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Tracking malaria transmission intensities through pregnancy-specific serology

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Tracking malaria transmission intensities through pregnancy-specific serology

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“Every morning in Africa, a gazelle wakes up.
It knows it must run faster than the fastest lion, or it will be killed.
Every morning in Africa a lion wakes up.
It knows it must outrun the slowest gazelle, or it will starve to death.
It doesn't matter whether you are a lion or a gazelle.
When the sun comes up, you better start running.”

-Thomas L. Friedman, in The New York Times

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ABSTRACT

Sustainable and cost-effective epidemiological surveillance approaches are needed to monitor progress of malaria control and elimination programs. Pregnant women are sensitive to changes in *Plasmodium falciparum* transmission and reflect malaria trends in the community. Moreover, women develop antibodies against VAR2CSA (*P. falciparum* pregnancy-specific protein) after very limited exposure to placental parasites during pregnancy. We thus hypothesized that detecting these antibodies would allow estimating the cumulative exposure to *P. falciparum* during a limited period of time (i.e., the pregnancy) amplifying signals of recent transmission, especially in areas of low endemicity where the chances of detecting antibodies is higher than detecting the parasite. This thesis aimed at: 1) understanding the determinants and consequences of malaria declines and resurgences as well as the timescales over which antimalarial immunity is gained and lost (**chapter 1**); 2) dissect VAR2CSA and develop a multiplex serological assay (**chapter 2**), and 3) assess the value of pregnancy-specific antibodies to reflect levels, recent changes and spatial clusters of malaria transmission in a variety of hyper- to hypo-endemic areas (**chapter 3**). *P. falciparum* infection and antibody responses against VAR2CSA antigens were measured in samples from malaria exposed and non-exposed individuals. The main findings show that in Mozambican pregnant women positive qPCR tests for *P. falciparum* decreased from 33% in 2003 to 2% in 2010 and increased to 6% in 2012, with antimalarial IgG responses mirroring these trends. Moreover, the adverse consequences of *P. falciparum* infections were increased in pregnant women after five years of a decline in the prevalence of malaria **(chapter 1)**. A multiplex bead-based immunoassay to detect antibodies against 25 VAR2CSA new-peptides was successfully implemented and antibody responses were specific among pregnant women and dependent on the level of exposure to malaria (**chapter 2)**. IgGs against two VAR2CSA peptides maximize the information on recent exposure during pregnancy and reflect differences by time, space and deliberated control efforts (**chapter 3)**. Sero-surveillance in easy accessible pregnant women through maternal clinics may have a profound impact in malaria endemic countries to derive transmission metrics for malaria control and elimination.

RESUMO

Monitorizar programas de controlo e eliminação de malária requer métodos de vigilância epidemiológica sustentáveis e eficazes. As grávidas são sensíveis a mudanças na transmissão de *Plasmodium falciparum* que refletem as tendências da malária na comunidade. Além disso as mulheres desenvolvem anticorpos contra VAR2CSA (proteína de *P. falciparum*) após exposição limitada a parasitas específicos da gravidez. Assim, formulou-se a hipótese de que a detecção destes anticorpos permitirá estimar não só a exposição cumulativa a *P. falciparum* durante um período especifico (uma gravidez) mas também amplificar sinais de transmissão recente, especialmente em áreas de baixa prevalência, onde a possibilidade de detectar anticorpos é mais elevada do que a de detectar o parasita. Os objetivos desta tese foram: 1) compreender os determinantes e consequências dos declínios e ressurgimentos da malária, bem como a aquisição e perca da imunidade antimalárica (**capítulo 1**); 2) descrever a proteína VAR2CSA e desenvolver um ensaio serológico em formato multiplex (**capítulo 2**); 3) avaliar anticorpos específicos de VAR2CSA para refletir níveis de transmissão, mudanças recentes e "hotspots" de malária em áreas híper- a hipo-endémicas (**capítulo 3**). A infecção por *P. falciparum* e os anticorpos contra os antigénios de VAR2CSA foram medidos em amostras de indivíduos expostos e não expostos à malária. Os principais resultados mostraram que, em grávidas moçambicanas, *P. falciparum* diminuiu de 33% em 2003 para 2% em 2010 tendo aumentado para 6% em 2012 (testado por qPCR), tendências que foram refletidas pelos anticorpos. Além disso, as consequências adversas das infecções por *P. falciparum* aumentaram em grávidas após cinco anos de um declínio na prevalência da malária (**capítulo 1**). Foi implementado com sucesso um ensaio imunológico para detectar anticorpos contra 25 novos péptidos de VAR2CSA, tendo as respostas de anticorpos sido específicas da gravidez e dependentes do nível de exposição à malária (**capítulo 2**). Anticorpos contra dois péptidos de VAR2CSA revelaram maximizar a informação sobre a exposição recente durante a gravidez e refletir as diferenças temporais, espaciais e resultantes de medidas de controlo (**capítulo 3**). Os resultados desta tese mostram que a sero-vigilância de grávidas acessíveis ao sistema de saúde pode ser utilizada para avaliar a transmissão da malária com vista ao seu controlo e eliminação.

LIST OF PUBLICATIONS

Alfredo Mayor, Azucena Bardaji, Eusebio Macete, Tacilta Nhampossa, Ana Maria Fonseca, Raquel Gonzalez, Sonia Maculuve, Pau Cistero, Maria Ruperez, Joe Campo, Anifa Vala, Betuel Sigauque, Alfons Jimenez, Sonia Machevo, Laura de la Fuente, Abel Nhama, Leopoldina Luis, John J. Aponte, Sozinho Acacio, Arsenio Nhacolo, Chetan Chitnis, Carlota Dobano, Esperanza Sevene, Pedro Luis Alonso, and Clara Menendez. (2015). ***Changing trends in P. falciparum burden, immunity, and disease in pregnancy***. The New England Journal of Medicine, 373:1607-17.

Ana Maria Fonseca, Llorenç Quinto, Alfons Jiménez, Raquel González, Azucena Bardají, Sonia Maculuve, Carlota Dobaño, Maria Rupérez, Anifa Vala, John J. Aponte, Esperanza Sevene, Eusebio Macete, Clara Menéndez, Alfredo Mayor. (2017) ***Multiplexing detection of IgG against Plasmodium falciparum pregnancy-specific antigens.*** Plos One, 12: e0181150.

Ana Maria Fonseca, Eusebio Macete, Raquel González, Azucena Bardají, Chenjerai Jairoce, Alfons Jiménez, Jennifer Hegewisch, Maria Rupérez, Llorenç Quintó, Pau Cisteró, Anifa Vala, Himanshu Gupta, Nicaise Tuikue Ndam, Simon Kariuki, Carlota Dobaño, Peter Ouma, Michael Ramharter, John J. Aponte, Achille Massougbodji, Valerie Briand, Ghyslain Mombo-Ngoma, Meghna Desai, Michel Cot, Arsenio Nhacolo, Esperanza Sevene, Clara Menéndez, Alfredo Mayor. ***Pregnancy-specific serology reveals temporal and spatial P. falciparum transmission patterns*****.** Under review.

ABBREVIATIONS

ACT	Artemisinin based combination therapy
AMA1	Apical membrane antigen 1
ATS	Cytoplasmic acid terminal segment
CIDR	Cysteine-rich inter-domain region
CSA	Chondroitin sulfate A
CSP	Circumsporozoite protein
DBL	Duffy biding like domain
DHAp	Dihydroartemisinin piperaquine
ELISA	Enzyme linked immunosorbent assay
EVI	European vaccine initiative
Fab	Antigen binding fragment
Fc	Crystallisable fragment
Fv	Variable fragment
HIV	Human immunodeficiency virus
HRP2	Histidine-rich protein 2
ID	Inter-domain
Ig	Immunoglobulin
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spraying
ITN	Insecticide-treated bed net
ISTp	Intermittent screening and treatment in pregnancy
LBW	Low birth-weight
LLITN	Long lasting insecticide treated bed net
MDA	Mass drug administration
MDG	Millennium development goal
mFOI	Molecular force of infection
MOI	Multiplicity of infection
MSP	Merozoite surface protein
NTS	N-terminal segment
PCR	Polymerase chain reaction
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfPR2-10	Proportion of 2-10 years old children with *P.falciparum* infection
PRBC	Parasite-infected red blood cells
RBC	Red blood cells
RDT	Rapid diagnostic test
SCR	Seroconversion rate
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
uORF	Upstream open reading frame
UpsE	VAR2CSA unique regulatory region
VSA	Variant surface antigens
WHO	World health organization

GENERAL INTRODUCTION

Overview

Malaria is a global disease affecting most tropical and subtropical regions and it is an important cause of mortality and morbidity. This chapter aims to provide a general background on this disease, with a special focus on *Plasmodium falciparum* infection during pregnancy. First, a brief description of malaria epidemiology is provided. Then the state of the art on *P. falciparum* pathophysiology, focusing on the life-cycle, the mechanisms involved in placental sequestration and the clinical symptoms of infection is presented. Third, the antibody production and maintenance is described under the immunity against malaria topic, followed by a section on malaria parasite and antibody detection methods. Finally, information on past and current malaria elimination efforts as well malaria control tools is provided and malaria surveillance is introduced, with special emphasis on serological tools for sero-surveillance and evidences pointing-out pregnant women as a promising group for malaria sentinel surveillance.

1. Malaria epidemiology

Malaria is widespread in tropical and subtropical regions in a broad area around the equator (Figure 1)[1]. This includes much of Sub-Saharan Africa, Asia, and Latin America. Children under five years of age, pregnant women and individuals with Human immunodeficiency virus (HIV) infection are at a considerably higher risk of contracting malaria infection and developing severe disease. Malaria infection has a significant impact on economic development of endemic countries due to increases in healthcare costs, loss of ability to work, and negative effects on tourism[2]. According to the latest World Health Organization (WHO) estimates[1], 91 countries and territories had ongoing malaria transmission in 2016 with 216 million cases worldwide and 445,000 deaths. Most cases (90%) and deaths (91%) occurred in the African region[1]. In addition, in areas with stable transmission, it is estimated that at least one in four pregnant women show parasitological evidence of malaria at delivery[3].

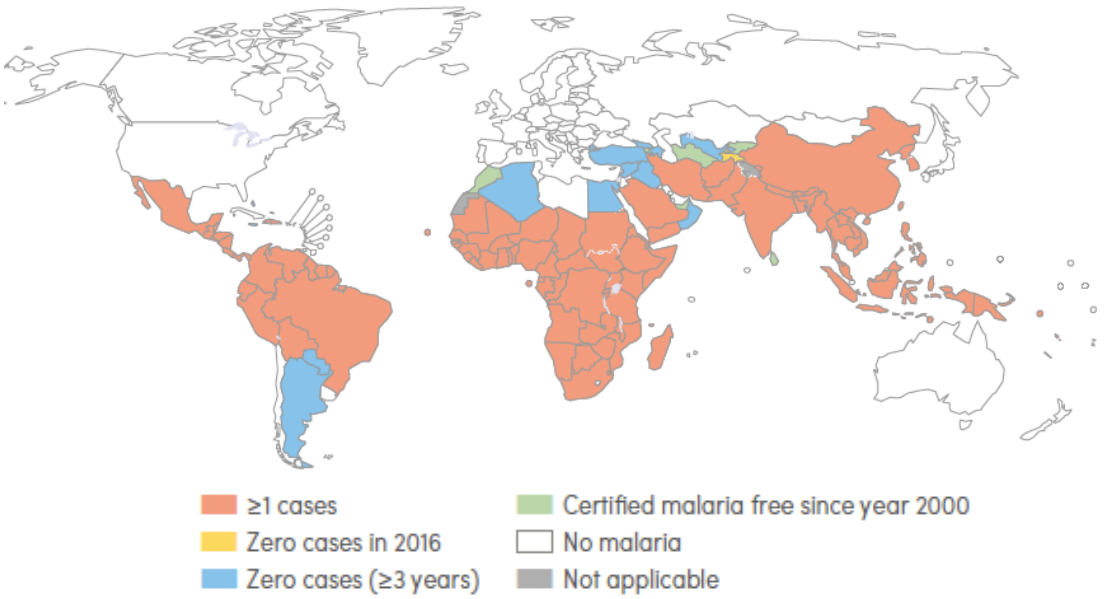

Figure 1. Countries and territories with indigenous malaria cases in 2000 and their status by 2016. Countries with zero indigenous malaria cases over at least the past three consecutive years are eligible to request certification of malaria free status from WHO. Source: WHO 2017[1].

Globally, the malarial incidence among African populations at risk fell considerably between 2000 and 2015 (*P. falciparum* parasite rate in 2-10 years old children [$PfPR_{2-10}$] = 33% in 2000 and 16% in 2015)[4]. As a result of increased enthusiasm for elimination, 17 countries eliminated malaria and a further 21 were identified as having the potential to eliminate malaria by 2020 (Figure 1)[1,4-7]. Despite this reduction, the incidence of malaria increased between 2014 and 2016 in the Americas, and marginally in the South-East Asia, Western Pacific and African regions (See: 4. Malaria control and elimination).

An overview of some studies performed in the African region[8] show that: 1) in the **horn of Africa**, Ethiopia experienced a 70% reduction in malaria from 2000 to 2007, in both outpatients with slide-confirmed malaria and children less than five years old admitted to hospital[9,10]; 2) in **east Africa** many reports have recorded substantial reductions in malaria transmission and morbidity. In the coastal area of Kenya, pediatric malaria admissions declined by as much as 75% between 2003 and 2007[11,12] and maternal delivery prevalence declined from 40% in 2000–2005 to 1% in 2009–2010[13].

In a district in central Kenya, the proportion of malaria outpatient visits declined from 40% in 2000 to 0% by the end of 2006[14]. In Rwanda, data from 20 facilities representing every district in the country showed a decline of more than 50% between 2005 and 2007 in both inpatient and outpatient slide-confirmed malaria cases[10]. In Korogwe district, Tanzania, the prevalence of malaria parasitemia among febrile patients fell substantially between 2003 and 2006 from 78% to 24% in lowland areas and from 25% to 7% in highland areas[15]. In Malawian women delivering in the hospital, the prevalence of malaria parasitemia decreased from 24% in 1997 to 5% in 2006[16]; 3) in **central Africa**, little progress has been documented, possibly because of lack of research initiatives and poor infrastructure to support routine case reporting data. The limited data available from, Sudan[17] show little change in the malaria burden from historical levels; 4) In **southern Africa,** sustained malaria control over many decades has succeeded in stopping transmission throughout most of the South Africa country, with the exception of the northeastern border regions adjacent to Mozambique and Swaziland[18]. In Zambia, pediatric malaria parasite prevalence declined by 53% between 2006 and 2008[19]; 5) In **west Africa,** surveillance at five health facilities across Gambia showed a 50‐85% decline in the prevalence of slide-confirmed malaria among outpatients and a 25‐90% decline in malaria-related hospital admissions[20]. In an urban hospital in Libreville, Gabon, was reported an 80% decline in the number of children with positive blood smears in the inpatient and outpatient services between 2003 and 2008[21] and the prevalence of malaria infection in maternal peripheral blood decreased from 25% in 2005 to 6% in 2011[22]. Data from one village in Senegal showed an increase in the incidence of slide-confirmed malaria between 1998 and 2001 followed by a slight decline of 20% in 2002[23].

2. Malaria pathophysiology

Malaria is caused by parasites from the genus *Plasmodium* and transmitted through female *Anopheles* mosquito bites, usually occurring between sunset and sunrise. There are more than 400 *Anopheles* species

with 30 malaria vectors of major importance. Five species of the genus *Plasmodium* cause all malarial infections in humans. Most cases are caused by either *Plasmodium falciparum* or *Plasmodium vivax*, but human infections can also be caused by *Plasmodium ovale*, *Plasmodium malariae*, and, in parts of southeast Asia, the monkey malaria *Plasmodium knowlesi*[24]. Among the *Plasmodium* species, *P. falciparum* (predominant in Africa with 99% of cases) causes the most severe form of malaria which can be fatal due to expression of antigens required for invasion of red blood cells (RBC)[25].

2.1. *Plasmodium falciparum* life-cycle

The *P. falciparum* life-cycle (Figure 2)[26] begins with the inoculation of motile sporozoites into the human dermis through the bite of an infectious female *Anopheles* mosquito. During this process, the circumsporozoite protein (CSP) plays an important role in dermal migration[27,28]. When in the blood stream, the motile sporozoites travel to the liver, invades hepatocytes and replicates. After about one week, the liver schizonts burst, releasing into the bloodstream thousands of merozoites that invade RBCs and begin the asexual cycle. RBCs invasion is an essential, complex, dynamic and tightly regulated process that involves many ligands and requires an orchestrated sequence of protein-protein interactions. Merozoite surface proteins (MSPs) and apical membrane antigen 1 (AMA1) are some of the merozoite proteins important to establish high affinity interactions with RBCs[29,30]. Once inside the RBC, the invading merozoite begins the intra-erythrocytic development, called schizogony. As schizogony proceeds, ring-stage (i.e. young trophozoite) evolve to trophozoite, a metabolically active stage that digests the hemoglobin, thus accumulating its end product, hemozoin, that can be seen under an optic microscope as granules in the cytoplasm of the parasite. *P. falciparum* undergoes asexual replication over 48 hours. When the RBC is nutritionally exhausted, they burst and release merozoites that can infect other RBCs, dramatically increasing the number of infected cells. Illness starts when total asexual circulation parasite numbers roughly reach 100 million. A small proportion of the young merozoites develop into male and female gametocytes that circulate in the blood and can be taken up by a mosquito

during its blood meal. In the mosquito gut, the gametocytes mature into gametes and reproduce sexually, forming an ookinete and finally an oocyst. The oocyst bursts and liberates sporozoites, which migrate to the salivary glands to await inoculation at the next blood feed to repeat the cycle.

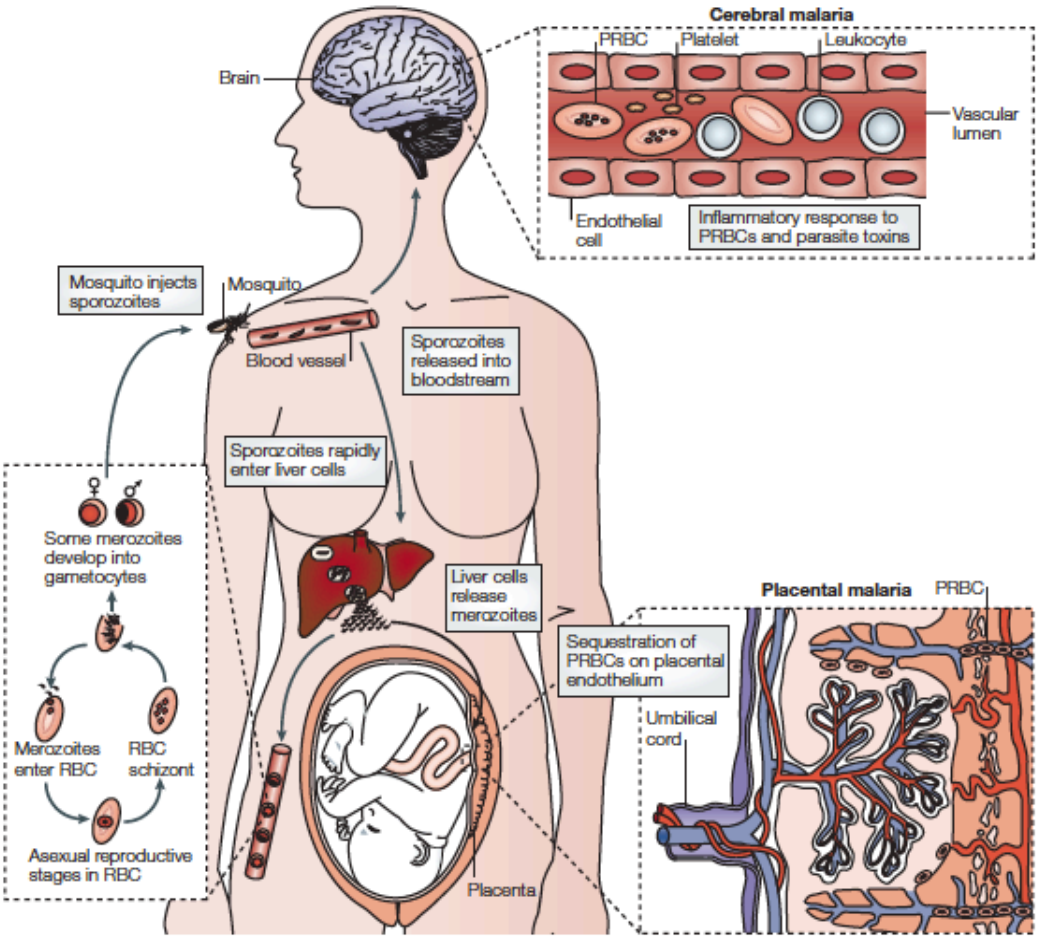

Figure 2. *Plasmodium falciparum* life-cycle. Mosquitoes carrying *P. falciparum* inject a small number of sporozoites into the bloodstream while feeding. Sporozoites are carried to the liver where they develop into merozoites that are released to the general circulation to establish the blood stage cycle. Merozoites invade red blood cells (RBC) and undergo asexual multiplication. During this asexual cycle parasite-infected red blood cells (PRBC) rupture and release more daughter merozoites. PRBC with mature merozoites express adherent ligands, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), and sequestration in organs such as the brain and placenta can occur. Some merozoites differentiate into gametocytes, which, when taken up by another feeding mosquito, perpetuate the sexual cycle in the insect**.** Source: Schofield and Grau, 2005[26].

P. falciparum genome have shown conservation in the central regions of the chromosomes but remarkable variability in the subtelomeric regions[31]. This high genetic variability can generate millions of new antigenic structures in a single infected individual, helping the parasite to escape immune recognition and adapt to new environments[32]. Importantly during blood stages, the parasite remodels the RBC by exporting proteins beyond the confines of its own membrane. *P. falciparum* variant surface antigens (VSA) are major parasite proteins expressed on the infected RBC surface[32]. Several VSA-encoding multigene families have been identified to date, such as *var*[33], *rif*[34] and *stevor*[35]. These variant surface antigens are suggested to mediate the sequestration of infected erythrocytes in the microvasculature and block the blood flow when binding is excessive. The most popular VSAs are from the *var* gene family, comprising around 60 members. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is the protein family involved in cytoadhesion encoded by *var* genes[36]. *var* genes are expressed in a mutually exclusive manner, with only one PfEMP1 protein expressed at a time by each parasite[37]. This is a group of high-molecular-weight proteins consisting of a single intracellular domain, a transmembrane domain, and several extracellular domains (Duffy Biding Like domains [DBLs] and Cysteine-Rich Inter-domain Regions [CIDRs]). The expression of these proteins can allow binding/sequestration to: 1) microvasculature; 2) uninfected RBCs; 3) infected RBCs; 4) platelets, and 5) syncytiotrophoblasts in the placenta[38-40]. This mechanism of sequestration of infected RBCs in host tissues is important for parasite survival because it removes infected RBCs from peripheral blood circulation avoiding the splenic clearance of the parasite[41].

2.2. VAR2CSA and placental sequestration

Various studies have proposed different PfEMP1 molecules as the adhesion ligands responsible for placental sequestration but the most consistent data implicate the PfEMP1 protein VAR2CSA encoded by the *var* gene *var2csa* (Figure 3)[42]. The *var2csa* transcript antigen was detected as the only *var* gene markedly up-regulated in infected RBCs selected to bind to chondroitin sulfate A (CSA) in the syncytiotrophoblast of the placenta[43,44].

Importantly, VAR2CSA has been detected on the surface of CSA adherent laboratory isolates and absent from non-CSA binding isolates[45,46]. Moreover, VAR2CSA was highly abundant in parasites isolated from infected placentas[47,48] and such a binding phenotype has been observed even among parasites collected in the first trimester, suggesting that sequestration can occur early in gestation[49-51]. This led to the hypothesis that *var2csa* might be subject to a unique regulatory mechanism that does not apply to the rest of the *var* gene family. It has also been suggested that it might even be specifically upregulated during pregnancy[52]. *var2csa* has a unique regulatory region (UpsE) not categorized for other *var* genes. The 5´ untranslated region of the mRNA contains an upstream open reading frame (uORF)[53]. uORFs were suggested as regulators of eukaryotic translation[54] acting as repressor of protein synthesis via several different mechanisms (stalling of the ribosome[55], prevention of re-initiation[56] and induction of nonsense mediated decay[57]). The presence of a uORF in the 5´ untranslated region of *var2csa* regulatory region (UpsE) raises the possibility that this element might function to prevent translation of *var2csa* mRNA in situations where the ligand for the encoded PfEMP1 is not available, for instance in the absence of a placenta[58]. This mechanism decreases the exposure of VAR2CSA protein to the immune system in the process of antigenic variation in non-pregnant individuals (See: 3.3. Immunology of malaria in pregnancy).

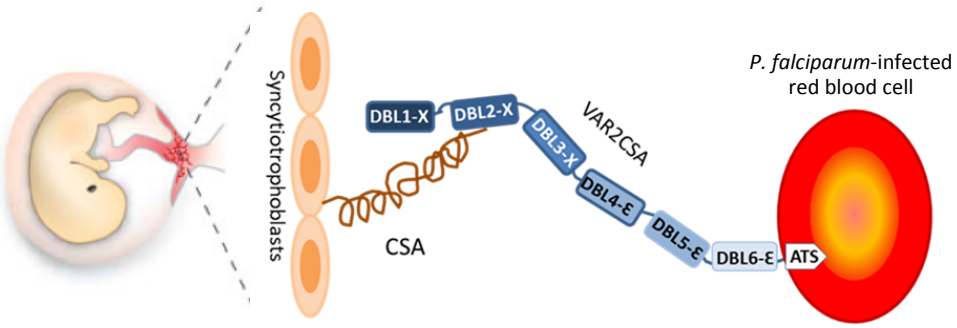

Figure 3. Representation of VAR2CSA PfEMP1 variant anchored to an infected red blood cell. VAR2CSA comprises a large extracellular N-terminal region with six Duffy Binding Like (DBL) domains, a single transmembrane segment and a cytoplasmic acid terminal segment (ATS) at the C-terminus. VAR2CSA binds to the chondroitin sulfate A (CSA) present on the surface of the syncytiotrophoblast cells in the placenta. DBL2X, DBL3X, DBL5Ɛ, and DBL6Ɛ have been associated with binding to CSA. Adapted from Bentley and Gamain, 2008[42].

VAR2CSA is a large protein (~350 kD) organized in extracellular, transmembrane, and intracytoplasmic regions (Figure 3). The extracellular region is uniquely structured among PfEMP1 family members including a large N-terminal sequence with six DBL domains (DBL1X, DBL2X, DBL3X, DBL4Ɛ, DBL5Ɛ and DBL6Ɛ), inter-domain (ID) regions and a cytoplasmic acid terminal segment (ATS) at the C-terminus. DBL2X, 3X, 5Ɛ, and 6Ɛ have been associated with binding to CSA[59]. In contrast to other PfEMP1 molecules, VAR2CSA lacks a DBL1α and a CIDR domain.

Compared to other *var* genes, *var2csa* is relatively conserved between clones and is present in all *P. falciparum* isolates[44,60], with nucleotide pairwise identity from 54% to 94%[47,61] and aminoacid identity average of 78% (range 75-83%)[62]. DBL domains are organized in a variable and conserved block structure and the sequence variation occurs mainly in regions under strong diversifying selection[62] (Figure 4). The first crystal structure of FCR3-DBL3X from VAR2CSA shows a conserved molecular architecture despite low sequence similarity[63,64]. In addition, natural acquired antibodies seem to target both polymorphic and conserved epitopes of VAR2CSA[65] (See: 3.3. Immunology of malaria in pregnancy).

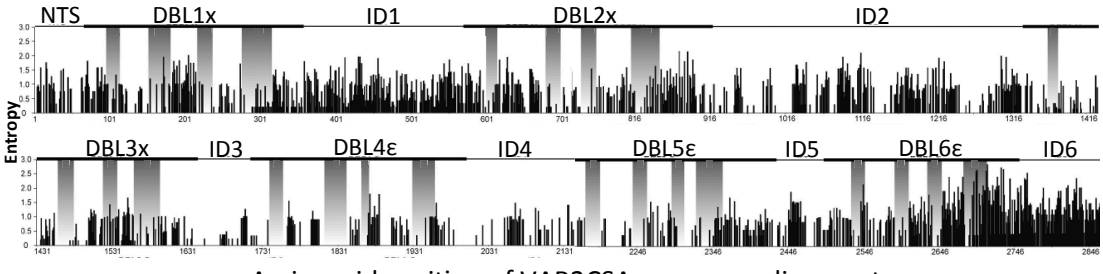

Figure 4. Positional entropy values across a VAR2CSA aminoacid alignment. Positional entropy values (measure of aminoacid diversity in each position of the alignment) calculated from a multiple alignment of 106 complete and partial VAR2CSA extracellular sequences. The N-terminal segment (NTS), Duffy Binding Like Domains (DBL) and inter-domain regions (ID) are labeled. Regions highlighted in grey correspond to the DBL semi-conserved blocks. Adapted from Bockhorst and colleagues, 2007[62].

2.3. Clinical presentation

Malaria-related symptoms and signs are observed during the asexual reproductive stage, after parasites have entered the bloodstream through RBC destruction, sequestration in vital organs, and delivery of parasite by-products[66]. Depending on the absence, presence and severity of symptoms and signs malaria infection can be characterized as asymptomatic, uncomplicated or severe.

Asymptomatic malaria is defined as a *Plasmodium* infection that does not lead to symptoms or signs. Although typically this presentation has been linked to elements acquired upon exposure (host defenses) and host genetic traits, parasite factors are also an important focus to explain asymptomatic infection[67]. **Uncomplicated malaria** has a non-specific clinical presentation with initial symptoms and signs including a general discomfort, headache, fatigue, myalgia, abdominal pain, fever and chills. After several days, the spleen becomes palpable (splenomegaly) and the liver can become enlarged (hepatomegaly), especially in young children. If properly diagnosed and treated, recovery success is common for patients with uncomplicated malaria[68]. In some cases, untreated infection can lead to **severe malaria**[69] the principal cause of malaria-related deaths, which is a common term used for a range of fatal manifestations, including severe anemia[70], acute respiratory distress[71] and cerebral malaria[72].

Malaria in pregnancy, characterized by sequestration of infected RBCs in the placenta**,** is a major cause of mother and offspring mortality and morbidity, such as maternal and infant anemia, increased risk of abortion, premature delivery, low birth-weight (LBW), and neonatal mortality[73-75]. The clinical presentation and complications of malaria in pregnancy differ depending on the transmission intensity, which is explained by the level of pre-pregnancy immunity acquired by the women against the infection[76] (See: 3.3. Immunology of malaria in pregnancy). In low or unstable transmission areas, malaria in pregnancy is frequently symptomatic. Most people in these areas, including pregnant women, have not acquired a significant level of

immunity against malaria and are at risk of developing severe disease. Maternal death may result directly from severe malaria or indirectly due to anemia[3]. In areas of high or stable malaria transmission, most adults have acquired sufficient immunity for protection from severe disease, and therefore malaria infection rarely results in complicated disease. During the first and second pregnancy women are more susceptible to the effects of malaria infection and may develop uncomplicated and even complicated disease. In subsequent pregnancies, the developed immunity usually prevents from severe disease unless the woman is co-infected with HIV[77]. The main effects of malaria in pregnancy in high transmission areas are maternal and fetal anemia, LBW, intra-uterus retardation growth and possibly fetal loss[3,76].

3. Malaria immunology

Individuals residing in malaria-endemic regions acquire clinical immunity to *P. falciparum* infections gradually, following repeated exposure to malaria parasites in childhood. Adults generally have gained sufficient immunity to reduce frequency and severity of infections[78]. Malaria **transmission intensity** and **host age** are the key determinants of immunity development and disease severity[79,80]. In areas of high transmission, the more susceptible groups to the harmful effects of malaria infection are infants, young children and pregnant women[73], rather than other adults due to development of naturally acquired protective immunity[81]. Most adults living in high transmission areas carry circulating parasites and demonstrate mild or no symptomatology. In regions with low transmission, both adults and children are susceptible to clinical malaria manifestations as exposure to *P. falciparum* infection is infrequent and inadequate for immunity to be acquired. However, despite decades of research, naturally acquired immunity to malaria is still poorly understood[82], especially regarding the reasons for immune responses being often lost upon cessation of malaria exposure and for sterile immunity being never achieved.

Acquired immune response is stimulated and regulated by the innate immunity (first line defense) via production of specific cytokines, ultimately resulting in antigen-specific humoral- and cell-mediated responses[83]. Previous

studies show that the humoral immune system appears to play a major role in anti-parasite immunity through production of antibodies[84]. Additionally, it is also suggested that host mechanisms of resistance to infection, mediated by the innate and acquired immune system, can reduce pathogen burden and ameliorate symptoms (Figure 5)[67].

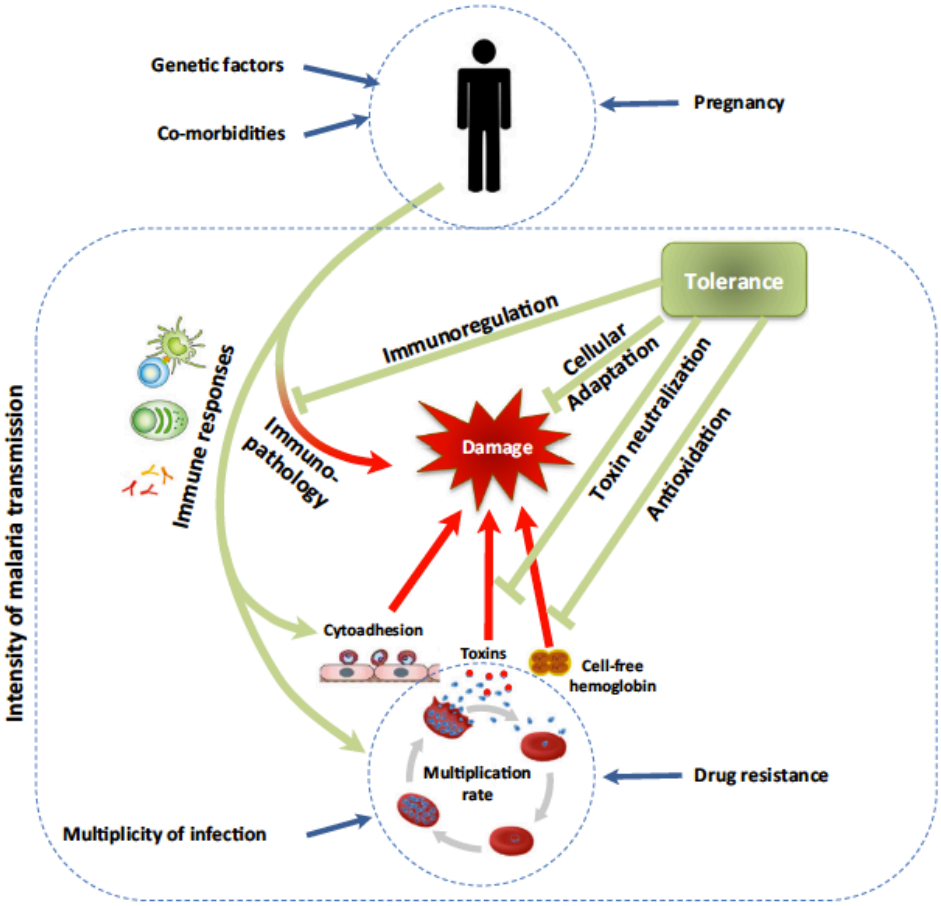

Figure 5. Host and malaria parasite interactions resulting in damage and disease. Disease is the clinical manifestation of body damage that results from host–parasite interactions. Damage may be caused directly by malaria parasites through RBC destruction or sequestration in vital organs or by parasite byproducts (toxins or oxidant agents such as hemozoin). However, damage may also arise as a result of the host immune response against infection, leading to inflammation. Host mechanisms of resistance to infection can reduce pathogen burden and ameliorate symptoms. Also, damage caused directly by the pathogen or indirectly by the host immune response can be minimized by immuno-regulation, cellular adaptation, or parasite toxin neutralization irrespective of pathogen burden (i.e., tolerance to malaria infection). Such mechanisms may be modulated by previous exposure to malaria parasites, leading to acquired responses, host genetic predisposition to combat disease, and comorbidities. Source: Galatas and colleagues, 2015[67].

3.1. Antibody responses

The 'standard' response of the immune system when exposed to a new foreign protein is to mount a specific antibody response within a couple of weeks. The first antibodies are secreted by short-lived plasma cells which are developed following interaction between B-cells and antigen-specific helper T-cells in the peripheral lymphoid organs. After a first exposure to parasites in the peripheral circulation, B-cells are triggered to respond[85]. B-cells display various antigen-receptors (membrane-bound immunoglobulins [Ig]) at their surface that are capable of capturing antigens. When a receptor has captured an antigen, B-cells rapidly undergo clonal expansion, and subsequently differentiate into plasma cells and memory B-cells. There are two types of plasma cells, short-lived and long-lived plasma cells[85]. At first exposure, these plasma cells secrete antibodies of relatively low affinity for several weeks, then develop apoptosis[86]. A few involved B- and T-cells migrate into the lymphoid follicles, where they form germinal centers. Here the B-cells undergo class-switching and affinity maturation that result in formation of additional antibody-secreting plasma cells and memory B-cells. Germinal center-derived plasma cells secrete high-affinity, appropriately class-switched antibodies, whereas the memory B-cells encode such antibodies but do not secrete them. Most germinal center-derived plasma cells following a primary response are also short-lived, making the overall primary antibody response transient. Upon re-exposure to the priming antigen, the memory B-cells ensure a secondary antibody response (recall) that is faster, stronger, and of better quality (high-affinity antibodies of appropriate class) than the primary response. This is because they can rapidly differentiate into plasma cells (effector memory B-cells) that secrete high-quality antibodies at high rates. As the infection is controlled, the expanded antigen-specific B-cell population contracts very substantially, leaving behind memory B-cells and plasma cells (Figure 6)[87]. Some of the latter are long-lived, and survive in the bone marrow and continue to secrete antibody for months or years[88,89]. These long-lived plasma cells are the main source of the sustained antibody levels that are often seen after repeated antigen exposure, even in the absence of persistent antigen.

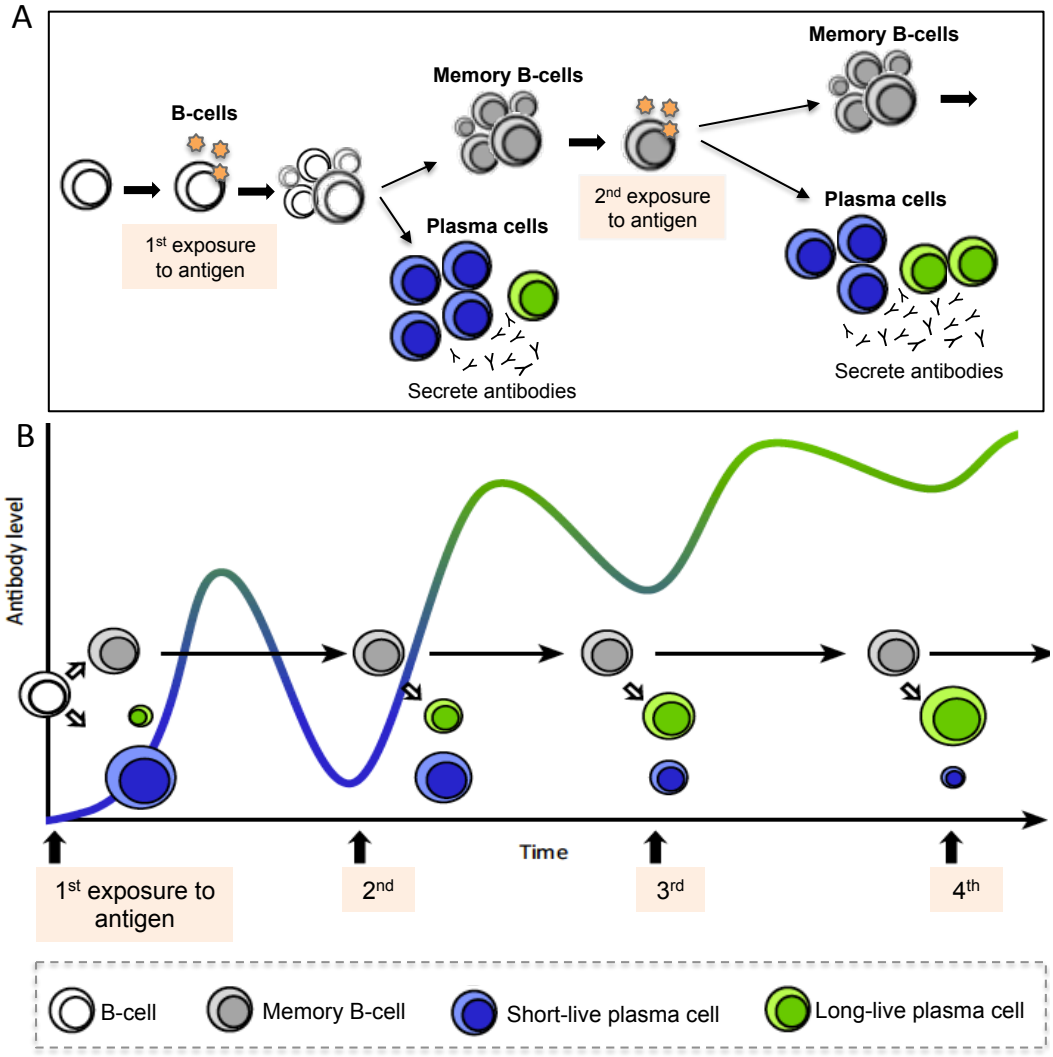

Figure 6. Process of memory build-up and time-exposure dependent changes in antibody longevity and plasma cell compositions. A) B-cells have different antigen-receptors that, in a 1st exposure tries to bind to antigens present on the parasites. When an antigen-receptor captures an antigen, B-cells divide rapidly, after which they start to produce memory B-cells and plasma cells. Plasma cells rapidly attack the parasite by production of antibodies. In a 2nd exposure the present memory B-cells will recognize the antigen. Plasma cells that already exist will respond instantly to the parasite by production of antibodies, while at the same time the memory B-cells will rapidly divide and produce new plasma cells. B) Exposure to *P. falciparum* parasites appears to result in formation of adequate B-cell memory and antibody-secreting short-lived plasma cells early on. As protective immunity is acquired, the establishment of increasing numbers of long-lived plasma cells is facilitated (perhaps as a result of decreased intensity and frequency of malaria-associated inflammation), thereby reducing the reliance on short-live plasma cells, and hence increasing the overall longevity of the antibody responses (wavy line). Adapted from Hviid and colleagues, 2015[87].

During an infectious process, B-cells control the antibody functions through different ways: 1) by selecting the Fc-domains via class switch recombination to establish the isotype (IgD, IgM, IgG, IgA or IgE) or 2) by differentially glycosylating antibodies aimed at modulating their affinity for individual Fc-receptors (Figure 7). IgG is the most abundant antibody isotype in mammals, including humans, and is associated with protective immunity. There are four subclasses of IgG. They are classified according to their IgG affinity to Fc-receptors and the complement and effector cell activation. They are further classified by the IgG amount present in the blood circulation. IgG responses dominated by IgG1 and IgG3 are related to malaria protection, whereas IgG2 and IgG4 are not related to malaria protection[84,90].

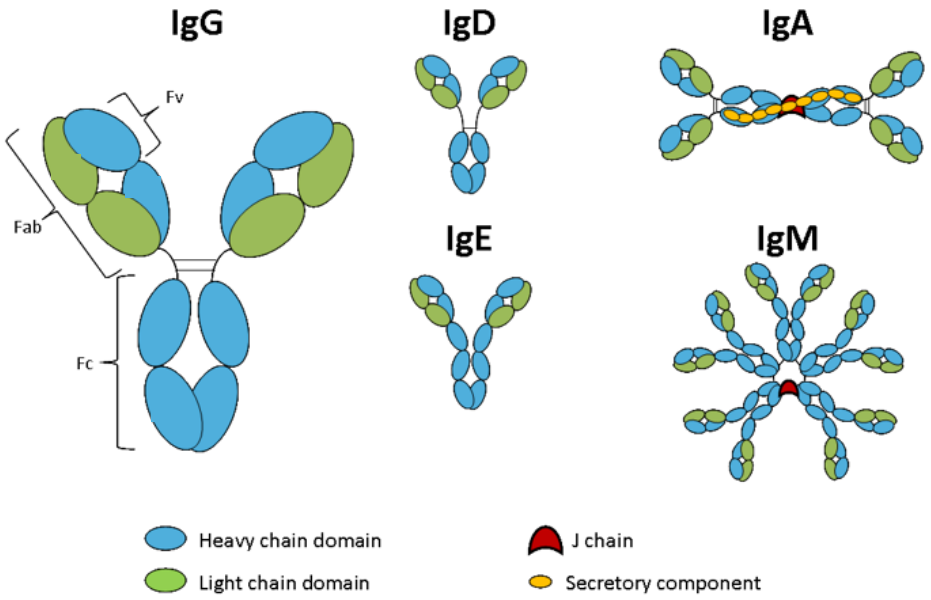

Figure 7. Schematic representation of the five antibody (immunoglobulin- Ig) isotypes in mammals. In mammals, antibodies are classified into five main isotypes – IgA, IgD, IgE, IgG and IgM. They are classified according to the heavy chain, which differ in the sequence and number of constant domains, hinge structure and the valency of the antibody. Briefly, antibody structure comprises the antigen binding fragment (Fab) that recognizes the antigen and the crystallisable fragment (Fc) that interacts with other elements of the immune system, such as phagocytes or components of the complement pathway, to promote removal of the antigen. The Fab domains consist of two variable and two constant domains, with the two variable domains making up the variable fragment (Fv), which provides the antigen specificity of the antibody with the constant domains acting as a structural framework. Adapted from Polijak and colleagues, 1973[91].

3.2. Immunological memory

It is widely perceived that immunity to malaria is short-lived, rendering people susceptible to repeated malaria infections[92]. However, there have been very few studies on "memory" responses and how the human immune system recognizes previously encountered malaria parasites. In particular, very little is known about the durability of malaria-specific B-cells and antibodies.

Antibody titers are thought to rapidly decline in the absence of re-infection or when individuals leave an endemic area, supporting the notion that malaria infection fails to induce durable immunological memory responses. In a 12-week study of Kenyan children following acute malaria[93], the half-lives of IgG responses specific for five merozoite antigens were estimated to be ten days for IgG1 and six days for IgG3 (much shorter than for tetanus [11 years] or measles vaccines [>300 years]). In longitudinal studies, malaria-specific antibody responses have been reported to decline to undetectable levels within three to nine months of documented malaria episodes in children[94-103]. Short-lived malaria-specific IgGs have been also observed in older children and adults[95,96,104]. A recent study showed that children exposed to intense seasonal malaria transmission generated IgG responses to hundreds of *P. falciparum* antigens each transmission season, although these antibodies declined rapidly during the next six months dry season[105]. In contrast, there are studies that point toward more stable IgG responses with increasing age in areas of stable and perennial *P. falciparum* transmission[94,97,99,106,107], although it is difficult to separate the effects of age and cumulative exposure to the parasite on malaria immunity. Similarly, there was no evidence of any significant decline in antibody titers to over periods of more than five years since the last known malaria infection among Thai individuals. Estimates of the half-life of this maintenance phase of antibody responses to malaria antigens ranged from around five to ~16 years, putting them in the same range as the half-lives estimated for antigens such as tetanus or diphtheria toxoids[108]. One reason for discrepancies may be that some studies characterized the decay of the antibody response in the first few

days/weeks after resolution of an acute malaria infection[94,103], which is likely to capture the initial very rapid decay in antibody titer associated with contraction of the pool of short-lived plasma cells, whereas other studies[107], where the most recent malaria infection occurred many months or years ago, are capturing the long term "maintenance" phase of the antibody response[89]. It is not clear, from these studies, whether the rapid decline of antibody concentrations observed in children is related to removal of antigen by chemotherapy, by consumption of antibodies and formation of antigen-antibody complexes during parasite clearance or due to limitations in the ability of the bone marrow compartment to support differentiation and/or survival of plasma cells[109,110]. Other factors contributing to diverging results and conclusions amongst studies are different: 1) methodologies (study design, analysis, antigen/antibody investigated) 2) malaria transmission, and 3) study populations[111].

3.3. Immunology of malaria in pregnancy

Pregnant women have an increased risk of malaria infection independent of their previous level of acquired immunity. Several studies have proposed specific physiological changes associated with gestation as one of the possible causes[112]. It has been suggested that the particular, immunological situation of pregnancy could favor malaria infection, due to the hormonal and immune adaptations to avoid fetus rejection[113-115]. This immune-tolerant state could be one of the mechanisms involved in placental malaria[116]. In addition, the increased susceptibility is also attributed to an augmented attraction of mosquitoes during pregnancy[117] and the singularity of *P. falciparum* infections in the context of gestation[38]. Specific antibodies against placental isolates that bind to CSA (anti-VAR2CSA antibodies) are more common in multigravid than in primigravid women in endemic areas[118], which could explain the decreased susceptibility to infection after successive pregnancies[119], and are not present in men or women that have never been pregnant[120]. Antibodies against VAR2CSA cross-react between geographically diverse placental isolates suggesting conserved epitopes[121], overlap of polymorphisms[62] or polymorphic conformational epitopes[122]. High

antibody levels have been associated with reduced risk of placental infection[123-125], low birth weight[46,124,126-128], and maternal anemia[127]. Moreover, antibodies against VAR2CSA are affected by variables that influence the risk of exposure to *P. falciparum* such as season, neighborhood, proximity to a river or use of control measures[128-130]. These findings reinforce the efforts of several studies to design a VAR2CSA-based vaccine to prevent placental malaria[131] (See: 4.2. Malaria control in pregnant women). In addition, it was recently suggested that anti-VAR2CSA antibodies at delivery reflect exposure to *P. falciparum* in pregnancy[40,125,128,132-136], and could be used as markers of exposure to the parasite and risk of infection especially in primigravid women[77,137]. In high-transmission areas, primigravida are at greater risk of infection than multigravida since women develop increasing resistance to placental malaria over successive pregnancies[73,121]. In low transmission areas, the antibody-mediated response is weaker and multigravida are likely to be serologically equivalent to primigravida regarding placental-type parasites[138,139]. Immunity to the non-pregnancy antigens is associated with a reduction in placental malaria infections as well, which explains young age as an independent risk factor for malaria during pregnancy when immunity is not fully developed yet[140,141].

In the particular case of malaria in pregnancy, antibodies measurements against VAR2CSA, possess complex dynamics. Ampomah *et al*[142] reported that antibodies seem to start increasing 150 days before delivery, showing greater rates of increasing levels in multigravida compared to primigravida. After delivery, levels showed a decrease until 250 days within delivery where levels go down again. In other study by Fowkes *et al*[143] VAR2CSA antibodies showed a half-life of 36-157 years, suggesting that indeed antibodies can be maintained for further pregnancies.

It has been shown that HIV infection reduces antibody levels against *P. falciparum* antigens in pregnant women[144] and attenuates the parity-dependent increase of antibodies[128] observed in HIV-negative women[40]. A longer duration of HIV infection in multigravida may impair "memory-mechanisms" responsible for the maintenance of antibody production[145,146],

explaining why antibody responses do not increase with parity in HIV-infected women. Such a waning of antibodies in HIV-immune-suppressed women[145,147] may contribute to increase the risk of malaria associated with HIV infection observed in multigravida compared to primigravida[148] and also to accelerate the decay of antibodies in absence of parasite exposure. In contrast, HIV-negative women may maintain elevated levels of antibodies for longer periods[94], overcoming fluctuations in parasite exposure associated with seasonality or the use of effective malaria control tools[129]. Actually, as well as known modifiers such as HIV infection[149], there are many other factors that could influence the level of acquired immunity including the duration of infection, the stage of infection and the time between pregnancies.

4. Malaria control and elimination

Malaria control has been the main goal of global programs since the 60s. However, in 2007 Bill & Melinda Gates (founders of one of the major economic sources for malaria research) gave a speech where they rescued the "malaria eradication" concept[150]. Consequently, in the last decade, researchers, policy makers and even the WHO have switched their minds from malaria control to this highly ambitious goal of eradication (See definitions in Box 1).

Box 1: Definitions of malaria Control, Elimination and Eradication

- **Control** of malaria means reduction of disease incidence, prevalence, morbidity, or mortality to a locally acceptable level as a result of deliberate efforts. Continued intervention measures are required to maintain control.
- **Elimination** of malaria means the interruption of local transmission (reduction to zero incidence of indigenous cases) of a specified malaria parasite in a defined geographical area as a result of deliberate activities. Continued measures to prevent re-establishment of transmission are required.
- **Certification of malaria elimination:** Countries that achieve at least three consecutive years of zero indigenous cases are eligible to apply for a WHO certification of malaria-free status.
- **Eradication** of malaria means the permanent reduction to zero of the worldwide incidence of infection caused by human malaria parasites as a result of deliberate activities and interventions are no longer required.

Adapted from Alonso and colleagues, 2011 and Rabinovich and colleagues, 2017[7,151].

Considerable declines in malaria have accompanied increased funding for control since the year 2000, catalyzed by the Roll Back Malaria initiative and the wider development agenda around the United Nations Millennium Development Goals (MDGs). The 15 years since have seen an increase of approximately 20-fold in international funding for malaria control[152], enabling widespread but uneven coverage scale-up of the main contemporary malaria control interventions that are based on vector control and chemotherapy (See: 4.1. Tools: diagnostics, drugs, vector control and vaccines). As part of this reinvigorated effort, a series of international goals were set with a target year of 2015, in particular the MDG to "halt and begin to reverse the incidence of malaria" and the more ambitious target defined later by the WHO to reduce cases incidence by 75% relative to 2000 levels[153]. While these targets were important for motivating action and mobilizing funds, no explicit plan was put in place to reliably measure progress towards them. Now that the benchmark year of 2015 has been reached, the international community defined a post-2015 agenda for malaria control and elimination. This agenda was defined around two key policy initiatives for the 2016-2030 period: the Global Technical Strategy[5] and Action and Investment to Defeat Malaria[154], led by WHO and the Roll Back Malaria Partnership. This technical strategy defines a clear and ambitious path for countries where malaria is endemic, and their global partners in malaria control and elimination for the next 15 years. The visions of this new strategy towards 2030 relative to 2015 include: 1) a 90% reduction in malarial mortality rates; 2) a 90% reduction in new malarial case incidence; 3) the elimination of malaria in at least 35 countries, and 4) prevention of re-establishment of malaria in all countries that are considered malaria-free. To achieve these goals the strategy is built on three pillars: 1) ensure universal access to malaria prevention, diagnosis and treatment; 2) accelerate efforts towards elimination and attainment of malaria-free status; 3) transform malaria surveillance into a core intervention (See: 5. Malaria surveillance). However, it is important to consider that historical failures to maintain gains against the disease underscore the fragility of successes of control programs (Figure 8). Although malaria transmission can be suppressed by effective control measures, in the absence of active intervention malaria will return to an intrinsic equilibrium determined by factors

related to ecology, efficiency of mosquito vectors, and socioeconomic characteristics[155].

Understanding where and why resurgence has occurred historically can help current and future malaria control programs avoid the mistakes of the past. A systematic review of the literature[155] identified 75 resurgence events in 61 countries, occurring from the 1930s through the 2000s. Almost all resurgence events (68/75 = 91%) were attributed to the weakening of malaria control programs because resource constraints, complacency and other issues with poor execution, war or disaster, purposeful cessation of control activities and community non-cooperation. Over half of the events (44/75 = 59%) were attributed to increases in the intrinsic potential for malaria transmission for a variety of reasons, of which the movement of humans or mosquitoes and the parasites they carry were the most common. Finally, only 24/75 (32%) were attributed to vector or drug resistance.

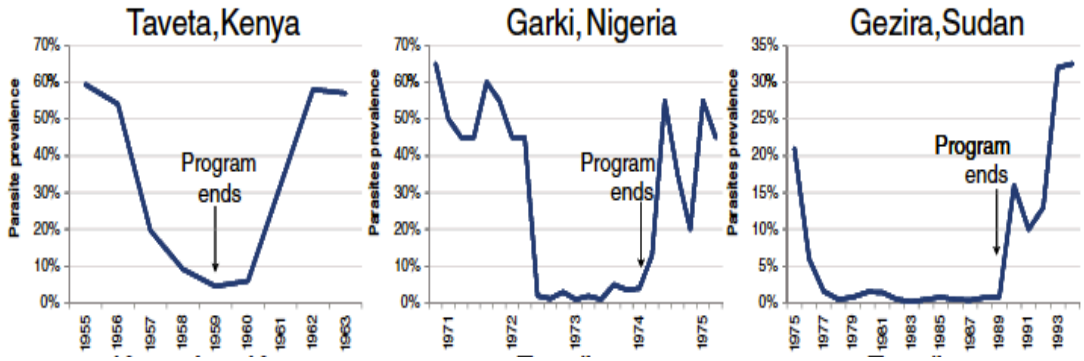

Figure 8. Malaria resurgence in Africa following cessation of pilot programs and relaxation of control activities. Adapted from Cohen and colleagues, 2012[155].

