Investigation of factors influencing *var* gene expression in *Plasmodium falciparum* parasites from acute and chronic infections

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I dedicate my thesis to Laura Annika Nykäin.

Life is about more than just surviving.

Kurzfassung:

Im Jahre 2015 gab es weltweit 214 Millionen neue Malariafälle und 438.000 Todesfälle durch Malaria. Dabei traten 88% der Krankheitsfälle und 90% der Todesfälle in Afrika auf. In malaria-endemischen Ländern besteht für Kinder unterhalb von fünf Jahren ein sehr hohes Risiko an schwerer Malaria zu erkranken und zu sterben. Kinder dieses Alters haben noch keine Semi-Immunität gegen die schweren Formen der Malaria entwickelt, da sich diese erst nach mehreren aufeinanderfolgenden Infektionen im Lauf des ersten Lebensjahrzehnts ausbildet. In semi-immunen Bewohnern endemischer Regionen kommt es zu chronischen, asymptomatischen Infektionen mit mikroskopisch nicht nachweisbaren Infektionen. Eine Ausnahme sind Frauen in der ersten Schwangerschaft, bei denen sich trotz Semi-Immunität eine plazentare Malaria entwickeln kann, die häufig zum Abort, zu Geburtsschäden oder zur Totgeburt führt.

Der einzellige Parasit *Plasmodium falciparum* (*P. falciparum*) verursacht die schwerste Form der Malaria und ist verantwortlich für die Mehrheit der auftretenden Todesfälle. Durch die Expression des *Plasmodium falciparum* erythrocyte membrane proteins 1 (PfEMP1) auf der Oberfläche von infizierten Erythrozyten können diese an Endothelrezeptoren des Wirts adhärieren. Das verhindert die Beseitigung der infizierten Erythrozyten durch die Milz, trägt aber auch zur Entstehung der schweren Symptome der Malaria durch Beeinträchtigung des Blutflusses in den Kapillaren bei. PfEMP1 wird von der Familie der hypervariablen *var*-Gene kodiert. Jeder Parasit besitzt etwa 60 verschiedene *var*-Gene, von denen aber jeweils nur eins pro Parasit exprimiert wird. Ein ständiger Wechsel des aktiv transkribierten *var*-Lokus führt zur Antigenvariation, die es dem Parasit ermöglicht, der Immunantwort des Wirtes zu entgehen.

Bisherige Untersuchungen haben die Expression der *var*-Gene vor allem *in vitro*, anhand von *P. falciparum* Laborstämmen, untersucht. Es gibt jedoch nur wenige Untersuchungen darüber, wie die *var*-Gen Expression durch die Moskito- und Wirtspassage beeinflusst wird.

Im ersten Projekt dieser Dissertation konnte gezeigt werden, dass die Moskito- und Humanpassage bei malaria-naiven Individuen die *var*-Gen Transkription grundlegend verändert. Die *in vitro var*-Gen Transkription wird maßgeblich durch die Replikationsdauer von *P. falciparum* im Wirt beeinflusst. Je länger eine Parasitenpopulation der Rezeptorselektion im Wirt ausgesetzt ist, umso mehr verschiebt sich die *var*-Gen Transkription zugunsten von wenigen, sehr stark transkribierten *var*-Genen. Nach längerer *in vitro* Kultivierung in Abwesenheit eines Selektionsdrucks werden dann wieder viele *var*-Gene in geringer Kopienzahl transkribiert. Dieses Transkriptionsprofil ist fast identisch mit dem *in vitro var*-Gen Transkriptionsprofil vor der Moskito- und Wirtpassage. Je länger eine Parasitenpopulation im Wirt war, desto länger dauert es bis sie zu diesem „prä-Moskito“ *in vitro* Transkriptionsprofil zurückkehrt. In Abwesenheit eines Selektionsdrucks scheint die *var*-Gen Transkription daher vor allem durch ein festgelegtes genetisches Programm definiert zu sein.

Die Daten aus dem zweiten Projekt dieser Dissertation weisen darauf hin, dass PfEMP1 nicht das einzige variable Oberflächenprotein ist, das chronische, asymptomatische *P. falciparum* Infektionen bei semi-immunen Personen ermöglicht. Um dies herauszufinden, wurden Parasiten von einer chronischen, asymptomatischen Infektion eines Patienten aus Gabun *in vitro* kultiviert und aus der Primärkultur klonale Kulturen einzelner Parasiten erstellt. Diese klonalen Kulturen wurden mittels Durchflusszytometrie (fluorenscence activated cell sorting (FACS)) mit konvaleszentem Serum des Patienten untersucht. Bei allen Parasiten wurde zudem die *var*-Gen Transkription charakterisiert. Hierbei zeigte sich, dass unterschiedliche Parasitenklone unterschiedliche FACS-Signale besaßen, die jedoch nicht mit der *var*-Gen Transkription korreliert waren. Darüber hinaus wurde das FACS-Signal nicht durch einen experimentellen PfEMP1-knock down in transgenen Feldisolaten verändert. Dies zeigt, dass PfEMP1 nicht für das variable Oberflächensignal von Parasiten in chronischen Infektionen verantwortlich ist, sondern andere variable Oberflächenproteine, wie z.B. die STEVOR und RIFIN Proteinfamilien, am variablen Obeflächensignal beteiligt sind. Zudem scheint die Rezeptorselektion in naiven Wirten und die Antikörperantwort in semi-immunen Wirten die *var*-Gen Expression zu beeinflussen.

Die *var*-Gen Familie ist hypervariabel und unterschiedliche Parasiten besitzen fast komplett unterschiedliche *var*-Gen-Repertoire. Bisher wurde nur ein konserviertes *var*-Gen beschrieben, dass bei allen *P. falciparum* Stämmen vorzukommen scheint: *var2csa*. Dieses *var*-Gen scheint aufgrund seiner speziellen Funktion, nämlich der Adhäsion an Chondroitinsulfat A (CSA) in der Plazenta, in allen *P. falciparum* Stämmen konserviert zu sein.

Im dritten Projekt dieser Dissertation konnte in zwei von 10 Feldisolaten aus verschiedenen afrikanischen Regionen ein *var*-Gen gefunden werden, welches ebenfalls konserviert ist. Die Feldisolate wiesen ansonsten eine hohe Diversität in den nichtkodierenden Regionen auf. Dies weist auf eine Selektion gegen die Diversität dieses spezifischen *var*-Genes hin.

In zukünftigen Untersuchungen mit kontrollierten Malariainfektionen (controlled human malaria infections (CHMI)) von malaria-naiven und semi-immunen Individuen könnte der Einfluss der Selektionsdrücke des Wirtes auf die *var*-Gen-Expression und die Rolle der anderen variable Oberflächenprotein bei der Antigenvariation untersucht werden.

Abstract

In 2015, 214 million new malaria cases and 438,000 malaria deaths occurred worldwide. About 88% of cases and 90% of deaths occurred in African. In endemic African countries, children under the age of five are at high risk of severe malaria infections and death. Children of that age have not yet developed a so called semi-immunity against severe forms of malaria, which is only established after several consecutive infections in the first decade of life. In semi-immune individuals living in endemic regions, malaria parasites persist in chronic, asymptomatic infections with parasitemias usually undetectable by microscopic analysis. The only exceptions are women during their first pregnancy, who, despite of semi-immunity, are at risk of placental malaria, which can lead to abortion, birth defects or stillbirth.

The protozoan parasite *Plasmodium falciparum* (*P. falciparum*) causes the most severe form of malaria and is responsible for the majority of malaria deaths. By expressing *Plasmodium falciparum* membrane protein 1 (PfEMP1) on their surface, infected erythrocytes are able to adhere to host endothelial receptors. This avoids clearance of the infected erythrocytes by the spleen but also contributes to the severe symptoms of malaria by blocking blood flow in the deep capillaries. PfEMP1 is encoded by a hypervariable, multi-copy gene family called *var*. Each parasite possesses about 60 different *var* genes which are transcribed in a mutually exclusive manner. Activation of a different *var* locus results in antigenic variation and enables the parasite to circumvent the host's immune response.

So far, expression of *var* genes has been studied mostly *in vitro*, using laboratory *P. falciparum* strains,while so far few investigations have analyzed how *var* gene expression is influenced by mosquito and human passage as well as by host pressures in acute and chronic infections.

The first project of this dissertation shows that in non-immune volunteers, mosquito- and host passage has a profound effect on *var* gene transcription. *In vitro var* gene transcription is influenced by the duration of intra-host receptor selection. The longer a parasite population is exposed to receptor selection, the fewer *var* loci are transcribed, though in higher relative copy numbers. After prolonged *in vitro* cultivation in the absence of selective pressures, *var* gene transcription returns to a default state where many *var* loci are transcribed at low relative copy numbers. This default transcription pattern is almost identical to the "premosquito" *var* gene transcription pattern. The longer parasites replicate within the human host, the longer it takes to return to this *in vitro* "premosquito" *var* gene transcription pattern. This default transcription pattern is almost identical to the "premosquito" *var* gene transcription pattern. The longer parasites replicate within the human host, the longer it takes to return to this *in vitro* "premosquito" *var* gene transcription pattern. In the absence of selection pressures, *var* gene transcription thus appears to be defined by a determined genetic program.

The data from the second project of this thesis indicate that PfEMP1 is not the only variable surface antigen (VSA) in chronic, asymptomatic *P. falciparum* infections. To investigate this, parasites from a chronic asymptomatic infection of a semi-immune gabonese individual were adapted to in vitro cultivation, from where clonal cultures were generated by limiting dilution. To determine the expressed surface signal of the cloned parasites, fluorescent activated cell sorting (FACS) with convalescent serum from the infected individual was performed. Additionally, *var* gene transcription was characterized by real-time PCR in all clonal cultures. Interestingly, individual clonal cultures exhibited different surface signals uncorrelated with *var* gene transcription. Even an experimental PfEMP1 knock down in transgenic field isolates did not affect surface signals expression. This demonstrates that PfEMP1 is not exclusively responsible for the variable surface signals of parasites from chronic infections. Indeed, the data suggest that non-PfEMP1 VSAs, such as the RIFIN and STEVOR protein families, contribute to the variable surface signals of parasites from chronic *P. falciparum* infections. Furthermore, *var* gene expression in non-immune individuals is influenced by receptor binding and in semi-immune individuals by antibodies against PfEMP1.

The *var* gene family is hypervariable and different *P.fal*ciparum strains carry almost completely distinct *var* gene repertoires. So far, only one highly conserved *var* gene has been described: *var2csa* that appears to be conserved due to its specific binding phenotype to chondroitinsulfate A (CSA) in the human placenta.

In the third project of this thesis, a *var* gene that is conserved in two out of ten field isolates from different African regions was identified. In contrast, the genetic diversity of non-coding regions in the field isolates was very high. This suggests a selection against the diversity of this particular *var* gene.

Future investigations of controlled human malaria infections (CHMI) of naïve volunteers and semi-immune individuals should further clarify the role of host selective pressures for *var* gene expression and the role of non-PfEMP1 VSAs in antigenic variation.

List of publications

This doctoral thesis is based on the following publications. For a detailed description of methods, please refer to the publications.

Pub. I: **Sporozoite Route of Infection Influences *In vitro var* Gene Transcription of *Plasmodium falciparum* Parasites From Controlled Human Infections.**

J Infect Dis. 2016 Sep 15;214(6):884-94. doi: 10.1093/infdis/jiw225. Epub 2016 Jun 7.

Pub. II: ***In Vitro* Variant Surface Antigen Expression in *Plasmodium falciparum* Parasites from a Semi-Immune Individual Is Not Correlated with *Var* Gene Transcription.**

PLoS One. 2016 Dec 1;11(12):e0166135. doi: 10.1371/journal.pone.0166135. eCollection 2016.

Pub. III: **Identification of a Conserved *Var* Gene in Different *Plasmodium falciparum* Strains**

Dimonte, S.; Bruske, E.; Enderes, C.; Turner, L.; Kremsner, PG.; Frank, M. Manuscript in final stages of preparation

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1. Introduction

1.1. Malaria

Malaria is a vector-borne, infectious disease that is endemic in more than 100 countries. These countries are located in sub Saharan Africa, South-America and Asia (Fig.1). There are approximately 214 million cases and an estimated 438000 deaths per year, mostly in children below the age of 5 and in pregnant women in Africa ('WHO | World Malaria Report 2016' 2017).

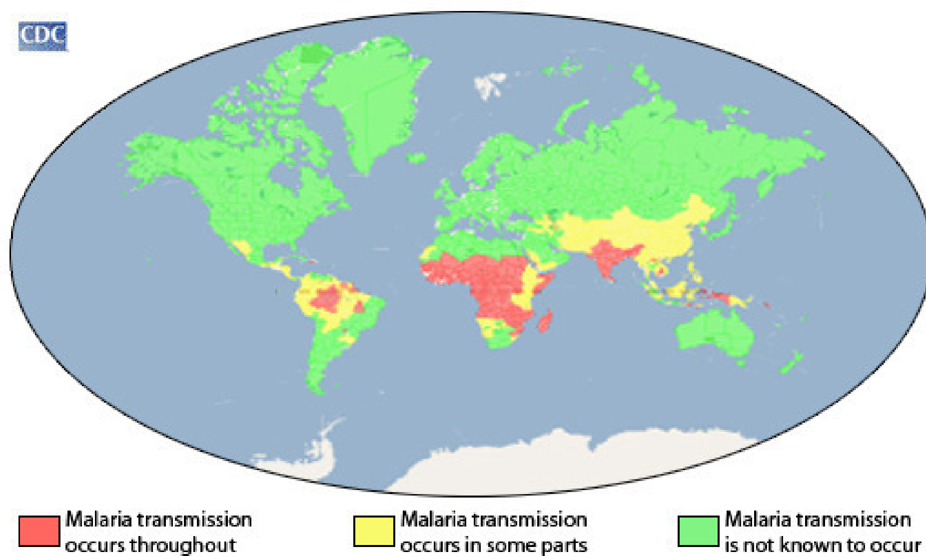


Fig. 1: Map of malaria transmission. Transmission occurs mostly in sub-Saharan African countries, followed by South America and Asia ('CDC - DPDx - Malaria' 2016)**.**

Causative agents of malaria are protozoan parasites of the genus *Plasmodium*. There are five different *Plasmodium* species pathogenic to humans, causing 4 different types of Malaria: *Plasmodium falciparum* (Malaria tropica), *Plasmodium ovale* and *Plasmodium vivax* (Malaria tertiana), *Plasmodium malariae* (Malaria quartana) and *Plasmodium knowlesi* (Ape malaria). Malaria tropica is the most severe form and is responsible for the majority of deaths, which is why this work focuses on *Plasmodium falciparum* (*P. falciparum*) ('CDC - DPDx - Malaria' 2016)

1.1.1 Geographical distribution

Geographical distribution of *P. falciparum* is linked to the presence of the vectors, which are female mosquitoes of the genus *Anopheles*. Different species of *Anopheles* have different requirements for breeding and can be found in almost all habitats, but the development of the parasite inside them requires high temperatures that are often reached in the tropics and subtropics. In the equatorial states of Africa, transmission of *P. falciparum* occurs perennially. Areas with seasonal transmission extend from there to the north, reaching to

below the Sahara as well as to the south including Namibia, Botswana, Zimbabwe, and Mozambique. Altitudes above 1500m are usually malaria free, because temperatures are too low for *Plasmodia* development (Lucius and Loos-Frank 2008; 'CDC - DPDx - Malaria' 2016)

1.1.2. Life cycle of *Plasmodium falciparum*:

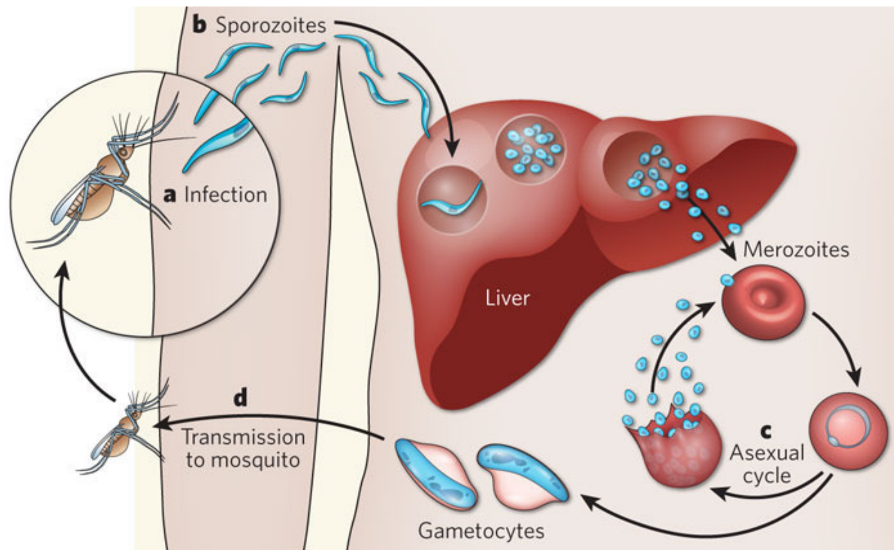


Fig. 2: Life cycle of the malaria parasite *Plasmodium falciparum* (blue) in the human host (Michalakis and Renaud 2009)

P. falciparum is a eukaryotic protist that belongs to the phylum Apicomplexa. Members of this phylum are characterized by the apicoplast, an organelle used for host cell invasion. The life cycle of these endoparasites is typically divided into 3 phases: Schizogony (asexual replication), gamogony (sexual development) and sporogony (development of infectious stages). Schizogony and gamogony occur in the human intermediate host, whereas sporogony takes place in the final host, which are mosquitoes of the genus Anopheles. During the blood meal of an infected mosquito, the infectious stages of the parasite, called sporozoites, are injected into the human host with the mosquito's saliva (Fig2). It has long been thought that a mosquito probes the skin several times until it finds a blood vessel and sprozoites are directly injected into the bloodstream via the saliva (Fairley 1947; Vanderberg 1975). However, there are also studies showing that some sporozoites remain in the skin and never reach the bloodstream, suggesting that the mosquito releases sporozoites already while probing the skin for a blood vessel. It is thus possible that some sporozoites remain in the skin (Sidjanski and Vanderberg 1997; Matsuoka et al. 2002; Medica and Sinnis 2005). The sporozoites that reach the bloodstream are transported into the liver within 2-30 minutes, where each of them invades one hepatocyte. After one cycle of asexual replication, up to 10,000 merozoites per infected liver cell are released into the bloodstream. The merozoites invade erythrocytes in which the parasite replicates asexually via a so called ring stage to throphozoites and finally to schizonts. The respective erythrocytes rupture and release blood stage merozoites, which, in turn, infect more erythrocytes. One schizont releases

approximately 20 merozoites. After a few blood stage cycles, sexual stages, called gametocytes, develop within some infected erythrocytes. Gametocytes are taken up by another mosquito during a blood meal. In the mosquito midgut, sexual replication occurs and sporozoites are produced, which migrate into the salivary glands to begin a new cycle (Lucius and Loos-Frank 2008; 'CDC - DPDx - Malaria' 2016).

