significantly higher levels of antibodies. Age and neighborhood of residence were the two other main factors affecting antibody values. IgG subclass responses to EBA-175 were differentially associated with incidence of malaria in follow-up period. Two-fold increments of cytophilic IgG1 and IgG3 levels independently were significantly correlated with decreased incidence of malaria (incidence rate ratio, IRR, 0.49, 95% confidence interval, CI, 0.25-0.97, P=0.026, and IRR 0.44, CI 0.19-0.98, P=0.037, respectively), while 2-fold increments in levels of non-cytophilic IgG4 were significantly correlated with increased malaria (IRR 3.07, CI 1.08-8.78, P=0.020). No significant associations were found between antibodies to MSP-1_{19} or AMA-1 and incidence of malaria.

Conclusions

Previous episodes and neighborhood were the main factors influencing levels of antibodies to all merozoite antigens, and having taken those into account only cytophilic antibodies to EBA-175 were associated with reduced incidence of malaria. Deeper understanding of the acquisition of antibodies against vaccine target antigens in early infancy is crucial for the rational development and deployment of malaria control tools in this vulnerable population.

Background

In areas where the intensity of transmission of *Plasmodium falciparum* is high, the greatest burden of malaria occurs in children under 5 years [1] and of severe malaria in infants under 12 months [2]. Natural immunity is acquired with age and exposure, protecting quite effectively from disease and high parasitemia [3]. The exact immune mediators, mechanisms and targets underlying such protection are unknown, but immunoglobulin passive transfer studies demonstrated that IgG antibodies are important effectors in protection [4, 5].

Blood stage antigens such as the 19 KDa fragment of the merozoite surface protein (MSP)-1 [7,8,9,10,11,12,13], the apical membrane antigen (AMA)-1 [6-8], and the 175 kDa erythrocyte binding antigen (EBA-175) [9-13] are considered important targets of naturally acquired immunity [14], and antibodies against these parasite proteins inhibit invasion of erythrocytes *in vitro* [9,23,24]. However, conflicting evidence in immuno-epidemiological studies and unsuccessful phase IIb trials of AMA-1 and MSP-1 experimental vaccines [15] question the extent of their relevance in protection against malaria. The antibody isotype elicited by *P. falciparum* antigens is considered to be important, and the protective effect of IgG has been attributed to the cytophilic (IgG1 and IgG3) rather than the non-cytophilic subclasses (IgG2 and IgG4) [16-20]. A meta-analysis of anti-merozoite antibodies supported the protective effect of total IgG responses to particular antigens against symptomatic falciparum malaria in humans, and highlighted the requirement for more prospective cohort studies in different populations, examining multiple antigens at multiple time-points [21]. A recent article has shown an association between IgG to EBA-175 and protection from malaria [22].

Several trials of malaria control strategies [23-25] are being conducted in Manhiça, a malaria endemic area of Southern Mozambique. As no previous data were available on naturally-acquired immune responses to blood stage *P. falciparum* antigens in the study area, a detailed analysis of the development of antibody responses was conducted during the first two years of life in the context of a randomized, placebo-controlled trial of IPTi with sulfadoxine-pyrimethamine (SP) [24, 26]. This paper sets out to report the evaluation of the age pattern of naturally-acquired antibodies to the leading vaccine candidates MSP-1_{19}, AMA-1, EBA-175, the description of the decay of maternal IgG, the pattern of IgG isotype responses, the effect of past and present parasite exposure and neighborhood in the antibody response, and the role of these antibodies in protection against clinical malaria. The understanding of the acquisition of antibody-mediated natural immunity in early infancy is crucial for the rational development and deployment of malaria control tools, including vaccines, in the most vulnerable population.

Methods

Study area and participants

The study was conducted at the Centro de Investigação em Saúde da Manhiça (CISM), Manhiça District (Maputo Province), in southern Mozambique. Adjacent to CISM is the Manhiça Health Center, a 110-bed referral health-care facility that provides curative and preventive services to the area population. The characteristics of the area have been described in detail elsewhere [27]. Malaria transmission is perennial with marked seasonality and an entomological inoculation rate for 2002 of 38 for the whole study area. There is a continuous demographic surveillance system in place covering 36,000 inhabitants of the same ethnicity.

Children included in this analysis participated in an IPTi randomized, placebo-controlled trial [24, 26] with registration number is NCT00209795 (http://clinicaltrials.gov). Infants were recruited at age 3 months when attending the EPI clinic between September 2002 and February 2004. Permanent residents of the CISM study area who did not report allergies to sulfa drugs and did not require admission to the hospital were eligible for inclusion. IPTi-SP or placebo was administered at 3, 4 and 9 months of age to the study participants. Antibody measurements were done at 5, 9, 12 and 24 months of age in 302 children (50% under IPTi-SP) out of the total of 1,498 participating in the efficacy trial. At each visit, capillary blood was collected into EDTA microtainers to obtain plasma and erythrocyte pellet. Clinical surveillance for malaria morbidity during the whole study was done through round-the-clock (24 h a day, 7 days a week) passive case detection, documented with standardized questionnaires and databases. Written informed consent for the study was obtained from parents/guardians. Ethical approval was obtained from the ethics review committee of Mozambique (National Health and Bioethics Committee, CNBS) and the ethics review committee of the Hospital Clinic of Barcelona (Ethical Committee of Clinical Investigation, CEIC) in Spain.

Malaria parasitemia

P. falciparum infections were diagnosed at 12 and 24 months of age and during the passive morbidity surveillance by thick and thin blood films, stained and read according to quality-control procedures [28]. In addition, submicroscopic infections were assessed by nested PCR from erythrocyte pellets collected at all four cross-sectional visits, as described [29].

Antibody Assays

Immunofluorescence Antibody Test (IFAT) with asynchronous cultures of *P. falciparum* (3D7) and enzyme-linked immunosorbent assays (ELISA) were done as described [26]. For the ELISA, plasma samples were assayed for IgG (including isotypes) and IgM to the recombinant proteins MSP-1_{19} (19 KDa fragment, C-terminus, 3D7 [30]), AMA-1 (3D7 [31]) and EBA-175 (region F2, CAMP [32]) from ICGEB (New Delhi, India) expressed in *Escherichia coli* and purified with a Histidine tag. IgG isotypes were measured in the samples in which a positive IgG response was detected for the corresponding antigen. Identical sets of antigen-coated plates were prepared for the determination of IgG, IgG1, IgG2, IgG3, or IgG4 in assays performed with any one plasma sample (1:200, duplicates) in parallel on the same day. Wells were incubated for 3 h with peroxidase-conjugated sheep anti-human IgG1 (1:6000), IgG2 (1:3000), IgG3 (1:6500), or IgG4 (1:5000) (Binding Site, Birmingham, UK) or peroxidase-conjugated rabbit anti-human IgG specific for gamma chains (1:6000 DAKO, Glostrup, Denmark). In parallel, purified human myeloma proteins IgG1, IgG2, IgG3, and IgG4 (Binding Site) were coated on plates at 2-fold dilutions from 2 to 0.001 μg/ml. Peroxidase-conjugated antibodies to each IgG subclass (Binding Site), at the same dilutions as used for plasma samples, were reacted with the myeloma proteins and used as positive controls.

Positive controls were a pool from 8 adults with lifelong exposure to malaria, and negative controls were 9 non-exposed adults plasmas in both IFAT and ELISA. For the IFATs, the highest dilution giving green positive fluorescence was scored and data are presented as end-point IgG titers: reciprocal of last plasma dilution causing positive fluorescence above the negative control levels. For the ELISA, specific reactivity of plasmas was obtained as optical density (OD) values at 492 nm. For total IgG and IgM, OD_{492} data was normalized against a positive control (1:200) run in the same experiment and used as continuous variables

(arbitrary unit, %). IgG isotype data was reported as OD_{492}. Positive antibody responses were those with an OD value above the cut-off (arithmetic mean of negative controls plus 3 standard deviations).

Definitions and statistical methods

Malaria infection was defined as the presence of asexual *P. falciparum* parasites of any density in a blood smear or assessed by PCR. A clinical malaria episode was defined as a positive blood smear plus an axillary temperature ≥37.5ºC. The sensitivity and specificity of these definitions are ~100% and 84%, respectively, in infants and 100% and 79.4% in children 1–4 years old [33]. Months were defined as 30.4 days. Incidence was expressed as episodes per Person Years at Risk (PYAR). To estimate the incidence, the time at risk was calculated as the number of PYAR since the beginning of the time at risk until the end of follow-up or migration or death or withdrawn consent, whatever was first. Children were not considered to be at risk for 28 days after the start of each episode of clinical malaria.

Antibody variables were logarithmically transformed. Averages within ages are presented as geometric means (GM) plus 95% confidence intervals (CI). Distribution of antibody responses to each antigen at each time point are presented as weighted scatter plots. To identify variables independently associated with antibody levels, multivariate random-effect regression models using a forward stepwise procedure (p-enter = 0.05, from Likelihood Ratio test) were estimated: intervention group, age, neighborhood of residence, previous clinical malaria episodes and present infection. In these models, the subject variable was a random effect, thus that we were able to take the sampling variability into account. Because of the strong effect of previous malaria episodes [26] we used a second model including only children with at least one episode before contact to further evaluate the effect of additional

variables on antibody values: intervention group, age, number of previous malaria episodes, time from previous episodes, present infection and parasite density.

To evaluate the relationship between the level of each of the antibody variables individually at 5 and 12 months and the incidence of clinical malaria episodes to 12 or 24 months, respectively, negative binomial regression models were estimated. Two periods were defined because of the differences between the two years (IPTi and maternal antibodies in year 1). IgG responses were treated as continuous values, or categorised by tertiles. The incidence rate ratio (IRR) of children with antibody levels in the highest tertile against those in the lowest tertile was estimated, as well as the IRR per 2-fold increase in the value of antibodies. Regression analyses were done crude and adjusted by intervention group, sex, neighborhood, present infection, and previous clinical episodes, for each antibody variable.
In an advanced analysis to evaluate the relationship between all the antibody variables together (IgG and IgM to all antigens) at 5 and 12 months and the incidence of clinical malaria episodes to 12 or 24 months, respectively, negative binomial regression models using a forward stepwise procedure (p-enter = 0.05, from Likelihood Ratio test) were estimated and adjusted by intervention group, sex, neighborhood, present infection, and previous clinical episodes. Only children who had at least 70% of the antibody variables measured were included in this advanced analysis; complete ELISA data was obtained from all samples, whereas IFAT data was obtained from 78.35% of samples, due to limited plasma volumes in some children.

Data analysis was performed using Stata 11 (StataCorp. 2007. *Stata Statistical Software: Release 10.* College Station, TX: StataCorp LP). Statistical significance was defined at $P<0.05$.

Results

Age pattern of naturally-acquired IgG, IgM and IgG subclass antibodies

IgG titers to *P. falciparum* blood-stages measured by IFAT were very high during the first year of life (Figure 1A), decreasing at age 2 years. A table in the Additional file summarizes the number of samples that were available per each time point to be analyzed by ELISA and the frequency of positive antibody responses. The age patterns of IgG responses against each merozoite antigen were different. IgG values to MSP-1_{19} remained constant during the first year of life, and then increased at 2 years (Figure 1B). IgG responses to AMA-1 were the highest at age 5 months, then gradually decreased up to age 1 year, and increased at age 2 years (Figure 1C); the association between age and IgG levels to AMA-1 was significant ($P<0.0001$). Total IgG responses to EBA-175 remained quite constant over the 2 years (Figure 1D) and were not significantly associated with age. IgM responses to the three merozoite antigens were significantly associated with age ($P<0.0001$), increasing from 5 to 9 months, and from 12 to 24 months (Figure 1 B-D).

The pattern of IgG isotypes varied depending on the antigen and the age of the child (Figure 2). MSP-1_{19} and AMA-1 predominantly elicited cytophilic IgG1 and IgG3 subclasses. EBA-175 primarily induced IgG1 and IgG3 followed by induction of IgG4 at 2 years. IgG2 levels were negligible for all antigens, and IgG4 were very low for MSP-1_{19} and AMA-1. The kinetics of IgG1, IgG3 and IgG4 were different for each antigen (Figure 2). IgG1 responses to MSP-1_{19} were quite constant during the first year of life, and then increased at age 2 years ($P=0.0207$), while IgG3 appeared to decrease over the first year and then increased at 2 years of age ($P<0.0001$) (Figure 2A). In contrast, IgG1 responses to AMA-1 were high at 5 months

of age, then decreased up to age 1 year, and increased to the highest levels at age 2 years (P<0.0001), whereas IgG3 remained constant in year 1, and then increased by 2 years (Figure 2B). IgG1 responses to EBA-175 remained similar over the first year, and then increased in the second year (P=0.0031) (Figure 2C). IgG3 to EBA-175 remained moderate at all time points, IgG4 was low in the first year but markedly increased at age 2 years (P<0.0001).

Effect of exposure to *P. falciparum* on the magnitude of antibody responses

There was a strong association between previous clinical malaria episodes or present infection and the magnitude of antibody responses, particularly for MSP-1_{19} and AMA-1 (Table 1). Children with a previous clinical malaria episode had on average 1.78 times higher IgG to AMA-1 than children without (CI 1.54-2.05, P<0.0001). Similarly, children with current parasitemia had 1.26 times higher IgG to AMA-1 than uninfected children (CI 1.10-1.43, P=0.0007). IgM levels were also strongly associated with present infections but not with past clinical episodes (Table 1). Results were different for EBA-175 IgG subclasses. Total IgG and IgM levels were significantly associated with previous episodes and current infection, respectively. However, IgG1 levels were significantly associated with previous episodes only, and IgG3 levels with current infection only, but IgG2 and IgG4 levels were not significantly associated with past or present exposure to *P. falciparum* (Table 1). All these analyses were adjusted by age and neighborhood of residence, and also by IPTi treatment group, since for some antigens SP affected antibody levels, as reported previously [26]. In all cases when neighborhood was associated with antibody levels to an antigen, current infection also appeared significantly associated in the same model. There were no geographical, sociological or entomological documented characteristics different among the neighbourhoods (e.g. proximity to swamps, rivers, housing type, etc) that could explain these spatial associations.

In a subgroup analysis including only children with previous malaria episodes (103 subjects and 212 antibody measurements) levels of antibodies in children with current parasitaemia were significantly higher than in those who were aparasitaemic (Table 2).

Association between antibody responses and incidence of clinical malaria.

We analysed the association between individual antibody responses at 5 and at 12 months and subsequent incidence of malaria at two different times at risk intervals: 5 to 12, and 12 to 24 months. Table 3 summarises the relationship between magnitude of antibodies and incidence of malaria, in the two time periods. Results could be grouped into five different patterns, the most common being: (i) no significant association between antibody levels and risk of malaria (e.g. IgM to AMA-1), and (ii) high antibody levels significantly associated with increased risk of malaria in the crude analysis, but significance disappearing in the adjusted analysis (e.g. IgG to MSP-1_{19}). In general, pattern (ii) was the trend for all responses, but in (i) it did not reach statistical significance. There was only one case (pattern iii) in which the association between high antibody levels at age 5 months and increased risk remained significant after adjusting for previous episodes (IgG1 to $MSP1_{19}$ at 5 months). We observed a differential pattern for IgG subclasses to EBA-175 in the adjusted analysis, particularly in year 2: (iv) 2-fold increments in levels of cytophilic IgG1 and IgG3, each one independently, were significantly correlated with decreased incidence of malaria (IgG1 IRR 0.49, CI 0.25-0.97, P=0.026; IgG3 IRR 0.44, CI 0.19-0.98, P=0.037), and (v) 2-fold increments in the levels of the non-cytophilic IgG4 were correlated with increased incidence of malaria (IRR 3.07, CI 1.08-8.78, P=0.020). Analyses by antibody tertiles were consistent with these results (data not shown).

In an advanced multilevel regression analysis including all antibody, clinical, epidemiological and demographic variables together, the single most significant risk factor for having a malaria episode was having had a previous episode (at 5 months: IRR 3.52, CI 1.41-8.80, P=0.007; at 12 months IRR 3.39, CI 1.58-7.29, P=0.0018). In addition, neighborhood of residence was the only other variable significantly associated with clinical malaria (P<0.0001) (Figure 3).

Discussion

This was a prospective study measuring antibody responses to merozoite antigens at multiple time points, performed in a malaria endemic area with established demographic and morbidity surveillance systems, which allowed to have a rigorous and complete documentation of clinical malaria cases during the 2 years study period. This type of design provides useful insights into the acquisition and duration of specific antibody responses, how they are boosted by *P. falciparum* exposure, and the correlation between these responses and protection from symptomatic malaria. The most important findings are that previous episodes and neighborhood of residence were the main factors influencing levels of antibodies to merozoite antigens, and having taken those into account only IgG1 and IgG3 antibodies to EBA-175 appeared to be significantly associated with less malaria incidence.

Overall, there was an age-related build-up of antigen-specific IgM and IgG antibodies to the *P. falciparum* merozoite antigens examined, supporting the notion that these antigens may be important targets of immunity. In addition, an important contribution from maternally-transmitted antibodies was suggested in early infancy, particularly for AMA-1 (IgG, IgG1) and for IgG to whole *P. falciparum* blood stages (IFAT), lasting for up to a year. The kinetics

of decay of maternal IgG antibodies and the kinetics of acquisition of infant IgG antibodies, but not of IgM, varied for each antigen studied. The isotype patterns resembled what has been described previously, but the significant induction of non-cytophilic IgG4 by EBA-175 at age 2 years represents a novel finding [10, 34].

Past and present exposure to *P. falciparum* is widely known to affect antibody responses. On the one hand, the occurrence of previous episodes was the strongest factor affecting IgG antibody values. On the other hand, the occurrence of previous episodes was the single most significant risk factor for having a malaria episode, consistent with data of IPTi studies in Tanzania [35] and Manhiça [24]. Consistent with this, different neighborhoods of residence had diverse malaria incidences and this was reflected in antibody levels. Age-adjusted *P. falciparum* antibody levels have been used as markers of micro-geographical variations in exposure to malaria infection [36]. Therefore, analyses of the relationship between antibody levels and risk of malaria must be adjusted for the effect of previous malaria episodes and neighborhood. Indeed, in the crude analysis, a common finding was that high antibody levels were associated with increased risk of malaria. In another study in Manhiça, high maternal and fetal IgG were associated with higher risk of malaria in the infant [37] and this was consistent with other similar studies [38, 39]. Nevertheless, when the analyses were adjusted for prior malaria episodes, the association disappeared, indicating that the main risk factor was the occurrence of previous episodes and that the antibodies were mere markers of the prior exposure. In only one case (MSP-1_{19} IgG1 at 5 months), the positive association between IgG1 and risk of malaria up to 12 months remained significant after adjustment. This might be explained by exposure to malaria occurring at the pre-natal and/or peri-natal period happening before enrollment and not captured in this study. It was hypothesized that if the

placental/maternal infections had been registered and adjusted for, this significance would have disappeared.

Studies restricted to infants are considered less adequate to analyze correlations between antibodies and malaria risk because of the confounding effect of maternal *in utero* exposure and of maternal transferred immunity. Therefore more weight is given to the analyses in the 12-24 month period. In most cases there was no association between antibody levels and incidence of malaria in the adjusted analysis. Inconsistencies in the associations between antibodies to MSP-1_{19} and AMA-1 and protection among different studies could partially be explained because of the different recombinant antigen reagents used. It was only for EBA-175 antibody responses that a significant pattern emerged. IgG1 and IgG3 at 12 months had a 51% and 56% protective effect, respectively, up to 24 months, whereas IgG4 was associated with increased risk of malaria in this age period. In fact, IgG1 and IgG3 to MSP-1_{19} and IgG3 to AMA-1 also showed a protective trend (IRR <1) but this did not reach statistical significance. The same conclusions were reached when we analyzed data by 2-fold increments or by tertiles. Another study also reported that high IgG4 levels specific for blood stage antigens were associated with an enhanced risk of infection and malaria attacks [40].

Findings are consistent with a recently published study from Papua New Guinea in which high levels of IgG (particularly IgG1 and IgG3) to EBA antigens were associated with protection from malaria [22]. Other previous studies of EBA-175 failed to find an association between responses to EBA-175 and incidence of *P. falciparum* malaria [10, 11, 13], although Okenu et al. reported a protective trend for high levels of IgG to region II (which includes F2 included in our study). However these studies examined total IgG whereas subclass-specific responses to merozoite antigens provide further insights into protective targets and

mechanisms of acquired immunity in children. Other studies have found an association between IgG1 and IgG3 to merozoite proteins and protection [18, 19]. Indeed, in this study an association between total IgG and incidence of malaria was not found; the effects of predominant IgG cytophilic vs. low level non-cytophilic isotypes going in opposite directions could partially compensate any small effect with total IgG.

IgG antibodies function in vivo by inhibiting merozoite invasion of erythrocytes, opsonizing merozoites for phagocytosis, and antibody-dependent cellular inhibition (ADCI). Antibodies against region II of EBA-175 may mediate protection against *P. falciparum* by blocking the binding of native EBA-175 to erythrocytes and subsequent invasion [14]. In addition, cytophilic IgG1 and IgG3 antibodies may be involved in opsonising merozoites for phagocytosis, activating complement, and/or operate through ADCI mechanisms, as proposed for GLURP, SERA or MSP-3 [16]. However, not all parasites use EBA-175 for invasion and there is substantial variability in the levels of the expression of this ligand compared to other ligands in endemic populations [41]. Therefore, it is possible that antibodies are partially protective depending on the expression of EBA-175 and use of this ligand in invasion, a variable that may contribute to differences among populations.

In this study there are a number of puzzling findings about EBA-175 (F2) antibodies, particularly with regards to IgG4. There is a significant rise in IgG4 at 24 months, and two distinct groups can be identified in the weighted scattered plots (very high and very low responders) but their significance remains unknown. Intriguingly, levels of non-cytophilic IgG4 to EBA-175 were not associated with either previous malaria episodes or present infection, IgG1 and IgG3 were only associated with previous or current infections, and there was a general trend that IgG4 levels were higher in placebo compared to SP recipients [26];

all these results contrast to the patterns found for MSP-1_{19} and AMA-1. Therefore, it appears overall that IgG subclass responses to EBA-175 may have intrinsic differences compared to IgG subclass responses to MSP-1_{19} or AMA-1 and this might have functional implications towards the mechanisms of protection and development of natural immunity.

Future studies need to investigate in more depth how different antibody isotypes induced by *P. falciparum* are transferred from the mother to the infant, and the kinetics of production in early life [42]. Different IgG isotypes appear to inherently vary in their capacity to transfer to the foetus [43], with IgG1 being the most efficiently transported subclass and IgG2 the least [44], and these differences may partly explain the susceptibility of newborns to various pathogens. *P. falciparum*-specific IgG1 and IgG3 isotypes appear to transfer to the offspring more often and more efficiently than IgG2 and IgG4 [45]. Transplacental IgG subclass transfer is also influenced by malaria infection and maternal hypergammaglobulinemia [46]. During placental malaria there is production of more IgG in the mother [37] and it appears that less IgG1 is transferred to the fetus, while IgG3 seems unaffected [46].

Conclusions

Considering the very few studies examining the association of IgG responses to EBA-175 with incidence of *P. falciparum* malaria, the findings reported here are remarkable. However, the relatively small number of children having high IgG1, IgG3 and IgG4 responses to EBA-175, and the multiple comparisons performed, lead to interpreting the protection data with caution and require further confirmation in different and larger studies. Advanced investigations are required at the cellular and molecular level to better understand what the implications of the data might be in relation to the mechanisms by which EBA-175 antibodies

mediate protection. This will help in the rational design and development of a vaccine against this leading target candidate.