The global malaria community holds tools that have been proven to work in reducing illness and death from malaria (See: 4.1. Tools: diagnostics, drugs, vector control and vaccines). Finding ways to maintain the funding, political will, and strong operational capacity to use malaria control tools over the long-term is imperative to ensure that the dramatic progress that has been achieved through international investment is sustained and extended.

4.1. Tools: diagnostics, drugs, vector control and vaccines

Diagnostic methods for individuals (including those who are pregnant) are directly performed on blood samples to detect the living parasite, antigens or DNA. These methods include: 1) conventional optic microscopic diagnosis by staining thin and thick blood smears; 2) rapid diagnostic tests (RDTs) and 3) molecular diagnostic methods (i.e. polymerase chain reaction - PCR)[156]. In pregnant women, the tests are performed in peripheral- and placental-blood samples when available at delivery. In placental specimens, histopathology can also be used to identify parasites and resultant inflammatory responses.

Optic microscopy is the oldest method, and still routinely used for malaria diagnosis in most large health clinics and hospitals[156]. Though only minimal equipment is required (staining solution and microscope with immersion lens), this technique must be carried out with precision. Intensive training is required in order to ensure accuracy. This method has a sensitivity between 50 and 500 parasites/μL with thick blood films[157]. Parasitemia can be counted by optic microscopy, but involves the analysis of many fields and it should imply at least two expert microscopists.

Rapid diagnostic test is an alternative to optic microscopy in remote areas as the test is easy to perform directly on the field (15-30 minutes after a finger-prick blood collection) it has a great value as point-of-care test[157]. In regions with no available microscopes, it is a frequently used method that contributes greatly to early diagnosis and subsequent treatment, as it can be performed in the rural areas itself, and by village health volunteers. The most widely used RDTs for malaria are typically based on the detection of the parasite protein HRP2 (Histidine-Rich Protein 2). RDTs currently have a sensitivity of ~100 parasites/μL[157] and ultrasensitive RDT (around 20 parasites/μL) are undergoing evaluation. Limitations in using this technique, include false positives (HRP2 persists in blood after infection clearance for several days)[158] and, more concerning in an elimination scenario, false negatives (gene deletions)[159,160]. Contrary to optic microscopy, it does not

allow to quantify parasitemia. However, studies in the field demonstrate that malaria infection prevalence measured by optic microscopy or RDTs is similar[161].

Polymerase chain reaction is a molecular diagnostic test that involves detection of specific DNA regions within the parasite genome. Compared to the conventional methods, this technique is the most sensitive method available to detect parasitemia to as low as two-five parasites/μL[162]. It is useful to accurately assess parasite density, measure the multiplicity of infection, evaluate the infecting species, identify drug-resistant parasites, and to process large numbers of samples when automated[156]. However, despite these advantages, it is not appropriate for use in the field due to the complex technique requiring special training with sophisticated equipment and high cost. Still, it is recommended by the WHO for epidemiological research and surveys to map asymptomatic individuals (i.e. usually individuals with submicroscopic infections and absence of symptoms[67]). Due to the difficulty of diagnosing this group of individuals, malaria elimination is greatly challenged if asymptomatic infections cannot be detected and treated. In a high endemic area of Senegal, a study suggested that more than 90% of exposed individuals are likely infected with chronic asymptomatic malaria[163].

Placental histopathology is considered the main approach to know whether the placenta has been exposed to malaria infection[164]. It is the most accurate indicator of ongoing placental sequestration and past infection and can also provide an indication of the stage of infection through assessment of the histological changes, which have occurred within the placenta. However, it can only be performed after delivery, when the placenta is available for examination and consequently it is too late to prevent adverse effects on the mother and fetus. Histology of a stained biopsy from the maternal side of the placenta can be examined for the presence of malaria parasites and hemozoin pigment (Figure 9) and is classified as acute (only parasite observed), chronic (parasite and pigment) or past (only pigment)[164,165]. Major barriers to the implementation of histology are: 1) its applicability only in hospital-based studies due to difficulties for placenta collection in areas where

many women deliver at home; 2) the requirement of skilled laboratory personnel and qualified histologists; 3) the cost of sample processing and 4) lack of standardization between laboratories[166,167].

Throughout pregnancy, the prevalence of placental infection can only be assessed indirectly in samples from peripheral blood. Peripheral infection at delivery is usually a good indicator of an ongoing placental infection, however, such measures generally underestimate the prevalence of sequestration and woman with placental infection often remain asymptomatic[168,169]. As a result, many placental infections are likely to go undetected until delivery and it is not possible to accurately assess the timing and duration of infection during pregnancy. Additionally, the past stage of infection is very rarely cleared before delivery, suggesting that, in the absence of treatment, the existence of pigment may have good sensitivity as an indicator of previous placental infection and that placental histology is an effective approach to determine the proportion of women who have experienced placental malaria at any stage of pregnancy[119].

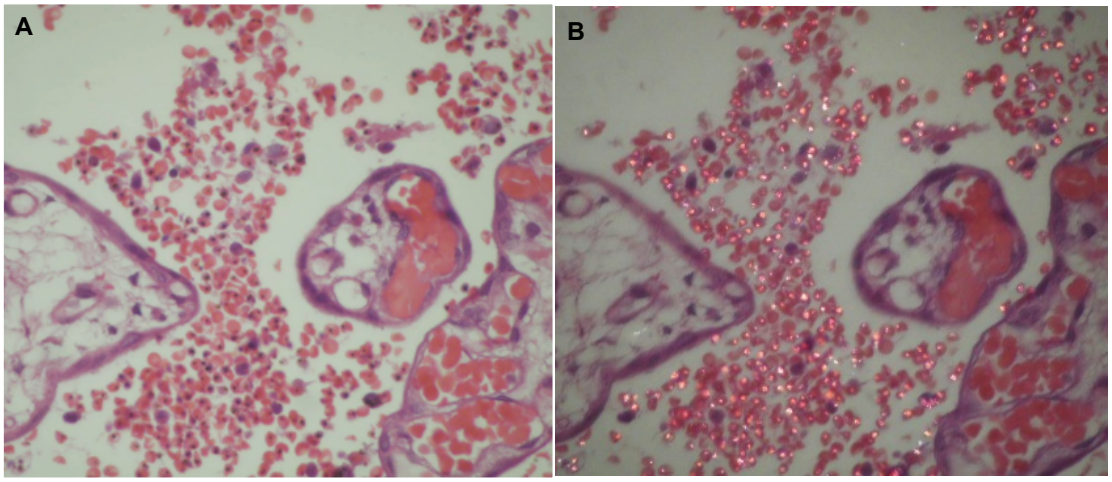

Figure 9. Histology of a *P. falciparum* infected placenta. A huge accumulation of infected red blood cells is observed. A) Infected red blood cells are those with a dark dot inside that correspond to hemozoin pigment or B) Hemozoin also emits fluorescence when observed using polarized light. Photography from Jaume Ordi.

Antimalarial drugs are used for treatment, but also for prevention in vulnerable populations, such as pregnant woman (See: Malaria control in pregnant women) and infants, that follow programs called intermittent

preventive treatment in pregnancy (IPTp) or in infants (IPTi). Artemisinins are superior to the classically used quinine and are available in a wide range of formulations[170]. Current recommended treatment of uncomplicated malaria involves artemisinin-based combination therapies (ACTs) as a three-day course (except in pregnant women in their first trimester). Treatment of severe malaria requires intravenous artesunate for at least 24 hours followed by three days of ACT. The choice of ACTs in a country or region should be based on optimal efficacy, safety and adherence[170]. Antimalarial drugs are the pillar of mass drug administration (MDA) campaigns, defined as the empiric administration of a therapeutic antimalarial regimen to an entire population at the same time. This strategy has been proven to rapidly reduce the prevalence and incidence of malaria in the short term[171]. However, if malaria transmission is not interrupted (or its importation not prevented), transmission eventually returns to its original levels, unless the vectorial capacity is reduced and maintained at very low levels during the post MDA period[172].

Vector control, implies targeting the *Anopheles* mosquito to interrupt malaria transmission. Indoor Residual Spraying (IRS) and Insecticide-Treated Bed Nets (ITN) are the main strategies. ITNs are thought to be the main tool contributing to base decrease in Africa, particularly long-lasting ITNs (LLITN), highlighting the importance of ITN distribution and population awareness of its use[4].

Vaccines can represent a complementary intervention to the current call for malaria eradication. It would supplement the waning natural immunity against malaria that is expected after elimination campaigns that would lead to the aforementioned shift towards more severe forms of malaria. A pre-erythrocytic vaccine to interrupt malaria transmission that completely prevents liver-stage infection for a significant duration (e.g., at least one transmission season) would prevent parasitemia and gametocyte generation and therefore interrupt onward transmission. The RTS,S/AS02 is the only vaccine against malaria that has been authorized. It is a pre-erythrocytic vaccine that targets

the sporozoite protein CSP. Unfortunately, it shows only modest efficacy upon vaccination in children (28-36%) and in infants (18-26%)[173]. Still, WHO recommended its pilot implementation and classified it among the support elements complementary to existing effective interventions. Other promising vaccination strategies are based on sporozoites inoculation, which show high efficacy against homologous challenge. However, heterologous protection has been reported to be moderate[174]. Blood-stage vaccines are an alternative and complementary approach to pre-erythrocytic vaccines. These types of vaccines interrupt malaria parasite transmission by efficiently clearing blood-stage infections, limiting gametocyte densities and the duration that a person is infectious, thus reducing human-to-mosquito malaria parasite transmission. Several promising *P. falciparum* blood-stage vaccine candidates are in clinical development, including the unstructured peptide P27A, the well-studied PfRH5, and the two placental malaria vaccine candidates based on VAR2CSA protein[175] (See: 4.2. Malaria control in pregnant women). Innovative new concepts in next-generation malaria vaccine protein subunit design are being explored to develop highly effective multicomponent/ multistage/ multiantigen formulations[176].

4.2. Malaria control in pregnant women

In areas in Africa where malaria is endemic, WHO recommends a combination of ITNs and IPTp with sulfadoxine–pyrimethamine (SP) for women who are HIV-negative or daily co-trimoxazole prophylaxis for HIV-positive women[177] plus management with ACT or parenteral artesunate for uncomplicated and complicated malaria respectively[170]. In sub-Saharan Africa, effectiveness of IPTp with SP is threatened by parasite resistance[178]. In the past decade, several trials have aimed to find alternative drugs for IPTp or alternative strategies to potentially replace IPTp such as screen-and-treat approaches. Of all the candidates studied, dihydroartemisinin-piperaquine has the potential to replace SP for IPTp[179-181]. However, further confirmatory studies of the safety, efficacy, cost-effectiveness, and feasibility of this three-day regimen are needed in HIV-negative women and in HIV-positive women on antiretroviral therapy. Additionally, intermittent screening and treatment in

pregnancy (ISTp) consists of intermittent RDT for malaria and treatment of RDT-positive cases with an effective artemisinin-based combination therapy, such as artemether-lumefantrine[182] or dihydroartemisinin-piperaquine[183]. The results from a meta-analysis suggest that, at the current level of sensitivity of RDTs, these test-and-treat strategies are not a suitable alternative to IPTp, even in areas with high resistance to SP[177]. Despite the benefits, coverage of ITNs and IPTp in pregnant African women is inadequate (38.8% and 21.5%, respectively; Figure 10)[184]. Moreover, chemotherapy approaches for malaria prevention exclude pregnant women at first trimester given contraindications or limited efficacy and safety data of antimalarials at early periods of gestation[185,186]. Although the use of chloroquine in the treatment of malaria prophylaxis during early pregnancy is probably safe and efficacious in some regions[187], it is not recommended by WHO. Therefore, the only option currently available for protecting pregnant women during this vulnerable period is the use of ITNs, whose coverage and use in sub-Saharan Africa, is less than optimal[188]. Targeting interventions to the period when malaria causes the most damage can ensure optimal pregnancy outcomes in resource constrained settings[189]. The risk of placental infection is higher early in gestation, mainly in primigravid women, and before women typically seek obstetric care, pointing to the urgent need to increase antenatal care attendance as early as possible or expand antimalarial interventions outside of the antenatal clinic (Figure 11)[149].

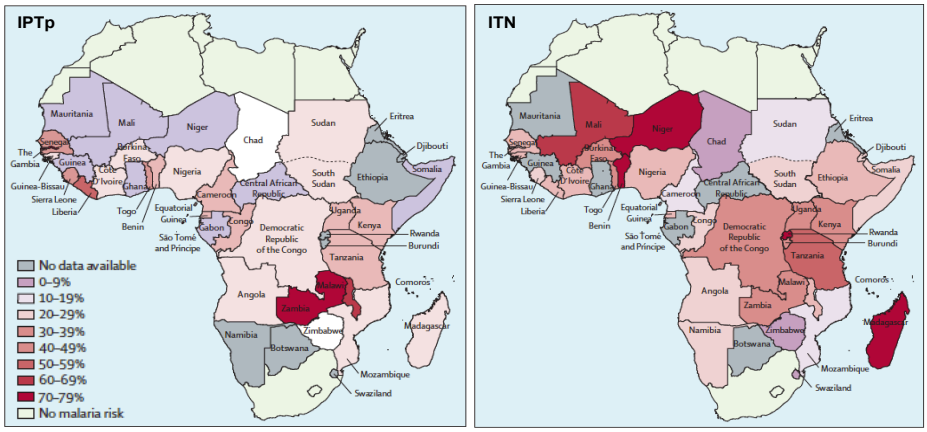

Figure 10. Coverage of Intermittent preventive treatment (IPTp) and insecticide-treated nets (ITN) in pregnant women during 2009-2011. Adapted from van Eijk and colleagues, 2013[184].

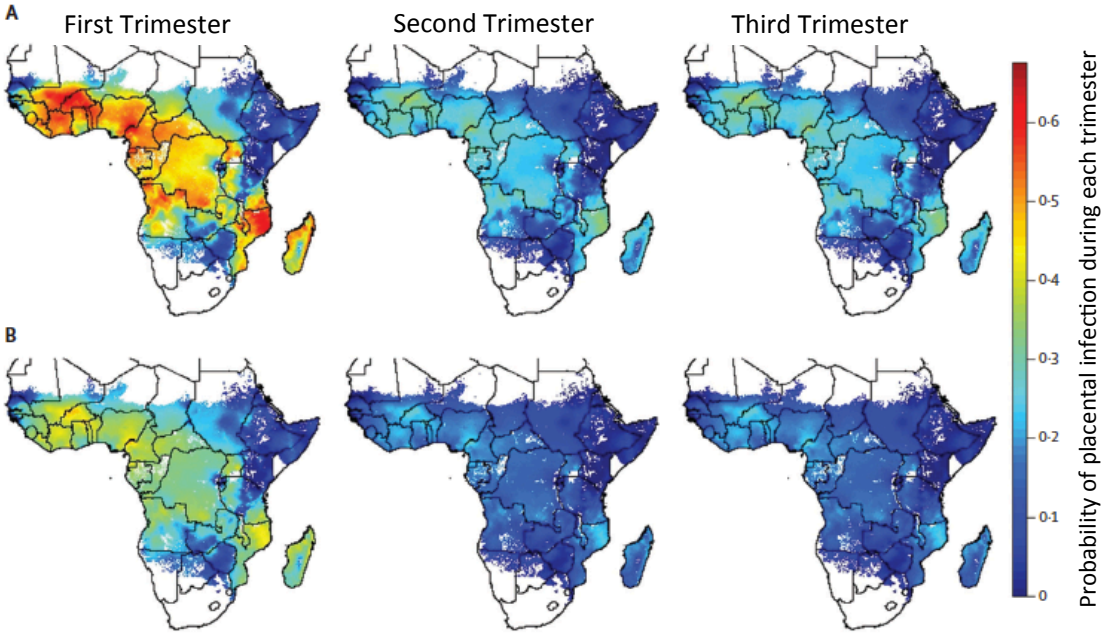

Figure 11. Risk of placental infection during pregnancy. A) Risk of primigravid women to have at least one incidental infection during each trimester in the absence of interventions; B) Risk averaged over all pregnancies. Adapted from Walker and colleagues 2014[149].

In the future, a vaccine against pregnancy associated malaria parasites to reduce the risk of malaria infection just before or during pregnancy, could be specifically efficacious on early pregnancy before IPTp administration. The European Vaccine Initiative (EVI) and its partners have been mobilizing funds for the development of a vaccine against placental malaria[190]. Both, the PRIMVAC and PAMVAC projects currently have VAR2CSA-based vaccine candidates in Phase Ia/b clinical trials. Although the two vaccine candidates are based on the same protein VAR2CSA, the selected antigens encompass different VAR2CSA regions and sequences with potentially distinct antigenic properties that might complement each other in terms of immunogenic potency and protective efficacy. While the PRIMVAC project has selected DBL1X–DBL2X, a 105-kDa domain of VAR2CSA from the *P. falciparum* strain 3D7, the PAMVAC project focus on ID1- DBL2X-ID2a, a 73-kDa derivative of VAR2CSA from the *P. falciparum* strain FCR3[190].

5. Malaria surveillance

Monitoring changes in malaria transmission intensity and disease prevalence through surveillance is a key pillar of the Global Technical Strategy 2016-2030[5] (See: 4. Malaria control and elimination). Effective surveillance of malaria cases is essential for identifying areas or population groups that are most affected by malaria, and for targeting resources for maximum impact. A strong surveillance system requires high levels of access to care and case detection, and complete reporting by all health sectors, public or private[1].

As transmission declines, surveillance becomes increasingly important to identify populations experiencing ongoing transmission[191,192]. Surveillance is particularly important in a pre-elimination context to identify asymptomatic carriers who might still transmit infection. Once elimination has been achieved, surveillance must continue in order to confirm a country or region's elimination status (defined as at least three consecutive years of zero indigenous cases) and to ensure that outbreaks resulting from re-introduced infections are quickly identified and controlled.

Conventional epidemiological metrics of malaria transmission in mosquito diagnosed via entomological estimates and in human diagnosed via passive (patients attending hospital or health care points) and active (cases actively searched in patients' houses) systems using microscopy, RDTs and PCRs, remain key for national malaria control programs in tracking progress in the reduction of malaria cases and identifying outbreaks and epidemics[193]. However, as transmission declines to low intensity, infected mosquitos and clinical cases become rare, slide and RDT positivity rates low, and transmission patterns increasingly heterogeneous[194,195]. In these cases, more sensitive metrics and/or combinations of approaches are needed to generate practical estimates of infection without excessive sampling.

Recent technological advances have produced a number of metrics that are suitable for low-transmission settings (Table 1)[193]. Molecular force of

infection (mFOI) and multiplicity of infection (MOI) both use parasite genotyping methods to assess the complexity of parasite infections[196]. Sequencing to determine parasite population structure can also be used to characterize transmission by measuring the genetic relatedness between infections in space and time. Other measures, such as allelic richness, can indicate the level of genetic diversity, which is expected to decline as transmission declines[197,198]. More refined sequencing approaches may be capable of assigning parasites as imported or local for monitoring the origin of infections. Antibody seroprevalence[199] and the seroconversion rate (SCR)[200] exploit human antibody responses to characterize previous or recent parasite exposure and are specific to a particular antigen or combination of antigens[201]. Antibodies are a sensitive marker of population-level malaria exposure in low-transmission settings and reflect exposure over a period of time, which is useful in areas with highly seasonal transmission[191,195,202,203] (See: 5.1. Serological tools for malaria surveillance).

Table 1. New metrics to measure malaria transmission[193]

Metrics	Definition	Measure of transmission	Method	Discriminatory power
Force of infection	Rate at which susceptible individuals contract malaria	• Probability of transmission	Time from birth to first malaria episode; microscopic detection of parasites following successful antimalarial treatment	• Difficult to measure • Difficult to standardize • Depends on diagnostic sensitivity • Cannot differentiate super infections
mFOI	The number of new parasite clones acquired by a host over time	• Population-level transmission intensity • Transmission heterogeneity	Cohort study >6 months with parasite genotyping	• Highly sensitive for monitoring changes in malaria exposure • Super infections can be differentiated
MOI	The number of different parasite strains co-infecting a single host	• Population-level transmission intensity • Transmission heterogeneity	Parasite genotyping of positive samples	• Saturates at high transmission • Restricted by age dependency • Insensitive at low transmission • Highly sensitive to spatial heterogeneity • Highly sensitive to increases in imported infection • Less sensitive to changes in seasonality
Genotyping SNPs or antigen sequencing	• Genetic diversity • Parasite signatures to map geographical relatedness of infection	• Population-level transmission intensity • Transmission heterogeneity • Geographical tracking of transmission patterns	• Haplotypes composed of >12 informative SNPs from single clone infections • Haplotypic signatures from highly variable loci	• Sensitive to changes in malaria exposure and spatial-temporal flow of infection • Standardization of measures needed • Methods for analysis and interpretation of data needed
Antibody seroprevalence	The percentage of seropositive individuals in a population	• Population-level transmission intensity	Seronegative or seropositive defined using appropriate cutoff points	• Dependent on antibody target tested • Saturates at high transmission • Sensitive at low transmission
SCR	The rate (typically annual) by which seronegative individuals became seropositive upon malaria exposure	• Population-level transmission intensity • Temporal changes in transmission can be detected from single sampling time point	Detection of antibodies in sera using serological assay	• Dependent on antibody target tested • Restricted by age dependency • Saturates at high transmission • Sensitive at low transmission • Sensitive to risk of malaria in absence of transmission

Importantly, the increasing availability of spatial databases on parasite rate[4,204], serology [205], vectors[206], malaria genetic epidemiology[207], and human population movements[208,209], together with the increased flexibility and

computational efficiency of mathematical and statistical modeling methods[210,211], have led to substantial advances in the spatial-temporal characterization of malaria transmission intensity. For these technologies to achieve the greatest impact, they will need to be linked to, and used by control programs to inform operational decision-making in real time.

5.1. Serological tools for malaria surveillance

Sero-surveillance is the detection of antibodies against antigens of malaria parasites and provides a sensitive tool for evaluating the impact of control interventions on transmission, particularly when transmission intensity is already low (Figure 12)[212]. While parasite-prevalence (measured by optic microscopy, RDTs or PCR) provides a snapshot of the exposure to malaria at a certain moment, seroprevalence offers a picture of malarial transmission over a long or short time period, depending on the serological marker used[201-203]. Antibody seroprevalence provides a general indication of the level of endemicity[213] and SCR indicates the rate at which seronegative individuals became seropositive by modeling age-specific seroprevalence data[192,200]. Serological measures correlate well with parasitological and entomological measures in describing transmission levels and spatial and demographic risk[192,200]. The potential applications of sero-surveillance as part of malaria control programs are: 1) estimate level of malaria transmission in populations; 2) monitor trends in transmission over extended periods of time (over years); 3) evaluate the impact of malaria control interventions; 4) detect recent changes in transmission; 5) identify focal areas with ongoing transmission (hotspots); 6) confirm malaria elimination and monitor for re-emergence; 7) screen for asymptomatic carriers, and 8) identify populations at high risk[214].

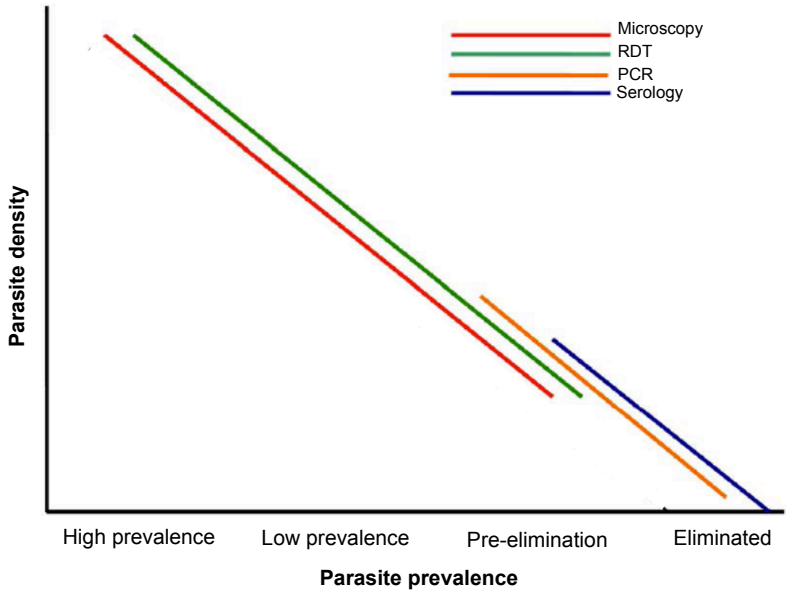

Figure 12. Position of different diagnostic approaches in relation to parasite densities and different stages towards malaria elimination. Adapted from malaEra, 2011[215].

A number of **platforms or methodologies** are currently being explored for application in sero-surveillance. Enzyme Linked Immunosorbent Assay (**ELISA)**, is the most widely established and used method[216,217]. A single antigen is used and the anti-antibody specific globulin forms a complex with an enzyme or colorant that changes the substrate into a detectable state (Figure 13A). New techniques, such as microarrays and multiplex bead based-assays have been developed. With these techniques, antibodies against multiple antigens can be detected in a large population, thus enabling its use as high throughput screening. **Protein microarray chip** consists of a support surface to which an array of antigens is bound. Anti-antibodies, typically labeled with a fluorescent dye, are added to the array after the serum antibodies. The reaction between the probe and the immobilized antigen emits a fluorescent signal that is read by a laser scanner (Figure 13B)[202,218]. **Multiplex bead based-assays** combines microspheres (polystyrene paramagnetic beads), lasers and a detection system to measure more analytes at the same time. This differs from an ELISA in that antigens are coupled to the surface of the beads instead of to the well. This creates the potential to multiplex the different beads into one assay, enabling various antibodies to be captured and quantified in a single blood sample.[219] Each

bead is labeled with a different color code based on a specific ratio of two fluorochromes that can be detected by the three readout systems. These techniques are more rapid, precise, sensitive and reproducible than the traditional serological methods (Figure 13C).

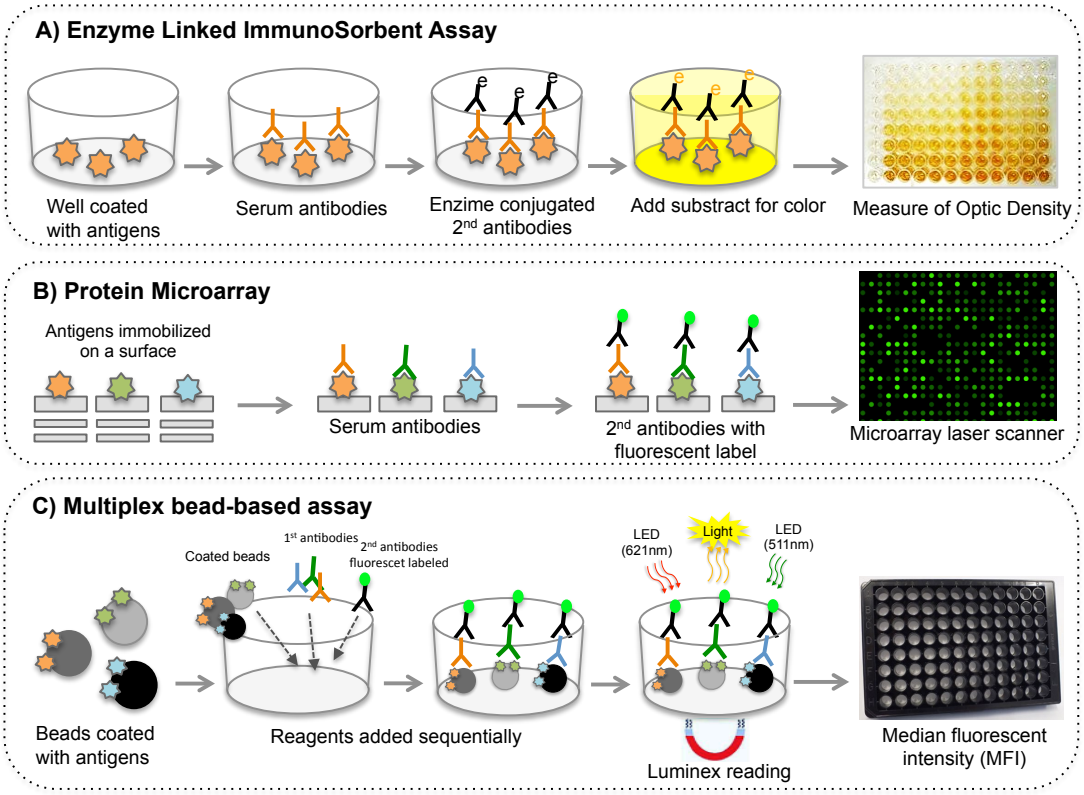

Figure 13. Representation of serological platforms. A) The enzyme linked immunosorbent assay (ELISA) detects antibodies against a single antigen and the optic density is measured. B & C) The microarray and multiplex bead-based assay can measure multiple antigens simultaneously by fluorescence using a microarray laser scanner or a luminex reader, respectively.

Each of these platforms must be carefully evaluated and standardized in sero-surveillance applications. An additional approach is the use of point-of-care tests, which provide rapid results at the site of clinical services or screening so that timely treatment decisions can be made[220], and are often in the form of simple lateral flow immunochromatographic devices. This methodology is valuable for the diagnosis and management of infectious diseases, such as HIV and malaria, and show potential for infectious disease surveillance[221]. Advantages and disadvantages of these methods are shown in table 2.

Table 2. Advantages and disadvantages of different sero-surveillance platforms[214]

Platform	Advantages	Disadvantages
ELISA	• Established and widely used technology • Efficient, high-throughput • Relatively low-cost • Limited technical expertise required • Semi-automated technology available	• Typically tests only a single antigen at a time; • Requires laboratory facilities.
Bead arrays	• Efficient, high-throughput • Able to test multiple antigen-specific responses simultaneously	• Relatively costly • Requires a high level of technical expertise • Requires laboratory facilities • Extensive optimization of antigen-coated beads required
Protein microarrays	• Evaluate antibodies to an extensive array of antigens	• Costly • Requires a very high level of technical expertise • Requires laboratory facilities • Requires production of a large number of antigens
Point-of-care test	• Allows testing in communities or at health facilities • Result within minutes provides real-time data • Low cost • No technical expertise or lab facilities required	• Limited to testing two or three antigens • Semi-quantitative only • Lower sensitivity than other methods

A comprehensive **evaluation of candidate antigens** is required to identify those antibody responses that are most sensitive for detecting changes in transmission. Antigen selection for sero-surveillance assays is influenced by specific properties of the antigen, including immunogenicity, antibody longevity (Figure 14), polymorphism, and cross-reactivity. Antigens should be selected according to target population, setting and application[214]. To date, antibody responses to the merozoite antigens $MSP1_{19}$, AMA1 and circumsporozoite antigen CSP have been most studied as markers of exposure to *P. falciparum*[192,199]. $MSP1_{19}$ and AMA1 are highly immunogenic antigens and antibodies against AMA1 were shown to be long-lived[143], which limit its value for detecting recent changes in transmission. Another important consideration is that antigens included in malaria vaccines are not suitable candidates for sero-surveillance tests because they are long-lived and it is not possible to differentiate between vaccine-induced and naturally acquired antibodies. Thus, CSP is not an ideal candidate for sero-surveillance tests if the RTS,S vaccine was to be licensed[222]. Recently, studies using protein microarrays to profile antibody responses have identified promising candidate antigens that require further investigation as potential biomarkers[105,202,223,224].

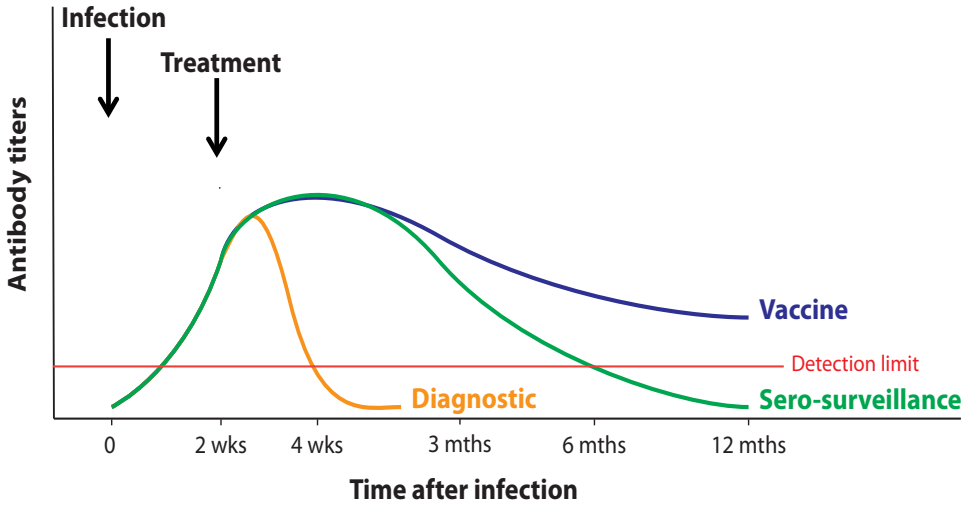

Figure 14. Optimal antibody kinetic profiled for vaccines (blue), diagnostic (orange) and sero-surveillance antigens (green).

In studies in a low-transmission setting, antibodies to VAR2CSA were strongly associated with *P. falciparum* infection during pregnancy[143]. Suggesting the potential of measure these antibodies in sentinel populations of pregnant women[225].

5.2. Pregnant women for sentinel surveillance

Based on the 2015 population growth and fertility patterns in Africa, 30.6 million pregnancies would have occurred in malaria endemic areas in 2015 with an estimated 9.5 million of those malaria-infected in the absence of protection[226]. These estimates represent a 37% reduction in the average risk of infection since the year 2000[226]. The pattern of malaria infection estimated in pregnant women in 2015 is similar to the pattern of *P. falciparum* parasite rate in 2-10 years old children in Africa ($PfPR_{2-10}$) in the same year (Figure 15)[4,226].

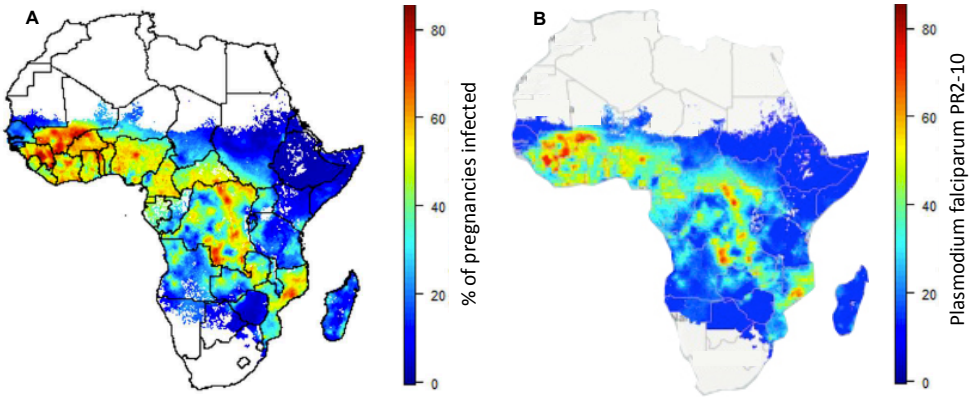

Figure 15. Malaria burden. A) Percent of pregnancies infected in the absence of intervention in 2015. B) *P. falciparum* parasite rate in 2-10 years old children ($PfPR_{2-10}$) in 2015. Adapted from Bhatt and colleagues, 2015 and Walker and colleagues, 2017[4,226].

The peculiarities of *P. falciparum* biology during gestation leads to an increased risk and density of infection[77] (See: 2.2. VAR2CSA and placental sequestration) together with imperfect prevention due to IPTp and ITN limitations (See: 4. Malaria control in pregnant women), support the new but promising concept of pregnant women as an optimal target population to generate useful estimates of malaria and to flag critical steps in the path towards elimination.

Pregnant women at maternal care clinics together with school children[227] and infants at immunization programs[228], represent a promising convenience group to monitor malaria transmission due to their easy accessibility at a central location[225]. It was reported that in Sub-Saharan Africa, 78% of pregnant women have at least one antenatal care visit, even in some difficult-to-reach rural areas[184] and, an increase in health care attendance was observed along the years. These observations suggest that pregnant women attending maternal care may be a representative group of the overall pregnant population (Figure 16). Moreover, maternal clinics are well distributed geographically offering a rapid and cost-effective method for monitoring spatial malaria trends and guide operational decision-making. Continuous (year-round) surveillance at maternal clinics can provide early warning signals to rises in transmission while accurately determining a denominator of populations at risk, thus reducing biases introduced by

increases in reporting rates associated with strengthening of surveillance systems. Comparing with other approaches (i.e., cross-sectionals in the community), integrating malaria surveillance into the maternal care routine system (together with screening for HIV, syphilis, and anemia) would require fewer human and financial resources[229]. Importantly, pregnant women attending maternal clinics for sentinel surveillance depend on the level and equity of maternal health care. Non-attendance at maternal clinics may be linked to demographic characteristics that could also be related to malaria exposure. The impact of malarial infection on fertility may also affect clinics attendance rates (as has been suggested for HIV infection[230]). However, malaria is less likely to lead to the strong fertility biases observed for sexually transmitted infections[231]. While the biology and epidemiology of malaria and HIV differ substantially, lessons can be learned from the extensive experience with the use of antenatal data as a convenience sample for HIV-infection surveillance[232-236]. There are several potential biases in inferring general population HIV prevalence from findings among maternal care attenders. Not all pregnant women attend antenatal care and maternity for delivery, and attendance is likely to vary by age, locality, socioeconomic status, education level, parity, ethnic group and religion. However a study from Glynn and colleagues[233] showed that the only important factors were age, marital status, having a previous child, education status and contraceptive use.

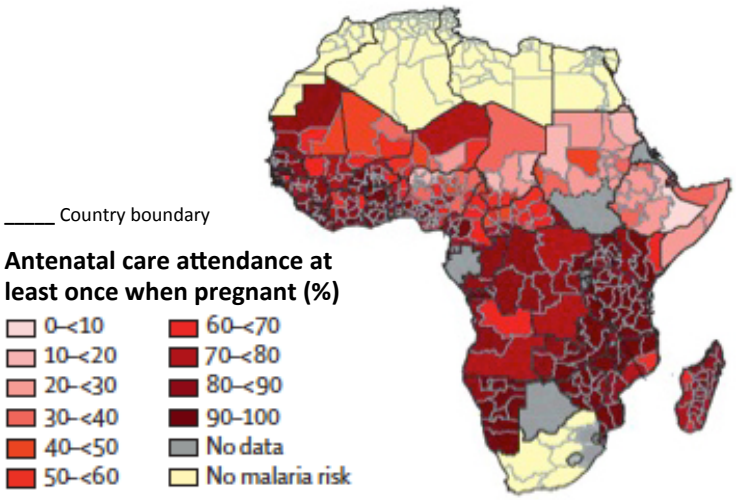

Figure 16. Women who attended antenatal care clinics at least once during pregnancy. Adapted from van Eijk and colleagues, 2011[237].

Data for malaria prevalence in children obtained from household surveys, such as malaria indicator surveys or school-based surveys, are used to measure transmission intensity and success of malaria control activities in a region[204]. Household surveys are logistically demanding and expensive. School surveys, by contrast, are do less expensive and often include larger sample populations[227]. However, neither approach provides a simple routine real-time measure of malaria in the community, as pregnant women attending maternal clinics. Studies conducted in the 1950s in Sierra Leona (Figure 17A)[238] and more recently in Tanzania (Figure 17B)[239], as well as with a meta-analysis (Figure 17C)[225] suggest that patterns of malaria prevalence in pregnant women may reflect transmission intensity in their communities. In this meta-analysis[225] the prevalence of malaria infection, as detected by microscopy or RDT in pregnant women, was compared with its prevalence in children included in the same study sampled in the same location or region during the same period (data from 18 sources including 57 data-points). These data demonstrated that the prevalence of malaria infection in pregnant women was lower than that in children aged 0–59 months from the same population, although prevalence estimates in both groups were closely correlated, with a strong linear relation (r=0.87) across the endemicity spectrum (Figure 17C). The difference in prevalence between children and pregnant women was smaller when the pregnant women were primigravid and in areas of low malaria transmission. This is not surprising, as parasites can sequester in the placenta, avoiding detection by diagnostic tests, and the concomitant peripheral parasite prevalence can be lower than that in the placenta. A meta-analysis by Kattenberg and colleagues[240] reported a sensitivity of peripheral maternal blood of 72% based on microscopy detection of placental malaria. If all placental malaria infections had been detected in the peripheral blood, the prevalence in pregnant women might have approached that recorded in children.

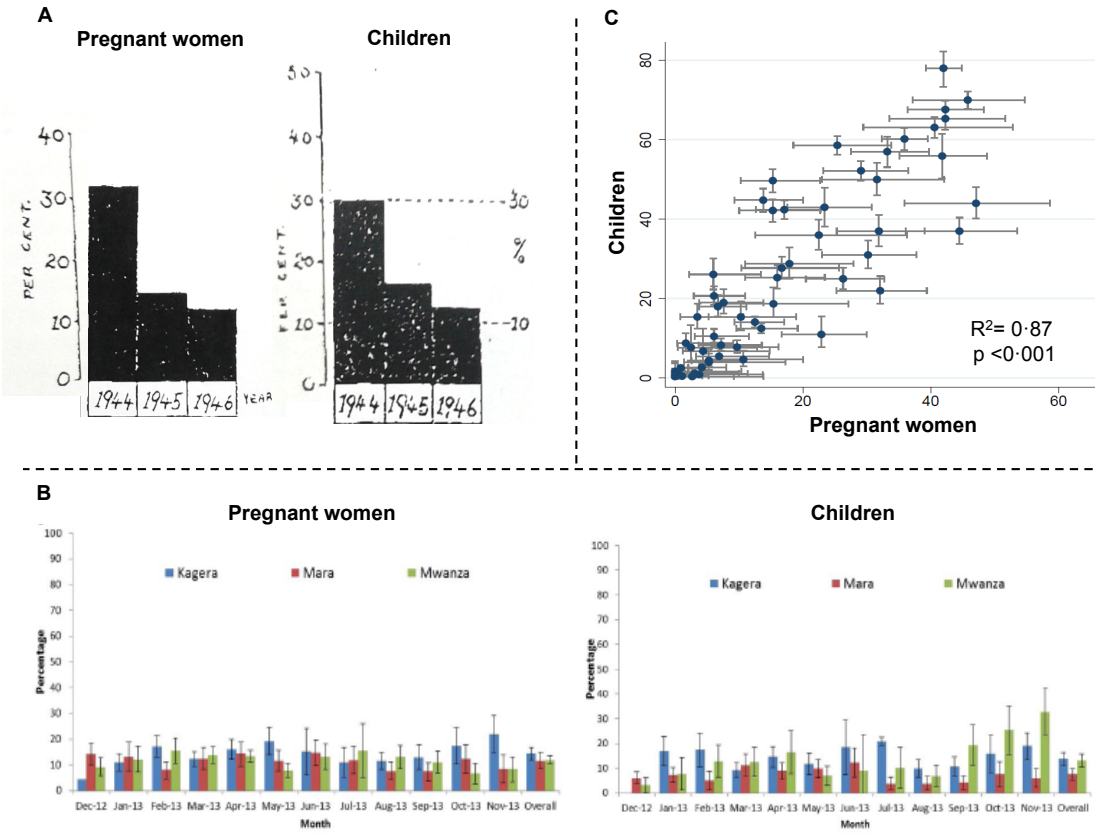

Figure 17. Malaria burden in pregnant women and children. A) Parasite rate of pregnant women attending the antenatal clinic and annual malaria incidence of schoolchildren in Freetown, Sierra Leona, adapted from Walton, 1948[238]; B) Distribution of malaria positivity among pregnant women and infants (9-12 months) by region, in Tanzania, December 2012–November 2013, adapted from Willilo and collegues, 2016[239]; C) Scatter plots of malaria prevalence in pregnant women versus children (0–59 months), sub-Saharan Africa, 1983–2012, adapted from van Eijk, 2015[225].

Measure anti-VAR2CSA antibodies during pregnancy integrate malaria exposure over time (a pregnancy) thus, increasing the chances to detect any malaria exposure, overcoming the difficulty to detect active infection in peripheral blood resulted from placental sequestration. However, the longevity of antibody responses against VAR2CSA and the effect of *var2csa* genetic diversity need to be evaluated to determine the utility of such a serological approach. Although further validation is needed, the use of pregnant women as a sentinel group for malaria transmission, and the use of antibodies against VAR2CSA as markers of cumulative exposure to *P. falciparum* during pregnancy, might be a feasible alternative to the generation of sensitive metrics of malaria transmission intensity for the elimination of malaria.

HYPOTHESIS AND OBJECTIVES

Hypothesis

In recent years there has been a decline in malaria transmission in many regions, leading to optimism that malaria elimination could potentially be achieved in many countries. As transmission declines, surveillance becomes increasingly important and metrics used to estimate malaria exposure in a community need to account for dynamic changes over space and time. These are essential indicators to guide strategic planning, implementation and evaluation of interventions. Pregnant women are at higher risk of malaria compared to non-pregnant women or men. The massive accumulation of *P. falciparum*-infected RBC in the placenta is mediated by VAR2CSA, a multi-domain variant antigen of approximately 350 kDa expressed on the surface of *P. falciparum* infected RBC that binds to CSA in the syncytiotrophoblast. Antibodies against VAR2CSA increase with exposure during successive pregnancies and are affected by variables that influence the risk of exposure to *P. falciparum* such as season, proximity to a river, use of IPTp or ITNs. Relatively low serological diversity of VAR2CSA and development of antibodies after single or very limited exposures to placental parasites suggest the potential of these antibodies as markers of exposure to *P. falciparum* during pregnancy. Importantly, several studies showed that the pattern of malaria prevalence in pregnant women was similar with malaria prevalence detected in children. Moreover, pregnant women in maternal clinics are a convenient and easy-to-access group for surveillance of infectious diseases (i.e., HIV) and most women (78%) attend antenatal care at least once during pregnancy, even in some hard-to-reach rural areas. Finally, data on malaria infection or exposure can be collected at maternal clinics as part of routine services without requiring any additional sampling.

Stemming from the data outlined above, the **hypothesis** driving this PhD thesis was that the prevalence of antibody responses against VAR2CSA-derived antigens can reflect pregnant women exposure to *P. falciparum* in low transmission settings. If so, serological measurements in pregnant women can be an important tool to monitor the intensity of malaria transmission in the general population.

Objectives

The **main objective of this thesis** was to understand the malarial trends as well as the timescales over which antimalarial immunity is gained and lost to identify VAR2CSA antigens to be used as a sero-surveillance tool of malaria exposure during one pregnancy. This approach applied to pregnant women can provide information about malaria changes over time and space, which can be used as sentinel for malaria surveillance in the surrounding community.

Specific objective 1: To understand the malarial trends and the consequences of malaria resurgences in parasitological, immunologic and clinical outcomes among pregnant women delivering between 2003 and 2012 in the Manhiça District in southern Mozambique.

Specific objective 2: To develop a multiplex-bead array assay to detect antibodies against a panel of new peptides covering VAR2CSA and recombinant domains. These antibodies could be used to identify immune correlates of protection against pregnancy-associated malaria and malaria adverse consequences, as well as serological markers for surveillance of malaria transmission. To fulfill this objective, it was essential:

- To design VAR2CSA-derived peptides from conserved and semi-conserved regions of the entire protein.

- To develop a multiplex-bead array to measure antibodies from plasma samples or dried blood spots and explore methodologies to define seropositivity thresholds.

- To discard antigens recognized by malaria never exposed individuals and exposed men.

Specific objective 3: To identify and characterize a set of VAR2CSA-derived antigens to be used in a serological assay to detect gradual changes in *P. falciparum* exposure during pregnancy. To fulfill this objective, it was essential:

- To calibrate the assay by selecting antigens targeted by IgG responses that are rapidly acquired after *P. falciparum* infection during pregnancy, reflect historical trends of malaria and lack persistence in blood circulation.

- To validate the assay by analyzing the seropositivity to selected antigens in pregnant women from different African regions with different levels of transmission, and assessing temporal, spatial as well as intervention-driven differences in exposure.

STUDY AREAS AND CONTEXT

The women included in this study were from Mozambique (Manhiça and Maragra), Benin (Allada, Sekou, and Attogon), Gabon (Lambarene and Fougamou), Kenya (Siaya) and Tanzania (Makole and Chamwino). These are regions from Western, Eastern, Central and Southern sub-Saharan Africa where malaria transmission is stable but displays distinctly varying characteristics according to the site. In these regions perennial malaria transmission with some seasonality is mostly attributable to *P. falciparum* (>95% of malaria infections in all sites). Transmission intensity was classified as holoendemic in Kenya, hyperendemic in Benin and Gabon and mesoendemic in Mozambique and Tanzania[241-243]. The prevalence of malaria, estimated by the proportion of 2-10 year old children infected with *P. falciparum* ($PfPR_{2-10}$) and derived from the Malaria Atlas Project geostatistical prediction model[4], decreased considerably from 2003 to 2012 (Figure 18).

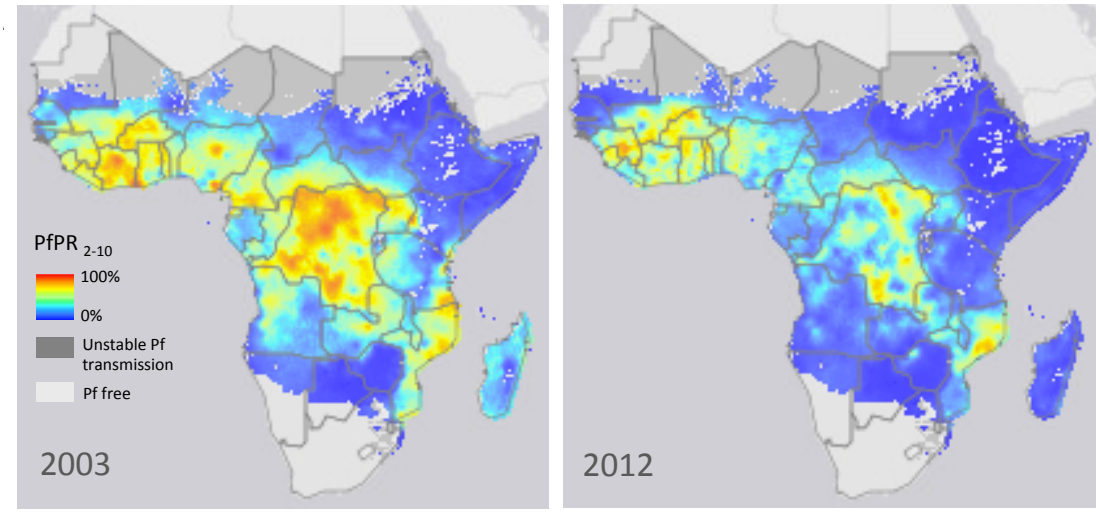

Figure 18. Changes in estimated rate of infection prevalence by *P. falciparum* in 2-10 years old children from 2003 to 2012. Maps are available from the Malaria Atlas Project (http:// www.map.ox.ac.uk/).[4]

Pregnant women included in the studies were recruited during three clinical trials of IPTp between 2003-2005 (Clinical trials.gov NCT00209781)[241] in Mozambique and between 2010-2012 (NCT00811421)[242,243] in Mozambique but also Benin, Gabon, Kenya and Tanzania. Women recruited during 2003-2005 received two doses of SP[241] and women recruited between 2010-2012 received two doses of mefloquine (MQ) or SP, if the women were

HIV-negative[243] or three doses of MQ or placebo, if they were HIV-positive receiving cotrimoxazol prophylaxis[242]. All women included in the study received bed nets treated with a long-lasting insecticide. Adult men from Mozambique as well pregnant women and men never exposed to *P. falciparum* from Barcelona were also included.

This study was a cooperative project between Institute of Global Health (ISGLOBAL), Barcelona, Spain; Centro de Investigação em Saúde da Manhiça (CISM), Mozambique; Ifakara Health Institute (IHI), Tanzania; Medical Research Unit, (MRU) Albert Schweitzer Hospital, Lambaréné, Gabon; Kenya Medical Research Institute/Centre for Global Health Research (KMRI), Kisumu, Kenya; Faculté des Sciences de la Santé, Université d'Abomey-Calavi, Benin; Institute for Tropical Medicine, Tübingen, Germany and Institut de Recherche pour le Développement, Paris, France (Figure 19).

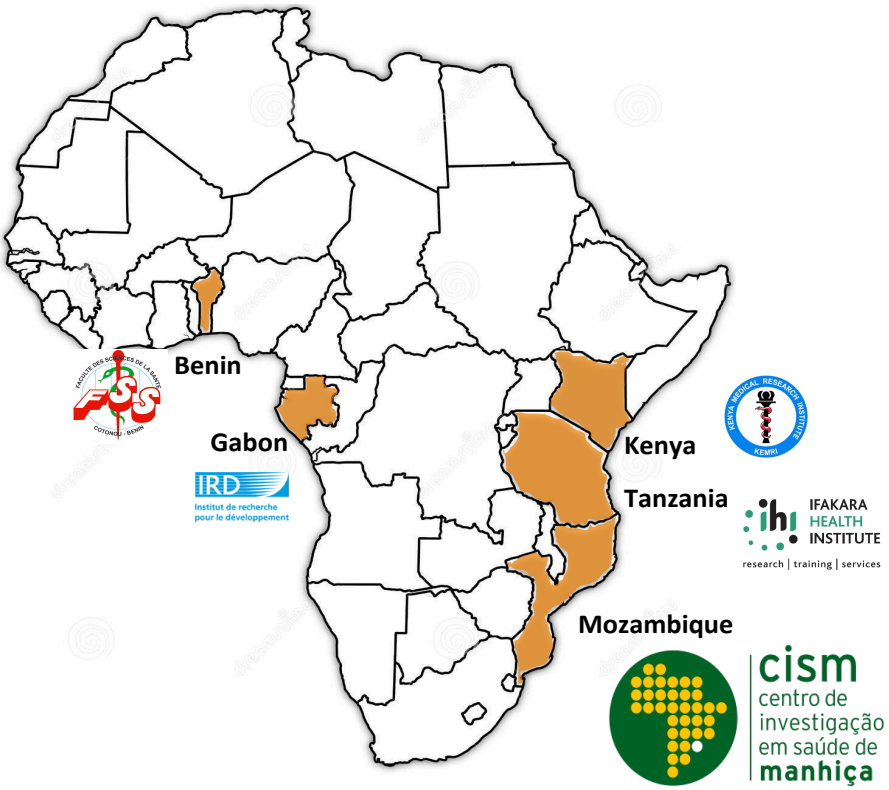

Figure 19. Study regions and institutions.

This study was approved by the Ethics Committees from the Hospital Clínic of Barcelona (Spain), the Comité Consultatif de Déontologie et d'Éthique from the Institut de Recherche pour le Développement (France), the Centers for Disease Control and Prevention (USA), and National Ethics Review Committees from each malaria endemic country participating in the study. Written informed consent was obtained from all the participants.

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RESULTS

CHAPTER 1

Changing trends in *P. falciparum* burden, immunity and disease in pregnancy

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ORIGINAL ARTICLE

Changing Trends in *P. falciparum* Burden, Immunity, and Disease in Pregnancy

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ABSTRACT

BACKGROUND

Prevention of reinfection and resurgence is an integral component of the goal to eradicate malaria. However, the adverse effects of malaria resurgences are not known.

METHODS

We assessed the prevalence of *Plasmodium falciparum* infection among 1819 Mozambican women who delivered infants between 2003 and 2012. We used microscopic and histologic examination and a quantitative polymerase-chain-reaction (qPCR) assay, as well as flow-cytometric analysis of IgG antibody responses against two parasite lines.

RESULTS

Positive qPCR tests for *P. falciparum* decreased from 33% in 2003 to 2% in 2010 and increased to 6% in 2012, with antimalarial IgG antibody responses mirroring these trends. Parasite densities in peripheral blood on qPCR assay were higher in 2010–2012 (geometric mean [±SD], 409±1569 genomes per microliter) than in 2003–2005 (44±169 genomes per microliter, P=0.02), as were parasite densities in placental blood on histologic assessment (50±39% of infected erythrocytes vs. 4±6%, P<0.001). The malaria-associated reduction in maternal hemoglobin levels was larger in 2010–2012 (10.1±1.8 g per deciliter in infected women vs. 10.9±1.7 g per deciliter in uninfected women; mean difference, −0.82 g per deciliter; 95% confidence interval [CI], −1.39 to −0.25) than in 2003–2005 (10.5±1.1 g per deciliter vs. 10.6±1.5 g per deciliter; difference, −0.12 g per deciliter; 95% CI, −0.67 to 0.43), as was the reduction in birth weight (2863±440 g in women with past or chronic infections vs. 3070±482 g in uninfected women in 2010–2012; mean difference, −164.5 g; 95% CI, −289.7 to −39.4; and 2994±487 g vs. 3117±455 g in 2003–2005; difference, −44.8 g; 95% CI, −139.1 to 49.5).

CONCLUSIONS

Antimalarial antibodies were reduced and the adverse consequences of *P. falciparum* infections were increased in pregnant women after 5 years of a decline in the prevalence of malaria. (Funded by Malaria Eradication Scientific Alliance and others.)