1.1.3. Pathology

Depending on the immune status of the host, a *P. falciparum* infection may take different courses. Uncomplicated or mild forms of malaria present themselves with rather unspecific symptoms such as fever, chills, headache, weakness, myalgias, arthralgias, and occasionally vomiting and diarrhea. Thrombocytopenia, hypoglycemia and anemia may also be included.

If untreated, *P. falciparum*-malaria can progress to severe forms which are potentially fatal, because the trophozoites of *P. falciparum* are able to adhere to the host's endothelial cells by cytoadhesion. As a result, throphozoites sequester from blood circulation and avoid clearance of infected erythrocytes by the spleen. Throphozoites are also able to bind to each other (platelet-mediated clumping) and to non-infected erythrocytes (rosetting). Cytoadhesion, platelet-mediated clumping and rosetting lead to the blocking of deep capillaries, which reduces blood flow in tissues and organs, causing low oxygen levels, edema, bleeding and eventually organ failure. If kidney, lungs, heart, brain, or other visceral organs are affected, an acute renal failure, severe anemia, acute respiratory distress syndrome, loss of brain functions, coma and death may occur. In pregnant women, placental malaria can lead to reduced birth weight, abortions and still births (Lucius and Loos-Frank 2008; Rowe et al. 2009; 'CDC - DPDx - Malaria' 2016).

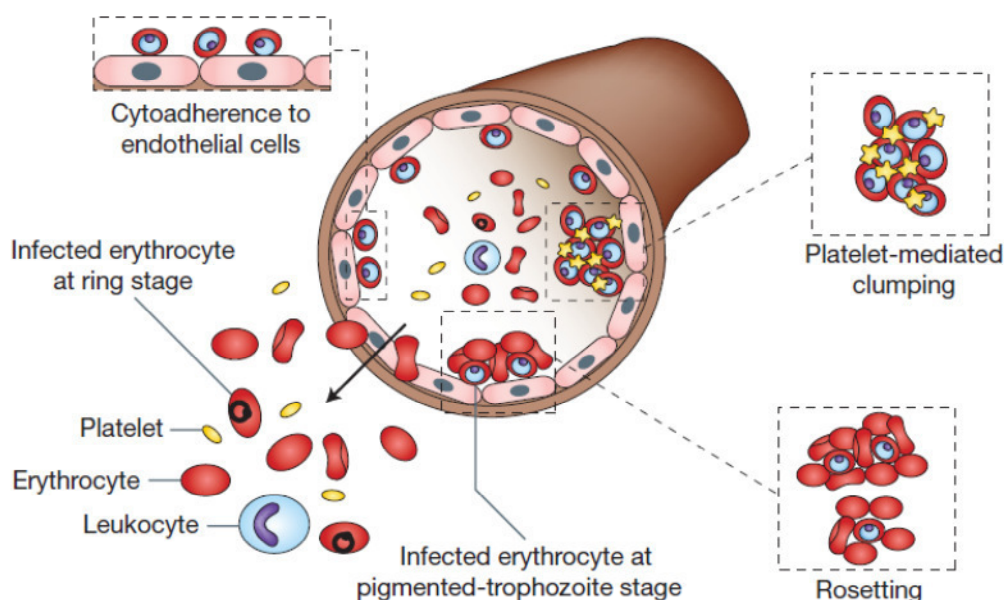


Fig.3: Forms of cytoadhrence of *P. falciparum* with host tissues. Infected erythrocytes at pigmented-trophozoite stage can adhere to endothelial cells, platelets (platelet-mediated clumping), or uninfected erythrocytes (rosetting) (Rowe et al. 2009).

1.2. Semi-immunity

In endemic regions with perennial malaria transmission, the majority of the population is infected with *P. falciparum* (Dal-Bianco et al. 2007). However, the incidence of severe malaria decreases with increasing age (Cham et al. 2009). After several consecutive infections in childhood, an unsterile immunity (semi-immunity) against severe forms of the disease is established (Cham et al. 2009; Chan et al. 2012; Giha et al. 1999; Gupta et al. 1999; Ofori et al. 2002). This semi-immunity is based on antibodies against specific parasite antigens (P. C. Bull et al. 1998). As a result, the parasites persist in a chronic, asymptomatic infection with submicroscopic parasitemia (L. H. Miller, Good, and Milon 1994). Transmission, however, can still take place. This shows that semi-immunity against *P. falciparum* can be acquired, but doesn't protect against new infections and isn't able to eliminate the parasites completely from the human host (Hviid 2010). Semi-immune individuals who lived outside of malaria endemic regions for some time and did not have constant re-infection with the parasite are fully sensitive to malaria infections upon their return to an endemic country. This suggests that semi-immunity is maintained by constant re-infection (Doolan, Dobaño, and Baird 2009; Moncunill et al. 2013; Owusu-Agyei et al. 2001; Bunn et al. 2004; Keenihan et al. 2003).

In 1961 and 1962, serum transfer experiments conducted in African children showed that the passive transfer of immunoglobulines via intramuscular injection has a protective effect against *P. falciparum* malaria (Cohen, McGREGOR, and Carrington 1961; Edozien, Gilles, and Udeozo 1962). The serum used in those studies was prepared from adult donors living in hyperendemic areas who were most likely semi-immune. In 1991, Sabchareon et al. demonstrated that serum from semi-immune Africans decreases the parasitemia of malaria patients in Thailand, suggesting that malaria antigens are relatively conserved across the world (Sabchareon et al. 1991).

Together, these findings strongly indicate that proteins of the malaria parasite act as antigens and induce an immune response in the human host. This ultimately leads to an antibody based protective immunity in the serum of semi-immune individuals (P. C. Bull et al. 1998, 1).

1.3 Escaping the immune system by antigenic variation

Despite the evidence for antibodies against *P. falciparum*, semi-immune malaria patients are not able to cure an infection via antibody response and parasites persist in submicroscopic levels in the blood (L. H. Miller, Good, and Milon 1994). Thus, the parasite must have developed a strategy to escape the human immune system.

P. falciparum uses antigenic variation to avoid the immune system of the human host. The trophozoites express several variant surface antigens (VSAs) on the surface of infected erythrocytes (P. C. Bull et al. 1998, 1998; Ofori et al. 2002) that are subject to constant changes so that immunologically new variants of the pathogen are ceaselessly generated (Su

et al. 1995). Among these VSAs are RIFIN (Repetitive interspersed family) (Fernandez et al. 1999), STEVOR (subtelomeric variable open reading frame) (Cheng et al. 1998), PfMC-2TM (*Plasmodium falciparum* maurer's clefts two transmembrane) (Sam-Yellowe et al. 2004), surfins (surface associated interspersed genes) (Winter et al. 2005) and PfEMP1 (*Plasmodium falciparum* Erythrocyte Membrane Protein 1) (Baruch et al. 1995). To date, PfEMP1 is the most studied of these VSAs(Baruch et al. 1995; Gardner et al. 2002; Smith et al. 2013; Su et al. 1995). This protein is predominantly presented on protruding structures of the erythrocyte surface called knobs (Gruenberg, Allred, and Sherman 1983; Langreth et al. 1978; Luse and Miller 1971), and is able to adhere to endothelial host cell receptors such as CD36 (cluster of differentiation 36) (Oquendo et al. 1989), EPCR (Endothelial protein C receptor) (Lau et al. 2015; Turner et al. 2013), ICAM1 (intracellular adhesion molecule 1) (Berendt et al. 1989), CSA (Chondroitin-Sulfat A) (Fried and Duffy 1996), thrombospondine (Roberts et al. 1985), and E-selectin (Ockenhouse et al. 1992). The disease pattern may vary, depending on which receptors the parasites bind to.

PfEMP1 is encoded by a hypervariable, multicopy gene family called *var* (Su et al. 1995). It consists of about 60 different *var* genes per parasite genome (Gardner et al. 2002), but only one is expressed at a time while all other loci are transcriptionally silenced (Chen et al. 1998; Dzikowski, Frank, and Deitsch 2006; Scherf et al. 1998; Voss et al. 2006). This is called mutually exclusive expression or allelic exclusion. If the parasite switches between the active *var* locus, it changes the PfEMP1-variant on the surface of infected erythrocytes and thereby possibly changes the adhesion-phenotype (Louis H. Miller et al. 2002) (Fig. 4A). While the immune system is fighting and eliminating the population of parasites expressing one PfEMP1 variant, a subpopulation of parasites expressing another variant is able to grow until the immune system has had time to build antibodies against the new variant. This leads to the typical wave like pattern of parasitemia in an infection and is a way of the parasite to trick the immune system (L. H. Miller, Good, and Milon 1994). Fig. 4B shows parasite levels in the blood of an individual that has been subjected to a single infection with *P. falciparum* via mosquito bite. The infection was monitored, but remained untreated until a curative dose of chloroquine was given on day 260 (see symbol C) (Miller et al., 1994).

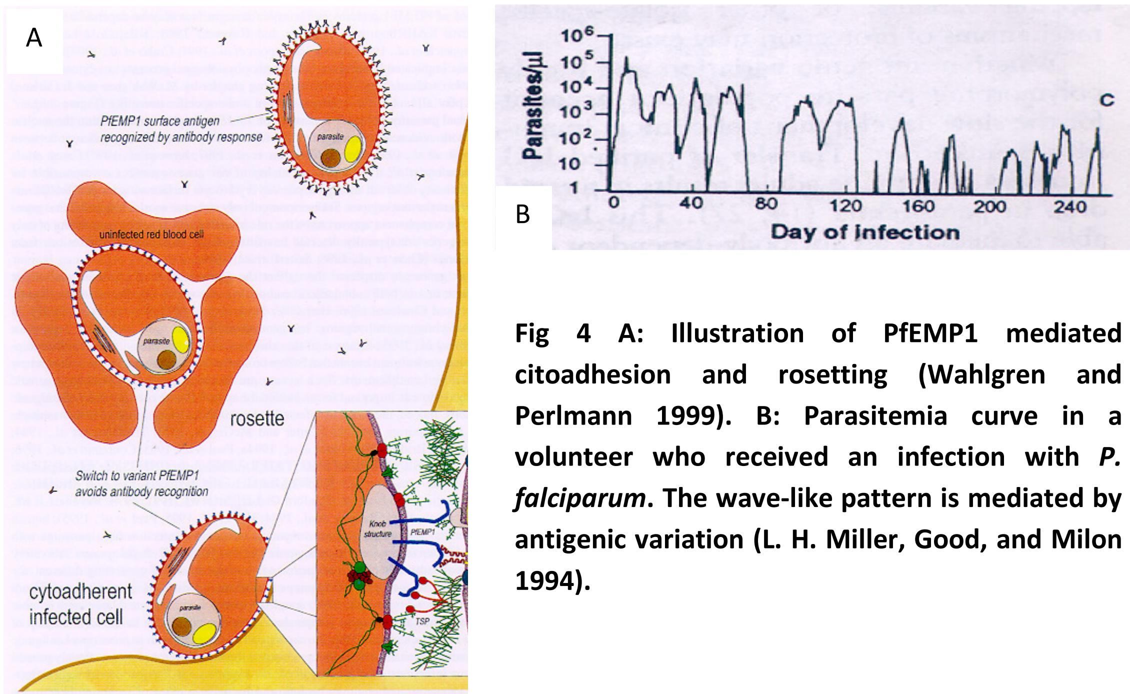


Fig 4 A: Illustration of PfEMP1 mediated citoadhesion and rosetting (Wahlgren and Perlmann 1999). B: Parasitemia curve in a volunteer who received an infection with *P. falciparum*. The wave-like pattern is mediated by antigenic variation (L. H. Miller, Good, and Milon 1994).

1.4. *var* genes

1.4.1. Structure and classification

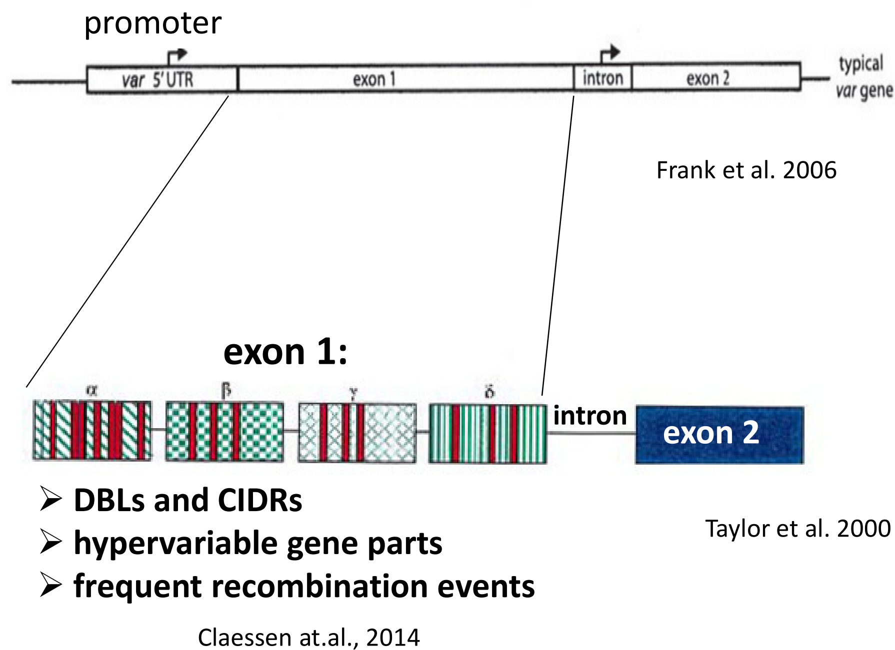


Fig. 5: schematic *var* gene structure with UTR, exon1, intron and exon 2. As well as details of exon 1 consisting of DBLs (Frank et al. 2006; Taylor, Kyes, and Newbold 2000; Claessens et al. 2014)

Despite being the most variable gene family of *P. falciparum*, *var* genes share conserved motives and have similar structures. The malaria genome project sequencing the *P. falciparum* laboratory strain NF54 3D7 (Gardner et al. 2002) showed that they are composed of a promoter within an untranslated region (5' UTR), exon 1 (4-10kb), an intron and exon 2 (2kb). Exon 2 is semi-conserved and encodes for the trans-membrane part of the protein. Exon 1 encodes for the extracellular part of the protein and consists of an N-terminal segment, followed by so called domain cassettes (DCs), consisting of several Duffy Binding Like (DBL) and Cysteine Rich Interdomain Regions (CIDR) domains. There are six major DBL domain classes (DBL α, β, γ, δ, ε, ζ) and four CIDR classes (CIDR α, β, γ, δ). These classes can be further divided into subclasses. (Claessens et al. 2014; Kraemer and Smith 2003; Kraemer et al. 2007; Lavstsen et al. 2003). Within the DBLs are conserved sequence blocks that are similar in all parasite strains as well as hypervariable sequence blocks for which high frequencies of recombination have been reported (Peter C. Bull et al. 2005, 2008; Kraemer et al. 2007; Taylor, Kyes, and Newbold 2000).

var genes can be sorted into groups according to their chromosomal position and promoter type (Lavstsen et al. 2003; Rask et al. 2010). UPS A type *var* genes are located at the telomere and are transcribed towards the centromer. PfEMP1 variants encoded by UPS A type *var* genes are associated with malaria infections in early childhood (Cham et al. 2009; Jensen et al. 2004; Lavstsen et al. 2012; Rowe et al. 2009). Many of them have recently been shown to have head structures that bind to EPCR (Endothelial protein C receptor) (Turner et al. 2013, 2), which is associated with severe malaria (Turner et al. 2013) and has been linked to cerebral malaria (Moxon et al. 2013). Other UPS A type PfEMP1 variants form rosettes with uRBCs. These variants are likely to be encoded by CIDR β, γ, and δ domains (Ghumra et al. 2012).

UPS B and B/A type *var* genes are located at the telomere and are transcribed towards the centromere. UPS C and B/C type *var* genes are located centrally and are transcribed towards the telomere. Most of the *Pf*EMP1 encoded by UPS B and C type *var* genes have a head structure that binds to CD36, which is associated with uncomplicated malaria (Almelli, Ndam, et al. 2014; Almelli, Nuel, et al. 2014; Kaestli et al. 2006; Merrick et al. 2012; Rottmann et al. 2006; Rowe et al. 2009). CD36 binding is mediated by the CIDRα domain in the PfEMP1 head structure (Hsieh et al. 2016). It is a common binding phenotype that is facilitated by many *var* genes (Baruch et al. 1997). In very recent studies, Lennartz et al. and Metwally et al. suggested synergetic binding of some *var* genes to several receptors, which was even associated with cerebral malaria (Lennartz et al. 2017; Metwally et al. 2017).

There is one unique *var* gene which possesses a UPS E type promoter. This *var* gene is called *var2csa* and was first described by Salanti et al. in 2003 (Salanti et al. 2003). It is highly conserved among all *P. falciparum* strains and encodes for the only PfEMP1 variant that is able to bind to chondroitin sulfate A (CSA) in the placenta of pregnant women, thus causing placental malaria. Its specific and unique binding phenotype poses a clear selection

advantage for this *var* gene in the presence of a placenta, which is most likely the reason for its high grade of conservation.

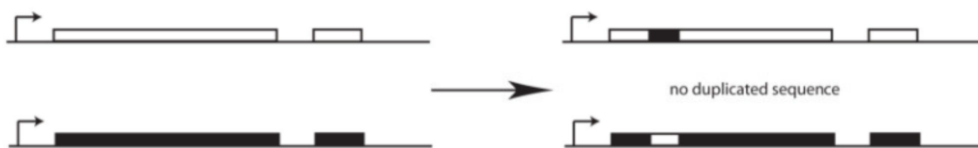
1.4.2 Hypervariability and Recombination

Frequent recombination of *var* genes is believed to be the main cause for *var* gene diversity among *P. falciparum* strains (Claessens et al. 2014; Frank et al. 2008; Freitas-Junior et al. 2000).

By analyzing the *var* genes of a genetic cross between two laboratory *P. falciparum* strains, Freitas-Junior showed in 2000 that *var* genes undergo recombination (Freitas-Junior et al. 2000). They observed recombination events between subtelomeric regions of heterologous chromosomes that cluster together near one pole of the nuclei in sexual parasite forms. These recombination events include reciprocal recombination and duplicative transposition (gene conversion). While reciprocal recombination leads to changes in both contributing *var* genes, gene conversion events leave one *var* gene intact, but a duplication of part of its sequence replaces a similar sequence in a related *var* gene (Fig. 6). Since these recombination events have been observed in the background of a genetic cross, it is very likely that they occurred during meiosis of *P. falciparum* in the mosquito. Interestingly, the heterologous chromosomes that underwent recombination have been shown to cluster together in the nuclear periphery in asexual blood stage parasites. This led the authors to the assumption that recombination between these chromosomes might also take place during mitotic division of asexual blood stages (Freitas-Junior et al. 2000).