List of abbreviations

ADCI, Antibody-dependent cellular inhibition; AMA-1, apical membrane antigen 1; BSA, bovine serum albumin; CI, confidence intervals; CISM, Centro de Investigação em Saúde da Manhiça; DNA, deoxyribonucleic acid; EBA-175, erythrocyte binding antigen 175; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EPI, expanded program on immunization; GM, geometric mean; IFAT, Immunofluorescence Antibody Test; Ig, immunoglobulin; IPTi, Intermittent Preventive Treatment in infants; IRR, incidence rate ratio; MSP-1, merozoite surface protein 1; NAI, naturally acquired immunity; *P. falciparum, Plasmodium falciparum;* PBS, phosphate buffered saline; PCR, polymerase chain reaction; SP, sulfadoxine-pyrimethamine

Author's contributions

CD, CM, JJA and PLA conceived and designed the study. CM, EM, PA and PLA carried out the clinical trial and IM coordinated the laboratory determinations at CISM. CD, DQ, TN and ES processed the blood samples. SP, BS, PG, AB, VC and CC produced the recombinant proteins. AM, ES, LP and CD developed the immunoassays. DQ and LP analyzed the samples. LQ, SS and JJA performed the data management and statistical analyses. CD wrote the first draft of the paper. All authors read, critically reviewed and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1 - IgG and IgM responses to asexual blood stage whole *P. falciparum* parasites, MSP-1_{19}, AMA-1 and EBA-175 in children up to 2 years of age.

For IFAT data (A), IgG levels (y axes in log scale) are expressed as end-point titers. For ELISA data (B-D), IgG (left) and IgM (right) levels are expressed as normalized OD values (y axes, percentages). In the weighted scatter plots, the area of the symbol is proportional to the number of observations. Geometric mean IgG titers and 95% confidence intervals are indicated by horizontal continuous and dashed lines, respectively. Red symbols correspond to IgG levels in children with previous or present infection.

Figure 2 - IgG subclass responses to MSP-1_{19}, AMA-1 and EBA-175 in children up to age 2 years of age.

Only children receiving IPTi with placebo are depicted here. IgG levels (y axes) are expressed as OD values. In the weighted scatter plots, the area of the symbol is proportional to the number of observations. Geometric mean IgG levels and 95% confidence intervals are indicated by horizontal continuous and dashed lines respectively.

Figure 3 - Map of the Manhiça area with the neighborhoods colored as a function of malaria incidence during the study period

The map illustrates the geographical microheterogeneity and the significant effect of neighborhood on malaria episodes and antibodies in the multilevel regression models. The study area expands a distance of less than 20 Km from north to south, and approximately 10 Km from east to west, and has two study clinics (Manhiça and Maragra). The number of children by groups of neighborhoods is indicated in the table.

Tables

Table 1. Effect of previous clinical malaria episodes and present infections on antibody levels to whole parasite (IFAT), MSP-1_{19}, AMA-1, and EBA-175. Multivariate random-effect models estimated by stepwise procedure and adjusted by age and neighborhood.

* Number of children who had previous clinical episodes: 18 at 5 months, 54 at 9 months, 60 at 12 months and 82 at 24 months. ** Number of children with current parasitemia (detected by microscopy or PCR): 36 children at 5 months, 62 at 9 months, 37 at 12 months and 78 at 24 months. NS = not significant in the forward stepwise procedure (p-enter = 0.05, from Likelihood Ratio test); *Pf* = *P. falciparum.*

Antigen	Antibody	Exposure to Malaria					
		Previous malaria episode *			Present infection **		
		Proportional difference	95% CI	P value	Proportional difference	95% CI	P value
Whole *Pf*	IgG	2.85	2.10-3.86	<0.0001	1.84	1.37-2.47	<0.0001
$MSP\text{-}1_{19}$	IgG	2.05	1.76-2.39	<0.0001	1.31	1.14-1.51	0.0001
	IgG1	2.56	2.05-3.19	<0.0001	1.94	1.59-2.37	<0.0001
	IgG2	1.14	1.06-1.23	0.0003	1.13	1.06-1.21	0.0003
	IgG3	2.31	1.89-2.83	<0.0001	1.74	1.42-2.14	<0.0001
	IgG4	1.16	1.10-1.23	<0.0001	1.09	1.03-1.16	<0.004
	IgM	NS	NS	NS	1.28	1.11-1.48	0.0005
AMA-1	IgG	1.78	1.54-2.05	<0.0001	1.26	1.10-1.43	0.0007
	IgG1	1.49	1.24-1.81	<0.0001	1.44	1.22-1.71	<0.0001
	IgG2	1.06	1.00-1.12	0.0444	1.10	1.04-1.15	0.0006
	IgG3	1.86	1.57-2.20	<0.0001	1.52	1.28-1.82	<0.0001
	IgG4	1.06	1.01-1.12	<0.0111	1.09	1.04-1.15	0.0004
	IgM	NS	NS	NS	1.25	1.12-1.39	0.0001
EBA-175	IgG	1.18	1.06-1.31	0.0025	1.12	1.01-1.24	0.026
	IgG1	1.22	1.00-1.49	0.0448	NS	NS	NS
	IgG2	NS	NS	NS	NS	NS	NS
	IgG3	NS	NS	NS	1.32	1.08-1.62	0.007
	IgG4	NS	NS	NS	NS	NS	NS
	IgM	NS	NS	NS	1.15	1.02-1.29	0.023

Table 2. Current infection was significantly associated with increased antibody levels in the subgroup of children who had previous episodes of malaria, only for the antigens and IgG isotypes included in the table. Multivariate random-effect regression models using a forward stepwise procedure (p-enter = 0.05, from Likelihood Ratio test), including the following variables: intervention group, age, number of previous malaria episodes, time from previous episodes, current infection and parasite density

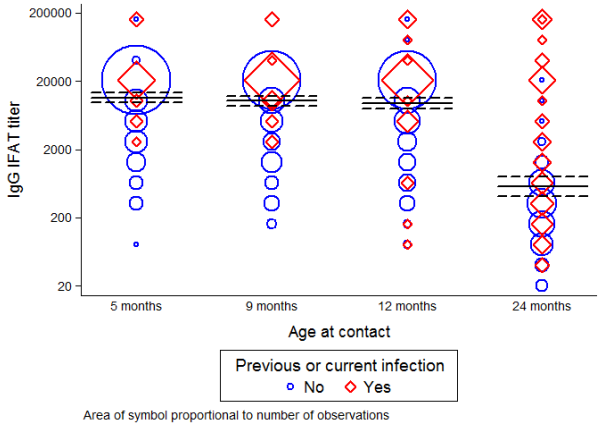
Antigen	Antibody	Proportional difference	95% CI	P value
$MSP\text{-}1_{19}$	IgG	1.61	1.24-2.09	0.0003
	IgG1	1.28	1.02-1.60	0.0332
	IgG3	1.73	1.32-2.26	<0.0001
AMA-1	IgG1	1.44	1.12-1.85	0.0044
	IgG3	1.82	1.37-2.42	<0.0001
	IgG4	1.11	1.02-1.22	0.0222
EBA-175	IgG3	1.46	1.15-1.86	0.0020

Table 3. Association between levels of antibodies (2-fold increment*)* at 5 and 12 months and the incidence of malaria at two time periods. Negative binomial regression model using likelihood ratio test, crude and adjusted by gender, IPTi treatment, previous malaria episodes, present infection, and neighborhood. The sample size at the two study periods are those indicated in the Additional File (table of antibody prevalences) for each antigen and antibody type, for months 5 and 12. IRR = Incidence rate ratio.

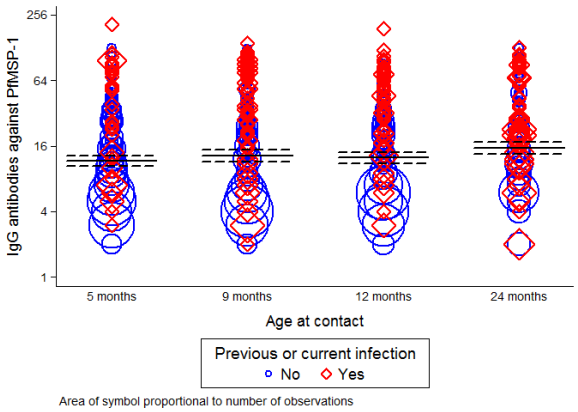
Antigen	Antibody	5-12 months						12-24 months					
		Crude			Adjusted			Crude			Adjusted		
		IRR	95% CI	P value	IRR	95% CI	P value	IRR	95% CI	P value	IRR	95% CI	P value
Whole *Pf*	IgG	1.23	0.96-1.58	0.100	0.99	0.79-1.24	0.923	1.25	0.05-1.50	0.016	1.05	0.90-1.24	0.528
$MSP\text{-}1_{19}$	IgG	1.35	1.10-1.67	0.005	1.19	0.95-1.50	0.129	1.46	1.21-1.75	<0.001	1.07	0.88-1.29	0.486
	IgG1	1.91	1.39-2.64	<0.001	1.74	1.20-2.52	0.003	1.69	1.20-2.38	0.002	0.85	0.54-1.32	0.464
	IgG2	3.65	1.35-9.84	0.013	3.41	1.36-8.53	0.015	2.37	0.83-6.71	0.096	0.73	0.30-1.82	0.504
	IgG3	1.62	1.12-2.35	0.010	1.17	0.73-1.88	0.514	1.39	0.88-2.18	0.135	0.74	0.48-1.16	0.191
	IgG4	2.91	0.36-23.8	0.299	0.85	0.13-5.42	0.863	4.58	0.89-23.4	0.045	1.13	0.39-3.31	0.824
	IgM	1.17	0.99-1.38	0.058	1.07	0.90-1.27	0.426	1.08	0.92-1.27	0.362	1.02	0.89-1.17	0.770
AMA-1	IgG	1.20	0.94-1.53	0.148	1.03	0.81-1.31	0.795	1.23	1.01-1.51	0.042	0.93	0.76-1.13	0.446
	IgG1	1.31	0.91-1.88	0.140	1.22	0.80-1.86	0.358	1.84	1.29-2.63	0.001	1.10	0.67-1.56	0.598
	IgG2	1.33	0.27-6.42	0.724	0.86	0.17-4.24	0.853	1.62	0.43-6.09	0.469	0.92	0.31-2.70	0.878
	IgG3	1.24	0.81-1.89	0.326	1.15	0.71-1.86	0.565	1.35	0.92-1.98	0.111	0.86	0.63-1.18	0.356
	IgG4	1.42	0.33-6.20	0.636	1.71	0.35-8.28	0.503	1.12	0.28-4.53	0.871	0.71	0.24-2.08	0.530
	IgM	1.14	0.89-1.47	0.308	1.07	0.85-1.35	0.579	1.06	0.84-1.35	0.609	1.03	0.85-1.26	0.750
EBA-175	IgG	1.43	1.03-1.99	0.029	1.34	0.99-1.81	0.059	1.11	0.81-1.53	0.510	0.90	0.68-1.18	0.430
	IgG1	1.48	0.75-2.94	0.257	1.77	0.98-3.21	0.052	0.87	0.35-2.13	0.755	0.49	0.25-0.97	0.026
	IgG2	2.50	0.07-84.0	0.609	2.66	0.30-23.5	0.384	0.46	0.03-6.10	0.561	0.37	0.06-2.19	0.264
	IgG3	1.58	0.86-2.91	0.139	1.12	0.75-1.67	0.581	1.50	0.73-3.08	0.269	0.44	0.19-0.98	0.037
	IgG4	0.69	0.34-1.40	0.300	0.85	0.51-1.39	0.484	0.77	0.31-1.88	0.579	3.07	1.08-8.78	0.020
	IgM	1.32	1.02-1.69	0.032	1.24	0.97-1.59	0.086	1.09	0.87-1.39	0.450	1.03	0.85-1.25	0.757

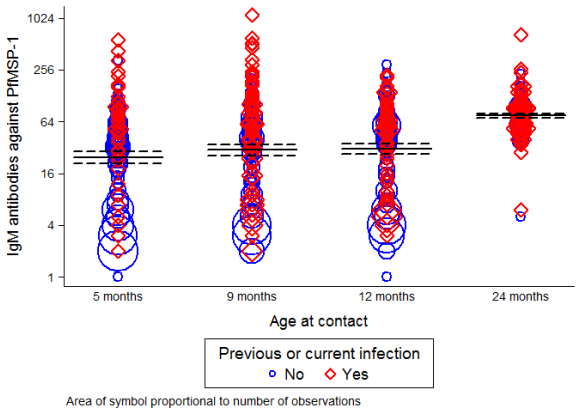
Figure 1.

A. Whole blood stage parasites by IFAT

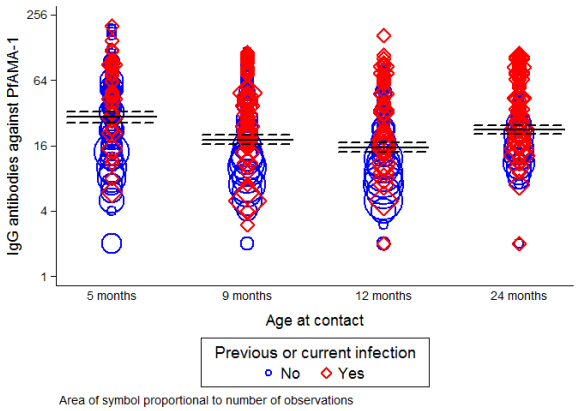


B. MSP-1





C. AMA-1



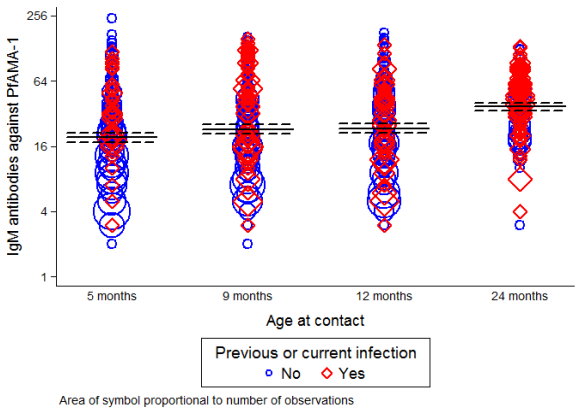
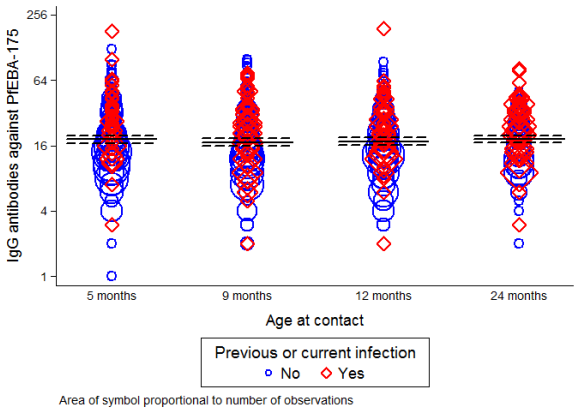


Figure 1

D. EBA-175


IgG antibodies against PfEBA-175
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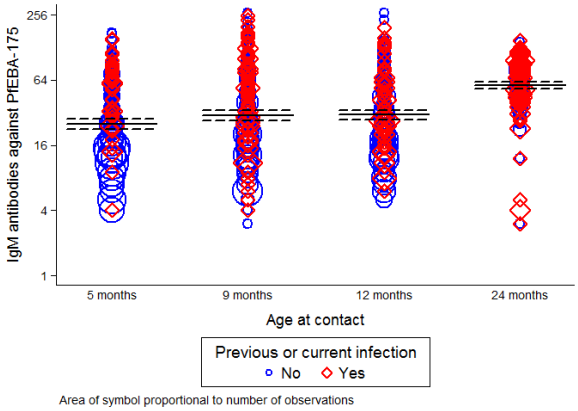
64
16
4
1
5 months
9 months
12 months
24 months
Age at contact
Previous or current infection
No
Yes
Area of symbol proportional to number of observations


IgM antibodies against PfEBA-175
256
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16
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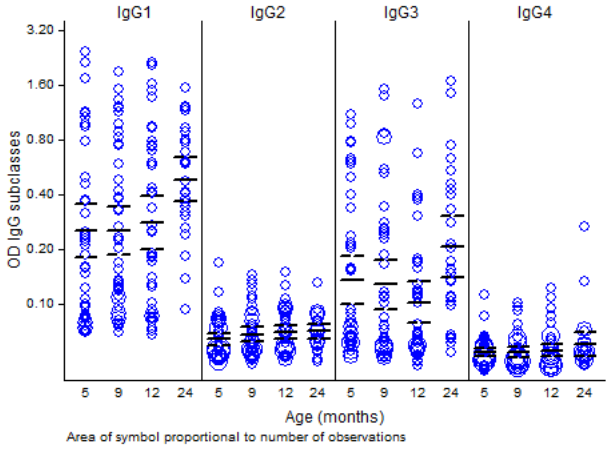
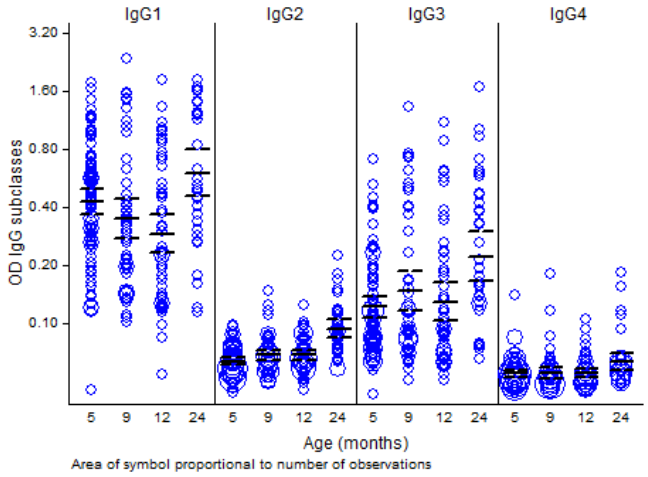
5 months
9 months
12 months
24 months
Age at contact
Previous or current infection
No
Yes
Area of symbol proportional to number of observations

Figure 2.

A. MSP-1



B. AMA-1



C. EBA-175

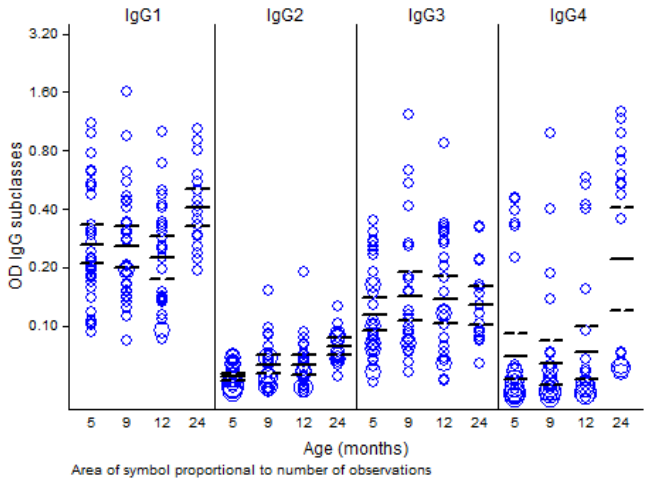
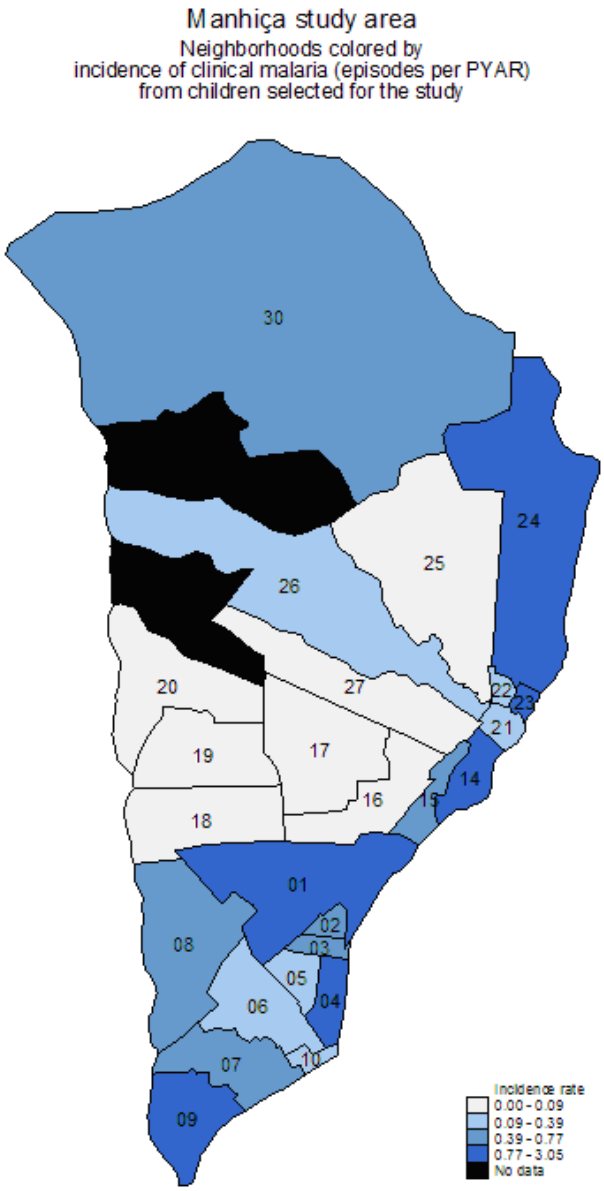


Figure 2

Figure 3.



Groups of Neighborhoods	Frequency	Percentage	Cummulative Percentage
1-2	41	11.10%	11.10%
3-4	22	6.00%	17.10%
5-6	72	19.50%	36.60%
7-8, 10	48	13.00%	49.60%
9	15	4.10%	53.70%
14-15	30	8.10%	61.80%
16-20	34	9.20%	71.00%
21-24	36	9.80%	80.80%
25	31	8.40%	89.20%
26-27,30	40	10.80%	100.00%

Figure 3

Additional file

Table of prevalence (number and percentage) of positive antibody responses against each merozoite antigen as measured by ELISA.