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The malaria parasite can persist and reappear in areas where infection is no longer circulating or is circulating at very low levels.[1] Since prevention of reinfection and resurgence is an integral component of the current goal to eradicate malaria,[2] understanding the determinants and clinical consequences of malaria declines and resurgences, as well as the timescales for gain and loss of antimalarial immunity, has become a priority.

Malaria is an infectious disease that requires boosting to maintain immunity over time[3]; a reduction in parasite exposure can lead to a loss of population-level immunity and an increase in the harmful effects of malaria infections during resurgences.[4] Because of the increased susceptibility to malaria during pregnancy,[5] the consequences of a reduction in malaria immunity among pregnant women could be particularly severe, especially in the context of the human immunodeficiency virus (HIV) pandemics in Africa,[6] which can impair the maintenance of effective immune responses.[7] However, declining malaria transmission among pregnant women[8-10] may have an effect on malaria-related clinical outcomes that has not yet been assessed.

In areas where malaria is endemic, antibodies against *Plasmodium falciparum* VAR2CSA[11] develop in pregnant women. VAR2CSA is a variant surface antigen that mediates placental accumulation of infected erythrocytes[12] and leads to the deleterious effects on the mothers and their offspring, especially in primigravid women.[13] Antibodies against VAR2CSA are acquired after exposure to placental parasites in a parity-dependent manner,[14] contribute to a reduction in the clinical consequences of malaria in pregnancy,[15] and are affected by variables that influence the risk of exposure to *P. falciparum*.[16-18] Therefore, decreases in this pregnancy-specific immunity after reductions in transmission might affect the disease burden during resurgences.

Although the relationship between the prevalence of malaria among pregnant women and the prevalence in the general community is still incompletely understood,[19] malaria testing in pregnant women at health care facilities can provide information about the effect of changes in malaria transmission on the disease burden. To better understand the consequences of malaria resurgences, we examined parasitologic, immunologic, and clinical trends among pregnant women delivering between 2003 and 2012 in the Manhiça District in southern Mozambique.

METHODS

STUDY SITES AND POPULATION

Pregnant women who provided written informed consent to participate in two clinical trials of antimalarial drugs between 2003 and 2005[20] and between 2010 and 2012[21,22] at antenatal clinics in the Manhiça District were included in the study. Biases due to pooling of data from these two clinical trials, as well as to changes from the earlier to the later period in the health care provided to the women, were minimized by the use of similar protocols and procedures during the two trials. The intensity of malaria transmission in this region used to be moderate[23] and has decreased in the past few years, as evidenced by a reduction in hospital admissions for malaria. The area has been under continuous demographic surveillance by the Centro de Investigação em Saúde da Manhiça (CISM) since 1996.[24]

All women included in the study received bed nets treated with long-lasting insecticide, as well as intermittent preventive treatment during pregnancy, which in 2003–2005 consisted of two doses of sulfadoxine–pyrimethamine[20] and in 2010–2012 consisted of two doses of mefloquine or sulfadoxine–pyrimethamine, if the women were HIV-negative,[22] or three doses of mefloquine or placebo, if they were HIV-positive and were receiving trimethoprim–sulfamethoxazole prophylaxis.[21] At delivery, maternal hemoglobin was measured with the use of a HemoCue or Sysmex KX21 analyzer, and newborns were weighed (with the use of weekly calibrated scales, which were either digital scales[20] or triple-beam balances[21,22]). Tissue samples from the maternal side of the placenta, as well as 50 μl of maternal peripheral-, placental-, and cord-blood samples on filter papers, were collected for parasitologic assessments. At delivery, peripheral blood was collected into EDTA vacutainers and centrifuged, with the plasma stored at −20°C. Clinical malaria episodes were treated according to national guidelines at the time of the study, and parenteral quinine was administered for severe malaria. The study protocols and informed-consent forms were reviewed and approved by the National Ethics Review Committee of Mozambique and the Ethics Review Committee of the Hospital Clinic of Barcelona.

PARASITOLOGIC ASSESSMENTS

Thick and thin blood films, as well as placental-biopsy samples in 10% neutral buffered formalin, were assessed for detection of plasmodium species according to standard, quality-controlled procedures.[25-27] A random selection of 50% of the peripheral-blood samples (958 samples) and placental-blood samples (941) on filter papers was used for detection of *P. falciparum* in duplicate by means of a real-time quantitative polymerase-chain-reaction (qPCR) assay targeting 18S ribosomal RNA (rRNA)[28] (see the Methods section in the Supplementary Appendix, available with the full text of this article at NEJM.org).

MEASUREMENT OF ANTIMALARIAL IGG ANTIBODIES

A random selection of 35% of the peripheral-blood plasma samples (654 samples) collected at delivery was used for flow-cytometric measurement of IgG[16] (expressed as mean fluorescence intensity [MFI]) against a chondroitin sulfate A–binding parasite line expressing VAR2CSA (CS2), indicating pregnancy-specific antimalarial immunity,[29] and a rosetting parasite line (R29), indicating general antimalarial immunity[30] (see the Methods section in the Supplementary Appendix). The *P. falciparum* CS2 (MRA-96) laboratory strain was obtained through the Malaria Research and Reference Reagent Resource Center (MR4) as part of the BEI Resources Repository, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

STUDY DEFINITIONS

Pregnant women were included in the analysis if they received sulfadoxine–pyrimethamine, mefloquine, or trimethoprim–sulfamethoxazole, alone or in combination with mefloquine, during pregnancy and if all information was available on the date of delivery, HIV status, age, and parity, as well as results of peripheral-blood microscopic examination and placental histologic assessment. Women were classified a priori as primigravid (first pregnancy) or multigravid (at least one previous pregnancy), and age was categorized as younger than 20 years, 20 to 24 years, or 25 years of age or older.[16,17] Maternal microscopic infection at delivery was defined as the presence of *P. falciparum* parasites in peripheral blood or in the placenta on either microscopic or histologic examination, qPCR-positive infection was defined as a positive result of qPCR testing in peripheral or placental blood, and submicroscopic infection was defined as the presence of malaria parasites on qPCR testing but not on microscopic examination. Past placental infection was defined by the presence of malaria pigment (i.e., hemozoin[27]) without parasite detection on placental histologic examination, and chronic placental infection was defined by the presence of malaria pigment in combination with the detection of parasites.

STATISTICAL ANALYSIS

Our primary hypothesis was that declines in the prevalence of malaria would be associated with reductions in antimalarial immunity, and our secondary hypothesis was that reduced immunity would lead to higher parasite densities, smaller differences in parasite densities and antibody levels between primigravid and multigravid women, and a larger adverse effect of malaria. Proportions were compared by means of Fisher's exact test, and continuous variables by means of Student's t-test. The primary outcome variables — maternal microscopic infection and level of IgG antibodies against CS2 — were compared between the two study periods (2003–2005 and 2010–2012) with the use of logistic- and linear-regression models, respectively, adjusted according to parity, age, and HIV infection status. Secondary variables included qPCR positivity for maternal infection, peripheral-blood and placental infections, submicroscopic infections, and levels of IgG antibodies against R29, as well as data according to year, with observations from 2003 and 2004 combined because of the small sample in 2003 (28 observations) and with the use of the first year of the second period (2010) as the reference year for comparisons. To test the secondary hypothesis, we used linear regression to analyze the effect of study period on log-transformed parasite densities, as well as the effect of malaria on maternal hemoglobin levels and newborn weight in each of the two periods. To determine whether a modification of the associations with parity was attributable to the study period, we included an interaction term in the regression models and assessed separately the effect of parity for each period. The data were analyzed with the use of Stata software, version 11.0 (Stata). P values of less than 0.05 were considered to indicate statistical significance for primary outcomes, with Bonfer-

roni corrections applied for the secondary outcome variables tested.

RESULTS

STUDY POPULATION

A total of 2259 women received bed nets treated with long-lasting insecticide and were given prophylaxis with sulfadoxine–pyrimethamine, mefloquine, or trimethoprim–sulfamethoxazole, alone or in combination with mefloquine, in the context of the two clinical trials of intermittent preventive treatment of malaria in pregnancy that were conducted in Manhiça.[20-22] Of these women, 440 were excluded because of missing data on HIV status, peripheral-blood microscopic examination, or placental histologic examination (Fig. S1 and Table S1 in the Supplementary Appendix). The 1819 women included in this study delivered between October 27, 2003, and August 14, 2012, and were similar in terms of baseline characteristics with all 2259 women participating in the randomized trials (Table S1 in the Supplementary Appendix), as were women in both study periods (Table 1).

TRENDS IN MALARIA DURING PREGNANCY

The prevalence of maternal microscopic infection at delivery (primary outcome) decreased from 11% in 2003–2005 to 2% in 2010–2012 (Table 1) (adjusted odds ratio, 0.19; 95% confidence interval [CI], 0.12 to 0.30; P<0.001). Similar decreases were observed in secondary parasitologic outcomes (P<0.001 in all cases) (Table 1). Microscopic examination of peripheral blood and histologic examination of the placenta, as compared with qPCR testing, are described in Table S2 in the Supplementary Appendix. The prevalence of peripheral-blood infections that were submicroscopic was higher during the 2003–2005 period than during the 2010–2012 period (70% [26 of 37 infections] vs. 47% [14 of 30]; adjusted odds ratio, 3.81; 95% CI, 1.17 to 12.46; P=0.03).

Secondary analysis by year showed that the prevalence of microscopic maternal infection decreased from 12% in 2003–2004 to 1% in 2010 (P<0.001, adjusted), and the prevalence of qPCR-positive maternal infection decreased from 33% to 2% (P<0.001, adjusted), with increases in 2012 to 4% for microscopic infections (P=0.003, adjusted) and to 6% for qPCR-positive infections (P=0.03, adjusted) (Fig. 1, and Fig. S2 in the Supplementary Appendix). Similar trends were observed after the exclusion of women who received mefloquine as intermittent preventive treatment of malaria during pregnancy in the 2010–2012 period (Table S3 in the Supplementary Appendix). Cord-blood microscopic infections were consistently low (≤1%) throughout the study period.

ANTIMALARIAL IMMUNITY

Geometric mean levels of IgG against CS2 (primary outcome) were lower in 2010–2012 than in 2003–2005 (Table 1) (adjusted ratio, 0.44; 95% CI, 0.36 to 0.54; P<0.001), with similar reductions observed for IgG antibodies against R29 (adjusted ratio, 0.43; 95% CI, 0.33 to 0.58; P<0.001). Secondary analysis by year showed that IgG levels decreased from 2003 to 2010 and gradually increased between 2010 and 2012 (Fig. 2). Maternal qPCR-positive infections at delivery were associated with an increase in IgG levels against CS2 (P<0.001, adjusted) but not against R29 (P=0.30, adjusted) (Fig. S3 in the Supplementary Appendix).

PARASITE DENSITIES AND CLINICAL OUTCOMES

Parasite densities in peripheral blood on qPCR assay were higher in 2010–2012 (geometric mean [±SD], 409±1569 genomes per microliter) than in 2003–2005 (44±169 genomes per microliter, P=0.02), as were parasite densities in placental blood on histologic examination (50±39% of infected erythrocytes vs. 4±6%, P<0.001) (Fig. 3). Similar increases in parasite densities, although not significant, were observed by means of microscopic examination in peripheral blood and qPCR assay in placental blood. Parasite densities quantified by means of microscopic examination in cord blood were also higher in 2010–2012 (geometric mean, 38,249±65,809 parasites per microliter) than in 2003–2005 (208±551 parasites per microliter; P=0.046; P=0.23 after Bonferroni correction).

The average maternal hemoglobin concentration increased slightly from 2003–2005 to 2010–2012, although no difference was observed in mean newborn weight (Table 1). A larger reduction in hemoglobin levels associated with microscopic maternal infections was observed in 2010–2012 (10.1±1.8 g per deciliter in infected women vs. 10.9±1.7 g per deciliter in uninfected women; mean difference, −0.82 g per deciliter; 95% CI, −1.39 to −0.25; P=0.005, adjusted; P=0.01 after Bonferroni correction) than in 2003–2005

Table 1. Characteristics of Study Participants and Parasitologic, Immunologic, and Clinical Outcomes, According to Study Period.*

Variable	2003–2005 (N=374)	2010–2012 (N=1445)	P Value
Year — no. (%)			
2003	28 (7)	0	
2004	232 (62)	0	
2005	114 (30)	0	
2010	0	358 (25)	
2011	0	803 (56)	
2012	0	284 (20)	
IPTp — no. (%)†			
Sulfadoxine–pyrimethamine	374 (100)	324 (22)	
Mefloquine	0	894 (62)	
Trimethoprim–sulfamethoxazole	0	227 (16)	
HIV status — no. (%)‡			
Negative	274 (73)	998 (69)	0.13
Positive	100 (27)	447 (31)	
CD4+ T-cell count at recruitment — per mm^3§	526.8±237.3	462.9±255.7	0.13
Parity — no. (%)			
Primigravid	113 (30)	422 (29)	0.70
Multigravid	261 (70)	1023 (71)	
Age — no. (%)			
<20 yr	129 (34)	458 (32)	
20–24 yr	106 (28)	358 (25)	0.08
≥25 yr	139 (37)	629 (44)	
Maternal hemoglobin — g/dl	10.5±1.5	10.8±1.7	0.02¶
Newborn birth weight — g	3055±473	3061±480	0.80
Primary outcomes			
Maternal microscopic infection — no. (%)‖	41 (11)	33 (2)	<0.001¶
IgG antibodies against CS2, geometric mean ±SD — MFI**	6055±8742	3001±3319	<0.001¶
Secondary outcomes			
Maternal qPCR-positive infection — no./total no. (%)††	50/165 (30)	33/753 (4)	<0.001¶
Peripheral-blood infection on microscopic assessment — no./total no. (%)	25/374 (7)	32/1445 (2)	<0.001¶
Peripheral-blood infection on qPCR testing — no./total no. (%)	37/169 (22)	30/789 (4)	<0.001¶
Active placental infection on histologic assessment — no./total no. (%)	37/374 (10)	18/1445 (1)	<0.001¶
Past or chronic placental infection on histologic assessment — no./total no. (%)	179/374 (48)	57/1445 (4)	<0.001¶
Placental-blood infection on qPCR testing — no./total no. (%)	39/165 (24)	27/776 (3)	<0.001¶
IgG antibodies against R29, geometric mean ±SD — MFI**	7820±9139	3610±5859	<0.001¶

* Plus–minus values are means ±SD unless otherwise noted. Dates of sample collection were as follows: 2003, October 27 through December 31; 2004, January 1 through December 26; 2005, January 1 through October 7; 2010, March 28 through December 31; 2011, January 1 through December 31; and 2012, January 1 through August 14. Percentages may not sum to 100 because of rounding. qPCR denotes quantitative polymerase chain reaction.

† IPTp denotes intermittent preventive treatment during pregnancy, with mefloquine given alone or in combination with trimethoprim–sulfamethoxazole.

‡ Maternal human immunodeficiency virus (HIV) status was determined with the use of the Determine HIV-1/2 test (Abbott Laboratories) and was confirmed by means of the Uni-Gold HIV Rapid Test (Trinity Biotech).

§ CD4+ T-cell counts were performed in BD Trucount tubes (BD Biosciences).

¶ The P value is significant according to multivariate analysis adjusted for HIV status, parity, and age.

‖ Maternal microscopic infection was defined by the presence of *P. falciparum* parasites in peripheral blood or in the placenta on microscopic or histologic examination, respectively.

** MFI (mean fluorescence intensity) was measured in arbitrary fluorescence units.

†† Maternal qPCR-positive infection was defined by a positive result on qPCR testing in peripheral or placental blood.

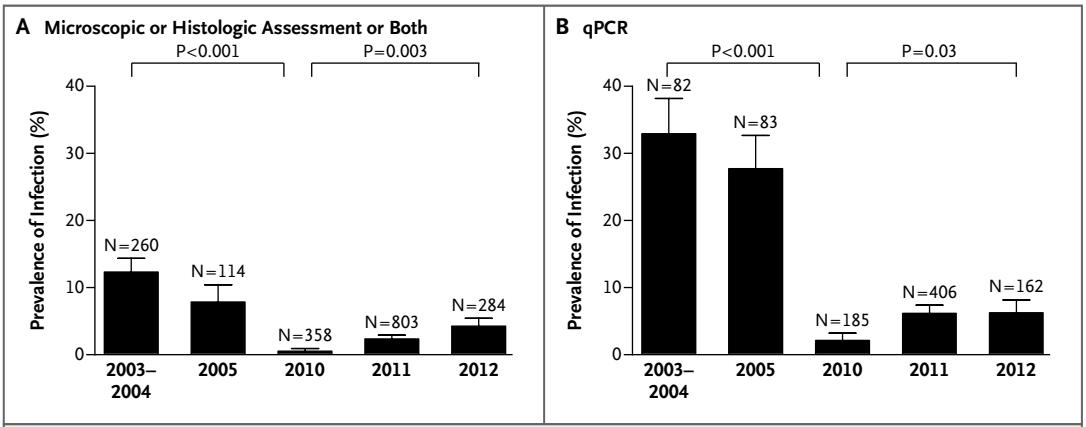

Figure 1. Prevalence of *Plasmodium falciparum* Infection among Pregnant Women at Delivery, According to Year.

Panel A shows the prevalence of infection on the basis of microscopic assessment, histologic assessment, or both, and Panel B shows the prevalence on the basis of quantitative polymerase-chain-reaction (qPCR) testing. Maternal infection was defined by the detection of *P. falciparum* in either peripheral blood or the placenta. Results of qPCR testing of both peripheral and placental blood were available for 918 women. Observations from 2003 (28 for microscopic assessment, histologic assessment, or both and 14 for qPCR testing) and 2004 (232 for microscopic assessment, histologic assessment, or both and 68 for qPCR testing) were combined because of the small sample in 2003. P values are based on a multivariate analysis adjusted for human immunodeficiency virus (HIV) status, parity, and age. T bars represent standard errors.

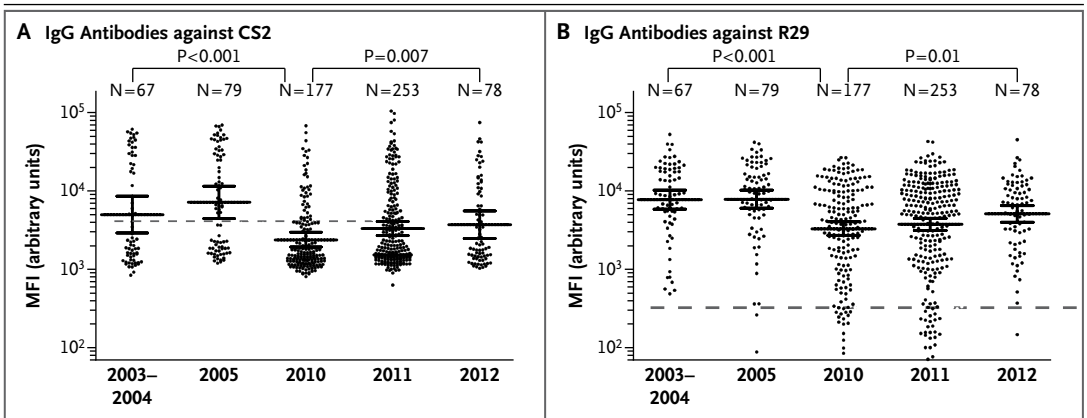

Figure 2. IgG Antibodies against Two *P. falciparum* Lines in Pregnant Women at Delivery, According to Year.

Panel A shows IgG antibodies, expressed as mean fluorescence intensity (MFI, measured in arbitrary fluorescence units), against a chondroitin sulfate A–binding parasite line (CS2), and Panel B shows MFI for IgG antibodies against a rosetting parasite line (R29). I bars represent geometric means plus 95% confidence intervals. Dashed lines indicate the mean plus 3 SD from the negative control values. P values are based on a multivariate analysis adjusted for HIV status, parity, and age.

(10.5±1.1 g per deciliter vs. 10.6±1.5 g per deciliter; mean difference, −0.12 g per deciliter; 95% CI, −0.67 to 0.43; P=0.67, adjusted). Similarly, past or chronic placental infections, which have been shown to lead to low birth weight because of fetal growth restriction,[5] were associated with larger reductions in the weight of newborns during the 2010–2012 period (2863±440 g in infected women vs. 3070±482 g in uninfected women; mean difference, −164.5 g; 95% CI, −289.7 to −39.4; P=0.01, adjusted; P=0.02 after Bonferroni correction) than during the 2003–2005 period (2994±487 g vs. 3117±455 g; mean difference, −44.8 g; 95% CI, −139.1 to 49.5; P=0.35, adjusted).

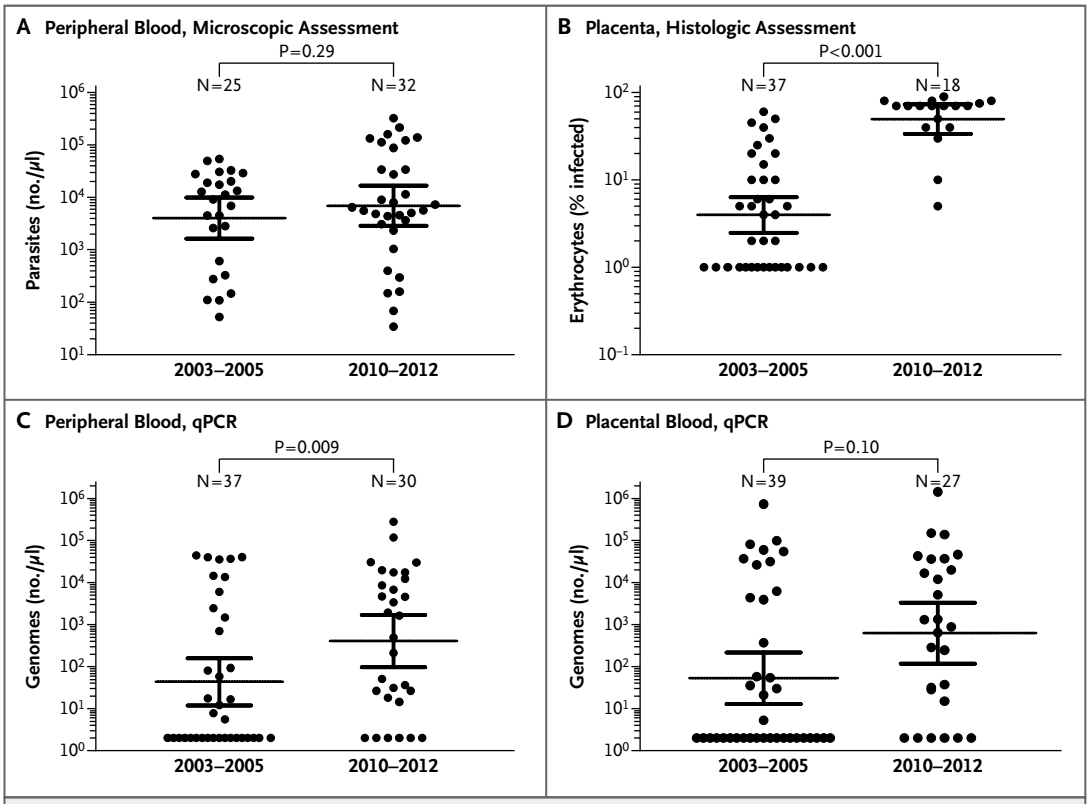

Figure 3. *P. falciparum* Densities in Pregnant Women Infected at Delivery, According to Study Period.

Results are shown for microscopic assessment of peripheral blood (Panel A), histologic assessment of the placenta (Panel B; lower limit of quantification, ≤1%), and qPCR testing of peripheral and placental blood (Panels C and D, respectively). For Panels C and D, the lower limit of quantification was 2 genomes per microliter, which was the number assigned if qPCR amplification was observed outside the lower range of the standard curve (i.e., 5 genomes per microliter). I bars represent geometric means plus 95% confidence intervals. P values are based on a multivariate analysis adjusted for HIV status, parity, and age.

EFFECT OF PARITY ON PARASITOLOGIC AND IMMUNOLOGIC OUTCOMES

The relationship of parity to parasite density, submicroscopic infections, and IgG levels differed between the two study periods. In the 2003–2005 period, higher parity was associated with a decrease in parasite densities in peripheral blood on microscopic examination and qPCR testing, as well as in placental blood on qPCR testing (Table 2). In contrast, parasite densities were similar between primigravid and multigravid women in the 2010–2012 period. The prevalence of peripheral-blood infections that were submicroscopic was higher among multigravid women (87% [20 of 23 infections]) than among primigravid women (43% [6 of 14], P=0.008) in 2003–2005 but was similar in primigravid women and multigravid women (38% [3 of 8 infections] and 50% [11 of 22], respectively; P=0.55) in 2010–2012. Finally, the increase in levels of pregnancy-specific IgG antibodies (i.e., IgG antibodies against CS2 parasites) with increasing parity was more marked among women delivering in 2003–2005 (adjusted ratio of mean MFI in multigravid women to mean MFI in primigravid women, 4.38; 95% CI, 2.30 to 8.36; P<0.001) than among those delivering in 2010–2012 (adjusted ratio of mean MFI, 1.49; 95% CI, 1.11 to 2.01; P=0.009) (Table 2), with similar results for the level of IgG antibodies against DBL3X and DBL5ε from VAR2CSA (Table S4 in the Supplementary Appendix). No parity effect was observed for levels of IgG antibodies against R29.

Table 2. Densities of *P. falciparum* and Levels of IgG Antibodies against *P. falciparum* Lines, According to Study Period and Parity.*

Outcome	2003–2005					2010–2012					P Value for Interaction†
	Primigravid		Multigravid		P Value	Primigravid		Multigravid		P Value	
	no. of patients	*geometric mean*	*no. of patients*	*geometric mean*		*no. of patients*	*geometric mean*	*no. of patients*	*geometric mean*		
Parasite density in the placenta											
Histologic assessment (% of infected erythrocytes)	20	5.9±8.4	17	2.5±3.2	0.06	9	47.7±43.8	9	51.8±33.9	0.83	0.18
qPCR testing (no. of genomes/μl)	13	857.3±4146.1	26	13.2±44.4	0.003‡	12	200.9±786.2	15	1548.4±6787.2	0.22	0.004
Parasite density in peripheral blood											
Microscopic assessment (no. of parasites/μl)	15	13,825.6±17,879.4	10	631.9±1228.5	<0.001	15	4269.0±13,248.4	17	10,647.9±18,047.6	0.30	0.001
qPCR testing (no. of genomes/μl)	14	546.6±2540.5	23	9.4±21.7	<0.001‡	8	988.3±4271.4	22	296.3±1098.3	0.46	0.14
IgG antibodies (MFI)§											
CS2 parasite line	45	2307.8±2097.3	101	9306.1±13,336.6	<0.001‡	131	1763.4±1198.7	376	3611.2±4206.7	<0.001‡	0.003
R29 parasite line	45	8390.4±8545.1	101	7579.1±9346.3	0.63	131	3071.0±5157.8	376	3819.4±6115.9	0.19	0.13

* Plus–minus values are geometric means ±SD.
† P values are for the interaction between study period and parity. P values for the interaction after Bonferroni correction were as follows: P=0.02 for placental parasite density on qPCR testing, P=0.005 for peripheral parasite density on microscopic assessment, and P=0.02 for IgG antibodies against the CS2 parasite line.
‡ The P value is significant according to a multivariate analysis adjusted for HIV status, parity, and age.
§ Mean fluorescence intensity (MFI) was measured in arbitrary fluorescence units.

DISCUSSION

Substantial declines in the prevalence of malaria among Mozambican pregnant women (from 11% in 2003–2005 to 2% in 2010–2012 on the basis of peripheral-blood microscopic assessment or placental histologic assessment, and from 30% to 4% on the basis of qPCR assay) were paralleled by reductions in levels of antimalarial IgG antibodies. These changes over a 10-year period were associated with increasing parasite densities and a larger adverse effect of infection on maternal hemoglobin levels and on the weight of newborns during the period of low malaria prevalence. Taken together, the data suggest that a weakening of antimalarial immunity as a result of infrequent parasite exposure can increase the occurrence of high-density infections and their harmful effect among women who become infected, as well as among their newborns.

The decline in the prevalence of malaria between 2003 and 2010, which was associated with a slight increase in the mean maternal hemoglobin level, is consistent with reductions in other areas of sub–Saharan Africa over similar time frames,[9,10,31,32] suggesting a continent-wide trend during this period. Intermittent preventive treatment with mefloquine during pregnancy in a number of the women between 2010 and 2012 was not responsible for the decrease in the prevalence of malaria, since similar declines were observed when these women were excluded from the analysis. Also, the numbers of malaria-susceptible primigravid women, rates of HIV infection, and CD4+ T-cell counts in HIV-infected women were relatively constant throughout the study period, and all the women received bed nets treated with a long-lasting insecticide, suggesting that these factors do not fully account for the changes observed. The introduction of artemisinin-based combination therapies in Mozambique in 2009, together with increasing use of intermittent preventive treatment with sulfadoxine–pyrimethamine during pregnancy and insecticide-treated bed nets, as recommended by the World Health Organization in 2005, may have contributed to the observed decline in the prevalence of malaria, together with socioeconomic development, improved accessibility to health care facilities, and continuous conduct of clinical trials and other investigations in the study area. The increases in prevalence between 2010 and 2012 without evident changes in malaria-control efforts suggest that climate factors, as well as increasing mosquito and parasite resistance to insecticides and antimalarial agents, respectively, may have played a role. Although similar trends have been observed in malaria-related hospital admissions in the area,[33] it remains unclear whether health care facility–based trends in malaria among pregnant women who are targets of intensive preventive measures can be extrapolated to the general community.

Levels of IgG antibodies against CS2 parasites (pregnancy-specific immunity) and R29 parasites (general immunity) mirrored changes in the prevalence of malaria infection. Moreover, levels of IgG antibodies against CS2 were increased in women with qPCR-positive *P. falciparum* infection at delivery.[16,17] These results indicate a close relationship between antibody levels and the intensity of malaria transmission[16-18,34-36] and suggest that antibodies against VAR2CSA may be a marker for cumulative exposure to the parasite during pregnancy.[19] More important, this study shows that 5 years of a marked decline in the prevalence of malaria was accompanied by reductions in levels of IgG antibodies against CS2 parasites by a factor of 2.8 and by reductions in IgG antibodies against antigens that are not specific to pregnancy, as well as by an increase in the geometric mean of parasite densities and a larger adverse effect of these infections on maternal hemoglobin levels and the weight of newborns. Reduced opportunities to acquire immunity during pregnancy because of low infection rates may render multigravid women as susceptible to malaria as primigravid women, as suggested by the similar parasite densities and levels of IgG antibodies against CS2 in the two groups of women during the period of low malaria prevalence. In accordance with previous reports on pregnant women[9] and children[37,38] residing in areas where malaria has declined substantially, these observations suggest that infrequent parasite exposure can weaken immune regulation of parasite density and increase the occurrence of adverse clinical outcomes among women who become infected, as well as among their newborns. However, the increase in antibody levels between 2010 and 2012 after relatively modest increments in the prevalence of infection suggests that immunity may be regained as exposure increases.

Our study has several limitations. Although similar procedures were followed in both study periods, possible confounding factors, such as changes in health care (i.e., access to antiretroviral agents and prenatal care) and in other vectorborne infections, were not measured, and we did not control for pooling of data from separate cohorts in this study. Moreover, estimates of the prevalence of malaria among women undergoing malaria-prevention measures at the hospital may not be representative of the prevalence in the community at large.

In conclusion, this study shows a rise in malaria with increased parasite densities and a larger adverse clinical effect after a marked fall in the prevalence of malaria and antimalarial immunity among pregnant women. These data reinforce the importance of sustaining efforts to avoid rebounds of malaria associated with reductions in naturally acquired immunity.[39]

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Mayor A, Bardají A, Macete E, et al. Changing trends in *P. falciparum* burden, immunity, and disease in pregnancy. N Engl J Med 2015;373:1607-17. DOI: 10.1056/NEJMoa1406459

Changing Trends in *P. falciparum* Burden, Immunity, and Disease in Pregnancy

SUPPLEMENTARY APPENDIX

Table of contents:

1. Supplementary methods

1.1. Quantitative polymerase chain reaction

DNA extracted from filter papers were used for detection of *P. falciparum* in duplicate by real-time quantitative PCR (qPCR) targeting 18S rRNA.[1] Parasitemia was quantified by extrapolation of cycle thresholds (Ct) from a standard curve of *P. falciparum* ring-IEs. Samples without amplification (no Ct detected) were considered negative, and a density of 2 genomes/μl was assigned if amplification was observed out of the lower range of the standard curve (5 genomes/μl). A negative control with no template DNA was run in all reactions.

1.2. Measurement of antimalarial IgG antibodies by flow citometry

Cryopreserved O+ erythrocytes infected by CS2 and R29 parasite lines were thawed and matured to trophozoite stage. Erythrocytes at 1% hematocrit were sequentially incubated with test plasma (1:20), rabbit anti–human IgG (1:200) and AlexaFluor donkey anti–rabbit IgG (1:1000) plus 10 μg/mL of ethidium bromide. IgG reactivity was expressed as the difference between the mean fluorescence intensity (MFI) of 1000 infected erythrocytes and the MFI of uninfected erythrocytes obtained in a BD LSRFortessa-4laser flow cytometer. A pool of plasma samples from twenty Mozambican pregnant women was used to normalize the data from different assays.[2] Thirty-five plasma samples from pregnant Spanish women who had never been exposed to malaria and 50 men from Manhiça were used as negative controls for R29 and CS2, respectively.

1.3. Measurement of antimalarial IgG antibodies by Luminex

A multiplex suspension array technology panel was constructed to quantify IgG responses against *P. falciparum* recombinant antigens using Luminex MagPlex™ (Luminex Corp., Austin, Texas) and the Bio-Plex 100 platform (Bio-Rad, Hercules, CA), following manufacturer's instructions. Recombinant antigens used were VAR2CSA domains (DBL3X, DBL2X, DBL5ε and DBL6ε from 3D7 strain), merozoite surface protein-1 ($MSP\text{-}1_{19}$; 19-kD fragment, 3D7 strain), erythrocyte-binding antigen-175 (EBA-175; region F2, field strain), apical membrane antigen-1 (AMA-1; full ectodomain, 3D7 strain), DBLα (CR1-binding minimal domain of the erythrocyte membrane protein 1 expressed by *P. falciparum* R29-rosetting+ line), circumsporozoite protein (CSP), sporozoite surface protein 2 (SSP2) and liver stage antigen (LSA1) (purchased from Protein Potential, LLC., Rockville, MD). Briefly, 10^6 magnetic carboxylated

microspheres (MagPlex^{TM}-C, Luminex) were washed and activated with 5 mg/ml of sulfo-NHS and EDC in 100 nM monobasic sodium phosphate buffer, pH 6.2, and coupled overnight at 4ºC with 3 μg of recombinant protein. After blocking with 1% bovine serum albumin (BSA) in PBS for 30 min and washing, 1,000 microspheres per analyte per well were added to a 96-well mylar flat-bottom plate. Microspheres were sequentially incubated 1 h with 100 µL of plasma (dilutions 1:500, 1:20,000 and 1:800,000 in duplicate for each sample), 45 min with 100 µL of biotinylated anti-human IgG (diluted 1:2,500) and 25 min with 100 µl of streptavidin-conjugated R-phycoerythrin (diluted 1:1,000). The plate was immediately read using Bio-Plex Manager version 4.0, and at least 50 microspheres per analyte were acquired per sample. Crude mean fluorescent intensity (MFI) was exported with background fluorescence from blank wells already subtracted. In parallel, 10^6 microspheres were coupled with 5 µg of anti-Fab Human IgG, and incubated with a 2-fold serial dilution curve starting at 500 ng/ml of human IgG serum (Sigma) in triplicate. The 5-parameter, weighted logistic regression equation for the dilution curve of human IgG was automatically obtained by Bio-Plex Manager software and used to extrapolate the MFI from sample dilution closer to inflexion point of the IgG curve, allowing extrapolation of the relative antibody units of IgG against each antigen.

1.4. Missing data. Sources of missing information are two-fold: HIV-status and *P. falciparum* infection (the later mainly in the placenta, due to deliveries in the way to the maternity that made not possible to collect the placenta for histological examination).

a) Women with missing data on *P. falciparum* infection in peripheral blood and/or in the placenta at delivery (primary outcome in the study) were equally distributed in 2003-2005 (16% [83/515]; 67 from women with HIV data and 16 from women with unknown HIV status]) and 2010-12 (17% [299/1744], p=0.640).

b) Among the 440 women with missing data excluded from the study, 304 had partial information on malaria infection (at least from one of the compartments: peripheral blood or placenta). Prevalence of maternal infection was similar among the women excluded because of missing data (5% [14/304]) and those included in the study (4% [76/1819]; p=0.758).

c) Parity of the pregnant women with missing data and those included in the study was similar along both study periods: mean parity of 3.3 (SD 1.9) in women with missing data and 3.0 (SD 2.0) in women included in the study from 2003-5 (p=0.135); mean parity of 2.9 (SD 2.7) in women with missing data and 2.8 (SD 2.7) in included women from 2010-12 (p=0.218)..

2. Author's contributions

AM, AB, RG and CM designed the study. AM, AB, EM, TN, AMF, RG, SM, PC, MR, JC, AN, BS, AJ, SM, LF, AN, LL, SA, CC, CD, ES and CM were involved in data acquisition. AM, JA, AN, RG, AB, PA and CM contributed to the analysis and/or interpretation of data. AM wrote the first draft of this manuscript. RG, AB, CD and CM reviewed and approved the final version of this manuscript

3, Supplementary Figures

Figure S1. Study profile.

PG, Primigravidae; MG, Multigravidae, qPCR, real-time quantitative Polymerase Chain Reaction

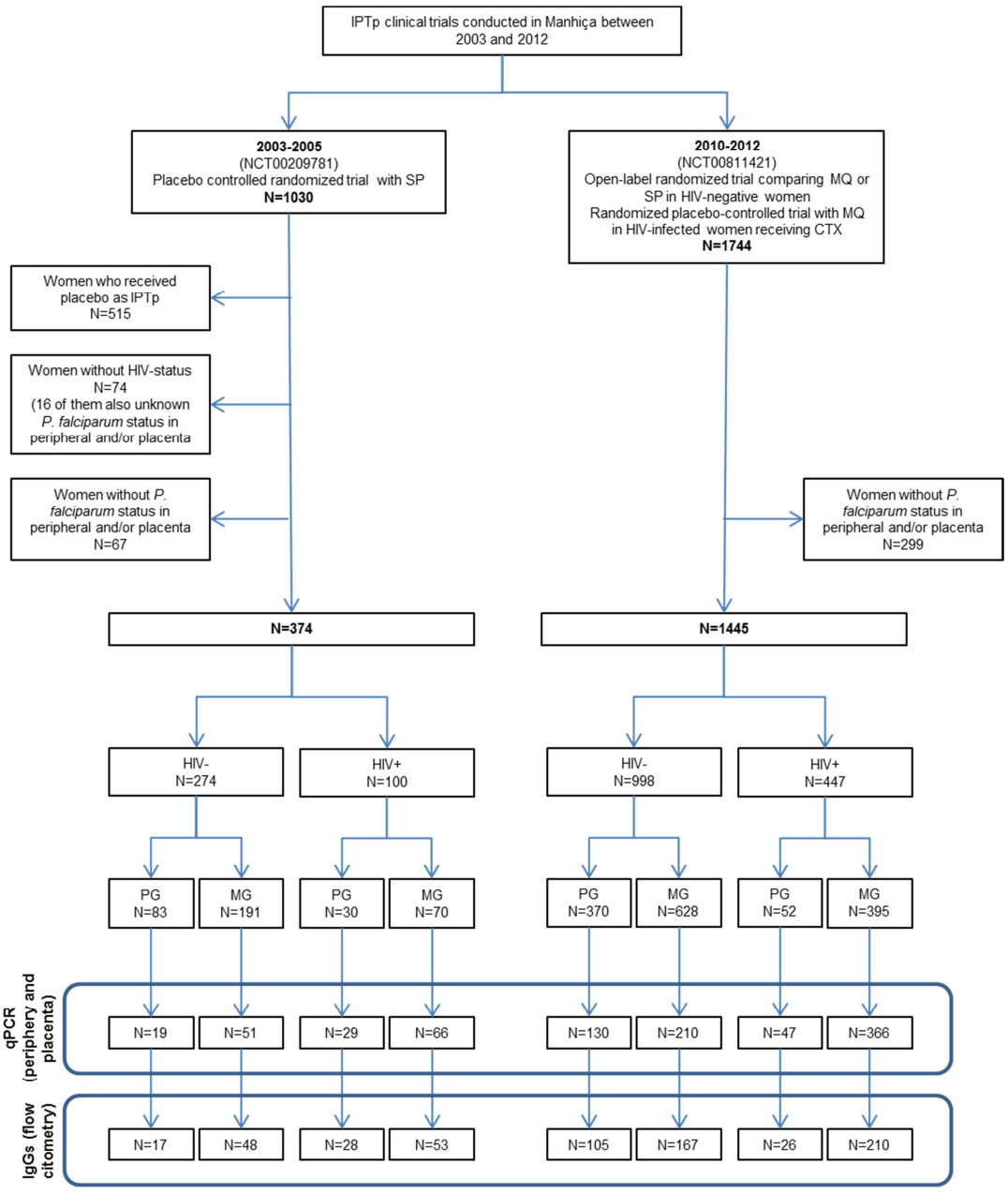

Figure S2. Prevalence of *P. falciparum* infection in peripheral blood or the placenta of pregnant women by year.

P values correspond to the multivariate analysis adjusted for HIV, parity, and age. Placental active infection was defined as the presence of parasites in placental sections after histological examination. T bars indicate standard error.

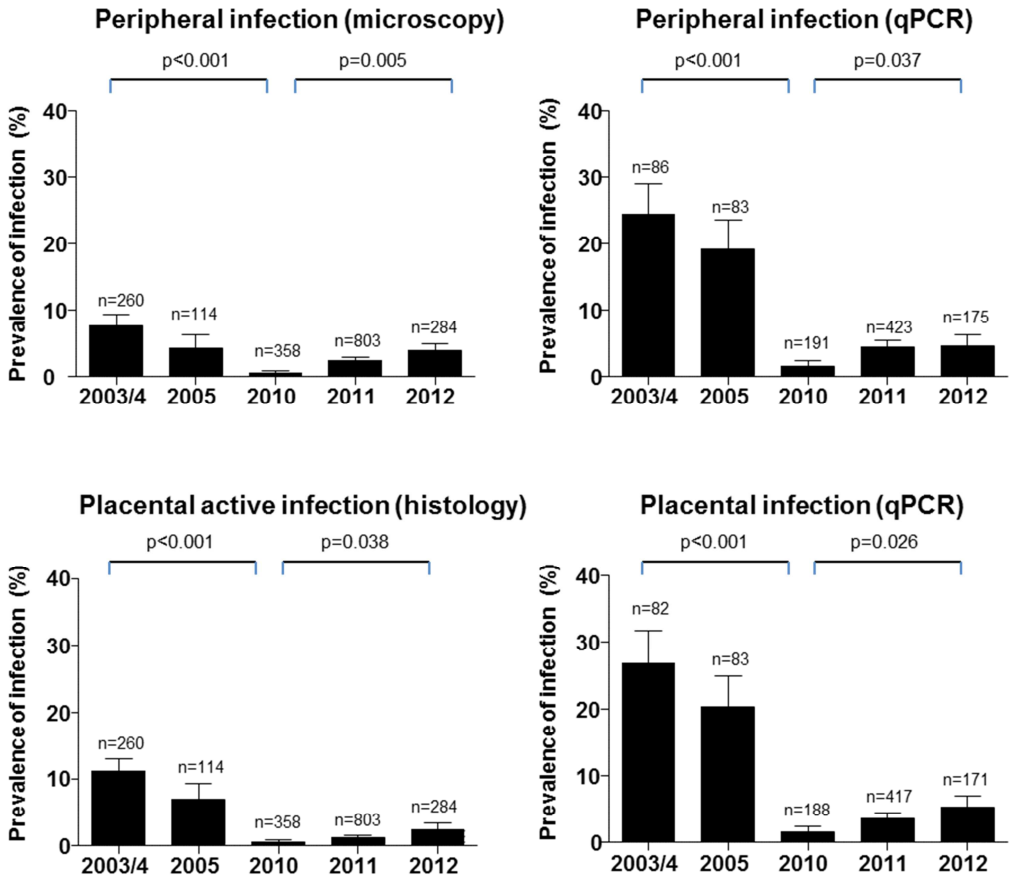

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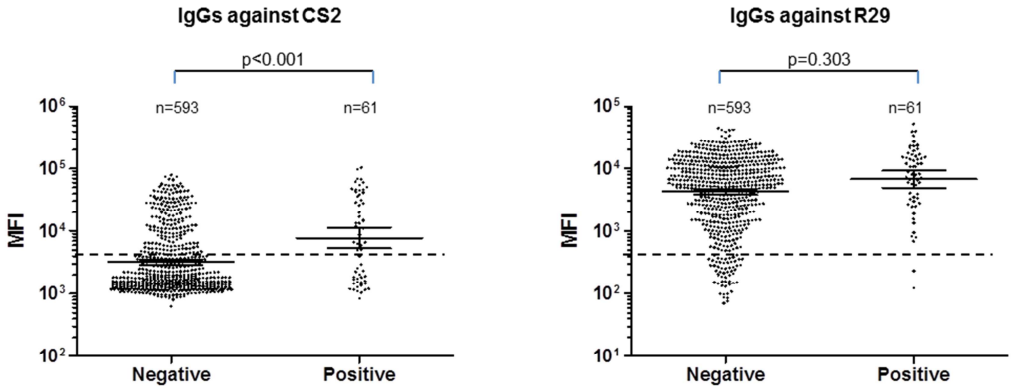
Figure S3. Levels of IgG antibodies (MFI) against parasite lines among pregnant women by qPCR-detected *P. falciparum* maternal infection.

Line and bars correspond to geometric means plus 95% confidence intervals, and P values to the multivariate analysis adjusted for HIV, parity, age, and time period. Dashed lines indicate the mean plus 3 standard deviations of the negative controls. CS2: CSA-binding parasite line; R29: Rosetting parasite line.



4. Supplementary Tables

Table S1. Characteristics of the women participating in the intermittent preventive treatment trials and those included in the study.

	IPTp trial 2003-2005[3]*			IPTp trial 2010-12[4, 5]		
	All	**Included**		**All**	**Included**	
	n=515†	**n=374**	**p¶**	**n=1744‡**	**n=1445**	**p¶**
Year, n (%)						
2003	40 (8)	28 (8)	0.970	0 (0)	0 (0)	0.910
2004	321 (62)	232 (62)		0 (0)	0 (0)	
2005	153 (30)	114 (30)		0 (0)	0 (0)	
2010	0 (0)	0 (0)		412 (24)	358 (25)	
2011	0 (0)	0 (0)		956 (56)	803 (56)	
2012	0 (0)	0 (0)		328 (19)	284 (20)	
Age (years), n (%)						
15-20	164 (32)	129 (35)	0.667	529 (30)	458 (32)	0.699
20-25	147 (29)	106 (28)		445 (26)	358 (25)	
≥25	204 (39)	139 (37)		770 (44)	629 (43)	
Parity, n (%)						
Primigravidae	137 (27)	113 (30)	0.257	505 (28)	422 (29)	0.579
Multigravidae	378 (73)	261 (70)		1239 (72)	1023 (71)	
Treatment, n (%)						
SP	515(100)	374 (100)	1.000	392 (22)	324 (32)	0.963
MQ	0 (0)	0 (0)		1072 (62)	894 (62)	
CTX	0 (0)	0 (0)		280 (16)	227 (16)	
HIV, n (%)						
Negative	324 (73)	274 (73)	1.000	1183 (68)	998 (69)	0.467
Positive	117 (27)	100 (27)		561 (32)	447 (31)	
Peripheral infection, n (%)						
Positive	35 (7)	25 (7)	0.893	34 (2)	32 (2)	0.901
Negative	458 (93)	349 (93)		1585 (98)	1413 (98)	
Placental histology, n (%)						
Acute	16 (4)	15 (4)	0.992	13 (1)	13 (1)	0.997
Chronic	28 (6)	22 (6)		5 (0)	5 (0)	
Past	174 (41)	157 (42)		54 (4)	52 (4)	
Negative	208 (49)	180 (48)		1399 (95)	1375 (95)	
Hemoglobin (g/dL), mean (SD)	10.5 (1.5)	10.5 (1.5)	0.678	10.8 (1.7)	10.8 (1.7)	0.579
Newborn weight (gr), mean (SD)	3020 (508)	3055 (473)	0.303	3038 (496)	3061 (480)	0.193

IPTp, Intermittent preventive treatment during pregnancy; SD, Standard deviation; SP, Sulphadoxine-pyrimethamine; MQ, mefloquine; CTX: Cotrimoxazole.

*, Women from the placebo arm excluded

†, Missing data: 74 for HIV; 22 for peripheral infection; 89 for placental infection

‡, Missing data: 125 for peripheral infection and 273 for placental infection

¶, Fisher's tests (binary outcomes) or t Student test (continuous outcomes)

Table S2. Performance of microscopy in peripheral blood (A) and placental histology (B) compared with quantitative Polymerase Chain Reaction.

A)

	PCR peripheral blood			
	Negative (n=891)		Positive (n=67)	
	n	%	n	%
Peripheral microscopy				
Negative (n=928)	888	100	40	60
Positive (n=30)	3	0	27	40

B)

	PCR placental blood			
	Negative (n=875)		Positive (n=66)	
	n	%	n	%
Placental histology				
Negative (n=821)	791	90	30	45
Past (n=94)	80	9	14	21
Acute (n=12)	3	0	9	14
Chronic (n=14)	1	0	13	20

Table S3. Prevalence of *P. falciparum* maternal infection by study period, including all the women and excluding those that received mefloquine as IPTp.

Microscopic infections at delivery were considered if *P. falciparum* parasites were observed in peripheral blood or in the placenta either by microscopy and histology, and qPCR if infections were detected by qPCR in peripheral or placental blood samples.

All the pregnant women delivering between 2003 and 2005 received SP as IPTp.

	2010		2011		2012	
	N	**n (%)**	**N**	**n (%)**	**N**	**n (%)**
All pregnant women						
Microscopic infections	358	2 (0.6)	803	19 (2.4)	284	12 (4.2)
qPCR infections	185	4 (2.2)	406	25 (6.2)	162	10 (6.2)
Excluding women that received mefloquine						
Microscopic infections	138	0 (0)	295	12 (4.1)	118	6 (5.1)
qPCR infections	73	2 (2.7)	174	14 (8.0)	77	7 (9.1)

Table S4. IgG responses against recombinant antigens from *P. falciparum* VAR2CSA, merozoite, and sporozoite/liver stages in 2003-5 and 2010.

	2003-5	2010				
	GM (SD)*	GM (SD)†	p‡	Ratio	95%CI	p¶
DBL2X	5.79 (7.56)	3.08 (3.56)	<0.001	0.559	0.424; 0.739	<0.001
DBL3X	12.88 (27.44)	4.68 (7.04)	<0.001	0.389	0.257; 0.588	<0.001§
DBL5ε	45.79 (113.64)	11.76 (23.42)	<0.001	0.263	0.159; 0.435	<0.001‖
DBL6ε	41.54 (80.42)	12.53 (20.83)	<0.001	0.321	0.213; 0.483	<0.001
DBLα	14.24 (15.64)	6.91 (8.05)	<0.001	0.485	0.375; 0.627	<0.001
AMA1	3883.26 (8443.12)	701.34 (1898.10)	<0.001	0.179	0.101; 0.317	<0.001
$MSP1_{19}$	59.48 (166.75)	14.65 (37.27)	<0.001	0.252	0.138; 0.459	<0.001
EBA175	17.21 (37.43)	4.78 (8.77)	<0.001	0.285	0.181; 0.448	<0.001
CSP	7.96 (12.01)	4.32 (5.73)	<0.001	0.563	0.409; 0.775	<0.001
LSA1	13.01 (24.71)	6.92 (11.95)	0.004	0.550	0.366; 0.827	0.001
SSP2	13.62 (18.94)	12.14 (17.80)	0.467	0.932	0.673; 1.291	0.644

GM, Geometric mean of relative units; SD, Standard deviation

*, n=132; †, n=173; ‡, univariate; ¶, adjusted for HIV infection, parity and age; §, Interaction between time period and parity on IgG levels (pI=0.062): adjusted ratio of mean MFIs by parity=4.30, 95%CI[1.48; 12.58], p=0.008 in 2003-5 and 1.33, 95%CI[0.61; 2.92], p=0.472 in 2010; ‖, Interaction between time period and parity on IgG levels (pI=0.079): adjusted ratio of mean MFIs by parity=7.77, 95%CI[2.29; 26.30], p=0.001 in 2003-5 and 1.21, 95%CI[0.44; 3.35], p=0.698 in 2010.

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CHAPTER 2

Multiplexing detection of IgG against *Plasmodium falciparum* pregnancy-specific antigens

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RESEARCH ARTICLE

Multiplexing detection of IgG against *Plasmodium falciparum* pregnancy-specific antigens

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Abstract

Background

Pregnant women exposed to *Plasmodium falciparum* generate antibodies against VAR2CSA, the parasite protein that mediates adhesion of infected erythrocytes to the placenta. There is a need of high-throughput tools to determine the fine specificity of these antibodies that can be used to identify immune correlates of protection and exposure. Here we aimed at developing a multiplex-immunoassay to detect antibodies against VAR2CSA antigens.

Methods and findings

We constructed two multiplex-bead arrays, one composed of 3 VAR2CSA recombinant-domains (DBL3X, DBL5ε and DBL6ε) and another composed of 46 new peptides covering VAR2CSA conserved and semi-conserved regions. IgG reactivity was similar in multiplexed and singleplexed determinations (Pearson correlation, protein array: $R^2 = 0.99$ and peptide array: $R^2 = 0.87$). IgG recognition of 25 out of 46 peptides and all recombinant-domains was higher in pregnant Mozambican women (n = 106) than in Mozambican men (n = 102) and Spanish individuals (n = 101; $p<0.05$). Agreement of IgG levels detected in cryopreserved plasma and in elutions from dried blood spots was good after exclusion of inappropriate filter papers. Under heterogeneous levels of exposure to malaria, similar seropositivity cutoffs were obtained using finite mixture models applied to antibodies measured on pregnant Mozambican women and average of antibodies measured on pregnant Spanish women never exposed to malaria. The application of the multiplex-bead array developed here, allowed the assessment of higher IgG levels and seroprevalences against VAR2CSA-derived antigens in women pregnant during 2003–2005 than during 2010–2012, in accordance with the levels of malaria transmission reported for these years in Mozambique.

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Competing interests: The authors have declared that no competing interests exist.

Conclusions

The multiplex bead-based immunoassay to detect antibodies against selected 25 VAR2CSA new-peptides and recombinant-domains was successfully implemented. Analysis of field samples showed that responses were specific among pregnant women and dependent on the level of exposure to malaria. This platform provides a high-throughput approach to investigating correlates of protection and identifying serological markers of exposure for malaria in pregnancy.

Introduction

Serological studies provide useful data for the identification of immune correlates of protection against infectious pathogens [1] and for the epidemiological surveillance of the infection [2]. The measurement of antibodies in plasma can guide the selection of malaria vaccine candidates through the identification of antibodies that last long in the blood and correlate with protection. As antibodies can circulate for weeks to months after active malaria infections, they can also be used to determine the history of exposure over time. Such serological biomarkers of malaria exposure can allow a more sensitive quantification of low malaria transmission when detecting the parasite becomes impractical due to the need for large sample sizes [3].

Malaria in pregnancy is characterized by the sequestration of infected erythrocytes in the placenta through the adhesion to chondroitin sulfate-A on syncytiotrophoblasts [4]. Adhesion is mediated by VAR2CSA [5], a member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family encoded by the *var* multigene family [6]. VAR2CSA is a large protein of 350 kDa composed of an intracellular and an extracellular region organized in 6 Duffy binding-like domains (3DBLX and 3DBLℰ) [4]. Among the members of the *var* multigene family, *var2csa* presents the lowest variability between parasite strains, with nucleotide pairwise identity from 54% to 94% [7,8] and aminoacid identity average of 78% (range 75–83%) [9].

Anti-VAR2CSA antibodies are acquired after exposure to *P. falciparum* infection during pregnancy [10], increase with successive pregnancies (parity-dependent) [11] and cross-react between geographically diverse placental isolates suggesting conserved epitopes [12], overlap of polymorphisms [9,13] or polymorphic conformational epitopes [14]. High levels of antibodies against VAR2CSA have been associated with reduced risk of placental infection [15–17], but also with low birth weight [6,16,18–20], and maternal anemia [19], supporting their dual role in protection and as marker of exposure. Anti-VAR2CSA antibodies have also been shown to mirror changes in the prevalence of infection in pregnant women from a rural area in Mozambique [21], suggesting the potential of these antibodies to provide information about changing trends in malaria transmission intensity [22,23].