In 2008, Frank et al. confirmed these observations by analyzing recombination events between two *P. falciparum* sibling strains. The authors found reciprocal recombination events as well as one case of duplicative transposition (Frank et al. 2008).

A. Reciprocal exchange



B. Gene conversion

Fig.6 A: Reciprocal gene exchange. Two genes exchange a part of their sequence. No duplication of sequence. B: Gene conversion. Part of a gene sequence is duplicated and inserted into another gene (Frank et al. 2008).

Claessens et al. recently proposed a model in which the diversity of *var* genes is mainly generated during mitotic divisions in the asexual part of the life cycle (Claessens et al. 2014). The authors performed whole genome sequencing on several generations of asexually replicating parasite strains. They found that structural mutations such as deletions, translocations or duplications occur frequently around *var* genes, yet the sequences of all newly generated *var* genes were in frame and domain architecture remained intact. They proposed that the high recombination rate of *var* exon 1 (up to 0.2% per iRBC per life cycle) could lead to the generation of millions of new antigenic structures each day in a single malaria patient (Claessens et al. 2014).

An exception to the highly variable *var* genes is *var2csa*, which has been described in chapter 1.4.1. This *var* gene can be found in all *P. falciparum* strains (Salanti et al. 2002; Gardner et al. 2002), which is most likely due to positive selection of its specific binding phenotype.

1.4.3. Transcriptional regulation

It is not entirely known how *var* gene switching or mutually exclusive expression is regulated. Most likely there is a whole complex of control mechanisms on a genetic, epigenetic as well as nuclear level. It has been shown that two promoters are necessary to silence a *var* gene (Deitsch, Calderwood, and Wellems 2001; Frank et al. 2006; Gannoun-Zaki et al. 2005). One promoter is located in the UPS region of a *var* gene and one within the intron. Only if both promoters are present, the gene is subject to mutually exclusive expression (Kyes et al. 2007). Amit-Avraham et al. showed recently that so called IncRNAs (long noncoding RNAs) play an important role in *var* gene activation and mutually exclusive expression (Amit-Avraham et al. 2015). IncRNAs originate from *var* introns and are associated with the active *var* locus at the same time as the UPS promoter of that *var* gene is active. They are incorporated into chromatin and if expressed *in trans*, activation of a silent *var* gene is triggered. If there is interference of the IncRNAs, the active *var* locus is down regulated (Amit-Avraham et al. 2015).

Epigenetic control mechanisms also have an important role in transcriptional control, particularly histone modification. In 2007, Chookajorn et al. showed that trimethylation of histone H3 at lysine 9 (H3K9me3) is highly enriched in silent *var* genes (Chookajorn et al. 2007). H3K9me3 was however not detectable in the coding region of active *var* genes. Lopez-Rubio et al. showed in 2009 that silencing of several variant gene families is associated with H3K9me3 (Lopez-Rubio, Mancio-Silva, and Scherf 2009). In 2 of these gene families, *var* and *rif*, the disruption of histone deacetylase PfSir2 causes changes in H3K9me3, leading to the disruption of mutually exclusive expression. It has also been shown recently that the *P. falciparum* variant-silencing SET gene (PfSETvs) encodes for an enzyme that controls histone H3 lysine 36 trimethylation (H3K36me3) (Jiang et al. 2013). H3K36me3 acts globally on all variant gene families. If the PfSETvs gene is knocked down, all variable gene families, including the *var* genes, transcribe all their variants at the same time in a single parasite. Jiang at al. propose a model where, in order for a *var* gene to be silenced, the PfSETvs-

dependent H3K36me3 has to be present along its entire length, including the transcription start site. In 2014, Ukaegbu et al. also highlight the importance of PfSET2 for *var* gene regulation and antigenic variation, however the authors found the PfSET2 induced H3K36me3 at all *var* genes, regardless of their transcriptional state (Ukaegbu et al. 2014). The authors suggest that H3K36me3 is not simply a silencing mark, but hypothesize that it marks *var* genes for inclusion into the mutually exclusive pathway. A knock down of PfSET2 (PfSETvs) would then loosen this mark which disrupts mutually exclusive expression, leading to transcription of all *var* genes at the same time. This is exactly what has been observed by Jiang at al. (Jiang et al. 2013). More importantly, Ukaegbu et al. propose a model in which PfSET2 is recruited to *var* loci by binding to the C terminal domain of RNA Polymerase II, possible while noncoding RNA (ncRNA) is produced (Ukaegbu et al. 2014). This finding not only provides deeper understanding of how histone modifications coordinate *var* gene expression, but also links epigenetic control mechanisms to transcriptional control mechanisms like ncRNAs.

Spatial conditions in the nucleus also seem to play a role in *var* gene transcription as well. *P. falciparum* has different compartments for active and inactive *var* gene promoters in the peripheral nucleus (Dzikowski et al. 2007; Ralph, Scheidig-Benatar, and Scherf 2005; Voss et al. 2006). This can, however, not be the only reason for mutually exclusive expression, because more than one active promoter can fit in such a compartment (Dzikowski et al. 2007).

There has to be a combination of these and maybe other mechanisms leading to the tight and specific control of *var* gene transcription.

It is still not known how the parasite chooses the *var* locus which is to be activated next, but there is evidence for a hierarchical switching pattern *in vitro* as well as *in vivo*. Long term *in vitro* cultures of laboratory strains express the same UPS C *var* genes over a long period of time without switching (Enderes et al. 2011; Frank et al. 2007; Noble et al. 2013; Recker et al. 2011). UPS C *var* genes are also associated with chronic long term infection. UPS A and B *var* genes can be found to be actively transcribed in acute infections, but almost never in culture adapted *P. falciparum* strains.

1.5. Host factors affecting *var* gene expression

Mutually exclusive expression of a single *var* gene at a time is a mechanism occurring at the individual parasite level (Dzikowski, Frank, and Deitsch 2006). During an infection, there are millions of parasites in the human host. In this big population, it is possible that all *var* genes are transcribed. This *P. falciparum* population then faces two kinds of selective pressures: the positive selective pressure of cytoadhesion, which favors parasites with a higher affinity to host receptors and the negative selective pressure of the immune system. On the one hand, the parasite wants to protect as many *var* gene variants as possible from exposure to the immune system, on the other hand it has to switch loci to not be eliminated by antibody

responses. Which factors contribute to keeping the balance between these two selective forces and do these factors come from host or parasite?

Controlled human malaria infections (CHMIs) have proven to be ideal for studying the dynamics of *var* gene transcription in natural infections. CHMIs have been conducted for the past 30 years (Rieckmann 1990) and have proven to be safe and effective (Verhage et al. 2005). So far, only two CHMI experiments have addressed possible factors affecting *var* gene transcription (Peters et al. 2002; Lavstsen et al. 2005; Wang et al. 2009). These investigations both showed that *var* gene transcription is reset after transmission from mosquito to humans, but propose two different models about how it is further regulated. Peters et al. 2002 suggest that *var* gene transcription in parasites exiting the liver follows an genetic program by which they first transcribe one dominant *var* locus with a high switching rate and then rapidly switch to other *var* loci with lower switching rates (Peters et al. 2002). Lavstsen et al. 2005 and Wang et al. 2009 propose that parasites exciting the liver first transcribe a broad repertoire of *var* genes which is then reduced to *var* loci that encode PfEMP1-variants which confer a growth advantage for the parasites. In this model, more efficient replication of parasites transcribing specific *var* loci leading to more efficient receptor binding, rather than genetically programmed switching, determines the *var* gene transcription profile (Lavstsen et al. 2005; Wang et al. 2009).

2. Aims of the thesis:

The purpose of this thesis was to investigate factors that influence *var* gene expression in *P. falciparum* infections.

The CHMI trial (TÜCHMI 001) at the Institute of Tropical Medicine in Tübingen provided a unique opportunity to obtain parasite samples from malaria-naïve, human volunteers infected with sprorozoites of the *P. falciparum* laboratory strain NF54. In the first part of the thesis, these samples were used to analyze *var* gene transcription of *P. falciparum* parasites that had passed through mosquitoes and non-immune human hosts. In this model, possible factors influencing *var* gene transcription patterns and switching are restricted to the selection of the *var* locus by the parasite and the presented receptors on host cells.

In the second part of the thesis, *var* gene transcription analysis in a culture adapted field isolate from a semi-immune individual was investigated. The field isolate was collected during an antigenic diversity study in Lambaréné, Gabon, 2006/2007. Here, positive selection pressure in terms of in-host receptor selection and negative selection pressure by the immune system were present. Since semi-immune individuals have a global antibody response against their *P. falciparum* infection, we were able to assess the importance of serum antibodies against all VSA families of *P. falciparum*.

In the third project of this thesis, *P. falciparum* field isolates from different African regions were screened for the presence of a *var* gene that has been found to be identical in the field isolate from the second project. The grade of conservation of this gene was assessed in the context of *var2csa* as a positive marker of conservation and the diversity of microsatellite DNA located within the non-coding regions of the genomes of the field isolates.

3. Results:

3.1 Analysis of *var* gene transcription before and after mosquito and liver passages in naïve human volunteers (Pub.I).

While *var* gene transcription profiles have been studied abundantly in laboratory strains, it is still unclear how they are influenced by mosquito and liver passages. Controlled human malaria infection studies using *P. falciparum* sporozoites provide an ideal basis to investigate this in more detail. Here, malaria naïve volunteers were infected intradermally or intravenously using increasing doses of cryopreserved *P. falciparum* sporozoites from the same mosquito feed, generated from the same premosquito culture. *In vitro* parasite cultures were established of blood samples taken from the volunteers who developed a microscopically detectable parasitemia. The *var* transcription profile was then determined at an early and a late time point post infection as well as in pre-mosquito parasites.

At the early time point post infection, all parasites taken from in vitro cultures transcribed the same group of subtelomeric *var* genes, clearly indicating that a fixed order of transcription exists. Parasites of intradermal infections showed a much stronger transcription signal than those of intravenous infections. There was one exception: parasites obtained from a culture initiated with parasites from the volunteer who received the lowest intravenous dose (50 sporozoites (50 IV)) behaved like parasites obtained from volunteers who were intradermally infected with 2500 sporozoites. This result suggests that, following intradermal infections, many sporozoites remain in the skin, thus resulting in a low effective liver dose.

The difference in the observed transcription signal intensities between intradermal and high dose intravenous infections may reflect the fact that parasites of low liver dose infections had a significantly longer prepatent period and thus were exposed to host-receptor selection for a longer time. The prepatent period was defined as the time from infection to the first positive microscopic reading analyzing a thick blood smear.

At the late time point, all parasite cultures (except for 50 IV) showed low *var* gene transcription levels with a uniform pattern.

Pre-mosquito *var* gene transcription profiles also showed low transcription levels of the same set of *var* genes as at the late time point. This profile seems to be a default program of *var* gene transcription activated after a longer time period without any selective pressure under culture conditions. Interestingly, we discovered that parasites with a long prepatent period took longer to reach this default state of *var* gene transcription. This suggests that longer durations of receptor selection may lead to a stronger selection for *var* loci that have a strong epigenetic mark, making them switch away slower.

Taken together, the data suggest that in non-immune volunteers, mosquito and host passages reset *var* gene transcripton, but the genetically determined program is influenced by the duration of host receptor selection. In the absence of selective pressures, the program returns to its default state.

3.2 Analysis of antibody response against *P. falciparum* VSAs in a semi-immune infection (Pub. II).

In this project, *var* gene transcription and the immune response to PfEMP1 were analyzed in a semi-immune individual, called MOA, from Gabon, Africa – a country with perennial *P. falciparum* transmission. There is a lack of studies with semi-immune malaria patients because close clinical monitoring is necessary, which is difficult to establish in rural areas of Africa.

Analysing *var* gene transcription via DBL cloning revealed that there were only 5 active *var* genes transcribed *in vivo* and that the same parasite populations stayed stable in the semi-immune individual over a long time. After cell culture adaptation of the parasite population, limiting dilution was performed to obtain parasite clones. After establishing a qRT-PCR primer set of the *var* genes of this field isolate via DBL cloning and sequencing, several parasite clones were identified expressing one *var* gene that was also expressed *in vivo.* Several other clones expressed *var* genes found *in vitro*.

FACS with convalescent serum of the semi-immune individual then showed that not all clones expressing the same *var* gene were recognized by the immune system of the host. Furthermore, the introduction of a PfEMP1 knock down did not affect the FACS signal. Transmission electron microscopy and immune-electron microscopy showed knobs on the surface of infected erythrocytes and antibody binding to knobby and knobless parts of the membrane. Trypsinisation of surface proteins led to a loss of the FACS signal in all clones. These results suggested that non-PfEMP1, trypsine sensitive VSAs might be the main targets of the immune response in chronic *P. falciparum* infections.

3.3 Identification of a conserved *var* gene in different *P. falciparum* strains (Pub. III).

During DBL cloning of the *var* genes of the MOA field isolate investigated in the second publication included in this work, one DBL was found that was 100% identical with the DBL of the *var* gene PF3D7_0617400 in the laboratory strain NF54 3D7. Sanger sequencing along the entire open reading frame (ORF) showed that the whole Exon 1 is identical to the 3D7 reference sequence. A clone of NF54 3D7 as well as a clone of the MOA field isolate from Gabon was identified that transcribe PF3D7_0617400 in a stable manner and immunofluorescence essays showed the presence of the corresponding PfEMP1 on the surface of infected erythrocytes. However, CD36 binding capacity and surface recognition signals differ between the two clones, suggesting a difference in PfEMP1 display. To

investigate whether or not PF3D7_0617400 is conserved in other *P. falciparum* strains, nine additional freshly culture adapted field isolates from Central Africa (Congo, Cameroon), West Africa (Gambia, Ghana and Togo) and East Africa (Kenya and Sudan) were screened by targeted PCR fragment Sanger sequencing of Exon 1 of PF3D7_0617400. To assess the degree of genetic conservation of PF3D7_0617400, Sanger sequencing was also performed for *var2csa*, which is highly conserved. As a marker for diversity of the non-coding genome, fragment length analysis of 57 microsatellites (MS) scattered across all 14 *P. falciparum* chromosomes was performed on all strains.

Altogether, PF3D7_0617400 could be detected in two field isolate, in which Exon 1 was 99-100% identical to the reference sequence on 3D7, except for a small insertion of 196 bp. The diversity of non-coding regions was high across all *P. falciparum* strains investigated in this work, but *var2csa* could be amplified in all field isolates. Some areas of *var2csa* were highly conserved, while others were highly variable. The data obtained from this project suggests that there is a purifying selection on the diversity of individual *var* genes, possibly driven by individual binding phenotypes.

4. Discussion:

Despite a reduction in *P. falciparum* infections in recent years due to intervention strategies such as treatment with artemisinin combination therapies (ACTs) or insecticide treated bed nets, malaria is still a high burden for people, especially children, who live in endemic countries as well as for travelers (WHO | World Malaria Report 2015' 2016).

Antigenic variation of virulence genes such as *var* genes and the ability of the parasite to hide from the immune system are still not fully understood. While extensive research has been done on the individual cell level regarding *var* gene transcription and PfEMP1 expression, little is known about the behavior of parasite populations during acute and chronic infections and the factors that influence virulence and disease outcome.

A good opportunity to study parasite populations in natural infections are CHMI studies such as the TÜCHMI 001 (NCT01624961) study that took place at the Institute of Tropical Medicine in 2012 (Mordmüller et al. 2015). While CHMI studies have been done for over 30 years (Rieckmann 1990), the TÜCHMI 001 study was especially interesting, because intravenous (IV) inoculation of viable *P. falciparum* sporozoites was used for the first time. Intradermal infections were used as a control group. There are many CHMIs performing infections via mosquito, but this poses the disadvantage that the amount of transmitted sporozoites is unknown. IV inoculation provides the advantage that the exact infectious dose is known because all sporozoites reach the bloodstream.

In the first publication included in this dissertation, it is shown for the first time that route and dose of infection play a role in determining *var* gene transcription patterns of parasites from naïve human hosts grown in culture (Dimonte et al. 2016). Low dose IV infections led to a pattern of few but highly transcribed *var* loci. The same is true for high dose ID infections. In contrast, high dose IV infections led to a pattern of many but low transcribed *var* genes. Low dose IV and high dose ID infections show the same prepatent period, which is significantly longer than high dose IV infections. These results led us to hypothesize that not all sporozoites intradermally injected reach the liver, leading to a low effective liver dose, a longer prepatent period and consequently to a lower starting parasitemia in the blood. Given the hypothesis can be substantiated, the data show that the duration of exposure to receptor selection, while inside the host, influences *var* gene transcription profiles of *in vitro* parasite populations from naïve volunteers. Parasites from low dose IV and high dose ID infections have been exposed to the host environment for a longer time than parasites from high dose IV infections. Since naïve volunteers do not have an antibody response against *P. falciparum*, a major selective force influencing the *var* gene transcription pattern is the binding to host receptors. Receptor selection then prefers *var* loci with a binding phenotype that best ensures the parasite's survival, leading to a limited transcription pattern of *var* genes.

The data revealed that parasite populations that were exposed to receptor selection for a longer time took longer to return to a default *var* gene transcription pattern which they exhibit under cell culture conditions without selection pressure. This default pattern was also detected in pre-mosquito cultures and is characterized by low transcription of a certain set of UPSB and UPS C *var* loci. Collectively, the results showed that mosquito and human passages result in a significant change in *var* gene transcription pattern and strength.

Bachmann et al. investigated ex vivo *var* gene transcript levels of TÜCHMI 001 volunteers on the day of blood smear positivity (Bachmann et al. 2016). The authors showed that the *var* gene transcription pattern was identical in most of the volunteers. In contrast to the data obtained from the first project of this thesis, a broad variety of mainly UPS B and UPS A *var* genes were transcribed at high levels. By comparing the results from the first project of this thesis with raw data obtained by Bachmann et al., a strong decrease between *ex vivo* and *in vitro var* gene transcription levels could be observed. The decrease in transcription levels in IV infections was most pronounced among subtelomeric UPS A and UPS B genes. This suggests that removing host receptor selection by transferring the parasites into cell culture had an immediate effect on *var* gene expression, lowering their transcription rate.

In a previous study, Lavstsen et al. proposed a model in which parasites transcribe a broad variety of *var* genes after liver release (Lavstsen et al. 2005). The then following changes of transcription patterns were probably due to a more efficient replication of parasites transcribing a *var* gene encoding for a specific PfEMP1 variant. Data from the first project presented here, as well as the data of Bachmann et al. are clearly consistent with the model of Lavstsen et al.. Bachmann et al. showed an initially broad transcription pattern of *var* genes and the first project of this thesis showed that longer duration of host receptor selection of the same parasite population leads to a restriction of active *var* loci. Most likely, the remaining active *var* loci are favored because they encode for PfEMP1 variants that confer better cytoadhesion to host receptors, resulting in a growth advantage for this parasite.