[*] Average geometric mean values for IgG in negative controls were: AMA-1 = 15.96; MSP-1_{19} = 15.17; EBA-175 = 21.75. [**] Only samples that were positive for IgG and with plasma remaining were analysed for IgG isotypes

Age (n samples)	Antigen	Ig isotypes	N total	n positives[*]	% Positives
5 months (n = 293)	MSP-1_{19}	IgG[**]	293	73	24.91
		IgG1	72	35	48.61
		IgG2	72	0	0
		IgG3	72	29	40.28
		IgG4	72	9	12.50
		IgM	293	36	12.29
	AMA-1	IgG	289	179	61.94
		IgG1	176	148	84.09
		IgG2	176	0	0
		IgG3	176	19	10.80
		IgG4	176	21	11.93
		IgM	293	65	22.18
	EBA-175	IgG	293	71	24.23
		IgG1	69	44	63.77
		IgG2	69	0	0
		IgG3	69	4	5.80
		IgG4	69	6	8.70
		IgM	293	61	20.82

9 months (n = 290)	MSP-1_{19}	IgG	290	88	30.34
		IgG1	87	48	55.17
		IgG2	87	0	0
		IgG3	87	42	48.28
		IgG4	87	19	21.84
		IgM	290	50	17.24
	AMA-1	IgG	290	116	40.00
		IgG1	110	71	65.55
		IgG2	110	0	0
		IgG3	110	27	24.55
		IgG4	110	15	13.64
		IgM	290	87	30.00
	EBA-175	IgG	290	57	19.66
		IgG1	55	29	52.73
		IgG2	55	0	0
		IgG3	55	2	3.64
		IgG4	55	6	10.91
		IgM	290	75	25.86

12 months (n = 291)	MSP-1_{19}	IgG	291	77	26.46
		IgG1	74	41	55.41
		IgG2	74	0	0
		IgG3	74	22	29.73
		IgG4	74	13	17.57
		IgM	291	44	15.12
	AMA-1	IgG	291	94	32.30
		IgG1	93	50	53.76
		IgG2	93	0	0
		IgG3	93	22	23.66
		IgG4	93	12	12.90
		IgM	291	92	31.62
	EBA-175	IgG	291	58	19.93
		IgG1	55	26	47.27
		IgG2	55	0	0
		IgG3	55	1	1.82
		IgG4	55	7	12.73
		IgM	291	77	26.46

24 months (n = 232)	MSP-1_{19}	IgG	230	51	22.17
		IgG1	49	43	87.76
		IgG2	49	0	0
		IgG3	49	38	77.55
		IgG4	49	11	22.45
		IgM	230	44	19.13
	AMA-1	IgG	230	63	27.39
		IgG1	60	47	78.33
		IgG2	60	0	0
		IgG3	60	28	46.67
		IgG4	60	9	15.00
		IgM	230	70	30.43
	EBA-175	IgG	230	39	16.96
		IgG1	36	30	83.33
		IgG2	36	0	0
		IgG3	36	1	2.78
		IgG4	36	4	11.11
		IgM	230	89	38.70

ARTICLE 3

IgG against *Plasmodium falciparum* variant surface antigens and growth inhibitory antibodies in Mozambican children receiving intermittent preventive treatment with sulfadoxine-pyrimethamine

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ABSTRACT

This study aimed to evaluate whether intermittent preventive treatment in infants with sulfadoxine-pyrimethamine (IPTi-SP) had an effect on the acquisition of IgG against *Plasmodium falciparum* variant surface antigens (VSA) and growth-inhibitory antibodies in Manhiça, Mozambique. In addition, we assessed factors affecting the magnitude of these responses and the association between antibody levels and protection against malaria.

IgG to VSA expressed by MOZ2, R29 and E8B parasite isolates were measured in plasma samples collected at 5, 9, 12 and 24 months of age by flow cytometry. Growth-inhibitory antibodies in dialyzed plasmas using GFP-D10 parasites were measured by flow cytometry at 12 and 24 months.

IPTi-SP did not significantly modify the levels of IgG against VSA nor the growth-inhibitory capacity of antibodies up to 2 years of age. Age but not previous episodes of malaria influenced the magnitude of these responses. In addition, anti-VSA IgG levels were 7% higher in children with current *P. falciparum* infection and were associated with neighborhood of residence. Children aged 24 months had 10% less parasite growth than those aged 12 months (95% CI 0.88–0.93, $P < 0.0001$). Growth-inhibitory antibodies correlated with levels of IgG against AMA-1, when evaluating the 10% ($R^2 = 0.444$, $P = 0.049$) and 20% ($R^2 = 0.230$, $P = 0.037$) highest inhibitory samples. None of the responses were associated with subsequent risk of malaria.

In conclusion, IPTi-SP does not negatively affect the development of antibody responses thought to be major contributors to the acquisition of immunity to malaria in infancy.

Introduction

The intermittent preventive treatment in infants (IPTi) consisting in the administration of a full dose of an antimalarial drug within the expanded program on immunization (EPI), has been shown in different settings to reduce the risk of malaria in this vulnerable group that suffers the highest burden of the disease (Aponte et al. 2009; Gosling et al. 2009; Odhiambo et al. 2010). In the context of a randomized, placebo-controlled trial of IPTi with SP (IPTi-SP) in Mozambique (Macete et al. 2006), we previously evaluated the impact of this intervention on antibody responses to *Plasmodium falciparum* merozoite antigens thought to be involved in

Abbreviations: AMA-1, apical membrane antigen 1; BSA, bovine serum albumin; CI, confidence intervals; DNA, deoxyribonucleic acid; DTP/OPV/Hep B, diphteria + tetanus + pertussis/oral polio vaccine/hepatitis B; EBA-175, erythrocyte binding antigen 175; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EPI, expanded program on immunization; GFP, green fluorescence protein; GIA, growth inhibition assay; GM, geometric means; IE, infected erythrocytes; Ig, immunoglobulin; IPTi, intermittent preventive treatment in infants; ITC, insecticide-treated curtains; ITN, insecticide-treated bed nets; MFI, mean fluorescence intensity; MSP-1, merozoite surface protein 1; NAI, naturally acquired immunity; NIE, non-infected erythrocytes; *P. falciparum*, *Plasmodium falciparum*; PBS, phosphate buffered saline; PCR, polymerase chain reaction; pLDH, *Plasmodium* lactate dehydrogenase; SP, sulfadoxine-pyrimethamine; VSA, variant surface antigens.

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erythrocyte invasion and considered important targets of naturally acquired immunity (NAI) and prime malaria vaccine candidates (Quelhas et al. 2008). We showed that IPTi-SP did not modify the levels of antibodies to such antigens in the first 2 years of life, and found some evidence that IPTi-SP may lead to enhanced IgG responses to certain antigens at certain time points (Quelhas et al. 2008). Our previous study did not include assessment of the effect of this intervention on other antibodies that are considered important, such as IgG to *P. falciparum* variant surface antigens (VSA), or growth inhibitory antibodies.

VSAs are considered major targets of protective immunity (Bull et al. 1998; Hviid 2010), mediating processes like parasite cytoadherence and sequestration (David et al. 1983) and antigenic variation (Smith et al. 1995; Beeson and Brown 2002) possibly contributing to immune evasion and malaria pathogenesis. Prior studies have evaluated antibodies to VSAs as a measurement of NAI to malaria, initially by agglutination assays (Forsyth et al. 1989; Bull et al. 1998) and in recent years by flow cytometry (Staalsoe et al. 1999). Earlier studies showed that the plasma titer of agglutinating activity was the only predictive index for protection against clinical malaria (Marsh et al. 1989). Subsequent studies showed that the expansion of antibody repertoire against VSA types between 1 and 5 years was important for developing NAI (Bull et al. 1998; Hviid and Staalsoe 2004) and that levels of IgG to VSA correlated with protection from clinical malaria (Dodoo et al. 2001; Ofori et al. 2002). Fewer studies have evaluated the impact of malaria control interventions on IgG responses to VSAs. Children using insecticide-treated bed nets (ITN) had lower VSA antibody levels and recognized a smaller proportion of VSA types (Askjaer et al. 2001). Pregnant women receiving IPT had reduced levels of anti-VSA IgG (Staalsoe et al. 2004b; Serra-Casas et al. 2010), that have been associated with protection against low birth weight and maternal anemia (Staalsoe et al. 2004a). Altogether, these findings indicate that responses to VSA may be modified by these specific interventions.

Functional antibodies that inhibit parasite invasion of erythrocytes (Cohen and Butcher 1969; Egan et al. 1999; Hodder et al. 2001) or act in conjunction with monocytes to inhibit parasite growth (Bouharoun-Tayoun et al. 1995) are likely to contribute to NAI. The magnitude of the antibody response does not fully reflect the functional activity, and growth inhibition assays (GIA) are available for determining the ability of antibodies to inhibit parasite growth *in vitro* (Khusmith and Druilhe 1983; Lunel and Druilhe 1989). At present, measurement of *Plasmodium* lactate dehydrogenase (pLDH) by spectrophotometry (Bergmann-Leitner et al. 2008) and of parasite stained DNA by flow cytometry (Persson et al. 2006), coupled to advances in transfection, have facilitated the development of assays to measure functional inhibitory antibodies to specific antigens, such as MSP-1 (O'Donnell et al. 2001; Saul and Miller 2001), AMA-1 (Dutta et al. 2003; Healer et al. 2004) and EBA-175 (Persson et al. 2008), suitable for high-throughput application in large pediatric studies. Only a few prior studies have applied GIA to assess naturally acquired and vaccine induced immunity in humans (Bolad et al. 2003; Crompton et al. 2010; Dent et al. 2008; McCallum et al. 2008; Roestenberg et al. 2008; Thera et al. 2008; Courtin et al. 2009). In one study, very little inhibitory responses were found in young children, possibly reflecting the late and complex acquisition of such functional capacity (Bolad et al. 2004). In other studies growth inhibitory activity appeared to decrease or remain relatively unchanged with age (Dent et al. 2008; Courtin et al. 2009) and exposure (McCallum et al. 2008). In one of these studies, time-to-infection was significantly associated with degree of growth inhibition, suggesting a protective effect for such antibodies (Dent et al. 2008), but other studies have shown no clear correlation with resistance to subsequent malaria (Marsh et al. 1989; McCallum et al. 2008; Crompton et al. 2010).

A limited number of studies have used GIA for functional assessment of antibody responses in relation to malaria control interventions. In one study, the use of insecticide-treated curtains (ITC) in children did not translate into a reduction of growth inhibition (Bolad et al. 2004), but others have reported that malaria transmission intensity does influence the level of growth-inhibitory antibodies (McCallum et al. 2008) suggesting that malaria control interventions may impact the acquisition of growth inhibitory antibodies.

As none of the previous studies have evaluated how the acquisition of growth-inhibitory or VSA antibodies might be affected by IPTi we addressed this question as part of our studies in Mozambique, and compared measured functional responses to those reported in our previous serological studies (Quelhas et al. 2008). We examined whether the level of anti-VSA antibodies or the percentage of growth-inhibitory antibodies during the first 2 years of life differed significantly in children receiving IPTi-SP compared to those receiving placebo.

Materials and methods

Study area

The study was conducted at the Centro de Investigação em Saúde da Manhiça, Manhiça District, southern Mozambique. The climate is subtropical, with a warm rainy season from November to April and a cool dry season during the rest of the year. Perennial malaria transmission is mostly due to *P. falciparum*. *Anopheles funestus* is the main vector, and the estimated average number of infective mosquito bites per person per year was 38 at the time of the study (Alonso et al. 2002). Efficacy of SP in children in this area showed a combined (early and late) therapeutic efficacy rate of 83%, with an *in vivo* parasitological sensitivity of 78.6% at day 14 (Abacassamo et al. 2004).

Study design

The efficacy study was an individually randomized, placebo-controlled trial (Macete et al. 2006). Infants were recruited from those attending the EPI clinic to receive dose 2 of the DTP/OPV/Hep B vaccines between September 2002 and February 2004. Treatment with SP or placebo was administered at 3, 4, 9 months of age alongside the routine vaccinations. Cross-sectional visits were scheduled at 5, 9, 12 and 24 months of age, when 1 ml of blood was collected by fingerprick into EDTA microtainers, to obtain plasma and erythrocyte pellet. Clinical surveillance for malaria morbidity was done through a passive case detection system based on the reporting of all malaria cases detected in children attending the Manhiça outpatient clinic.

For the immunological studies, we included the last 501 children recruited in the main IPTi trial. Among those, 302 were selected for analysis of antibody responses because they fulfilled the following criteria: (i) having received all 3 doses of SP or placebo, (ii) having plasma available to conduct all the serological determinations, and (iii) having an equal representation of SP- and placebo-recipients. Those children who came for all 4 visits were prioritized. For anti-VSA antibody determinations plasmas from all four visits were tested, and for GIA plasmas from the 12 and 24 months visits were tested. All immunological assays were performed by personnel who were blinded.

Informed consent was obtained from all parents or guardians and ethical approval for the protocol was granted from the ethics review committees of Mozambique and the Hospital Clinic, Barcelona, Spain. The trial registration number is NCT00209795 (http://clinicaltrials.gov).

Antibodies specific to P. falciparum variant surface antigens

Parasite isolates and in vitro culture

A pool of three *P. falciparum* isolates were used: MOZ2 (Serra-Casas et al. 2010), a Mozambican pediatric isolate adapted to *in vitro* culture (Trager and Jensen 1976), and two laboratory lines, R29 (Rowe et al. 1997) and E8B (Beeson et al. 2004). These parasites are different at the transcriptional level for *var* gene expression and at the phenotypic level for cytoadhesion patterns (Mayor A, unpublished data). Mature trophozoites of each isolate grown in group O erythrocytes were cryopreserved in multiple aliquots to minimize interassay variations among different experiments (Kinyanjui et al. 2004). The required amount of aliquots from the three isolates were gradually thawed, pooled together and adjusted to a parasitemia level of 1–3% and 1% hematocrit prior to starting each assay.

Measurement of P. falciparum anti-VSA specific IgG

Anti-VSA plasma IgG levels were measured by flow cytometry (Staalsoe et al. 1999). Plasma samples obtained from the same child at visits 5, 9 and 12 months were processed in parallel and those from visit at 24 months were then processed all together. In each well of a 96-well round-bottom plate, 95 μl of parasite suspension were incubated with 5 μl of test plasma at room temperature for 30 min and sequentially incubated for another 30 min with 100 μl of polyclonal rabbit anti-human IgG (Dako, Glostrup, Denmark) diluted at 1:200 and 100 μl of alexa fluor-conjugated donkey anti-rabbit IgG diluted at 1:1000 (Invitrogen, Carlsbad, CA, USA) plus 10 μg/ml ethidium bromide. All dilutions were made in phosphate buffered saline (PBS, SIGMA) + 1% bovine serum albumin (BSA, SIGMA). Cells were washed three times in the same PBS-BSA solution between each incubation step. We verified that no significant agglutination was occurring under the assay conditions.

Data was acquired with a FACSCalibur and analyzed using CellQuest Pro software (BD Biosciences, USA). Infected erythrocytes (IE) and non-infected erythrocytes (NIE) were gated according to their ethidium bromide fluorescence and reactivity against the IE surface was expressed as the ratio between the mean fluorescence intensity (MFI) of IEs and the MFI of NIEs. A pool of plasma samples obtained from 15 malaria-exposed Mozambican adults was used to normalize the data from different assays. Plasma from 11 Spanish individuals with no previous exposure to malaria served as negative controls.

P. falciparum growth inhibitory antibodies

Sample treatment

GIA assays were conducted at the Walter and Eliza Hall Institute, Melbourne, Australia. All plasma samples were first heated on a block at 56 °C for 20 min to inactivate complement. To remove non-specific inhibitors such as antimalarial drugs and antibiotics and to equilibrate pH, plasma aliquots of 100 μl were dialyzed against PBS (pH 7.3) in 50 kDa-MWCO microdialysis tubes (Chemicon, Temecula, CA, USA) over 48 h and then re-concentrated to the original starting volume using centrifugal concentration tubes (100-kDa MWCO; Pall Corp., Ann Arbor, MI, USA), as described (Persson et al. 2006).

Parasite line and culture synchronization

The D10 strain transfected to express a green fluorescent protein (GFP) and a pyrimethamine-resistant plasmid insert (Wilson et al. 2010), was cultured in RPMI-HEPES with 5% pooled serum and 0.25% Albumax II. The culture medium was supplemented with glutamine (2 mM) and hypoxanthine (50 μg/ml). Pyrimethamine 200 μM was added to maintain the PfM3' wildtype plasmid (O'Donnell et al. 2001) and blasticidin-S-HCL 5 mg/ml was added to maintain the fluorescent plasmid. To prevent bacterial contamination, gentamicin 50 μg/ml was added to the culture medium. Two weeks prior to performing the assays, IE with a starting parasitemia of 2% were kept synchronous in culture by adding heparin (Pharmacia, Perth, Australia) at a final concentration of 50 IU/ml after feeding (Boyle et al. 2010a,b). Heparin was washed off with RPMI medium during the invasion window period (4–8 h) at the end of which heparin was added back and culture proceeded. Late pigmented trophozoite or schizont stage parasites were harvested for the assays.

Growth inhibition over two cycles of parasite replication

With a starting parasitemia of 0.1–0.3% and haematocrit at 1%, a two-cycle assay was performed as described (Persson et al. 2006). To evaluate immune sera, duplicate cultures were set up on U-bottom 96-well tissue culture plates (BD Biosciences, San Jose, CA, USA). Positive inhibition controls (0.25 mg/ml heparin inhibiting >90% of growth (Boyle et al. 2010a,b); 0.5 mg/ml anti-AMA1 monoclonal antibody 1F9 inhibiting around 50% of growth) (Coley et al. 2006) were included for each assay and negative controls (3 individual plasmas from non-exposed Australian donors) were incorporated for each of the multiple plates.

To each well, 5 μl of sample or control and 45 μl of parasite suspension were added. All outer wells were filled with PBS to prevent evaporation of inner wells. Plates were placed in a sealed humidified chamber, gassed with a mix of 95% N_2, 4% CO_2, 1% O_2, and incubated at 37 °C for 80–96 h. At 48 h of culture, 10 μl fresh medium were added to each well, and the chamber was gassed again. Initial parasitemia and parasite stage were monitored by microscopy of a smear prepared at set-up, and a sample culture was kept in parallel in the incubator to monitor developmental stage of parasites and guide the timing of harvesting parasites. Extreme care was taken at all steps to avoid yeast or bacteria contamination of the cultures leading to nonspecific inhibition.

Measurement of growth inhibition

At the end of two complete cycles, when parasites were at the late-ring to mid-pigmented trophozoite stage, parasitemia was measured on a FACSCalibur cytometer, using a high-throughput plate sampler. All samples were tested in duplicate and in two separate assays. Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). GIA values are expressed as percentage of parasite growth in study subjects relative to non-exposed Australian donors [(mean parasitemia of test sample/mean parasitemia of control sample) × 100].

Definitions and statistical methods

Infection was defined as the presence of asexual *P. falciparum* parasites of any density in a blood smear measured by microscopy and/or by PCR. A clinical malaria episode was defined as the latter plus an axillary temperature of 37.5 °C or history of fever within the prior 24 h.

A Shapiro-Wilk normality test revealed a non-normal distribution of all data, therefore values were log transformed, a more symmetrical distribution was obtained and data was analyzed using parametric methods. Averages were expressed as geometric means (GM) plus 95% confidence intervals (CI). The distribution of antibody responses at each time point in SP- and placebo-recipients are presented as (i) weighted scattered plots and (ii) reverse cumulative distribution plots (Reed et al. 1995).

T-test and chi-square or fisher exact test were used to compare mean levels or prevalence (tertiles of distribution), respectively, between treatment groups. Crude and adjusted linear regression models were used to estimate the effect of IPTi-SP on the antibody levels at each visit.

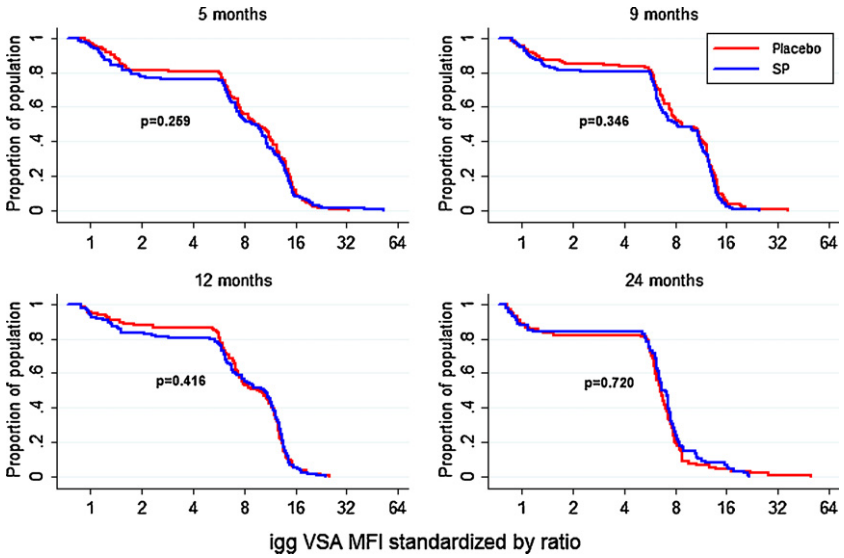

Fig. 1. IgG responses to VSA in infants receiving IPTi with SP (blue) or placebo (red), at ages 5, 9, 12 and 24 months, illustrated as reverse cumulative distribution functions. IgG levels (X axis) are expressed as mean fluorescence intensity (MFI) values standardized by ratio. Average value for negative controls was 7.8. The Y axis represents the proportion of children from each group that has a given MFI value or higher. Statistical significance between the two treatment groups was analyzed by linear regression.

A multivariate random-effect linear model was estimated to evaluate the proportional differences in VSA IgG levels between treatment groups adjusted by the following variables: IgG VSA MFI in non-infected red cells, age at visit, treatment, clinical malaria episodes before visit, malaria infection at visit. For these models, variance was discomposed in between-batch (an experiment run on a given day) and between-subject, as well as the residual standard deviation. Intraclass correlations were estimated based on the results of the above mentioned components of variance.

Linear regression models with random intercept were estimated to identify variables independently associated with growth inhibitory activity of antibodies. Selection of the variables for the final model was done through a stepwise procedure, where the criterion for including or excluding a variable was having a *P*-value $\leq$0.05 or $\geq$0.10, respectively from the Likelihood ratio test. The models took into account repetitive measurements in the same individual and estimated both inter-individual and intra-individual variability. In a first model that included all children, the variables were intervention group (SP or placebo), age at visit, the occurrence of previous clinical episodes (yes/no) and neighborhood of residence. The 30 neighborhoods located in the Manhiça study area were grouped into 10 for the estimation of multivariate models. In a second model, including only children with at least one clinical malaria episode, the variables were treatment group, age, number of previous episodes, parasite density, time from last episode, and current infection.

A Pearson correlation coefficient was used to measure the degree of correlation between *P. falciparum* growth and levels of IgG against recombinant antigens MSP-1, AMA-1, EBA-175 assessed by ELISA (Quelhas et al. 2008).

Crude and adjusted negative binomial regression models were estimated using a stepwise procedure to evaluate the relationship between the level of antibodies at ages 5 and 12 months and the incidence of clinical malaria episodes from 5 to 12 or from 12 to 24 months, respectively. Co-variates included were: sex, neighborhood, treatment group and previous episodes. An additional model including all antibody measurements in this and the previous study (IgG against whole parasite, IgG [total or isotypes] and IgM against merozoite antigens) was estimated to identify if any of the antibody variables was independently associated with the incidence of subsequent clinical malaria.

To estimate the incidences, the time at risk was calculated as the number of person years at risk (PYAR; episodes per 365.25 days) since the beginning of the time at risk until the end of follow-up, migration, death or withdrawn consent, whichever occurred first. An arbitrary lag of 21 days was applied after a case of clinical malaria. Children did not contribute to the time at risk or the clinical malaria cases during the lag periods. Months were defined as 30.4 days.

Data analysis was performed using Stata 11 for Windows (Stata Corporation, College Station, TX). Statistical significance was defined as a *P* of <0.05.

Results

Antibody responses to P. falciparum VSA in relation to IPTi-SP

We found no significant differences in IgG responses to VSA between the SP and the placebo groups, at any of the time points (Fig. 1), when analyzed by GMs or linear regression (Table 1), or by tertiles (data not shown). Similarly, no significant differences in IgG responses were observed after adjusting the linear regression analyses by previous episodes of clinical malaria (Table 1).

Multivariate analysis to evaluate factors associated with anti-VSA antibodies showed that levels of IgG against VSA decreased significantly with age ($P<0.0001$), and were 7% higher in children with *P. falciparum* infection at the time of sample collection (Table 2). Previous malaria episodes were not associated with the levels of VSA antibodies (Table 2). Neighborhood was also independently associated with levels of anti-VSA antibodies and, consistent with the first analysis, IPTi treatment did not affect VSA IgG levels (Table 2). All of the above analyses took into account the variability between batches and between subjects and according to the intraclass correlations, the measures of the same batch showed to be more similar than the measures of the same individual.