Measuring antibodies against native VAR2CSA protein present in the surface of infected erythrocytes by flow cytometry [12] and recombinant proteins by ELISA (enzyme linked immunosorbent assay) [16,20,24] is time-consuming and does not allow dissection of the fine specificity of interactions involved in immune protection. As a result of the growing demand for rapid, precise and cost-effective techniques for the detection of antibodies, multiplex-bead array assays have been developed to provide measures using large numbers of antigens [25,26]. However, very few studies have used multiplex assays to measure antibodies against malaria antigens specifically expressed during pregnancy [17,27–30].

We aimed at developing a multiplex-bead array assay to detect antibodies against a panel of new peptides covering VAR2CSA and recombinant domains that could be used for the identification of immune correlates of protection against placental malaria and its adverse consequences, as well as to identify serological markers for surveillance of malaria transmission. To achieve this, antigens were coupled to microspheres, and the effect of multiplexing as well as the assay performance were assessed using plasma samples collected from a population in South Mozambique and Spanish individuals never exposed to malaria. VAR2CSA peptides were selected based on the pregnancy-specific recognition by pregnant women naturally exposed to malaria in Mozambique, compared to non-pregnant Mozambican adults. We tested this serological platform on plasma samples but also on blood collected on filter paper as a convenient alternative for serological surveys in remote locations [31]. We also compared the use of plasmas from pregnant women never exposed to malaria [27] and finite mixture models (FMM) [32] to provide seropositivity thresholds. Finally, we applied the multiplex-bead array to measure IgG responses in pregnant Mozambican women from two time periods (2003–2005 and 2010–2012) with different intensities of malaria transmission [21].

Methodology

Ethics statement

The Mozambican National Health and Bioethics Committee and the Hospital Clinic of Barcelona Ethics Committee approved the study and the use of non-identifiable plasma and serum samples for the immunological studies described [33–36]. Written informed consent was obtained from all the adult participants and from parents or guardians of healthy children participating in the RTS,S vaccine study.

Antigens

Recombinant proteins used were VAR2CSA Duffy binding-like domains (DBL3X, DBL5Ɛ and DBL6Ɛ, from 3D7 strain) [20,37], apical membrane antigen 1 (AMA1, from 3D7 strain) [38], merozoite surface protein-1, 19-kDa, ($MSP1_{19}$, from 3D7 strain) [39], all produced at ICGEB (New Delhi, India); circumsporozoite protein (rCSP) from *P. falciparum* purchased from Sanaria, Rockville; and *Clostridium tetani*, tetanus toxin purchased from Santa Cruz Biotechnology (Dallas, Texas). Forty-six synthetic peptides covering conserved and semi-conserved regions from VAR2CSA were designed after alignment of 18 full-length sequences from different geographic origins (Asia, Africa, Central and South America; Table A in S1 File and design strategy detailed in Text A in S1 File). A circumsporozoite peptide (pCSP) of 64 aminoacids ($NVDP[NANP]_{15}$) was also included (adapted from [40]). Peptides were synthesized by GL Biochem (Xangai, China) and median purity was estimated as 79% (range: 71–91%) by HPLC (high performance liquid chromatography) and mass spectrometry.

Development of multiplex arrays to measure IgG

Two multiplex suspension array panels were constructed to quantify IgG responses against *P. falciparum* recombinant proteins and synthetic peptides, using the xMAP ™ technology and the Luminex® 100/200™ System (Luminex® Corp., Austin, Texas). MagPlex® microspheres (magnetic carboxylated polystyrene microparticles, 5.6 μm) with different spectral signatures were selected for each protein (DBL3X, DBL5Ɛ, DBL6Ɛ, AMA1, $MSP1_{19}$ and rCSP), peptide (46 VAR2CSA peptides and pCSP), tetanus toxin and bovine serum albumin (BSA). Antigens were covalently coupled to beads following a modification of the Luminex® Corporation protocol (Text B in S1 File). Antigen amounts in the coupling reaction for one million beads were

determined after a titration experiment, and were as follows: 2 μg of rCSP and tetanus toxin, 4 μg of DBL3X, DBL5Ɛ, DBL6Ɛ, AMA1, 8 μg of $MSP1_{19}$, 170 μg of each peptide and for BSA a 1% solution in PBS. To determine if coupling was effective, beads were tested with two hyper-immune plasma pools (HIP). One pool was composed of 23 plasmas from pregnant Mozambican women hyperimmune against VAR2CSA (HIP-VAR2CSA) and the other was composed of 6 RTS,S/AS02-vaccinated children [41] hyperimmune against the circumsporozoite (CSP) protein (Luminex® assay described in Text C in S1 File). Protein and peptide multiplex arrays were prepared by pooling together equal volumes of coated beads. All samples were analyzed in duplicate at dilution 1:400 for the protein array and 1:100 for the peptide array. A positive control (HIP-VAR2CSA) was included in each assay plate, in addition to blanks (wells without plasma sample) to assess background levels. A minimum of 50 microspheres were read per spectral signature and results were exported as crude median fluorescent intensity (MFI). Duplicates were averaged and background MFIs were subtracted. Results were normalized (nMFI) to account for plate-to-plate variation by dividing the background subtracted MFI of each sample by the value of the positive pool in the same plate and multiplying with the median of positive pools in all plates.

Study population

For the development of both multiplex arrays, we measured IgGs in 550 pregnant women (550 plasma samples [106/550 immune against VAR2CSA as assessed by flow cytometry against native VAR2CSA expressed on CS2 parasite line] and 240 dried blood spots [DBS]) residing in Manhiça district (Southern Mozambique) who participated in two clinical trials of intermittent preventive treatment conducted between 2003–2005 [35] and 2010–2012 [33,34] (Table 1).

Manhiça is a region in southern Mozambique under continuous demographic surveillance by the Centro de Investigação em Saúde de Manhiça (CISM) [42]. Pregnancy-associated malaria in this region used to be intense (2003–2005) and has decreased in the past few years (2010–2012) [21]. Parasite detection by qPCR and anti-CS2 IgG levels were measured in a previous study [21]. Plasma samples from 102 men (50% malaria positive by microscopy) recruited at the Manhiça District Hospital during 2006 were included [43,44]. In addition, 49 plasma samples were collected during 2010 from pregnant women recruited at delivery at the Hospital Clinic in Barcelona and 52 were collected during 2008 from healthy adult men in Barcelona. Finally, we also included serum samples from 6 RTS,S/AS02-vaccinated children obtained during scheduled study visits as specified per protocol to measure antibodies against CSP antigen.

Plasma and serum samples were cryopreserved at -80˚C. DBS were prepared and stored avoiding humidity at -20˚C.

Reconstitution of dried blood spots

DBS collected from 240 pregnant Mozambican women, as well as freshly prepared DBS using plasma from positive and negative controls resuspended in full blood, were tested for antibody elution. Briefly, four spots of approximately 3 mm in diameter were cut from the filter papers using a punch (McGill® round punch, 3 mm) and transferred to individual wells of a 96-well polystyrene U-bottom plate. Antibodies were eluted with 200 μl Luminex® assay buffer (1% BSA, 0.05% sodium azide in filtrated PBS [Phosphate-buffered saline]) at room temperature overnight with gentle mixing which, assuming a hematocrit of 50% gives a concentration of eluted blood proteins equivalent to a 1:50 plasma dilution (adapted from [32,45]). To assess the quality of the elution, hemoglobin levels in the eluted DBS were measured by

Table 1. Characteristics of pregnant Mozambican women included in the study.

Variable	VAR2SSA-immune[¶]	2003–2005	2010–2012	p[§]
	N = 106	N = 204	N = 240	
Parity, n (%)				
Primigravidae	10 (9)	55 (27)	70 (29)	0.607
Multigravidae	96 (91)	149 (73)	170 (71)	
Age, n (%)				
<20yr	22 (21)	64 (31)	69 (29)	0.195
20-24yr	31 (29)	59 (29)	56 (23)	
≥25yr	53 (50)	81 (40)	115 (48)	
HIV status, n (%)				
Negative	81 (76)	144 (71)	197 (82)	0.004
Positive	25 (24)	60 (29)	43 (18)	
Malaria status, n(%)*				
Negative	24(36)[#]	125 (61)	228 (95)	<0.001
Positive	43 (64)	79 (39)	12 (5)	

[¶] Immune pregnant women from Mozambique against the VAR2CSA native protein measured by cytometry using the CS2 laboratory strain, collected between 2003–2005

*Positive if peripheral and/or placental blood were positive for *P. falciparum* by qPCR targeting the 18s rRNA

[#] qPCR (periphery and placenta) performed in 67 (63%) of 106 samples CS2 IgG positive

[§] Comparison between pregnant women from 2003–2005 with 2010–2012 by Fisher exact test

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spectrophotometry (wavelengths 415, 380 and 450) and calculated using the Harboe method with the Allen correction (Hb[mg/l] = 167.2 x A415–83.6 x A380–83.6 x A450) x dilution factor). Three criteria to discard DBS improperly eluted were established. First, reddish-brown spots against a pale background were discarded after visual examination of spots reconstitution [32]. Second, DBS were also discarded if hemoglobin levels measured in the elutions were below the upper quartile of hemoglobin value among samples considered with inappropriate visual aspect. Finally, samples were also discarded if anti-tetanus antibodies measured in the elutions were below the lowest quartile obtained from anti-tetanus IgG among samples with appropriate visual aspect and hemoglobin levels. To test if DBS can be considered an alternative to plasma, antibodies eluted from filter paper spots and correspondent antibodies from cryopreserved plasma were measured against the protein array and a subset of the peptide array (15 VAR2CSA-based peptides + pCSP).

Definition of seropositives

Pregnant women were classified as seropositive against the recombinant domains and peptides by 2 methods: 1) nMFI above the mean plus 3 standard deviations (SD) from 49 pregnant Spanish women never exposed to malaria; 2) nMFI above the mean plus 3 SD from a seronegative population of pregnant Mozambican women delivering between 2003–2012 identified by a FMM that uses maximum likelihood estimation to fit a two-component model of seronegatives and seropositives [32]. To assess the performance of the model to fit the two-components in samples from pregnant Mozambican women from different malaria transmission intensities, the FMM was calculated from nMFIs against the protein array and a subset of the peptide array (15 VAR2CSA-based peptides + pCSP) obtained among plasma samples collected during 2003 to 2005 as a period of intense malaria transmission (high, n = 204), 2010 to 2012 as a

period of low malaria transmission (low, n = 240) and from a group of samples from both periods (high & low, n = 444) (samples detailed in Table 1) [21].

Statistical analysis

Intra-assay variation was calculated as the mean coefficient of variation (CV) from replicates of 7 plasma samples analyzed in each of the 37 plates. The inter-assay variation was calculated as the CV of the median MFI from all antigens included in each multiplex array measured in the positive pool (HIP-VAR2CSA) repeated in the 37 consecutive plates, before normalization [46,47]. Data was fitted to a normal distribution by logarithmic transformation of nMFIs. Pearson's rank coefficient was used to assess the correlation between nMFI values for each antigen in singleplex versus multiplex. nMFIs were compared between study groups through linear regression analysis. Agreement of nMFIs measured in plasma and in correspondent blood eluted from DBS was assessed by Bland-Altman plots. Results were expressed as bias (mean of the differences) and 95% confidence level of agreement wide (CLw) and interpreted as suggested by Giavarina et all [48]. Agreement of seroprevalence defined by cutoffs calculated using nMFI values from pregnant women never exposed to malaria or FMM were evaluated by Kappa statistic and interpreted as suggested by Landis and Koch [49]. Proportions were compared by Fisher's exact test. Linear and logistic regression models adjusted by age, parity and HIV were estimated to compare nMFIs and seroprevalences obtained from pregnant Mozambican women recruited during 2003–2005 and 2010–2012. Statistical analyses were performed with Stata/SE software (version 12.0; StataCorp) and Graphpad Prism (version 6, Graphpad, Inc). P-values of less than 0.05 were considered to indicate statistical significance.

Results

Characteristics of VAR2CSA peptides

The alignment of 18 VAR2CSA aminoacid sequences yielded 34% positions that were polymorphic with positional Shannon entropy values above 0.43 (second tercile) (Figure A in S1 File). On average, DBL3X and DBL5Ɛ domains showed lower entropy (mean entropy [SD] of 0.20 [0.28] for DBL3X and 0.21 [0.31] for DBL5Ɛ) than DBL6Ɛ (0.52 [0.50]). Forty-six synthetic peptides with lengths between 35 to 65 amino acids from conserved and semi-conserved regions of VAR2CSA were designed for the multiplex-bead array (Figure A in S1 File and Table B in S1 File). Five peptides were from the DBL1X domain, 4 from DBL2X, 6 from DBL3X, 6 from DBL4Ɛ, 5 from DBL5Ɛ, 4 from DBL6Ɛ and 16 from N-terminal segment (NTS) and inter-domain regions (ID). Peptides corresponding to the C-terminal region of the protein (ID5-DBLƐ-ID6) showed on average higher entropy values than peptides from the other regions (mean entropy [SD] from C-terminal region = 0.37 [0.42] and mean entropy [SD] from other regions = 0.15 [0.26]; $p<0.001$).

Coupled beads are recognized by IgG from hyperimmune samples

Correct coupling of antigens on beads was confirmed by measuring the IgG recognition from hyperimmune samples. Beads coupled with rCSP and pCSP showed maximum nMFIs (≈30,000) when the least diluted pool from RTS,S/AS02-vaccinated children was used (10 points two-fold dilution curve, 1:400 to 1:20,000) (Fig 1A). The analysis of the protein array (3 VAR2CSA recombinant domains + AMA1, $MSP1_{19}$, rCSP and tetanus toxin) and peptide array (46 VAR2CSA-based peptides + pCSP) against HIP-VAR2CSA resulted in a 15 points two-fold dilution curve (1:50 to $1:1.6x10^6$) for each antigen (Fig 1B and 1C) and nMFIs at least 10 times higher compared with the recognition by negative controls (except p7, p14 and p32

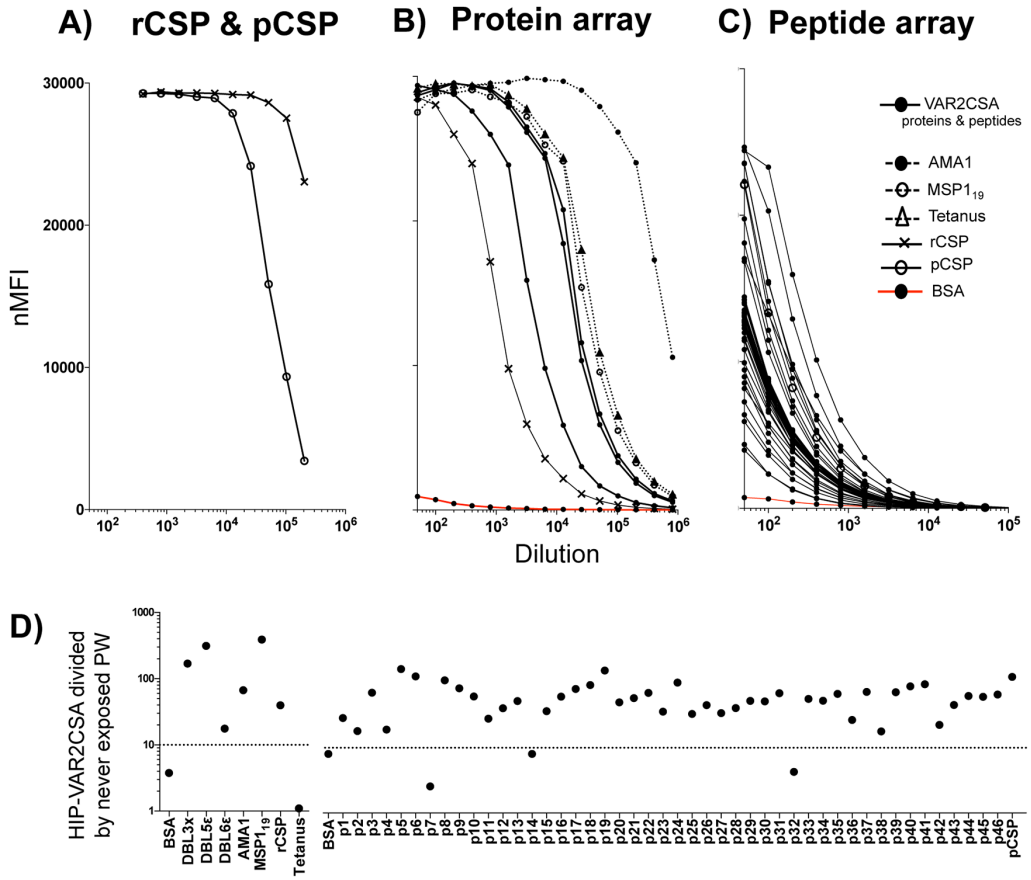

Fig 1. Antigens coupled on beads were highly recognized by IgGs in hyperimmune samples compared with negative controls. A) nMFIs obtained from 10 two-fold dilutions (1:400 to 1:20,000) of CSP hyperimmune serum measured against the CSP recombinant protein (rCSP) and peptide (pCSP). B) and C) nMFIs obtained from 15 two-fold dilutions (1:50 to 1:1.6x10^6) of VAR2CSA hyperimmune plasma (HIP-VAR2CSA) measured against the protein array (3 VAR2CSA recombinant domains + AMA1, MSP1$_{19}$, rCSP, tetanus toxin) and the peptide array (46 VAR2CSA-based peptides + pCSP). D) nMFIs measured in HIP-VAR2CSA divided by mean nMFIs measured in 5 pregnant Spanish women never exposed to malaria (PW) (dilution 1:100 for peptides array and 1:400 for proteins array).

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with a quotient <10) (Fig 1D). High nMFIs measured in hyperimmune pools compared with lower recognition by plasmas from negative controls and low background confirmed that all beads were properly coupled.

Multiplexing does not affect IgG antibody reactivity

nMFIs measured in HIP-VAR2CSA using the singleplex beads were highly correlated with nMFI values obtained in multiplex for all antigens tested (protein array [3 VAR2CSA recombinant domains + AMA1, MSP1$_{19}$, rCSP and tetanus toxin] and peptide array [46 VAR2CSA-based peptides + pCSP]) (Fig 2). Pearson′s rank correlation coefficient was $R^2 = 0.99$ ($p < 0.001$) for the protein array and $R^2 = 0.87$ ($p < 0.001$) for the peptide array. These results indicate that in the multiplex format the individual antigens on the beads do not compete for the available antibodies in the plasma sample.

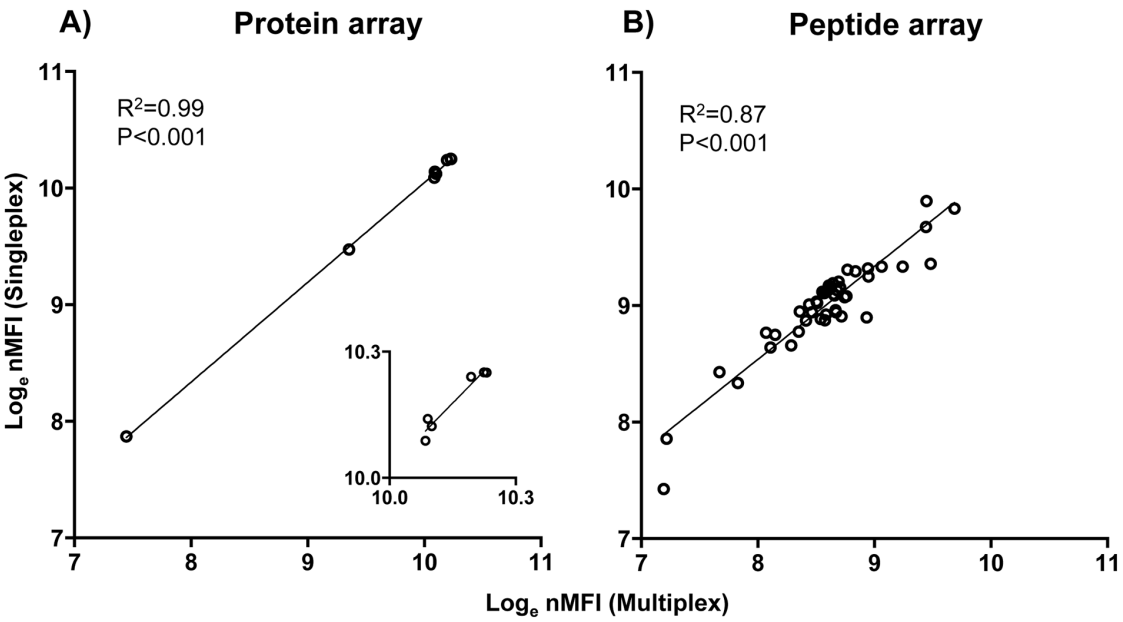

Fig 2. Correlation of nMFIs measured in VAR2CSA hyperimmune plasma in singleplex and multiplex. A) Protein array (3 VAR2CSA recombinant domains + AMA1, $MSP1_{19}$, rCSP and tetanus toxin). B) Peptide array (46 VAR2CSA-based peptides + pCSP). Each dot corresponds to the nMFI value for each antigen of the array.

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Intra and inter-assay variation

The intra-assay variation (mean CV of replicates from 7 plasma samples per plate) ranged from 2.6% to 6.5% for the protein array (3 VAR2CSA recombinant domains + AMA1, $MSP1_{19}$, rCSP and tetanus toxin) and from 3.8% to 7.6% for the peptide array (46 VAR2CSA-based peptides + pCSP) in a total of 37 plates. The inter-assay variation (variability of positive pool [HIP-VAR2CSA] between 37 plates) was 3.8% for the protein array and 16.7% for the peptide array. The normalization strategy reduced the variability between the 37 plates as shown by a decrease of CV obtained for the median MFI of anti-tetanus IgG after normalization (CV reduction from 4.4% to 2.3%) (Figure B in S1 File).

IgGs against VAR2CSA recombinant proteins and peptides in individuals exposed and non-exposed to malaria

IgGs from 106 pregnant Mozambican women immune against VAR2CSA, assessed by cytometry against native VAR2CSA expressed on CS2 parasite line, recognized all the recombinant proteins and 34/46 peptides at levels above BSA recognition (mean nMFI from each malaria antigen above mean nMFI from BSA plus 3 SD). All antigens were recognized at higher levels by plasma IgGs from VAR2CSA-immune pregnant women than from never exposed individuals ($p < 0.05$), with the exception of peptide 7 ($p = 0.269$) and tetanus toxin ($p = 0.097$). Levels of recognition of 34/46 of VAR2CSA peptides and all recombinant proteins were higher among VAR2CSA-immune, pregnant Mozambican women than among Mozambican men exposed to malaria ($p < 0.05$). The non-pregnancy-specific malaria antigens were equally recognized by IgGs from VAR2CSA-immune pregnant women and men exposed to malaria (rCSP, $p = 0.206$; pCSP, $p = 0.217$ and $MSP1_{19}$, $p = 0.247$). Overall, 25 out of 46 VAR2CSA peptides (3 peptides from DBL1X, 3 from DBL2X, 5 from DBL3X, 2 from DBL4Ɛ, 5 from

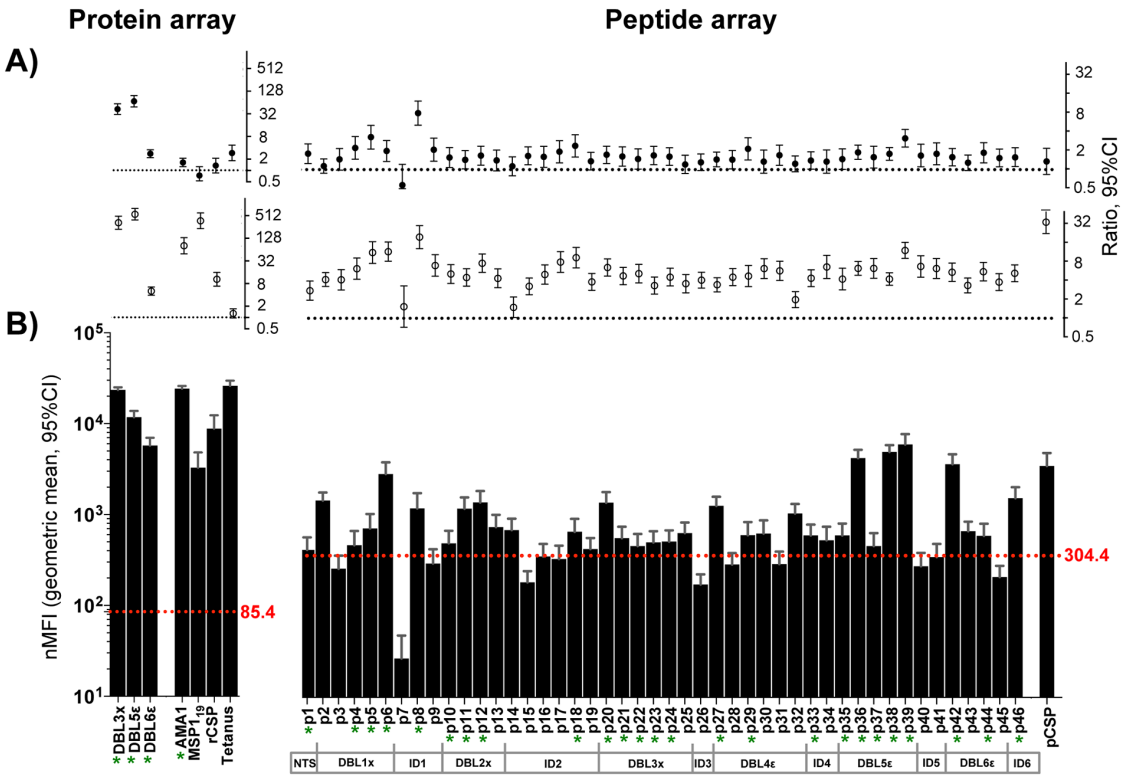

Fig 3. IgG antibodies measured in plasma from VAR2CSA-immune, pregnant Mozambican women compared with plasma from Spanish individuals and Mozambican men. A) Ratio of nMFIs against the protein array (3 VAR2CSA recombinant domains + AMA1, $MSP1_{19}$, rCSP and tetanus toxin) and the peptide array (46 VAR2CSA-based peptides + pCSP) measured in VAR2CSA-immune pregnant Mozambican women and Spanish individuals (open circle) or Mozambican men (black circle), obtained by linear regression. T-bars correspond to the 95% confidence interval (CI). B) Bars represent the mean nMFIs from VAR2CSA-immune pregnant women. T-bars correspond to the 95% CI. Red dashed line represents the mean nMFI from BSA plus 3 standard deviations (SD) (BSA reactivity threshold). Asterisk indicates that IgG level from VAR2CSA-immune pregnant women was above the BSA reactivity threshold and statistically higher from both never exposed individuals and exposed men ($p < 0.05$ by linear regression).

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DBL5Ɛ, 2 from DBL6Ɛ and 5 from NTS and ID regions) and all VAR2CSA recombinant proteins (DBL3X, DBL5Ɛ and DBL6Ɛ) were recognized by IgGs from VAR2CSA-immune pregnant women above the BSA threshold and at higher levels than by plasma from never exposed Spanish individuals and exposed Mozambican men (Fig 3A and 3B).

Dried blood spots as a source of IgG antibodies

The agreement between nMFIs against the protein array (3 VAR2CSA recombinant domains + AMA1, $MSP1_{19}$, rCSP and tetanus toxin) and the peptide array (subset comprised by 15 VAR2CSA-based peptides + pCSP) obtained with cryopreserved plasma pooled from women with high levels of IgGs against VAR2CSA and the correspondent elution product of freshly prepared DBS showed a bias of -0.03 (CLw = 0.26) for protein array and 0.27 (CLw = 0.29) for peptide array (Fig 4A). Similar agreement was obtained for anti-tetanus toxin nMFIs measured in samples from 37 pregnant Spanish women (bias = 0.02 and CLw = 0.1) (Fig 4A). Twenty-nine out of the 240 DBS from pregnant Mozambican women had an inappropriate visual aspect after spot reconstitution (reddish-brown spots against a pale background, Fig 4B). Elutions from additional 11 DBS revealed low hemoglobin levels (below the highest quartile of

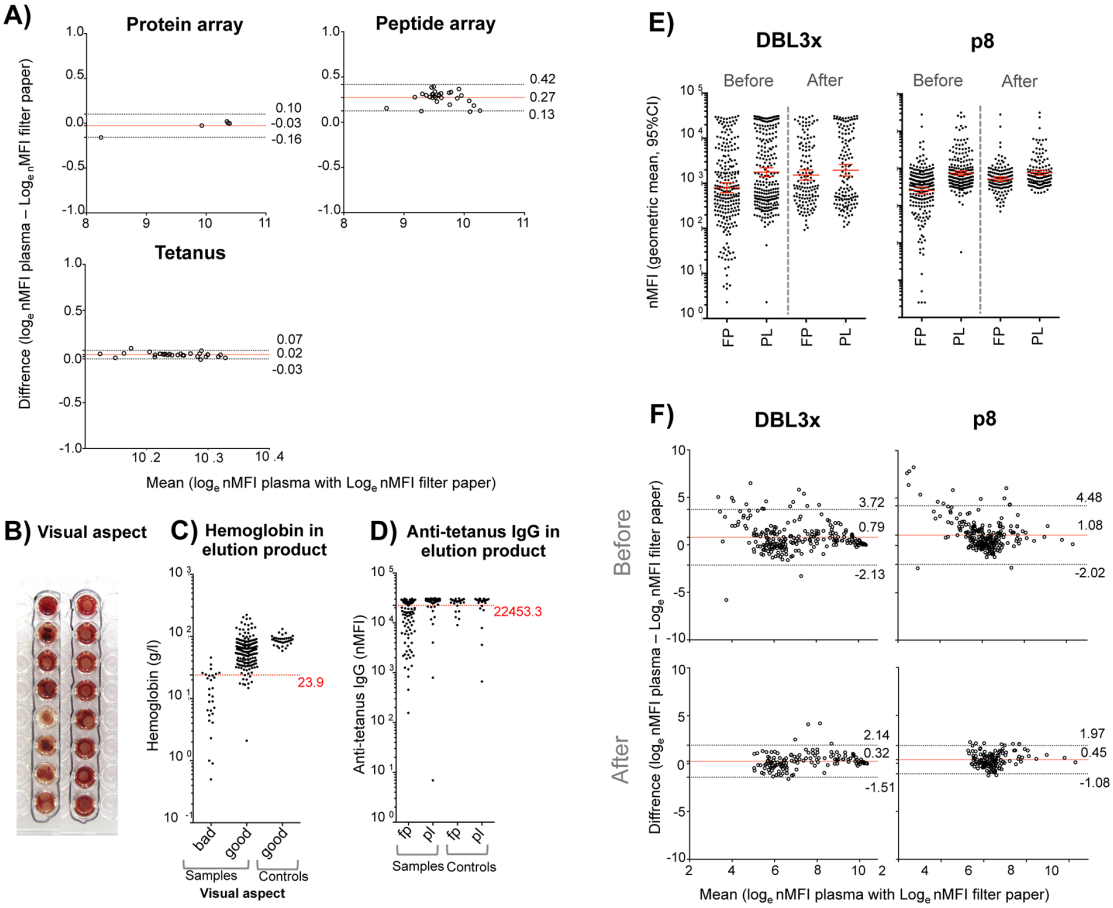

Fig 4. Performance of the multiplex assay to measure IgGs eluted from dried blood spots. A) Bland-Altman plots showing the agreement of nMFIs from VAR2CSA-hyperimmune pregnant women (HIP-VAR2CSA) measured in cryopreserved plasma (PL) and the corresponding elution product from dried blood spots (DBS) against protein array (3 VAR2CSA recombinant domains + AMA1, $MSP1_{19}$, rCSP and tetanus toxin) and peptide array (subset comprised by 15 VAR2CSA-based peptides + pCSP) and from pregnant Spanish women against tetanus toxin. Red lines indicate the bias (mean of the differences) and dashed lines the 95% confidence level of agreement (CL). B) Visual aspect after DBS reconstitution. Reddish-brown spots against a pale background indicate inappropriate elution. C) Dot-plot representing the hemoglobin (g/dl) measured in the product of DBS elution from pregnant Mozambican and Spanish (control) women separated by quality of visual inspection. Red dashed line represents the upper quartile of hemoglobin level measured in the eluted DBS from samples with inappropriate visual aspect. D) Dot-plot representing nMFIs against the tetanus toxin measured in the product of DBS elution and corresponding cryopreserved plasma from pregnant Mozambican women and Spanish controls. Red dashed line represents the lower quartile of anti-tetanus IgG measured in the product of DBS elution among samples with appropriate visual aspect and hemoglobin levels. E) Dot plots of nMFIs against DBL3X and p8 from VAR2CSA inter-domain 1 measured in the product of DBS elution and in corresponding cryopreserved plasma from pregnant Mozambican women before and after discarding DBS of inappropriate quality. Red line represent nMFI means and T-bars the 95% confidence interval (CI) and, F) agreement represented by Bland-Altman plots.

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samples with inappropriate visual aspect, Fig 4C), and a further 50 revealed low anti-tetanus toxin levels (below the lowest quartile, Fig 4D). Altogether, 63% (150/240) of DBS collected from pregnant Mozambican women yielded appropriate elution based on visual inspection of spot reconstitution as well as adequate hemoglobin levels and anti-tetanus toxin nMFIs measured in the eluted product. The range of Bland-Altman plot CLw obtained from protein array before exclusion of improperly eluted samples varied from 5.25 for AMA1 to 6.59 for $MSP1_{19}$

Table 2. Agreement of nMFIs measured in plasma and corresponding dried blood spots before and after discarding improperly eluted samples.

	BEFORE (n = 240)			AFTER (n = 150)		
	bias	CL	CLw	bias	CL	CLw
Protein array						
DBL3x	0.79	(-2.13; 3.72)	5.85	0.32	(-1.51; 2.14)	3.65
DBL5ε	0.64	(-2.44; 3.72)	6.15	0.15	(-1.82; 2.12)	3.94
DBL6ε	1.20	(-1.43; 3.82)	5.26	0.72	(-0.60; 2.05)	2.65
AMA1	0.57	(-2.06; 3.19)	5.25	0.29	(-0.79; 1.37)	2.16
MSP119	1.34	(-1.96; 4.63)	6.59	0.75	(-1.07; 2.57)	3.64
rCSP	1.53	(-1.69; 4.76)	6.45	0.89	(-0.64; 2.43)	3.07
Peptide array						
p1	0.84	(-1.86; 3.53)	5.39	0.35	(-1.41; 2.12)	3.53
p4	0.81	(-1.69; 3.31)	5.00	0.38	(-1.37; 2.13)	3.5
p5	0.36	(-2.62; 3.34)	5.96	-0.17	(-1.94; 1.60)	3.54
p6	1.30	(-1.82; 4.41)	6.22	0.72	(-0.99; 2.42)	3.41
p8	1.08	(-2.02; 4.18)	6.21	0.45	(-1.08; 1.97)	3.05
p10	-0.01	(-2.75; 2.73)	5.47	-0.59	(-2.22; 1.04)	3.26
p12	0.38	(-2.52; 3.28)	5.80	-0.23	(-1.70; 1.25)	2.95
p18	0.18	(-2.81; 3.17)	5.98	-0.39	(-2.25; 1.46)	3.71
p20	0.78	(-2.29; 3.86)	6.15	0.16	(-1.38; 1.70)	3.08
p22	-0.05	(-3.05; 2.06)	5.11	-1.05	(-2.42; 0.33)	2.75
p24	0.23	(-2.35; 2.82)	5.17	-0.24	(-1.72; 1.24)	2.96
p33	0.53	(-1.98; 3.03)	5.02	-0.01	(-1.40; 1.42)	2.82
p36	0.21	(-3.08; 3.49)	6.57	-0.37	(-2.28; 1.54)	3.82
p37	0.30	(-2.45; 3.06)	5.51	-0.22	(-2.16; 1.71)	3.87
p44	0.58	(-2.95; 4.11)	7.06	-0.06	(-2.30; 2.18)	4.48
pCSP	0.29	(-1.99; 2.57)	4.57	-0.18	(-1.53; 1.18)	2.71

https://doi.org/10.1371/journal.pone.0181150.t002

and decreased after exclusion to 2.16 for AMA1 to 3.94 for DBL5Ɛ. Regarding the peptide array, the range before exclusion was from 4.57 for pCSP to 7.06 for p44 and also decreased after exclusion (2.75 for pCSP to 4.48 for p44) (Fig 4E and 4F and Table 2). All freshly prepared samples from 37 pregnant Spanish women (control) showed appropriate elution by visual inspection, hemoglobin levels above the threshold (Fig 4C), and the majority (84% [31/37]) showed nMFIs above the tetanus threshold (Fig 4D).

Definition of seropositivity

Seroprevalence against VAR2CSA antigens (DBL3X, DBL5Ɛ, DBL6Ɛ and 15 VAR2CSA-based peptides) obtained by cutoffs derived from never exposed pregnant Spanish women and FMM (Table C in S1 File) agreed fairly for protein array (mean Kappa score [standard error of the mean—SEM] = 0.35 [0.05], $p < 0.001$) and substantially for peptide array (mean Kappa score [SEM] = 0.69 [0.07], $p < 0.001$) in plasma from 204 pregnant Mozambican women from a period of high malaria intensity, 2003–2005 (Fig 5A, Table D in S1 File). In contrast, the two arrays agreed almost perfectly in plasma from 240 pregnant Mozambican women collected during the period of low malaria intensity between 2010 and 2012 (mean Kappa score [SEM] of protein array = 0.93 [0.06], $p < 0.001$ and of peptide array = 0.85 [0.06], $p < 0.001$ (Fig 5B, Table D in S1 File). When samples from both periods (high & low) were pooled, the arrays also agreed almost perfectly (mean Kappa score [SEM] of protein array = 0.90 [0.05], $p < 0.001$ and peptide array = 0.83 [0.05], $p < 0.001$) (Fig 5C, Table D in S1 File). Regarding

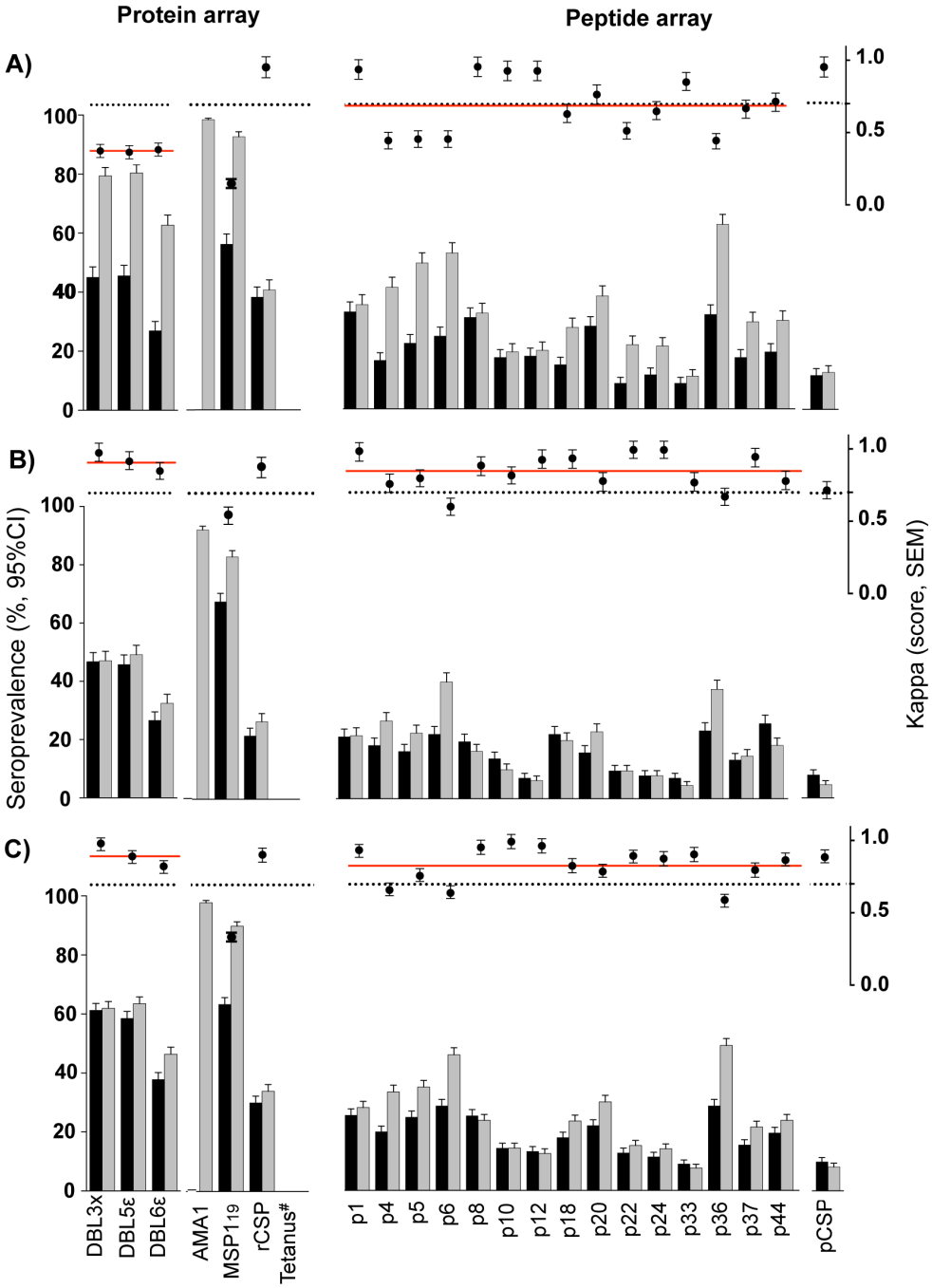

Fig 5. Agreement of seroprevalences defined by finite mixture models and pregnant women never exposed to malaria in periods of different malaria transmission intensity. Three epidemiological scenarios were considered to test the agreement of seroprevalences against the protein array (3 VAR2CSA recombinant domains + AMA1, $MSP1_{19}$, rCSP and tetanus toxin) and the peptide array (subset comprised by 15 VAR2CSA-based peptides + pCSP): A) high malaria intensity; B) low malaria intensity; C) both periods together (high & low). Black bars correspond to seroprevalence obtained by finite mixture models (FMM), grey bars to seroprevalence obtained by pregnant women never exposed to malaria and T-bars indicate the 95% confidence interval (CI). Dots are the Kappa statistic of agreement of seroprevalences obtained by both methods for each antigen, red line represents the

mean of Kappa score, dashed line represents 0.7 Kappa score threshold for good agreement and T-bars represent standard errors of the mean (SEM).

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the non-pregnancy specific malaria antigens, good and almost perfect agreement of seroprevalences was observed for the less immunogenic pCSP (high: Kappa [SEM] = 0.95 [0.07]; low: 0.72 [0.06]; high & low: 0.89 [0.05]; $p < 0.001$) and rCSP (high: 0.95 [0.07]; low: 0.87 [0.06]; high & low: 0.91 [0.05]; $p < 0.001$). In contrast, the more immunogenic AMA1 (Kappa = 0 in all scenarios) and $MSP1_{19}$ (high: 0.16 [0.04]; low: 0.56 [0.06]; high & low: 0.34 [0.04]; $p < 0.001$) failed to produce a good agreement between arrays. It was not possible to define seropositivity against tetanus toxin because all individuals had high IgG levels and no population could be identified as negative.

Anti-VAR2CSA IgGs measured in pregnant Mozambican women from two periods of different malaria transmission intensity

Prevalence of malaria infection was assessed by qPCR in peripheral and placental blood from pregnant Mozambican women (Table 1). In samples from 2003 to 2005, prevalence was 39% (79/204). In samples from 2010 to 2012, prevalence was 5% (12/240). nMFIs against the protein array (DBL3X, DBL5Ɛ, and DBL6Ɛ) and the peptide array (subset comprised by 15 VAR2CSA-based peptides) were higher in women from 2003–2005 (high malaria intensity) than from 2010–2012 (low malaria intensity) (Fig 6A). The ratio of mean nMFI measured in women during high malaria intensity and mean nMFIs measured in women during low malaria intensity assessed by linear regression ranged from 2.05 (95%CI: 1.68, 2.49 for DBL6Ɛ) to 4.57 (95%CI: 3.28, 6.37 for DBL5Ɛ) for proteins and from 1.21 (95%CI: 1.06, 1.38 for p22) to 2.34 (95%CI: 1.82, 2.99 for p5) for peptides. The same difference between malaria intensity periods was observed for seroprevalences when we used the FMM as seropositivity cutoff in all samples (high & low) (Fig 6B). The odds ratio of seropositivity between high malaria intensity and low intensity assessed by logistic regression ranged from 3.64 (95%CI: 2.41, 5.51 for DBL6Ɛ) to 5.91 (95%CI: 3.68, 9.47 for DBL3X) for protein array and from 1.33 (95%CI: 0.81, 2.19 for p18) to 4.45 (95%CI: 2.36, 8.36 for p12) for peptide array. Differences in nMFIs and seroprevalences were statistically significant ($p < 0.05$) between study periods for all antigens, except for seroprevalences against p18 ($p = 0.265$).

Discussion

Here we developed a bead-based immunoassay that multiplexes the measurement of IgGs against synthetic peptides from the *P. falciparum* pregnancy-specific antigen VAR2CSA. The assay allows the detection of antibody responses against 46 new VAR2CSA peptides and additionally 3 recombinant domains (DBL3X, DBL5Ɛ and DBL6Ɛ). All VAR2CSA recombinant domains and 25 peptides coated onto beads were more highly recognized by IgGs from plasma of malaria-exposed pregnant women than from Spanish individuals and malaria-exposed Mozambican men, in accordance with the pregnancy-restricted exposure to VAR2CSA-expressing malaria parasites. Similar nMFI values were obtained when coated beads were tested alone or in combination, thus discarding interference by multiplexing the measurements. Intra-assay variation was 4.3% and inter-assay variation was 3.8% for protein array, whereas for peptide array it was 4.9% and 16.7%, which are acceptable ranges [47]. Moreover, plate-to-plate variation decreased after normalization with the positive control.

Results of this study confirm that DBS can be an alternative to plasma for studies on malaria serology during pregnancy [32,45]. However, it is necessary to discard samples in inappropriate

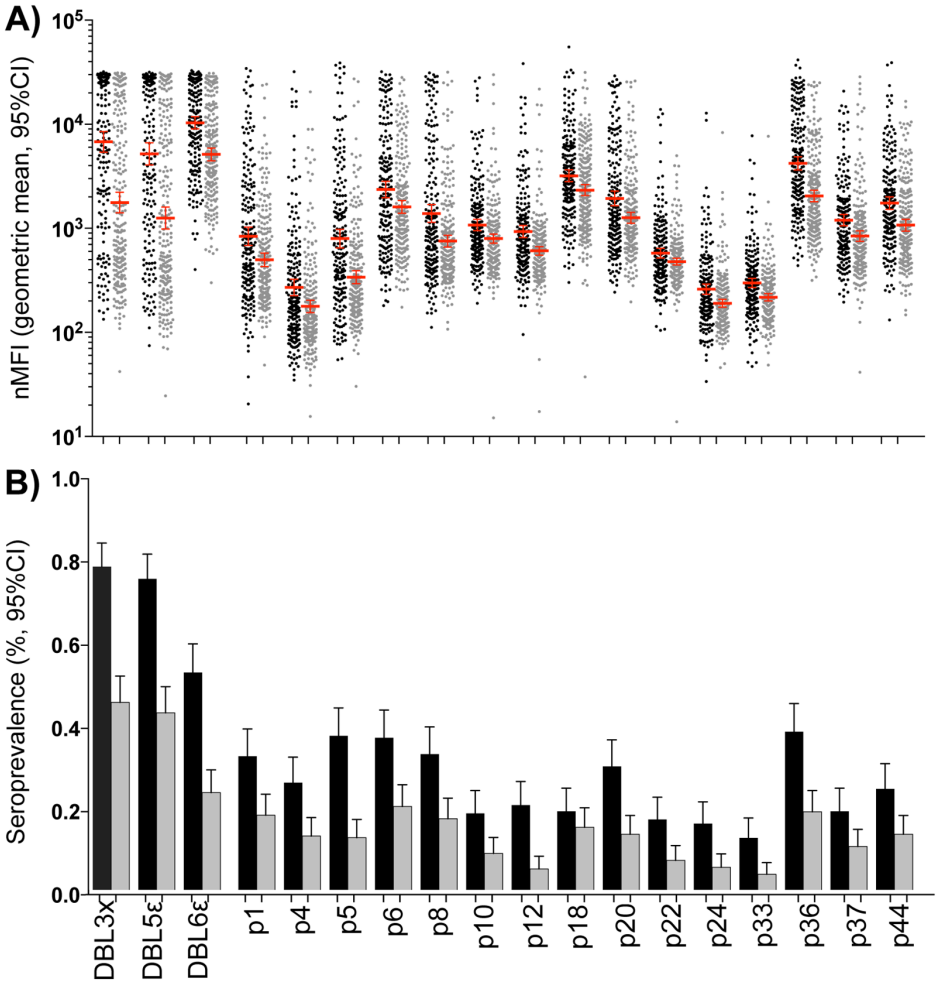

Fig 6. IgGs in pregnant women from two periods of different malaria transmission intensity. A) The dot plot shows the nMFIs against the protein array (DBL3X, DBL5ε, and DBL6ε) and the peptide array (subset comprised by 15 VAR2CSA-based peptides) in periods of high (2003–2005; black dots) and low (2010–2012; grey dotes) malaria transmission intensity. Dots represent nMFI of each pregnant woman, red lines correspond to geometric mean and T-bars represent the 95% confidence interval (CI). B) Seroprevalence obtained in periods of high and low malaria intensity (cutoff: FMM high & low). Black bars correspond to seroprevalence in the period of high intensity, grey bars to seroprevalence in the period of low intensity and T-bars to 95%CI. Differences of nMFIs or seroprevalences between high and low transmission periods assessed by linear and logistic regressions were statistically significant ($p < 0.05$) for all antigens, except for seroprevalences against p18 ($p = 0.265$).

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conditions due to incorrect handling or storage of blood samples on filter papers. Although this can be achieved by visual inspection of the elution product after spot reconstitution [32], subjectivity can be avoided by quantitative approaches based on the measurement of hemoglobin levels and anti-tetanus IgGs in the elution product.

This study also showed that a two-component finite mixture model of seronegatives and seropositives can be fitted to the population from malaria endemic areas to assess the serological status of pregnant women, as has been done for seroprevalences to general malaria antigens (usually AMA1 and $MSP1_{19}$) from populations composed of all age groups [50]. Such an

approach can be applied in scenarios of heterogeneous antibody distributions where a subgroup of the study population is seronegative and another seropositive avoiding additional sampling of malaria never exposed pregnant women, which could be logistically complex. Importantly, seropositivity cutoffs to general malaria antigens (e.g. AMA1 and $MSP1_{19}$) must be obtained from means of never exposed pregnant women as a consequence of the absence of younger age groups. In scenarios of extreme transmission (very low or very high), discrimination of both populations by FMM may be inaccurate due to an overlap or absence of seropositive and seronegative groups [51] and cutoffs defined based on means of never exposed pregnant women should be considered. Together with malaria transmission intensity, characteristics of the study population (percentage of primigravidae) or antigens (immunogenicity and longevity of antibody responses) can have an important influence on the performance of FMM and must be taken into account for a correct interpretation of the results.

Among the antigens used in this study, 3 serological profiles were observed. Merozoite (AMA1, $MSP1_{19}$) and circumsporozoite (CSP recombinant and peptide) antigens were not recognized by Spanish individuals but similarly highly recognized by malaria-exposed men and pregnant women. Tetanus toxin was highly recognized by malaria exposed and non-exposed pregnant women as a consequence of vaccination required during pregnancy [52]. VAR2CSA domains and 25 out of 46 peptides were recognized by VAR2CSA-immune pregnant women but not by individuals never exposed to malaria and exposed men, in line with the specificity of VAR2CSA expressed by placental parasites [10,53]. Different dilution curves obtained against VAR2CSA-derived antigens suggested that both arrays were composed of antigens with different degrees of immunoreactivity. DBL3X and DBL5Ɛ domains were more recognized by malaria-exposed pregnant women than DBL6Ɛ, in accordance with previous studies showing that DBL3X and DBL5Ɛ are more conserved [24,54] than the C-terminal domain DBL6Ɛ [55,56]. Aminoacid variability obtained by the alignment of VAR2CSA sequences from parasites in different geographic area also showed low variability for DBL3X and DBL5Ɛ ($entropy_{DBL3X}$ = 0.197, $entropy_{DBL5Ɛ}$ = 0.208) compared with DBL6Ɛ (entropy = 0.522). Interestingly, among the 25 peptides selected as highly immunoreactive and pregnancy-specific, 10 (40%) were from DBL3X and DBL5Ɛ domains, also supporting the existence of conserved epitopes in these domains [24,54].

The VAR2CSA multiplex assay developed in this study was used to measure antibodies from a set of samples from pregnant Mozambican women collected between 2003 and 2012. This period was characterized by a substantial decline of malaria prevalence paralleled by a reduction of antimalarial antibody levels [21]. Here, by applying the FMM to this IgG heterogeneous population of pregnant women we were able to identify a high percentage of seropositive individuals in the period of high transmission (2003–2005) compared with a low percentage of seropositives in the period of low transmission (2010–2012), demonstrating the ability of this immunoassay to distinguish different malaria transmission intensity periods.

In conclusion, the VAR2CSA multiplex technology allows the measurement of pregnancy specific antibodies against a set of 25 VAR2CSA new peptides and 3 recombinant proteins (DBL3X, DBL5Ɛ, and DBL6Ɛ) in small volumes of plasma or DBS using a high-throughput approach. Anti-VAR2CSA IgG levels and seroprevalences assessed by FMM cutoff were related with the intensity of malaria in pregnancy, suggesting this technology as a valuable tool for investigating correlates of protection and identifying serological markers of exposure.

Supporting information

S1 File. Figure A. Entropy plot of the multi-sequence alignment and peptide position. Bars correspond to Shannon entropy values calculated on the multiple sequence alignment of 18

VAR2CSA aminoacid sequences from field isolates. VAR2CSA domains and regions (NTS: N-terminal segment; DBL, Duffy-binding like; ID: inter-domain region) are indicated. Dotted horizontal lines indicate second tercile of all Shannon entropy values (0.43). Green-lines indicate each peptide position and correspondent Shannon entropy mean (standard deviation [SD]). **Figure B. Effect of normalization on plate-to-plate variation**. Coefficient of variation (CV) before (A) and after (B) normalization by positive pool, as assessed by the median MFI of anti-tetanus toxin IgG measured in plasma from 7 pregnant women from Mozambique per plate in 37 consecutive plates. Black dots correspond to MFI of each women and red line to median MFI from 7 women per plate. **Table A. Geographic origin, Genebank accession number and reference of 18 VAR2CSA sequences used in the alignment. Table B. Amino acid sequences of 46 VAR2CSA peptides (N-terminal to C-terminal). Table C. Seropositivity thresholds obtained by pregnant Spanish women never exposed to malaria and finite mixture models in high, low and high & low malaria transmission intensity periods. Table D. Seroprevalences defined by pregnant Spanish women never exposed to malaria and finite mixture models in pregnant women from high, low and both (high & low) malaria transmission periods and correspondent kappa agreement. Text A. Peptides design. Text B. Coupling of microspheres. Text C. Luminex assay to measure total IgG**. (DOCX)

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Multiplexing detection of IgG against *Plasmodium falciparum* pregnancy-specific antigens

Supporting information

Text A. Peptides design

Peptides were designed after alignment by Clustal W of 18 VAR2CSA full-length sequences from *Plasmodium falciparum* isolates of different geographic origins (Asia, Africa, Central and South America) (S1 Table). Shannon entropy values (measure of aminoacid diversity in each position of the alignment) [1] were obtained in Bioedit and a threshold of 0.43 (second tercile) was defined to distinguish the highly polymorphic regions from the conserved and semi-conserved regions (S1 Fig). Briefly, peptides correspond to sequences of at least 20 sequential aminoacids with an entropy value bellow the second tercile. A maximum of 2 positions with entropy values above threshold interspersed between conserved regions were included. In those cases with a position having more than 2 polymorphisms, the most frequent aminoacid was selected (random selection in case of equal prevalence). In those peptides less than 35 aminoacids-long (recommended length for optimal coupling [2,3]) polymorphic lateral positions were also included.

Text B. Coupling of microspheres

Two hundred µl of beads ($2{,}5x10^6$) were transferred into a 1.5 mL eppendorf tube and resuspended by sonication and vortexing. The supernatant was removed after precipitation of the beads by a magnetic separator during 60 seconds. Beads were washed twice with 250 µl of distilled water and pellets were resuspended in 80 µl of activation buffer (0.1 M NaH2PO4, pH 6.2). Sulfo-NHS (N-hydroxysulfosuccinimide) and EDC (1-Ethyl- 3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (Pierce, Thermo Fisher Scientific Inc., Rockford, IL) were simultaneously added to reaction tubes at 5 mg/mL each in activation buffer, and reaction tubes were incubated at room temperature with gentle agitation, protected from light for 20 minutes. Activated beads were washed twice with 250 µl of coupling buffer (MES 50 mM, 2-[N-

morpholino] ethanesulfonic acid monohydrate pH 5, Sigma- Aldrich). Proteins and peptides were added to correspondent beads and volume was adjusted with coupling buffer until a maximum of 400 µl. Beads and antigens were vortexed, sonicated and then incubated over night at 4ºC in the dark, with shaking. Coupled beads were blocked with 500 µl of 1% bovine serum albumin (BSA) in PBS for 30 minutes on a shaker at room temperature, avoiding light and then washed twice with 500 µl of assay buffer (1% BSA, 0.05% sodium azide in PBS filtrated) and resuspended in 400 µl of the same buffer. Beads were quantified on a Guava PCA desktop cytometer (Guava, Hayward, CA), and stored at 4°C in the dark.