As mentioned before, PfEMP1 is mostly, but not exclusively, presented on electron dense structures called knobs (Horrocks et al. 2005). Stanisic et al. recently (July 2016) discovered an NF54 strain that lost the ability to form knobs *in vitro* (Stanisic et al. 2016). The authors studied the behavior of knob-less infected erythrocytes in human volunteers and observed a reduction in infectivity. However, the parasite population recovers knob formation and cytoadhesion capacity in the host. The authors suggested that very few parasite clones within the population were still able to form knobs and therefore have been selected due to their ability to cytoadhere that avoids clearance by the spleen; this shows that "the human environment can modulate virulence". It also supports our hypothesis that, in acute infections, infected erythrocytes with strong cytoadhesion abilities are favored by receptor selection. Instead of sporozoites, Stanisic et al. used parasitized erythrocytes to infect human volunteers, thus skipping the passage of the parasites through mosquito and human liver, of which the influence on knob recovery is unknown. Nevertheless, in the work

presented here, transmission electron microscopy was performed on the NF54 strain used to feed the mosquitoes which produced the sporozoites used to infect the human volunteers described in the first publication included in this thesis. In contrast to Stanisic et al., knobs were clearly detected on the surface of infected erythrocytes.

In 2015, Tilly et al. showed that *in vitro* culture conditions have an influence on knob structure and cytoadhesion (Tilly et al. 2015). The authors investigated two common laboratory *P. falciparum* strains, NF54 3D7 and FCR3, cultivated with either AlbuMAX or human serum as additives. They observed a significantly reduced knob amount in NF54 3D7 if AlbuMAX instead of human serum was used. There was also a reduced cytoadhesion to various host receptors in the presence of AlbuMAX, including CD36. However, the reduction in CD36 binding was not correlated to the presence of knobs, but to the amount of PfEMP1 on the surface of infected erythrocytes, which was also reduced in the presence of AlbuMAX compared to human serum. The authors suggested that this reduction of PfEMP1 is rather due to a defective PFEMP1 membrane transfer or an altered surface conformation than due to changes in *var* gene transcription (Frankland et al. 2007). For the *var* transcription analysis in the projects included in this thesis, AlbuMAX was used in the culture medium. Parasite cultures investigated in the second project of this thesis have been exposed to human serum prior to FACS analysis (ensuring that the appropriate amount of PfEMP1 is on the surface). Tilly et al. also assumed that knobs are not essential for receptor binding under static conditions, but are essential for adhesion under flow conditions, which has been demonstrated earlier by Crabb et al. in 1997 (Crabb et al. 1997). These findings highlight one advantage of CHMIs, where parasites are exposed to physiological shearing forces in the blood flow of the human volunteers.

CHMIs are usually done with malaria-naïve volunteers, who have no antibodies against *P. falciparum* VSAs. However, most of the population at risk of *P. falciparum* malaria infections live in endemic countries and are semi-immune. Semi-immune individuals have chronic asymptomatic infections and have protective antibodies against many *P. falciparum* antigens (Cham et al. 2009). Unfortunately, it is difficult to conduct proper studies in endemic countries, because the settings are usually rural and lack equipment and infrastructure. It was therefore most convenient to analyze samples of a research study investigating antigenic diversity in chronic *P. falciparum* infections in Lambarene, Gabon (Enderes et al. 2011; Bruske et al. 2016). These analysis showed that in a chronic *P. falciparum* infection, where parasites have been exposed to host pressures for a long time, only very few *var* genes were transcribed *in vivo*. The results were conform with the observations from the first publication included in this thesis, which showed that parasite cultures of acute infections in naïve volunteers switch slower, the longer they were exposed to receptor selection (Dimonte et al. 2016).

FACS analysis of parasites from acute infections (TÜCHMI 001) with convalescent serum of the same semi-immune individual showed clear recognition of parasites that have been exposed to receptor selection for a longer time and transcribe few *var* loci with a high

relative copy number. Parasites that have been exposed to receptor selection for a shorter time transcribed many *var* loci, but with a low relative copy number and thus showed a low FACS signal. Interestingly, FACS analysis of clonal parasites obtained from the semi-immune individual probed with serum from the same individual showed that the FACS signal did not correlate with transcription of individual *var* genes or with their relative copy number. Furthermore, transgenic PfEMP1 knockdown parasites exhibited no difference in FACS reactivity, while trypsinisation of the parasites abolished the surface signal. Immune electron microscopy performed on the MOA field isolate showed that gold particles bound to membrane areas with and without knobs. These results led to the hypothesis that other, non-PfEMP1 VSAs, which are membrane bound, trypsine-sensitive and knob independent, also take part in mediating immune escape in semi-immune infections (Bruske et al. 2016). The STEVORS and RIFINS are the largest families of non-PfEMP1 VSAs that are displayed on the surface of *P. falciparum*-infected erythrocytes and are trypsine sensitive. Both VSAs seem to mediate rosetting (Niang, Yan Yam, and Preiser 2009; Goel et al. 2015) and RIFINS could even be able to adhere to endothelial cells (Goel et al. 2015).

Alltogether, the combination of receptor selection, expression of PfEMP1s with high binding affinities, and the antibody response of semi-immune hosts, may favor parasites possessing VSAs that restricts *var* gene transcription to specific loci. This may lead to a selection of individual *var* genes by conserving their sequence. One example of such a positive selection could be *var2csa*, which is highly conserved among all *P. falciparum* strains due to its specific function and unique binding phenotype (Salanti et al. 2003). Another example might be the *var* gene PF3D7_0617400, which was assessed in the third project described in this dissertation. It was found to be conserved in 2 out of 10 investigated field isolates from different African regions with 99-100% identity to the NF54 3D7 reference sequence. One field isolate was from Togo in East Africa and one from Gabon in Central Africa (The MOA field isolate described in publication I and II of this work). Analysis of *var* gene transcription identified one NF54 and one MOA clone transcribing PF3D7_0617400 and immunofluorescence assays showed that the corresponding PfEMP1 is expressed on infected erythrocytes of both clones. The NF54 clone transcribing PF3D7_0617400 successfully bound to the CD36 receptor, suggesting that the expressed PfEMP1 exhibits a CD36 binding phenotype. Furthermore, PF3D7_0617400 has a CIDR α2.1 domain (Metwally et al. 2017) which possesses a recently described hydrophobic pocket that confers CD36 binding (Hsieh et al. 2016). Despite an overall low sequence similarity of the CIDRα2-6 domains, the hydrophobic pocket is present in virtually all of them, raising the question why the sequence of PF3D7_0617400 is conserved in 2 field isolates and thus seems to give the parasite a selective advantage. Metwally et al. recently suggested that PF3D7_0617400 is able to bind to CD36, ICAM, P-selectin, E-selectin, CD9, CD15 and a yet unidentified receptor(Metwally et al. 2017). The ability to bind to multiple receptors has recently been shown to confer more efficient binding (Lennartz et al. 2017), providing a potential explanation why PF3D7_0617400 might lead to a selective advantage.

A model in slight contrast to purifying selection was recently proposed by Cleassens et al. (Claessens et al. 2014), in which frequent recombination events of *var* genes during mitotic divisions in the asexual blood stage phase of the parasite could lead to the generation of millions of new antigenic structures each day in a single malaria patient. However, the authors investigated parasites from *in vitro* cultures that do not face any selection pressures. In a recent publication, Hamilton at al. investigated mutation rates in clone trees of long term culture-adapted laboratory strains as well as culture adapted field isolates from Cambodia (Hamilton et al. 2016). The results showed that there are significantly higher *var* gene exon 1 recombination rates in the culture-adapted Cambodian field isolates than in the laboratory isolates. The authors suggest that this is due to higher *var* gene transcriptional activity in field isolates, as active *var* gene transcription could somehow open the chromosome to facilitate recombination. This might also explain the lower recombination rates of UPS A *var* genes *in vitro*, where they are much less expressed than *in vivo*. However, the authors remark that *var* gene recombination *in vivo* is currently uninvestigated and further research is needed to find a correlation between *var* gene transcription mechanics and recombination rates.

In 2013, Noble et al. investigated *in vitro var* gene transcription of the *P. falciparum* laboratory strain HB3 (Noble et al. 2013). The authors propose that in acute infections of malaria naïve hosts with no anti-PfEMP1 antibodies, subtelomeric UPS A genes are predominantly transcribed, because they consist of relative long and conserved sequences, conferring a growth advantage due to higher receptor binding affinities (Avril et al. 2012; Claessens et al. 2012; Lavstsen et al. 2012). The acquisition of an immune response against these genes leads to the transcription of more variant, centrally located *var* genes with low off rates, their variability mediating immune escape in chronic infections.

The findings presented in this work as well as in previous publications suggest that the surface of *P. falciparum* infected erythrocytes is composed of different VSAs in acute and chronic infections. Bull et al. and Nielsen at al. suggested a model that differentiates between VSAs expressed on parasites from individuals with severe malaria (VSA_{SM}) and VSAs expressed on parasites from individuals with uncomplicated malaria (VSA_{UM}) (P. C. Bull et al. 2000; Nielsen et al. 2002). According to this model, expression of VSA_{SM} may generate high parasitemias and possibly a strong humoral immune response, because parasites expressing VSA_{SM} have a growth advantage in children (that are not yet semi-immune). The strong humeral immune response restricts the growth of parasites expressing VSA_{SM} during infections of semi-immune individuals, resulting in growth of parasites expressing VSA_{UM} that in turn replicate in a less efficient manner. There is strong evidence that UPS A *var* genes may be part of VSA_{SM} because they mediate disease associated phenotypes such as binding to endothelial protein C receptor. Additionally, a recent report by Abdi et al. suggests that anti-PfEMP1 antibodies not only select against specific PfEMP1 variants, but also against the

amount of PfEMP1 on the surface of infected erythrocytes in chronic infections (Abdi et al. 2016). One could combine the model by Bull and the report by Abdi and expand them.

At the early onset of an acute infection in a naïve host, a broad repertoire of *var* genes is transcribed with high copy numbers, leading to many VSAs (in this case PfEMP1) on the surface of infected erythrocytes to bind to suitable host receptors. Favoring of parasites transcribing *var* genes that encode for PfEMP1 with high receptor binding affinities leads to better cytoadherance, less clearing of trophozoites in the spleen and a better replication rate, causing higher parasitemias and severe symptoms. These PfEMP1s most likely constitute the majority of VSA_{SM} and *var* genes encoding for them belong to the UPS A and UPS B type (Avril et al. 2012; Claessens et al. 2012; Lavstsen et al. 2012; Turner et al. 2013).

The acquisition of antibodies against VSA_{SM} leads to a shift in *var* gene transcription to a few *var* loci with low copy numbers and low switching rates, leading to only few PfEMP1s on the surface of infected erythrocytes, making them adhere less which leads to more clearance in the spleen and a slower replication rate. According to Noble at al 2013, the *var* genes encoding for these PfEMP1s are centrally located (UPS B or C type) and highly variable, thus mediating immune escape in chronic infections (Noble et al. 2013). Their high variability might be due to frequent recombination events occurring during mitosis (Claessens et al. 2014). Hsieh et al. showed in a recent publication that there is a hydrophobic pocket in the PfEMP1 protein that confers CD36 binding, a common binding phenotype in centrally located *var* genes (Hsieh et al. 2016). This hydrophobic pocket seems to be well conserved among CD36 binding *var* genes. Its concave shape might protect the structure from being discovered by the host's immune system, while the genetic sequence of the *var* gene and thus the amino acid sequence of the resulting PfEMP1 around the hydrophobic pocket may change and recombine to avoid the host's antibody response against visible parts of the protein. This might be a reason for the successful concept of CD36 binding. But why are parasitemias in semi-immune individuals so low that they cannot be microscopically detected if the parasites possess a conserved CD36 binding motive? Considering the results of publication II of this thesis, PfEMP1s on the surface of infected erythrocytes most likely constitute the minority of VSA_{UM}. This may cause a reduced CD36 binding capacity, while other, non-PfEMP1 VSAs such as STEVORS and RIFINS or even yet unknown VSAs play a significant role in mediating an immune escape in chronic infections. In this way, a balance between parasites being cleared by the spleen and parasites binding to receptors to circumvent the host's immune system in achieved.

Synergistic binding of a PfEMP1 variant to several host receptors adds a new variable to these dynamics. In acute infections this could increase virulence of the parasites and it has indeed been associated with cerebral malaria (Lennartz et al. 2017). In chronic infections the presence of such a PfEMP1 clearly provides an advantage for the parasite's survival, as it is harder to be cleared from the bloodstream by the spleen.

Malaria is considered a relatively young parasitic disease in humans due to its ability to kill the host if it remains untreated and in this process also kills itself. Yet, the goal of a parasite is to co-exist with the host to ensure its own survival, like in semi-immune malaria patients.
In conclusion, receptor selection in naive hosts and antibody response in semi-immune hosts leads to transcription of *var* loci that are profitable for the survival of the parasite, but keep harmful effects for the host to a minimum. This could lead to a purifying selection and the conservation of certain *var* gene sequences and VSA composition.

5. Own contribution

Pub I:

Fort the first publication I shared equally the work on cell culture parasite harvesting, RNA extraction and cDNA production with Dr. Ellen Bruske. I did 75% of realtime PCR runs. I analysed the results of all realtime PCR runs and processed them for statistic analysis. Statistics have been done by Johanna Haß.

Pub II:

For the second project, I designed, established and validated the primer set for the MOA field isolate and performed realtime PCR for all new MOA clones. I also cultivated C32 cells that were needed to perform the CD36 binding essays which I shared with Dr. Ellen Bruske. I performed genotyping on all MOA clones.

Pub III:

For the third publication, I designed the primer set for the *var* gene PF3D7_0617400. I performed PCR on 10 field isolates and sequenced the PCR product.

The overall project was developed by my advisor Dr. Matthias Frank and carried out together with Dr. Ellen Bruske, University of Tübingen.

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8. Resumé

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9. Appendix

i. Publications

The Journal of Infectious Diseases

MAJOR ARTICLE

Sporozoite Route of Infection Influences In Vitro *var* Gene Transcription of *Plasmodium falciparum* Parasites From Controlled Human Infections

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Background. Antigenic variation in *Plasmodium falciparum* is mediated by the multicopy *var* gene family. Each parasite possesses about 60 *var* genes, and switching between active *var* loci results in antigenic variation. In the current study, the effect of mosquito and host passage on in vitro *var* gene transcription was investigated.

Methods. Thirty malaria-naive individuals were inoculated by intradermal or intravenous injection with cryopreserved, isogenic NF54 *P. falciparum* sporozoites (PfSPZ) generated from 1 premosquito culture. Microscopic parasitemia developed in 22 individuals, and 21 in vitro cultures were established. The *var* gene transcript levels were determined in early and late postpatient cultures and in the premosquito culture.

Results. At the early time point, all cultures preferentially transcribed 8 subtelomeric *var* genes. Intradermal infections had higher *var* gene transcript levels than intravenous infections and a significantly longer intrahost replication time ($P = .03$). At the late time point, 9 subtelomeric and 8 central *var* genes were transcribed at the same levels in almost all cultures. Premosquito and late postpatient cultures transcribed the same subtelomeric and central *var* genes, except for *var2csa*.

Conclusions. The duration of intrahost replication influences in vitro *var* gene transcript patterns. Differences between premosquito and postpatient cultures decrease with prolonged in vitro growth.

Keywords. malaria; *var* genes; transcription; *Plasmodium falciparum*; controlled human malaria infections; epigenetics.

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The protozoan parasite *Plasmodium falciparum* causes the most severe form of malaria and is responsible for the death of approximately half a million African children per year [1]. *P. falciparum* malaria is a consequence of cytoadhesion of infected red blood cells to host endothelial receptors [2]. Cytoadhesion is mediated by a polymorphic family of surface proteins called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [3–5], encoded by the multicopy *var* gene family, which consists of approximately 60 different *var* genes [6]. In mutually exclusive expression, only a single member of the *var* gene family [7–10] is expressed at a time, and switches in *var* gene transcription provide the basis for antigenic variation [2]. Epigenetic modifications, such as the histone mark H3K9me3 [11–13] of the *var* gene promoter and the conserved *var* intron [14–17], are essential to mediate silencing of individual *var* genes.

The *var* genes can be grouped according to their chromosomal position and promoter type [18, 19]. Of the 59 *var* genes in the 3D7 clone of *P. falciparum,* 36 are located in subtelomeric areas of the 14 *P. falciparum* chromosomes and 23 in central *var* clusters. Central *var* genes possess promoters of the UpsC or UpsB/C type and are transcribed toward the telomere. Subtelomeric *var* genes belong to the UpsA type, if transcribed toward the telomere and to the UpsB or UpsB/A type, if transcribed toward the centromere [6, 16, 18]. The subtelomeric *var* gene PF3D7_1200600 (*var2csa*) [20] and the pseudo *var* gene PF3D7_0533100 (*var1csa*) [8, 21] have a unique UpsE and UpsD promoter. In culture-adapted parasites, *var* gene transcription follows a hierarchy favoring transcription of central UpsC genes [22–24], indicating that chromosomal position is important for the epigenetic control of *var* gene transcription.

So far, 2 controlled human malaria infection (CHMI) studies have tried to determine the forces that shape *var* transcript patterns in natural *P. falciparum* infections. Both investigations proposed that *var* gene transcription is reset after transmission from mosquito to humans, but the investigators proposed different explanations. Peters et al [25] suggested that transcription is biased toward individual *var* genes at liver release as a

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consequence of epigenetic imprint. In contrast, Lavstsen et al and Wang et al [26, 27] propose that almost all *var* transcripts are detected early after liver release but that transcription changes during intrahost replication owing to more efficient replication of parasite populations expressing specific PfEMP1 variants.

In 2012, the first CHMI trial comparing intravenous and intradermal inoculation of aseptic, purified, cryopreserved sporozoites (PfSPZ) was conducted at the Institute of Tropical Medicine of the University of Tübingen (TÜCHMI-001) [28]. All sporozoites were generated during a single mosquito feed with gametocytes deriving from a single vial of an NF54 cell bank.

Recently, Bachmann et al [29] analyzed *var* gene transcript levels in ex vivo RNA obtained from the volunteers of the TÜCHMI-001 trial. The authors showed that all parasite populations had a virtually identical *var* gene transcript pattern, dominated by subtelomeric UpsB and some UpsA *var* genes. This pattern was dramatically different from the premosquito transcript pattern.

In the current work, we analyzed *var* gene transcript levels of in vitro culture-adapted parasites from successfully infected volunteers as well as from the original premosquito culture used to produce the PfSPZ. We observed a clear bias toward expression of subtelomeric UpsB genes with a marked difference in *var* gene transcript levels and switch rates between intradermal and intravenous infections. After prolonged in vitro growth, the transcript profiles of the cultures returned to the premosquito transcript profile.

MATERIAL AND METHODS

Ethics Statement

Ethical approval for the TÜCHMI-001 study was obtained from the ethics committee of the University Clinic of the University of Tübingen. Written informed consent was obtained from the study participants. The study methods were carried out in accordance with the Declaration of Helsinki in the sixth revision as well as International Conference on Harmonization–Good Clinical Practice guidelines.