Antibody-mediated growth inhibition of blood stage P. falciparum in relation to IPTi-SP

The growth inhibition assay results were divided into three equal groups (tertiles) to define categories of parasite growth.

Table 1
Comparison of levels of IgG against variant surface antigens (VSA) and antibody-mediated growth inhibition (GIA) between IPTi treatment groups for each cross-sectional visit.

Variable	Age (months)	Treatment group	Subjects	Geometric mean	95% confidence interval	*P*-value[a]	Crude analysis[b]		Adjusted analysis[b,c]	
							Proportional difference	*P*-value	Proportional difference	*P*-value
IgG-VSA (MFI)	5	Placebo	124	7.23	6.14–8.51	0.4216	1	0.4194	1	0.2595
		SP	134	6.57	5.54–7.78		0.91		0.87	
	9	Placebo	119	7.18	6.13–8.42	0.3573	1	0.3548	1	0.3464
		SP	122	6.46	5.48–7.61		0.90		0.90	
	12	Placebo	112	7.50	6.45–8.72	0.4373	1	0.4348	1	0.4160
		SP	116	6.86	5.80–8.11		0.92		0.91	
	24	Placebo	89	5.26	4.39–6.30	0.6503	1	0.6480	1	0.7204
		SP	88	5.57	4.66–6.66		1.06		1.05	
GIA (% growth)	12	Placebo	126	87.29	84.79–89.85	0.5450	1	0.5429	1	0.6573
		SP	117	86.27	84.18–88.41		0.99		0.99	
	24	Placebo	95	78.09	74.98–81.34	0.6703	1	0.6683	1	0.6653
		SP	104	78.96	76.49–81.50		1.01		1.01	

MFI = mean fluorescence intensity.
[a] *T* test.
[b] Linear regression.
[c] Adjusted by previous episodes of clinical malaria.

Tertiles for growth were defined as "low" (from 38.50 to 79.81), "medium" (from 79.81 to 90.76), and "high" (from 90.76 to 125.20) percent growth, i.e. those with highest inhibition will be in the "low" growth tertile. According to this definition, at 12 months 23.9% (39 of 163) and at 24 months 49.6% (68 of 137) of the subjects were in the "low" growth tertile.

No significant differences were found in GIA activity between IPTi treatment groups at 12 or at 24 months, either in the crude or the adjusted analyses (Table 1). The two most inhibitory plasmas (38.50% and 38.66% growth, within the 10 percentile) belonged to each of the treatment groups (1 = SP and 1 = placebo).

When assessing what other factors could affect GIA activity in children receiving IPTi-SP, only age was significantly associated with growth inhibition activity. When including all subjects, children aged 24 months had 10% less parasite growth than those aged 12 months (95% CI 0.88–0.93, $P < 0.0001$) (Fig. 2), taking into account intra-individual variation. When looking only at the subjects with previous malaria episodes, children aged 24 months had 14% less parasite growth than those aged 12 months (95% CI 0.80–0.93, $P = 0.0002$).

Correlation between GIA activity and antibody levels by ELISA

Although we did not assess antigen-specific inhibitory activity, we compared GIA responses to those found in previous ELISA studies measuring antibodies to merozoite antigens putatively involved in invasion processes (Quelhas et al. 2008). A significant inverse correlation was observed between *P. falciparum* growth and levels of IgG against AMA-1 by ELISA, when evaluating the 10% ($R^2 = 0.444$, $P = 0.049$) and 20% ($R^2 = 0.230$, $P = 0.037$) highest inhibitory samples (Fig. 3). The strength of the correlation decreased when more samples (less inhibitory) were included in the analysis. No significant correlation was found between *P. falciparum* growth and levels of IgG against VSA (data not shown).

Level of antibodies to VSA and growth inhibitory antibodies and incidence of clinical malaria

Association of anti-VSA IgG levels or GIA activity with incidence of subsequent malaria episodes was evaluated both by crude and adjusted analyses, but only the latter is reported here. Anti-VSA IgG levels were not associated with future risk of malaria

Table 2
Variables that affect the levels of VSA antibodies, analyzed by linear regression.

Variable	Proportional difference	95% CI	*P* value	Overall *P* value[a]
Age at visit				
5 months	1	–	–	<0.0001
9 months	0.93	0.89–0.96	0.0001	
12 months	0.94	0.91–0.98	0.0038	
24 months	0.68	0.61–0.76	<0.0001	
Treatment				
Placebo	1	–	–	–
SP	0.98	0.94–1.02	0.3422	
Previous clinical malaria episodes[b]				
No	1	–	–	–
Yes	0.97	0.93–1.03	0.3306	
Present malaria infection[c]				
No	1	–	–	–
Yes	1.07	1.01–1.12	0.011	
Neighborhood	–	–	–	0.0256

[a] Likelihood-ratio test.
[b] Number of children who had previous clinical episodes: 18 at 5 months, 54 at 9 months, 60 at 12 months and 82 at 24 months.
[c] Number of children with current parasitemia (detected by microscopy or PCR): 36 children at 5 months, 62 at 9 months, 37 at 12 months and 78 at 24 months.

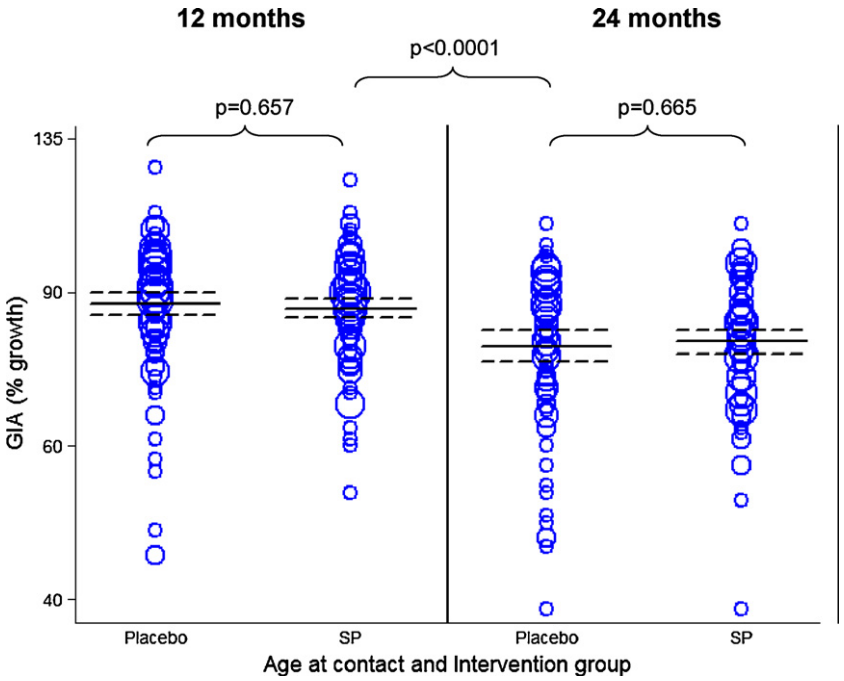

Fig. 2. *P. falciparum* growth at ages 12 and 24 months in children who received IPTi with SP or placebo. Y axis shows parasite growth (%). Area of the symbol is proportional to the number of observations. Geometric mean and 95% confidence intervals are indicated by horizontal continuous and dashed lines, respectively. *P* values shown correspond to comparisons between treatment groups, and between ages (grouping placebo and SP) by linear regression.

during the first or second years of life. For antibodies measured at 5 months of age, the incidence rate ratio (IRR) for clinical malaria between 5 and 12 months of age per a 2-fold increment in the value of anti-VSA antibodies was 1.12 (95% CI 0.89–1.41, $P = 0.3416$). For antibodies measured at 12 months of age, the IRR for malaria in the period between 12 and 24 months per a 2-fold increment in the value of anti-VSA antibodies was 0.98 (95% CI 0.78–1.22, $P = 0.8363$). Both crude and adjusted analyses by group-

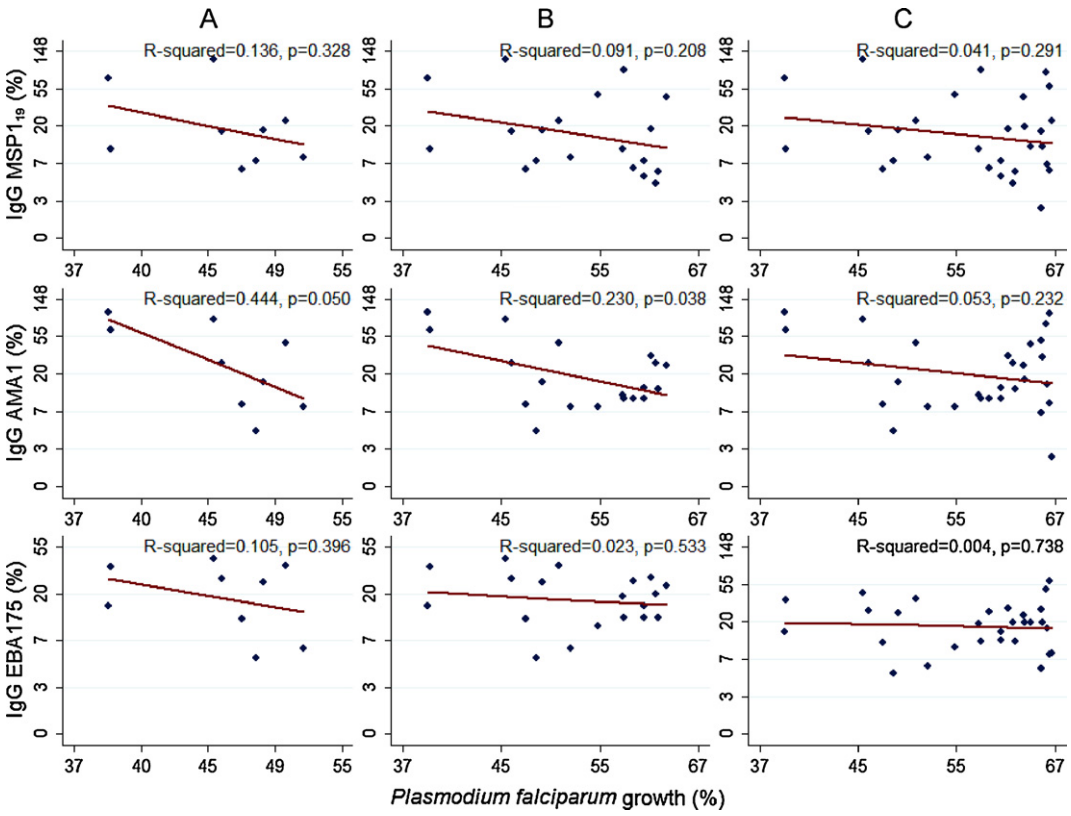

Fig. 3. Correlation between *P. falciparum* growth and levels of IgG against MSP-1 (top row), AMA-1 (middle row), EBA-175 (bottom row) measured by ELISA (Quelhas et al., 2008) by Pearson coefficient analysis. (A) 10% highest inhibitory samples; (B) 20% highest inhibitory samples; (C) 30% highest inhibitory samples. Y axis shows IgG levels (normalized OD values, %) and X axis shows parasite growth (%).

ing antibody levels into tertiles yielded the same results (data not shown).

No association was observed between GIA activity at 12 months and subsequent incidence of malaria during the interval 12–24 months. The malaria IRR per 2-fold increment in the levels of inhibitory antibodies at 12 months was 3.20 (95% CI 0.72–14.23, $P = 0.1226$). Analyses by tertiles yielded the same results (data not shown).

A global regression analysis including all the different antibody variables measured at 5 and 12 months was used to assess the relationship between these and the risk of subsequent clinical malaria (between 5–12 and 12–24 months, respectively). Significant associations were only observed for factors other than antibody levels; these were the neighborhood of residence ($P < 0.0001$) at both 12 and 24 months of age, and the occurrence of previous episodes of clinical malaria in the 12–24 months period ($P = 0.0035$). There were no documented characteristics in the neighborhoods (e.g. proximity to swamps, rivers, etc.) that could explain these spatial associations.

Discussion

This study shows that IPTi-SP administered at 3, 4, and 9 months of age does not substantially modify the levels of antibodies to *P. falciparum* variant surface antigens in the first 2 years of life in Mozambican children. In addition IPTi does not affect the development of growth inhibitory antibodies in the second year of life. Combined with the findings in our previous study (Quelhas et al. 2008), the current work further supports the hypothesis that this intervention does not negatively affect the development of naturally acquired antibody responses to malaria.

It has been suggested that the reduction in exposure to *P. falciparum* by malaria prevention strategies might interfere with the development of malaria-specific immunity in children and adults. This has been the case for the use of extended chemoprophylaxis in various settings (Bjorkman et al. 1986; Bradley-Moore et al. 1985; Geerligs et al. 2003; Greenwood et al. 1995; Hogh et al. 1994; Laing 1984; Menendez et al. 1997; Pringle and Avery-Jones 1966), where a significant reduction of malaria antibody responses was observed after termination of prophylaxis. Neither anti-VSA nor growth-inhibitory antibodies were measured in such studies. Other interventions that have been evaluated in children include the use of ITN, which after 4 years resulted in a significantly lower magnitude and breadth of VSA antibody responses (Askjaer et al. 2001), and the use of ITC, which did not affect growth-inhibitory antibodies (Bolad et al. 2004). Two studies have assessed the impact of IPT with SP for malaria prevention during pregnancy (IPTp) on the development of VSA responses. Kenyan primigravidae women receiving IPTp had significantly lower levels of IgG to CSA-binding VSA than those receiving a placebo (Staalsoe et al. 2004b). A study conducted in Mozambique also observed that IgG against VSA in mothers at delivery were lower in the SP group than in the placebo group, although statistical difference was lost when the overall analysis was adjusted according to maternal HIV status and parity (Serra-Casas et al. 2010). Our study is the first to evaluate such responses in children receiving IPTi. In contrast to the IPTp studies, we did not observe any reduction of responses in the SP treatment group. In our IPTi study there was no knowledge of the HIV status of the infants, which could also be a factor influencing antibody responses under IPTi treatment, although the prevalence of HIV is expected to be lower in this age group.

In the current study, increased anti-VSA IgG levels were associated with concurrent infection, which is consistent with what we observed for antibodies against merozoite antigens or whole *P. falciparum* lysate (Quelhas et al. 2008). In contrast, anti-VSA IgG levels were not associated with previous malaria episodes. In Tanzania, VSA-specific antibody responses were strongly related to the level of *P. falciparum* exposure (transmission intensity), and increased with age between 5 and 24 months (Vestergaard et al. 2008). In Manhiça, VSA levels were low throughout the 2 years and decreased significantly from age 12 to 24 months, suggesting that these were predominantly maternal antibodies. In fact, the very low signal-to-noise ratio of VSA MFI values obtained in these young subjects represented an experimental challenge and led us to explore new approaches to analyze VSA data. We considered MFI values of non-infected erythrocyte as one of the fixed parts of our statistical models in which the infected erythrocyte MFI value was the dependent variable. This was mathematically equivalent to using a ratio between VSA values in infected and uninfected erythrocytes, as done in other studies (Beeson et al. 2007). These values were then normalized against a factor of variability between batches. In our study children, antibodies to VSA remained low up to 2 years of age, therefore their acquisition appears to happen at a slower rate at older ages, as observed in other studies conducted in the area (Campo JJ, Dobaño C, unpublished data). Low IgG levels could explain why no association was observed between anti-VSA IgG levels and risk of malaria. Our choice of a pool of three *P. falciparum* isolates (MOZ2, R29 and E8B) was believed to give reasonable representation of the variants expressed in the population. Other studies in different age groups have found variable results; some reported associations between VSA antibodies and protection but others have not (Marsh et al. 1989; Bull et al. 1998; Giha et al. 2000; Dodoo et al. 2001; Feng et al. 2009), therefore the role of VSA antibody responses in protection in infancy and early childhood needs to be further investigated.

Antibodies capable of inhibiting growth of *P. falciparum* are thought to be one of the main functional responses against proliferation of the parasite during erythrocytic stages (Richards et al. 2009). Levels of growth-inhibitory antibodies in Manhiça appeared not to be associated with past or present malaria exposure, but were significantly associated with age. Children aged 24 months had more inhibitory activity than those aged 12 months. The study was not designed to assess GIA on earlier time-points (5, 9 months) therefore we do not have information of maternally transferred inhibitory antibodies. Few studies have assessed growth inhibitory antibodies in small children (Marsh et al. 1989; Dent et al. 2006; McCallum et al. 2008) as most studies have evaluated older individuals in whom different antibody kinetics are observed. In a cohort exposed to moderate malaria transmission in Kenya, growth-inhibitory antibodies could be acquired at an early age, but were not higher in older children and adults (up to 81 years old), which suggests that ongoing boosting of responses with further exposure did not occur (McCallum et al. 2008). In a separate cohort of children in a low transmission setting, inhibitory antibodies were overall lower and associated with the level of exposure to blood-stage infection (McCallum et al. 2008). In a different study, growth inhibition decreased with age with the highest median levels among children under 4 years compared to adults (Dent et al. 2008). Overall these studies support the idea that functional antibodies are acquired at an early age, but are not boosted further and may even decrease with time. However, in our young study population functional antibodies increased with age up to 2 years. It is possible that this early acquisition of moderately inhibitory antibodies could represent the development of immunity to severe malaria, thought to be predominant during the first and second years of life in highly exposed populations, as suggested previously (McCallum et al. 2008).

A number of merozoite antigens have been shown to be targets of vaccine-induced invasion inhibitory antibodies, mostly localized on the merozoite surface: MSP-1 (John et al. 2004; Ockenhouse et al. 2006), AMA-1 (Thera et al. 2008; Spring et al. 2009), or

EBA-175 (Duraisingh et al. 2003). We observed a significant correlation between levels of IgG against AMA-1 and GIA in the samples with the 20% highest *P. falciparum* growth inhibition, suggesting that AMA-1-specific antibodies could be an important component of the growth inhibitory activity. A strong correlation between ELISA and functional assay responses has been observed in vaccine studies in malaria naïve volunteers (Ockenhouse et al. 2006; Polhemus et al. 2007), however in malaria endemic regions no consistent correlations between these two assays have been found. Interference of non-AMA-1 IgGs on the growth-inhibitory activity of AMA-1 antibodies has been reported (Miura et al. 2008). It has been suggested that functional and serological assays of antibody responses to merozoite proteins measure different aspects of antibody-mediated immunity in naturally exposed populations (O'Donnell et al. 2001; John et al. 2004; Dent et al. 2006; McCallum et al. 2008). Nevertheless, we found some evidence that the two can also correlate in pediatric field samples with the highest GIA activity. Consistent with this, a study in Senegal with children aged 7–19 years found that the growth inhibitory effects of purified IgG measurable *in vitro* reflected levels of anti-AMA-1 and MSP-1 (Courtin et al. 2009).

The lack of correlation between GIA activity and protection in our study is consistent with several studies carried out in other settings and conditions (Marsh et al. 1989; Perraut et al. 2005; McCallum et al. 2008; Spring et al. 2009; Crompton et al. 2010). One study found a significant association between level of GIA (controlling for age) and time-to-infection and risk of infection (Dent et al. 2008) and another reported that inhibitory activity correlated with a reduction in the odds of experiencing malaria (Crompton et al. 2010). However it is puzzling how the age related decrease in GIA reported in the literature parallels the age-related decrease in the prevalence of high density parasitemia.

In our infant cohort, the only factors that were associated with risk of subsequent clinical malaria were having had previous episodes in the second year of life and neighborhood of residence, which is thought to reflect the microheterogeneity in transmission intensity within the study area.

The considerable amount of variability in results from studies that assessed GIA in field studies could be related to the assay used and the choice of parasite strain. Greater sensitivity and reproducibility is achieved over conventional GIA assays by testing inhibitory activity over two cycles of replication to amplify any inhibitory effects of samples and by evaluating parasitemia by flow cytometry (Bergmann-Leitner et al. 2006; Wilson et al. 2010). The use of the GFP fluorescent parasites removes the need to use a nucleic acid stain for parasite detection by flow cytometry, thereby reducing handling and giving better resolution between ring stage parasites and non-infected erythrocytes by flow cytometry compared to ethidium bromide stained rings. Dialysis of samples effectively removes most antimalarials and nonspecific inhibitory factors present in human plasma when using small volumes (Persson et al. 2006). There is variability in inhibitory activity against different parasite isolates in some studies (McCallum et al. 2008; Dent et al. 2008; Spring et al. 2009) and it is therefore suggested that GIA capacity is strain specific. Variation in invasion phenotype might have evolved as a mechanism that facilitates immune evasion by *P. falciparum* and a broad inhibitory response against multiple ligands may be required for effective immunity (Persson et al. 2008). In our study a genetically modified D10 clone was used as it has proven to be efficiently transfected to express GFP (Wilson et al. 2010), but it may be valuable to perform GIA studies with local field strains in future studies.

In conclusion, despite the reduction in exposure to *P. falciparum* during infancy in SP recipients relative to placebo recipients, this and our previous study collectively show that IPTi-SP does not negatively affect the development of antibody responses to *P. falciparum* blood stage antigens. Variant antigens expressed on the surface of the infected erythrocyte, considered as major targets of immunity to malaria, and growth-inhibitory activity, have remained unchanged after IPTi treatment. Being the first study to longitudinally assess the development of anti-VSA and inhibitory responses during IPTi-SP, this report provides additional insights into the complex effects of prospective interventions on NAI, the caveat being as is always the case, within the limitations of the detection tools to measure broadly relevant responses.

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ARTICLE 4

Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine does not modify plasma cytokines and chemokines or intracellular cytokine responses to *Plasmodium falciparum* in Mozambican Children

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Immunobiology (submitted)

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Running title: Cytokine responses after IPTi with SP

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Abbreviations: APC, allophycocyanin; CD, cluster of differentiation; CI, confidence interval; CV, coefficient of variation; DTP/OPV/Hep B, diphteria+tetanus+pertussis/oral polio vaccine/hepatitis B; EDTA, ethylenediaminetetraacetic acid; EPI, expanded program on immunization; FITC, fluorescein isothiocyanate; FSC, forward scatter; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; GM, geometric means; HIV, human immunodeficiency virus; ICS, intracellular cytokine staining; Ig, immunoglobulin; IL, interleukin; IFN, interferon; IPTi, Intermittent Preventive Treatment in infants; IRR, incidence rate ratio; MCP, monocyte chemotactic protein; MCAF, monocyte chemotactic and activating factor; MIP, macrophage inflammatory protein; *P. falciparum, Plasmodium falciparum;* PBS, phosphate buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PerCP, Peridinin chlorophyll protein; PYAR, person years at risk; SSC, side scatter; SP, sulfadoxine-pyrimethamine; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor; WBA, whole blood assay

Abstract

Cytokines and chemokines are key mediators of anti-malarial immunity. We evaluated whether Intermittent Preventive Treatment in infants with Sulfadoxine-Pyrimethamine (IPTi-SP) had an effect on the acquisition of these cellular immune responses in Mozambican children exposed to *Plasmodium falciparum* infections. Multiple cytokines and chemokines were quantified in plasma by luminex, and antigen-specific cytokine production in whole blood was determined by intracellular cytokine staining and flow cytometry, at ages 5, 9, 12 and 24 months.