Text C. Luminex assay to measure total IgG

Immediately before use, stock suspensions of antigen-coated microspheres were thoroughly resuspended by vortexing and sonication. Frozen plasma samples were thawed at room temperature, mixed by vortexing, and spin at 16000 g for 5 minutes to remove particles. 50 µl of diluted microspheres (1000 microspheres/analyte/well) were added to a 96-well mylar flat-bottom plate following the addition of 50 µl of diluted plasma in duplicates to a final concentration of 1:400 for protein array and 1:100 for peptide array and incubated for 1 hour in agitation, protected from light at room temperature. After incubation, the plates were washed 3 times with 200 µl of washing buffer (0.05% Tween 20 in PBS) by pelleting in a magnetic 96 well separator. 100 µl of biotinylated anti-human IgG (Sigma, Tres Cantos, Spain) diluted 1:1000 in assay buffer was added to each well, and plates were incubated for 45 minutes in agitation, protected from light at room temperature. After the incubation period, the plates were washed as before and 100 µl of streptavidin-conjugated R-phycoeryhtrin (Invitrogen, Carlsbad, CA) at a 1:1000 dilution in assay buffer was added and incubated for 25 minutes in the same conditions previously mentioned. Finally the plates were washed as before and the beads were resuspended in 100 µl of assay buffer and analyzed using the Luminex® 100/200™ System.

Figure A. **Entropy plot of the multi-sequence alignment and peptide position**. Bars correspond to Shannon entropy values calculated on the multiple sequence alignment of 18 VAR2CSA aminoacid sequences from field isolates. VAR2CSA domains and regions (NTS: N-terminal segment; DBL, Duffy-binding like; ID: inter-domain region) are indicated. Dotted horizontal lines indicate second tercile of all Shannon entropy values (0.43). Green-lines indicate each peptide position and correspondent Shannon entropy mean (standard deviation [SD]).

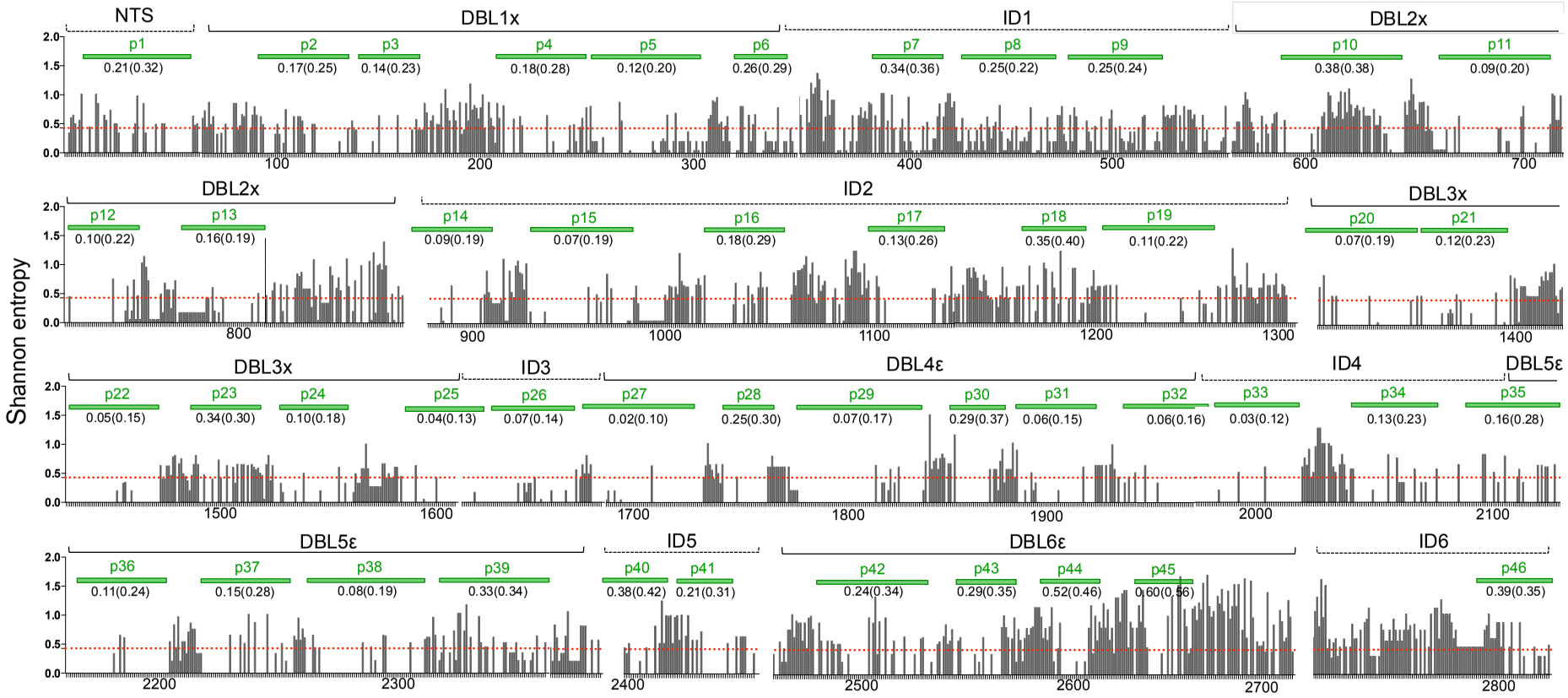
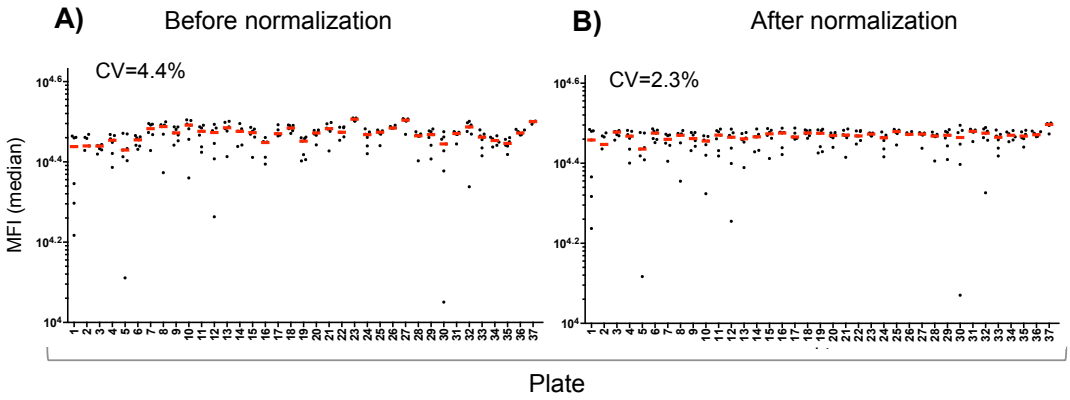


Figure B. **Effect of normalization on plate-to-plate variation.** Coefficient of variation (CV) before (A) and after (B) normalization by positive pool, as assessed by the median MFI of anti-tetanus toxin IgG measured in plasma from 7 pregnant women from Mozambique per plate in 37 consecutive plates. Black dots correspond to MFI of each women and red line to median MFI from 7 women per plate.



4

Table A. Geographic origin, Genebank accession number and reference of 18 VAR2CSA sequences used in the alignment.

Geographic origin	**Accession number** (protein)	**Reference**
SE Asia	AAQ73930	Kraemer and Smith, 2003
SE Asia	AAQ73926	
SE Asia	AAQ73924	
SE Asia	ABS79813	Bockhorst, et al, 2007
SE Asia	ABS79814	
SE Asia	ABS79815	
SE Asia	ABS79816	
SE Asia	ABS79817	
E Africa	XP_001350415	Gardner, et al, 2002
E Africa	ABS79818	Bockhorst, et al, 2007
E Africa	ABS79819	
E Africa	ABS79820	
W Africa	ABS79821	
S America	ABS79822	
C America	HB3sc1-295	http://www.broad.mit.edu
C America	HB3sc1-262	
C America	HB3var2csaA	http://www.cbs.dtu.dk/cgi-bin/webface
C America	HB3var2csaB	

Table B. Amino acid sequences of 46 VAR2CSA peptides (N-terminal to C-terminal)

Position	Peptide	Lenght	Aminoacid sequence (N-terminal to C-terminal)	S.entropy (mean)
NTS	p1	56	I A D K I E A Y L G A K S N D S K I D Q S L K A D P S E V Q Y Y R S G G D G Y Y L K N N I C K I T V N H S D S G	0.21
DBL1X	p2	45	N I F V P P R R Q R M C I N N L E K L N V D K I R D K H A F L A D V L L T A R N E G E R I	0.17
	p3	35	V Q N H P D T N S S N V C V A L E R S F A D L A D I I R G T D E N K C	0.14
	p4	52	D E N Y K K L R E D W W N A N R Q K V W E V I T C G A R S N D L L I K R R W R T S K E S N G E N K L E L	0.18
	p5	51	C R K C G H Y E E K V P T K L D Y V P Q F L R W L T E W I E D L Y R E K Q N L I D D M E R H R E E C T	0.12
	p6	38	E G T S Y C N M C K E K C K K Y C E C V K K W K S E W E N Q K N K Y K D L Y	0.26
ID1	p7	35	N Y S S L D D Y I K D D P Y S A E Y V T K L S F I L N S S D A N T S S	0.34
	p8	48	D E V C N C N E S E I S S V G Q A Q T S G P S S N K T C I T H S S I K A N K K K V C K D V K L G	0.25
	p9	48	N D K V L R V C V I E D T S L S G V D N C C C Q D L L G I L Q E N C S D K N K S G S S S N G S C	0.25
DBL2X	p10	64	G L Q K E Y A N T I G L P P R T Q S L Y L G N L P K L E N V C K G V T D I N F D T K E K F L A G C L I A A F H E G K N L K T S Y Y	0.38
	p11	50	K L C K A L E Y S F A D Y G D L I K G T S I W D N E Y T K D L E L N L Q K I F G K L F R K Y I K K N	0.09
	p12	36	T S Y S S L D E L R E S W W N T N K K Y I W L A M K H G A G M N S T T C	0.10
	p13	47	S G S S C D D I P T I D L I P Q Y L R F L Q E W V E H F C K Q R Q E K V K D V I E N C N S C K	0.16
ID2	p14	36	G T A G S S W S K R W D Q I Y K R Y S K Y I E D A K R N R K A G T K N C	0.09
	p15	51	E N K C V Q S D I D S F F K H L I D I G L T T P S S Y L S I V L D D N I C G A D K A P W T T Y T T Y T	0.07
	p16	35	T L V V V N V P S P L G N T P H G Y K Y A C Q C K I P T T E E T C D D	0.18
	p17	42	K L D G N D V M F F N L F E Q W N K E I Q Y Q I E Q Y M T N T K I S C N N E K N V L	0.13
	p18	35	C E C Y E L W I E K I K E Q W D K Q K D N Y N K F Q S K Q I Y D A N K	0.35
	p19	63	K K V V S L S N F L F F S C W E E Y I Q K Y F N G D W S K I K N I G S D T F E F L I K K C G N N S G D G E T I F S E K L N N A	0.11
DBL3X	p20	52	Y I R G C Q P K R Y D G F I F P G K G G E K Q W I C K D T I I H G D T N G A C I P P R T Q N L C V G E L	0.07
	p21	51	W D K S Y G G R S N I K N D T K E S L K N K L K N A I Q K E T E L L Y E Y H D K G T A I I S R N P M K	0.12
	p22	48	N N S N G L P K G F C H A V Q R S F I D Y K N M I L G T S V N I Y E Y I G K L Q E D I K K I I E	0.05
	p23	35	S G A D K V N D W W K G I E G E M W G A V K C G I T K I N K K Q K K N	0.34
	p24	45	E C G V S P P T G N D E D Q F V S W F K E W G E Q F C I E R L R Y E Q N I R E A C T I N G	0.10
	p25	43	G A C K R K C E K Y K K Y I S E K K Q E W D K Q K T K Y E N K Y V G K S A S D L L K E	0.04
ID3	P26	43	N Y P E C I S A N F D F I F N D N I E Y K T Y Y P Y G D Y S S I C S C E Q V K Y Y K Y	0.07
DBL4ε	P27	52	N N K S L C H E K G N D R T W S K K Y I K K L E N G R T L E G V Y V P P R R Q Q L C L Y E L F P I I I K	0.02
	P28	35	D M E N A K E E L L E T L Q I V A E R E A Y Y L W K Q Y N P T G K G I	0.25
	P29	65	A N K K A C C A I R G S F Y D L E D I I K G N D L V H D E Y T K Y I D S K L N E I F G S S N K N D I D T K R A R T D W W E N E T I	0.07
	P30	36	N G T D R K T I R Q L V W D A M Q S G V R K A I D E E K E K K K Q N E N	0.29
	P31	36	F P P C M G V E H I G I A K P Q F I R W L E E W T N E F C E E Y T K Y F	0.06
	P32	45	K G A D D C G D N S N I E C K K A C A N Y T N W L N P K R I E W N G M S N Y Y N K I Y R K	0.06
ID4	P33	44	S N K E S E D G K D Y S M I M E P T V I D Y L N K R C N G E I N G N Y I C C S C K N I G	0.03
	P34	53	C E D N K G P L D L M N E V L N K M D E K Y S A H K M K C T E V Y L E H V E E Q L K E I D N A I K D Y Q L	0.13
DBL5ε	P35	53	Y P L D R C F D D K S K M K V C D L I G D A I G C K D K T K L D E L D E W N D M D M R D P Y N K Y K G V L	0.16
	P36	53	I P P R R R Q L C F S R I V R G P A N L R N L N E F K E E I L K G A Q S E G K F L G N Y Y N E D K D K E K	0.11
	P37	47	K E K A L E A M K N S F Y D Y E Y I I K G S D M L T N I Q F K D I K R K L D K L L T K E T N N	0.15
	P38	57	D W W K T N K K S I W N A M L C G Y K K S G N K I I D P S W C T I P T T E K P P Q F L R W I K E W G T N V C I Q K	0.08
	P39	65	K E Y V K S E C S N V T N I N L G S Q A S E S N N C T S E I R K Y Q E W S R K R S I Q W E A I S E R Y K K Y K G M D E F K N V K N	0.33
ID5	P40	35	E P D A N E Y L K E H C S K C P C G F N D M E E I T N N T D N E K E A	0.38
	P41	35	F N Q I I E K V K I P A E L E D V I Y R L K H H E Y N K S N D Y I C N	0.21
DBL6ε	P42	62	T D L L V K N S S D I N K G V L I P P R R K N L F L N I D P S D I C K Y K K N P K L F N D F I Y S S A F T E V E R L K K V Y	0.24
	P43	35	K V A N A M K Y S F A D I G N I I K G D D M M E K N S S D K I G K I L	0.29
	P44	35	T D G Q N E K R K T W W D M N K Y H I W E S M L C G Y K Q A G G D T K	0.52
	P45	35	C R F P D T D G V P Q F L R W F Q E W T E T F C V T R Q E L Y E K L N	0.60
ID6	P46	35	P L P P Q N D E P I T S D I L Q K T I P F G I A L A L G S I A F L F M	0.39

Table C. Seropositivity thresholds obtained by pregnant Spanish women never exposed to malaria and finite mixture models in high, low and high & low malaria transmission intensity periods.

	Never exposed PW*	FMM		
		high	low	High & low
	mean+3SD	mean+3SD	mean+3SD	mean+3SD
Protein array				
DBL3x	1368.67	20202.95	1400.37	1447.82
DBL5ε	737.25	12602.27	969.02	1144.02
DBL6ε	8093.25	25800.74	9909.14	11220.63
AMA1	5056.25	44542.36	48089.15	47910.02
$MSP1_{19}$	366.93	8670.87	1392.78	3869.55
rCSP	6259.87	7099.31	8104.68	7717.40
Tetanus	48703.14	40693.42	46539.19	43531.98
Peptide array				
p1	1024.2	1298.71	1078.42	1237.06
p4	226.38	730.88	335.73	448.75
p5	603.13	2085.36	800.75	1003.13
p6	1829.77	5667.09	3272.26	3516.23
p8	1721.95	1951.08	1332.21	1609.94
p10	1953.74	2288.22	1623.94	1938.41
p12	1671.68	1924.04	1376.54	1557.93
p18	5147.87	7941.2	4987.13	6426.76
p20	2125.35	3235.13	2692.81	2882.63
p22	914.71	1460.81	890.62	1052.43
p24	418.46	613.22	406.68	502.15
p33	620.18	717.24	478.38	577.00
p36	2531.42	6866.72	3968.99	4966.87
p37	1652.05	2604.36	1793.35	2186.54
p44	2383.95	3797.17	1864.48	3063.01
pCSP	1008.19	1106.09	763.92	909.98

PW, Pregnant women; FMM, Finite mixture model; SD, Standard deviation
*2 or less plasma samples were considered outliers and excluded by showing nMFI above decile 9 plus 1.5 times the inter-decile range for each antigen

Table D. Seroprevalences defined by pregnant Spanish women never exposed to malaria and finite mixture models in pregnant women from high, low and both (high & low) malaria transmission periods and correspondent Kappa agreement.

	High transmission (n=204)					Low transmission (n=240)					High&Low transmission (n=444)				
	Sero+ (C-) % (n)	Sero+ (FMM) % (n)	Kappa	SEM	p	Sero+ (C-) % (n)	Sero+ (FMM) % (n)	Kappa	SEM	p	Sero+ (C-) % (n)	Sero+ (FMM) % (n)	Kappa	SEM	p
Protein array															
DBL3x	79 (162)	45 (92)	0.35	0.05	< 0.001	47 (113)	47 (112)	0.99	0.06	< 0.001	62 (275)	61 (272)	0.99	0.05	< 0.001
DBL5ε	80 (164)	46 (93)	0.34	0.05	< 0.001	49 (118)	46 (110)	0.93	0.06	< 0.001	64 (282)	59 (260)	0.90	0.05	< 0.001
DBL6ε	63 (128)	27 (55)	0.36	0.05	< 0.001	33 (78)	27 (64)	0.86	0.06	< 0.001	46 (206)	38 (168)	0.83	0.05	< 0.001
AMA1	99 (203)	0 (0)	NA	NA	NA	95 (229)	0 (0)	NA	NA	NA	97 (432)	0 (0)	NA	NA	NA
MSP1$_{19}$	94 (191)	57 (116)	0.16	0.04	< 0.001	86 (206)	70 (168)	0.56	0.06	< 0.001	89 (397)	63 (280)	0.34	0.0355	< 0.001
rCSP	41 (84)	39 (79)	0.95	0.07	< 0.001	28 (66)	23 (54)	0.87	0.06	< 0.001	34 (150)	30 (133)	0.91	0.05	< 0.001
Tetanus	0 (0)	0 (0)	NA	NA	NA	0 (0)	0 (0)	NA	NA	NA	0 (0)	0 (0)	NA	NA	NA
Peptide array															
p1	36 (73)	33 (68)	0.95	0.07	< 0.001	22 (52)	21 (51)	0.99	0.06	< 0.001	28 (125)	26 (114)	0.94	0.05	< 0.001
p4	42 (85)	17 (34)	0.44	0.06	< 0.001	27 (64)	18 (44)	0.76	0.06	< 0.001	34 (149)	20 (89)	0.66	0.04	< 0.001
p5	50 (102)	23 (46)	0.45	0.06	< 0.001	23 (54)	16 (39)	0.80	0.06	< 0.001	35 (156)	25 (111)	0.76	0.05	< 0.001
p6	53 (109)	25 (51)	0.45	0.06	< 0.001	40 (96)	22 (53)	0.60	0.06	< 0.001	46 (205)	29 (128)	0.64	0.04	< 0.001
p8	33 (67)	31 (64)	0.97	0.07	< 0.001	16 (39)	20 (47)	0.89	0.06	< 0.001	24 (106)	26 (113)	0.96	0.05	< 0.001
p10	20 (40)	18 (36)	0.94	0.07	< 0.001	10 (24)	14 (33)	0.82	0.06	< 0.001	14 (64)	14 (64)	1.00	0.05	< 0.001
p12	20 (41)	18 (37)	0.94	0.07	< 0.001	6 (15)	7 (17)	0.93	0.06	< 0.001	13 (56)	13 (59)	0.97	0.05	< 0.001
p18	28 (57)	15 (31)	0.63	0.07	< 0.001	20 (48)	22 (53)	0.94	0.06	< 0.001	24 (105)	18 (80)	0.83	0.05	< 0.001
p20	39 (79)	28 (58)	0.77	0.07	< 0.001	23 (55)	16 (38)	0.78	0.06	< 0.001	30 (134)	22 (98)	0.79	0.05	< 0.001
p22	22 (45)	9 (18)	0.51	0.06	< 0.001	10 (23)	10 (23)	1.00	0.06	< 0.001	15 (68)	13 (57)	0.90	0.05	< 0.001
p24	22 (44)	12 (24)	0.65	0.07	< 0.001	8 (19)	8 (19)	1.00	0.06	< 0.001	14 (63)	12 (51)	0.88	0.05	< 0.001
p33	11 (23)	9 (18)	0.86	0.07	< 0.001	5 (11)	7 (17)	0.77	0.06	< 0.001	8 (34)	9 (40)	0.91	0.05	< 0.001
p36	63 (129)	32 (66)	0.44	0.06	< 0.001	38 (90)	23 (56)	0.67	0.06	< 0.001	49 (219)	29 (128)	0.59	0.04	< 0.001
p37	30 (61)	18 (36)	0.67	0.07	< 0.001	15 (35)	13 (32)	0.95	0.06	< 0.001	22 (96)	16 (69)	0.80	0.05	< 0.001
p44	30 (62)	20 (40)	0.72	0.07	< 0.001	18 (44)	26 (62)	0.78	0.06	< 0.001	24 (106)	20 (87)	0.87	0.05	< 0.001
pCSP	13 (26)	12 (24)	0.95	0.07	< 0.001	5 (11)	8 (19)	0.72	0.06	< 0.001	8 (37)	10 (45)	0.89	0.05	< 0.001

Sero+, seropositive; C-, Spanish malaria never exposed pregnat women; FMM, Finite mixture models; SEM, Standard error of mean

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CHAPTER 3

Pregnancy-specific serology reveals temporal and spatial *P. falciparum* transmission patterns

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Pregnancy-specific serology reveals temporal and spatial *P. falciparum* transmission patterns

Short title: Malaria transmission through VAR2CSA serology

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ABSTRACT

Pregnant women constitute a promising sentinel group for the continuous monitoring of malaria transmission. Detecting antibodies developed by pregnant women against VAR2CSA, the *Plasmodium falciparum* antigen that mediates parasite sequestration in the placenta, may allow estimating the cumulative prevalence of exposure to *P. falciparum* during pregnancy and thus amplify signals of transmission, especially in low endemic areas where the chance of detecting the parasite is low. To identify antibody signatures that are quantitative measures of recent *P. falciparum* exposure in pregnancy, we dissected IgG responses against VAR2CSA using a multiplex peptide-based suspension array in 2354 pregnant women delivering between 2004 and 2012 in Mozambique, Benin, Kenya, Gabon, Tanzania and Spain. Two VAR2CSA peptides of limited polymorphism were immunogenic, accessible to naturally-acquired antibodies and targeted by IgG responses readily boosted upon infection during pregnancy and with estimated half-lives below two years. Seroprevalence against these peptides reflected declines of transmission in Southern Mozambique from 2003-2010 with a significant rebound in 2010-2012, as well as geographical differences in malaria burden and reductions in exposure associated with the use of preventive measures during pregnancy. Finally, VAR2CSA serology identified local clusters of transmission that were missed by detection of *P. falciparum* infections. These data suggest that VAR2CSA serology in easy accessible pregnant women allow a fine scale estimation of exposure over time and space, providing a useful adjunct metric for malaria surveillance and response aiming to interrupt malaria transmission.

SIGNIFICANCE STATEMENT

Timely monitoring of malaria transmission underpins the success of malaria control and elimination programs. Malaria surveillance in pregnant women at maternal facilities has the potential to provide an easy-to-implement complement for tracking malaria trends in the general population. Detection of antibodies against VAR2CSA, the parasite antigen that mediates sequestration of parasites in the placenta, may allow quantifying women who had at least one infection during pregnancy. Here we show that these antibodies reflect changes in malaria transmission, either natural or driven by deliberate efforts, and identify areas of high malaria burden. Serological screening in pregnancy would offer a cost-effective method to track malaria burden and flag critical steps in programmatic environments where year-to-year changes in transmission need to be monitored.

INTRODUCTION

Agile malaria surveillance and response systems which can be sustained over time are needed for the optimal design of control programs (1, 2). Rates of *Plasmodium falciparum* (*Pf*) infection in pregnant women are sensitive to changes in transmission (3, 4) and correlate well with infection in infants at routine health services (5) as well as in children at schools (6) and household surveys (7). Thus, passive detection of malaria cases at maternal clinics constitutes a promising approach to provide contemporary data on the level, and changes in levels, of malaria burden in the population for successful malaria control and elimination (8).

After exposure to *Pf* parasites that sequester in the placenta (9), women develop antibodies against VAR2CSA (10), a multi-domain variant antigen of the erythrocyte membrane protein 1 (PfEMP1) family. VAR2CSA is expressed on the surface of infected erythrocytes and mediates placental sequestration of parasites through binding to Chondroitin Sulphate A (CSA) (11). Levels of antibodies against VAR2CSA are affected by variables that influence the risk of *Pf* exposure (12-14) and mirror malaria trends in pregnancy (3). Moreover, levels of IgG against VAR2CSA at delivery correlate with the risk of malaria in the offspring (14), suggesting their value to pinpoint areas of high malaria transmission (15). As antibodies against VAR2CSA persist after the infection is cleared (16), they can provide a sensitive adjunct for *Pf* monitoring, especially in low endemic areas where the chances of detecting antibodies is higher than detecting the parasite (17).

The utility of sero-surveillance is mainly dependent on the longevity of the antibodies, with short half-lived ones being the most informative to identity rapid changes in recent transmission (18). As different VAR2CSA domains elicit IgG responses with varying magnitudes and dynamics (16, 19, 20), we hypothesized that short lived antibodies against immunogenic and non-polymorphic VAR2CSA epitopes would allow a fine-scale estimation of recent *Pf* transmission during pregnancy (17). We probed plasma from 2354 pregnant women living in areas from high to low and absent *Pf* transmission (Benin, Gabon, Mozambique, Kenya, Tanzania and Spain) against a quantitative suspension array containing VAR2CSA-based antigens (Duffy binding-like recombinant domains DBL3X, DBL5Ɛ, DBL6Ɛ and 25 peptides) as well as general parasite antigens (apical membrane antigen 1 [AMA1], 19-kDa fragment of the merozoite surface protein-1 [$MSP1_{19}$] and a circumsporozoite peptide [pCSP]). To identify antibody signatures that are informative of

recent *P. falciparum* exposure, we dissected anti-VAR2CSA antibody responses naturally-acquired by malaria-exposed African pregnant women with the aim of selection peptides targeted by IgGs that were rapidly acquired after *Pf* infection, lacked long persistence in circulation and were sensitive to the level of parasite exposure. Finally, we quantified the relationship between VAR2CSA antibody responses and temporal, spatial as well as intervention-driven changes in malaria burden. The selection strategy presented here identified biomarkers of recent exposure for pregnant women living in malaria endemic areas and illustrates the utility of this approach to flag critical steps in programmatic environments where year-to-year changes in transmission need to be monitored.

RESULTS

Study participants and *Pf* burden

Study participants consisted of 2354 pregnant women (Table 1 and Fig. S1) recruited in 2004-2005 (n=146) and 2010-2012 (n=2208) in the context of intermittent preventive treatment during pregnancy (IPTp) clinical trials (21-23). Among them, 993 were from Mozambique, 854 from Benin, 131 from Gabon, 296 from Kenya, 31 from Tanzania and 49 from Spain. The women randomly selected for this study were similar in terms of baseline characteristics with the 6216 women participating in the randomized clinical trials (Table S1).

The study areas represent five sub-Saharan African sites with different intensities of malaria transmission. Prevalence of *Pf* infection detected by real-time quantitative polymerase chain reaction (qPCR) at delivery, either in peripheral or placental blood (averaged from 2010 to 2012), was 46% (332/725) in Benin, 10% (9/89) in Gabon and 6% (28/452) in Mozambique among HIV-uninfected women, and 8% (22/273) in Kenya and 4% (13/327) in Mozambique among HIV-infected women (Table 1). The prevalence of *Pf* infection among pregnant Mozambican women decreased from 25% (37/146) in 2004-2005 to 2% (3/176) in 2010 and increased to 6% (4/72) in 2012. The same temporal pattern was observed in the proportion of 2-10 years old children with *Pf* infection ($PfPR_{2-10}$; 27% in 2004-2005, 5% in 2010 and 11% in 2012), derived from the Malaria Atlas Project geostatistical prediction model (24). A subset of 239 pregnant Mozambican women recruited between 2011 and 2012 was followed during pregnancy, with prevalence of *Pf* infection detected by qPCR of 16% (38/239) at first antenatal visit (mean gestational age: 20.7 weeks, standard deviation [SD]=5.45), 3% (8/239)

at the second IPTp administration (25.9 weeks, SD=4.98) and 5% (13/239) at delivery (mean gestational age: 38.4, SD=2.26). 2% (5/239) of the women experienced a *Pf* infection at unscheduled visits during the study follow-up. Overall, *Pf* infection was detected at any of these time-points in 21% (49/239) of the Mozambican women.

***Pf*-specific antibody profiles depend on parasite exposure in pregnancy**

Mean antiparasite IgG levels in pregnant Mozambican women delivering between 2010 and 2012 were above levels against bovine serum albumin (BSA) plus three SD and higher than IgG levels in pregnant Spanish women for all antigens except for DBL6Ɛ and three of the 25 VAR2CSA peptides (Fig. 1A and Table S2 and S3). Five VAR2CSA peptides, together with DBL6Ɛ and pCSP, were recognized by IgGs from 5% or more of the pregnant Spanish women never exposed to malaria (Fig. 1B), suggesting unspecific recognition, and were thus excluded from subsequent analysis. The decline in *Pf* infection rates assessed by qPCR in peripheral or placental blood of pregnant Mozambican women delivering in 2004-2005 and 2010-2012 (from 25% to 5%; Fig. 1C) (3) was mirrored by drops of IgG levels against ten (p1, p5, p8, p10 p12, p20, p27, p36, p38, p39) of the 18 VAR2CSA peptides previously selected (Fig. 1D and Table S4).

Acquisition and decay of IgG responses against VAR2CSA

Dynamics of IgG responses were assessed in a longitudinal cohort of 239 pregnant Mozambican women (Fig. 2A). Compared to uninfected women, the 49 (21%) women with a *Pf* infection during pregnancy had at delivery higher IgG levels against the ten down-selected peptides (Fig. 2B and Table S5). At delivery, seroprevalences against p1 (23%), p5 (26%), p8 (26%) and p39 (31%) were above the cumulative prevalence of *Pf* infection during pregnancy (Fig. 2C and Table S5). No difference in IgG levels was observed between Mozambican primigravid and multigravid women (Fig. 2D and Table S5). Time to double IgG levels (T_{2x}) in women experiencing a *Pf* infection during follow-up ranged from 0.45 years (95% confidence interval [CI] [0.31-0.80]) for p5 to 1.07 years (95%CI [0.60-5.23]) for p27 (Fig. 3E and Table S6). Half-life of IgGs ($T_{1/2}$) among seropositive women at recruitment without evidences of *Pf* infection during follow-up ranged from 0.55 years (95%CI [0.38-1.02]) for p8 to 3.66 years (95%CI [0.98-∞]) for p1 (Fig. 2E and Table S6). In the case of recombinant antigens, IgGs against DBL5Ɛ showed the lowest T_{2x} (0.31 years, 95%CI [0.21-0.61]) and $T_{1/2}$ (0.66 years, 95%CI [0.42-1.65]), while the highest T_{2x} and $T_{1/2}$ were observed for IgGs against AMA1 (1.76 years, 95%CI [0.76-∞] and 4.18 years, 95%CI [1.86-∞], respectively).

Among the down-selected VAR2CSA peptides (p1, p5, p8 and p39), IgGs against p5 (51 aminoacids) and p8 (48 aminoacids) showed the lowest half-lives (p8: 0.55 years, 95%CI [0.38-1.02]; p5: 1.33 years, 95%CI [0.65-∞]) and the largest increase in women exposed to *Pf* during pregnancy (n=49) compared to uninfected women (n=190; adjusted ratio $[AR]_{p5}$: 2.15, 95%CI [1.39-3.31] and AR_{p8}: 2.17, 95%CI [1.46-3.23]; Fig. 2B and Fig. S4). 3D modeling mapped both sequences on the exposed surface of DBL1X-ID1 region of VAR2CSA (Fig. 2F). Aminoacid variability of peptide sequences obtained from amplicon-based Sanger sequencing of 50 *Pf* isolates collected in Mozambique, Benin, Kenya and Gabon was 5% (SD 2) for p5 and 16% (SD 5) for p8, compared to the consensus peptide sequence included in the array (Fig. 2G, fig. S2 and S3).

Performance of selected VAR2CSA peptides to assess spatial and temporal *Pf* trends

In pregnant Mozambican women at delivery, p5 and p8 seroprevalences, as well as the composite of both (p5+8), decreased from 2004-2005 to 2010 (adjusted odds ratio $[AOR]_{p5+8}$: 0.27, 95%CI [0.11-0.68]), followed by an increase from 2010 to 2012 (AOR_{p5+8}: 2.49, 95%CI [1.34-4.61]; Fig. 3A and Table S7). This decrease and subsequent increase mirrored *Pf* prevalence by qPCR. HIV infection and parity did not modify the associations observed (p-value for interaction>0.05 in all cases; Table S7). Similar to *Pf* prevalence determined by qPCR, seroprevalences were the highest in HIV-uninfected women from Benin, followed by Gabon (AOR_{p5+8}: 0.31, 95%CI [0.21-0.47]) and Mozambique (AOR_{p5+8}: 0.21, 95%CI [0.16-0.28]; Fig. 3B and Table S8). Pregnant women living in an area from Tanzania where no *Pf* infection was detected by qPCR were seronegative at delivery against p5, p8 and p5+8 while 42% and 48% of them were seropositive against AMA1 and $MSP1_{19}$, respectively (Fig. 3B). Among HIV-infected women, seroprevalences of p8 and p5+8 were lower in Mozambique than in Kenya (AOR_{p5+8}: 0.58, 95%CI [0.38-0.88]; Fig. 3C and Table S8). p5 and p5+8 seroprevalences were higher in anemic than non-anemic women (AOR_{p5+8}: 1.26, 95%CI [1.03-1.55]; Fig. 3D and Table S9). Seroprevalences were lower in HIV-uninfected women who received IPTp with mefloquine (MQ) compared to those who received sulfadoxine-pyrimethamine (SP) (AOR_{p5+8}: 0.74, 95%CI [0.59-0.94]; Fig. 3E and Table S10). Seroprevalences in HIV-infected women were lower in women who received MQ compared with those who received placebo although differences were not statistically significant (AOR_{p5+8}: 0.76, 95%CI [0.50-1.15];Fig 3F and Table S10).

Geographical patterns of malaria transmission through VAR2CSA serology

Spatial geocoordinates were available from 698 pregnant Mozambican women residing in Manhiça District (Southern Mozambique). Geographical areas experiencing significantly higher seroprevalences than would be expected by chance were observed for p5 (radius=2.82 Km; p=0.024) and p5+8 (radius=1.06 Km; p=0.049; Fig. 4 and Table S11). The serological hotspot was identified far from the main road crossing the Manhiça village, and close to the Incomati river and the Maragra sugar cane plantations which are adjacent to the village. Seroprevalences were two to three times higher in pregnant women inside than in those outside the local clusters identified. The distribution of HIV infection, parity, age and IPTp treatment was similar in women inside and outside the serological hotspot (p>0.05; Table S12), thus excluding unbalanced distribution of susceptible pregnant women as a cause of the hotspots observed. Women who were seropositive for p8 as well as those with active *Pf* infection detected by microscopy or qPCR at delivery, tended to localize in the same region although statistical significance was not reached. Hotspots of $MSP1_{19}$ and AMA1 seropositive women were not observed due to high prevalence of seroresponders (88% and 95%, respectively; Fig. 4 and Table S11).

DISCUSSION

Routine *P. falciparum* testing of easily accessible pregnant women at maternal clinics has the potential to offer a rapid, consistent and cost-effective method to evaluate malaria burden in different communities and to track progress of interventions. IgGs against two VAR2CSA peptides, selected based on their ability to maximize the information on recent *Pf* exposure in pregnancy, reflected differences in malaria burden over time and space in multiple African scenarios, as well as changes in parasite rates associated with the use of different preventive regimens. Overall, our results point that in areas with high attendance to maternal health services this pregnancy-specific serology may serve as a useful sentinel surveillance tool for risk stratification as well as to flag changes in malaria burden and progress in the path towards elimination.

p5 (51 aminoacids) and p8 (48 aminoacids) are localized in DBL1X domain and ID1 region of VAR2CSA, respectively. Limited diversity of p5 sequence (5%) was observed in *Pf* isolates from a variety of African regions, in accordance with estimates from previous studies for the DBL1X domain (25). p8 corresponds to a more diverse (16%) variant of the ID1 region in

VAR2CSA (26). Both peptides are exposed on the N-terminal region of VAR2CSA protein (DBL1X-ID1), a domain previously reported to be accessible to naturally-acquired antibodies (27) and to contain immuno-dominant epitopes driving humoral immunity (19). In accordance with this localization on the surface of VAR2CSA and its exposure restricted to pregnancy (10, 20), our results show that both peptides are recognized by IgGs from malaria-exposed pregnant women at levels above the recognition by pregnant Spanish women and Mozambican men (20). IgG responses against both VAR2CSA peptides increased upon *Pf* infection in pregnancy, with two-fold higher levels among women infected by *Pf* compared to uninfected women. Moreover, p5 and p5+8 seroresponders were at higher risk of anemia than seronegative women, supporting the concept that antibodies against these VAR2CSA peptides are markers of recent infection, which impacted adversely the health of the women (3). In contrast to slow decay of IgG responses against AMA1, which persisted for more than four years, half-life of IgGs against p5 and p8 was below two years, the average time reported in Mozambique for a second pregnancy to occur (25). The short half-life of IgGs against p5 and p8, together with the observation of similar IgG levels in multigravid and primigravid women, suggests that antibodies acquired in one pregnancy are not maintained over multiple pregnancies and can be used as a reliable indicator of recent exposure in pregnant women of all parities.

Seroprevalences to p5, p8 and the composite of both peptides (p5+8) were able to mirror trends in *Pf* prevalence among pregnant Mozambican women delivering between 2004 and 2012 (3), a temporal pattern which was also observed in the $PfPR_{2-10}$ (24). Temporal trends were similar among HIV-uninfected and infected women, suggesting that impairment of *Pf*-specific antibody responses driven by viral infection (28) may not affect short-lived IgG responses against p5 and p8. Seroprevalence also reflected the burden of malaria among pregnant women residing in a variety of African settings, as well as reductions in infection rates resulting from the use of MQ as IPTp among HIV-uninfected women (23). Similar trends, although not statistically significant, were observed among HIV-infected women receiving cotrimoxazol prophylaxis alone or in combination with MQ (22), possibly due to the longer duration of protection provided by three IPTp doses in HIV-infected women compared to the two doses in HIV-uninfected women. Finally, pregnant women living in an area from Tanzania where no *Pf* infection was detected by qPCR as well as pregnant Spanish women never exposed to malaria were seronegative against p5 and p8, suggesting that pregnancy-specific serology might be used to confirm the eventual interruption of transmission.

Geographical distribution of seropositives to p5, p8 and p5+8 among pregnant Mozambican women revealed a serological hotspot in an area close to the river and sugar cane plantations where the density of anopheline mosquito can be expected to be higher. In contrast, antibodies against $MSP1_{19}$ and AMA1 were not able to identify these malaria transmission patterns due to saturation of antibody responses after lifelong exposure to *Pf*. Although acute *Pf* infections detected by microscopy or qPCR were concentrated in the same region than the serological hotspot, numbers were not enough to reach statistical significance. Pregnancy-specific serology may increase the strength of evidence of exiting hotspots at very low transmission (2). These results support the value of VAR2CSA serology to amplify signals of recent exposure and suggest their potential to trigger targeted interventions to individuals living in close proximity to passively detected seropositive pregnant women.

This study has several limitations. First, the peptide array used may have missed some conformational non-linear epitopes. Second, persistence of IgGs against VAR2CSA was assessed among pregnant Mozambican women after substantial declines in malaria burden (3). However, different transmission dynamics and host genetic factors may affect the frequency and duration of infections and thus the dynamic of antibodies (16). Third, steeper decay of antibodies may be observed out of pregnancy when infecting parasites express non-VAR2CSA variants. Fourth, small numbers of pregnant women from malaria-free areas in Tanzania and Spain limit the generalizability of our data to support p5 and p8 serology as a tool to confirm interruption of transmission. Finally, antibody assessments in this study were mainly conducted at delivery, and further studies need to assess the performance of this serology at antenatal visits or soon after delivery (i.e., during infant immunization).

In summary, this study shows that IgGs against two VAR2CSA peptides from the DBL1X-ID1 domain reveal temporal and spatial differences in malaria burden, as well as reductions in exposure associated with the use of preventive measures during pregnancy, and allow the identification of local clusters of transmission that are missed by detection of *Pf* infections. Inferring recent exposure in readily accessible women at highly-attended maternal health centers would allow amplifying signals of ongoing malaria transmission and increase the power to detect changes, either natural or driven by deliberate efforts, as well as hotspots in areas of low endemicity (2). Moreover, peptides such as p1 targeted by long-lasting IgG responses may be useful to capture past changes in transmission by sampling women of child-

bearing age and relating seroprevalence with the number and timing of previous pregnancies. Development of operationally suitable serological tests (29) capable to detect antibodies against VAR2CSA synthetic peptides may be used in programmatic environments to stratify areas based on the burden of malaria, measure the impact of interventions and document year-to-year changes in transmission. Further validation studies are needed to describe the relationship between pregnancy-specific serological data and malaria transmission in the general population, as well as its value to confirm interruption of malaria transmission (30) and to provide early signals of *Pf* resurgence after local elimination.

MATERIALS AND METHODS

Study design, population and procedures

Pregnant women who provided written informed consent to participate in three IPTp randomized clinical trials evaluating the efficacy and safety of antimalarial drugs between 2003 and 2005 in Mozambique (NCT00209781) (21) and between 2010 and 2012 in Mozambique, Benin, Gabon, Kenya and Tanzania (NCT00811421) (22, 23) were included in the study. Participants were recruited at the first antenatal visit and all received a long-lasting insecticide treated bednet, as well as IPTp. Women from 2003–2005 received two doses of SP (21) and women from 2010–2012 received two doses of MQ or SP if they were HIV-uninfected (23), and three doses of MQ or placebo plus daily cotrimoxazol prophylaxis if they were HIV-infected (22). At delivery, tissue samples from the maternal side of the placenta, as well as 50 µl peripheral and placental dried blood spots (DBS) were collected. Peripheral and placental blood from pregnant Mozambican and Beninese women were also collected into EDTA vacutainers and centrifuged, with the plasma stored at −20°C. From a subset of pregnant Mozambican women delivering between 2011 and 2012, peripheral blood samples were also collected at first antenatal visit and before administration of the second IPTp dose. Households of Mozambican women were geocoded using a global information system. Clinical malaria episodes were treated according to national guidelines at the time of the study. DBS and plasma samples were also collected from 49 pregnant women never exposed to *Pf* who delivered in 2010 at the Hospital Clinic of Barcelona. The study was approved by the Ethics Committees from the Hospital Clínic of Barcelona, the Comité Consultatif de Déontologie et d'Éthique from the Institut de Recherche pour le Développement (France), the

Centers for Disease Control and Prevention (USA), and National Ethics Review Committees from each malaria endemic country participating in the study. Written informed consent was obtained from all the participants, which included permission to conduct testing for immune markers using stored biological samples.

Laboratory determinations

HIV serostatus at recruitment was assessed using rapid diagnostic tests following national guidelines and hemoglobin at delivery was determined in capillary blood sample using mobile devices (HemoCue, Hemocontrol and Sysmex KX analyzer). Thick and thin blood films, as well as placental biopsies, were read for *Plasmodium* species detection according to standard, quality-controlled procedures (3). Blood onto filter papers was tested for the presence of *Pf* in duplicate by means of a qPCR assay targeting 18S ribosomal DNA (3).

Antibody measurements

IgGs from plasma (Benin and Mozambique) or DBS (Gabon, Kenya and Tanzania) were measured using the xMAP^{TM} technology and the Luminex® 100/200™ System (Luminex® Corp., Austin, Texas) in a 40% random selection of the pregnant women participating in the clinical trials. Two multiplex suspension array panels were constructed (20), one including *Pf* recombinant proteins (DBL3X, DBL5Ɛ and DBL6Ɛ, AMA1 and $MSP1_{19}$, from 3D7 strain), and another consisting of synthetic peptides (25 VAR2CSA peptides covering conserved and semi conserved regions of VAR2CSA [Table S2] and pCSP) (20). BSA was used in both arrays to assess unspecific IgG recognition (20). Peptides were synthetized by Gl Biochem (Xangai, China) and median purity was estimated as 79% (range: 71-91%) by high performance liquid chromatography (HPLC) and mass spectrometry. The recombinant proteins were all produced at ICGEB, New Delhi, India. *Clostridium tetani*, tetanus toxin was purchased from Santa Cruz Biotechnology (Dallas, Texas) to control for the amount of IgGs eluted from DBS. Procedures for reconstitution of DBSs and quality control, bead-based immunoassay, data normalization and definition of seropositivity cutoffs are described in SI Methods.

***var2csa* sequencing and 3D protein modeling**

DNAs extracted from 50 DBS that were *Pf* positive by qPCR were used for Sanger sequencing of *var2csa* PCR amplification products covering peptides of interest (SI Methods). Sequence variability with respect to the peptide included in the array was assessed after aminoacid

alignment. The 3D-structure of DBL1X-ID1 was obtained by submitting the 3D7 sequence, with domain limits previously defined (25), to the HHPred server (http://toolkit.tuebingen.mpg.de/hhpred). The structure with highest HHPred score, corresponding to the DBL1α domain of the VarO strain (Protein Data Bank [PDB] 2yk0 (31)), was selected for homology modeling in MODELLER based on the default alignment. Molecular graphics were generated in UCSF Chimera version 1.5.3. (32).

Definitions and statistical analysis

Pregnant women were included in the analysis if all information was available on IPTp treatment, date of delivery, HIV status, age, parity and antibody responses. Women were classified as primigravid (first pregnancy) and multigravid (at least one previous pregnancy). Age was categorized as younger than 20 years, 20 to 24 years, or 25 years of age or older (13). Anemia was defined as hemoglobin level at delivery below 11 mg/l. Malaria episodes were defined on the basis of a *Pf*–positive blood smear and an axillary temperature ≥37.5°C and/or fever reported within the previous 24 hours. Proportions were compared by means of Fisher's exact test. We estimated linear and logistic regression models to evaluate the association of log-transformed IgG levels and seropositivity with study periods (2004–2005 and 2010–2012) and country, *Pf* infection, parity, anemia and IPTp intervention. Models were adjusted by age, parity, HIV, IPTp drug and country when applicable. The modification of the associations by HIV infection or parity was assessed by including interaction terms into the regression models. To control the false discovery rate in the selection of antigens, adjusted p-values (q values) were computed using the Simes procedure (33). The fixed-effect slope component of the multilevel mixed-effect linear regression analysis of the longitudinal cohort of pregnant Mozambican women was used to estimate the time in years to double antibody levels (T_{2x}) in women experiencing a *Pf* infection during follow up, and half-life ($T_{1/2}$) in seropositive women at recruitment with no evidence of *Pf* infection at follow-up (SI Methods). Statistical analyses were performed with Stata/SE software (version 12.0; StataCorp), and Graphpad Prism (version 6, Graphpad, Inc).

Geospatial analysis

Spatial hotspots of *Pf* infection and seropositivity among pregnant Mozambican women living in Manhiça district were designated using hierarchical cluster analysis with Ward's minimum variance method (34). Kulldorff spatial scan method (35-37) was used to identify the most likely hotspot for *Pf* infection and seropositivity, setting our significance threshold (alpha) as

0.05. Hotspots of greater size than one cluster were identified by consecutively aggregating nearest-neighboring areas until a proportion of the total study population was included. In order to ascertain statistical significance, we employed Monte Carlo sampling, using Poisson likelihoods, and following the original Kulldorff method for identification of clusters (38). Analysis were performed using the R statistical software (version 3.2.1) (39) and maps were generated using OpenStreetMap (40). The key R packages used were SpatialEpi (41), deldir (42), geosphere (43), rgeos (44), raster (45) and leaflet (46).

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FIGURE LEGENDS

Fig. 1. IgGs against *P. falciparum* VAR2CSA in malaria exposed and non-exposed pregnant women. (**A**) nMFIs measured in pregnant Mozambican and Spanish women. Red dashed line represents the mean nMFI from BSA plus three standard deviations (SD) and black lines the seropositivity cutoffs. (**B**) Seroprevalence among pregnant Spanish (blue) and Mozambican women (black). Asterisks indicate antigens recognized by pregnant Mozambican women at levels above IgGs against BSA plus three SD and above levels in pregnant Spanish women (q-value <0.05 by Simes procedure), as well as those antigens poorly recognized by pregnant Spanish women (seroprevalence<5%). Prevalence of *P. falciparum* infection in peripheral and placental blood by qPCR (**C**) and nMFIs (**D**) among pregnant Mozambican women delivering in 2004-2005 and 2010-2012. Red lines represent the geometric mean and T-bars the 95%CI. Asterisks indicate antigens recognized by IgG whose levels dropped between 2004 and 2012, as assessed by linear regression adjusted by IPTp treatment, parity, age and HIV (q-value <0.05 by Simes procedure).

Fig. 2. IgG responses during pregnancy against selected VAR2CSA antigens and polymorphism in target sequences. (**A**) *P. falciparum* prevalence by qPCR in 239 pregnant Mozambican women at recruitment, second IPTp administration and delivery. Cumulative prevalence at delivery refers to peripheral or placental infection detected by microscopy, qPCR or histology at any time-point. (**B**) Ratio of nMFIs at delivery in Mozambican women infected during pregnancy compared with uninfected women. (**C**) Seroprevalence at delivery, showing the cumulative prevalence of infection during pregnancy (red-dashed line) and the prevalence at delivery by qPCR (light-blue line). (**D**) Ratio of nMFIs at delivery in multigravid compared with primigravid women, adjusted by IPTp, parity, age and HIV. T-bars correspond to 95%CI. (**E**) IgG dynamics during pregnancy with estimates of time to double (T_{2x}) and half-life ($T_{1/2}$) obtained from linear mixed-effect regression model. Red points represent *P. falciparum* infection, dark-grey lines the seropositivity cutoff, red lines the fitted-estimation and dashed lines the 95%CI. (**F**) Space-feeling representation of DBL1X-ID1 showing p5 (blue) and p8 (red). (**G**) Logo representation of p5 and p8 sequences obtained from 50 *P. falciparum* isolates (20 from Mozambique, 10 from Benin, 10 from Gabon and 10 from Kenya).

Fig. 3. IgG seroprevalence against VAR2CSA selected antigens according to study period, country, anemia status and intermittent preventive treatment group. (**A**) Pregnant Mozambican women delivering in 2004-2005, 2010, 2011 or 2012; (**B**) HIV-uninfected pregnant women from Benin (B), Gabon (G), Mozambique (M) and Tanzania (T); (**C**) HIV-infected pregnant women from Kenya (K) and Mozambique. (**D**) Non-anemic (NA) and anemic (A) pregnant women. (**E**) HIV-uninfected pregnant women receiving mefloquine (MQ) or sulfadoxine-pyrimethamine (SP) as IPTp and (**F**) HIV-infected pregnant women receiving MQ or placebo (PL) as IPTp. Maternal microscopic infection was defined by the presence of *P. falciparum* parasites in peripheral blood or in placenta on microscopic or histologic examination, respectively. Maternal qPCR-positive infection was defined by a positive result on qPCR testing in peripheral or placental blood. P-values were obtained from multivariate regression models adjusted for HIV, parity, age, IPTp treatment and country when applicable (* and ** indicate crude and adjusted p-values <0.05, respectively). T-bars represent 95%CI. N/E, Not estimated due to low prevalence of *P. falciparum* infection; OM, Optical microscopy.

Fig. 4. Geographical patterns of *P. falciparum* infection and IgG seropositivity in pregnant women living in southern Mozambique. Geographical distribution of (sero)positives among pregnant Mozambican women (HIV-uninfected and -infected) living in Manhiça District who delivered between 2010-2012 with microscopy, qPCR and spatial geocoordinates available. Distribution of pregnant women with and without *P. falciparum* infection at delivery, either in peripheral or in placental blood, detected by microscopy or histology (**A**), or by qPCR (**B**). Distribution of AMA1 (**C**), $MSP1_{19}$ (**D**), p5 (**E**), p8 (**F**) and p5+8 (**G**) seropositive and seronegative pregnant women at delivery. Grey dots indicate (sero)negatives, blue dots indicate (sero)positives, red dots indicate (sero)positives selected by the hotspot cluster algorithm, red circle indicate the most likely hotspot (continuous and dashed line if p-value<0.05 or >0.05, respectively). N/E, Not estimated due to high prevalence of seroresponders. Maps were generated using the OpenStreetMap.

FIGURES

Figure 1.

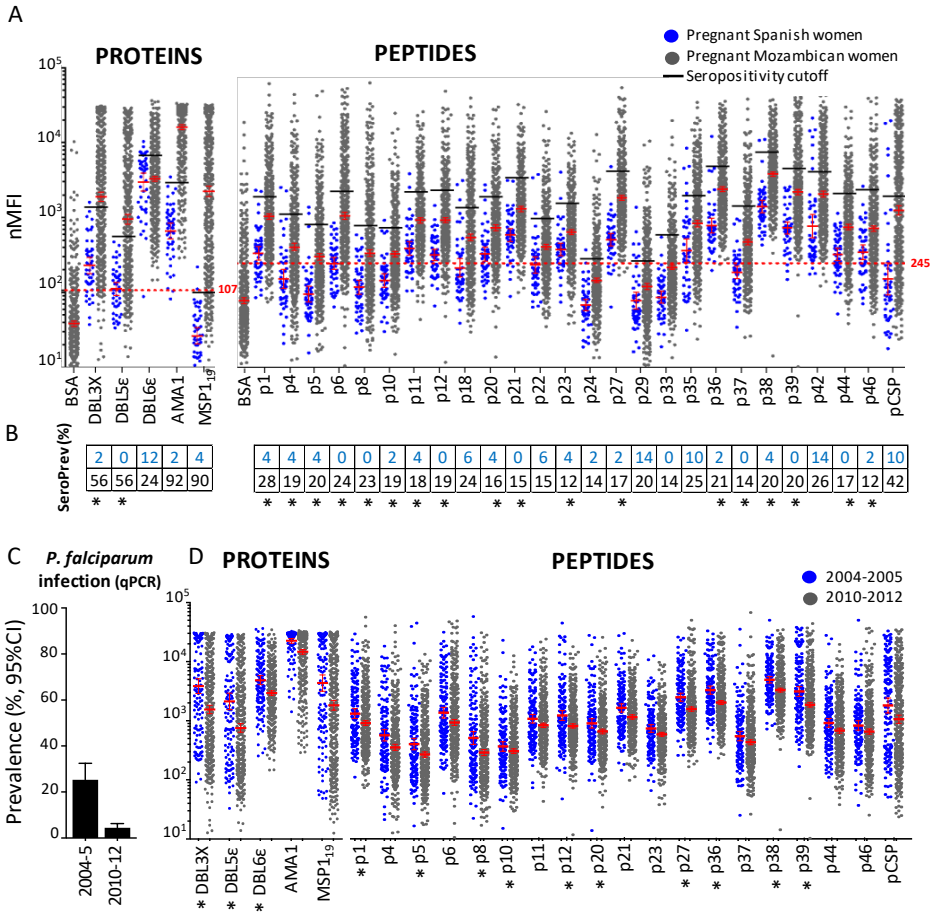

Figure 2.