Study Participants and Sample Collection

All samples were obtained from individuals participating in the CHMI trial at the Institute of Tropical Medicine in Tübingen (TÜCHMI-001; ClinicalTrials.gov identifier NCT01624961 [28]). The study population consisted off 30 healthy individuals aged 18–45 years without any history of malaria. Participants received either an intradermal inoculation of 2500 PfSPZ (n = 6) (identified as 2500.1–6 ID), or an intravenous inoculation of 50 (n = 3), 200 (n = 3), 800 (n = 9) or 3200 (n = 9) PfSPZ (identified as 50.1–3, 200.1–3, 800.1–9, and 3200.1–9 IV). Twenty-two individuals became parasitemic. Four of 6 intradermally inoculated individuals developed asexual blood stage parasitemia (2500.1–4 ID). The number of positive cultures in the different intravenous inoculations were as follows: 50 PfSPZ, 1 of 3 (50.1 IV); 200 PfSPZ, 1 of 3 (200.1 IV); 800 PfSPZ, 7 of 9 (800.1–7 IV); and 3200 PfSPZ, 9 of 9 (3200.1–9 IV). All volunteers were treated with artemether/lumefantrine on the first day of microscopically detectable parasitemia (day of positivity) or 21 days after inoculation [28]. RNA extraction on the day of positivity was performed as described in the Supplemental Methods.

Parasite Cultures

On the day of positivity, 0.25 mL of patient blood was used to establish 10-mL in vitro cultures. Twenty-two cultures were inoculated, but only 21 were passed on to the culture laboratory. At a parasitemia of 3%–4%, 4 mL was expanded to a 20-mL culture and maintained in continuous in vitro culture for a period of 8 weeks. In addition, 2 cryopreserved vials of the original NF54 strain WCB SAN02-073009 used to generate the PfSPZ were provided by Sanaria Inc. Both vials were thawed and taken into in vitro culture under the same conditions described above. Parasites were cultivated as reported elsewhere [30].

Sorbitol Synchronization, RNA Extraction and Complementary DNA Synthesis

RNA was harvested from a 20-mL parasite culture with a parasitemia of 3%–5%, and every 2–3 generations thereafter. Parasite synchronization, RNA extraction, and complementary DNA synthesis were conducted as described elsewhere [30].

var Gene Transcriptional Profiling by Quantitative Reverse-Transcription Polymerase Chain Reaction

The earliest *var* gene transcription analysis (time point 1 [t1]) reflects the time point when the first 20-mL culture reached a parasitemia of 3%–5%. Time point 2 (t2) reflects the *var* gene transcriptional profile at the end of the in vitro cultivation period. For 5 cultures, *var* gene transcription was characterized at 2 additional time points. Transcription analysis of the 2 NF54 WCB SAN02-073009 cultures was performed after 21 generations of in vitro growth (time point 3 [t3]).

For transcriptional profiling of all 59 *var* genes in the 3D7 genome, we used the primer set of Salanti et al [20], with modifications described elsewhere [30]. All reactions included the same 5 housekeeping genes and were performed under conditions described elsewhere [30]. All runs were done in triplicates, and *var* gene transcription was measured as the relative copy number of the housekeeping gene arginyl-tRNA synthetase (PF3D7_1218600), using the delta cycle threshold method, as described elsewhere [20].

Electron Microscopy and Flow Cytometry

Electron microscopy and flow cytometry with semi-immune serum were performed as described in Supplemental methods.

Data Analysis and Statistics

Data were analyzed using Microsoft Excel (version 2007) and R (version 3.2.0) software [31]. Individual *var* gene transcript copy numbers were averaged over triplicates of reverse-transcription polymerase chain reaction runs and scaled to the

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highest mean *var* gene transcriptional signal in the entire population (PF3D7_0223500 of 2500.3 ID). For both data sets and time points, we conducted hierarchical cluster analysis in R [32, 33] to analyze the transcriptional profiles of the *var* gene family in different cultures. Overlay peak plots were created for each cluster. To determine the total *var* gene transcription signal, the area under the curve (AUC) was calculated. The AUC was calculated using natural spline interpolation (R package MESS; [34]) and compared between clusters or time points. Transcription profiles of premosquito (t3) NF54 WCB SAN02-073009 cultures where compared with those of time-points t1 and t2, visually using mean overlay plots, and statistically using 1-sample Wilcoxon rank sum tests.

RESULTS

Successful In Vitro Adaptation of Parasites From all Study Participants

After 21 inoculated cultures were successfully adapted to in vitro culture, *var* gene transcriptional profiling was performed on 18 of the 21 in vitro cultures at the earliest possible time point (t1) and at the end (t2) of in vitro culture. Patient characteristics, infectious dose, route of infection, and prepatent period are shown in Table 1. The experimental setup is shown in Figure 1. At t1, the mean growth time was 16 generations after infection. Seventeen of 18 cultures were analyzed after 13–19 generations (mean, 16 generations). Culture 3200.9 IV was delayed to 29 generations after infection, owing to technical difficulties. At t2, the mean growth time was 41 generations after infection. Seventeen of 18 cultures were analyzed after 36–45 generations (mean, 42 generations). For 1 culture, t2 was at 33 generations after infection, because the culture stopped growing (50.1 IV). For detailed parasitological data see Supplementary Table 1. Transcript level analysis of 2 NF54 WCB SAN02-073009 cultures was performed after 21 generations (t3).

Table 1. Patient Characteristics, Infectious Dose, Route of Infection, and Prepatent Period

Patient Identifier	Patient Age, y	Patient Sex	Dose, PfSPZ/Route	Prepatent Period, d[a]
2500.1	22	Female	2500/Intradermal	15
2500.2	21	Male	2500/Intradermal	14
2500.3	42	Male	2500/Intradermal	13
50.1	27	Male	50/Intravenous	13
200.1	27	Male	200/Intravenous	14
800.1	29	Male	800/Intravenous	11
800.2	28	Male	800/Intravenous	11
800.3	22	Male	800/Intravenous	12
800.4	24	Female	800/Intravenous	12
800.5	25	Male	800/Intravenous	12
3200.2	27	Male	3200/Intravenous	11
3200.3	26	Male	3200/Intravenous	11
3200.4	30	Female	3200/Intravenous	11
3200.5	28	Male	3200/Intravenous	11
3200.6	28	Male	3200/Intravenous	11
3200.7	24	Male	3200/Intravenous	10
3200.8	25	Male	3200/Intravenous	11
3200.9	27	Female	3200/Intravenous	12

Abbreviation: PfSPZ, *Plasmodium falciparum* sporozoites.

[a] The prepatent period is the time from infection to the first positive thick blood smear (in days).

Analysis of Postpatient In Vitro *var* Gene Transcription

At t1, cultures from individuals infected with 2500 PfSPZ given intradermally or 50 PfSPZ given intravenously transcribed only a few subtelomeric *var* genes at high relative copy numbers (mean maximum signal, 4.33 relative copy numbers). In contrast, cultures obtained from individuals infected with 200 or 3200 PfSPZ intravenously (Figure 2) transcribed subtelomeric and central *var* loci at low signals (mean maximum signal, 0.61 relative copy number). However, a subset of subtelomeric *var* genes was transcribed most actively across all cultures (ie, PFD3D7_ 0223500 and PF3D7_1100100).

To assess *var* gene transcript levels in the entire population, a t1 heat map was generated. Hierarchical cluster analysis (Figure 3) revealed 8 subtelomeric *var* genes being transcribed most actively in all cultures (PF3D7_0223500, PF3D7_0400100, PF3D7_0733000 PF3D7_0632500, PF3D7_1100100, PF3D7_0800100, PF3D7_1255200, and PF3D7_1373500). Two distinct clusters were identified. Cultures from cluster 1 (2500.1–3 ID, 50.1 IV, and 3200.4 IV) displayed high individual *var* transcript levels (20%–80% of the maximum relative copy number) of the set of 8 subtelomeric *var* loci. Cultures from cluster 2 (200.1 IV, 800.1–5 IV, 3200.2 IV, 3200.3 IV, and 3200.5–9 IV) displayed overall lower transcript levels of these genes (between 10%–20% of the maximum relative copy number). Moreover, a subset of central *var* genes was transcribed with equal strength in cluster 2. Furthermore, the total *var* gene transcript level was higher in cluster 1 than in cluster 2 (median AUC, 209.42 and 81.45, respectively). Cluster 2, which consisted exclusively of cultures from individuals infected intravenously with doses ≥200 PfSPZ, thus seemed to be associated with a lower total *var* gene transcript level and a more diverse *var* gene transcript profile.

To determine whether the different transcript patterns persisted over time, a second heat map for all cultures was generated at t2 (Figure 4). Hierarchical cluster analysis of the individual *var* loci now identified 1 large cluster of 17 cultures with a low total transcript level (median AUC, 62.16). However, the culture obtained from the individual infected with 50 PfSPZ intravenously (50.1 IV) continued to transcribe the same subtelomeric *var* genes at high levels and thus formed its own cluster (Supplementary Figure 1) (AUC, 194.13).

At t2, *var* gene transcription favored 17 *var* genes in the entire population (Figure 4). This cluster contained 7 of 8 subtelomeric *var* loci with the highest transcript levels identified at t1. Interestingly, 8 highly transcribed central *var* genes were the same genes transcribed in the cultures forming cluster 2 at t1

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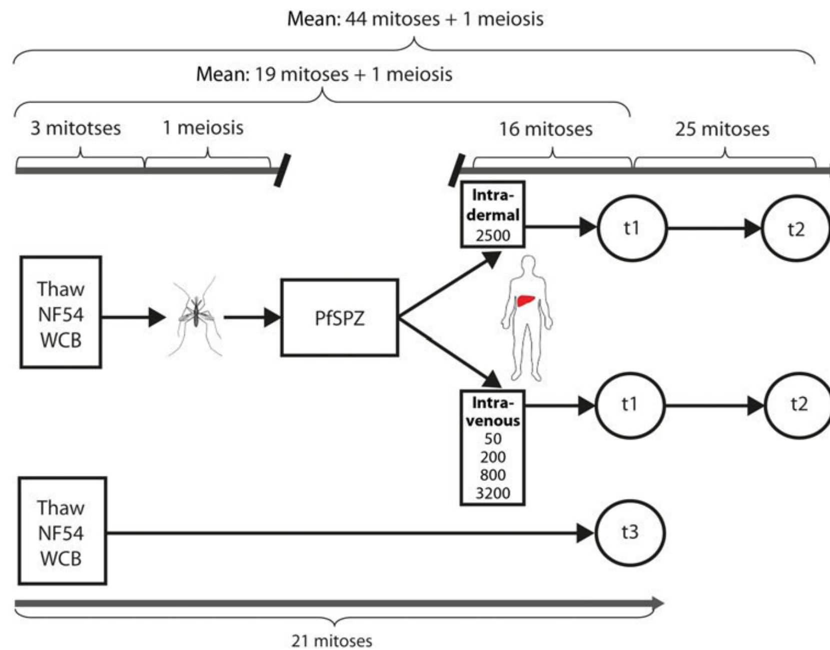

Figure 1. Transcription analysis of NF54 parasites with or without passage through mosquito and human host. The upper part of the figure depicts the generation of sporozoites, controlled human infection by the intradermal or intravenous route, and subsequent in vitro culture. Transcription analysis was performed at time points 1 (t1; mean, 16 generations after infection) and 2 (t2; mean, 41 generations after infection). Gametocytogenesis was induced after 3 mitotic divisions. The lower part of the figure shows an aliquot of the original NF54 culture, maintained in continuous in vitro culture. This was done with 2 independent vials of the same NF54 WCB SAN02-073009 frozen stock. Transcriptional analysis was performed after 21 mitotic divisions (time point 3 [t3]). Abbreviation: PfSPZ, *Plasmodium falciparum* sporozoites.

(PF3D7_0712300, PF3D7_0712000, PF3D7_0712800, PF3D7_0712600, PF3D7_0808700, PF3D7_0809100, PF3D7_0421300, and PFD3D7_0808600). Cluster analysis according to *var* gene promoter type showed that this corresponded to a shift from preferred transcription of UpsB promoters at t1 to a more diversified transcription profile of UpsB and UpsC promoters at t2.

var Gene Transcription and Sporozoite Route of Infection

All cultures in cluster 2 were from individuals infected intravenously with doses ≥200 PfSPZ. Cluster 1 was composed of all cultures from intradermally infected individuals (2500.1–3 ID) as well as cultures from the lowest-dose and highest-dose intravenous infections (50 .1IV and 3200.4 IV). To assess whether the duration of intrahost replication differed between the 2 clusters, prepatent periods were analyzed. The mean prepatent period of the individuals in cluster 1 (13.2 days) was significantly longer ($P = 0.03$) than the mean prepatent period of individuals in cluster 2 (11.4 days). (Figure 5*A*). Importantly, the prepatent period in individuals infected with 2500 PfSPZ intrademally (13–15 days) was the same or longer than in those infected with 50 PfSPZ or 200 PfSPZ intravenously (13–14 days), whereas the prepatent period of those infected with 800 or 3200 PfSPZ intravenously was significantly shorter (11.2 days) ($P = .03$). However, the cluster 1 culture 3200.4 IV had a short prepatent period.

To characterize the switch rates of cluster 1 cultures, transcription profiles at 4 time points were analyzed for the respective cultures: 50.1 IV at 19, 21, 28, and 33 generations after infection; 2500.1 ID at 19, 21, 35, and 38 generations; 2500.2 ID at 15, 21, 34, and 42 generations; 2500.3 ID at 15, 22, 34, and 42 generations; and 3200.4 IV at 16, 21, 35, and 43 generations (Figure 5*B*). This analysis revealed that parasites of the individual infected with 50 PfSPZ (50.1 IV) displayed consistently high transcript levels at all time points and that cultures from intradermally infected individuals showed gradually decreasing transcript levels over time. By contrast, transcript levels of the individual infected with 3200 PfSPZ intravenously (3200.4 IV) rapidly decreased to background levels, suggesting a high switch rate.

To investigate whether cluster 2 cultures exhibited a faster switch rate than cluster 1 cultures, *var* gene transcription levels in ex vivo RNA obtained from the volunteers on the day of blood smear positivity was compared with *var* gene transcript levels at t1 and t2 [29]. Strikingly, the total ex vivo *var* transcript level was higher in cluster 2 than in cluster 1. However, between ex vivo and t1, the total transcript level decreased significantly ($P < .001$) in cluster 2 but did not decrease in cluster 1 (Figure 5*C*). Together, these data show that parasites from individuals infected intravenously with doses ≥200 PfSPZ exhibit a faster switching rate than parasites from intradermally infected individuals.

Comparison of Premosquito and Postpatient *var* Gene Transcript Patterns

To assess the influence of meiosis and host passage on *var* gene transcription, we compared the transcript pattern of the

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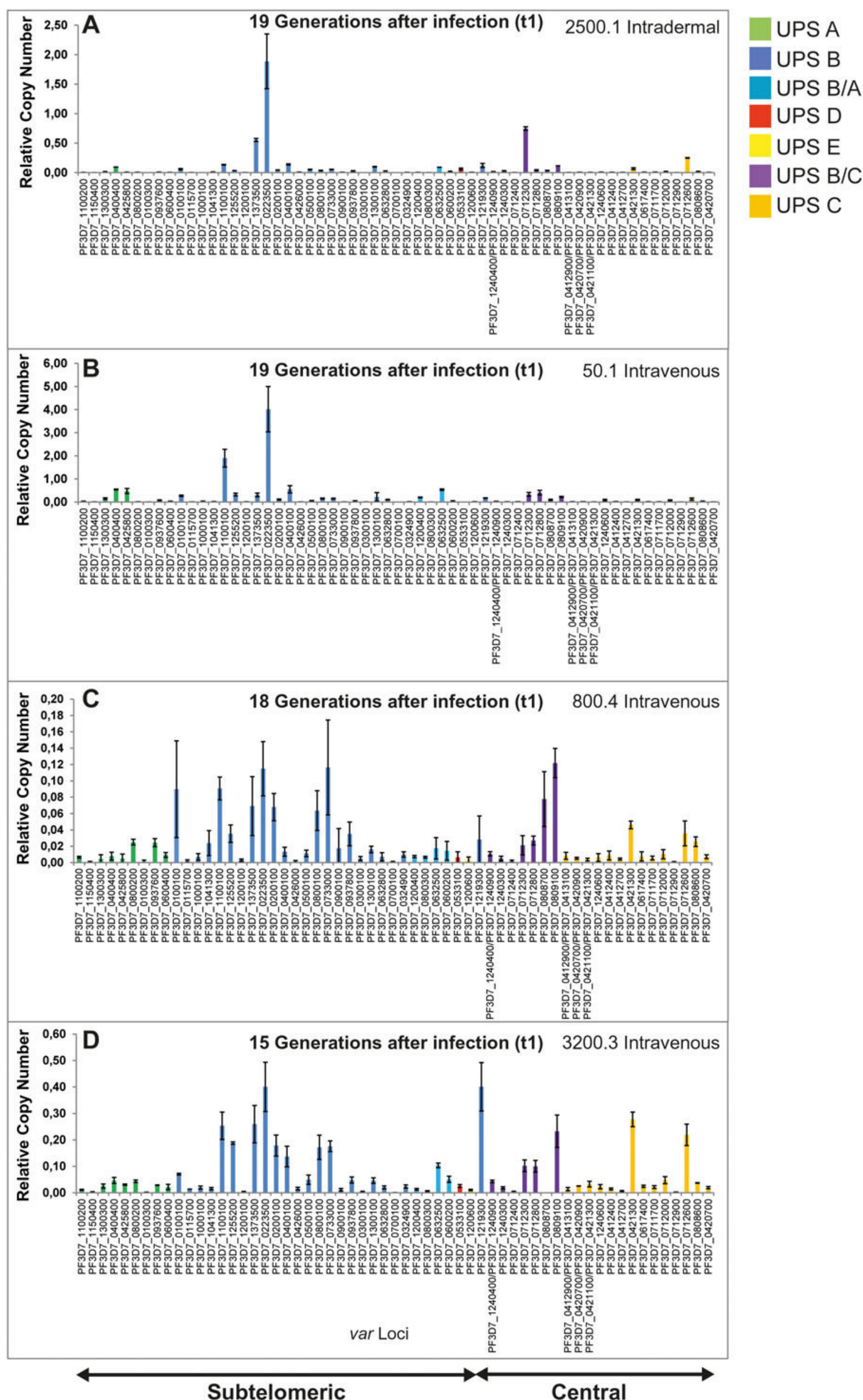

Figure 2. Transcription analysis of NF 54 parasites at time point 1 (t1) after intradermal or intravenous infection. Representative individual transcription profiles of NF54 parasites obtained from individuals infected intradermally (2500 *Plasmodium falciparum* sporozoites [PfSPZ]) (*A*) or intravenously (50, 800, or 3200 PfSPZ) (*B*–*D*). Patient identifiers (eg, 2500.1 ID) refer to dosage and route of administration (ID, intradermal; IV, intravenous); see Table 1 for details. The transcription signal is quantified in relative copy numbers (y-axis), and *var* genes are arranged according to chromosomal position (centrally located [central] or in subtelomeric region) and promoter type (UPSA, *B*, *C*, B/A, B/C, *E*, and *D*) and annotated according to the reannotation at www.GeneDB.org. (For previous annotation see Supplementary Table 2).