The administration of IPTi-SP did not significantly affect the proportion of CD3+ cells producing IFN-γ, IL-4 or IL-10 at any of the time points. Overall, plasma cytokine or chemokine concentrations did not differ between treatment groups. Th1 and pro-inflammatory responses were higher than Th2 and anti-inflammatory responses, respectively, and IFN-γ:IL-4 ratios were higher for placebo than for SP recipients. Levels of cytokines and chemokines varied according to age, declining from 5 to 9 months. Plasma concentrations of IL-10, IL-12 and IL-13 were associated with current infection or prior malaria episodes. Higher frequencies of IFN-γ and IL-10 producing CD3+ cells and elevated concentrations of IL-10, IFN-γ, MCP-1 and IL-13 in plasma were individually associated with increased incidence of malaria, at different time points. When all cytokines and chemokines were analyzed together, only higher IL-17 at 12 months was associated with lower incidence of malaria up to 24 months.

Our work has further confirmed that IPTi-SP is a safe malaria control intervention that does not negatively affect the development of cellular immune response during early childhood. This study has also provided new insights as to how these cytokine responses

are acquired upon age and exposure to the *P. falciparum* parasite, as well as their associations with malaria susceptibility.

Introduction

In 2009 there were an estimated 68,925,435 cases of malaria in the African region (78% of worldwide estimates) and 111,885 malaria deaths (91% of worldwide estimates) (World Health Organization 2010). These figures demonstrate that *Plasmodium falciparum* malaria remains a major threat to the health of Africans, in particular children under 5 years of age. In malaria endemic areas, older children and adults develop immunity to severe morbidity and death, though remaining susceptible to infection (Marsh 1992; Doolan, Dobaño, and Baird 2009). Passive transfer studies in humans suggest that antibodies are a key mediator of naturally acquired immunity (Cohen, McGregor, and Carrington 1961). More recent data (Pombo et al. 2002) suggest that cellular immunity also plays an important role in the protection against *P. falciparum* disease in humans (Good et al. 2005).

Cytokines and chemokines are considered key mediators of anti-malarial immunity. For example, the production of cytokines and chemokines by activated leukocytes, including CD8+ and CD4+ T cells, mediate mechanisms such as phagocytosis and killing of parasitized erythrocytes (Fritsche et al. 2001) and inhibition of parasite growth and development inside hepatocytes (Tsuji, Rodrigues, and S Nussenzweig 2001).

It has been also shown that cytokines play an important role in the immunopathology of malaria and many field studies have described an association of specific cytokines with severity of disease, in particular IL-2 (Ramharter et al. 2003), IL-12 (Luty et al. 2000; Perkins, Weinberg, and Kremsner 2000), IFN-γ (Day et al. 1999; Ramharter et al. 2003), IL-1β (Brown et al. 1999), IL-6 (Kern et al. 1989; ME Molyneux

et al. 1991), TNF (Kern et al. 1989; Grau et al. 1989; Kwiatkowski et al. 1989; Kwiatkowski et al. 1990; Shaffer et al. 1991; Molyneux et al. 1991; Othoro et al. 1999; Brown et al. 1999; Ramharter et al. 2003; Körner et al. 2010; Scuderi et al. 1986), IL-4 (Kumaratilake and Ferrante 1992; Troye-Blomberg 1994; Eisenhut 2010), IL-10 (Winkler et al. 1998; Kurtzhals et al. 1998; Day et al. 1999; Othoro et al. 1999; Ramharter et al. 2003), MIP-1β (Ochiel et al. 2005), and TGF-β (Perkins, Kremsner, and Weinberg 2001).

There is less field data available on the relevance of individual cytokines in naturally acquired immunity to malaria, e.g. IL-12 (Sedegah, Finkelman, and Hoffman 1994; Dodoo et al. 2002; Torre et al. 2002) , IFN-γ (Chizzolini et al. 1990; Riley et al. 1992; Harpaz et al. 1992; Luty et al. 1999; Torre et al. 2002; Dodoo et al. 2002), TNF (Harpaz et al. 1992; Bouharoun-Tayoun et al. 1995; Dodoo et al. 2002), or IL-10 (John et al. 2000). Previous studies have investigated individual or a few cytokines but it is more likely that a network of cytokine and chemokines determines protection or susceptibility from *P. falciparum* infections (Prakash et al. 2006). Most studies of cytokine responses have been cross-sectionals done after the onset of symptoms and at the initial stages of clinical illness (Kremsner et al. 1990; Deloron, Lepers, and Coulanges 1989; Nguyen-Dinh and Greenberg 1988; Rhodes-Feuillette et al. 1985) only a few studies have assessed longitudinally the evolution of cellular responses to infection, and those have been mostly in adult populations (Peyron et al. 1990; Riley et al. 1993; Dodoo et al. 2002). Furthermore, while a number of studies have evaluated newborn cytokines responses in cord blood (Fievet et al. 1996; King et al. 2002; Bouyou-Akotet et al. 2004; Malhotra et al. 2005; Brustoski, Moller, et al. 2005; Brustoski, Kramer, et al. 2005;

Brustoski et al. 2006; Breitling et al. 2006; Metenou et al. 2007), very few have measured prospectively cellular responses to *P. falciparum* in infants (Hesran et al. 2006) or children (Peyron et al. 1990; Mshana et al. 1991; Dodoo et al. 2002).

In the context of a randomized, placebo-controlled trial of intermittent preventive treatment in infants with sulfadoxine-pyrimethamine (IPTi-SP) in Mozambique (Macete et al. 2006), we have previously shown that this intervention had no impact on the antibody responses to *P. falciparum* erythrocytic stage antigens and to variant surface antigens in Mozambican infants, nor on the capacity of antibodies to inhibit parasite growth (Quelhas et al. 2008; Quelhas et al. 2010). Only a handful of studies have evaluated the impact of any malaria control interventions on the development of cellular immune responses. In one study, continuous chemoprophylaxis for 3 years in Gambian children resulted in higher lymphoproliferative responses and IFN-γ production (Otoo et al. 1989), and there was no clinical rebound of malaria one year after termination of prophylaxis (despite a decrease in anti-malarial antibody levels). In another study, permethrin-treated bednets showed a significant impact on percent lean body mass in Kenyan school children, thought to be due to decreased production of pro-inflammatory cytokines TNF, IL-1 and IL-6 in this group (Friedman et al. 2003). However, no study has yet assessed the impact on the child's cellular immunity of IPTi-SP, a safe and efficacious malaria control strategy consisting in the administration of a full dose of this antimalarial drug within the Expanded Program on Immunization (EPI) (Aponte et al. 2009).

In the current study, we have determined prospectively the effect of IPTi-SP on levels of *P. falciparum* antigen-specific intracellular cytokines using flow cytometry in

children up to 2 years of age. To partially overcome the difficulty to study this age group because of limited blood volumes, we used whole blood assays (WBA) instead of isolated peripheral blood mononuclear cells for measuring antigen-specific responses by intracellular cytokine staining (ICS). This approach also provides an environment more similar to that existing *in vivo*, and it is faster and more economical (Struik et al. 2004; Malhotra et al. 2005; Walther et al. 2006; Barbosa et al. 2009). In addition, we have used luminex-based microsphere suspension array methods to measure multiple cytokines and chemokines free in small volumes of plasma (Jason et al. 2001; Metenou et al. 2007). Finally, we assessed the factors influencing the levels of cytokines and chemokines at different time points and the associations between these and the incidence of malaria in the first and second years of life. This type of work has not been as comprehensively conducted in this age group with a longitudinal design in previous field studies.

Materials and methods

Study area

The study was conducted at the Centro de Investigação em Saúde da Manhiça, Manhiça District, southern Mozambique. This area is characterized by a perennial malaria transmission mostly due to *Plasmodium falciparum. Anopheles funestus* is the main vector, and the estimated average number of infective mosquito bites per person per year is 38 (IDRC and INDEPTH network 2002). For children in this area, SP showed a combined (early and late) therapeutic efficacy rate of 83%, with an *in vivo* parasitological sensitivity of 78.6% at day 14 (Abacassamo et al. 2004).

Study design

The efficacy study was an individually randomized, placebo-controlled trial (Macete et al. 2006). Infants were recruited from those attending the EPI clinic to receive dose 2 of DTP/OPV/Hep B between September 2002 and February 2004. Treatment with SP or placebo was administered at 3, 4, 9 months of age alongside the routine vaccinations. Cross-sectional visits were scheduled at 5, 9, 12 and 24 months of age. For the ancillary immunological studies within IPTi, we included the last 501 children recruited in the main trial. At each cross-sectional visit, capillary blood collected by fingerprick was placed into EDTA microtainers (1 ml), to obtain plasma for extracellular cytokines, and into heparin microtainers (0.5 ml) for intracellular cytokines determinations. All immunological assays were performed by personnel who were blinded to the children's study group. Clinical surveillance for malaria morbidity was done through passive case detection. Written informed consent was obtained from all parents or guardians and ethical approval for the protocol was obtained from the ethics review committees of Mozambique and the Hospital Clinic, Barcelona, Spain. The trial registration number is NCT00209795 (http://clinicaltrials.gov).

For intracellular cytokine staining (ICS) assays, among those recruited, children with the following criteria were selected: (i) having received all 3 doses of SP or placebo, (ii) having a full set of months 5, 9 and 12 blood samples, and (iii) having an equal representation of SP- and placebo-recipients. Among those, 258 samples were analyzed from month 5, 208 samples from month 9 and 144 samples from month 12. Once month 24 samples were collected, 143 that had the previous set were analyzed.

For luminex assays, those children who came for all 4 visits were prioritized, and plasmas were selected for analysis when there were sufficient volumes available: 229 children samples from month 5, 221 from month 9, 225 from month 12, and 158 from month 24.

Multiplex flow cytometric assay to measure cytokines and chemokines in plasma

Plasma cytokines and chemokines IL-2, IL-12 (p70), IFN-γ, IL-1β, IL-6, TNF, IL-4, IL-5, IL-13, IL-10, IL-7, IL-17, G-CSF, GM-CSF, MCP-1 (MCAF), MIP-1β were determined using a Bio-plex 100 System (Bio-Rad, CA, USA) and following manufacturer's instructions. According to supplier availability, two types of cytokine detection kits were used (10-plex and 17-plex). Most samples (all except those from 26 children) were tested using the 17-plex kit; the first 27 children (visits 5, 9, 12 months) were tested using the 10-plex kit (which did not contain IL-1β, IL-5, IL-7, IL-12, IL-13 IL-17, G-CSF, GM-CSF).

Assays were conducted in 96-well Millipore multiscreen filter plates (Millipore, MA, USA), running in duplicate 8 standards and 40 plasmas per plate. Plasma samples previously stored at -80 ºC were thawed in a water bath at 37ºC, and centrifuged at 1,000 g at 4°C for 10 min. Antigen-coated luminex beads were incubated with patient plasma, in conjunction with manufacturer's reagents. Plasma samples were diluted in Bio-Plex human sample diluent as 1:3 volumes (30µl sample + 90µl diluent). To each sample well, the following were added in a step wise manner: 50 µl premixed beads (1x), 50 µl of diluted standard or sample, and 25 µl Bio-Plex detection antibody (1x), and 50 µl streptavidin-PE preparation (1x).The mixture was incubated for 30 min in the dark, at

room temperature, on a rotating platform. The wells were then washed 3 times to remove unbound excess serum using a vacuum filtration system and a final incubation was done with streptavidin-PE for 10 min at room temperature while shaking at 300 rpm. Beads were finally resuspended in 125 µl assay buffer, and shaken on a microplate shaker at 1100 rpm at room temperature for 30 sec immediately before reading the plate on the Luminex 100, in accordance with manufacturer's instructions. Calibration was done using the CAL2 high RP1 target value. Concentrations of unknown cytokines were calculated by plotting unknowns against a 5-parameter logistic regression standard curve of 8 points that determine the limits of detection for each cytokine, expressed as pg/ml. The coefficient of variation (%CV, reproducibility between duplicate values) needed to be <20% to accept the value for analysis. Results were analyzed using the Bio-Rad Bioplex Manager Software v4.0.

Intracellular cytokine staining to measure P. falciparum-specific responses

CD3+ specific intracellular cytokines IFN-γ, IL-4 and IL-10 were measured by ICS. Heparinized whole blood was aliquoted into two tubes to test in parallel the stimulated and the non-stimulated (negative/ background control) samples. These were respectively incubated with 20 µl extract of *P. falciparum* schizont lysate at $1x10^8$schizonts/ml or 20 µl uninfected erythrocyte extract, in the presence of 1 µg/ml co-stimulatory antibodies to CD28 and CD49d (BD Biosciences, San Jose, CA) for 16 h at 37^oC and 5% CO_2. To block cytokine secretion, 1 µM Brefeldin A (GolgiPlug™, Pharmingen, San Diego, CA) was added during the final 4 h of incubation. To arrest activation and avoid loss of activated cells by adhesion to polystyrene tubes, 2 mM

EDTA/PBS solution was added for 15 min at room temperature, and tubes were vigorously vortexed before and after the incubation. In order to lyse erythocytes and fix lymphocytes, 5 ml 1x FACS Lysing solution were added. These were vortexed, incubated for 10 min at room temperature, and immediately placed at –80^{o}C. Frozen samples were thawed in batches at a later time for parallel processing and staining, hereby avoiding loss of function or increased background staining. Thawing was done by briefly placing samples in a 37^{o}C water bath and immediately washing off lysing solution using 7 ml Cell Wash buffer. Samples with clumping/debris of dead cells were previously filtered through 70 μm cell sieve filters (Cell Strainer BD Falcon), and pellet was resuspended with a pipette in 2 ml permeabilizing solution before vortexing for 10 min at RT. Prior to staining, frozen cells were washed with 8 ml wash buffer and centrifuged at 1500 rpm for 10 min at room temperature. Supernatant was decanted, residual pellet was resuspended in 500 μl wash buffer, and 100 μl of each sample were transferred to 96 well round bottom microtiter plates. Plates were centrifuged at 2000 rpm for 5 min, then flicked to discard the supernatants and finally pellets were resuspended by vortexing before adding the staining antibodies. Samples were stained with a pool of fluorescence-conjugated antibodies: 5 μl CD3 PerCP, 7 μl IFN-γ FITC, 3 μl IL-4 PE, and 4 μl IL-10 APC (BD Biosciences, San Jose, CA). After incubating at room temperature in the dark for 30 min, samples were washed twice and supernatants discarded. Samples were brought up to a total volume of 300 μl by adding Cell Wash buffer before acquisition. Stained samples were acquired on a 4-color FACS Calibur (BD Biosciences) and 50000-100000 total events were collected. Isotype controls were included to evaluate autofluorescence and to assist in setting the quadrants. The data were analyzed with CELLQuest software (BD

Biosciences). Lymphocytes were gated using FSC vs. SSC plots and then SSC vs. CD3+ T cells plots were used to isolate CD3+ cells (Fig 1). We analyzed samples in which > 1000 CD3+ events could be acquired, and in which defined lymphocyte and CD3 populations could be identified, thus excluding those with compromised cell recovery that could confound the readings. Each intracellular cytokine was assessed within its respective fluorescence channel (PerCP vs. FITC or PE or APC). Non-stimulated control samples were used to establish the threshold quadrants of background responses that were applied to quantify % of positive cytokine-producing cells in paired antigen-stimulated samples; results were expressed as the percentage of cytokine (IL-4, IL-10, IFN-γ)-producing CD3+ cells. Samples were considered positive if there was a true difference in response proportions (95% confidence interval [CI]) between the response to test antigens (stimulated) and the background responses to the non-stimulated samples: if the CI was entirely below 0.05%, the response was negative; if the CI was entirely above 0.05%, the response was positive; if the CI overlapped with 0.05%, the response was indeterminate.

Definitions and statistical methods

Malaria infection was defined as the presence of asexual *P. falciparum* parasites of any density in a blood smear measured by microscopy and/or by PCR (Quelhas et al. 2008). A clinical malaria episode was defined as a positive blood smear plus an axillary temperature of 37.5°C or history of fever within the prior 24 h. The sensitivity and specificity of these definitions are ~100% and 84%, respectively, in infants and 100% and 79.4% in children 1–4 years old (Saúte et al. 2003). To estimate the incidences, the time

at risk was calculated as the number of person years at risk (PYAR; episodes per 365.25 days) since the beginning of the time at risk until the end of follow-up, migration, death or withdrawn consent, whichever occurred first. Children did not contribute to the time at risk or the clinical malaria cases during the lag periods (an arbitrary lag of 21 days was applied after a case of clinical malaria). Months were defined as 30.4 days.

A Shapiro-Wilk normality test revealed a non-normal distribution of data, therefore all analyses were carried out in log scale and expressed as Geometric Means (GM). A Pearson correlation coefficient was used to measure the degree of correlation among cytokines. Correlations were considered "low" if $Rho < 0.33$, "moderate" if *Rho* was between 0.33 and 0.66, or "high" if $Rho > 0.66$. Ratios between cytokines were obtained by dividing the values from each child, transforming that value to its natural log, then calculating the mean for the total number of children, and finally transforming that value to its exponential (GM). T-test and chi-square or fisher exact test were used to compare mean levels or prevalence (tertiles of distribution) respectively, between IPTi treatment groups. Crude and adjusted linear regression models were also used to estimate the effect of IPTi-SP on the cytokine levels at each visit.

To identify what variables were independently associated with cytokine responses (intervention group [SP or placebo], age at visit, neighbourhood of residence, current infection and the occurrence of previous clinical episodes [yes/no]), linear regression models with random intercept were estimated. Selection of the variables for the final model was done through a stepwise procedure, where the criterion for including or excluding a variable was having a p-value ≤ 0.05 or ≥ 0.10 respectively from the Likelihood ratio test.

To evaluate the relationship between each of the individual cytokines at ages 5 and 12 months and the incidence of clinical malaria episodes from 5 to 12 or from 12 to 24 months, respectively, crude and adjusted negative binomial regression models were estimated using a stepwise procedure. Cytokine responses were treated as continuous values, or categorised by tertiles. The incidence rate ratio (IRR) of children with cytokine levels in the highest tertile against those in the lowest tertile was estimated, as well as the IRR per 2-fold increase in the value of cytokines. Co-variates included were: sex, neighbourhood of residence, treatment group and previous malaria episodes.

Two additional separate negative binomial regression models were estimated including (i) all the cytokine responses together and (ii) all the cytokine together plus antibody variables assessed in our previous studies (IgG against whole parasite, IgG [total or isotypes] and IgM against merozoite antigens) (Quelhas et al. 2008; Quelhas et al. 2010) to identify what immunological variables were independently associated with the subsequent incidence of clinical malaria.

Data analysis was performed using Stata 11 for Windows (Stata Corporation, College Station, TX). Statistical significance was defined as a *P* of <0.05.

Results

Intracellular and plasma cytokine responses in relation to IPTi-SP

The administration of IPTi-SP did not significantly affect the proportion of CD3+ cells producing IFN-γ, IL-4 or IL-10 intracellularly in peripheral whole blood samples

stimulated ex-vivo with *P. falciparum* extract antigens, at any of the time points, neither by crude analysis nor by adjusting for previous episodes of clinical malaria (Table 1). Also, in general, no significant differences were found in plasma cytokine or chemokine responses between treatment groups. There were a three isolated exceptions (IL-12 at 9 months, IL-7 at 12 months, G-CSF at 24 months) in which cases cytokine/chemokine concentrations were consistently higher in children who received placebo (Table 1). The complete raw data depicting levels of each cytokine and chemokine studied per age and treatment group is shown in supplementary Figure 1.

Factors associated with individual cytokine responses

Cytokine/chemokine levels varied according to the age of the child. All of them showed a decline from 5 to 9 months of age. Thereafter, some cytokines continued decreasing gradually throughout all measurements (plasma IL-2, TNF, IL-4, IL-10), others reached their highest levels at 24 months (plasma IL-12, IL-13, IL-17, MCP-1, G-CSF), and the remaining varied without any specific pattern (Table 2, supplementary Fig 1).

In addition to age, plasma IL-10 levels were also associated with having had previous episodes of clinical malaria (1.45 [1.14-1.86], $P= 0.0028$) and having current infection (1.80 [1.41-2.29], $P < 0.0001$). IL-12 and IL-13 were also associated with having had previous malaria episodes (1.35 [1.02-1.77], $P= 0.0340$; 1.32 [1.04-1.67], $P= 0.0234$). None of the other factors analysed in the multivariate analyses (gender, neighbourhood of residence, treatment) had any significant association with the cytokines assessed here.

Correlations and ratios between cytokines

We observed no correlation between the magnitude of IL-4, IL-10 or IFN-γ responses measured intracellularly upon antigen-stimulation and plasma concentrations (data not shown). A few correlations were found between plasma cytokines when analysed against each other (Supplementary figure 2). Concentrations of Th1 (IL-2, IL-12, IFN-γ) and Th2 (IL-4, IL-5, IL-13) cytokines correlated moderately within themselves, and both these two types showed a moderate/ high correlation in between them (Th1 vs Th2). Pro-inflammatory (IL-1β, IL-6, TNF) and anti-inflammatory/ regulatory (IL-10, IL-7, IL-17) cytokines correlated moderately within themselves, and both these showed a moderate/ low correlation between them (pro- vs anti-inflammatory). Chemokines G-CSF and GM-CSF both correlated moderately/highly to Th1 and Th2 type cytokines, whereas MCP-1 correlated with most anti-inflammatory cytokines and MIP-1 β correlated with most pro-inflammatory cytokines.

We examined whether there was a transition from Th2 to Th1 (Hesran et al. 2006) or from anti-inflammatory to pro-inflammatory cytokines with age in early infancy by analyzing the ratios. Overall, Th1 responses were higher than Th2 responses over the 2 years of life (Supplementary figure 1), and the ratio of IFN-γ:IL-4 increased from 5 to 24 months (Table 3). When data was stratified by treatment, IFN-γ:IL-4 ratios were higher for placebo than for SP recipients at all ages (Table 3), particularly at age 24 months coinciding with the highest prevalence of *P. falciparum* infection (7.7% at 5 months, 13.5% at 9 months, 8% at 12 months and 26.6% at 24 months). Overall, the pro-

inflammatory responses were higher than anti-inflammatory responses over the 2 years of life (Suppl. Fig 1), and the ratio of TNF:IL-10 decreased from 5 to 24 months (Table 3).

Individual cytokines and incidence of malaria

Elevated cytokine responses, both antigen-specific and non-specific, were not associated with a reduction in the subsequent incidence of clinical malaria in crude or adjusted analyses (Table 4). In some cases there was an association between higher cytokine levels and higher malaria incidence and this was manifested at certain age intervals (5-12 months or 12-24 months). In particular, intracellular IFN-γ and IL-10 production by CD3+ cells was associated with higher incidence of malaria during the first year of age but not in the second year (Table 4). Adjusting for previous episodes did not affect these results.

Associations between individual plasma cytokines and incidence of malaria were observed throughout the two years, but mostly in the second year. High concentrations of IL-10 and MCP-1 in plasma were associated with higher malaria incidence in both time intervals and by all analysis methods (Table 4). High IL-13 was also positively associated with incidence of malaria as analysed by all methods but only during the second year. A few disperse significant associations for plasma IFN-γ, IL-1β, IL-4, IL-5, IL-7, G-CSF and GM-CSF were also observed during the second year, when adjusted the analyses by the variables included in the multivariate model; all were in the same direction of higher concentrations correlating with increased malaria risk (Table 4).