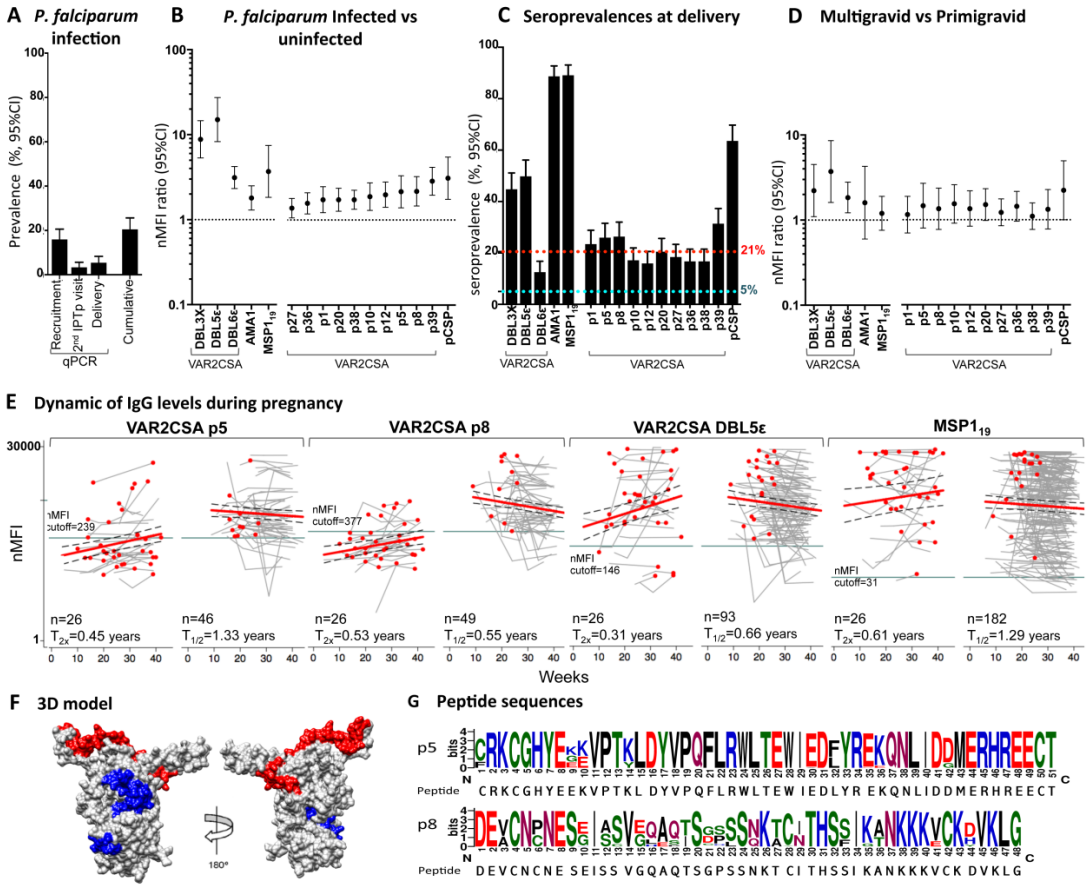
A P. falciparum infection
Prevalence (%, 95%CI)
Recruitment
2nd IPTp visit
Delivery
Cumulative
qPCR
B P. falciparum Infected vs uninfected
nMFI ratio (95%CI)
DBL3X
DBL5ε
DBL6ε
AMA1
MSP1 19
p27
p36
p1
p20
p38
p10
p12
p5
p8
p39
pCSP
VAR2CSA
C Seroprevalences at delivery
seroprevalence (%, 95%CI)
21%
5%
D Multigravid vs Primigravid
E Dynamic of IgG levels during pregnancy
VAR2CSA p5
VAR2CSA p8
VAR2CSA DBL5ε
MSP1 19
nMFI
cutoff=239
cutoff=377
cutoff=146
cutoff=31
n=26
T2x=0.45 years
n=46
T1/2=1.33 years
n=26
T2x=0.53 years
n=49
T1/2=0.55 years
n=26
T2x=0.31 years
n=93
T1/2=0.66 years
n=26
T2x=0.61 years
n=182
T1/2=1.29 years
Weeks
F 3D model
180°
G Peptide sequences
p5
Peptide
CRKCGHYEEKVPTKL DYVPQFLRWLTEW IEDLYR EKQNLIDDMERHREECT
p8
Peptide
DEVCNCNE SEISS VGQAQTSGPSSNKT CI THSSI KANKKKVCK DVKLG

Figure 3.

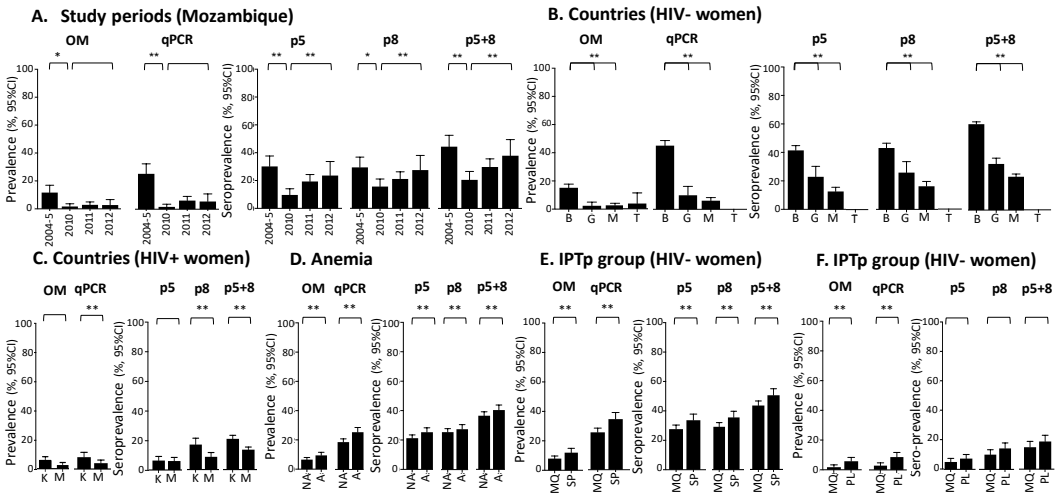
A. Study periods (Mozambique)
B. Countries (HIV- women)
C. Countries (HIV+ women)
D. Anemia
E. IPTp group (HIV- women)
F. IPTp group (HIV- women)
OM
qPCR
p5
p8
p5+8
Prevalence (%, 95%CI)
Seroprevalence (%, 95%CI)
2004-5
2010
2011
2012
B G M T
K M
NA A
MQ SP
MQ PL

Figure 4.

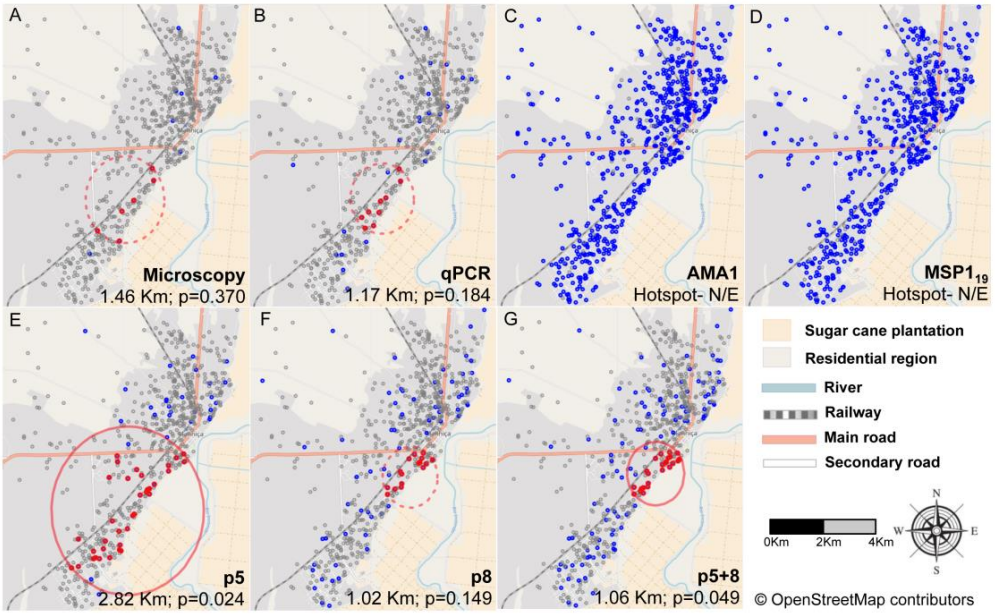
A
Microscopy
1.46 Km; p=0.370
B
qPCR
1.17 Km; p=0.184
C
AMA1
Hotspot- N/E
D
MSP1$_{19}$
Hotspot- N/E
E
p5
2.82 Km; p=0.024
F
p8
1.02 Km; p=0.149
G
p5+8
1.06 Km; p=0.049
Sugar cane plantation
Residential region
River
Railway
Main road
Secondary road
0Km
2Km
4Km
N
W
E
S
© OpenStreetMap contributors

Table 1. Study participants by country and HIV status.

	HIV-uninfected					HIV-infected		
	2004-2005	2010-2012				2004-2005	2010-2012	
	Mozambique	Mozambique*	Benin	Gabon	Tanzania	Mozambique	Mozambique*	Kenya
Variable	N=65	N=485	N=854	N=131	N=31	N=81	N=362	N=296
Parity, n(%)[†]								
Primigravidae	17 (26)	181 (37)	188 (22)	38 (29)	16 (52)	28 (35)	46 (13)	22 (7)
Multigravidae	48 (74)	304 (63)	666 (78)	93 (71)	15 (48)	53 (65)	316 (87)	274 (93)
Age, n(%)[‡]								
<20	19 (29)	181 (37)	86 (10)	42 (32)	5 (16)	27 (33)	41 (11)	15 (5)
20-24	17 (26)	123 (25)	281 (33)	45 (34)	14 (45)	26 (32)	84 (23)	96 (32)
≥ 25	29 (45)	181 (37)	487 (57)	44 (34)	12 (39)	28 (35)	237 (65)	185 (62)
IPTp, n(%)								
Sulfadoxine-pyrimethamine	65 (100)	151 (31)	288 (34)	55 (42)	11 (35)	81 (100)	0 (0)	0 (0)
Mefloquine	0 (0)	334 (69)	566 (66)	76 (58)	20 (65)	0 (0)	178 (49)	139 (47)
Placebo[§]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	184 (51)	157 (53)
Microscopy, n(%)[¶, ǁ]								
Positive	9 (14)	13 (3)	110 (15)	3 (2)	1 (3)	8 (10)	8 (2)	15 (5)
Negative	56 (86)	468 (97)	616 (85)	125 (98)	30 (97)	73 (90)	323 (98)	268 (95)
qPCR, n(%)[#, ǁ]								
Positive	16 (25)	28 (6)	332 (46)	9 (10)	0 (0)	21 (26)	13 (4)	22 (8)
Negative	49 (75)	424 (94)	393 (54)	80 (90)	31 (100)	60 (74)	314 (96)	251 (92)

IPTp, intermitent preventive treatment during pregnancy; qPCR, quantitative polimerase chain reaction.

*40% (196/485) of HIV-uninfected and 12% (43/362) of HIV-infected were pregnant women with samples collected also at recruitment and second IPTp administration.

[†]Women were classified as primigravid (first pregnancy) and multigravid (at least one previous pregnancy).

[‡] Age was categorized as younger than 20 years, 20-24 years, or 25 years or older.

[§]All HIV-infected women who received placebo were under cotrimoxazol prophylaxis.

[¶]Maternal microscopic infection defined by the presence of *P. falciparum* parasites in peripheral blood or in placenta on microscopic or histologic examination, respectively.

[#]Maternal qPCR-positive infection was defined by a positive result on qPCR testing in peripheral or placental blood.

[ǁ]Not determined: 179 microscopy and 196 qPCR.

Supplementary Information for

Pregnancy-specific serology reveals temporal and spatial *P. falciparum* transmission patterns

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This PDF file includes:

Supplementary text
Figs. S1 to S4
Tables S1 to S12
References for SI reference citations

Supplementary Information Text

Materials and Methods

Bead-based immunoassay. Two multiplex suspension array panels were constructed to quantify IgG responses against *P. falciparum* recombinant proteins and synthetic peptides, using the xMAP™ technology and the Luminex® 100/200™ System (Luminex® Corp., Austin, Texas). MagPlex® microspheres (magnetic carboxylated polystyrene microparticles, 5.6 μm) with different spectral signatures were selected for each protein (DBL3X, DBL5Ɛ, DBL6Ɛ, AMA1 and $MSP1_{19}$), peptide (25 VAR2CSA peptides and pCSP), tetanus toxin and bovine serum albumin (BSA). Antigens were covalently coupled to beads following a modification of the Luminex® Corporation protocol (1). Briefly, 200 μl of beads ($2{,}5x10^6$) were transferred into a 1.5 mL eppendorf tube and resuspended by sonication and vortexing. The supernatant was removed after precipitation of the beads by magnetic separation during 60 seconds. Beads were washed twice with 250 μl of distilled water and pellets were resuspended in 80 μl of activation buffer (0.1 M NaH2PO4, pH 6.2). Sulfo-NHS (N-hydroxysulfosuccinimide) and EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; Pierce, Thermo Fisher Scientific Inc., Rockford, IL) were simultaneously added to reaction tubes at 5 mg/mL each in activation buffer, and reaction tubes were incubated at room temperature with gentle agitation, protected from light for 20 minutes. Activated beads

were washed twice with 250 µl of coupling buffer (MES 50 mM, 2-[N-morpholino] ethanesulfonic acid monohydrate pH 5, Sigma-Aldrich). Antigen amounts in the coupling reaction for one million beads were determined after a titration experiment, and were as follows: 2 µg of tetanus toxin, 4 µg of DBL3X, DBL5Ɛ, DBL6Ɛ, AMA1, 8 µg of $MSP1_{19}$, 170 µg of each peptide and for BSA a 1% solution in PBS (Phosphate-buffered saline) and volume was adjusted with coupling buffer until a maximum of 400 µl. Beads and antigens were vortexed, sonicated and then incubated over night at 4ºC in the dark, with shaking. Coupled beads were blocked with 500 µl of 1% BSA in PBS for 30 minutes on a shaker at room temperature, avoiding light and then washed twice with 500 µl of assay buffer (1% BSA, 0.05% sodium azide in PBS filtrated) and resuspended in 400 µl of the same buffer. Beads were quantified on a Guava PCA desktop cytometer (Guava, Hayward, CA), and stored at 4°C in the dark. Protein and peptide multiplex arrays were prepared by pooling together equal volumes of coated beads.

Immediately before use, stock suspensions of antigen-coated microspheres were thoroughly resuspended by vortexing and sonication. Frozen plasma or the product of DBS elution were thawed at room temperature, mixed by vortexing, and spun at 16000 g for 5 minutes to remove particles. 50 µl of diluted microspheres (1000 microspheres/analyte/well) were added to a 96-well Mylar flat-bottom plate following the addition of 50 µl of diluted sample in duplicates to a final concentration of 1:400 for protein array and 1:100 for peptide array and incubated for 1 hour in agitation, protected from light at room temperature. After incubation, the plates were washed three times with 200 µl of washing buffer (0.05% Tween 20 in PBS) by pelleting in a magnetic 96 well separator. 100 µl of biotinylated anti-human IgG (Sigma, Tres Cantos, Spain) diluted 1:1000 in assay buffer was added to each well, and plates were incubated for 45 minutes in agitation, protected from light at room temperature. After the incubation period, the plates were washed and 100 µl of streptavidin-conjugated R-phycoeryhtrin (Invitrogen, Carlsbad, CA) at a 1:1000 dilution in assay buffer was added and incubated for 25 minutes. Finally the plates were washed as before and the beads were resuspended in 100 µl of assay buffer and analyzed using the Luminex® 100/200™ System.

A hyperimmune plasma pool composed by 23 plasmas from *P. falciparum* infected Mozambican pregnant women was tested to determine if the coupling was effective and was included in each assay plate as positive control, in addition to blanks (wells without sample) to assess background levels. A minimum of 50 microspheres were read per spectral signature and results were exported as crude median fluorescent intensity (MFI). Duplicates were averaged and background MFIs were subtracted. A total of 224 plates were analyzed and the intra-assay variation (mean CV of replicates from 20 plasma samples per plate) ranged from 1.4% to 7.3% for the protein array and from 2.5% to 12.4% for the peptide array. The inter-assay variation (variability of positive pool between 224 plates) was 5% for the protein array and 26% for the peptide array (1). Results were normalized (nMFI) to account for plate-to-plate variation by dividing the background subtracted MFI of each sample by the value of the positive pool in the same plate and multiplying by the median of positive pools in all plates.

Definition of IgG seropositivity. Seropositivity cutoffs were obtained using finite mixture models (FMM) for pregnancy-specific malaria antigens (VAR2CSA peptides and recombinant domains). FMM can be applied in scenarios of heterogeneous IgG distributions where a subgroup of the study population is seronegative and another seropositive avoiding additional sampling of malaria never exposed pregnant women. However, seropositivity cutoffs to general malaria antigens (AMA1, $MSP1_{19}$ and pCSP) were obtained from means plus three standard deviation (SD) of IgG levels from never exposed pregnant women as a consequence of absence of heterogeneous IgG distribution against *P. falciparum* general antigens in malaria exposed adult pregnant women (1).

Reconstitution of dried blood spots. Antibodies were eluted from a total of 880 DBS from Gabon (n=310), Kenya (n=408) and Tanzania (n=162) as previously described (1, 2). Briefly, four spots of approximately 3 mm in diameter were cut from the filter papers using a punch (McGill® round punch, 3 mm) and transferred to individual wells of a 96-well polystyrene U-bottom plate. Antibodies were eluted with 200 µl Luminex® assay buffer (1% BSA, 0.05% sodium azide in filtrated PBS) at room temperature overnight with gentle mixing which, assuming a hematocrit of 50%, gives a concentration of eluted blood proteins equivalent to a 1:50 plasma dilution (2, 3).

To assess the quality of the elution, hemoglobin levels in the eluted DBS were measured by spectrophotometry (wavelengths 415, 380 and 450) and calculated using the Harboe method with the Allen correction (Hb [mg/l] = 167.2 x A415 – 83.6 x A380 – 83.6 x A450) x dilution factor). Three criteria to discard DBS improperly eluted were followed as previously described (1). First, 259 reddish-brown spots against a pale background were discarded after visual examination of reconstituted spots (2). Second, 10 DBS were also discarded because hemoglobin levels measured in the elutions were below the upper quartile of hemoglobin value among samples considered with inappropriate visual aspect (hemoglobin upper quartile = 7.4m/l). Finally, 153 samples were also discarded because anti-tetanus antibodies measured in the elutions were below the lowest quartile obtained from anti-tetanus IgG among samples with appropriate visual aspect and hemoglobin levels (anti-tetanus lowest quartile = [11563,5 nMFI]). Finally, 458/880 samples (131 from Gabon, 296 from Kenya and 31 from Tanzania) were considered as correctly eluted.

Sequencing of *var2csa* p5 and p8 in *P. falciparum* isolates. A total of 50 *P. falciparum* isolates collected on filter paper (Whatman 903) from infected individuals in Mozambique (n=20), Benin (n=10), Gabon (n=10) and Kenya (n=10) were selected for DNA sequencing. A half of the filter paper containing a 25 µL of blood drop was used for DNA extraction using a QIAamp DNA Mini kit (Qiagen), as per the manufacturer's instructions. Finally, DNA was eluted in 100 µL of AE buffer given in the kit. The presence of *P. falciparum* infection was detected using a previously described method (4, 5).

We designed a single polymerase chain reaction (PCR) based assay to amplify purified DNA templates using 2720 Thermal Cycler (Applied Biosystems) followed by Sanger sequencing for *var2csa* gene. In brief, a 25 µl reaction was set up, containing 0.5 µM of each forward (p5_F-

5'aaggtgtgggaagttattac-3') and reverse (p8_R- 5'attagttaaagatgcaagtact-3') primers, 1x HOT FirePol Master Mix (Solis BioDyne; Cat. No. 04-27-00125) and 5 µl of template DNA. The reaction volume was raised by PCR-grade water. The template DNA was denatured at 95°C for 15 minutes in a thermocycler, followed by 35 cycles of amplification (95°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute) and a final extension at 72°C for 10 minutes. A reaction using 5 µl of PCR-grade water instead of template DNA was included as a negative control. PCR products were run on 1.5% agarose (Invitrogen) gels in 1× TBE buffer (Tris/Borate/EDTA; Thermo Fisher Scientific) to determine the presence and size of the amplified DNA. PCR products were visualized using a ultraviolet trans-illuminator. The PCR primer set was also tested with human gDNA to check their specificity. The expected size of the PCR was 960 bp covering amino acid positions 220 to 539 of *var2csa* gene. PCR products were quantified using EPOCH Biotech system. Approximately 1200 ng of PCR products were sent to Genewiz, following safety instructions for the accurate shipment of PCR amplicons. In order to sequence the p5 and p8 fragments of *var2csa* gene, three new sequencing primers were used (Seq_p5_R - 5'ccatttcttcacacattcac-3'; Seq_p8_F - 5'gggtgatccttatttcgcagaa-3'; Seq_p8_R- 5'cgcaagaaatcttggcaacaaca-3') along with PCR p5_F primer. This allowed us to sequence 960 bp *var2csa* amplicons in both directions. The bi-directional sequencing with PCR p5_F and Seq_p5_R, and Seq_p8_F and Seq_p8_R primer sets covered 220-335 and 389-499 amino acids respectively.

The variations in the test sequences were identified by sequence alignment (Blastn, NCBI:https://blast.ncbi.nlm.nih.gov) against reference sequence of 3D7 (PF3D7_1200600) retrieved from PlasmoDB. The nucleotide sequences obtained from field isolates were translated using ExPASy online tool (http://web.expasy.org/translate/) and represented as a logo figure using the Weblogo Version 2.8.2 online tool (http://weblogo.berkeley.edu).

IgG dynamic analysis through mixed-effects regression models. Summary statistics according to the longitudinal design were calculated by trimester. Time-at-risk was estimated using gestational age at recruitment as the time when subjects first came under observation, and gestational age at delivery as the latest time under which the subjects were both under observation and at risk. Antibody levels were analyzed assuming a lognormal distribution, and therefore they were described by the geometric means and the overall, between and within-subjects standard deviations.

The crude and adjusted effect of *P. falciparum* infection on antibody levels was analyzed using log-linear mixed-effects regression models incorporating Gaussian random intercepts. This resulted in an estimate of the rates of antibody dynamics (boosting or decay), assuming a single exponential model. Time to twofold increase were calculated from the estimated rates and the boundaries at 95% confidence interval obtained from mixed-effects models for women with *P. falciparum* infection at follow-up (6, 7). Similarly, half-lives were calculated from models including women who were seropositive at recruitment with no *P. falciparum* infection at follow-up (6, 7). Where the boosting rate is a negative value (rate below 1) or the decay rate is a positive value (rate above 1), the calculated time to twofold increase or half-life was reported

as infinity. Statistical comparisons were performed at two-sided significance level of 0.05 and 95% Confidence Intervals were calculated for all estimations.

Consider our longitudinal dataset consisting of antibody measurements of 239 pregnant women on three successive gestational ages. Because we were not really interested in these particular 239 women per se, we treated them as a random sample from a larger population and modeled the between-woman variability as a random effect, as a random-intercept term at the woman level. We thus fitted the model:

$$\ln(C_{ij}) = \beta_0 + \beta_1 T_{ij} + \upsilon_j + \varepsilon_{ij}$$

where C_{ij} are the concentrations for i = 1 .. 3 measurement of gestational age (T) and j = 1 ... 239 women. The fixed portion of the model, $\beta_0 + \beta_1 T_{ij}$, simply states that we wanted one overall regression line representing the population average. The random effect υ_j serves to shift this regression line up or down according to each woman. Back-transforming the measurements to the original scale we obtain the following overall regression line:

$$C_{ij} = e^{\beta_0 + \beta_1 T_{ij}} = e^{\beta_0} e^{\beta_1 T_{ij}} = C_0 e^{\beta_1 T_{ij}}$$

where C_0 is the baseline concentration. We can estimate the time required to obtain a value Δ times C_{ij}

$$\Delta C_{ij} = C_0 e^{\beta_1 (T_{ij} + t_\Delta)} = C_0 e^{\beta_1 T_{ij}} e^{\beta_1 t_\Delta} = C_{ij} e^{\beta_1 t_\Delta}$$

and therefore

$$\Delta = e^{\beta_1 t_\Delta}$$
$$\ln(\Delta) = \beta_1 t_\Delta$$
$$t_\Delta = \ln(\Delta) / \beta_1$$

In particular, for half-life $\Delta = ½$ and then $t_{½} = \ln(½) / \beta_1 = -\ln(2) / \beta_1$. Similarly, time to twofold increase can be calculated ad $t_{2x} = \ln(2) / \beta_1$.

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Fig. S1. Study profile

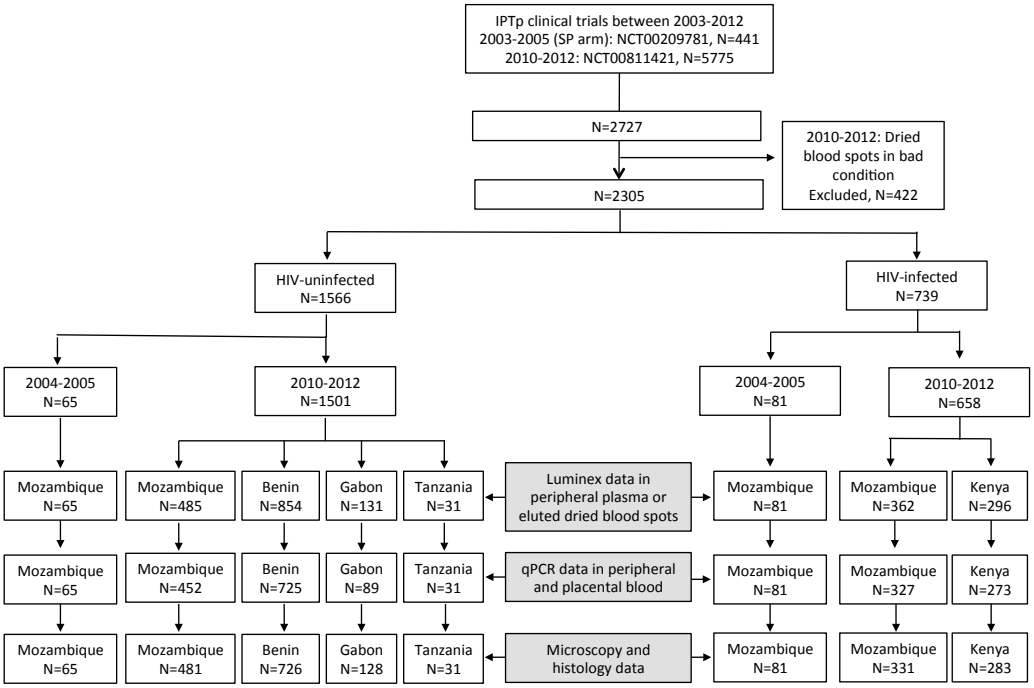

Fig. S2. VAR2CSA peptide p5 sequences from *P. falciparum* isolates from Mozambique, Benin, Kenya and Gabon.

Grey fill means conserved regions, red indicate aminoacid change, blue indicate nucleotide change but same aminoacid and positions with two aminoacids correspond to mixed infecti

Peptide p5	C	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mozambique																																																						
MTP1	F	R	K	C	G	H	Y	E	G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
MTP2	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
MTP3	C	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	G	M	E	R	H	R	E	E	C	T
MTP4	C	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
MTP5	C	R/Q	K	C	G	H	Y	E	E/K	K/E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E/D	K	Q	N	L	I	D	G	M	E	R	H	R	E	E	C	T
MTP6	C	R	K	C	G	H	Y	E	G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
MTP7	C	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
MTP8	C	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
MTP9	F	R	K	C	G	H	Y	E	E/R	K/E	V	P	T	K/Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
MTP10	C	R	K	C	G	H	Y	E	G	K	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	G	M	E	R	H	R	E	E	C	T
Mç1	F	R	K	C	G	H	Y	E	G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mç2	C/F	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	G	M	E	R	H	R	E	E	C	T
Mç3	F	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mç4	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mç5	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mç6	C	R	K	C	G	H	Y	E	G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	G	M	E	R	H	R	E	E	C	T
Mç7	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mç8	F	R	K	C	G	H	Y	E	G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mç9	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Mç10	C	R	K	C	G	H	Y	E	G	K	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	G	M	E	R	H	R	E	E	C	T
Benin																																																					
B1	C	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L/F	Y	R	E/D	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B2	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B3	C	R	K	C	G	H	Y	E	E	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	A	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B4	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B5	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B6	C	R	K	C	G	H	Y	E	K	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	E	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B7	C	R	K	C	G	H	Y	E	G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	E	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B8	F	R	K	C	G	H	Y	E	G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B9	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
B10	C	R	K	C	G	H	Y	E	G	K	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	E	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Kenya																																																				
K1	C/F	R	K	C	G	H	Y	E	E/G	K	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	G/D	M	E	R	H	R	E	E	C	T
K2	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
K3	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
K4	C	R	K	C	G	H	Y	E	G	K	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
K5	F	R	K	C	G	H	Y	E	K	E	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	A	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
K6	C/F	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
K7	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
K8	F	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	G	M	E	R	H	R	E	E	C	T
K9	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
K10	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
Gabon																																																			
G1	C	R	K	C	G	H	Y	E	E/G	K	V	P	T	K/Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	E	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G2	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G3	C	R	K	C	G	H	Y	E	E/G	K/E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L/F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G4	C	R	K	C	G	H	Y	E	E/G	K	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L/F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G5	C	R	K	C	G	H	Y	E	G	K	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G6	C	R	K	C	G	H	Y	E	E/G	K	V	P	T	K/Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L/F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G7	F	R	K	C	G	H	Y	E	G	K	V	P	T	Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G8	F	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T
G9	C	R	K	C	G	H	Y	E	E/G	K	V	P	T	K/Y	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	L/F	Y	R	E	K	Q	N	L	I	D	G/D	M	E	R	H	R	E	E	C	T
G10	C	R	K	C	G	H	Y	E	K	E	V	P	T	K	L	D	Y	V	P	Q	F	L	R	W	L	T	E	W	I	E	D	F	Y	R	E	K	Q	N	L	I	D	D	M	E	R	H	R	E	E	C	T

Fig. S3. VAR2CSA peptide p8 sequences from *P. falciparum* isolates from Mozambique, Benin, Kenya and Gabon.

Grey fill means conserved regions, red indicate aminoacid change and blue indicate nucleotide change but same aminoacid.

Peptide p8	D	E	V	C	N	C	N	E	S	E	I	S	S	V	G	Q	A	Q	T	S	G	P	S	S	N	K	T	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
Mozambique																																																			
MTP1	D	E	V	C	N	C	N	E	S	E	I	S	S	V	E	Q	A	Q	T	S	R	P	S	S	N	K	T	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
MTP2	D	E	V	C	N	P	N	E	S	E	I	S	S	V	G	Q	A	Q	T	S	G	S	L	S	Q	K	A	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
MTP3	D	E	V	C	N	P	N	E	S	G	I	A	S	V	E	H	A	Q	T	S	G	S	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	H	V	K	L	G
MTP4	D	E	V	C	N	P	N	E	S	G	I	A	S	V	E	H	A	Q	T	S	G	S	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	H	V	K	L	G
MTP5	D	E	A	C	N	C	N	E	S	G	I	S	S	V	E	Q	A	Q	T	S	D	P	S	S	N	K	T	C	N	T	H	S	S	I	K	A	N	K	K	K	E	C	K	D	V	K	L	G
MTP6	D	E	V	C	N	C	N	E	S	E	I	S	S	V	E	Q	E	Q	I	S	G	P	S	S	N	K	T	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	H	V	K	L	G
MTP7	D	E	V	C	N	C	N	E	S	G	I	A	S	V	G	Q	A	S	I	S	D	R	S	S	Q	K	A	C	I	T	H	S	F	I	G	A	N	K	K	K	V	C	K	D	V	K	L	G
MTP8	D	E	A	C	N	C	N	E	S	E	I	S	S	V	G	Q	A	S	I	S	D	S	S	S	Q	K	A	C	N	T	H	S	F	I	K	T	N	K	K	K	E	C	K	D	V	K	L	G
MTP9	D	E	A	C	N	P	N	E	S	E	I	S	S	V	G	Q	A	S	I	S	D	S	S	S	Q	K	A	C	N	T	H	S	F	I	K	T	N	K	K	K	E	C	K	D	V	K	L	G
MTP10	D	E	A	C	N	P	N	E	S	G	I	A	S	V	E	H	A	Q	T	S	G	S	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	H	V	K	L	G
Mç1	D	E	V	C	N	P	N	E	S	G	I	S	S	V	G	Q	A	S	T	S	C	P	S	S	N	K	T	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
Mç2	D	E	V	C	N	P	N	E	S	E	I	S	S	V	G	Q	A	Q	T	S	D	P	S	S	N	K	T	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	H	V	K	L	G
Mç3	D	E	V	C	N	P	N	E	S	E	I	S	S	V	E	Q	A	Q	T	S	D	P	S	S	N	K	T	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	H	V	K	L	G
Mç4	D	E	A	C	N	C	N	E	S	E	I	A	S	V	G	Q	E	Q	I	S	D	S	S	S	N	K	T	C	N	T	H	S	S	I	K	T	N	K	K	K	V	C	K	H	V	K	L	G
Mç5	D	E	V	C	N	C	N	E	S	E	I	A	S	V	G	Q	A	S	I	S	D	P	S	S	N	K	T	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
Mç6	D	E	A	C	N	P	N	E	S	G	I	A	S	V	E	H	A	Q	T	S	G	S	S	S	N	K	T	C	I	T	H	S	F	I	K	T	N	K	K	K	V	C	K	D	V	K	L	G
Mç7	D	E	V	C	N	C	N	E	S	G	I	A	C	V	E	L	A	P	I	S	D	S	S	S	Q	K	A	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
Mç8	D	E	V	C	N	P	N	E	S	G	I	S	S	V	G	Q	A	Q	T	S	C	P	S	S	N	K	T	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
Mç9	D	E	V	C	N	P	N	E	S	E	I	S	S	V	G	Q	A	Q	T	S	G	S	L	S	Q	K	A	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
Mç10	D	E	A	C	N	P	N	E	S	E	I	S	S	V	E	Q	A	S	I	S	G	S	S	S	N	K	T	C	N	T	H	S	S	I	K	T	N	K	K	K	E	C	K	D	V	K	L	G
Benin																																																			
B1	D	E	A	C	N	C	N	E	S	E	I	A	S	V	G	Q	E	Q	I	S	G	P	S	S	N	K	T	C	N	T	H	S	F	I	G	A	N	K	K	K	V	C	K	Y	V	K	L	G
B2	D	E	V	C	N	C	N	E	S	G	I	S	S	V	G	Q	A	Q	T	S	G	S	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	E	C	K	D	V	K	L	G
B3	D	E	V	C	N	P	N	E	S	E	I	S	S	V	E	H	A	Q	T	S	V	L	L	S	Q	K	A	Y	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	Y	V	K	L	G
B4	D	E	V	C	N	P	N	E	S	E	I	S	S	V	G	Q	A	Q	T	S	G	S	L	S	Q	K	A	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
B5	D	E	V	C	N	P	N	E	S	E	I	S	S	V	G	Q	A	Q	T	S	G	S	L	S	Q	K	A	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
B6	D	E	V	C	N	P	N	E	S	E	I	A	C	V	E	L	A	Q	T	S	D	S	S	S	Q	K	A	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
B7	D	E	V	C	N	C	N	E	S	G	I	A	S	V	G	Q	A	Q	T	S	D	A	L	S	Q	K	A	C	I	T	H	S	F	I	G	A	N	K	K	K	E	C	K	D	V	K	L	G
B8	D	E	A	C	N	C	N	E	S	G	I	S	S	V	E	L	E	Q	T	S	C	P	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	H	V	K	L	G
B9	D	E	V	C	N	C	N	E	S	G	I	S	S	V	G	Q	A	Q	T	S	D	S	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	E	C	K	D	V	K	L	G
B10	D	E	V	C	N	P	N	E	S	E	I	A	S	V	G	L	A	Q	T	S	V	L	L	S	Q	K	A	Y	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	Y	V	K	L	G
Kenya																																																		
K1	D	E	V	C	N	C	N	E	S	K	I	A	C	V	E	L	A	P	I	S	D	P	S	S	N	K	T	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	H	V	K	L	G
K2	D	E	V	C	N	C	N	E	S	G	I	S	S	V	E	L	A	Q	T	S	C	P	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	D	V	K	L	G
K3	D	E	V	C	N	C	N	E	S	G	I	S	S	V	E	L	A	Q	T	S	C	P	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	D	V	K	L	G
K4	D	E	A	C	N	P	N	E	S	E	I	A	S	V	E	Q	A	S	I	S	G	S	S	S	N	K	T	C	N	T	H	S	F	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
K5	D	E	A	C	N	C	N	E	S	E	I	A	S	V	E	Q	A	Q	T	S	G	P	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	H	V	K	L	G
K6	D	E	V	C	N	P	N	E	S	G	I	A	S	V	E	L	A	Q	T	S	D	P	S	S	N	K	T	C	I	T	H	S	S	I	K	A	N	K	K	K	V	C	K	H	V	K	L	G
K7	D	E	A	C	N	C	N	E	S	K	I	A	S	V	G	Q	E	Q	I	S	G	S	S	S	N	K	T	C	N	T	H	S	S	I	K	T	N	K	K	K	E	C	K	Y	V	K	L	G
K8	D	E	V	C	N	P	N	E	S	G	I	A	S	V	E	Q	A	Q	T	S	D	P	S	S	N	K	T	C	T	T	H	S	S	I	K	A	N	K	K	K	V	C	K	H	V	K	L	G
K9	D	E	A	C	N	P	N	E	S	G	I	A	C	V	G	Q	A	Q	T	S	G	P	S	S	N	K	T	C	I	T	H	S	F	I	G	A	N	K	K	K	V	C	K	D	V	K	L	G
K10	D	E	V	C	N	C	N	E	S	G	I	S	S	V	E	L	A	Q	T	S	C	P	S	S	N	K	T	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	D	V	K	L	G
Gabon																																																		
G1	D	E	A	C	N	P	N	E	S	G	I	A	C	V	G	Q	A	Q	T	S	G	S	S	S	N	K	T	C	I	T	H	S	S	I	K	A	N	K	K	K	E	C	K	H	V	K	L	G
G2	D	E	V	C	N	C	N	E	S	E	I	A	S	V	E	Q	A	P	I	S	D	R	S	S	Q	K	A	C	I	T	H	S	S	I	K	T	N	K	K	K	V	C	K	H	V	K	L	G
G3	D	E	V	C	N	P	N	E	S	G	I	A	S	V	G	Q	E	S	I	S	D	R	S	S	Q	K	A	C	I	T	H	S	F	I	G	A	N	K	K	K	V	C	K	D	V	K	L	G
G4	D	E	A	C	N	C	N	E	S	E	I	A	S	V	G	Q	E	Q	I	S	G	P	S	S	N	K	T	C	N	T	H	S	F	I	G	A	N	K	K	K	V	C	K	Y	V	K	L	G
G5	D	E	A	C	N	P	N	E	S	G	I	S	S	V	E	Q	E	Q	I	S	G	P	S	S	N	K	T	C	I	T	H	S	F	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
G6	D	E	A	C	N	P	N	E	S	E	I	A	S	V	E	Q	A	S	I	S	G	S	S	S	N	K	T	C	N	T	H	S	F	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
G7	D	E	V	C	N	P	N	E	S	G	I	S	S	V	E	L	A	Q	I	S	D	R	L	S	Q	K	A	C	N	T	H	S	S	I	K	A	N	K	K	I	V	C	K	H	V	K	L	G
G8	D	E	A	C	N	C	N	E	S	E	I	S	S	V	G	Q	A	Q	I	S	G	S	S	S	N	K	T	C	N	T	H	S	S	I	K	T	N	K	K	K	E	C	K	D	V	K	L	G
G9	D	E	A	C	N	P	N	E	S	E	I	A	S	V	E	Q	A	P	I	S	G	S	S	S	N	K	T	C	N	T	H	S	F	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G
G10	D	E	V	C	N	P	N	E	S	E	I	S	S	V	G	Q	A	Q	T	S	G	S	L	S	Q	K	A	C	N	T	H	S	S	I	K	A	N	K	K	K	V	C	K	D	V	K	L	G

Fig. S4. Diagram of peptide selection.

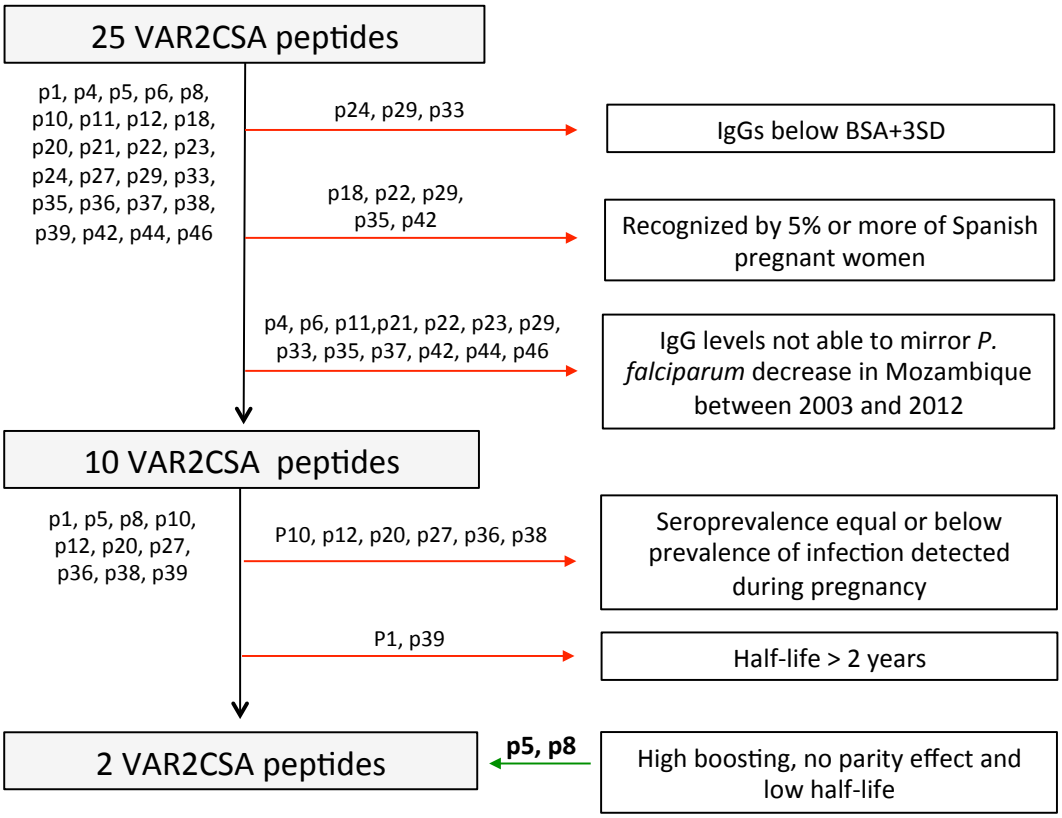

Table S1. Characteristics of the women participating in the intermittent preventive treatment trials and those included in the study.

	HIV-uninfected														HIV-infected						
	2003-2005			2010-2012											2003-2005			2010-2012			
	Mozambique			Mozambique			Benin			Gabon			Tanzania			Mozambique			Mozambique			Kenya		
Variable	included N=65	all N=324	p	included N=485	all N=1183	p	included N=854	all N=1183	p	included N=131	all N=1183	p	included N=31	all N=1200	p	included N=81	all N=117	p	included N=362	all N=561	p	included N=296	all N=465	p
Year delivery, n(%)																								
2003	0 (0)	22 (7)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	12 (10)		0 (0)	0 (0)		0 (0)	0 (0)	
2004	22 (34)	210 (65)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		45 (56)	56 (48)		0 (0)	0 (0)		0 (0)	0 (0)	
2005	43 (66)	91 (28)	<0.001	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)		36 (44)	49 (42)	0.005	0 (0)	0 (0)		0 (0)	0 (0)	
2010	0 (0)	0 (0)		99 (20)	301 (26)		457 (54)	614 (55)		22 (17)	242 (23)		4 (13)	344 (32)		0 (0)	0 (0)		100 (28)	111 (21)		57 (19)	81 (19)	
2011	0 (0)	0 (0)		293 (60)	672 (58)		335 (39)	425 (38)		60 (46)	535 (52)		27 (87)	539 (50)		0 (0)	0 (0)		188 (52)	284 (52)		144 (49)	222 (51)	
2012	0 (0)	0 (0)		93 (19)	182 (16)	0.028	62 (7)	72 (6)	0.659	49 (37)	250 (24)	0.013	0 (0)	204 (19)	<0.001	0 (0)	0 (0)		74 (20)	146 (27)	0.014	95 (32)	129 (30)	0.752
Parity, n(%)																								
Primigravidae	17 (26)	94 (29)		181 (37)	447 (38)		188 (22)	272 (23)		38 (29)	352 (30)		16 (52)	480 (40)		28 (35)	32 (27)		46 (13)	78 (14)		22 (7)	46 (10)	
Multigravidae	48 (74)	230 (71)	0.764	304 (63)	736 (62)	0.868	666 (78)	911 (77)	0.629	93 (71)	831 (70)	0.920	15 (48)	720 (60)	0.199	53 (65)	85 (73)	0.345	316 (87)	483 (86)	0.623	274 (93)	419 (90)	0.297
Age, n(%)																								
<20	19 (29)	114 (35)		181 (37)	464 (39)		86 (10)	122 (10)		42 (32)	357 (30)		5 (16)	162 (14)		27 (33)	32 (27)		41 (11)	65 (12)		15 (5)	33 (7)	
20-24	17 (26)	91 (28)		123 (25)	289 (24)		281 (33)	393 (33)		45 (34)	333 (28)		14 (45)	437 (36)		26 (32)	38 (32)		84 (23)	156 (28)		96 (32)	145 (31)	
≥ 25	29 (45)	119 (37)	0.486	181 (37)	430 (36)	0.764	487 (57)	668 (56)	0.968	44 (34)	492 (42)	0.161	12 (39)	601 (50)	0.407	28 (35)	47 (40)	0.639	237 (65)	340 (61)	0.267	185 (62)	285 (62)	0.538
IPTp, n(%)																								
Sulfadoxine-pyrimethamine	65 (100)	324 (100)		151 (31)	392 (33)		288 (34)	394 (33)		55 (42)	393 (33)		11 (35)	399 (33)		81 (100)	117 (100)		0 (0)	0 (0)		0 (0)	0 (0)	
Mefloquine	0 (0)	0 (0)		334 (69)	791 (67)		566 (66)	789 (67)		76 (58)	790 (67)		20 (65)	801 (67)		0 (0)	0 (0)		178 (49)	280 (50)		139 (47)	231 (50)	
*Placebo**	0 (0)	0 (0)	N/E	0 (0)	0 (0)	0.455	0 (0)	0 (0)	0.849	0 (0)	0 (0)	0.052	0 (0)	0 (0)	0.847	0 (0)	0 (0)	N/E	184 (51)	281 (50)	0.788	157 (53)	234 (50)	0.503
Microscopy peripheral blood, n(%)†																								
Positive	4 (6)	22 (7)		10 (2)	23 (2)		70 (8)	84 (8)		2 (2)	46 (5)		0 (0)	0 (0)		6 (7)	6 (5)		8 (2)	10 (2)		10 (3)	22 (5)	
Negative	61 (94)	289 (93)	1.000	475 (98)	1080 (98)	1.000	770 (92)	908 (92)	0.933	128 (98)	921 (95)	0.110	31 (100)	1059 (100	N/E	75 (93)	106 (95)	0.563	350 (98)	502 (98)	0.812	283 (97)	391 (95)	0.273
Microscopy placental blood, n(%)†																								
Positive	4 (6)	21 (7)		12 (2)	23 (2)		67 (9)	74 (9)		1 (1)	37 (4)		0 (0)	2 (0)		5 (6)	6 (6)		7 (2)	8 (2)		10 (4)	19 (5)	
Negative	60 (94)	263 (93)	1.000	471 (98)	974 (98)	0.856	659 (91)	776 (91)	0.724	124 (99)	880 (96)	0.075	31 (100)	1034 (100	1.000	76 (94)	97 (94)	1.000	329 (98)	453 (98)	0.794	275 (96)	381 (95)	0.450
Placental histology, n(%)†																								
Active infection	9 (14)	30 (11)		4 (1)	12 (1)		78 (11)	10 (83)		3 (2)	27 (3)		1 (3)	17 (2)		7 (9)	9 (9)		6 (2)	6 (1)		9 (3)	14 (4)	
Negative‡	56 (86)	248 (89)	0.515	477 (99)	997 (99)	0.604	649 (89)	769 (90)	0.559	126 (98)	878 (97)	1.000	30 (97)	993 (98)	0.414	74 (91)	93 (91)	1.000	328 (98)	456 (99)	0.571	276 (97)	386 (96)	1.000

IPTp, intermitent preventive treatment during pregnancy; qPCR, quantitative polimerase chain reaction.

*All HIV-infected women who received placebo were under cotrimoxazol prophylaxis.

†Not determined: 747 microscopy peripheral blood, 1170 microscopy placental blood and 1224 histology in all.

‡No *P. falciparum* parasites observed.

N/E, Not estimated due to absence of sample in one of the groups.

Comparisons were performed by Fisher exact text.

Table S2. Amino acid sequences of the 25 VAR2CSA peptides included in the peptide array.

Position	Peptide	Length	Aminoacid sequence (N-terminal to C-terminal)
NTS	p1	56	I A D K I E A Y L G A K S N D S K I D Q S L K A D P S E V Q Y Y R S G G D G Y Y L K N N I C K I T V N H S D S G
DBL1X	p4	52	D E N Y K K L R E D W W N A N R Q K V W E V I T C G A R S N D L L I K R R W R T S K E S N G E N K L E L
	p5	51	C R K C G H Y E E K V P T K L D Y V P Q F L R W L T E W I E D L Y R E K Q N L I D D M E R H R E E C T
	p6	38	E G T S Y C N M C K E K C K K Y C E C V K K W K S E W E N Q K N K Y K D L Y
ID1	p8	48	D E V C N C N E S E I S S V G Q A Q T S G P S S N K T C I T H S S I K A N K K K V C K D V K L G
DBL2X	p10	64	G L Q K E Y A N T I G L P P R T Q S L Y L G N L P K L E N V C K G V T D I N F D T K E K F L A G C L I A A F H E G K N L K T S Y Y
	p11	50	K L C K A L E Y S F A D Y G D L I K G T S I W D N E Y T K D L E L N L Q K I F G K L F R K Y I K K N
	p12	36	T S Y S S L D E L R E S W W N T N K K Y I W L A M K H G A G M N S T T C
ID2	p18	35	C E C Y E L W I E K I K E Q W D K Q K D N Y N K F Q S K Q I Y D A N K
DBL3X	p20	52	Y I R G C Q P K R Y D G F I F P G K G G E K Q W I C K D T I I H G D T N G A C I P P R T Q N L C V G E L
	p21	51	W D K S Y G G R S N I K N D T K E S L K N K L K N A I Q K E T E L L Y E Y H D K G T A I I S R N P M K
	p22	48	N N S N G L P K G F C H A V Q R S F I D Y K N M I L G T S V N I Y E Y I G K L Q E D I K K I I E
	p23	35	S G A D K V N D W W K G I E G E M W G A V K C G I T K I N K K Q K K N
	p24	45	E C G V S P P T G N D E D Q F V S W F K E W G E Q F C I E R L R Y E Q N I R E A C T I N G
DBL4ε	P27	52	N N K S L C H E K G N D R T W S K K Y I K K L E N G R T L E G V Y V P P R R Q Q L C L Y E L F P I I I K
	P29	65	A N K K A C C A I R G S F Y D L E D I I K G N D L V H D E Y T K Y I D S K L N E I F G S S N K N D I D T K R A R T D W W E N E T I
ID4	P33	44	S N K E S E D G K D Y S M I M E P T V I D Y L N K R C N G E I N G N Y I C C S C K N I G
DBL5ε	P35	53	Y P L D R C F D D K S K M K V C D L I G D A I G C K D K T K L D E L D E W N D M D M R D P Y N K Y K G V L
	P36	53	I P P R R R Q L C F S R I V R G P A N L R N L N E F K E E I L K G A Q S E G K F L G N Y Y N E D K D K E K
	P37	47	K E K A L E A M K N S F Y D Y E Y I I K G S D M L T N I Q F K D I K R K L D K L L T K E T N N
	P38	57	D W W K T N K K S I W N A M L C G Y K K S G N K I I D P S W C T I P T T E K P P Q F L R W I K E W G T N V C I Q K
	P39	65	K E Y V K S E C S N V T N I N L G S Q A S E S N N C T S E I R K Y Q E W S R K R S I Q W E A I S E R Y K K Y K G M D E F K N V K N
DBL6ε	P42	62	T D L L V K N S S D I N K G V L I P P R R K N L F L N I D P S D I C K Y K K N P K L F N D F I Y S S A F T E V E R L K K V Y
	P44	35	T D G Q N E K R K T W W D M N K Y H I W E S M L C G Y K Q A G G D T K
ID6	P46	35	P L P P Q N D E P I T S D I L Q K T I P F G I A L A L G S I A F L F M

Table S3. Ratio of IgG levels against *P. falciparum* VAR2CSA and general antigens in pregnant Mozambican and Spanish women.

	Pregnat Spanish women (n=49) vs pregnat Mozambican women (n=637)		
Variable	Ratio (95%CI)*	p	q
Proteins			
DBL3X	8.24 (4.85; 14)	<0.001	<0.001
DBL5ε	8.5 (4.9; 14.75)	<0.001	<0.001
DBL6ε	1.11 (0.82; 1.49)	0.503	0.503
AMA1	24.51 (18.53; 32.43)	<0.001	<0.001
$MSP1_{19}$	85.07 (46.72; 154.9)	<0.001	<0.001
Peptides			
p1	3.08 (2.22; 4.25)	<0.001	<0.001
p4	2.65 (1.79; 3.9)	<0.001	0.001
p5	3.15 (2.2; 4.52)	<0.001	<0.001
p6	4.32 (2.96; 6.31)	<0.001	<0.001
p8	2.85 (1.92; 4.23)	<0.001	<0.001
p10	2.24 (1.65; 3.04)	<0.001	<0.001
p11	2.37 (1.78; 3.15)	<0.001	<0.001
p12	2.92 (2.18; 3.91)	<0.001	<0.001
p18	2.53 (1.74; 3.68)	<0.001	0.001
p20	2.24 (1.64; 3.06)	<0.001	<0.001
p21	2.23 (1.68; 2.95)	<0.001	<0.001
p22	1.74 (1.31; 2.31)	<0.001	<0.001
p23	1.71 (1.33; 2.2)	<0.001	<0.001
p24	2.13 (1.63; 2.78)	<0.001	<0.001
p27	3.58 (2.71; 4.75)	<0.001	<0.001
p29	1.55 (1.1; 2.18)	0.012	0.012
p33	2.5 (1.91; 3.28)	<0.001	<0.001
p35	2.29 (1.6; 3.27)	<0.001	0.007
p36	3.03 (2.33; 3.95)	<0.001	<0.001
p37	2.53 (1.84; 3.49)	<0.001	<0.001
p38	2.7 (2.11; 3.46)	<0.001	<0.001
p39	3.01 (2.2; 4.1)	<0.001	<0.001
p42	2.67 (1.93; 3.70)	<0.001	<0.001
p44	2.32 (1.71; 3.15)	<0.001	<0.001
p46	2.07 (1.49; 2.89)	<0.001	<0.001
pCSP	8.2 (4.84; 13.87)	<0.001	<0.001

CI. confidence interval; p, p-value; q, q-value obtained by the Simes procedure.

*Ratio of normalized median fluorescence intensity (nMFIs) measured in pregnant Spanish women compared with those from Mozambique, assessed by linear regression.

Table S4 Ratio of IgG levels against *P. falciparum* VAR2CSA and general antigens in pregnant Mozambican women recruited between 2004 and 2012.

2004-2005 (n=146) vs 2010-2012 (n=491)

Variable	Ratio (95%CI)*	p	q
Proteins			
DBL3X	0.35 (0.25; 0.51)	<0.001	0.001
DBL5ε	0.31 (0.21; 0.46)	<0.001	<0.001
DBL6ε	0.56 (0.45; 0.70)	<0.001	<0.001
AMA1	0.69 (0.59; 0.82)	0.084	0.109
$MSP1_{19}$	0.43 (0.28; 0.65)	0.083	0.109
Peptides			
p1	0.55 (0.44; 0.70)	0.004	0.013
p4	0.59 (0.46; 0.77)	0.091	0.112
p5	0.46 (0.36; 0.59)	0.013	0.029
p6	0.58 (0.44; 0.75)	0.027	0.052
p8	0.44 (0.33; 0.58)	0.002	0.006
p10	0.74 (0.59; 0.93)	0.008	0.021
p11	0.79 (0.65; 0.96)	0.137	0.158
p12	0.55 (0.44; 0.68)	0.001	0.004
p18	0.58 (0.44; 0.75)	0.020	0.041
p20	0.60 (0.48; 0.74)	0.008	0.021
p21	0.67 (0.56; 0.81)	0.033	0.057
p22	0.77 (0.64; 0.94)	0.096	0.114
p23	0.78 (0.65; 0.93)	0.047	0.069
p24	0.64 (0.53; 0.78)	0.013	0.029
p27	0.62 (0.51; 0.75)	<0.001	0.001
p29	0.72 (0.56; 0.92)	0.183	0.189
p33	0.75 (0.62; 0.91)	0.176	0.188
p35	0.64 (0.50; 0.82)	0.200	0.199
p36	0.55 (0.46; 0.66)	<0.001	<0.001
p37	0.65 (0.53; 0.81)	0.044	0.068
p38	0.66 (0.56; 0.77)	<0.001	<0.001
p39	0.52 (0.42; 0.65)	<0.001	<0.001
p42	0.65 (0.52; 0.81)	0.158	0.175
p44	0.67 (0.55; 0.81)	0.031	0.056
p46	0.67 (0.54; 0.84)	0.036	0.059
pCSP	0.48 (0.33; 0.70)	0.068	0.096

CI. confidence interval; p, p-value; q, q-value obtained by the Simes procedure.

*Ratio of normalized median fluorescence intensity (nMFIs) measured in pregnant Mozambican women delivering during 2004-2005 compared with those delivering during 2010-2012 corresponding to a trend of malaria decrease assessed by linear regression adjusted by parity, age, treatment and HIV.