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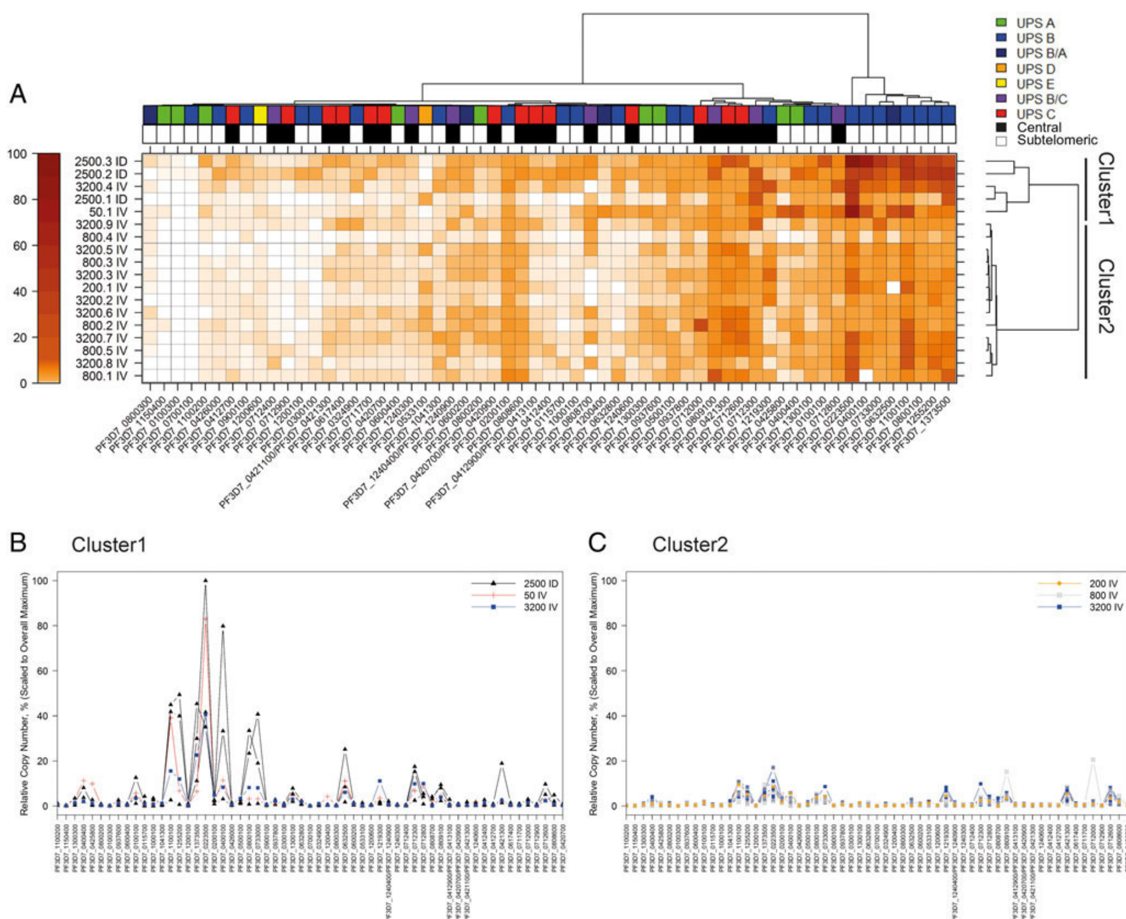

Figure 3. Transcription analysis of all cultures at time period 1 (t1). *A*, *var* Gene transcription heat map. The transcription signal is expressed as the percentage of the highest individual *var* gene transcriptional signal in the entire population (PF3D7_0223500 of 2500.3 ID) and reflected in the color of the boxes of the heat map (*bar at far left*). Patient identifiers with corresponding infection route and dose are displayed to the left of the heat map; individual *var* loci are listed at the bottom. Patient identifiers (eg, 2500.3 ID) refer to dosage and route of administration (ID, intradermal; IV, intravenous); see Table 1 for details. Promoter type and chromosomal position are indicated by the boxes above the heat map. Promoter types of *var* genes are marked with colored boxes. Subtelomeric *var* genes are marked as white and central *var* genes as black boxes. Note that a cluster of 8 subtelomeric *var* genes exhibits the highest transcription levels in all cultures. The 2 clusters of cultures from the different study participants are depicted on the right. Cluster 1: 2500.1–3 ID, 50.1 IV, and 3200.4 IV. Cluster 2: 200.1 IV, 800.1–5 IV, 3200.2 IV, 3200.3 IV, and 3200.5–9 IV. *B*, *C*, Overlay peak plots of clusters 1 and 2. Cultures from patients with the same dose and route of infection were collapsed to a single peak plot. The relative copy number as a percentage (of PF3D7_0223500 of 2500.3 ID) is indicated on the y-axis, and the individual *var* gene loci on the x-axis. The order of *var* genes is the same as in Figure 2.

premosquito strain at t3 (after 21 mitotic divisions) with the mean transcription signal of all patient profiles at t1 (after 19 mitotic divisions plus 1 meiotic division plus host passage) and t2 (after 44 mitotic divisions plus 1 meiotic division plus host passage) (Figure 6*A*). Despite an almost identical number of mitotic divisions at t3 and t1, the most pronounced difference in *var* gene transcript patterns was found between these 2 time points. Interestingly, the transcript pattern at t3 and t2 showed that the same subtelomeric and central *var* loci were preferentially transcribed. The exception was *var2csa*, which was highly transcribed at t3 but not at t1 and t2. To quantify the change in transcript levels of all *var* genes, a fold change analysis was performed based on t3. Prolonged in vitro culture resulted in a *var* gene transcript pattern that was almost identical to the premosquito pattern (Figure 6*B*).

Analysis of PfEMP1 Expression by Electron Microscopy and Flow Cytometry

In the premosquito strain WCB SAN02-073009, scanning and transmission electron microscopy showed the presence of knobs on the surface of infected red blood cells, supporting intact PfEMP1 presentation (Supplementary Figure 2*A* and 2*B*). In the postpatient cultures, PfEMP1 presentation was assessed by means of fluorescence-activated cell sorting (FACS) with semi-immune serum of a Gabonese individual [36]. All investigated cultures displayed surface reactivity, supporting PfEMP1 expression (Supplementary Figure 2*C*). Interestingly, the FACS signal was higher in cluster 1 than in cluster 2.

DISCUSSION

Several investigations have shown that in vitro antigenic variation in *P. falciparum* exhibits a conserved transcription pattern

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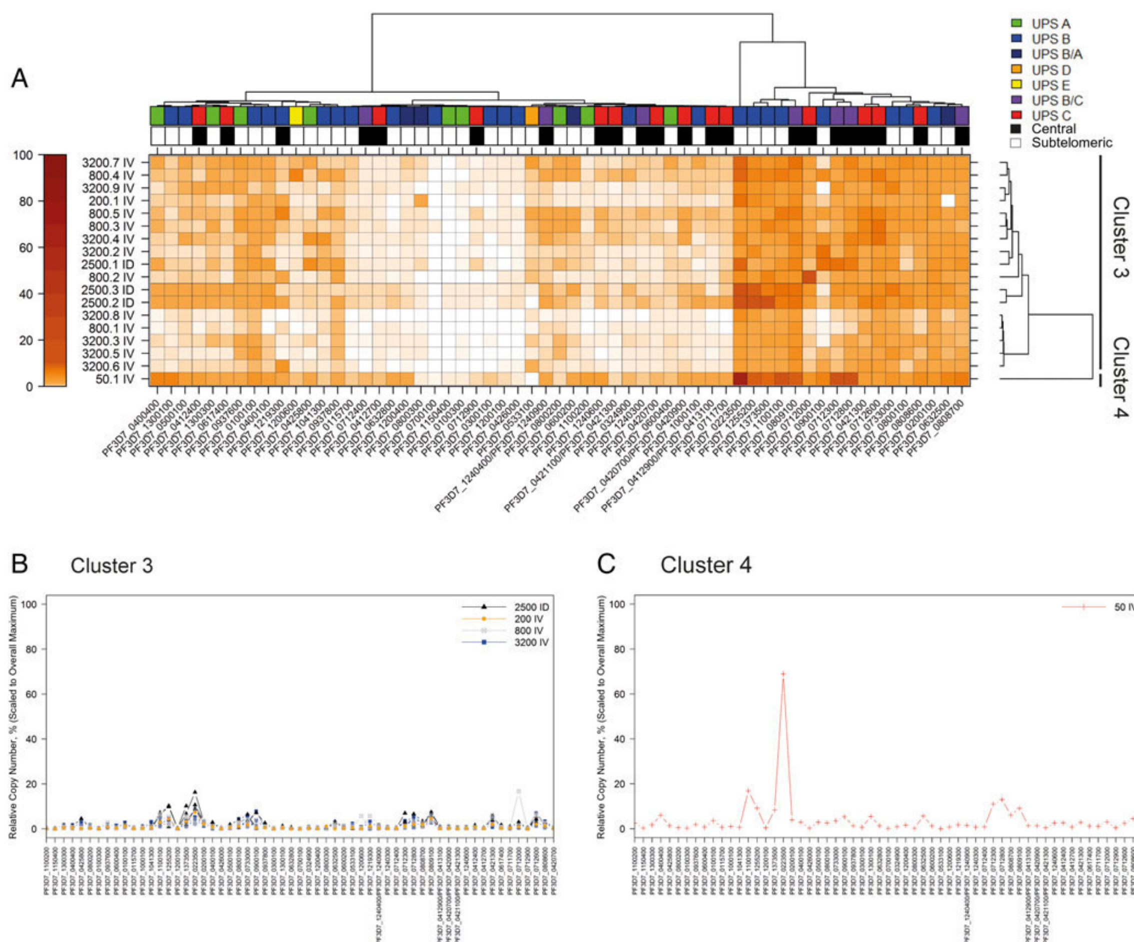

Figure 4. Transcription analysis of all cultures at t2. *A*, *var* Gene transcription heat map, following the same scheme as in Figure 3. Note that the cluster of *var* genes with the highest transcriptional levels is now composed of 10 subtelomeric and 7 central *var* genes. *B*, *C*, Overlay peak plot of cultures comprising cluster 3 and 4 following the same scheme as in Figure 3; *var* transcription levels have decreased across all cultures, except the 50.1 IV culture. Patient identifiers (eg, 3200.7 ID) refer to dosage and route of administration (ID, intradermal; IV, intravenous); see Table 1 for details.

in different laboratory strains and newly adapted field isolates [22–24, 30]. This raises the question how such a conserved profile can result in successful antigenic variation. Here, we show for the first time that the route of infection influences *var* gene transcript levels and switch rates of in vitro culture-adapted *P. falciparum* strains.

Ex vivo transcript level analysis of TÜCHMI-001 patients on the day of blood smear positivity [29] showed that the same UpsB and UpsA *var* genes were transcribed at high levels in the majority of patients. This is consistent with a model proposed by Lavstsen et al [26], suggesting that parasites transcribe a broad variety of *var* genes after liver release and that subsequent transcriptional changes are due to more efficient replication of parasite populations transcribing specific PfEMP1 variants. We observed a strong decrease in transcript levels between ex vivo and in vitro, indicating that the removal of host receptor selection had an immediate effect on *var* gene transcription. This decrease was most pronounced among subtelomeric UpsA and UpsB genes.

Previous investigations have shown that subtelomeric UpsB genes have high in vitro off-rates [22], and recently UpsA *var* genes have been shown to possess an additional silencing mechanism consisting of exonuclease-mediated RNA degradation [35], providing a potential explanation for the rapid decrease of transcript levels observed in this group of *var* genes. Prolonged in vitro culture was associated with a further decrease in transcript levels of subtelomeric UpsB genes, in contrast to the constant transcript levels of central UpsC genes. This is consistent with multiple previous reports showing higher off rates of subtelomeric UpsB genes compared with central UpsC genes [22–24].

Interestingly, the transcriptional signal of individual subtelomeric UpsB genes (ie, PF3D7_0223500) decreased faster in cultures from intravenous infections than in cultures from intradermal infections, and this was correlated with a shorter intrahost replication time in intravenous infections. In a detailed previous investigation of CHMI with mosquito-delivered sporozoites, Cheng et al [37] showed that not all intradermally

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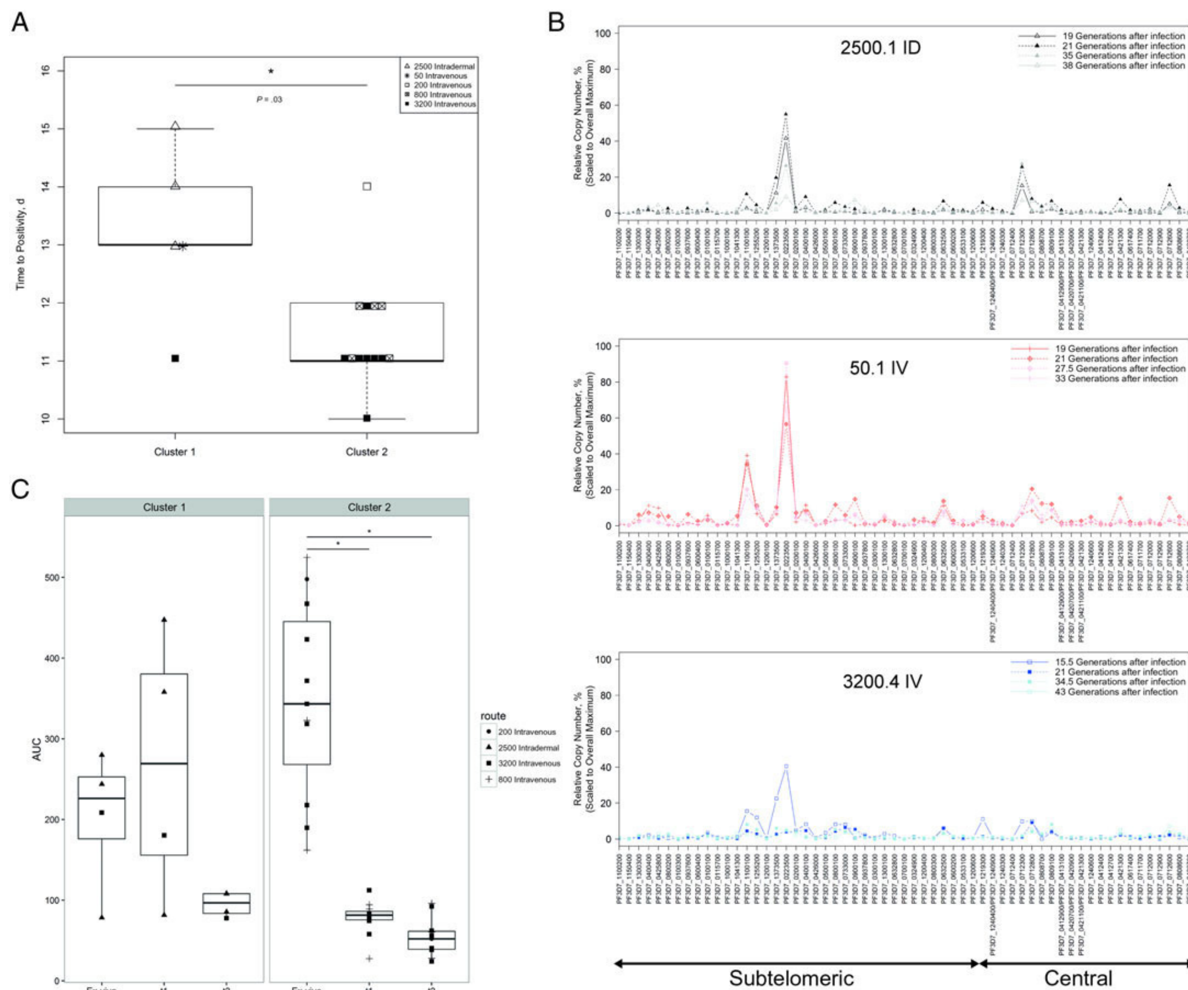

Figure 5. *var* Gene transcription profiles are influenced by route of infection and duration of intrahost replication. *A*, Prepatent periods of clusters 1 and 2; y-axis denotes the time from infection to blood smear positivity (prepatent period). *B*, Representative overlay peak plots of individual parasite populations (top, 2500.1 ID; middle, 50.1 IV; bottom, 3200.4 IV) at different times of in vitro culture. Relative copy number as a percentage (scaled to PF3D7_0223500 of 2500.3 ID) is indicated on the y-axis, individual *var* gene loci on the x-axis. Note that the transcriptional signal level for 3200.4 IV decreases faster than in the 2500.1 ID and 50.1 IV cultures. Patient identifiers (eg, 2500.1 ID) refer to dosage and route of administration (ID, intradermal; IV, intravenous); see Table 1 for details. *C*, Comparison of total *var* gene signal (AUC) of cluster 1 and 2 between the day of blood smear positivity (ex vivo) and in vitro (t1 and t2). Note that cluster 2 exhibits a statistically significant decrease of total the *var* gene signal (AUC) between ex vivo and in vitro (t1 and t2) (indicated by the asterisks). Cluster 1 shows no statistically significant decrease in total *var* signal (AUC) between ex vivo and in vitro. The same housekeeping gene (arginyl-tRNA synthetase; PF3D7_1218600) was used to analyze ex vivo and in vitro t1 and t2 real-time polymerase chain reaction. In clusters 1 and 2, 50.1 IV and 800.5 IV are removed because no RNA was available for this analysis at the ex vivo time point. Abbreviation: AUC, area under the curve.

inoculated sporozoites reach the liver. Consequently, despite a high dose of 2500 sporozoites in the intradermal infections, only a small proportion may have reached the liver, resulting in a lower starting parasitema, leading to a longer prepatent period. The fact that the culture from the lowest-dose intravenous infection (50 PfSPZ) also displayed a longer prepatent period than higher-dose intravenous infections (800 and 3200 PfSPZ) is consistent with this finding and with previous data from Cheng et al [37], showing that a 10-fold reduction in starting parasitemia prolongs intravascular growth by 2 days.

We hypothesize that the longer intravascular replication in intradermal infections resulted in increasing selection for parasites transcribing the most advantageous *var* genes for intravascular replication. After in vitro culture adaptation, this stronger epigenetic mark may have resulted in slower decay of transcript levels in cultures from intradermal infections.

Previous data generated with isogenic transgenic parasites have shown that increasing drug selection with the drug blasticidin generates parasite populations with higher transcript levels and lower switch rates than parasites selected with a lower blasticidin concentration [30], thus supporting the observation that selective pressures influence *var* gene transcription. The observation that prolonged postpatient in vitro growth resulted in a conserved transcriptional profile is equally consistent with the conserved transcriptional profile observed in isogenic transgenic parasites propagated without drug pressure [30]. Together, these observations support a conserved epigenetically determined transcription profile after prolonged in vitro culture.