Multiple cytokines and incidence of malaria

When all cytokines were analysed together in relation to incidence of malaria using a stepwise procedure, significant associations were only observed between higher levels of intracellular IFN-γ at 5 months and higher malaria incidence in the follow up interval 5-12 months (IRR 1.28, 95% CI 1.03-1.58, *P*=0.0234). Similarly, higher concentrations of plasma IFN-γ (IRR 1.15, 95% CI 1.05-1.27, *P*=0.0040) and MCP-1 (IRR 1.60, 95% CI 1.22-2.10, *P*=0.0007) at 12 months were associated with higher malaria risk during the 12-24 month period. In contrast, in this analysis higher plasma concentrations of IL-17 at 12 months were associated with lower malaria incidence during the interval 12-24 months (IRR 0.80, 95% CI 0.69-0.93, *P*=0.0035). Other co-variates that were significantly associated with malaria incidence, independently of the cytokine concentrations, were having had a previous episode in the second year of life (IRR 4.63, 95% CI 2.59-8.26, *P*< 0.0001), and the child's neighborhood of residence in both time intervals (*P*< 0.0001).

Finally, when antibody variables from prior studies were also included in the stepwise model together with all cytokines and co-variates, significant associations were only observed for higher intracellular IFN-γ at 5 months and higher malaria incidence during the follow up interval 5-12 months (IRR 1.26, 95% CI 1.01-1.57, *P*=0.0381). At 12 months, higher plasma IFN-γ (IRR 1.26, 95% CI 1.11-1.44, *P*=0.0004) and IL-5 (IRR 1.27, 95% CI 1.04-1.55, *P*=0.0203) correlated with higher incidence of malaria up to 24 months, while higher IL-17 plasma concentrations were associated with lower malaria risk during the 12-24 month period (IRR 0.84, 95% CI 0.71-0.98, *P*=0.0300). Likewise, the additional variables that independently showed significant associations with malaria

risk were having had a previous episode in the second year of life (IRR 3.16, 95% CI 1.64-6.09, $P< 0.0006$), and the neighborhood in both time intervals ($P< 0.0001$).

Discussion

In the context of a clinical trial evaluating the safety and efficacy of IPTi-SP as a malaria control tool during infancy, we studied prospectively the cytokine responses to *P. falciparum* antigens and the concentrations of plasma cytokines and chemokines at multiple time points during a 2 year follow up period. The longitudinal design and the established demographic and morbidity surveillance systems allowed for a rigorous and complete documentation of clinical malaria cases that could provide insights into the acquisition of cytokine/chemokine responses in children living in a malaria endemic area, how they are influenced by age and *P. falciparum* exposure, and the correlation between these responses and incidence of symptomatic malaria.

One of the main findings of this study was that overall there were no differences in the magnitude of intracellular or plasma cytokines/chemokines between IPTi treatment groups, except for some plasma cytokines and chemokines in which children receiving placebo had higher concentrations than those receiving SP (IL-12 at 9 months, IL-7 at 12 months and G-CSF at 24 months). In these cases, we hypotesize that placebo recipients, who suffered more *P. falciparum* infections than SP recipients owing to the efficacy of IPTi (Macete et al. 2006) had more systemic immune activation and higher levels of some plasma cytokines. However, of these, only IL-12 was directly associated to previous episodes of malaria in the multivariate analysis; to our knowledge, there is no

data in the literature on the influence of *P. falciparum* infection or malaria interventions in the concentrations of IL-7 or G-CSF in peripheral blood. The levels of these cytokines/chemokines were not associated with subsequent incidence of malaria.

Among the factors that significantly affected the levels of cytokines (both intracellular and plasma), age was the most prominent. Without exception, all cytokines varied significantly in magnitude from the first visit to at least one of the following visits. In all cases, there was a decrease of cytokine values from 5 to 9 months, and some had the highest levels at 24 months. This fluctuation is not surprising as cross-sectional samplings were not scheduled to pick up bursts of production upon infection and most plasma cytokines' half-lives are short (Harpaz et al. 1992), thus may not represent a long lasting response. In addition, cytokine production is stimulated and regulated by many different factors within the cellular responses network, and while some are specific to infection, other are innate or are produced in response to acute or systemic inflammatory conditions.

In contrast to antibody responses that are strongly affected by past or present malaria infection (Quelhas et al. 2008), parasite exposure appeared to have a minor effect on the concentrations of cytokines measured at the time of the cross-sectional visits. Plasma IL-10 increased significantly in children who have had previous episodes or current infection, and higher levels of IL-12 and IL-13 were also associated with previous episodes. Each of these cytokines belongs to a different family (immuno-regulatory, Th1 and Th2 respectively) therefore it seems that the limited effect of exposure to *P. falciparum* infection was generalised and not biased towards a specific type. Similar observations have been reported (Ramharter et al. 2003) where malaria patients

responded to *P. falciparum* infected erythrocytes with significant increases in the percentage of IL-2, IFN-γ, and TNF, but also IL-10, positive cells.

Considering that the synergistic and antagonistic effects that cytokines and chemokines have on each other are the key to how they regulate the immune system, we analyzed the correlations and ratios among some representative families. First, we observed no correlation between intracellular and plasma IL-4, IL-10 or IFN-γ, which is not uncommon as they represent two different measurements. Intracellular cytokines were produced by CD3+ cells after antigen stimulation in vitro (recall response) whereas plasma cytokines reflect what is present ex-vivo in peripheral blood (non malaria specific), and may be produced by many types of cells. In another study (Jason et al. 2001), the strength of associations between serum and cellular cytokines varied greatly, suggesting that serum cytokines at best only weakly reflect peripheral blood cell cytokine production and balances. We observed a high ratio between prototype Th1 (IFN-γ) and Th2 (IL-4) cytokines at all ages, and this proportion increased when looking only at the placebo group possibly indicating more exposure. We also observed the relative transition from Th2 to Th1 responses with age (PrabhuDas et al. 2011).

We finally assessed whether cytokine and chemokine levels were associated with subsequent incidence of malaria, and what factors affected these associations. Overall, high cytokine responses were not associated with a reduced incidence of clinical malaria when analyzed individually. Only when all cytokines where analyzed together in relation to risk of malaria, a significant association towards a decreased risk was found for IL-17 in the second year of life. IL-17 is a Th17 cytokine and its relation to various infectious agents has been described (van de Veerdonk et al. 2009). However to our knowledge,

there has not been any study reporting a role for IL- 17 in protection against human malaria.

In fact, the most common finding was that there were no associations, or that higher levels of some cytokines correlated with increased malaria incidence. In particular, IL-10 (intracellular and plasma) and MCP-1 were more consistently associated with incidence of malaria, and this association was not explained by age or previous episodes. In addition, IFN-γ (intracellular and plasma) and IL-13 were also associated with malaria incidence, but these were dependent on age and/or previous episodes. A few other disperse associations were observed during the second year for plasma IL-1β, IL-4, IL-5, IL-7, G-CSF, GM-CSF, when adjusted by previous episodes. These results suggest that the responses measured were not necessarily part of an acquired protective response, but rather could be interpreted as biomarkers of physiopathological processes.

IL-10, an immunoregulatory cytokine, is extensively reported in relation to malaria immunopathogenesis (Winkler et al. 1998; Kurtzhals et al. 1998; Day et al. 1999; Othoro et al. 1999; Ramharter et al. 2003) and not so much associated with immunity (John et al. 2000). Higher IL-10 has been associated with less effective parasite clearance (Hugosson et al. 2004). To our knowledge nothing has been reported for chemokine MCP-1 in children in relation to immunity, and it does not correlate with any of the other cytokines associated with increased incidence of malaria. In pregnant women, MCP-1 concentrations were higher in the placentas of primiparous women (more susceptible to malaria) than in those of multiparous women (Bouyou-Akotet et al. 2004). IL-13, IL-4 and IL-5, Th2-type cytokines, are associated with reduced parasite killing (Kumaratilake and Ferrante 1992). Production of IL- 4 but not IFN-γ by activated human T cells is

associated with elevated antibodies to malaria antigens (Troye-Blomberg et al. 1990), and consistent with this, our previous studies also found correlations between higher antibody levels and increased malaria risk (Dobaño et al. *submitted*).

Responses associated with reduced malaria incidence would more likely be expected in the antigen-specific cytokines, as measured by ICS. Blood cells from donors in malaria endemic areas stimulated with *Plasmodium* antigens are known to activate many types of cells, with production of both IFN-γ (Th1 type) and IL-4 (Th2 type) (Troye-Blomberg et al. 1990). IFN-γ is thought to be a central mediator of protective immune responses against blood stages of malaria (McCall and Sauerwein 2010). Surprisingly, in our study, increased frequency of IFN-γ positive CD3+ cells at 5 months was associated with higher incidence of malaria up to 1 year. This is in contrast with other studies since IFN-γ is more often reported to confer protection (Chizzolini et al. 1990; Riley et al. 1992; Harpaz et al. 1992; Luty et al. 1999; Dodoo et al. 2002; Torre et al. 2002) than pathology (Day et al. 1999; Miller et al. 2002; Ramharter et al. 2003). In most of these studies IFN-γ was measured in older children or adults and it is likely that the immature immune system of the infant responds differently. In the second year, however, there were no significant associations between intracellular cytokines and incidence of malaria. At a population level, memory-like IFN-γ responses have been measured following malaria infection (McCall et al. 2010) and it has been reported that IFN-γ responses are both more prevalent and of greater magnitude at the end of the rainy malaria transmission season (Riley et al. 1993), thus showing that IFN-γ might also be a marker of exposure.

The apparent predisposition of children with higher cytokine responses to increased malaria risk might also be influenced by genetic factors. Many studies have shown associations between genetic polymorphisms, immunoregulation, phenotypes and disease risk (Allison 1954; Riley 1996; Modiano et al. 1996; Hill 1998; Traoré et al. 1999; Stirnadel et al. 2000; McNicholl et al. 2000; Modiano et al. 2001; Mockenhaupt et al. 2006; Paganotti et al. 2006; Awandare et al. 2006; Ockenhouse et al. 2006; Aubouy, Migot-Nabias, and Deloron 2007; Allison 2009; Duah et al. 2009; Basu et al. 2010), but unfortunately this was not assessed in this study. Other factors not evaluated that could help explain these associations would be malnutrition (Mbugi et al. 2010), co-infection with HIV or other pathogens (Chaisavaneeyakorn et al. 2002), or prenatal exposure (Broen et al. 2007). The duration and/or the nature of antigen exposure *in utero* appears to govern the outcome with respect to neonatal immune responses, such that placental malaria induce antigen-specific IL-10-producing regulatory T cells that can inhibit Th1-type responses, while antigen-specific IFN-γ production predominate in babies born to mothers successfully treated for malaria during gestation (Broen et al. 2007). Prenatal infection could thus contribute to the *P. falciparum*-specific IFN-γ and IL-10 response pattern in 5-month old children seen in this study.

In conclusion, our work has further confirmed that IPTi-SP is a safe intervention that does not compromise the development of a wide range of cytokines and chemokines thought to be major contributors to anti-malarial immunity in infancy. This study has also given some insights as to how these responses are acquired upon age and exposure to the *P. falciparum* parasite. Despite the low and heterogeneous antigen-specific cytokine responses observed, infant field studies such as the current one help to advance the

understanding of the relationship between innate immune and adaptive cellular immune responses. Eventually, an effective orchestration of both types of immune responses is necessary for the generation of an efficient and non-pathogenic resolution of the malarial disease.

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TABLES AND FIGURES

Table 1. Comparison of levels of intracellular (*P. falciparum*-specific) and plasma cytokines and chemokines between IPTi treatment groups for each cross-sectional visit. The three analyses methods are presented and *P* values correspond to each one of these.

			Crude geometric mean					Crude linear regression[a]		Adjusted linear regression[a,b]	
			Placebo		SP		*P* value[c]	Proportional difference[d]	*P* value	Proportional difference	*P* value
Cytokines and chemokines		Age (months)	GM	95% CI	GM	95% CI					
Intracellular (% producing lymphocytes)	IFN-γ	5	0.17	0.13 - 0.23	0.13	0.10 - 0.18	0.2100	0.77	0.2078	0.80	0.2935
		9	0.05	0.04 - 0.07	0.05	0.04 - 0.07	0.9945	1.00	0.9944	1.01	0.9650
		12	0.07	0.05 - 0.11	0.05	0.03 - 0.08	0.3485	0.75	0.3442	0.76	0.3639
		24	0.05	0.04 - 0.08	0.04	0.03 - 0.06	0.3952	0.80	0.3911	0.82	0.4339
	IL-4	5	0.96	0.81 - 1.12	0.94	0.80 - 1.10	0.8645	0.98	0.8639	1.00	0.9956
		9	0.87	0.77 - 0.99	0.82	0.71 - 0.95	0.5447	0.94	0.5423	0.94	0.5467
		12	0.80	0.64 - 0.99	0.91	0.71 - 1.17	0.4270	1.14	0.4229	1.13	0.4532
		24	0.86	0.70 - 1.04	0.82	0.65 - 1.04	0.8140	0.96	0.8123	0.97	0.8482
	IL-10	5	0.37	0.30 - 0.45	0.36	0.31 - 0.43	0.9333	0.99	0.9330	1.01	0.9227
		9	0.15	0.12 - 0.19	0.18	0.15 - 0.21	0.2855	1.16	0.2826	1.15	0.2943
		12	0.23	0.17 - 0.31	0.22	0.17 - 0.31	0.8863	0.97	0.8853	0.96	0.8525
		24	0.32	0.25 - 0.42	0.35	0.27 - 0.45	0.6670	1.08	0.6642	1.07	0.6957
Plasma (pg/ ml)	IL-2	5	16.17	12.31 - 21.24	17.25	13.04 - 22.82	0.7427	1.07	0.7413	1.12	0.5697
		9	12.99	9.48 - 17.79	12.17	9.35 - 15.83	0.7520	0.94	0.7506	0.93	0.7421
		12	8.56	6.34 - 11.56	7.17	5.22 - 9.86	0.4249	0.84	0.4223	0.84	0.4383
		24	7.09	5.08 - 9.90	5.36	3.83 - 7.49	0.2410	0.76	0.2373	0.76	0.2540
	IL-12	5	2.36	1.80 - 3.10	1.87	1.34 - 2.61	0.2806	0.79	0.2776	0.79	0.2843
		9	2.37	1.74 - 3.22	1.54	1.21 - 1.96	0.0309	0.65	0.030	0.66	0.030
		12	1.43	1.14 - 1.78	1.31	1.08 - 1.58	0.5509	0.92	0.5483	0.93	0.5965
		24	2.35	1.83 - 3.01	2.22	1.74 - 2.83	0.7477	0.95	0.7458	0.97	0.8447

	IFN-γ	5	156.50	105.27 - 232.66	147.86	98.96 - 220.93	0.8422	0.94	0.8413	0.97	0.9285
		9	145.93	93.78 - 227.08	115.02	75.84 - 174.44	0.4377	0.79	0.4351	0.79	0.4345
		12	77.61	51.44 - 117.10	55.47	35.17 - 87.47	0.2807	0.71	0.2780	0.71	0.2679
		24	128.29	85.71 - 192.03	79.89	51.45 - 124.06	0.1162	0.62	0.1133	0.62	0.1069
	IL-1β	5	35.03	25.11 - 48.87	41.15	28.66 - 59.09	0.5169	1.17	0.5143	1.16	0.5470
		9	11.61	8.50 - 15.86	11.45	8.46 - 15.50	0.9503	0.99	0.9500	0.99	0.9575
		12	13.53	9.70 - 18.86	13.98	10.54 - 18.55	0.8804	1.03	0.8797	1.04	0.8692
		24	20.05	13.99 - 28.73	17.64	12.12 - 25.69	0.6256	0.88	0.6229	0.86	0.5662
	IL-6	5	99.22	72.21 - 136.34	127.60	97.52 - 166.95	0.2310	1.29	0.2284	1.27	0.2543
		9	59.24	43.36 - 80.95	56.47	41.93 - 76.05	0.8259	0.95	0.8249	0.95	0.8102
		12	76.28	54.51 - 106.75	70.96	51.65 - 97.48	0.7566	0.93	0.7553	0.93	0.7724
		24	82.64	55.45 - 123.14	80.68	54.88 - 118.62	0.9317	0.98	0.9311	0.97	0.9073
	TNF	5	53.64	38.35 - 75.02	54.50	40.40 - 73.51	0.9440	1.02	0.9437	1.00	0.9915
		9	31.91	22.10 - 46.06	27.30	19.11 - 39.00	0.5463	0.86	0.5440	0.85	0.5344
		12	21.72	15.16 - 31.12	20.49	14.29 - 29.37	0.8205	0.94	0.8195	0.95	0.8326
		24	12.36	7.72 - 19.81	9.86	6.58 - 14.76	0.4682	0.80	0.4646	0.78	0.4301
	IL-4	5	3.95	2.83 - 5.52	5.64	4.18 - 7.62	0.1170	1.43	0.1150	1.49	0.0785
		9	2.96	1.99 - 4.39	3.64	2.69 - 4.93	0.4038	1.23	0.4011	1.23	0.4064
		12	2.49	1.81 - 3.44	2.65	1.93 - 3.64	0.7851	1.06	0.7839	1.08	0.7470
		24	1.41	0.98 - 2.04	1.44	1.09 - 1.90	0.9394	1.02	0.9389	1.01	0.9726
	IL-5	5	2.65	2.14 - 3.28	2.66	2.16 - 3.29	0.9664	1.01	0.9662	1.03	0.8334
		9	2.57	1.97 - 3.35	2.16	1.73 - 2.69	0.3107	0.84	0.3075	0.84	0.3139
		12	2.05	1.68 - 2.50	1.62	1.32 - 2.00	0.1091	0.79	0.1069	0.80	0.1166

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		24	2.15	1.87 - 2.46	1.97	1.75 - 2.22	0.3499	0.92	0.3461	0.92	0.3512
	IL-13	5	0.81	0.66 - 0.99	0.78	0.61 - 0.99	0.8112	0.96	0.8100	0.97	0.8583
		9	0.71	0.54 - 0.92	0.57	0.46 - 0.71	0.2124	0.81	0.2095	0.81	0.2130
		12	0.56	0.45 - 0.69	0.41	0.32 - 0.52	0.0522	0.73	0.0507	0.74	0.0682
		24	1.36	1.11 - 1.67	1.36	1.11 - 1.67	0.9937	1.00	0.9937	1.01	0.9305
	IL-10	5	3.77	3.01 - 4.72	4.20	3.32 - 5.32	0.5107	1.11	0.5084	1.14	0.4313
		9	3.94	3.00 - 5.16	3.56	2.94 - 4.30	0.5408	0.90	0.5385	0.89	0.4838
		12	2.74	2.21 - 3.40	2.45	1.99 - 3.01	0.4519	0.89	0.4494	0.91	0.5360
		24	2.53	2.00 - 3.19	2.40	1.80 - 3.20	0.7811	0.95	0.7794	1.00	0.9978
	IL-7	5	3.79	3.13 - 4.59	3.37	2.80 - 4.04	0.3791	0.89	0.3761	0.91	0.4655
		9	3.98	3.13 - 5.05	3.20	2.71 - 3.79	0.1447	0.81	0.1422	0.81	0.1447
		12	3.01	2.55 - 3.54	2.33	1.96 - 2.79	0.0384	0.78	0.0372	0.78	0.0430
		24	2.41	2.11 - 2.75	2.29	2.02 - 2.59	0.5600	0.95	0.5569	0.94	0.5195
	IL-17	5	4.73	3.47 - 6.45	4.77	3.70 - 6.16	0.9689	1.01	0.9688	0.99	0.9509
		9	3.72	2.86 - 4.84	2.87	2.22 - 3.72	0.1677	0.77	0.1649	0.78	0.1690
		12	3.01	2.32 - 3.91	2.93	2.29 - 3.76	0.8871	0.97	0.8863	0.98	0.8903
		24	5.56	4.37 - 7.08	4.28	3.26 - 5.61	0.1526	0.77	0.1493	0.76	0.1345
	G-CSF	5	17.84	14.28 - 22.29	16.05	12.87 - 20.01	0.5020	0.90	0.4993	0.91	0.5400
		9	13.85	10.91 - 17.57	12.32	9.98 - 15.19	0.4649	0.89	0.4620	0.89	0.4672
		12	10.48	8.17 - 13.44	11.11	9.18 - 13.44	0.7125	1.06	0.7107	1.07	0.6476
		24	27.96	23.58 - 33.16	22.21	19.14 - 25.76	0.0442	0.79	0.0425	0.79	0.0435
	GM-CSF	5	18.29	11.87 - 28.19	19.95	13.03 - 30.54	0.7775	1.09	0.7761	1.12	0.7124
		9	18.34	11.82 - 28.46	12.78	8.34 - 19.59	0.2431	0.70	0.2400	0.70	0.2432
		12	10.62	7.05 - 16.00	8.10	5.37 - 12.24	0.3565	0.76	0.3535	0.77	0.3632
		24	10.91	7.05 - 16.87	8.77	6.23 - 12.35	0.4335	0.80	0.4298	0.81	0.4376
	MCP-1	5	43.17	36.47 - 51.10	46.18	38.41 - 55.51	0.5945	1.07	0.5925	1.08	0.5476
		9	41.14	33.21 - 50.95	41.49	35.24 - 48.85	0.9499	1.01	0.9496	1.01	0.9697
		12	39.93	33.47 - 47.62	32.71	27.32 - 39.16	0.1185	0.82	0.1165	0.82	0.1225
		24	56.52	48.17 - 66.32	50.18	41.82 - 60.22	0.3304	0.89	0.3266	0.89	0.3320
	MIP-1β	5	565.32	474.10 -	693.5	603.63 -	0.0709	1.23	0.0693	1.18	0.1313

				674.10	1	796.78					
		9	402.91	342.05 - 474.60	456.42	387.36 - 537.80	0.2878	1.13	0.2851	1.13	0.2917
		12	487.27	413.65 - 573.99	583.64	482.50 - 705.98	0.1574	1.20	0.1550	1.20	0.1537
		24	618.41	515.94 - 741.22	593.85	494.69 - 712.89	0.7543	0.96	0.7524	0.96	0.7708

[a] Linear regression
[b] Adjusted by previous episodes of clinical malaria
[c] *T* test
[d] Proportional difference in cytokine/chemokine concentrations in SP-recipients in relation to placebo-recipients

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Table 2. Intracellular and plasma cytokines and chemokines by age, analyzed by multivariate random-effect models estimated by stepwise procedure and adjusted by previous episodes, current infection, IPTi treatment, gender and neighborhood of residence. Intracellular IL-4 is not included (null model). The first *P* value corresponds to the comparison in between ages and the second corresponds to the overall comparison.