Table S5. Ratio of IgG levels against *P. falciparum* VAR2CSA and general antigens measured in pregnant Mozambican women at delivery according to *P. falciparum* infection during pregnancy, parity and seroprevalence at delivery.

	***P. falciparum* infected (n=49) vs uninfected (n=190)**			**Multigravid (n=175) vs Primigravid (n=64)**			**Seroprevalences at delivery (n=239)**
Variable	Ratio (95%CI)*	p	q	Ratio (95%CI)†	p	q	% (95%CI)
Proteins							
DBL3X	8.83 (5.33; 14.62)	<0.001	<0.001	2.22 (1.10; 4.51)	0.028	0.288	44.77 (38.42; 51.11)
DBL5ε	15.06 (8.29; 27.37)	<0.001	<0.001	3.72 (1.61; 8.59)	0.002	0.066	49.79 (43.41; 56.18)
DBL6ε	3.13 (2.33; 4.21)	<0.001	<0.001	1.84 (1.22; 2.79)	0.004	0.066	12.55 (8.32; 16.78)
AMA1	1.80 (1.30; 2.50)	<0.001	0.001	1.20 (0.76; 1.90)	0.439	0.621	88.70 (84.66; 92.75)
$MSP1_{19}$	3.69 (1.83; 7.46)	<0.001	0.001	1.60 (0.60; 4.29)	0.351	0.595	89.12 (85.15; 93.10)
Peptides							
p1	1.73 (1.22; 2.46)	0.002	0.003	1.15 (0.70; 1.88)	0.574	0.659	23.43 (18.02; 28.84)
p4	1.92 (1.30; 2.84)	0.001	0.002	1.40 (0.81; 2.42)	0.229	0.542	21.76 (16.49; 27.03)
p5	2.15 (1.39; 3.31)	<0.001	0.001	1.46 (0.80; 2.68)	0.219	0.542	25.94 (20.34; 31.54)
p6	1.62 (1.11; 2.37)	0.013	0.016	1.18 (0.69; 2.01)	0.543	0.659	18.83 (13.84; 23.82)
p8	2.17 (1.46; 3.23)	<0.001	<0.001	1.35 (0.77; 2.35)	0.293	0.542	26.36 (20.73; 31.99)
p10	1.88 (1.30; 2.73)	<0.001	0.002	1.54 (0.91; 2.58)	0.107	0.413	17.15 (12.34; 21.97)
p11	2.00 (1.37; 2.93)	<0.001	0.001	1.12 (0.66; 1.91)	0.675	0.698	20.92 (15.73; 26.11)
p12	1.98 (1.41; 2.80)	<0.001	<0.001	1.35 (0.84; 2.19)	0.220	0.542	15.90 (11.23; 20.57)
p18	2.24 (1.42; 3.53)	<0.001	0.001	1.29 (0.68; 2.43)	0.440	0.621	26.36 (20.73; 31.99)
p20	1.73 (1.27; 2.36)	<0.001	0.001	1.51 (0.98; 2.32)	0.065	0.389	20.50 (15.35; 25.66)
p21	1.49 (1.06; 2.10)	0.021	0.023	1.29 (0.80; 2.08)	0.289	0.542	14.64 (10.13; 19.16)
p22	1.52 (1.09; 2.11)	0.014	0.017	1.31 (0.82; 2.07)	0.261	0.542	16.74 (11.97; 21.50)
p23	1.33 (1.01; 1.75)	0.041	0.041	1.15 (0.79; 1.69)	0.461	0.622	15.48 (10.86; 20.10)
p24	1.73 (1.27; 2.35)	<0.001	0.001	1.46 (0.95; 2.23)	0.088	0.389	17.57 (12.71; 22.43)
p27	1.38 (1.06; 1.79)	0.016	0.018	1.22 (0.85; 1.76)	0.279	0.542	18.41 (13.46; 23.36)
p29	2.60 (1.62; 4.15)	<0.001	<0.001	1.18 (0.61; 2.28)	0.621	0.664	27.62 (21.91; 33.32)
p33	1.66 (1.19; 2.31)	0.003	0.004	1.21 (0.76; 1.93)	0.416	0.621	17.57 (12.71; 22.43)
p35	2.21 (1.38; 3.54)	0.001	0.002	1.36 (0.70; 2.63)	0.365	0.595	23.85 (18.41; 29.29)
p36	1.57 (1.18; 2.09)	0.002	0.003	1.44 (0.96; 2.15)	0.078	0.389	16.74 (11.97; 21.50)
p37	2.13 (1.39; 3.29)	<0.001	0.001	1.41 (0.77; 2.58)	0.270	0.542	18.41 (13.46; 23.36)
p38	1.73 (1.34; 2.24)	<0.001	<0.001	1.10 (0.77; 1.58)	0.595	0.659	16.74 (11.97; 21.50)
p39	2.86 (1.96; 4.17)	<0.001	0.001	1.33 (0.78; 2.26)	0.297	0.542	31.38 (25.46; 37.31)
p42	1.94 (1.32; 2.84)	<0.001	0.001	0.91 (0.53; 1.56)	0.739	0.739	25.94 (20.34; 31.54)
p44	1.61 (1.12; 2.32)	0.011	0.014	1.16 (0.69; 1.93)	0.576	0.659	21.34 (16.11; 26.57)
p46	1.45 (1.03; 2.04)	0.033	0.035	1.18 (0.73; 1.90)	0.496	0.640	18.83 (13.84; 23.82)
pCSP	3.10 (1.75; 5.49)	<0.001	<0.001	2.22 (1.00; 4.93)	0.052	0.389	63.60 (57.45; 69.74)

CI, confidence interval; p, p-value: q, q-value obtained by the Simes procedure.

*Ratio of normalized median fluorescence intensity (nMFIs) measured in Mozambican women experiencing infection during pregnancy compared with those not infected, assessed by linear regression adjusted by age, parity, treatment and HIV.

†Ratio of nMFIs measured in multigravid pregnant Mozambican women compared with primigravid, assessed by linear regression adjusted by malaria infectio, age, treatment and HIV.

Table S6. Dynamics of IgG levels against *P. falciparum* VAR2CSA and general antigens in Mozambican women during pregnancy.

Variable	Time to double Years (95%CI)*	Half-life Years (95%CI)*
Proteins		
DBL3X	0.40 (0.26; 0.88)	0.99 (0.56; 4.80)
DBL5ε	0.31 (0.21; 0.61)	0.66 (0.42; 1.65)
DBL6ε	0.53 (0.38; 0.90)	0.83 (0.46; 3.79)
AMA1	1.76 (0.76; ∞)	4.18 (1.86; ∞)
$MSP1_{19}$	0.61 (0.30; ∞)	1.29 (0.69; 9.12)
Peptides		
p1	0.62 (0.39; 1.45)	3.66 (0.98; ∞)
p4	1.14 (0.55; ∞)	1.25 (0.62; ∞)
p5	0.45 (0.31; 0.80)	1.33 (0.65; ∞)
p6	0.72 (0.42; 2.64)	0.95 (0.50; 9.50)
p8	0.53 (0.34; 1.14)	0.55 (0.38; 1.02)
p10	0.45 (0.32; 0.77)	0.90 (0.49; 5.91)
p11	0.73 (0.42; 2.66)	2.60 (0.80; ∞)
p12	0.66 (0.42; 1.52)	0.89 (0.52; 3.03)
p18	0.50 (0.32; 1.20)	0.63 (0.40; 1.45)
p20	0.47 (0.33; 0.79)	1.62 (0.70; ∞)
p21	0.62 (0.40; 1.43)	1.40 (0.57; ∞)
p22	0.61 (0.40; 1.22)	2.32 (0.72; ∞)
p23	0.65 (0.42; 1.47)	0.83 (0.44; 6.75)
p24	0.74 (0.43; 2.56)	0.97 (0.49; 151.42)
p27	1.07 (0.60; 5.23)	2.25 (0.85; ∞)
p29	0.41 (0.28; 0.78)	1.23 (0.58; ∞)
p33	0.66 (0.42; 1.53)	1.10 (0.55; 69.09)
p35	0.41 (0.28; 0.78)	0.69 (0.38; 4.05)
p36	0.76 (0.45; 2.47)	0.77 (0.46; 2.39)
p37	0.52 (0.35; 1.03)	1.25 (0.59; ∞)
p38	0.57 (0.40; 1.00)	1.20 (0.61; 68.73)
p39	0.46 (0.31; 0.90)	3.30 (0.87; ∞)
p42	0.83 (0.49; 2.71)	N/E
p44	0.61 (0.36; 1.96)	0.96 (0.49; 20.59)
p46	2.50 (0.74; ∞)	0.68 (0.43; 1.66)
pCSP	0.99 (0.43; ∞)	N/E

CI, confidence interval.

*Log linear mixed effect regression models adjusted by parity, age, treatment and HIV.

N/E, Not estimated due coefficient of regression >1.

Table S7. (Sero)Prevalence of *P. falciparum* among pregnant Mozambican women at delivery, according to year.

	2004-2005	2010	2011	2012	2010 vs 2004-2005			iHIV	iParity	2012 vs 2010			iHIV	iParity
Variable	% (n/N)	% (n/N)	% (n/N)	% (n/N)	OR (95%CI)*	Crude p	Adjusted p	Adjusted p	Adjusted p	OR (95%CI)†	Crude p	Adjusted p	Adjusted p	Adjusted p
Microscopy‡	12 (17/146)	2 (3/176)	3 (7/243)	3 (2/72)	N/E	0.002	N/E	N/E	N/E	2.13 (0.33; 13.65)	0.589	0.426	N/E	0.538
qPCR§	25 (37/146)	2 (3/176)	6 (15/243)	6 (4/72)	0.10 (0.01; 0.79)	<0.001	0.029	N/E	0.828	4.68 (0.93; 23.54)	0.116	0.061	0.997	0.158
p5	30 (44/146)	10 (17/176)	19 (47/243)	24 (17/72)	0.14 (0.04; 0.50)	<0.001	0.003	0.366	0.108	3.20 (1.48; 6.88)	0.005	0.003	0.772	0.355
p8	30 (44/146)	16 (28/176)	21 (52/243)	28 (20/72)	0.43 (0.17; 1.11)	0.003	0.081	0.690	0.875	2.17 (1.10; 4.28)	0.034	0.026	0.142	0.260
p5+8	46 (65/146)	20 (36/176)	30 (72/243)	38 (27/72)	0.27 (0.11; 0.68)	<0.001	0.005	0.415	0.406	2.49 (1.34; 4.61)	0.006	0.004	0.136	0.362
pCSP	53 (77/146)	36 (63/176)	40 (97/243)	46 (33/72)	0.44 (0.18; 1.06)	0.002	0.067	0.317	0.135	1.70 (0.94; 3.10)	0.142	0.081	0.440	0.086
DBL3X	71 (103/146)	49 (87/176)	49 (119/243)	62 (45/72)	0.25 (0.09; 0.65)	<0.001	0.004	0.765	0.123	1.60 (0.89; 2.87)	0.062	0.117	0.058	0.134
DBL5ε	70 (102/146)	49 (87/176)	51 (124/243)	60 (43/72)	0.21 (0.08; 0.56)	<0.001	0.002	0.456	0.268	1.42 (0.79; 2.54)	0.142	0.244	0.111	0.178
DBL6ε	38 (55/146)	16 (29/176)	22 (53/243)	21 (15/72)	0.29 (0.11; 0.82)	<0.001	0.019	0.683	0.752	1.45 (0.71; 2.97)	0.416	0.312	0.743	0.468
AMA1	98 (143/146)	93 (164/176)	88 (215/243)	92 (66/72)	N/E	0.057	N/E	N/E	N/E	0.79 (0.28; 2.24)	0.677	0.659	0.063	0.571
MSP1$_{19}$	95 (138/146)	94 (165/176)	84 (204/243)	90 (65/72)	0.48 (0.10; 2.34)	0.770	0.364	0.552	0.053	0.62 (0.22; 1.73)	0.343	0.362	0.471	0.861

OR, Odds ratio; CI, Confidence interval; p, p-value.

*Odds ratio of seroprevalences measured in pregnant Mozambican women delivering during 2004-2005 compared with those delivering during 2010 corresponding to a trend of malaria decrease, assessed by logistic regression adjusted by parity, age, treatment and HIV.

†Odds ratio of (sero)prevalences measured in pregnant Mozambican women delivering during 2012 compared with those delivering during 2010 corresponding to a trend of malaria increase, assessed by logistic regression adjusted by parity, age, treatment and HIV.

‡Maternal microscopic infection defined by the presence of *P. falciparum* parasites in peripheral blood or in placenta on microscopic or histologic examination, respectively.

§Maternal qPCR-positive infection defined by a positive result on qPCR testing in peripheral or placental blood.

iHIV, Interaction term included into the regression model to assess modification of the associations by HIV.

iParity, Interaction term included into the regression model to assess modification of the associations by parity.

N/E, Not estimated due to low and high prevalence of infection and seroresponders, respectivelly.

Table S8. (Sero)Prevalences of *P. falciparum* among pregnant women, according to country.

	HIV-uninfected								HIV-infected					
	Benin	Gabon	Mozambique	Gabon vs Benin	Mozambique vs Benin			iParity	Kenya	Mozambique	Mozambique vs Kenya			iParity
Variable	% (n/N)	% (n/N)	% (n/N)	OR (95%CI)*	OR (95%CI)*	Crude p	Adjusted p	Adjusted p	% (n/N)	% (n/N)	OR (95%CI)†	Crude p	Adjusted p	Adjusted p
Microscopy‡	15 (110/726)	2 (3/128)	3 (13/481)	0.10 (0.03; 0.32)	0.12 (0.06; 0.22)	<0.001	<0.001	0.132	5 (15/283)	2 (8/331)	0.47 (0.19; 1.14)	0.067	0.095	0.988
qPCR§	46 (332/725)	10 (9/89)	6 (28/452)	0.11 (0.05; 0.23)	0.06 (0.04; 0.10)	<0.001	<0.001	0.870	8 (22/273)	4 (13/327)	0.48 (0.23; 0.99)	0.037	0.046	0.119
p5	41 (354/854)	23 (30/131)	13 (61/485)	0.40 (0.26; 0.62)	0.20 (0.15; 0.28)	<0.001	<0.001	0.715	6 (19/296)	6 (22/362)	0.96 (0.50; 1.82)	0.857	0.894	0.311
p8	42 (359/854)	25 (33/131)	16 (77/485)	0.50 (0.32; 0.76)	0.29 (0.22; 0.39)	<0.001	<0.001	0.378	17 (50/296)	9 (31/362)	0.45 (0.28; 0.73)	0.002	0.001	0.181
p5+8	61 (522/854)	33 (43/131)	24 (115/485)	0.31 (0.21; 0.47)	0.21 (0.16; 0.28)	<0.001	<0.001	0.904	21 (62/296)	14 (49/362)	0.58 (0.38; 0.88)	0.012	0.011	0.128
pCSP	44 (380/854)	23 (30/131)	13 (63/485)	0.37 (0.24; 0.57)	0.19 (0.14; 0.26)	<0.001	<0.001	0.856	21 (61/296)	10 (36/362)	0.41 (0.26; 0.64)	<0.001	<0.001	0.340
DBL3X	89 (762/854)	49 (64/131)	46 (224/485)	0.11 (0.07; 0.16)	0.10 (0.08; 0.14)	<0.001	<0.001	0.945	48 (143/296)	36 (130/362)	0.62 (0.45; 0.85)	0.001	0.004	0.049
DBL5ε	89 (763/854)	37 (48/131)	45 (219/485)	0.06 (0.04; 0.10)	0.10 (0.07; 0.14)	<0.001	<0.001	0.846	31 (91/296)	36 (129/362)	1.33 (0.96; 1.86)	0.186	0.090	0.126
DBL6ε	54 (458/854)	24 (32/131)	15 (73/485)	0.28 (0.18; 0.43)	0.16 (0.12; 0.21)	<0.001	<0.001	0.147	0 (0/296)	10 (35/362)	N/E	N/E	N/E	N/E
AMA1	100 (854/854)	82 (108/131)	93 (450/485)	N/E	N/E	N/E	N/E	N/E	95 (280/296)	93 (335/362)	0.76 (0.40; 1.45)	0.291	0.404	N/E
MSP1$_{19}$	99 (843/854)	84 (110/131)	89 (431/485)	0.08 (0.04; 0.18)	0.13 (0.07; 0.26)	<0.001	<0.001	1.000	88 (261/296)	85 (307/362)	0.73 (0.46; 1.16)	0.212	0.177	0.928

OR, Odds ratio; CI, Confidence interval; p, p-value.

*Odds ratio of seroprevalences measured in HIV-uninfected pregnant women from Gabon and Mozambique compared with pregnant Beninese women assessed by logistic regression adjusted by parity, age and treatment.

†Odds ratio of seroprevalences measured in HIV-infected pregnant women from Mozambique compared with pregnant women from Kenya assessed by logistic regression adjusted by parity, age and treatment.

‡Maternal microscopic infection defined by the presence of *P. falciparum* parasites in peripheral blood or in placenta on microscopic or histologic examination, respectively.

§Maternal qPCR-positive infection defined by a positive result on qPCR testing in peripheral or placental blood.

iParity, Interaction term included into the regression model to assess modification of the associations by parity.

N/E, Not estimated due to low/high prevalence of seroresponders.

Table S9. (Sero)Prevalences of *P. falciparum* among pregnant women, according to anemi status.

	Non-anemic	Anemic	Anemic vs Non-anemic			iHIV	iParity
Variable	% (n/N)	% (n/N)	OR (95%CI)*	Crude p	Adjusted p	Adjusted p	Adjusted p
Microscopy†	7 (79/1206)	9 (70/743)	1.59 (1.12; 2.26)	0.021	0.010	0.319	0.740
qPCR‡	19 (223/1164)	26 (181/702)	1.65 (1.27; 2.14)	<0.001	<0.001	0.934	0.300
p5	21 (280/1315)	25 (206/813)	1.29 (1.03; 1.61)	0.031	0.029	0.467	0.371
p8	25 (330/1315)	27 (220/813)	1.18 (0.95; 1.46)	0.314	0.132	0.193	0.841
p5+8	36 (470/1315)	39 (321/813)	1.26 (1.03; 1.55)	0.083	0.024	0.069	0.650
pCSP	27 (348/1315)	27 (222/813)	1.11 (0.90; 1.37)	0.670	0.333	0.193	0.292
DBL3X	63 (826/1315)	61 (497/813)	1.00 (0.81; 1.24)	0.437	0.998	0.600	0.042
DBL5ε	59 (774/1315)	59 (476/813)	1.01 (0.81; 1.26)	0.888	0.923	0.305	0.063
DBL6ε	27 (355/1315)	30 (243/813)	1.15 (0.92; 1.44)	0.149	0.232	0.469	0.593
AMA1	96 (1256/1315)	95 (771/813)	1.01 (0.66; 1.55)	0.474	0.959	0.182	0.618
MSP1$_{19}$	92 (1213/1315)	91 (739/813)	0.91 (0.66; 1.27)	0.274	0.594	0.651	0.568

OR, Odds ratio; CI, Confidence interval; p, p-value.

*Odds ratio of seroprevalences measured in anemic pregnant women compared with those non-anemic assessed by logistic regression adjusted by country, HIV, parity, age and treatment.

†Maternal microscopic infection defined by the presence of *P. falciparum* parasites in peripheral blood or in placenta on microscopic or histologic examination, respectively.

‡Maternal qPCR-positive infection was defined by a positive result on qPCR testing in peripheral or placental blood.

iHIV, Interaction term included into the regression model to assess modification of the associations by HIV.

iParity, Interaction term included into the regression model to assess modification of the associations by parity.

Table S10. (Sero)Prevalences of *P. falciparum* among pregnant women, according to intermittent preventive treatment intervention group.

	HIV-uninfected						HIV-infected					
	SP	**MQ**	**MQ vs SP**			**iParity**	**Placebo**[¶]	**MQ**	**MQ vs Placebo**			**iParity**
Variable	% (n/N)	% (n/N)	OR (95%CI)*	Crude p	Adjusted p	Adjusted p	% (n/N)	% (n/N)	OR (95%CI)[†]	Crude p	Adjusted p	Adjusted p
Microscopy[‡]	12 (55/449)	8 (71/886)	0.58 (0.39; 0.85)	0.013	0.006	0.851	6 (18/322)	2 (5/292)	0.29 (0.11; 0.80)	0.017	0.017	0.353
qPCR[§]	35 (148/418)	26 (221/848)	0.62 (0.47; 0.83)	<0.001	0.001	0.997	9 (27/314)	3 (8/286)	0.29 (0.13; 0.66)	0.004	0.003	0.057
p5	34 (170/494)	28 (275/976)	0.75 (0.59; 0.96)	0.014	0.022	0.793	7 (25/341)	5 (16/317)	0.66 (0.35; 1.27)	0.228	0.215	0.684
p8	36 (179/494)	30 (290/976)	0.75 (0.59; 0.96)	0.011	0.021	0.765	14 (49/341)	10 (32/317)	0.68 (0.42; 1.10)	0.097	0.115	0.078
p5+8	51 (252/494)	44 (428/976)	0.74 (0.59; 0.94)	0.009	0.013	0.964	19 (64/341)	15 (47/317)	0.76 (0.50; 1.15)	0.178	0.198	0.079
pCSP	34 (166/494)	31 (307/976)	0.93 (0.73; 1.18)	0.405	0.548	0.486	17 (58/341)	12 (39/317)	0.69 (0.44; 1.07)	0.090	0.099	0.732
DBL3X	73 (360/494)	71 (690/976)	0.92 (0.69; 1.23)	0.383	0.589	0.641	44 (149/341)	39 (124/317)	0.85 (0.61; 1.16)	0.234	0.299	0.124
DBL5ε	72 (357/494)	69 (673/976)	0.83 (0.62; 1.11)	0.191	0.198	0.738	36 (122/341)	31 (98/317)	0.81 (0.58; 1.12)	0.187	0.204	0.069
DBL6ε	44 (216/494)	36 (347/976)	0.70 (0.55; 0.89)	0.002	0.004	0.878	6 (22/341)	4 (13/317)	N/E	N/E	N/E	0.726
AMA1	96 (475/494)	96 (937/976)	N/E	N/E	N/E	N/E	95 (324/341)	92 (291/317)	0.60 (0.32; 1.12)	0.099	0.109	N/E
MSP1$_{19}$	94 (465/494)	94 (919/976)	0.98 (0.60; 1.59)	0.981	0.927	0.804	87 (298/341)	85 (270/317)	0.84 (0.54; 1.32)	0.409	0.461	0.702

MQ, mefloquine ; SP, sulfadoxine-pyrimethamine; OR, Odds ratio; CI; Confidence interval; p, p-value.

[¶]All HIV-infected women who received placebo were under cotrimoxazol prophylaxis.

*Odds ratio of seroprevalences measured in HIV-uninfected pregnant women under sulfadoxine-pyrimethamine prophylaxis compared with those under mefloquine prophylaxis assessed by logistic regression adjusted by country, parity and age.

[†]Odds ratio of seroprevalences measured in HIV-infected pregnant women under mefloquine prophylaxis compared with those under placebo assessed by logistic regression adjusted by country, parity and age.

[‡]Maternal microscopic infection defined by the presence of *P. falciparum* parasites in peripheral blood or in placenta on microscopic or histologic examination, respectively.

[§]Maternal qPCR-positive infection defined by a positive result on qPCR testing in peripheral or placental blood.

iParity, Interaction term included into the regression model to assess modification of the associations by parity.

N/E, Not estimated due to low/high prevalence of seroresponders.

Table S11. (sero)Prevalences inside and outside serological hotspots.

	Peptide p5 hotspot r = 2.82 km				Peptide p8 hotspot r = 1.02 km				Composite p5+8 hotspot r = 1.06 km			
	Inside (n=254) n (%)	**Outside** (n=444) n (%)	OR (95%CI)	p	**Inside** (n=57) n (%)	**Outside** (n=641) n (%)	OR (95%CI)	p	**Inside** (n=66) n (%)	**Outside** (n=632) n (%)	OR (95%CI)	p
***P. falsiparum* infection by:**												
Microscopy*	8 (3)	6 (1)	2.58 (0.87; 7.61)	0.087	2 (4)	12 (2)	2.00 (0.42; 9.41)	0.381	3 (5)	11 (2)	2.87 (0.76; 10.86)	0.120
qPCR†	18 (7)	15 (3)	2.36 (1.15; 4.82)	0.019	4 (7)	29 (5)	1.51 (0.50; 4.57)	0.461	5 (8)	28 (4)	1.73 (0.63; 4.71)	0.287
Seropositivity for:												
p5	35 (14)	31 (7)	2.14 (1.28; 3.59)	0.004	11 (19)	55 (9)	2.44 (1.18; 5.05)	0.016	11 (17)	55 (9)	1.99 (0.97; 4.06)	0.059
p8	37 (15)	48 (11)	1.36 (0.85; 2.17)	0.195	16 (28)	69 (11)	3.41 (1.78; 6.53)	<0.001	18 (27)	67 (11)	3.25 (1.76; 6.02)	<0.001
p5+8	62 (24)	72 (16)	1.65 (1.12; 2.44)	0.011	23 (40)	111 (17)	3.27 (1.82; 5.85)	<0.001	25 (38)	109 (17)	2.91 (1.68; 5.06)	<0.001
AMA1	240 (94)	406 (91)	1.60 (0.84; 3.04)	0.151	55 (96)	591 (92)	2.56 (0.60; 10.94)	0.204	63 (95)	583 (92)	1.86 (0.56; 6.24)	0.312
$MSP1_{19}$	226 (89)	385 (87)	1.20 (0.74; 1.96)	0.458	54 (95)	557 (87)	3.05 (0.92; 10.13)	0.069	61 (92)	550 (87)	1.98 (0.76; 5.18)	0.161
Parity												
Primigravidae	62 (24)	128 (29)			16 (28)	174 (27)			17 (26)	173 (27)		
Multigravidae	192 (76)	316 (71)		0.217	41 (72)	467 (73)		0.877	49 (74)	459 (73)		0.885
Age												
<20	60 (24)	128 (29)			19 (33)	26 (169)			20 (30)	168 (27)		
20-24	66 (26)	102 (23)			17 (30)	151 (24)			20 (30)	148 (23)		
≥ 25	128 (50)	214 (48)		0.296	21 (37)	321 (50)		0.150	26 (39)	316 (50)		0.225
IPTp												
Sulfadoxine-pyrimethamine	42 (17)	83 (19)			14 (25)	111 (17)			16 (24)	109 (17)		
Mefloquine	163 (64)	261 (59)			35 (61)	389 (61)			39 (59)	385 (61)		
Placebo‡	49 (19)	100 (23)		0.376	8 (14)	141 (22)		0.220	11 (17)	138 (22)		0.316
HIV												
Uninfected	156 (61)	264 (59)			38 (67)	382 (60)			43 (65)	377 (60)		
Infected	98 (39)	180 (41)		0.630	19 (33)	259 (40)		0.325	23 (35)	255 (40)		0.429

r, Radius of hotspot; IPTp, intermitent preventive treatment during pregnacy; Odds ratio of (sero)prevalences measured inside the hotspot compared with outside, assessed by logistic regression, adjusted by HIV, parity, age and treatment; Comparisons of parity, age, IPTp and HIV inside and outside hotspots were performed by Fisher exact text.

*Maternal microscopic infection defined by the presence of *P.falciparum* parasites in peripheral blood or in placenta on microscopic or histologic examination, respectively.

†Maternal qPCR-positive infection was defined by a positive result on qPCR testing in peripheral or placental blood.

‡All HIV infected women who received placebo were under cotrimoxazol prophylaxis.

Table S12. Characteristics of study population inside and outside serological hotspots.

	Peptide p5 hotspot r = 2.81 km			Peptide p8 hotspot r = 1.02 km			Composite p5+8 hotspot r = 1.06 km		
	Inside (n=254) n (%)	**Outside** (n=444) n (%)	p	**Inside** (n=57) n (%)	**Outside** (n=641) n (%)	p	**Inside** (n=66) n (%)	**Outside** (n=632) n (%)	p
Parity									
Primigravidae	62 (24)	128 (29)		16 (28)	174 (27)		17 (26)	173 (27)	
Multigravidae	192 (76)	316 (71)	0.217	41 (72)	467 (73)	0.877	49 (74)	459 (73)	0.885
Age									
<20	60 (24)	128 (29)		19 (33)	26 (169)		20 (30)	168 (27)	
20-24	66 (26)	102 (23)		17 (30)	151 (24)		20 (30)	148 (23)	
≥ 25	128 (50)	214 (48)	0.296	21 (37)	321 (50)	0.150	26 (39)	316 (50)	0.225
IPTp									
Sulfadoxine-pyrimethamine	42 (17)	83 (19)		14 (25)	111 (17)		16 (24)	109 (17)	
Mefloquine	163 (64)	261 (59)		35 (61)	389 (61)		39 (59)	385 (61)	
*Placebo**	49 (19)	100 (23)	0.376	8 (14)	141 (22)	0.220	11 (17)	138 (22)	0.316
HIV									
uninfected	156 (61)	264 (59)		38 (67)	382 (60)		43 (65)	377 (60)	
infected	98 (39)	180 (41)	0.630	19 (33)	259 (40)	0.325	23 (35)	255 (40)	0.429

r. Radius of hotspot; IPTp, intermitent preventive treatment during pregnacy.
* All HIV infected women who received placebo were under cotrimoxazol prophylaxis.
Comparisons were performed by Fisher exact text.

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GENERAL DISCUSSION

Overview

Timely monitoring of malaria transmission and disease is a key pillar of the WHO 2016-2030 Global Malaria Strategy[5] and underpins the success of malaria control and elimination programs[244]. Studies conducted in Sierra Leona[238] and Tanzanian[239] communities, as well as a meta-analysis of prevalence data from African children and pregnant women[225], suggest that the patterns of *P. falciparum* infection in pregnancy reflect transmission intensity in their communities. Pregnant women develop antibodies to VAR2CSA (parasite antigen that binds to Chondroitin Sulphate A in the placenta[42]) even with only limited exposure to placental parasites[77]. Through VAR2CSA antibody detection, cumulative prevalence of *P. falciparum* exposure during pregnancy can be more accurately estimated. This is important especially in low endemic areas where the opportunity to detect antibodies is greater than detecting the actual parasite. Therefore, passive case detection systems among pregnant women at maternal health services may provide an easy-to-implement method for tracking malarial transmission in Africa, complementing the current traditional surveillance programs.

Our first intention with this thesis was to understand the malaria trends and the consequences of malaria resurgence in parasitological, immunologic and clinical outcomes among pregnant women delivering between 2003 and 2012 in the Manhiça District in southern Mozambique **(chapter 1)**. After the observation that the prevalence of malaria infection in pregnant women was paralleled by changes in levels of anti-VAR2CSA IgG antibodies, we focused on the development of a multiplex bead-based immunoassay to detect antibodies against a panel of new peptides covering conserved and semi-conserved regions of VAR2CSA **(chapter 2).** The multiplex bead-based immunoassay, developed in chapter 2 was successfully implemented and results showed that responses to 25 new VAR2CSA peptides were specific among pregnant women and were capable to distinguish periods of different malaria intensities in Mozambique between 2003 and 2012, suggesting their potential as serological markers for surveillance of malaria transmission. Stemming from previous evidence, we aimed to expand this concept in

chapter 3 by identifying a set of VAR2CSA-peptides for use in a serological assay to infer recent malaria exposure as well as to estimate temporal and spatial trends of *P. falciparum* exposure in pregnant women.

Main findings

In **chapter 1** we demonstrated the existence of marked changes over a 10-year period in the prevalence of *P. falciparum* parasitemia among pregnant Mozambican women attending for delivery at Manhiça District Hospital. *P. falciparum* prevalence by qPCR in peripheral blood declined from 33% in 2003-2004 to 2% in 2010 with a resurging trend reaching a parasite prevalence of 6% in 2012. The decline in the prevalence of malaria between 2003 and 2010, was consistent with reductions in other areas of sub-Saharan Africa over similar time frames[8,13,16,22], suggesting a continent-wide trend during this period. These changes over a 10-year period were associated with increasing parasite densities and a larger adverse effect of infection on maternal hemoglobin levels and on the weight of newborns during the period of low malaria prevalence. It was previously described that malarial immunity requires boosting to be maintained over time[245], and a reduction in parasite exposure can lead to a loss of population-level immunity and an increase in the harmful effects of malaria infections during resurgences[79]. Moreover, women when pregnant have an increased susceptibility to malaria infection[73], and the consequences of a reduction in malarial immunity could be particularly severe, especially in the context of HIV pandemics in Africa[246]. The fall and rise in the prevalence of malaria infection observed in our study was paralleled by changes in levels of antimalarial IgG antibodies. This is in accordance with previous results demonstrating that acquisition of antibodies against VAR2CSA followed the force of malaria transmission[247], with pregnant women living in rural areas having higher levels of antibodies than women in urban areas where transmission is lower[125,136,139,248]. Moreover, antibody responses to VAR2CSA in pregnant women are affected by variables that influence the risk of exposure to *P. falciparum* such as parity, season, neighborhood, IPTp and use of ITNs[128-130].

These findings have three main implications. Firstly, they show the relatively short timescales over which antimalarial immunity can be lost, leading to an increase in the susceptibility to severe malaria. In the present study, only five years of a marked malaria decline caused a 2.8-fold reduction in the IgG antibodies against malaria antigens. Secondly, these results demonstrate the importance of sustaining efforts when moving from control to elimination, as reappearance of malaria in areas where infection is either no longer circulating, or at very low levels, can be accompanied by increased harmful effects of malaria infection if population-level immunity is lost. Thirdly, regular sampling of VAR2CSA antibody levels in pregnant women at maternal health clinics could be more useful to assess the cumulative exposure during a particular pregnancy than measuring parasitemia at delivery, especially in low-transmission settings where the possibility of detecting current infection is low[192]. These findings suggest that antibody testing in pregnant women could provide a measure of malarial transmission and serve as an estimate of the risk for the detrimental consequences of malaria infection in a population, if malaria transmission recurred. Finally, if sensitive enough, a metric of malaria transmission based on VAR2CSA antibodies could help to guide malaria eradication activities by allowing assessment of transmission to at-risk populations in areas where malaria incidence has decreased substantially.

In **chapter 2** we describe the first high-throughput method to multiplex the measurement of antibodies in plasma or DBS against 46 peptides and three recombinant proteins (DBL3X, DBL5Ɛ and DBL6Ɛ) derived from the malaria in pregnancy specific protein VAR2CSA. The development of multiplex bead-based immunoassays (xMAP® system - Luminex), capable of screening more than one antigen simultaneously in one batch of serum sample volume, increased the number of studies aiming to discover novel serological markers. Several studies have implemented the multiplex immunoassay for serology and identified similar outcomes to ELISA[219,249,250]. In addition, the multiplex immunoassay proved to be reproducible[201,219,249] as observed in our study with a very good intra- and inter-plate reproducibility. Based on the analysis of field blood samples from Spanish and Mozambican participants and after optimization of the multiplex immunoassay, we identified

25 new VAR2CSA-derived synthetic peptides. Results showed that responses were specific among pregnant women and were capable to distinguish periods of different malaria intensities in Mozambique between 2003 and 2012. Moreover, we describe a new method to assess the quality of the elutions from DBS for serological purposes. To collect DBS instead of peripheral blood samples is logistically easy to implement in malaria endemic regions. However, visual inspection of the quality of antibody elution only depends on operator decisions being a limitation of this approach[200,251]. To overcome this limitation, we suggest a new method based on the measurement of hemoglobin and anti-tetanus antibodies on the product of elution. Measure these parameters together with visual inspection, allows a selection of correctly eluted DBS samples based on quantitative and not only on qualitative criteria. Additionally, we suggest that calculation of seropositivity cutoffs derived from a finite mixture model[192] applied to a field population is an alternative to using never exposed individuals in context of heterogeneous transmission. This results in a logistical simplification of studies, as no additional recruitment of naive individuals is needed. The VAR2CSA multiplex assay developed here allows an easy and rapid measurement of IgGs against several VAR2CSA-based peptides using small amounts of plasma or DBS. This technology can be applied to identify immune correlates of protection against malaria in pregnancy as well as for malaria surveillance in elimination contexts.

Although the role of antibodies against VAR2CSA has been extensively assessed to identify immune correlates of protection against malaria in pregnancy[131], to our knowledge the study reported in **chapter 3** is the first to address the potential of these antibodies to infer recent malaria exposure in pregnant African women. In this study (**chapter 3)** we used the multiplex suspension array developed in **chapter 2** to measure IgGs against a set of VAR2CSA peptides that maximize the information on previous exposure to *P. falciparum* during pregnancy. After calibrating the assay to provide measures of recent exposure[203] by selecting VAR2CSA peptides recognized by short-lived IgGs, we have applied this approach to blood samples from Mozambican, Beninese, Gabonese, Kenyan, Tanzanian and Spanish

pregnant women. We demonstrated that two VAR2CSA peptides (peptide p5 and p8) of limited diversity are immunogenic, accessible to antibodies naturally acquired by pregnant women and readily boosted upon infection during pregnancy but do not have long-lasting persistence in blood. IgG seroprevalences against these peptides reflect differences in malaria burden over time and space, changes in parasite exposure associated with the use of different IPTp regimens and allow the identification of local clusters of transmission that are missed by detection of active infections[214].

The selected peptides are exposed on the N-terminal region of VAR2CSA protein (DBL1X-ID1), a domain previously reported to be accessible in the full-length VAR2CSA[252] and to contain immuno-dominant epitopes driving the humoral immune response[65]. Sequences obtained from *P. falciparum* isolates from a variety of African settings show limited diversity of regions corresponding to the selected peptides, with a sequence identity similar to ranges obtained for the DBL1X domains in previous studies (between 69.5% and 89.2%[62]). Peptide p8 is included in a ID1 dimorphic region that is the most frequent variant in African parasites[253]. The results of this study show that the sequences covered by these peptides are accessible to antibodies acquired by pregnant women from different African regions, and that the genetic background of parasites may not affect the immunological responses.

Short half-lived antibodies have been described to be the most informative of recent exposure and allowing to identify rapid changes in local transmission[203,254]. Short half-life was estimated for antibodies against p5 and p8 (below two years, the average time reported in Mozambique for a second pregnancy to occur[255]) suggesting that antibodies acquired in one pregnancy may not be maintained over multiple pregnancies in most women. This observation is consistent with the similar IgG levels observed in multigravid compared with primigravid women. Moreover, previous studies showed that antibody levels against DBL1X increase slowly over pregnancy in Cameroonian pregnant women[125], suggesting limited or no stable long-lasting memory B-cell responses responsible for the maintenance of antibody

production[87]. In contrast, the parity-dependent increases of immunity typical of placental-type parasites[256] was observed for VAR2CSA recombinant domains (DBL3X, DBL5Ɛ and DBL6Ɛ) as reported previously[40]. The parity-dependent response agrees with the long half-life (36–57.6 years) of antibodies against the recombinant protein DBL5Ɛ domain that was reported in uninfected Thai pregnant women[143]. Several studies have shown that HIV infection can impair the *P. falciparum*-specific antibody responses in pregnant women[257] and also accelerate the decay of antibodies in the absence of parasite exposure compromising the long-term serologic memory[128]. However, our results show that temporal trends assessed by seroprevalences against p5 and p8 were similar among HIV-uninfected and infected women, suggesting that short-lived IgG responses against p5 and p8 may not be affected by HIV infection. These data suggest that immune biomarkers based on p5 and p8 from VAR2CSA might be reliable indicators of recent exposure independently of parity or HIV status of pregnant women. It is important to refer that peptides targeted by long-lasting IgG responses (e.g. peptide p1) may also be useful to capture past changes in transmission by sampling women of child-bearing age and relating seroprevalence with the number and timing of previous pregnancies. Our findings may allow the design of field-deployable rapid tests detecting antibodies against VAR2CSA peptides in pregnant women. Such tests may thus constitute a pragmatic complement for routine survey strategies to flag critical steps in programmatic environments where year-to-year changes in transmission need to be monitored and inform decision-making in real time.

Limitations

This study has several limitations. Firstly, being a hospital-based convenience sampling it cannot claim that the data are representative of changing epidemiology in the whole community. Secondly, we cannot discard differences in the parasitological and molecular measurements both along time and in space along the project. However, the impact of these differences was minimized by the fact that this study was performed in the context of clinical trials following standard procedures[241-243] and that the analysis was adjusted by potential confounders. Thirdly, the peptide array used may have

missed some conformational non-linear epitopes. Although, this limitation may have resulted in the lack of identification of some potential biomarkers, this concern is largely mitigated by the ability to screen peptides covering VAR2CSA simultaneously, many of which likely provide similar information. Indeed, the identification of antigen targets that may be subsequently easier to produce is of potential benefit. Of note, the array used in this study also included purified, validated recombinant proteins for commonly used antigens, such as AMA1 and MSP1, at a wide range of dilutions, and none of these constructs were identified as among the most informative in our study. Fourthly, dynamics of antibody acquisition and maintenance (non-linear mixed effects modeling) may not be generalizable to other settings with different intensities of transmission. This is because more frequent or prolonged infections might lead to greater boosting and maintenance of responses. Although the degree to which some serologic biomarkers can predict exposure will likely vary in these contexts [94,97,99,106,107], calibration of antibody responses was done in a low endemic setting in Southern Mozambique, mimicking low transmission in elimination setting. Different transmission dynamics and host genetic factors may affect the frequency and duration of infections and thus the dynamic of antibodies[143]. Fifthly, steeper decay of antibodies may be observed out of pregnancy when infecting parasites express non-VAR2CSA variants. Sixthly, small numbers of pregnant women from malaria-free areas in Tanzania and Spain may limit the generalizability of our data to support VAR2CSA-serology as a tool to confirm interruption of transmission. Finally, antibody assessments in this study were mainly conducted at delivery, and further studies need to consider the performance of this serology at first antenatal visits or soon after delivery (i.e., during infant immunization).

Further research

If future studies confirm our observations, development of simple antibody-detecting RDTs[258-260] based on synthetic peptides, will allow the use of VAR2CSA-serology on field samples[261] for routine surveillance[214]. Production of synthetic peptides for RDTs development can be easily

standardized to allow for consistent measurement of antibody seroprevalences using DBS[191,251] or whole blood obtained from finger pricks in appropriate settings. Moreover, measure anti-malarial seroprevalences could be a practical solution to estimate exposure to infection without excessive sampling particularly in low transmission setings[192]. Antibodies against the VAR2CSA peptides identified in this thesis (peptides p5 and p8) capture an infection during a defined period of time (one pregnancy) and sample sizes required to detect exposure to the antigen are lower than to detect active infection[262]. However, the choice of a particular sample size must involve weighing statistical precision with ethical issues, available human and economic resources, and possible time constraints[263]. Also, the sample size determination must have into account varying transmission intensities. Thus, optimizing sample size is crucial to avoid wasting valuable resources[264,265]. Implementation of optimized study designs is particularly important for countries on the brink of malaria elimination or eradication. Simple assays derived from VAR2CSA-based RDTs could reduce costs, simplify testing, avoid cold chain, reduce time for results to reach central agencies, and provide immediate feedback to individuals and communities about ongoing exposure. Finally, this approach has the potential to generate rich epidemiologic surveillance data that would be widely accessible to malaria control programs mainly in contexts of low burden.

The use of maternal health care platforms to describe and predict the levels of transmission in the general population does however require further validation and optimization. Notable areas of investigation that require attention include studies on the representativeness of women attending antenatal care and maternity and how this varies in relation to levels of malaria endemicity. Representativeness and external validity of the study will depend on the level and equity of maternal clinics demographic distribution and attendance. Women attending health care may indicate greater access to resources, better education or knowledge, social support, or even just geographic proximity to the clinic[233]. Consequently, non-attendance at maternal clinic may be linked to demographic characteristics that could also be related to malaria exposure. Another factor that can affect the

representativeness of data is the impact that malaria infection may have on fertility and attendance to maternal clinics (as has been suggested for HIV infection[230]). However, malaria is less likely to lead to the strong fertility biases observed for sexually transmitted infections[231]. Because of these reasons, caution should be employed in generalizing the findings of surveillance to all pregnant women, if a substantial proportion of pregnant women do not attend maternal clinics. Moreover, rates of maternal health care attendance in a country may not be uniform and could differ both among geographical regions and over time. Thus, a continuous and broad data collection is important to obtain reliable measures. Notably, the antenatal care coverage has increased in Sub-Saharan Africa during the last decade, with over 78% of pregnant women having at least one antenatal care contact during pregnancy[226]. It is likely that enrolment will continue to expand and maternal care platforms should increasingly provide a representative sample of community events to be generalizable to most *P. falciparum* endemic countries in Africa. Importantly, surveillance programs should carefully consider the impact of changes in maternal care attendance during data interpretation.

In spite of the adverse clinical impact of malaria in pregnant women, chemotherapy approaches for malaria prevention (IPTp with SP[241]) and elimination (MDA with DHAp) need to exclude women during the first trimester of pregnancy given contraindications or limited efficacy and safety data of antimalarials at early periods of gestation[185]. Such an increased risk of malaria and imperfect prevention during the first trimester makes the screening of pregnant women at first antenatal visit (before receiving preventive measures) not only a major health requirement but also an important source of information to assess malaria transmission while allowing the treatment of early infections. The results of our study were obtained with samples collected at delivery but validation of samples collected at first antenatal visits is still required. Moreover, assessment of serologic responses in communities before and after implementation of malaria control measures will be needed to validate the utility of serology in evaluating the impact of these interventions.

Here we developed a malaria in pregnancy-specific serological assay based on short-lived anti-VAR2CSA antibodies targeting recent malaria exposure. As discussed above, this technology should be transposed to an RDT format to be implemented in a representative group of pregnant women attending antenatal care before IPTp administration. To implement this serological surveillance in malaria endemic countries should be logistically easily and cost-effective compared to other currently used measures of malaria transmission (parasite and entomological rates). If proved that pregnant women attending maternal clinics are representative from surrounding community, this serological platform should be able to generate reliable and contemporary data to detect changes in levels of malaria burden in the population, to stratify areas into regions with ongoing or no recent transmission, to identify localized geographical areas with higher burdens of malaria (hotspots), to identify imported cases and to assess the impact of deliberate intervention in reducing malaria transmission. Finally, detection of antibodies in people such as pregnant women, who are most likely to be infected, can increase the sensitivity of the method to confirm interruption of malaria transmission (freedom from infection)[266] and detect reintroduction of *P. falciparum* transmission after local elimination. Moreover, the implementation of a surveillance system based on a sentinel population of pregnant women is also an opportunity to collect extra blood stored in DBS allowing to generate a biobank of biologic material that can be useful to monitor other factors such as antimalarial resistance and parasite gene flow.

Finally, programmatic research is essential to assess feasibility, acceptability, and cost-effectiveness, and to develop approaches to integrate this pregnancy-based sero-surveillance tool into malaria control and elimination programs as well as to link with other surveillance strategies.

CONCLUSIONS

The main conclusions of this thesis are the following:

1. Parasite prevalence in pregnant women from Manhiça (southern Mozambique) fell from levels consistent with stable transmission in the 2003-2005 period to levels in 2010 comparable to those reported from areas of low or unstable transmission, and then slightly increased until 2012.

2. The reduction of malaria in southern Mozambique was accompanied by increases in parasite densities and reductions of newborn weight in those women still infected.

3. Rise in malaria, with increased parasite densities and larger adverse clinical effects, after a marked fall in the prevalence of malaria and antimalarial immunity in pregnant women reinforces the importance of sustaining efforts to avoid rebounds of malaria associated with reductions in naturally acquired immunity.

4. Levels of IgG antibodies against the parasite strain that expresses VAR2CSA (CS2) mirrored changes in the prevalence of malaria infection. This suggests that measuring these antibodies may be useful to estimate changes in the intensity of malaria transmission during elimination activities.

5. A multiplex bead-based immunoassay to detect antibodies against 46 new-peptides and recombinant-domains (DBL3X, DBL5Ɛ and DBL6Ɛ) derived from the malaria in pregnancy protein VAR2CSA was successfully implemented. This platform provides a high-throughput approach to investigating correlates of protection and identifying serological markers of exposure for malaria in pregnancy.

6. A new method to assess the quality of the elutions from DBS for serological purposes was described. Moreover, calculation of anti-VAR2CSA IgG levels seropositivity cutoffs using a finite mixture model proved to be an alternative to using never exposed individuals in contexts of heterogeneous transmission.

7. IgG responses against 25 VAR2CSA new-peptides were specific of pregnant women and dependent on the level of exposure to malaria.

8. Two VAR2CSA peptides (p5 and p8) were selected as potential serological markers to infer recent malaria exposure as well as to estimate temporal and spatial trends of malaria transmission in pregnant women because:

    a) They are of limited diversity in their sequence, immunogenic, accessible to naturally acquired antibodies, readily boosted upon infection during pregnancy, but do not have long-lasting persistence in blood.

    b) IgG responses against these peptides reflect differences in malaria burden over time and space, changes in parasite exposure associated with the use of different IPTp regimens and allow the identification of local clusters of transmission that are missed by detection of active infections.

Final consideration

The findings of this thesis lay the foundation for the development of methods to describe, reconstruct and predict the relationship between *P. falciparum* exposure in pregnancy and malaria transmission in the general population. Antibody-detecting RDTs based on VAR2CSA synthetic peptides can provide consistent and contemporary data to assess the level, and changes in levels, of malaria burden in the population, as well as to quantify the impact of interventions and identify geographical areas with higher burdens of malaria. Routine serological screening of readily accessible pregnant women would offer a rapid and cost-effective method to optimize the timely deployment of interventions and the accurate tracking of progress, eventually confirming the interruption of malaria transmission and providing warning signals for *P. falciparum* resurgence after local elimination.

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ANNEX

Other publication

Resisting and tolerating *P. falciparum* in pregnancy under different malaria transmission intensities

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Resisting and tolerating *P. falciparum* in pregnancy under different malaria transmission intensities

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Abstract

Background: Resistance and tolerance to *Plasmodium falciparum* can determine the progression of malaria disease. However, quantitative evidence of tolerance is still limited. We investigated variations in the adverse impact of *P. falciparum* infections among African pregnant women under different intensities of malaria transmission.

Methods: *P. falciparum* at delivery was assessed by microscopy, quantitative PCR (qPCR) and placental histology in 946 HIV-uninfected and 768 HIV-infected pregnant women from Benin, Gabon, Kenya and Mozambique. Resistance was defined by the proportion of submicroscopic infections and the levels of anti-parasite antibodies quantified by Luminex, and tolerance by the relationship of pregnancy outcomes with parasite densities at delivery.

Results: *P. falciparum* prevalence by qPCR in peripheral and/or placental blood of HIV-uninfected Mozambican, Gabonese and Beninese women at delivery was 6% (21/340), 11% (28/257) and 41% (143/349), respectively. The proportion of peripheral submicroscopic infections was higher in Benin (83%) than in Mozambique (60%) and Gabon (55%; $P = 0.033$). Past or chronic placental *P. falciparum* infection was associated with an increased risk of preterm birth in Mozambican newborns (OR = 7.05, 95% CI 1.79 to 27.82). Microscopic infections were associated with reductions in haemoglobin levels at delivery among Mozambican women (−1.17 g/dL, 95% CI −2.09 to −0.24) as well as with larger drops in haemoglobin levels from recruitment to delivery in Mozambican (−1.66 g/dL, 95% CI −2.68 to −0.64) and Gabonese (−0.91 g/dL, 95% CI −1.79 to −0.02) women. Doubling qPCR-peripheral parasite densities in Mozambican women were associated with decreases in haemoglobin levels at delivery (−0.16 g/dL, 95% CI −0.29 to −0.02) and increases in the drop of haemoglobin levels (−0.29 g/dL, 95% CI −0.44 to −0.14). Beninese women had higher anti-parasite IgGs than Mozambican women ($P < 0.001$). No difference was found in the proportion of submicroscopic infections nor in the adverse impact of *P. falciparum* infections in HIV-infected women from Kenya (*P. falciparum* prevalence by qPCR: 9%, 32/351) and Mozambique (4%, 15/417).

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Conclusions: The lowest levels of resistance and tolerance in pregnant women from areas of low malaria transmission were accompanied by the largest adverse impact of *P. falciparum* infections. Exposure-dependent mechanisms developed by pregnant women to resist the infection and minimise pathology can reduce malaria-related adverse outcomes. Distinguishing both types of defences is important to understand how reductions in transmission can affect malaria disease.

Trial registration: ClinicalTrials.gov NCT00811421. Registered 18 December 2008.

Keywords: Malaria, Pregnancy, Immunity, Resistance, Tolerance

Background

As the rest of the population, pregnant women living in areas of different malaria endemicity experience varying degrees of exposure to infection, which affects the acquisition of antimalarial immunity and determines the course of disease [1, 2]. Protective immunity to *Plasmodium falciparum* in pregnancy has been suggested to rely mostly on antibodies against VAR2CSA that block adhesion of infected erythrocytes to placental chondroitin sulphate A, and thereby prevent parasite sequestration in the placenta [3]. Such immune resistance to *P. falciparum*, which is acquired after exposure to placental-type parasites, reduces parasite densities below the detection limit of microscopy (i.e. submicroscopic infections) [4–6] and can eventually clear placental infections.

Infected hosts may also tolerate the presence of *P. falciparum* by minimising parasite-induced damage without necessarily limiting the infection [7–9]. This type of host defence, not to be confused with immunological tolerance [7, 10], has been suggested by the frequent observation in malaria endemic areas of individuals, including pregnant women, who harbour levels of parasitaemia in their blood that would commonly be associated with fever in malaria-naïve individuals [11, 12]. Moreover, the higher risk of life-threatening disease in younger age groups [13] supports the notion that the ability to modulate host inflammation (anti-disease or clinical immunity) [14, 15] develops faster than the capacity to restrict parasite growth (anti-parasite immunity). However, other studies do not support the hypothesis that a special clinical immunity exists independently of parasitological immunity [16], but rather suggest that immunity resulting in decreased parasite densities reduces the severity of symptoms.

Resolving the role of resistance and tolerance could aid the development of host-directed therapies to reduce malaria-induced immunopathology and mitigate malaria disease [9]. However, quantitative analyses of tolerance to human malaria have been mainly limited to the assessment of peripheral *P. falciparum* parasitemia needed to trigger the onset of fever (i.e. pyrogenic threshold) [14, 15]. Alternative clinical outcomes and analytical frameworks are needed for pregnant women in whom parasitaemia is poorly associated with fever [17]. Here, we aimed to assess the variations in the clinical impact of *P. falciparum* infections and in host defences developed by pregnant women under different malaria transmission intensities. To achieve this, we compared the carriage of submicroscopic infections and antibodies against *P. falciparum* antigens as indicators of the level of parasitological immunity [1]. We assessed the correlation between health outcomes (haemoglobin levels and birthweight) and parasite densities at delivery for summarising tolerance [8], with a flat slope indicative of tolerance to infection. As immune resistance is strongly influenced by the number of previous pregnancies in areas of stable transmission [18] and pregnancy outcomes can be affected by the preventive measures used during pregnancy, analyses were adjusted for parity of the pregnant women and the antimalarials received as intermittent preventive treatment during pregnancy (IPTp).