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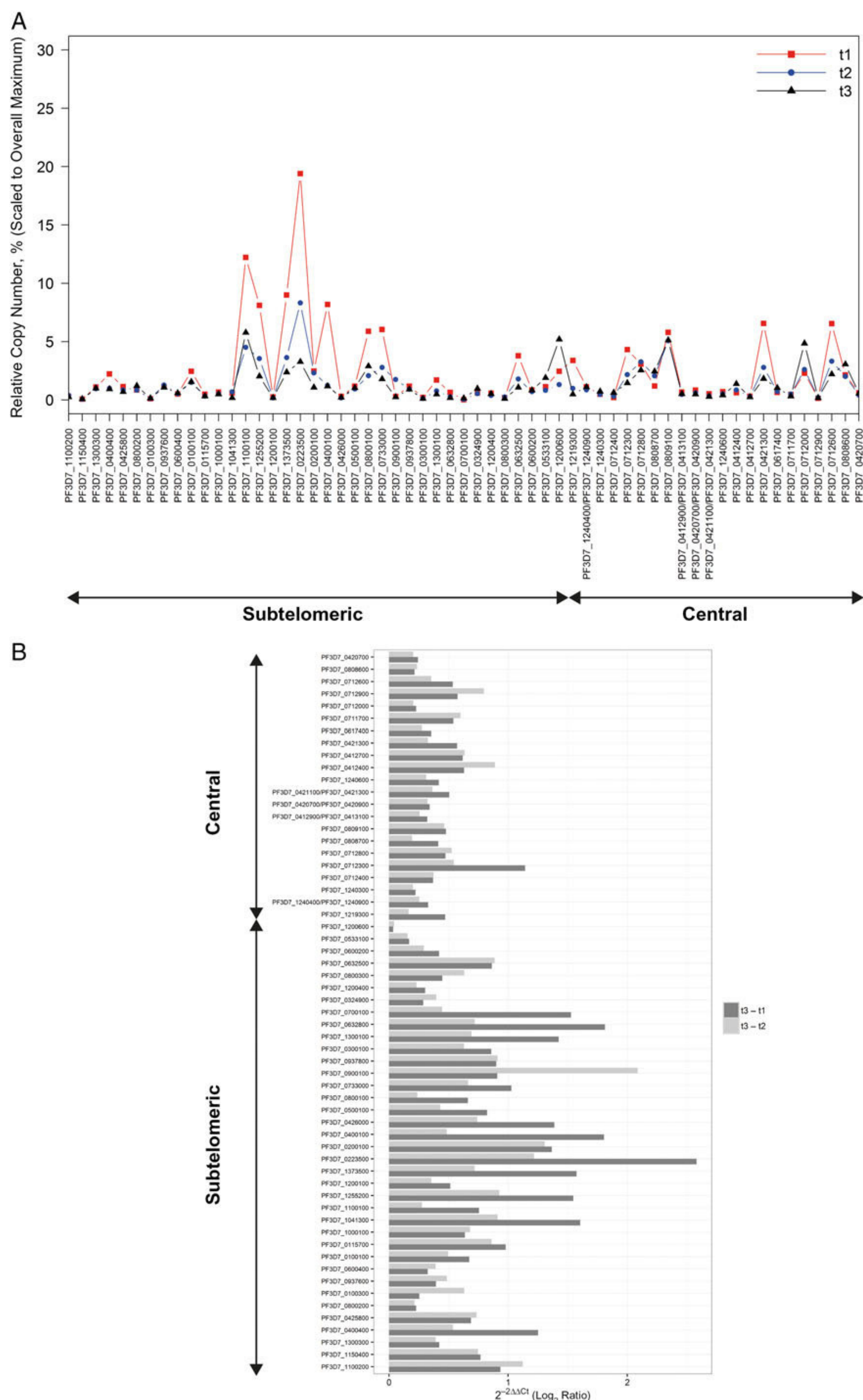

Figure 6. Transcription analysis of premosquito (time period 3 [t3]) and postpatient (time periods 1 and 2 [t1, t2) NF54 parasites. *A*, Transcription overlay plots of the same NF54 culture after exclusive mitotic replication for 21 generations compared with 19 and 40 mitotic replications plus 1 meiotic division and host passage. The transcription signal of the original NF54 parasites after t3 (21 mitotic divisions) is represented by triangles. The height of the transcription signal is indicated on the y-axis, and the individual *var* gene loci on the x-axis. *B*, *var* Gene transcription fold changes after mosquito and host passage. Log_2 ratios for t1 (*dark gray*) and t2 (*light gray*) compared with t3 were calculated using the delta delta cycle threshold method [35]; individual *var* loci are depicted on the left. Genes that are transcribed with stronger signals at t3 (premosquito culture) will have a value <1; *var2csa* and the majority of central *var* genes belong to this group. Subtelomeric *var* loci at t1 represent the majority of *var* genes transcribed at higher levels after passage through mosquito and human host (value, >1).

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Consistent with this, late postpatient cultures transcribed the same subtelomeric and central *var* genes as the premosquito culture used to generate PfSPZ. The only exception was *var2csa*, a locus responsible for binding infected red blood cells in the placenta [20]. *var2csa* was highly transcribed in the premosquito culture, consistent with reports that this gene occupies a unique position in the *var* gene switching network [38, 39]. We hypothesize that the absence of the placental receptor chondroitin sulfate A in our patient population resulted in a selective disadvantage for this locus.

FACS analysis with serum of a semi-immune individual showed a stronger recognition of cluster 1 than of cluster 2 parasites. Chan et al [40] have shown that the high surface reactivity of semi-immune serum with the 3D7 laboratory strain is due to CD-36 binding PfEMP1 variants. This finding would be consistent with the longer prepatent period of cluster 1 parasites, leading to more stable expression of CD36 binding PfEMP1 variants due to longer receptor selection.

One limitation of our investigation is that *var* gene transcriptional changes may have occurred as a consequence of in vitro culture adaptation. However, because the in vitro culture conditions were constant across all cultures, it seems unlikely that in vitro culture adaptation per se could be responsible for the observed differences in *var* gene transcription. Despite the observed changes in *var* gene transcription during in vitro growth of cultures from intravenous and intradermal infections, transcription of *var* genes was nevertheless highly conserved; that is, the same *var* genes were transcribed in almost all cultures.

A model by Recker et al [41] suggests that host pressure is critical for generating antigenic variation by mounting cross-reactive immune responses against a major and minor antigen. Although our study did not assess the importance of the immune response, the observation that differences in intrahost replication time influence *var* gene transcription is consistent with a model of antigenic variation based on host selective pressures. It remains an open question whether the major and minor antigenic determinants proposed by Recker et al [41] consist of different PfEMP1 epitopes or possibly also encompass other multicopy gene families of *P. falciparum* [42–44]. Future investigations with CHMI and with natural chronic infections in semi-immune individuals will be necessary to definitively answer these questions.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. B. K. L. S. is an employee of and S. L. H. the chief executive and scientific officer of Sanaria Inc. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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RESEARCH ARTICLE

In Vitro Variant Surface Antigen Expression in *Plasmodium falciparum* Parasites from a Semi-Immune Individual Is Not Correlated with *Var* Gene Transcription

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Abstract

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is considered to be the main variant surface antigen (VSA) of *Plasmodium falciparum* and is mainly localized on electron-dense knobs in the membrane of the infected erythrocyte. Switches in PfEMP1 expression provide the basis for antigenic variation and are thought to be critical for parasite persistence during chronic infections. Recently, strain transcending anti-PfEMP1 immunity has been shown to develop early in life, challenging the role of PfEMP1 in antigenic variation during chronic infections. In this work we investigate how *P. falciparum* achieves persistence during a chronic asymptomatic infection. The infected individual (MOA) was parasitemic for 42 days and multilocus *var* gene genotyping showed persistence of the same parasite population throughout the infection. Parasites from the beginning of the infection were adapted to tissue culture and cloned by limiting dilution. Flow cytometry using convalescent serum detected a variable surface recognition signal on isogenic clonal parasites. Quantitative real-time PCR with a field isolate specific *var* gene primer set showed that the surface recognition signal was not correlated with transcription of individual *var* genes. Strain transcending anti-PfEMP1 immunity of the convalescent serum was demonstrated with CD36 selected and PfEMP1 knock-down NF54 clones. In contrast, knock-down of PfEMP1 did not have an effect on the antibody recognition signal in MOA clones. Trypsinisation of the membrane surface proteins abolished the surface recognition signal and immune electron microscopy revealed that antibodies from the convalescent serum bound to membrane areas without knobs and with knobs. Together the data indicate that PfEMP1 is not the main variable surface antigen during a chronic infection and suggest a role for trypsin sensitive non-PfEMP1 VSAs for parasite persistence in chronic infections.

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Introduction

P. falciparum is responsible for the most severe form of human malaria and is a major cause for morbidity and mortality in sub-Saharan Africa [1]. In endemic areas, semi-immunity against *P. falciparum* is associated with the development of antibodies [2,3] against variant surface antigens (VSAs) expressed on infected red blood cells (iRBCs) [4–6].

To date, five multicopy gene families that encode VSAs have been described in *P. falciparum*: *stevor* (subtelomeric variable open reading frame) [7], *rif* (repetitive interspersed family) [8], *pfmc-2tm* (*P. falciparum* Maurer's clefts two transmembrane) [9], *surfin* (surface associated interspersed genes) [10] and *var* [11]. However, the antigenic importance of the corresponding VSA protein families is a question of ongoing research. The best investigated VSA is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [12–14]. PfEMP1 is a variant surface protein that is encoded by the multicopy *var* gene family and mediates cytoadherence of iRBCs to a broad repertoire of host endothelial receptors [14]. In the extracellular part, PfEMP1 possesses a semi-conserved structure consisting of a Duffy-binding like (DBL)-1α domain, a cysteine rich interdomain region (CIDR) located downstream, followed by a variable number of less conserved DBL stretches. Each individual parasite carries approximately 60 *var* genes but only expresses one *var* gene at a time [13–16], thereby ensuring that only one PfEMP1 variant is exposed to the immune system. Switches in *var* gene transcription provide a basis for antigenic variation and immune escape during chronic infections [17] and are tightly controlled on multiple layers. At the level of the individual *var* locus, silencing appears to be mediated by the interaction of the 5' promoter and the intron promoter as well as histone modifications [18–25]. Epigenetic memory appears to "mark" the active *var* locus [23,26] to ensure its continued expression in the next generation of offspring and it has been shown that individual active promoters are stably transcribed for prolonged periods of time [27]. *in vitro* switching investigations with long term laboratory strains and with parasites obtained from controlled human infections [28–32], provide evidence that *var* gene switching is highly structured and suggest a repeatable hierarchy of *var* gene activation. These observations raise the question of how such a stably inherited transcription pattern is compatible with antigenic variation during natural chronic infections in endemic areas.

Mathematical models [33,34] and serum transfer experiments [35,36] strongly support the seroepidemiological evidence that antibodies against surface antigens are of critical importance in the development of semi-immunity [6,37,38]. Strain-transcending immunity against PfEMP1 has been shown to develop early in life [39,40]. Consistent with this, parasitemia levels in adult residents of endemic areas are often submicroscopic [41]. In contrast, the parasitemia observed during infections of non immune individuals is continuously detected by light microscopy and displays a pattern of consecutive waves with sequential removal and subsequent expansion of parasite populations [33,42]. Hypervariability of the *var* gene family is thought to be necessary for the parasite to escape the human immune response [43,44]. Indeed, frequent recombination events have been documented within the *var* gene family at the individual strain [45–49] and at the population level [50].

In this work we examine the question of how *P. falciparum* achieves persistence during a chronic asymptomatic infection by conducting *in vivo* and *in vitro* investigations of a natural infection in an "asymptomatically infected" individual. The *in vivo* data demonstrated persistence of the same parasite strain throughout the infection. Fluorescent-activated cell sorting (FACS) analysis of cultured adapted parasites with convalescent sera identified a clonally variant surface recognition signal that was not associated with *var* gene transcription. This signal could be abolished by trypsinisation and immune electron microscopy revealed that antibody binding was in membrane areas with and without knobs. Together the data suggest that the

surface signal of infected red blood cells from a chronic infection is not exclusively composed of PfEMP1 and support a role for trypsin sensitive non-PfEMP1 VSAs in parasite persistence during chronic infections.

Results

A stable parasite population survives for an extended time period during an asymptomatic *P. falciparum* infection

We investigated the dynamics of a *P. falciparum* infection in an asymptomatically infected Gabonese individual (MOA) for a period of 42 days (Fig 1A). Parasitemia increased from 90 parasites/μl on day 0 to a maximum of ~ 450 parasites/μl on day 7. Between day 7 and day 14, blood parasitemia dropped in the absence of treatment and remained submicroscopic until day 42 of the infection. On day 42 the patient became symptomatic with a *P. ovale* infection and was treated according to local guidelines. Despite negative thick blood smears from day 14 to 42, we detected a continuous submicroscopic parasitemia by *msp2* FC allele specific PCR. To determine if the obtained *msp2* signals indeed represented the same parasite population, we sequenced the PCR products in MOA blood samples. The PCR products from day 7, 14, 21, 28 and 42 showed identical *msp2* FC allele sequences (accession numbers KC887547-KC887556). These data suggested that the parasitemia consisted of a stable parasite population. We have previously shown that parasites with identical *msp2* sequences can still represent different strains with different *var* gene repertoires [47]. To determine the presence of individual parasites throughout the infection we therefore characterized the *in vivo* and *in vitro var* gene repertoire. To this end, a MOA parasite isolate of day 7 (MOA bulk) was transferred into *in vitro* culture and 19 clones were generated in 2 independent limiting dilution experiments. The first limiting dilution experiment generated the 3 clones MOA D2, C3 and D5 and has been previously reported [30]. The second limiting dilution experiment generated 16 new MOA clones: H4, G3, E8, B5, J1, E10, C4, C8, G9, E1, B10, D11, F11, H6, A1 and G2.

We characterized the DBL alpha repertoire of the MOA clones with universal DBL primers [30]. This revealed 36 individual DBLα sequences (Fig 1B) (accession numbers KC887669-KC887743). All DBLα sequences were confirmed by PCR with DBL specific primers followed by Sanger sequencing. This MOA DBL primer set was used to compare the *var* gene repertoire of the MOA bulk culture and the 19 MOA clones. As expected, PCR on genomic DNA (gDNA) of the orginal MOA bulk culture resulted in PCR products for each of the 36 DBLα sequences. 35 primers produced PCR fragments on all 19 MOA clones (Fig 1B). In one clone (MOA D5), the MOA D2_18 DBL could not be amplified. In 9 clones (MOA C3, D2, H6, C8, D11, G9, B10, C4, E10) the MOA PD5 DBL could not be amplified and in the 9 remaining clones (MOA J1, G3, E8, H4, B5, F11, G2, A1, E1), all 36 primer pairs resulted in PCR products. Together, the data suggested that the clones were isogenic but recombination events had occurred in individual parasite clones.

To identify the relative proportion of *in vitro* MOA DBLs throughout the *in vivo* infection, we determined the *var* gene repertoire of DNA obtained on day 0, 7 and 28 (accession numbers KC887557-KC887668) of the infection by the same DBL cloning approach. Sequence comparison revealed that of the 44 *var* gene sequences identified on day 7, 26 (59%) corresponded to *var* genes identified within the repertoire of the MOA *in vitro* clones. Of the 33 *var* sequences that were identified on day 28, 18 (55%) corresponded to MOA clone *var* genes. The relative proportion of MOA *in vitro* DBL sequences thus remained stable over time (Fig 1C). The remainders of the sequences on day 7 or day 28 were not identified in the *in vitro* DBL MOA cloning experiment thus indicating the presence of clones, which were not identified in the *in vitro* limiting dilution experiment. We next wanted to investigate, if *var*

A

B

MOA Clone/ DBL	MOA C3_10	MOA C3_22	MOA C3_33	MOA C3_39	MOA C3_95	MOA C3_97	MOA D2_3	MOA D2_18	MOA D2_25	MOA D2_34	MOA D2_69	MOA D2_79	MOA D2_83	MOA P C3	MOA P D5	MOA P D2	MOA d0_1	MOA d0_2
MOA bulk	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D5	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
C3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
D2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
H6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
C8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
D11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
G9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
B10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
C4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
E10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
J1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

MOA Clone/ DBL	MOA d0_3	MOA d0_23	MOA d0_30	MOA d0_37	MOA D2_33	MOA D5_2	MOA D5_14	MOA D5_77	MOA D5_101	MAO C3_2	MOA C3_21	MOA C3_27	MOA C3_36	MOA C3_63	MOA C3_65	MOA T0_36	MOA D7_15	MOA D7_33
MOA bulk	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
J1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

C

Fig 1. *In vitro* and *in vivo* analysis of a chronic *P. falciparum* infection in a semi-immune individual. (A) Parasitemia curve of the asymptomatic chronic *P. falciparum* infection of an adult semi-immune Gabonese individual over 70 days and timeline of the *in vivo* investigation. The patient was asymptomatic from day 0 to day 42, at which point a new infection with *P. ovale* was diagnosed and treated according to local guidelines. DNA, RNA, cryopreserved parasites and sera employed in this analysis are indicated by arrows. Blood parasitemia is quantified on the y-axis. *P. falciparum* parasites were detected from day 0 to day 10 by light microscopy. From day 7 to day 38 sub microscopic parasitemia was documented by PCR twice per week. For *in vitro* analysis parasites from day 7 were brought into

tissue culture (MOA bulk) and 19 *in vitro* clones were generated by limiting dilution in two independent cloning experiments. (B) Matrix of PCR results with 36 DBL specific primers on DNA of *in vitro* MOA bulk culture and 19 MOA clones. 1 indicates amplification of the target sequence, 0 indicates absence of a reaction product. All 36 DBLs were amplified from MOA bulk and the clones MOA J1, G3, E8, H4, B5, F11, G2, A1 and E1. In the remaining clones 35 DBLs were successfully amplified. In the clone MOA D5 the DBL MOA D2_18 could not be amplified. In the clones MOA C3, D2, H6, C8, D11, G9, B10, C4, E10 the DBL MOA P D5 could not be amplified (grey). (C) Results of DBL shotgun cloning of DNA from patient blood of day 7 and day 28. Numbers in brackets shown below the x-axis refer to the total number of *var* DBL sequences identified on these days. Sequences that were identified exclusively in DNA from patient blood are designated as "*in vivo*" MOA (grey). The proportion of MOA "*in vitro*" DBLs is represented by the black part of the bars. MOA *in vitro var* DBLs were detected at the same proportion (59% (26 of 44)) on day 7 and day 28 (55% (18 of 33)).