Cytokines and chemokines		Age (months)	Proportional difference	95% CI	*P* value[a]	Overall *P* value
Intracellular	IFN-γ	5	1	-	-	< 0.0001
		9	0.36	0.26; 0.48	< 0.0001	
		12	0.41	0.29; 0.57	< 0.0001	
		24	0.32	0.23; 0.45	< 0.0001	
	IL-10	5	1	-	-	< 0.0001
		9	0.45	0.37; 0.55	< 0.0001	
		12	0.63	0.50; 0.78	< 0.0001	
		24	0.92	0.74; 1.15	0.4700	
Plasma	IL-2	5	1	-	-	< 0.0001
		9	0.74	0.58; 0.95	0.0193	
		12	0.47	0.37; 0.60	< 0.0001	
		24	0.38	0.29; 0.51	< 0.0001	
	IL-12	5	1	-	-	0.0001
		9	0.87	0.69; 1.10	0.2428	
		12	0.63	0.50; 0.79	0.0001	
		24	1.02	0.79; 1.31	0.9070	
	IFN-γ	5	1	-	-	< 0.0001
		9	0.84	0.59; 1.21	0.3510	
		12	0.43	0.30; 0.61	< 0.0001	
		24	0.69	0.47; 1.03	0.0703	
	IL-1β	5	1	-	-	< 0.0001
		9	0.30	0.22; 0.40	< 0.0001	
		12	0.36	0.27; 0.48	< 0.0001	
		24	0.51	0.38; 0.70	< 0.0001	
	IL-6	5	1	-	-	0.0001
		9	0.51	0.38; 0.68	< 0.0001	
		12	0.65	0.49; 0.87	0.0032	
		24	0.75	0.55; 1.03	0.0806	
	TNF	5	1	-	-	< 0.0001
		9	0.54	0.39; 0.73	0.0001	
		12	0.39	0.29; 0.53	< 0.0001	
		24	0.21	0.15; 0.30	< 0.0001	
	IL-4	5	1	-	-	< 0.0001
		9	0.69	0.53; 0.90	0.0073	
		12	0.54	0.41; 0.71	< 0.0001	
		24	0.31	0.23; 0.41	< 0.0001	
	IL-5	5	1	-	-	0.0001
		9	0.88	0.75; 1.04	0.1450	

		12	0.68	0.58; 0.81	< 0.0001	
		24	0.77	0.65; 0.93	0.0056	
	IL-13	5	1	-	-	< 0.0001
		9	0.77	0.64; 0.92	0.0045	
		12	0.58	0.49; 0.70	< 0.0001	
		24	1.59	1.30; 1.94	< 0.0001	
	IL-10	5	1	-	-	< 0.0001
		9	0.85	0.71; 1.03	0.0941	
		12	0.60	0.50; 0.73	< 0.0001	
		24	0.53	0.43; 0.66	< 0.0001	
	IL-7	5	1	-	-	< 0.0001
		9	0.99	0.86; 1.15	0.9244	
		12	0.74	0.64; 0.86	< 0.0001	
		24	0.66	0.56; 0.77	< 0.0001	
	IL-17	5	1	-	-	< 0.0001
		9	0.68	0.54; 0.85	0.0009	
		12	0.63	0.50; 0.79	0.0001	
		24	1.05	0.82; 1.35	0.6774	
	G-CSF	5	1	-	-	< 0.0001
		9	0.77	0.64; 0.91	0.0032	
		12	0.64	0.53; 0.76	< 0.0001	
		24	1.50	1.24; 1.81	< 0.0001	
	GM-CSF	5	1	-	-	< 0.0001
		9	0.78	0.56; 1.09	0.1525	
		12	0.49	0.35; 0.68	< 0.0001	
		24	0.52	0.36; 0.75	0.0004	
	MCP-1	5	1	-	-	< 0.0001
		9	0.92	0.80; 1.06	0.2724	
		12	0.80	0.70; 0.93	0.0025	
		24	1.22	1.04; 1.42	0.0156	
	MIP-1β	5	1	-	-	< 0.0001
		9	0.68	0.58; 0.80	< 0.0001	
		12	0.85	0.73; 1.00	0.0437	
		24	0.98	0.82; 1.16	0.7803	

[a] Each age in relation to 5 months

Table 3. Ratios between prototype Th1:Th2 and pro-: anti-inflammatory cytokines.

	5 months	9 months	12 months	24 months
Ratio IFN-γ:IL-4				
Overall	32	39	25	71
SP	26	32	21	56
Placebo	40	49	31	91
Ratio TNF:IL-10				
Overall	14	8	8	4
SP	13	8	8	4
Placebo	14	8	8	5

Table 4. Intracellular and plasma cytokines and chemokines and their association with incidence of malaria during the first and second years of age. For each time interval (5 to 12 months and 12 to 24 months) *P* values correspond to each one of the analyses methods used.

		5 - 12 months												12 - 24 months									
		Tertiles[a]						Two-fold increment[b]						Tertiles						Two-fold increment					
		Crude			Adjusted[c]			Crude			Adjusted			Crude			Adjusted			Crude			Adjusted		
Cytokines and chemokines		IRR	95% CI	*P* value[d]	IRR	95% CI	*P* value	IRR	95% CI	*P* value	IRR	95% CI	*P* value	IRR	95% CI	*P* value	IRR	95% CI	*P* value	IRR	95% CI	*P* value	IRR	95% CI	*P* value
Intracellular	IFN-γ	3.24	1.13 - 9.33	0.052	4.62	1.58 - 13.47	0.004	1.21	1.03 - 1.43	0.021	1.26	1.07 - 1.49	0.003	0.95	0.38 - 2.39	0.635	0.68	0.32 - 1.45	0.533	0.97	0.84 - 1.11	0.639	0.96	0.85 - 1.08	0.5226
	IL-4	1.49	0.64 - 3.48	0.645	1.31	0.62 - 2.77	0.783	1.19	0.90 - 1.58	0.212	1.17	0.91 - 1.49	0.213	0.53	0.20 - 1.37	0.404	0.78	0.35 - 1.72	0.816	0.81	0.60 - 1.10	0.178	0.95	0.75 - 1.20	0.6635
	IL-10	3.11	1.12 - 8.67	0.086	3.40	1.30 - 8.89	0.033	1.32	1.04 - 1.67	0.022	1.38	1.11 - 1.71	0.004	0.97	0.38 - 2.47	0.989	1.39	0.68 - 2.82	0.239	0.98	0.80 - 1.20	0.833	0.96	0.82 - 1.12	0.5904
204 Plasma	IL-2	2.71	0.94 - 7.83	0.131	2.13	0.83 - 5.42	0.219	1.19	0.97 - 1.45	0.083	1.11	0.95 - 1.29	0.200	1.54	0.66 - 3.59	0.423	1.98	1.01 - 3.90	0.055	1.12	0.98 - 1.29	0.090	1.11	1.00 - 1.24	0.051
	IL-12	1.38	0.56 - 3.41	0.309	1.53	0.71 - 3.30	0.080	1.10	0.91 - 1.34	0.330	1.06	0.90 - 1.25	0.453	1.63	0.63 - 4.21	0.528	1.87	0.91 - 3.82	0.223	1.09	0.88 - 1.35	0.422	1.10	0.92 - 1.32	0.266
	IFN-γ	0.92	0.37 - 2.32	0.929	0.99	0.44 - 2.21	0.378	1.00	0.89 - 1.13	0.992	0.98	0.89 - 1.08	0.706	1.28	0.56 - 2.93	0.836	3.60	1.79 - 7.23	0.001	1.05	0.95 - 1.16	0.316	1.16	1.07 - 1.26	0.0004
	IL-1β	1.42	0.43 - 4.73	0.654	1.24	0.41 - 3.71	0.653	1.00	0.84 - 1.19	0.975	1.02	0.87 - 1.20	0.813	1.52	0.63 - 3.68	0.050	2.11	1.11 - 4.01	0.009	1.11	0.94 - 1.31	0.194	1.18	1.04 - 1.33	0.011
	IL-6	1.03	0.40 - 2.69	0.967	1.05	0.45 - 2.45	0.967	1.00	0.85 - 1.18	0.979	1.02	0.88 - 1.19	0.747	1.52	0.67 - 3.46	0.609	1.86	0.95 - 3.63	0.184	1.10	0.95 - 1.26	0.194	1.16	1.03 - 1.30	0.013
	TNF	1.53	0.52 - 4.48	0.716	2.08	0.80 - 5.43	0.116	1.13	0.95 - 1.34	0.166	1.12	0.97 - 1.30	0.117	1.00	0.43 - 2.33	0.926	1.37	0.68 - 2.77	0.580	1.05	0.94 - 1.19	0.390	1.06	0.96 - 1.16	0.243
	IL-4	1.28	0.48 - 3.41	0.506	1.21	0.53 - 2.78	0.516	1.08	0.94 - 1.24	0.253	1.07	0.95 - 1.20	0.271	1.83	0.82 - 4.07	0.168	2.26	1.19 - 4.27	0.041	1.11	0.98 - 1.25	0.093	1.16	1.05 - 1.29	0.004
	IL-5	1.67	0.58 - 4.80	0.618	1.74	0.69 - 4.37	0.485	1.13	0.87 - 1.48	0.347	1.05	0.84 - 1.32	0.652	1.90	0.77 - 4.69	0.329	2.80	1.43 - 5.49	0.001	1.15	0.89 - 1.49	0.289	1.39	1.13 - 1.70	0.002
	IL-13	1.35	0.46 - 3.92	0.527	1.70	0.66 - 4.37	0.114	1.22	0.92 - 1.62	0.157	1.19	0.94 - 1.50	0.132	3.33	1.21 - 9.15	0.038	3.26	1.45 - 7.32	0.016	1.42	1.11 - 1.81	0.005	1.32	1.07 - 1.62	0.006
	IL-10	3.36	1.27 - 8.87	0.035	3.06	1.21 - 7.76	0.025	1.28	1.04 - 1.57	0.016	1.17	0.99 - 1.38	0.059	3.61	1.60 - 8.13	0.009	2.30	1.19 - 4.44	0.045	1.43	1.16 - 1.75	0.000 4	1.25	1.06 - 1.47	0.006
	IL-7	1.77	0.62 - 5.04	0.529	1.41	0.56 - 3.52	0.594	1.26	0.92 - 1.73	0.137	1.10	0.85 - 1.43	0.456	1.56	0.62 - 3.96	0.242	2.16	1.09 - 4.30	0.029	1.20	0.88 - 1.65	0.245	1.34	1.04 - 1.72	0.019
	IL-17	0.67	0.25 - 1.83	0.622	0.76	0.33 - 1.74	0.551	1.01	0.83 - 1.23	0.914	1.04	0.88 - 1.22	0.681	0.78	0.30 - 2.00	0.055	0.80	0.36 - 1.77	0.278	0.97	0.79 - 1.20	0.785	0.92	0.78 - 1.09	0.342
	G-CSF	1.83	0.64 - 5.27	0.439	2.42	0.95 - 6.20	0.074	1.25	0.95 - 1.64	0.102	1.21	0.96 - 1.53	0.085	2.41	0.90 - 6.46	0.207	3.10	1.46 - 6.56	0.013	1.25	1.00 - 1.55	0.048	1.35	1.13 - 1.63	0.001

	GM-CSF	1.19	0.41 - 3.42	0.945	1.19	0.48 - 2.97	0.490	1.05	0.92 - 1.21	0.475	1.04	0.93 - 1.16	0.517	1.63	0.64 - 4.10	0.187	2.41	1.16 - 5.05	0.011	1.11	0.99 - 1.26	0.077	1.13	1.03 - 1.24	0.010
	MCP-1	3.01	1.18 - 7.63	0.064	3.16	1.37 - 7.25	0.015	1.64	1.21 - 2.23	0.001	1.66	1.29 - 2.15	< .0001	3.07	1.34 - 7.06	0.008	2.85	1.43 - 5.69	0.007	1.31	1.02 - 1.69	0.038	1.36	1.11 - 1.67	0.003
	MIP-1β	0.52	0.20 - 1.35	0.113	0.83	0.34 - 2.07	0.341	0.92	0.67 - 1.27	0.620	1.10	0.80 - 1.51	0.571	1.50	0.67 - 3.40	0.396	0.98	0.50 - 1.94	0.329	0.98	0.78 - 1.24	0.859	0.93	0.76 - 1.13	0.430

[a] Incidence rate ratio in high tertile relative to low tertile

[b] Incidence rate ratio in two-fold increment

[c] Adjusted by treatment group, gender, previous episodes of clinical malaria, current infection, neighbourhood

[d] Negative binomial regression model using likelihood ratio test

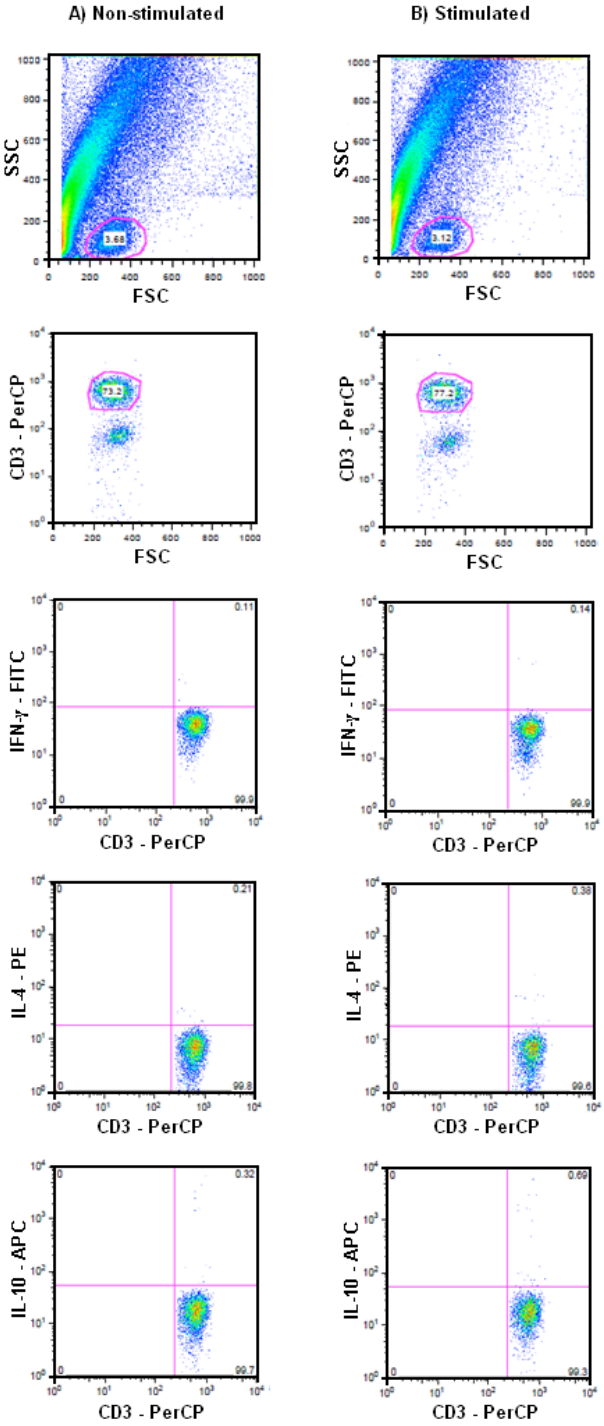
FIGURE LEGENDS

Figure 1. Representative FACS plots showing flow cytometric detection of *P. falciparum* antigen-specific IFN-γ (FITC-labeled), IL-4 (PE-labeled) and IL-10 (APC-labeled) following intracellular cytokine staining of $CD3^+$ lymphocytes. Non-stimulated control samples (A) were used to establish the threshold quadrants of background responses that were applied to quantify % of positive cytokine-producing cells in paired antigen-stimulated samples (B) of the same child. The UR quadrant represents $CD3^+$ $cytokine^+$ lymphocytes.

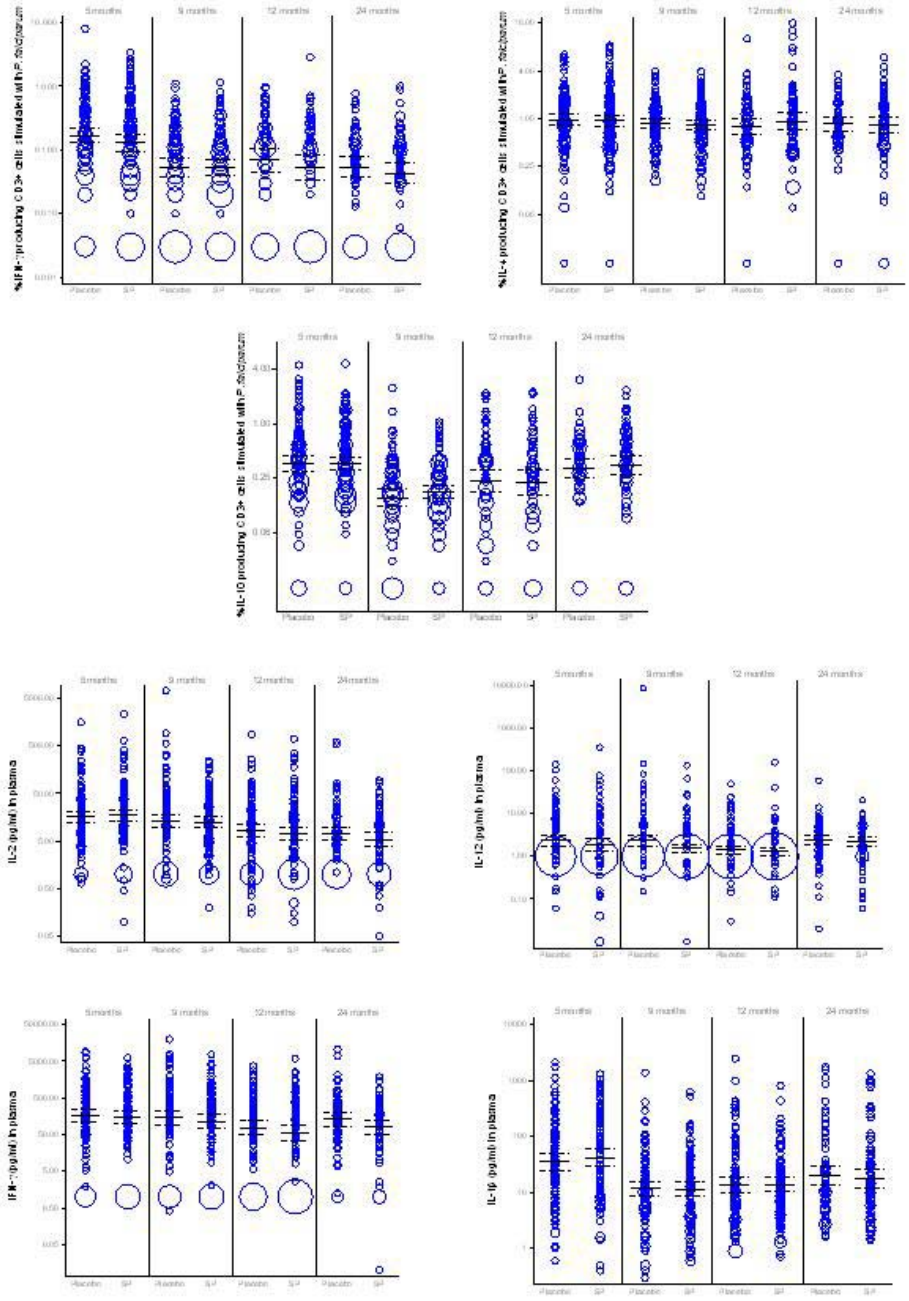
Supplementary Figure 1. Intracellular and plasma cytokines and chemokines in Mozambican infants receiving IPTi with SP (right) or placebo (left), at 5, 9 12 and 24 months. Cytokine values (Y axis) are expressed as % producing lymphocytes for intracellular cytokines, and as pg/ml for plasma cytokines/ chemokines. In the weighted scatter plots the area of the symbol is proportional to the number of observations. Geometric mean and 95% confidence intervals are indicated by horizontal continuous and discontinuous lines respectively.

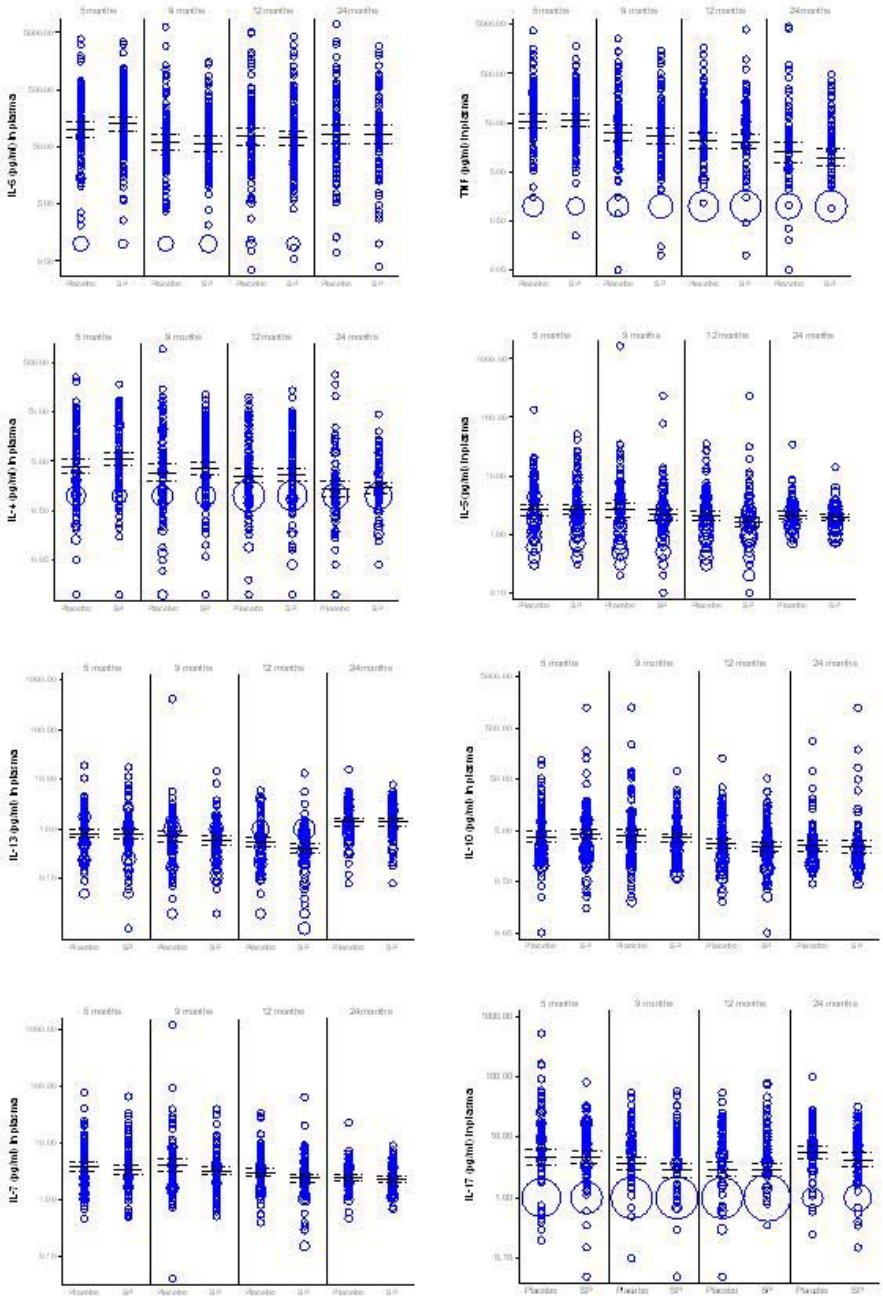
Supplementary Figure 2. A) Correlation among cytokines, B) Correlation coefficients and strength of correlation.

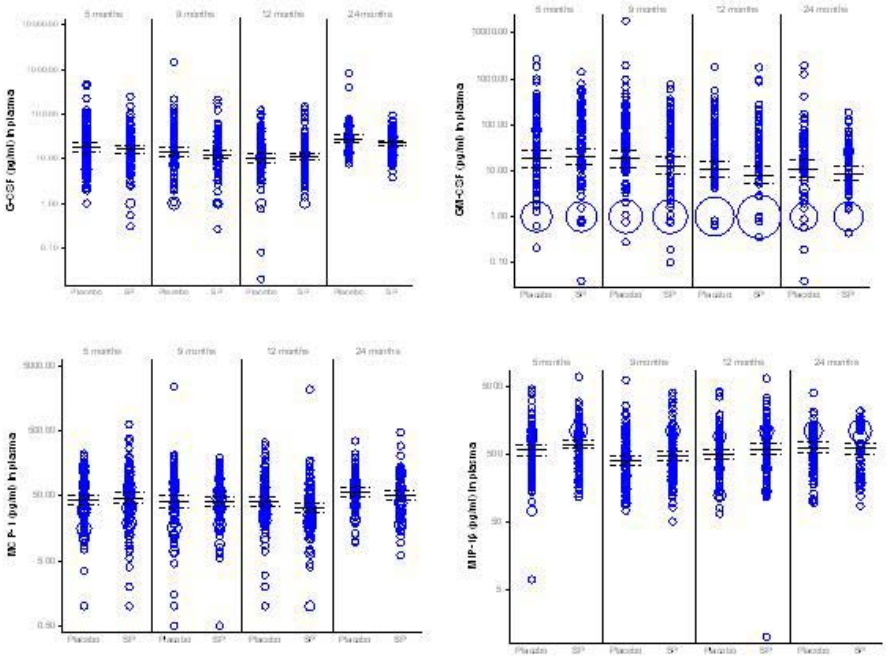
Figure 1.