Methods

Study populations

This study was conducted between 2010 and 2012 in four sub-Saharan countries (Additional file 1: Figure S1), namely Benin (Allada, Sékou and Attogon), Gabon (Lambaréné and Fougamou), Kenya (Siaya), and Mozambique (Manhiça and Maragra). Pregnant women were enrolled in the context of the Malaria in Pregnancy Preventive Alternative Drugs clinical trial (ClinicalTrials.gov NCT0081121; Table 1) [19, 20]. At enrolment, pregnant women received long-lasting insecticide-treated bed-nets. Following national guidelines in place, HIV status was assessed after voluntary HIV counselling and testing with an HIV rapid test and positive results were confirmed with a second rapid test [19, 20]. Haemoglobin and the syphilis rapid plasma reagin (RPR) test were assessed as part of routine antenatal care on finger-prick-collected capillary blood. Among HIV-infected women, 5 mL of venous blood were taken for CD4 + T cell count by flow cytometry after staining of whole blood with CD3, CD8 and CD4 fluorochrome-labelled antibodies. Flow cytometry acquisition was performed using FACSCalibur (BD Biosciences) and TruCOUNT tubes (Becton Dickinson). Additionally, viral load determination was performed using the COBAS

Table 1 Characteristics of participants by study area

	HIV-uninfected[a]				HIV-infected[b]		
	All	Benin	Gabon	Mozambique	All	Kenya	Mozambique
	n = 946	n = 349	n = 257	n = 340	n = 768	n = 351	n = 417
Age (years), mean (SD)							
	24.3 (6.3)	25.4 (5.2)	24.4 (6.7)	23.1 (6.8)	26.7 (5.7)	26.9 (5.4)	26.5 (5.9)
Parity, n (%)							
Primigravidae	265 (28)	69 (20)	66 (26)	130 (38)	81 (11)	30 (9)	51 (12)
Multigravidae	681 (72)	280 (80)	191 (74)	210 (62)	687 (89)	321 (91)	366 (88)
Gestational age at baseline (weeks), mean (SD)							
	21.0 (4.9)	22.0 (4.1)	19.5 (5.4)	21.3 (5.2)	20.3 (5.5)	19.9 (5.9)	20.6 (5.2)
MUAC at baseline (cm), mean (SD)							
	25.9 (3.0)	25.3 (2.3)	25.7 (3.6)	26.8 (3.0)	26.8 (2.7)	27.0 (2.7)	26.7 (2.7)
RPR at baseline, n (%)							
Positive	9 (1)	2 (1)	1 (0)	6 (2)	39 (5)	12 (3)	27 (7)
Negative	928 (99)	347 (99)	247 (100)	334 (98)	726 (95)	338 (97)	388 (93)
Literate, n (%)							
No	371 (39)	280 (80)	34 (13)	57 (17)	142 (18)	16 (5)	126 (30)
Yes	575 (61)	69 (20)	223 (87)	283 (83)	626 (82)	335 (95)	291 (70)
HIV viral load (copies/mL) at baseline, n (%)[c]							
< 399					181 (24)	125 (36)	56 (13)
400/999					183 (24)	76 (22)	107 (26)
1000/9999					243 (32)	97 (28	146 (35)
10,000/max					123 (16)	49 (14)	74 (18)
UNK					38 (5)	4 (1)	34 (8)
CD4 (cells/μL) at baseline, n (%)[d]							
< 350					279 (36)	137 (39)	142 (34)
350/max					460 (60)	212 (60)	248 (59)
UNK					29 (4)	2 (1)	27 (6)
Maternal haemoglobin at baseline (g/dL), mean (SD)							
	10.4 (1.5)	10.3 (1.2)	10.3 (1.4)	10.7 (1.8)	10.1 (1.7)	10.2 (1.9)	10.0 (1.6)
IPTp, n (%)							
Antimalarial 1[e]	629 (66)	230 (66)	172 (67)	227 (67)	390 (51)	174 (50)	216 (52)
Antimalarial 2[e]	317 (34)	119 (34)	85 (33)	113 (33)	378 (49)	177 (50)	201 (48))
Gestational age at delivery (weeks),[f] mean (SD)							
	39.6 (1.7)	39.2 (1.2)	41.1 (1.8)	38.9 (1.2)	38.8 (1.2)	ND	38.8 (1.2)
Preterm birth,[g] n (%)							
No	867 (95)	328 (95)	238 (95)	301 (95)	374 (96)	ND	374 (96)
Yes	44 (5)	16 (5)	13 (5)	15 (5)	17 (4)	ND	17 (4)

[a]The IPTp trial evaluated the efficacy and safety of two doses of Intermittent Preventive Treatment in pregnancy (IPTp) with mefloquine compared to IPTp with sulphadoxine-pyrimethamine
[b]Evaluated three doses of IPTp with MQ compared to placebo in HIV-infected women on cotrimoxazole prophylaxis
[c]38 missing values
[d]29 missing values
[e]Antimalarial: 1 = Mefloquine and 2 = Sulphadoxine-Pyrimethamine for HIV-uninfected; 1 = Placebo and 2 = Mefloquine for HIV-infected
[f]Gestational age was estimated by newborn physical examination using the Ballard score
[g]Preterm birth if gestational age was < 37 weeks
IPTp intermittent preventive treatment, *MUAC* mid-upper arm circumference, *RPR* rapid plasma reagin, *UNK* unknown

AMPLICOR or AmpliPrep (Roche Diagnostics) devices. Haemoglobin was determined in capillary blood samples using mobile devices (HemoCue and Hemocontrol) [19, 20]. Gestational age at enrolment was determined from symphysis fundal height measurement using a standard tape measure (centimetres) and McDonald's rule to transform the symphysis fundal height in centimetres into gestational weeks [19, 20]. Following physical examination, recruited women with gestational age ≥ 13 weeks received their first dose of IPTp (either sulfadoxine-pyrimethamine (SP) or mefloquine (MQ) for HIV-uninfected women and either placebo or MQ for HIV-infected women) under supervision. Women allocated to the SP group received standard IPTp (three tablets of the fixed combination therapy containing 500 mg of sulfadoxine and 25 mg of pyrimethamine), whereas participants allocated to the MQ groups received 15 mg/kg of the drug. The second IPTp-SP/MQ administration for HIV-uninfected women, and the second and third administrations of IPTp-MQ/placebo for HIV-infected women, were given at least 1 month apart. All HIV-infected women also received study co-trimoxazole tablets on a monthly basis for daily prophylaxis.

At delivery, maternal haemoglobin was determined and newborns were weighed using weekly calibrated scales (either digital or three beam balances) and their gestational age at birth (except for Kenyan newborns) was evaluated using the Ballard's score [21]. Newborn weights not captured at birth but within the first week of life were estimated using a linear regression model [22]. Peripheral and placental blood smears, as well as 50 μL of maternal peripheral and placental blood spotted onto filter paper, were collected for parasitological assessments. Tissue samples were also collected from the maternal side of the placenta and placed into 10% neutral buffered formalin. Biopsies were processed, stained and examined following standard procedures [23]. From the first antenatal visit, all study women were indicated to receive ferrous sulphate-folic acid supplements for prevention of anaemia in pregnancy. If a woman was diagnosed with anaemia, she was treated with oral ferrous sulphate 200 mg/8 hours for 3 months or blood transfusion for severe cases. Clinical malaria episodes were treated with oral quinine (first trimester) or artemether-lumefantrine (subsequent trimesters) for uncomplicated malaria; parenteral quinine was used for treatment of severe malaria [19, 20].

Parasitological determinations

Thick and thin blood films, as well as placental biopsies, were read for *Plasmodium* species detection according to standard, quality-controlled procedures [19, 20, 24]. The quality of the reading across sites was controlled through a quality control program during the study. Past placental infection was defined by the presence of *P. falciparum* pigment (i.e. hemozoin) without parasite detection on placental histologic examination, and chronic placental infection was defined by the presence of *P. falciparum* pigment in combination with the detection of parasites [1]. A 30% random selection of paired peripheral and placental blood onto filter papers from HIV-negative women [20] and all the paired filter papers from HIV-infected women [19] were tested for the presence and density of *P. falciparum* in duplicate by means of a real-time quantitative polymerase chain-reaction (qPCR) targeting 18S rDNA [1, 25]. Parasitemia was quantified by extrapolation of cycle thresholds from a standard curve of *P. falciparum* ring-infected erythrocytes. Samples without amplification (no cycle thresholds detected) were considered negative, and a density of 2 parasites/μL was assigned if amplification was observed out of the lower range of the standard curve (5 parasites/μL). A negative control with no template DNA was run in all reactions. *P. falciparum* infections were considered submicroscopic if parasites were detected by qPCR but not by microscopy [1]. A quality check program was established to ensure comparable performance of qPCR techniques in different laboratories [25].

Measurement of antimalarial IgGs and *P. falciparum* Histidine-Rich Protein 2 (PfHRP2)

A random selection of 50% of peripheral plasma samples collected at delivery from HIV-uninfected women in the extremes of the malaria transmission spectrum (n = 170 from Benin and n = 170 from Mozambique) was tested for IgG levels against *P. falciparum* recombinant VAR2CSA domain (DBL3X from 3D7 strain), and the merozoite surface protein-1 ($MSP\text{-}1_{19}$; 19-kD fragment, 3D7 strain) using a multiplex suspension array panel (xMAP™ technology) and the Luminex® 100/200™ System (Luminex Corp., Austin, TX, USA) [1]. Briefly, magnetic carboxylated microspheres (MagPlex™-C, Luminex) were coupled with recombinant protein. After blocking with bovine serum albumin in phosphate-buffered saline, microspheres were sequentially incubated with 100 μL of plasma (dilutions 1:500, 1:20,000 and 1:800,000 in duplicate for each sample), 100 μL of biotinylated anti-human IgG (diluted 1:2500) and 100 μL of streptavidin-conjugated R-phycoerythrin (diluted 1:1000). The plate was immediately read using Bio-Plex Manager version 4.0, and at least 50 microspheres per analyte were acquired per sample. Crude mean fluorescent intensity was exported with background fluorescence from blank wells already subtracted. PfHRP2 [26] was quantified in plasmas available from women with peripheral qPCR-detected *P. falciparum* infection at delivery (n = 42 in Benin and n = 13 in Mozambique) using a commercial HRP2 antigen-capture ELISA (Malaria Ag CELISA kit; Cellabs) [27].

Statistical analysis

Pregnant women were included in the analysis if they had all information on IPTp type of treatment, HIV infection, age, parity, newborn weight, maternal haemoglobin levels, as well as qPCR results in peripheral and placental blood at time of delivery. Women were classified as primigravid (first pregnancy) and multigravid (at least one previous pregnancy). Age was categorised as < 25 and ≥ 25 years on the basis of median maternal age in the study population. Preterm birth was defined as a gestational age < 37 weeks. Participant's baseline characteristics, parasitological outcomes, antimalarial IgG levels and PfHRP2 levels were compared between study areas by univariate analysis and logistic or lineal regression models. Changes in maternal haemoglobin levels and birthweight with increases in qPCR-peripheral parasite density were assessed by linear regression. The impact of *P. falciparum* infections on haemoglobin levels, birthweight and preterm birth was assessed in logistic regression models. Continuous outcome variables (qPCR-parasite densities, antimalarial IgGs and HRP2 levels) exhibiting a skewed distribution were log transformed. Regression models were estimated through a backward stepwise approach with a significance level for removal from the model of 0.1 and a significance level for addition of 0.05, adjusted by type of IPTp drug and baseline covariates at recruitment (season, age, gravidity, gestational age, anaemia, literacy, RPR result and mid-upper arm circumference (MUAC)), as well as CD4 + T cell count in the case of HIV-infected women [19, 20]. The modification of the associations by study area was assessed by including interaction terms into the regression models and combining the coefficients plus the interaction and the standard error by the delta method. *P* values of less than 0.05 were considered to indicate statistical significance. Stata version 13 was used for data analysis and GraphPad version 5.01 for graphical depiction of data.

Results

P. falciparum burden in the study populations

A total of 1714 pregnant women, 946 (55%) HIV-uninfected and 768 (45%) HIV-infected, were included in this study (Table 1 and Additional file 1: Figure S1). The 3428 peripheral and placental filter papers from these 946 HIV-uninfected and 768 HIV-infected women were tested for the presence and density of *P. falciparum* by qPCR [25, 28]. Covariates at recruitment were similar in the study subgroup and the rest of the women participating in the randomised trial [19, 20] (Additional file 1: Table S1). Prevalence of qPCR-detected *P. falciparum* at delivery, either in peripheral blood at delivery or placental blood (maternal qPCR infection in Table 2), was 41% (143/349) in Benin, 11% (28/257) in Gabon and 6% (21/340) in Mozambique ($P < 0.001$) among HIV-uninfected women, and 9% (32/351) in Kenya and 4% (15/417) in Mozambique ($P < 0.001$) among HIV-infected women (Fig. 1a and Table 2). Similar trends were observed for maternal microscopic infections (Fig. 1b and Table 2). The relationship between microscopy positivity and qPCR parasitaemia in the different study sites is presented in Additional file 1 (Table S2). The proportion of samples that were negative by microscopy but for which qPCR indicated a parasitaemia above 200 parasites/μL was not different between sites (Additional file 1: Table S3).

Effects of *P. falciparum* infection on health outcomes

Microscopic *P. falciparum* infection detected at delivery in peripheral blood and/or the placenta of Mozambican women was associated with a reduction of 1.17 g/dL (95% CI −2.09 to −0.24, $P = 0.014$) in their haemoglobin levels, but this reduction was not observed among Beninese nor Gabonese women ($P = 0.581$ and $P = 0.154$, respectively; Fig. 2a). Microscopic infections were also associated with larger drops in haemoglobin levels from recruitment to delivery in Mozambican (−1.66 g/dL, 95% CI −2.68 to −0.64, $P = 0.001$) and Gabonese (−0.91 g/dL, 95% CI −1.79 to −0.02, $P = 0.045$) women, but not among Beninese women ($P = 0.213$; Fig. 2c). No impact of microscopic *P. falciparum* infection in the mother was observed on the birthweight, irrespective of study area (Fig. 2e). However, past or chronic placental *P. falciparum* infection, defined by the presence of *P. falciparum* pigment (i.e. hemozoin) on placental histologic examination, was associated with a reduction of 247.2 g (95% CI −479.1 to −15.2, $P = 0.037$) in the birthweight of Mozambican babies, which was not observed in Benin ($P = 0.569$) and Gabon ($P = 0.486$). Given the strong association between birthweight and gestational age at delivery (Additional file 1: Table S4), the regression model assessing the relationship between birthweight and infection was adjusted by preterm birth, showing a loss of statistical significance ($P = 0.192$). However, past or chronic placental *P. falciparum* infection were associated with an increased risk of preterm birth in Mozambican newborns (OR = 7.05, 95% CI 1.79 to 27.82, $P = 0.005$), but not among Beninese ($P = 0.785$) and Gabonese newborns ($P = 0.639$). Microscopic *P. falciparum* infection was not associated with reductions in haemoglobin levels of HIV-infected women from Kenya and Mozambique nor with reductions of birthweight (Fig. 2b, d, f). Finally, no adverse clinical impact was observed for *P. falciparum* submicroscopic infections, defined by the detection of *P. falciparum* by qPCR but not by microscopy, irrespective of the HIV-infectious status of the pregnant women (Additional file 1: Figure S2). Adjusting variables that remained in the final regression models and interactions assessed with parity, age and IPTp treatment are detailed in Table S5 and S6 of Additional file 1.

Table 2 Parasitological outcomes in peripheral blood and placenta by study area

	HIV-uninfected				HIV-infected		
	Benin	Gabon	Mozambique	*P*	Kenya	Mozambique	*P*
Periphery at delivery							
Smear, n (%)							
Negative	320 (92)	245 (96)	333 (98)	0.002[a]	332 (95)	408 (98)	0.025[a]
Positive	27 (8)	11 (4)	7 (2)		17 (5)	8 (2)	
qPCR, n (%)							
Negative	242 (69)	235 (91)	325 (96)	<0.001[a]	326 (93)	405 (97)	0.007[a]
Positive	107 (31)	22 (9)	15 (4)		25 (7)	12 (3)	
qPCR parasites/μL, GM (SD)	280.5 (882.9)	684.2 (1703.2)	514.4 (1630.6)	0.484	141.4 (329.1)	3350.9 (9887.2)	0.003
Placenta							
Smear, n (%)							
Negative	322 (93)	244 (96)	332 (98)	0.018[a]	335 (95)	406 (98)	0.032[a]
Positive	23 (7)	9 (4)	8 (2)		16 (5)	7 (2)	
Histology, n (%)							
Acute	8 (2)	5 (2)	1 (0)	<0.001	6 (2)	4 (1)	<0.001
Chronic	23 (7)	2 (1)	2 (1)		5 (1)	1 (0)	
Past	37 (11)	28 (11)	13 (4)		64 (18)	14 (3)	
No infected	277 (80)	218 (86)	322 (95)		276 (79)	387 (95)	
qPCR, n (%)							
Negative	253 (72)	235 (91)	323 (95)	<0.001[a]	324 (92)	407 (98)	0.001[a]
Positive	96 (28)	22 (9)	17 (5)		27 (8)	10 (2)	
qPCR parasites/μL, GM (SD)	668.5 (2473.1)	1284.9 (5100.0)	1876.2 (5910.5)	0.609	379.5 (1255.4)	5851.3 (19950.4)	0.033
Maternal microcopy infection,[b] n (%)							
Negative	294 (87)	239 (95)	329 (97)	<0.001[a]	325 (93)	396 (98)	0.002[a]
Positive	45 (13)	12 (5)	9 (3)		24 (7)	9 (2)	
Maternal qPCR infection,[b] n (%)							
Negative	206 (59)	229 (89)	319 (94)	<0.001[a]	319 (91)	402 (96)	<0.001[a]
Positive	143 (41)	28 (11)	21 (6)		32 (9)	15 (4)	

[a]The *P* value is significant according to multivariate analysis adjusted for type of IPTp drug, season, age, gravidity, gestational age, anaemia, literacy, RPR result and MUAC at recruitment, as well as CD4 + T cell count in the case of HIV-positive women (Wald test) (ND for placental histology)
[b]Maternal infections were considered microscopic if *P. falciparum* parasites were observed in peripheral blood at delivery or in placental blood from the maternal side either by microscopy or histology and qPCR infections if peripheral blood at delivery or placental blood samples were positive by qPCR
GM geometric mean, *SD* standard deviation

Submicroscopic *P. falciparum* infections, antimalarial immunity and HRP2 levels

The proportion of peripheral submicroscopic infections among HIV-uninfected women was the highest in Benin (83%; 82/106), followed by Mozambique (60%, 9/15) and Gabon (55%, 12/22, $P_{adjusted} = 0.033$; Fig. 3a). Similar trends, although not statistically significant, were observed for placental submicroscopic infections (82% (78/95), 58% (10/17) and 71% (15/21), respectively, $P_{adjusted} = 0.674$; Fig. 3b). Among pregnant women with a qPCR-detected infection either in peripheral or placental blood, the proportion of submicroscopic infections in any of the two compartments was the highest in Benin (94%, 124/132), followed by Gabon (82%, 18/22) and Mozambique (74%, 14/19, $P_{adjusted} = 0.028$). PfHRP2 concentrations, indicative of overall parasite biomass [26], were higher in pregnant women with a peripheral qPCR-detected infection from Mozambique (n = 13) than from Benin (n = 42, $P = 0.048$; Fig. 3c). HRP2 levels decreased with parity of pregnant women in Benin (0.13, 95% CI 0.02 to 0.79, $P = 0.028$) but not in Mozambique (1.09, 95% CI 0.09 to 12.21, $P = 0.945$). In contrast, age was not associated with differences in HRP2 levels among Beninese ($P = 0.052$) and Mozambican women ($P = 0.123$). Levels of IgGs against DBL3X from VAR2CSA and MSP1 were higher in women from Benin than in women from Mozambique ($P < 0.007$; Fig. 3d). IgG responses against MSP1 increased with parity of Beninese pregnant women (2.39, 95% CI 1.49 to

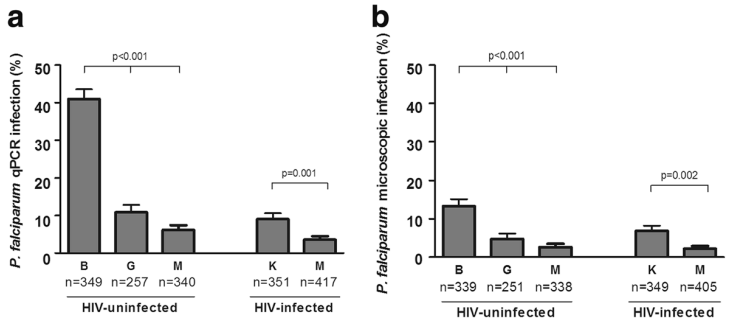

Fig. 1 Prevalence of *P. falciparum* maternal infection according to study area. Prevalence of *P. falciparum* at delivery in peripheral and/or placental blood as detected by qPCR (**a**) and microscopy (**b**). *P* value according to the multivariate analysis adjusted for type of IPTp drug, season, age, gravidity, gestational age, anaemia, literacy, RPR result and MUAC at recruitment, as well as CD4 + T cell count in the case of HIV-infected women. T bars represent standard errors. *B* Benin, *G* Gabon, *M* Mozambique, *K* Kenya

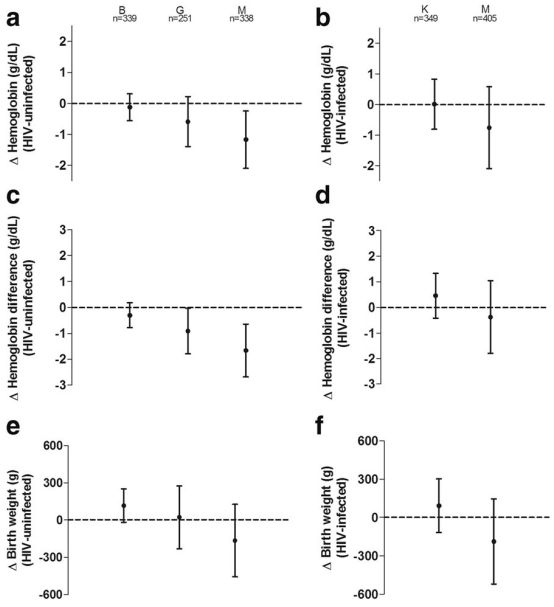

Fig. 2 Impact of microscopic malaria infections at delivery on pregnancy outcomes. Maternal microscopic infections were considered present if *P. falciparum* parasites were observed in peripheral blood at delivery and/or in the placenta either by microscopy or histology. The dot and T bar represents the mean difference and 95% confidence interval in haemoglobin levels at delivery (**a**, **b**), the difference of haemoglobin levels from recruitment to delivery (**c**, **d**), or birth weight (**e**, **f**) between malaria infected and uninfected women in the multivariate regression analysis adjusted for type of IPTp drug, season, age, gravidity, gestational age, anaemia, literacy, RPR result and MUAC at recruitment, plus CD4 + T cell count at recruitment in the case of HIV-infected women (**b**, **d**, **f**). Modification of the associations by study area (*B* Benin, *G* Gabon, *K* Kenya, *M* Mozambique) was determined through the inclusion of an interaction term in the regression models, and combination of the coefficients plus the interaction and the standard error was estimated by the delta method

3.83, $P < 0.001$) but not among Mozambican pregnant women ($P = 0.401$). IgG responses against DBL3x increased with parity of Beninese and Mozambican pregnant women although the magnitude of the increase was higher among Beninese (4.77, 95% CI 3.08 to 7.38, $P < 0.001$) than Mozambican women (1.86, 95% CI 1.23 to 2.79, $P = 0.003$; Fig. 3e). Age was not associated with differences in IG responses irrespectively of antigen and study area ($P = 0.223$ for MSP1 and $P = 0.171$ for DBL3X in Benin and $P = 0.959$ for MSP1 and $P = 0.380$ for DBL3X in Mozambique). No differences in covariates at recruitment were observed between the women with plasma available for serological tests and the rest of the women. The proportion of submicroscopic infections did not differ among HIV-infected women from Kenya and Mozambique (Fig. 3a, b).

P. falciparum densities and pregnancy outcomes

Doubling qPCR parasite densities in peripheral blood of HIV-uninfected Mozambican women was associated with a reduction of 0.16 g/dL (95% CI −0.29 to −0.02, $P = 0.023$) in their haemoglobin levels. In contrast, haemoglobin levels remained unaffected by increasing parasite densities in Beninese ($P = 0.287$) and Gabonese women ($P = 0.381$; Fig. 4a and Additional file 1: Figure S3). Similarly, doubling qPCR parasite densities in peripheral blood of HIV-uninfected Mozambican women was associated with a larger drop in the haemoglobin levels from recruitment to delivery (−0.29 g/dL, 95% CI −0.44 to −0.14, $P < 0.001$), while no relationship was found in Beninese ($P = 0.076$) and Gabonese women ($P = 0.176$; Fig. 4c and Additional file 1: Figure S3). No association was found between qPCR parasite densities and birthweight irrespectively of the country of women, nor with pregnancy outcomes in HIV-infected women (Fig. 4).

Discussion

Although the importance of tolerance in the context of *P. falciparum* infection was suggested early on [29], the

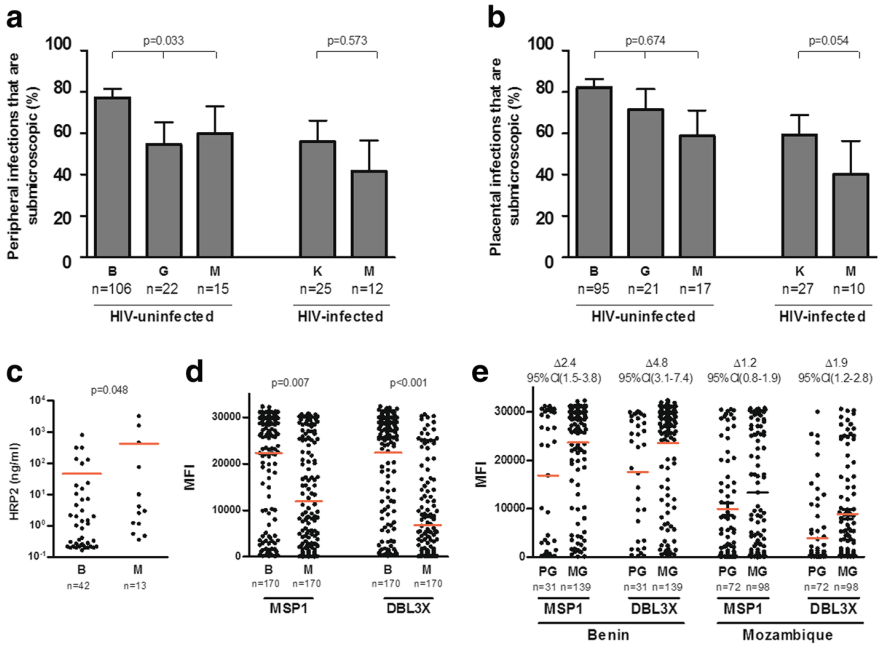

Fig. 3 Proportion of *P. falciparum* submicroscopic infections, PfHRP2 concentrations and antibody responses against *P. falciparum* antigens according to study area. Proportion of infections that are submicroscopic in peripheral (**a**) and placental (**b**) blood. *P* value according to the multivariate analysis adjusted for type of IPTp drug, season, age, gravidity, gestational age, anaemia, literacy, RPR result and MUAC at recruitment, as well as CD4 + T cell count in the case of HIV-infected women. T bars represent standard errors. *B* Benin, *G* Gabon, *M* Mozambique, *K* Kenya. **c** PfHRP2 concentrations, indicative of overall parasite biomass, among women with a peripheral qPCR-detected infection in Mozambique (*M*; n = 13) and Benin (*B*; n = 42). **d** Levels of IgG antibodies against MSP1 and DBL3X in Beninese (n = 170) and Mozambican (n = 170) women. **e** Levels of IgG antibodies against MSP1 and DBL3X by parity (primigravidae (*PG*) versus multigravidae (*MG*)), with the proportional increase (Δ) of the antibody levels plus 95% confidence interval in multigravidae versus primigravidae women. *P* values obtained from regression analysis adjusted for type of IPTp drug, season, gravidity, gestational age, anaemia, literacy, RPR result and MUAC at recruitment, as well as CD4 + T cell count in the case of HIV-infected women. *MFI* mean fluorescence intensity

link between such a host defence strategy and malaria disease has been obscured by the difficulties of quantifying the tolerance phenotype. We sought to reduce the complexity of tolerance to a single metric based on the relationship between parasite densities and health outcomes (haemoglobin levels and birthweight) [8], with a flat slope indicative of tolerance to infection. To assess for variations in the levels of resistance to *P. falciparum* among women from the different endemic areas, we used the proportion of submicroscopic infections at delivery and IgG levels against parasite antigens as an indicator of the ability of pregnant women to restrict parasite growth [1]. Here, we have demonstrated that the lowest levels of immune resistance and tolerance observed among HIV-uninfected Mozambican women, compared to women from Benin, were accompanied by the largest adverse impacts of *P. falciparum* infections. Thus, the reduced severity of infections observed in pregnant women from high endemic regions [1] may not be mediated entirely by an adaptive immune response to the parasite, but also by a tolerance to *P. falciparum*, as quantified by the slope of the relationship between parasite densities and haemoglobin levels [8]. Taken together, these results provide evidence that pregnant women develop exposure-dependent mechanisms to minimise malaria pathology which, in concert with immune resistance, can reduce the adverse impact of *P. falciparum* infections.

Variation in resistance to *P. falciparum* among HIV-uninfected women from three sub-Saharan African countries was suggested by different proportions of submicroscopic infections. Prevalence of qPCR-detected *P. falciparum* infections at delivery ranged from 41% in Benin to 11% in Gabon and 6% in Mozambique. Pregnant women from Benin had the highest proportion of submicroscopic infections at delivery, suggesting an increased capacity to maintain infections at densities bellow the detection limit of microscopy. These observations are in contrast with trends reported showing a significantly higher percentage of infections detected by microscopy in the general population residing in areas of high compared to those in low transmission areas [30]. These discrepancies may suggest a special dynamic and progression of *P. falciparum* infection during pregnancy compared to

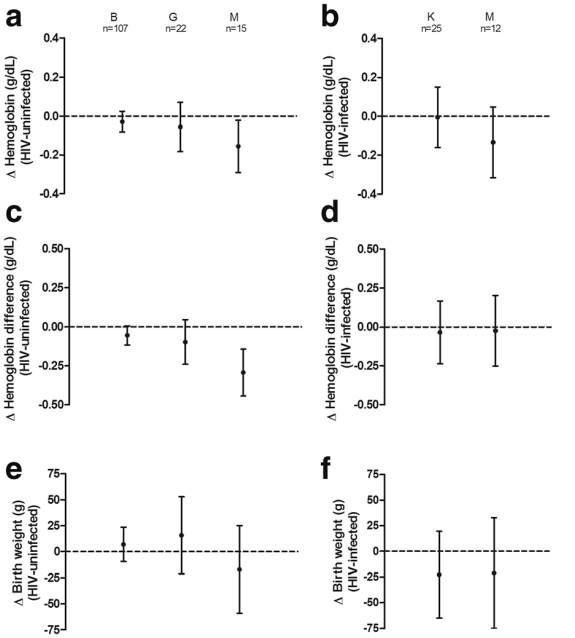

Fig. 4 Relationship of qPCR-detected parasite densities in peripheral blood with pregnancy outcomes. The dot and T bar represent the effect and 95% confidence interval on haemoglobin levels at delivery (**a**, **b**), the difference of haemoglobin levels from recruitment to delivery (**c**, **d**), or birth weight (**e**, **f**) due to a two-fold increase in the parasitemia levels in its natural scale. Modification of the associations by study area (*B* Benin, *G* Gabon, *K* Kenya, *M* Mozambique) was determined through the inclusion of an interaction term in the regression models, and combination of the coefficients plus the interaction and the standard error was estimated by the delta method. Analyses were adjusted for type of IPTp drug, season, age, gravidity, gestational age, anaemia, literacy, RPR result and MUAC at recruitment, as well as CD4 + T cell count in the case of HIV-infected women (**b**, **d**, **f**)

infections in non-pregnant hosts. In line with this, PfHPR2 levels in plasma, indicative of total parasite biomass [26], were lower among Beninese than Mozambican women, whereas levels of anti-parasite antibodies, as well as the increase with parity of IgGs against DBL3X from VAR2CSA, were higher in women from Benin than from Mozambique. Overall, these data supports the notion that the acquisition of antimalarial immunity after exposure to *P. falciparum* parasites can increase the capacity to resist *P. falciparum* growth during subsequent infections in pregnancy.

Variations in tolerance to *P. falciparum* were also observed in pregnant women living under contrasting levels of malaria transmission, as indicated by differences in the relationship between haemoglobin levels and increasing parasite burden. In this study, haemoglobin levels at delivery decreased as parasite densities increased in HIV-uninfected women from the lowest transmission setting in Mozambique. By contrast, the haemoglobin level in HIV-uninfected Beninese and Gabonese women were not affected by parasite density, indicating a better tolerance to the infection [7]. Parasite factors such as the level of resistance to SP, as well as the protective effect of IPTp with SP against adverse birth outcomes that are related to curable sexually transmitted and reproductive tract infections [31, 32], may affect the clinical impact of infections among pregnant women who received antimalarials as IPTp. However, no relationship was observed between the level of molecular markers of SP resistance in the parasite population based on the frequencies of dihydropteroate synthase (*Pfdhps*) K540E mutation [33, 34] previously reported (>90% *Pfdhps*-K540E in Kenya [33] but < 50% in Mozambique [35], Gabon [36] and Benin [37]) and the outcomes of the study. Moreover, the efficacy of IPTp with SP to clear peripheral parasites and prevent new infections during pregnancy has been suggested to be compromised only in areas with > 90% prevalence of *Pfdhps* K540E mutation [33]. Taken together, these results suggest that immunoregulatory responses that reduce pathogenic inflammation and potentially the risk of anaemia [38] may be developed by pregnant women exposed to *P. falciparum*, as has been suggested for children [39–41].

In absence of HIV infection, the adverse clinical impact of *P. falciparum* infections was the highest in pregnant women from the low transmission site in Mozambique, who had the lowest levels of immune resistance and tolerance to *P. falciparum*. This adverse impact was observed for microscopic *P. falciparum* infections, which were associated with reductions in maternal haemoglobin levels at delivery as well as with increased drops in haemoglobin levels from recruitment to delivery, but not with birthweight. Moreover, placental past/chronic infections among Mozambican women were associated with an increased risk of preterm births, in accordance with previous studies showing a larger impact of infections during pregnancy compared to infections detected only at delivery [42–44]. Such an adverse effect of placental past/chronic infections on the birthweight of newborns was not observed among Beninese or Gabonese women. *P. falciparum* microscopic infection in Gabonese women was also associated with an increased drop in haemoglobin levels from recruitment to delivery, although of a lower magnitude than the drop observed in Mozambican women. These data suggest that the malaria-related adverse impact in the health of pregnant women is higher in Gabon than in Benin, but lower than in Mozambique. Such an intermediate severity of the infections in Gabonese women might be explained by the development of tolerance to *P. falciparum* (as suggested by the lack of an association between haemoglobin levels with increasing parasite densities) rather than by immune resistance to the infection (as

indicated by the similar carriage of submicroscopic infections in Gabonese and Mozambican women). Overall, this evidence suggests that resistance and tolerance to malaria can be acquired after exposure to *P. falciparum* parasites in areas of high transmission, and can reduce the detrimental consequences of *P. falciparum* infections. Importantly, HIV-infected pregnant women from Kenya and Mozambique did not show any evidence of varying levels of resistance or tolerance, suggesting that the ability to limit the adverse impact of *P. falciparum* infection may be reduced when the immune system is suppressed by the viral infection [45].

This study has some limitations. First, *P. falciparum* infection at delivery in women who received IPTp most likely reflect a recently acquired infection. Thus, this study may under-estimate the adverse impact of *P. falciparum* infections during pregnancy, as compared to recent reports showing that submicroscopic *P. falciparum* infection at inclusion (16.5 weeks) increases the risk of low birth weight for primigravid and premature delivery for multigravid pregnant Beninese women [6]. Second, other factors apart from anti-parasite immunity may contribute to the carriage of submicroscopic infections, such as the stage of the infection, the chronicity of infections, which is most common among primigravid women without immunity to placental parasites [46], and the existence of suppressive levels of antimalarial drugs. Third, site-specific differences in the extent of healthcare provided and economic development, as well as other factors than can affect pregnancy outcomes (i.e. haemoglobinopathies), might contribute to variations in the clinical impact of infections among pregnant women from different countries. However, the impact of these differences was minimised by the fact that this study was performed in the context of a clinical trial following standard procedures [19, 20] and that the analysis was adjusted by potential confounders. Fourth, the immunological analyses were conducted only in a subset of plasma samples available from the women included in the study, which were similar in covariates with the rest of the women. Fifth, analyses were performed by group, rather than at the individual level, assuming uniform exposure and risk among all individuals at each site. Finally, the small prevalence of *P. falciparum* infection among HIV-positive women who were receiving cotrimoxazole during pregnancy [20] may have reduced the power to detect variations in resistance and tolerance to malaria.

Conclusions

This study shows that pregnant women may reduce the deleterious impact of *P. falciparum* infection through two conceptually different types of defence against malaria, namely, one that targets the parasite (resistance), and the other that prevents the damage induced by the infection (tolerance) [7, 8]. Down-regulation of malaria-induced inflammation may mediate this tolerance phenotype, although other mechanisms that increase the resilience to tissue injuries, such as metabolic adaptation to tissue repair and detoxification of pathogen by-products [11, 47], might still be involved. Making the distinction between resistance mechanisms that restrict parasite multiplication from those that minimise the harm caused by the infection [7, 8] is important to understand how reductions in transmission intensity can affect host defences and, therefore, the clinical presentation of *P. falciparum* infections, as well as for the design of new host-directed therapies [9, 48, 49].

Additional file

Additional file 1: Supplementary Tables and Figures. (DOCX 279 kb)

Abbreviations

DBL3X: Duffy-Binding Like 3 X; HRP2: Histidine-Rich Protein2; IPTp: intermittent preventive treatment during pregnancy; MQ: mefloquine; MSP1: Merozoite Surfce Protein 1; *P. falciparum*: *Plasmodium falciparum*; qPCR: quantitative polymerase chain-reaction; SP: sulfadoxine-pyrimethamine

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AM: Conception and design, acquisition of data, analysis and interpretation of data, drafting and revising the article. NDN, EM: acquisition of data, analysis and interpretation of data, drafting and revising the article. LQ: analysis and interpretation of data. PC, AMF, AJ, RG, SK, ES, MR, AV, SM, PO, MR, JJA, AN, AC, VB, PGK, GMN, MD, EM, MC, CM: acquisition of data and interpretation of data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was approved by the Ethics Committees from the Hospital Clínic of Barcelona (Spain), the Comité Consultatif de Déontologie et d'Éthique from the Institut de Recherche pour le Développement (France), the Centers for Disease Control and Prevention (USA), and National Ethics Review Committees from each malaria endemic country. Study participants signed a written informed consent form prior to enrolment.

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ADDITIONAL MATERIAL

Resisting and tolerating *P. falciparum* in pregnancy under different malaria transmission intensities

Table S1. Characteristics of the women participating in the intermittent preventive treatment trials and those not included in the study.

	HIV-uninfected			HIV-infected		
	Included			Included		
	No (n=2598)	Yes (n=946)	p	No (n=258)	Yes (n=768)	p
IPTp, n (%)						
Antimalarial 1	1737 (67)	629 (66)	0.840	124 (48)	390 (51)	0.472
Antimalarial 2	861 (33)	317 (34)		134 (52)	378 (49)	
Parity, mean (SD)	2.4 (1.7)	2.4 (1.6)	0.733	2.8 (1.5)	2.9 (1.5)	0.870
Age (years), mean (SD)	23.7 (6.2)	23.5 (6.0)	0.482	25.9 (5.4)	26.1 (5.6)	0.592
MUAC (cm), mean (SD)	25.8 (3.3)	25.8 (2.9)	0.804	26.7 (2.5)	26.7 (2.7)	
Anemia at recruitment, n (%)						
No	965 (37)	333 (35)	0.288	88 (34)	250 (33)	0.647
Yes	1631 (63)	613 (65)		170 (66)	518 (67)	
RPR, n (%)						
Positive	44 (2)	9 (1)	0.253	13 (5)	39 (5)	1.000
Negative	2522 (97)	928 (98)				
UNK	30 (1)	9 (1)		242 (95)	726 (95)	
Literate, n (%)						
No	969 (37)	371 (39)	0.309	51 (20)	142 (18)	0.646
Yes	1627 (63)	575 (61)		207 (80)	626 (82)	
Peripheral infection by microscopy, n (%)						
Negative	2008 (95)	898 (95)	0.788	153 (96)	740 (97)	0.477
Positive	108 (5)	45 (5)		7 (4)	25 (3)	
Placental infection by microscopy, n (%)						
Negative	1729 (95)	898 (96)	0.350	93 (96)	741 (97)	0.533
Positive	94 (5)	40 (4)		4 (4)	23 (3)	
Placental histology, n (%)						
Negative	1746 (96)	895 (96)	1.000	101 (96)	741 (98)	0.291
Active	81 (4)	41 (4)		4 (4)	16 (2)	
Viral Load (copies/mL) at baseline, n (%)						
<399				47 (18)	181 (24)	0.265
400/999				63 (24)	183 (24)	
1000/9999				82 (32)	243 (32)	
10000/max				47 (18)	123 (16)	
UNK				19 (7)	38 (5)	
CD4 (cells/µl) at baseline, n (%)						
<350				99 (38)	279 (36)	0.733
350/max				148 (57)	460 (60)	
UNK				11 (4)	29 (4)	

IPTp, Intermittent preventive treatment; MUAC, Mid-upper arm circumference; RPR, Rapid plasma reagin;

Antimalarial: 1=Mefloquine and 2=Sulphadoxine-Pyrimethamine for HIV-; 1=Placebo and 2=Mefloquine for HIV+

Table S2. Relationship between microscopy positivity and qPCR parasitaemia in the different study sites.

		HIV-uninfected									HIV-infected					
		Benin			Gabon			Mozambique			Kenya			Mozambique		
		Peripheral smear at delivery														
		Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total
Peripheral qPCR																
Negative																
	n	238	3	241	233	1	234	324	1	325	318	6	324	403	1	404
	%	68	1		91	0		95	0		91	2		97	0	
Positive																
	n	82	24	106	12	10	22	9	6	15	14	11	25	5	7	12
	%	24	7		5	4		3	2		4	3		1	2	
Total		320	27	347	245	11	256	333	7	340	332	17	349	408	8	416
		Placental smear														
		Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total
Placental qPCR																
Negative																
	n	244	6	250	229	3	232	322	1	323	319	5	324	402	1	403
	%	71	2		91	1		95	0		91	1		97	0	
Positive																
	n	78	17	95	15	6	21	10	7	17	16	11	27	4	6	10
	%	22	5		6	2		3	2		5	3		1	1	
Total		322	23	345	244	9	253	332	8	340	335	16	351	406	7	413

Table S3. Submicroscopic infections by qPCR parasite density (lower or higher than 200 parasites/µl) and study site.

		HIV-uninfected				HIV-infected		
		Benin	Gabon	Mozambique	p	Kenya	Mozambique	p
Submicroscopic in peripheral blood (delivery)								
≤200 p/µl								
	n	60	10	7	0.914	12	5	1.000
	%	73	83	78		86	100	
>200 p/µl								
	n	22	2	2		2	0	
	%	27	17	22		14	0	
Submicroscopic in placental blood								
≤200 p/µl								
	n	54	13	8	0.359	12	1	0.101
	%	69	87	80		75	25	
>200 p/µl								
	n	24	2	2		4	3	
	%	31	13	20		25	75	

Table S4. Relationship between gestational age at delivery (assessed by Ballard score) and pregnancy outcomes. a) describes the relationship assessed by Spearman correlation and b) the comparison of outcomes between normal and preterm births.

a

	Spearman HIV-uninfected			Spearman HIV-infected*		
	n	**rho**	**p**	**n**	**rho**	**p**
Birth weigth & Gestational age	909	0.161	<0.001	391	0.420	<0.001
Hemoglobin levels (delivery) & Gestational age	911	-0.0135	0.683	391	0.057	0.262
Δ hemoglobin levels & Gestational age	911	0.027	0.415	391	0.017	0.740

Δ hemoglobin: difference in hemoglobin levels from recruitment to delivery

* Only data from Mozambique

b

	HIV-uninfected				HIV-infected*			
	n	**Mean**	**SD**	**p**	**n**	**Mean**	**SD**	**p**
Birth weigth by preterm birth (grams)								
No	865	3051.1	422.1	0.001	374	3114.4	431.3	<0.001
Yes	44	2673.5	598.0		17	2467.6	494.6	
Hemoglobin levels (delivery) by preterm birth (g/dL)								
No	867	11.1	1.5	0.337	374	10.53	1.77	0.371
Yes	44	11.4	1.4		17	10.92	1.77	
Δ hemoglobin levels by preterm birth (g/dL)								
No	867	0.69	1.69	0.449	374	0.501	1.89	0.988
Yes	44	0.89	1.85		17	0.494	1.47	

Δ hemoglobin: difference in hemoglobin levels from recruitment to delivery

* Only data from Mozambique

Table S5. Adjusting variables that remained in the final regression models.

HIV-uninfected					HIV-infected				
	Effect	95%CI		p		Effect	95%CI		p
BIRTH WEIGHT-PAST/CHRONIC INFECTION									
Parity	161.62	89.34	233.90	<0.001	**PTB**	-323.75	-414.96	-232.54	<0.001
Age	105.72	40.92	170.51	0.001	**Gest2**	-152.27	-260.66	-43.88	0.006
PTB	-322.20	-451.91	-192.49	<0.001	**Gest3**	-167.24	-287.18	-47.30	0.006
BIRTH WEIGHT-MICROSCOPIC INFECTION									
Parity	159.88	88.08	231.67	<0.001	**PTB**	-318.52	-409.61	-227.44	<0.001
PTB	-314.21	-442.57	-185.84	<0.001	**Gest2**	-142.94	-251.66	-34.22	0.01
Age	131.29	65.81	196.77	<0.001	**Gest3**	-157.56	-278.11	-37.00	0.01
Gest2	-61.64	-119.58	-3.70	0.037					
Literacy	50.56	-9.31	110.43	0.098					
BIRTH WEIGHT-SUBMICROSCOPIC INFECTION									
Parity	163.37	89.98	236.75	<0.001	**Gest3**	-181.21	-303.38	-59.04	0.004
Gest2	-60.49	-120.09	-0.90	0.047	**Gest2**	-155.38	-266.09	-44.68	0.006
Age	104.86	39.11	170.62	0.002	**PTB**	-308.24	-401.22	-215.26	<0.001
PTB	-306.37	-436.67	-176.07	<0.001					
PRETERM BIRTH-PAST/CHRONIC INFECTION									
					Anemia at rec	0.58	0.38	0.86	0.008
					CD4	0.17	0.02	1.25	0.081
					Literacy	3.57	1.68	7.57	0.001
HEMOGLOBIN LEVELS-MICROSCOPIC INFECTION									
MUAC	-0.46	-0.80	-0.11	0.009	**Parity**	-0.88	-1.41	-0.35	0.001
Treatment	-0.19	-0.38	0.00	0.056	**PTB**	0.70	0.30	1.09	0.001
Gest2	0.25	0.06	0.44	0.011	**Age**	0.65	0.31	0.99	<0.001
RPR	-1.04	-1.97	-0.11	0.028	**CD4**	0.26	-0.04	0.57	0.088
Anemia at rec	-0.89	-1.08	-0.70	<0.001	**Anemia at rec**	-1.21	-1.52	-0.89	<0.001
Literacy	-0.27	-0.46	-0.08	0.005	**Literacy**	0.50	0.11	0.89	0.013
HEMOGLOBIN LEVELS-SUBMICROSCOPIC INFECTION									
MUAC	-0.48	-0.82	-0.14	0.006	**Parity**	-0.82	-1.36	-0.28	0.003
Treatment	-0.19	-0.39	0.01	0.058	**PTB**	0.76	0.36	1.16	<0.001
Gest2	0.24	0.05	0.44	0.015	**Anemia at rec**	0.61	0.27	0.95	<0.001
RPR	-0.92	-1.85	0.00	0.05	**CD4**	0.26	-0.04	0.57	0.093
Literacy	-0.26	-0.46	-0.07	0.008	**Literacy**	0.53	0.13	0.92	0.009
Anemia at rec	-0.90	-1.09	-0.71	<0.001	**Age**	-1.22	-1.54	-0.90	<0.001
DIFFERENCE HEMOGLOBIN LEVELS (DELIVERY-RECRUTIMENT)-MICROSCOPIC INFECTION									
Parity	-0.24	-0.47	-0.01	0.044	**Parity**	-1.04	-1.60	-0.48	<0.001
Treatment	-0.18	-0.39	0.03	0.097	**Literacy**	0.64	0.22	1.06	0.003
Literacy	-0.27	-0.49	-0.06	0.014	**Age**	0.58	0.23	0.93	0.001
RPR	-0.72	-1.49	0.05	0.067	**Anemia at rec**	1.51	1.17	1.84	<0.001
Anemia at rec	1.45	1.24	1.66	<0.001	**PTB**	0.68	0.26	1.10	0.001
Gest2	0.26	0.05	0.48	0.015					
MUAC	-0.44	-0.83	-0.05	0.025					
DIFFERENCE HEMOGLOBIN LEVELS (DELIVERY-RECRUTIMENT)-SUBMICROSCOPIC INFECTION									
Parity	-0.25	-0.49	-0.02	0.036	**Parity**	-0.87	-1.43	-0.30	0.003
Anemia at rec	1.40	1.19	1.62	<0.001	**Anemia at rec**	1.48	1.15	1.82	<0.001
Literacy	-0.22	-0.44	0.00	0.048	**Age**	0.55	0.19	0.90	0.002
RPR	-0.78	-1.57	0.01	0.052	**Literacy**	0.63	0.21	1.04	0.003
Gest3	0.45	-0.02	0.92	0.063	**PTB**	0.79	0.36	1.21	<0.001
MUAC	-0.43	-0.81	-0.05	0.028					
Gest2	0.59	0.15	1.04	0.009					
BIRTH WEIGHT-QPCR PARASITE DENSITIES									
Parity	324.64	158.50	490.78	0					
Gest2	555.75	116.38	995.13	0.014					
Gest3	728.21	264.64	1191.77	0.002					
HEMOGLOBIN LEVELS AT DELIVERY-qPCR PARASITE DENSITIES									
Parity	-0.89	-1.47	-0.30	0.003	**Parity**	-1.90	-3.71	-0.09	0.04
Age	0.74	0.24	1.25	0.004	**Anemia at rec**	-2.16	-3.30	-1.02	0.001
Anemia at rec	-0.71	-1.19	-0.22	0.005					
RPR	-6.05	-8.70	-3.40	<0.001					
DIFFERENCE IN HEMOGLOBIN LEVELS (DELIVERY - RECRUITMENT) -qPCR PARASITE DENSITIES									
Parity	-1.57	-2.25	-0.88	<0.001					
Anemia at rec	1.26	0.71	1.81	<0.001					
RPR	-8.37	-11.36	-5.37	<0.001					

Age (<25 and ≥25 years); parity (primigravid vs multigravid women); Gest2. Second trimestre at recrutiment; Gest3. Third trimester at recrutiment; MUAC. mid-upper arm circumference (<22 vs ≤ 22 cm) ; PTB. Pretertm birth; Rec. Recruitment; RPR. rapid plasma reagin.

Figure S1. Study profile.

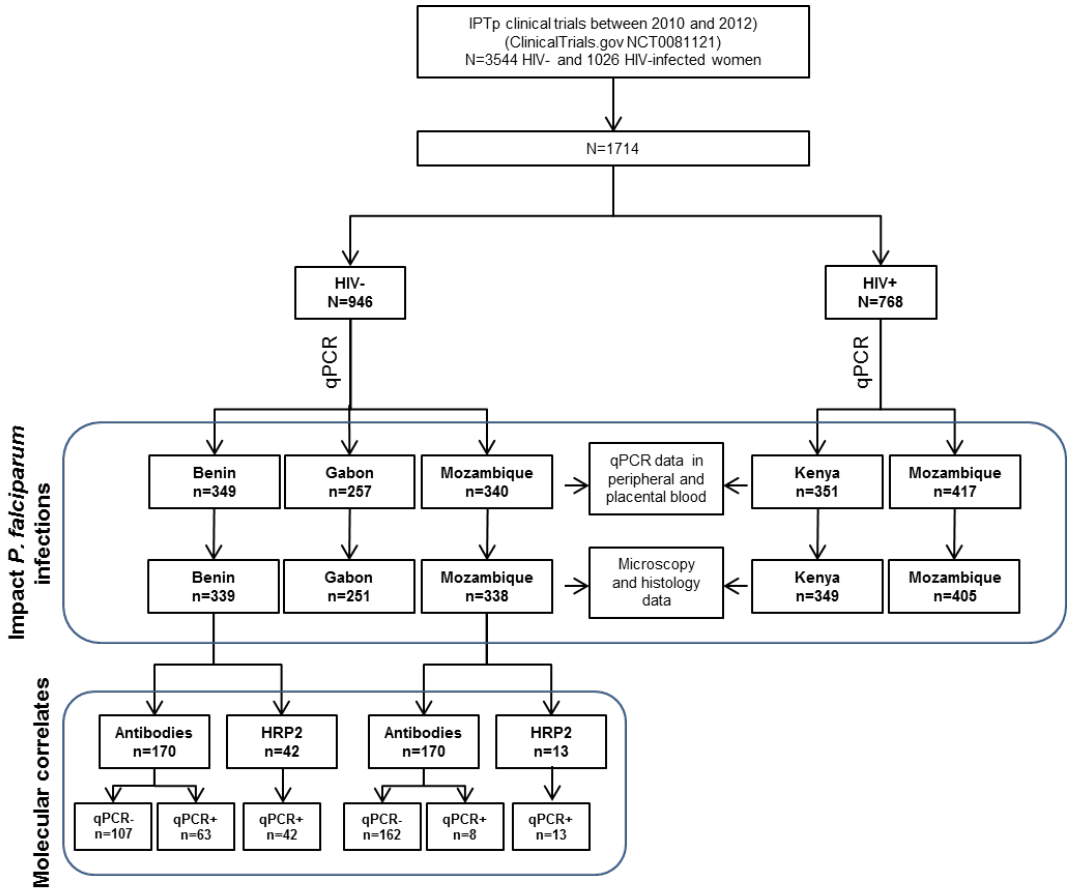

Figure S2. Impact of malaria submicroscopic infections in peripheral blood on pregnancy outcomes.

Submicroscopic infections were defined as *P. falciparum* infections detected by qPCR but negative by microscopy. The dot and T bar represents the mean difference and 95% confidence interval in hemoglobin levels (**a, d**), the difference of haemoglobin levels from recruitment to delivery (**b, e**), or birth weight (**c, f**) between malaria infected and uninfected women in the multivariate regression analysis adjusted for type of IPTp drug, season, age, gravidity, gestational age, anemia, literacy, RPR result and MUAC at recruitment, plus CD4+T cell count at recruitment in the case of HV-infected women (**d, e, f**). Modification of the associations by study area (B=Benin, G=Gabon, K=Kenya, M=Mozambique) was determined through the inclusion of an interaction term in the regression models, and combination of the coefficients plus the interaction and the standard error was estimated by the delta method.

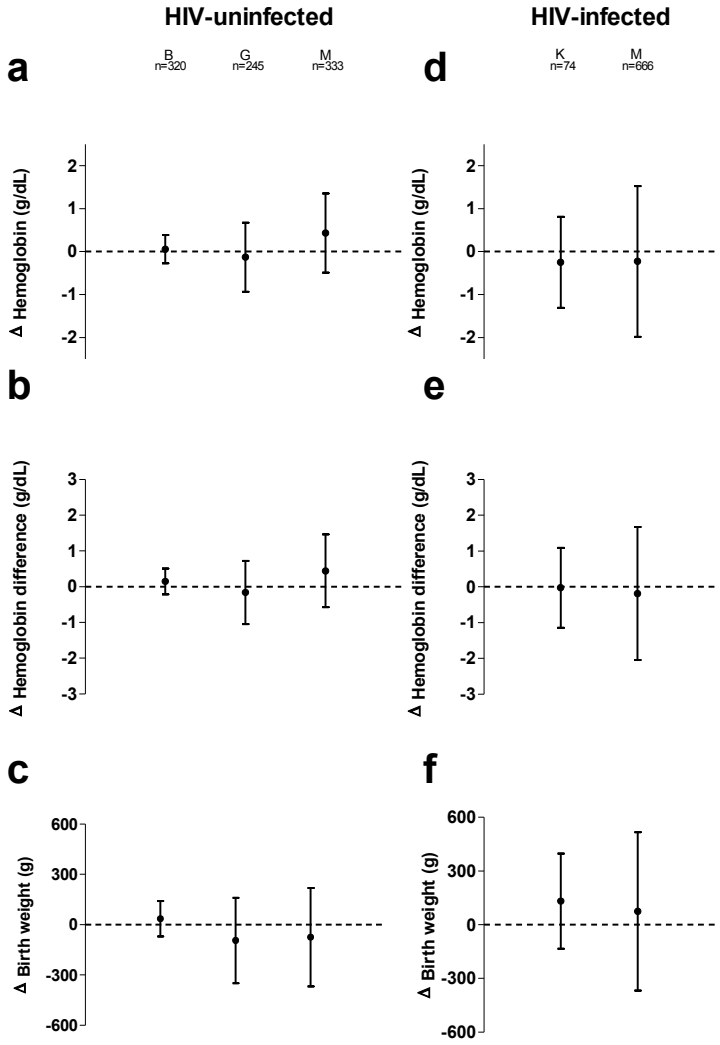

Figure S3. Relationship between parasite densities in peripheral blood measured by qPCR and haemoglobin levels and the difference in haemoglobin levels from recruitment to delivery (Δ Hemoglobin) in HIV-uninfected women from Benin (**a, d**), Gabon (**b, e**) and Mozambique (**c, f**). Solid red lines indicate best fit.