A) Non-stimulated
B) Stimulated
SSC
FSC
3.68
3.12
CD3 - PerCP
73.2
77.2
IFN-γ - FITC
0.11
99.0
0.14
99.0
IL-4 - PE
0.21
99.8
0.38
99.6
IL-10 - APC
0.32
99.7
0.69
99.3

Supplementary Figure 1

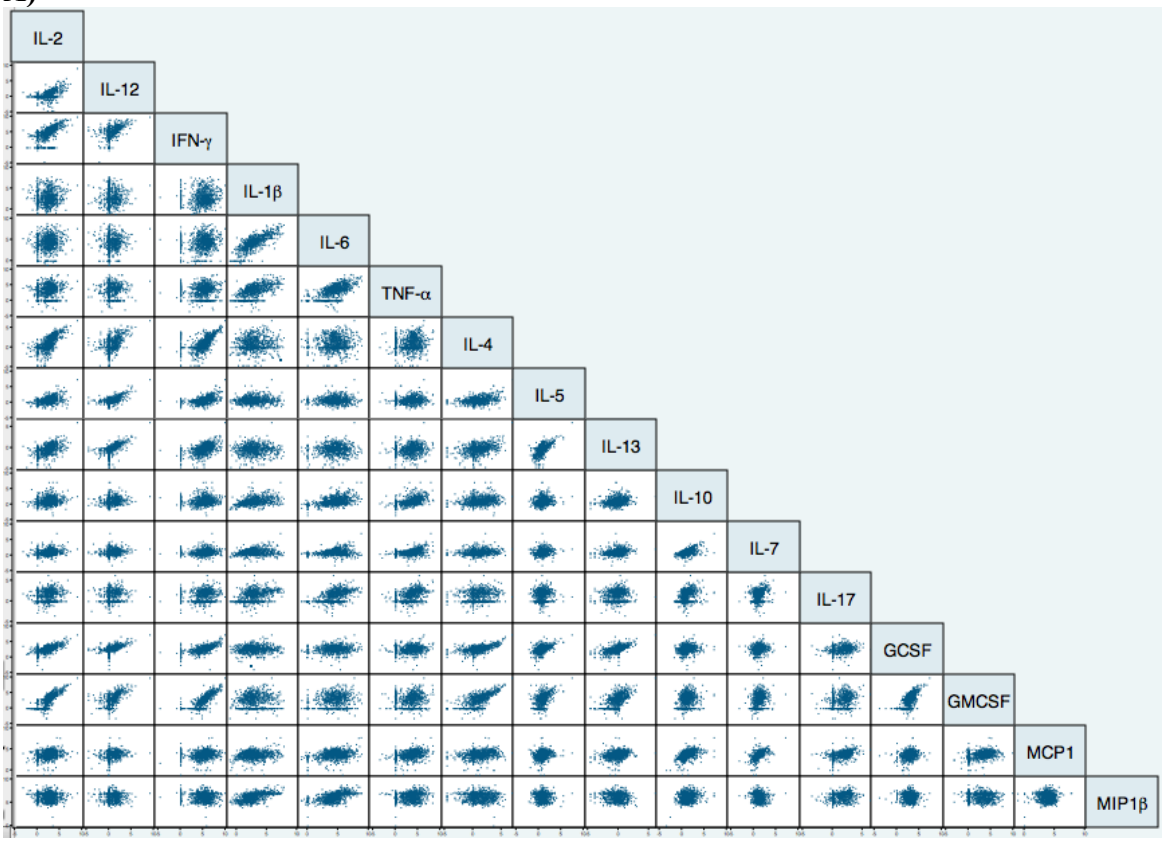



5 months
9 months
12 months
24 months
Placebo
SP
IL-6 (pg/ml) in plasma
TNF (pg/ml) in plasma
IL-4 (pg/ml) in plasma
IL-5 (pg/ml) in plasma
IL-13 (pg/ml) in plasma
IL-10 (pg/ml) in plasma
IL-7 (pg/ml) in plasma
IL-17 (pg/ml) in plasma


5 months
9 months
12 months
24 months
G-CSF (pg/ml) in plasma
GM-CSF (pg/ml) in plasma
MCP-1 (pg/ml) in plasma
MIP-1β (pg/ml) in plasma
Placebo
SP

Supplementary Figure 2. A: Correlation among cytokines; B: Correlation coefficients and strength of correlation.

A)



B)

	IL-2															
Rho	0.5067	IL-12														
P value	0.0000															
Rho	0.6141	0.4683	IFN-γ													
P value	0.0000	0.0000														
Rho	0.0535	0.0404	0.0423	IL-1β												
P value	1.0000	1.0000	1.0000													
Rho	0.1232	0.0520	0.1135	0.7641	IL-6											
P value	0.0514	1.0000	0.1423	0.0000												
Rho	0.1251	0.1024	0.0809	0.5698	0.6245	TNF										
P value	0.0415	0.6424	1.0000	0.0000	0.0000											
Rho	0.6900	0.4530	0.4654	0.0785	0.0613	0.1096	IL-4									
P value	0.0000	0.0000	0.0000	1.0000	1.0000	0.2079										
Rho	0.4387	0.5839	0.4602	0.0518	0.0618	0.0714	0.3695	IL-5								
P value	0.0000	0.0000	0.0000	1.0000	1.0000	1.0000	0.0000									
Rho	0.4903	0.6000	0.4927	0.0220	0.0704	0.1261	0.3626	0.6395	IL-13							
P value	0.0000	0.0000	0.0000	1.0000	1.0000	0.0711	0.0000	0.0000								
Rho	0.1960	0.1686	0.0998	0.3608	0.4501	0.5090	0.1959	0.1599	0.2315	IL-10						
P value	0.0000	0.0005	0.5265	0.0000	0.0000	0.0000	0.0000	0.0015	0.0000							
Rho	0.1973	0.1531	0.1685	0.1678	0.2204	0.4150	0.1530	0.1902	0.2639	0.5486	IL-7					
P value	0.0000	0.0045	0.0005	0.0004	0.0000	0.0000	0.0037	0.0000	0.0000	0.0000						
Rho	0.1645	0.1101	0.1369	0.4041	0.5033	0.4619	0.1438	0.0442	0.1094	0.3237	0.2928	IL-17				
P value	0.0009	0.3704	0.0233	0.0000	0.0000	0.0000	0.0109	1.0000	0.3954	0.0000	0.0000					
Rho	0.6281	0.5565	0.6359	0.0629	0.1018	0.1420	0.5400	0.4705	0.5924	0.1984	0.1676	0.1791	G-CSF			
P value	0.0000	0.0000	0.0000	1.0000	0.6750	0.0130	0.0000	0.0000	0.0000	0.0000	0.0007	0.0002				
Rho	0.8156	0.5813	0.7761	0.0549	0.1425	0.1583	0.6757	0.5051	0.5816	0.2171	0.2659	0.2193	0.6603	GM-CSF		
P value	0.0000	0.0000	0.0000	1.0000	0.0124	0.0019	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000			
Rho	0.2276	0.1977	0.1097	0.1841	0.2986	0.3715	0.2422	0.1967	0.2541	0.4904	0.5657	0.4275	0.2183	0.3114	MCP-1	
P value	0.0000	0.0000	0.2067	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		
Rho	-0.0256	-0.0653	-0.0235	0.5813	0.5685	0.2114	-0.0322	-0.0771	-0.1016	0.1364	-0.1751	0.2942	-0.0475	-0.0622	0.0154	MIP-1β
P value	1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	1.0000	1.0000	0.6872	0.0095	0.0002	0.0000	1.0000	1.0000	1.0000	

Strength of correlation:
Low ***Rho*** **< 0.33**
Moderate ***Rho*** **between 0.33 and 0.66**
High ***Rho*** **> 0.66**

V. SUMMARY OF RESULTS AND OVERALL CONCLUSIONS

ARTICLE 1

Antibody responses to *P. falciparum* blood stage antigens in relation to IPTi-SP – crude analysis.

- No significant differences were found in IgG IFAT titres between the SP and the placebo groups at either of the time points.
- Similarly, IgG responses to *P. falciparum* merozoite antigens MSP-1_{19}, AMA-1 and EBA-175, measured by ELISA, did not significantly differ between treatment groups, at any of the cross-sectional visits except for IgG responses to AMA-1 and EBA-175 at age 5 months (after two IPTi doses at 3 and 4 months) which were significantly higher in SP- compared to placebo-recipients (AMA-1 p=0.032, EBA-175 p=0.050).
- IgM responses to the same antigens did not significantly differ between the two treatment groups.
- IgG subclass responses to MSP-1_{19}, AMA-1 or EBA-175 did not significantly differ between SP- or placebo-recipients for most of the cross-sectional visits. In two cases, levels of cytophilic IgG1 antibodies were significantly higher in the SP than in the placebo group: AMA-1 at 5 months (p=0.035) and MSP-1_{19} at 9 months (p=0.029).

Antibody responses to *P. falciparum* blood stage antigens in relation to IPTi-SP – adjusted analysis.

- IgG responses to whole parasites, MSP-1_{19}, AMA-1, and EBA-175 adjusted by previous clinical malaria episodes or current parasitaemia did not generally differ between SP and placebo groups, except for a few cases in which IgG levels were significantly higher in the SP compared to the

placebo group.

- In children who have had previous clinical malaria episodes, IgG IFAT titres were significantly higher in the SP than in the placebo group at age 9 months (p=0.018), and ELISA antibody levels were also significantly higher in SP- than in placebo-recipients for MSP-1_{19} at 9 (IgG, p=0.0007; IgG1, p=0.024) and 24 (IgG, p=0.022) months of age, and for AMA-1 at 9 months (IgG, p=0.009).
- In children with present infection, levels of IgG1 to MSP-1_{19} were significantly higher in the SP than in the placebo group at 9 months (p=0.011).
- In children with no documented previous clinical episodes, significant differences were only observed at 5 months, when only a few malaria episodes have occurred, particularly in the SP group. Then, levels of IgG and IgG1 to AMA-1 were higher in the SP than in the placebo group (p=0.009 and p=0.006), and levels of IgG1 to EBA-175 were higher in the SP than in the placebo group at age 5 months (p=0.014), consistent with the above analyses.
- Different ages at first clinical episode or different parasite densities did not seem to explain the higher IgG levels after IPTi with SP.
- No other significant associations in relation to IPTi-SP treatment were found with the other antigens and antibodies studied.

ARTICLE 2

Effect of exposure to *P. falciparum* on the magnitude of antibody responses

- Previous episodes of clinical malaria and present *P. falciparum* infection resulted in significantly higher levels of IgG to blood stage merozoite antigens MSP-$1_{19,}$ AMA-1 and EBA-175, constituting the main factors affecting the magnitude of antibody responses.

- Age and neighbourhood of residence were the two other most significant factors affecting the magnitude of antibody responses to *P. falciparum* merozoite antigens.

Age pattern of naturally-acquired IgG, IgM and IgG subclass antibodies

- The age patterns of IgG responses differed depending on the antibody type and subclass and the malaria antigen, with an important contribution from maternally-transmitted antibodies in early infancy, particularly for AMA-1 (IgG, IgG1) and for IgG to whole *P. falciparum* blood stages
- *P. falciparum* merozoite antigens predominantly induced cytophilic isotypes IgG1 and IgG3. In addition, considerable levels of IgG4 for EBA-175 were detected at age 24 months.

Association between antibody responses and incidence of clinical malaria.

- After adjusting for the effect of past and present exposure to *P. falciparum*, IPTi treatment and neighborhood of residence, only IgG subclass responses to EBA-175 at age 12 months were differentially associated with incidence of malaria in the second year of life:
    - Two-fold increments of cytophilic IgG1 and IgG3 levels independently were significantly correlated with decreased incidence of malaria (incidence rate ratio, IRR, 0.49, 95% CI, 0.25-0.97, p=0.026, and IRR 0.44, 95% CI 0.19-0.98, p=0.037, respectively),
    - Two-fold increments in levels of non-cytophilic IgG4 were significantly correlated with increased malaria (IRR 3.07, 95% CI 1.08-8.78, p=0.020).
- No significant associations were found between IgG antibodies to MSP-1_{19} or AMA-1 and subsequent incidence of malaria.
- Deeper understanding of the acquisition of antibodies against vaccine candidate target antigens in early infancy is crucial for the rational

development and deployment of malaria control tools in this vulnerable population.

ARTICLE 3

Antibody responses to *P. falciparum* VSA in relation to IPTi-SP.

- IPTi treatment did not affect VSA IgG levels, at any of the time points, with or without adjusting by previous episodes of clinical malaria.
- In addition to the neighbourhood of residence, age and current infection were the only factors associated with anti-VSA antibodies: levels of IgG against VSA decreased significantly with age ($p<0.0001$), and were 7% higher in children with *P. falciparum* infection at the time of sample collection.
- Previous malaria episodes were not associated with the levels of VSA antibodies.

Antibody-mediated inhibition of blood stage *P. falciparum* growth in relation to IPTi-SP.

- IPTi treatment did not affect GIA activity at 12 or at 24 months, either in the crude or the adjusted analyses.
- At 12 months 23.9% (39 of 163) and at 24 months 49.6% (68 of 137) of the subjects were in the “low” growth tertile (from 38.50 to 79.81) percent growth, meaning the group with most inhibition of growth.
- When assessing what other factors could affect GIA activity in children receiving IPTi-SP, only age was significantly associated with growth inhibition activity:
    - When including all subjects, children aged 24 months had 10% less parasite growth than those aged 12 months (95% CI 0.88-0.93, $p<0.0001$).

- When looking only at the subjects with previous malaria episodes, children aged 24 months had 14% less parasite growth than those aged 12 months (95% CI 0.80-0.93, p=0.0002).

Correlation between GIA activity and antibody levels by ELISA

- Although we did not assess antigen-specific inhibitory activity, GIA responses were compared to those found in previous ELISA studies measuring antibodies to merozoite antigens putatively involved in erythrocyte invasion processes. A significant inverse correlation was observed between *P. falciparum* growth and levels of IgG against AMA-1 by ELISA, when evaluating the 10% (R2 = 0.444, p=0.049) and 20% (R2 = 0.230, p=0.037) highest inhibitory samples.

Level of antibodies to VSA and growth inhibitory antibodies and incidence of clinical malaria

- Anti-VSA IgG levels were not associated with future risk of malaria during the first or second years of life.
- Likewise, no association was observed between GIA activity at 12 months and subsequent incidence of malaria during the interval 12-24 months.
- The only factors associated with risk of subsequent clinical malaria were for factors other than antibody levels: neighbourhood of residence (p<0.0001) at 12 months of age, and the occurrence of previous episodes of clinical malaria in the 12-24 months period (p=0.0035). There were no documented characteristics in the neighbourhoods (e.g. proximity to swamps, rivers, etc) that could explain these spatial associations.

ARTICLE 4

Intracellular and plasma cytokine responses in relation to IPTi-SP

- IPTi-SP did not significantly affect the proportion of CD3+ cells producing IFN-γ, IL-4 or IL-10 intracellularly in peripheral whole blood.
- In general, no significant differences were found in plasma cytokine or chemokine concentrations between treatment groups. There were three isolated exceptions (IL-12 at 9 months, IL-7 at 12 months, G-CSF at 24 months) in which cases cytokine/chemokine concentrations were consistently higher in children who received placebo.

Factors associated with individual cytokine responses

- Cytokine/chemokine levels varied according to the age of the child. All of them showed a decline from 5 to 9 months of age. Thereafter, some cytokines continued decreasing gradually throughout all measurements (plasma IL-2, TNF, IL-4, IL-10), others reached their highest levels at 24 months (plasma IL-12, IL-13, IL-17, MCP-1, G-CSF), and the remaining varied without any specific pattern.
- In addition to age, plasma IL-10 levels were also associated with having had previous episodes of clinical malaria and having current infection. IL-12 and IL-13 were also associated with having had previous malaria episodes. None of the other factors analysed in the multivariate analyses (gender, neighbourhood of residence, treatment) had any significant association with the cytokines assessed here.

Correlations and ratios between cytokines

- No correlations were found between the magnitude of IL-4, IL-10 or IFN-γ responses measured intracellularly upon antigen-stimulation and plasma concentrations.

- A few correlations were found between plasma cytokines when analysed against each other.
    - Concentrations of Th1 (IL-2, IL-12, IFN-γ) and Th2 (IL-4, IL-5, IL-13) cytokines correlated moderately within themselves, and both these two types showed a moderate/ high correlation in between them (Th1 vs Th2).
    - Pro-inflammatory (IL-1β, IL-6, TNF) and anti-inflammatory/ regulatory (IL-10, IL-7, IL-17) cytokines correlated moderately within themselves, and both these showed a moderate/ low correlation between them (pro- vs anti-inflammatory).
    - Chemokines G-CSF and GM-CSF both correlated moderately/ highly to Th1 and Th2 type cytokines, whereas MCP-1 correlated with most anti-inflammatory cytokines and MIP-1 β correlated with most pro-inflammatory cytokines.
- Overall, Th1 responses were higher than Th2 responses over the 2 years of life, and the ratio of IFN-γ:IL-4 increased from 5 to 24 months.
- Overall, the pro-inflammatory responses were higher than anti-inflammatory responses over the 2 years of life, and the ratio of TNF:IL-10 decreased from 5 to 24 months.

Individual cytokines and incidence of malaria

- Elevated cytokine responses, both antigen-specific and non-specific, were not associated with a reduction in the subsequent incidence of clinical malaria in crude or adjusted analyses.
- In some cases higher cytokine levels were associated with higher malaria incidence: intracellular IFN-γ and IL-10 production by CD3+ cells was associated with higher incidence of malaria during the first year of life but not in the second year. Adjusting for previous episodes did not affect these results.
- Associations between individual plasma cytokines and incidence of malaria were observed throughout the two years, but mostly in the second year; all

associations were in the same direction of higher concentrations correlating with increased malaria risk:

- High plasma concentrations of IL-10 and MCP-1 were associated with higher malaria incidence in both time intervals and by all analysis methods.
- High IL-13 was also positively associated with incidence of malaria as analysed by all methods but only during the second year.
- A few disperse significant associations for plasma IFN-γ, IL-1β, IL-4, IL-5, IL-7, G-CSF and GM-CSF were also observed during the second year, when adjusted the analyses by the variables included in the multivariate model.

Multiple cytokines and incidence of malaria

- When all cytokines were analysed together in relation to incidence of malaria using a stepwise procedure, significant associations were only observed between higher levels of intracellular IFN-γ at 5 months and higher malaria incidence in the follow up interval 5-12 months. Similarly, higher concentrations of plasma IFN-γ and MCP-1 at 12 months were associated with higher malaria risk during the 12-24 month period.
- In contrast, in this analysis higher plasma concentrations of IL-17 at 12 months were associated with lower malaria incidence during the interval 12-24 months (IRR 0.80, 95% CI 0.69-0.93, p=0.0035).
- Other co-variates that were significantly associated with malaria incidence, independently of the cytokine concentrations, were having had a previous episode in the second year of life, and the child's neighbourhood of residence in both time intervals.

OVERALL CONCLUSIONS

- The magnitude of IgM, IgG or IgG subclass responses to *P. falciparum* blood stage antigens involved in erythrocyte invasion by the merozoite was not impaired by the administration of IPTi-SP in Mozambican children at ages 5, 9, 12 and 24 months. However, for some antibodies and at certain time points, the magnitude of IgG or IgG1 responses to AMA-1, EBA-175, MSP-1_{19} or whole blood stage parasites were higher in SP- compared to placebo-recipients, particularly in children with previous episodes of clinical malaria.

- *P. falciparum* merozoite antigens predominantly induced cytophilic isotypes IgG1 and IgG3. In addition, IgG4 for EBA-175 was detected at age 24 months.

- Previous episodes of clinical malaria and present *P. falciparum* infection were the main factors significantly increasing the magnitude of Ig responses to merozoite blood stage antigens. Neighbourhood of residence and age were the other two most significant factors affecting the levels of antibodies. The age patterns of IgG responses to merozoite proteins differed depending on the antibody type, Ig subclass and antigen, with an important contribution from IgG and IgG1 maternally-transmitted antibodies in early infancy.

- After adjusting for past and present infection, IPTi treatment and neighbourhood of residence, elevated IgG1 and IgG3 at 12 months correlated with decreased incidence of malaria, while elevated IgG4

correlated with increased malaria. No significant associations were found between IgG antibodies to MSP-1_{19} or AMA-1 and incidence of malaria.

- Similarly, the magnitude of IgG responses to VSA putatively involved in antigenic variation and cytoadhesion, or the capacity of functional antibodies to inhibit the growth of *P. falciparum* parasites in vitro, were not affected by the administration of IPTi-SP in Mozambican children up to age 2 years.

- Neighbourhood of residence, age and current infection were the only factors associated with anti-VSA IgG levels, with an important contribution from maternally-derived antibodies.

- GIA activity was positively correlated with levels of IgG to AMA-1 in the 10-20% highest inhibitory plasmas. Percent inhibition augmented from 12 to 24 months of age, and was not affected by past or present *P. falciparum* infection.

- Levels of VSA antibodies or growth-inhibitory antibodies were not associated with subsequent incidence of malaria. Only previous episodes and neighbourhood of residence were significantly associated with malaria risk.

- The magnitude of cytokine and chemokine responses was not generally affected by the administration of IPTi-SP in Mozambican children at ages 5, 9, 12 and 24 months. Only for IL-12, IL-7, G-CSF, at one time point, the plasma concentrations were higher in placebo- compared to SP-recipients.

- The magnitude of cytokine and chemokine responses varied according to age, declining from 5 to 9 months, and were not affected by any of the

- other factors analysed. Only for IL-10, IL-12, IL-13, plasma concentrations were affected by *P. falciparum* past or present infection.

- Th1 responses were higher than Th2 responses over the 2 years of life, and the ratio of IFN-γ:IL-4 increased from 5 to 24 months. Pro-inflammatory responses were higher than anti-inflammatory responses over the 2 years of life, and the ratio of TNF:IL-10 decreased from 5 to 24 months

- Higher frequencies of IFN-γ and IL-10 producing CD3+ cells and elevated concentrations of IL-10, IFN-γ, MCP-1 and IL-13 in plasma were individually associated with increased incidence of malaria, at different time points. When all cytokines and chemokines were analysed together, only IL-17 at 12 months was associated with lower incidence of malaria up to 24 months.

- OVERALL, this PhD thesis has contributed to the comprehensive research agenda developed by the IPTi Consortium, by confirming that IPTi-SP is a safe malaria control intervention that does not negatively affect the natural acquisition of antibody or cytokine immune response during early childhood, and thus has provided further evidence for IPTi implementation in malaria endemic countries. In addition, this work has contributed to a deeper understanding of the development of humoral and cellular responses in early infancy that is crucial for the rational development and deployment of this and other malaria control tools in this vulnerable population.

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O que faz andar a estrada? É o sonho. Enquanto a gente sonhar a estrada permanecerá viva.
É para isso que servem os caminhos, para nos fazerem parentes do futuro.
Mia Couto, in Terra Sonambula

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