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sequences, which had not been cloned on all days, were present throughout the course of the infection. We designed 11 primer pairs for DBL sequences (S1 Table). All primer pairs successfully amplified PCR products from DNA of all three days, which were confirmed by sequencing. DBL sequence analysis revealed a few chimeric sequences that were only identified on individual days of the infection. However, PCR with chimera specific primers could not verify the existence of the sequences (data not shown), indicating that they were generated during the PCR with conserved DBL primers, a problem that has been reported previously [46].

DBL cDNA cloning on day 0 of the infection in MOA blood samples revealed that in marked contrast to the sequence diversity detected in the DNA cloning experiments, only 6 different *var* transcripts were detected. All of the 6 *var* transcripts were identified within the *var* gene repertoire of MOA *in vitro* clones and are shown in Table 1. Furthermore, *in vivo* transcription was biased towards one specific DBL: d0_37. Subsequently, the transcription of these 6 *var* genes was assessed by quantitative Real-Time PCR with gene specific primers in the culture adapted MOA bulk strain at 30, 90 and 150 generations of *in vitro* growth. This revealed stable transcription of all 6 *var* loci at all time points, suggesting relatively low switch rates of these 6 *in vivo* transcripts during *in vitro* growth (S1 Fig).

Convalescent serum of the MOA individual displays a variable surface recognition signal on different MOA clones

To test whether a part of the parasite population was able to avoid the host´s immune system, we investigated the humoral immune response against surface antigens of the MOA bulk strain and the 19 MOA clones with convalescent serum obtained on day 70 of the investigation. As expected, surface recognition signals of the MOA clones in flow cytometry were variable -ranging from a mean fluorescence (MFI) of 54 to 377- and significantly different from

Table 1. MOA *in vivo* DBL transcripts and corresponding *in vivo* DNA DBLs.

	MOA *in vivo var* transcripts
1	d0_37 / Day7_18
2	d0_30 / Day7_CL24
3	d0_2 / Day7_92 / Day28_74
4	d0_3 / Day7_61
5	d0_1/ Day7_35 / Day28_2
6	d0_23

The 6 *in vivo* DBL-transcripts are denoted d0_37, d0_30, d0_2, d0_1 and d0_23. The corresponding *in vivo* MOA DNA DBLs from days 7 and 28 are also indicated. All *in vivo* transcripts were present in the 19 MOA clones (Fig 1B). Among 6 identified individual *var* transcripts, d0_37 was most abundant in two independent cloning experiments. Transcription of the cDNA *var* DBLs was confirmed by Quantitative Real-Time (qRT) PCR with gene specific primers (data not shown).

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each other (Fig 2). The signal of MOA bulk (MFI of 174) reflected the mean value of all clones contained in this culture.

PfEMP1 presentation in the membrane of the infected erythrocyte has been shown to differ in knob positive and knob negative parasite lines [51,52]. To ensure that the difference in FACS signal was not due to a loss of knobs in some strains, we performed raster- and scanning electron microscopy on two clones with a highly significant difference in FACS signal: MOA D2 (MFI = 377) and MOA D5 (MFI = 93). This demonstrated that both clones carried knobs. Observer blinded knob quantification showed no quantitative knob difference in the two clones. Furthermore, the *kahrp* gene could successfully be amplified from DNA of all clones (data not shown).

var gene transcription and surface reactivity do not correlate with each other

To assess if the differences in surface reactivity correlated with transcription of individual *var* loci, we investigated *var* gene transcription by DBL specific Real-Time PCR in all 19 clones and the MOA bulk culture. The transcription signal was quantified as the relative copy number (RCN) of the housekeeping gene arginyl-tRNA synthetase (PFL0900 c).

The 3 MOA clones D2, C3 and D5 that were generated in the first limiting dilution experiment were analyzed after > 65 generation of continued growth since limiting dilution. The 16 MOA clones generated during the second limiting dilution experiment were analyzed after approximately 35 generations of continued growth since limiting dilution.

In 5 of the 16 MOA clones generated in the second limiting dilution experiment the *in vivo* transcript d0_37 was the dominant *var* gene transcript (H4, G3, E8, B5, J1) but the height of the transcription signal was not correlated with surface reactivity (Fig 3A–3C). In order to investigate the correlation of surface reactivity and *var* gene transcription in the entire population, a heat map correlating the *var* transcription signal and the individual FACS signals of all MOA *in vitro* cultures was generated (Fig 3D). This showed that the 5 clones transcribing d0_37 showed high, mean and low FACS signals. Furthermore 5 clones (G2, C8, A1 H6 F11) transcribing T0_36 as the dominant transcript exhibited FACS signals from high (A1) to low (H6) (S2 Fig). Analysis of individual clone transcription profiles showed that neither transcription of two dominant *var* loci (indicating a transcription switch in part of the population) nor individual high *var* DBL transcription signals were correlated with the height of the surface signal (Fig 4 and S3 Fig).

We next compared the transcription signal of the dominant *var* loci in all 19 clones and the MOA bulk culture (S3 Table). The strength of the dominant *var* locus in 16 clones (35 generations of growth since limiting dilution) ranged from 67 RCN (DBL_D2_69, Clone E10) to 3 RCN (DBL C3_65, Clone E1). The strength of the individual *var* signals in the clones transcribing d0_37 as the dominant *var* locus ranged from 60–30 RCN and from 42–19 RCN for the clones transcribing T0_36,clearly indicating that these were the dominant transcripts.

As expected the MOA bulk culture and the clones C3 and D5 (> 65 generations of growth since limiting dilution) showed lower maximum individual *var* gene signals (3, 1 and 4 RCN). Despite lower transcription signals the surface signal of the MOA bulk culture and the clones C3 and D5 was high and medium (Fig 5A, 5C and 5D). However clone D2 (> 65 generations of growth since limiting dilution) displayed persistently high individual *var* gene signals of the DBLs PD2 and D2_18 and exhibited the highest surface recognition signal of the entire population (Fig 5B). Overall, there was no correlation between the height of the surface recognition signal and *var* gene transcription in the entire population.

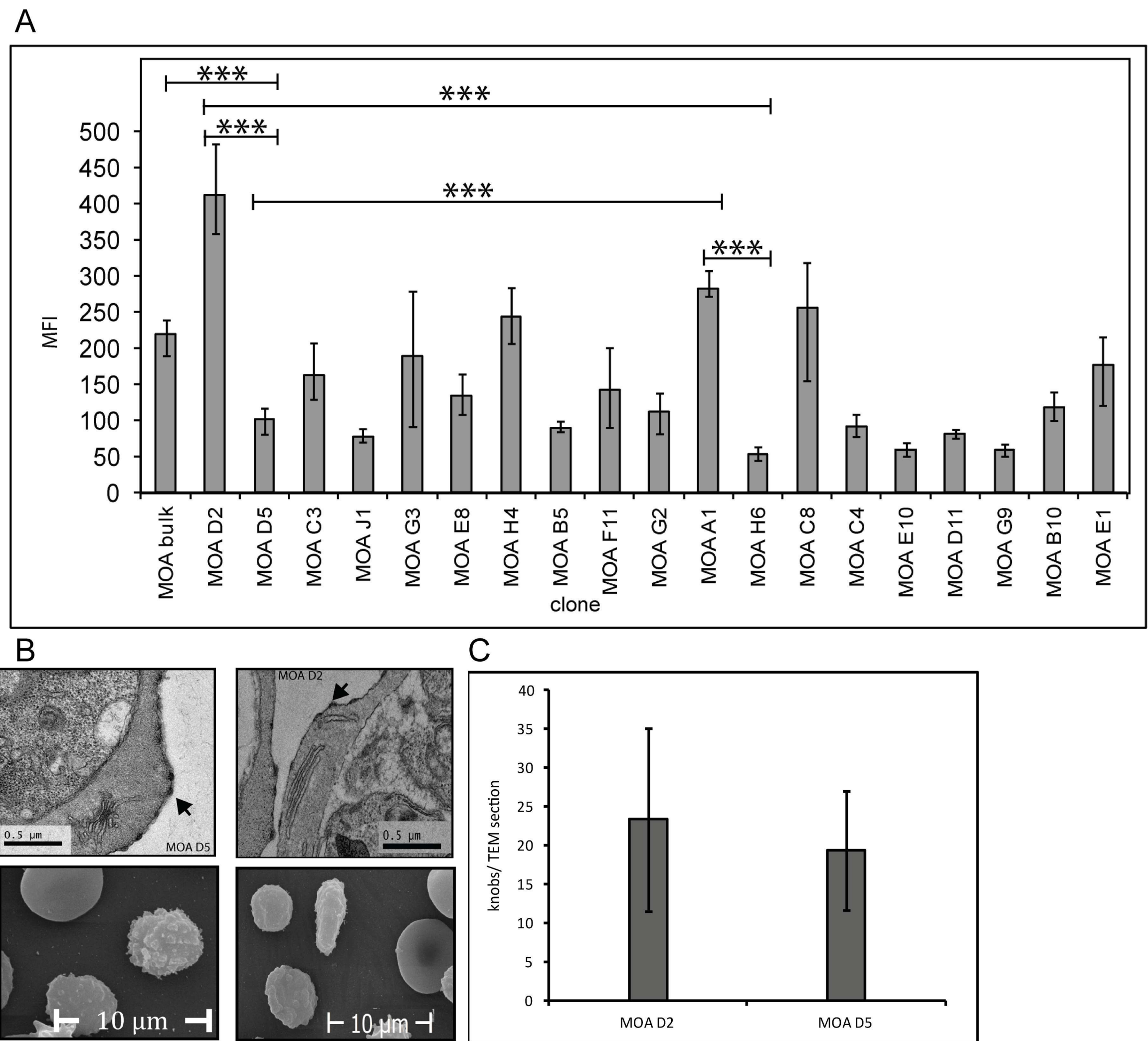

Fig 2. Flow cytometry signals with day 70 serum on MOA bulk and MOA clones are variable. (A) Mean fluorescence intensities (MFI) obtained with MOA serum of day 70 followed by staining with a secondary antibody attached to FITC shows variable surface recognition signals ranging from low (< 80 MFI) to medium (81–160 MFI) and high (> 160 MFI) in MOA bulk and in the MOA clones. Error bars reflect the mean error of at least three independent experiments (Student´s t-test: MOA bulk vs. D5: p = 0.008, A1 vs. H6: p = 0.0003, D2 vs. D5: p = 0.0001, D2 vs. H6: p = 0.008, D5 vs. A1: p = 0.0002). (B) and (C) show transmission and scanning electron microscopy graphs of two representative clones with a highly significant difference in surface recognition signal (MOA D2 and MOA D5) and the presence of knobs (arrows) in equal numbers. Knobs were quantified per TEM cut of each clone.

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Convalescent serum has strain-transcending anti-PfEMP1 immunity against laboratory strains

To investigate if the convalescent MOA serum had cross-reactive anti-PfEMP1 components, we selected E5 and NF54-C2, two sibling parasites of the original NF54 laboratory strain, in three consecutive CD36 receptor panning assays [31,47]. Unselected E5 had a comparatively low surface reactivity in flow cytometry using MOA serum of day 70 with a mean fluorescence of 114. CD 36 receptor binding selection resulted in an increased surface recognition signal of E5 CD36+ to MFI = 287 compared to wild type E5, when exposed to heterologous serum of the MOA individual from day 70 (Fig 6). The same increase in MFI was observed for NF54–

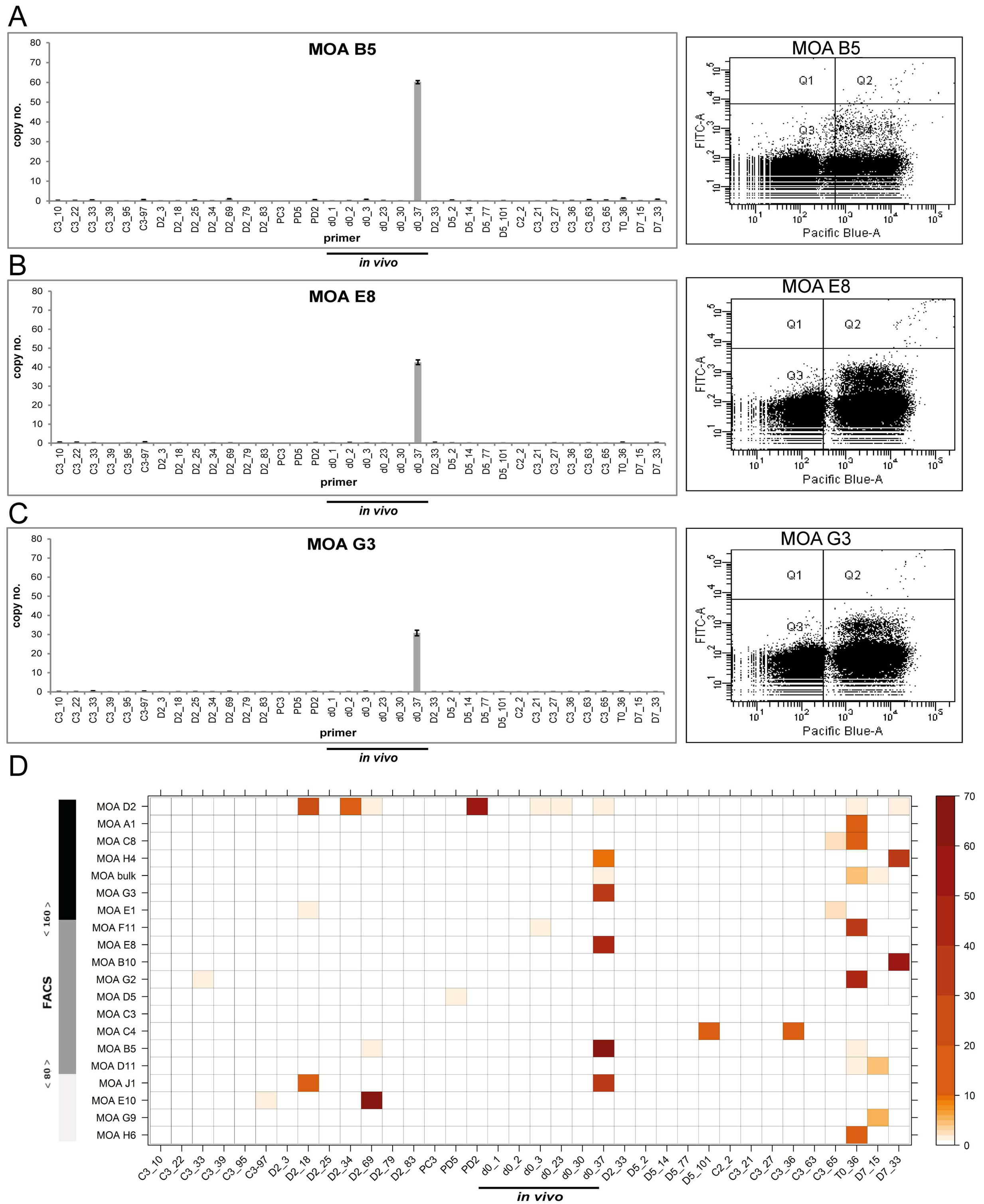

Fig 3. No correlation of *var* gene transcription and flow cytometry signals in clonal parasite populations. (A) Left panel: *var* gene transcription profile of the clone B5 transcribing the MOA *in vivo* transcript d0_37 with the highest transcription signal. The transcription signal is quantified as relative copy number (RCN) of the housekeeping gene arginyl-tRNA synthetase (PFL0900 c) (n = 3, standard errors are given) on the y-axis. The 36 primer pairs are depicted on the x-axis. The 6 primer pairs quantifying *in vivo* transcripts are underlined. Right panel: The corresponding dot plot after incubation with MOA serum of day 70. DNA signals by staining with Hoechst-33342 are depicted on the x-axis ("Pacific Blue-A"), the antibody recognition signal is depicted at the y-axis

("FITC-A"). Uninfected red blood cells are shown in area Q3, infected erythrocytes with both strong (upper cloud) and weak (lower cloud) signals accumulate in Q4. (B) and (C) *var* gene transcription profiles of clones MOA E8 and MOA G3 transcribing d0_37 at lower transcription signals but exhibiting higher surface signals than clone B5. (D) Heat map correlating *var* gene transcription and flow cytometry signal in 19 MOA clones and the MOA bulk culture. The FACS signal is quantified by the left bar with a colour code ranging from black (high: MFI > 160) to dark grey (medium: MFI 81–159) to light grey (weak: MFI < 80). All MOA clones and MOA bulk are sorted according to their corresponding flow cytometry signal and depicted on the y-axis. The MOA specific primer set for 36 *var* loci is listed on the x-axis. *var* gene transcription is colour coded as indicated by the bar on the right. *var* genes marked in red are those with the highest transcription signal and *var* genes in white are those with very low (<3%) or no transcription. Note that clones transcribing d0_37 and T0_36 are evenly distributed from high to low flow cytometry signals.

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C2 after CD36 selection (S4 Fig). *var* gene profiling in the E5 strain was done using an expanded primer set by designing primers based on previously published DBL sequences [47] and two newly identified DBLs (S3 Table) (accession number KC887546). For NF54-C2 transcriptional profiling we utilized a gene specific primer set [22,30]. CD36 binding selection changed the transcription profile in E5 and NF54–C2, indicating that the phenotype was likely mediated by PfEMP1 (Fig 6 and S4 Fig). In contrast to the laboratory strains, binding to the CD36 receptor in MOA D2, D5 and C3 field isolates was very weak and consecutive rounds of panning did not result in a significant increase in CD36 binding capacity (Table 2). Together these data show that laboratory strain CD36-binding PfEMP1 are recognized by the MOA serum antibodies.

The surface signal of the MOA D2 clone is PfEMP1 independent

To specifically determine the target of the MOA serum on the infected erythrocyte in the different parasite lines, the transgenic *var* knock-down parasites ΔE5E2, ΔMOA D2 and ΔNF54-C2 were generated. The MOA D2 line was chosen for this experiment because it was the clone with the highest surface recognition signal and showed constant transcription of the DBLs PD2 and D2_18 despite prolonged *in vitro* growth (Fig 5). In these parasites, application of blasticidin drug pressure leads to the transcriptional shutdown of the *var* gene family [22,53]. The transgenic lines ΔE5E2 and ΔNF54-C2 were selected for CD36 binding in the absence of blasticidin drug pressure. As expected, this generated strong surface reactivity with MOA d70-serum (Fig 7 and S5 Fig). Application of blasticidin resulted in transcriptional knock-down of the *var* gene family and a strong reduction of surface reactivity in ΔE5E2 (Student´s t-test p = 0.02) and ΔNF54-C2 (Fig 7 and S5 Fig). Furthermore, CD36 receptor binding selection of ΔE5E2s and ΔNF54-C2 was not possible under blasticidin pressure (S5 Fig) despite the presence of knobs in the membrane of the infected erythrocytes (Fig 7A).

Transcriptional profiling of the transgenic line ΔMOA D2 (grown without blasticidin pressure) displayed a strong surface signal and strong transcription of endogenous *var* DBLs PD2 and D2_18. After growth under blasticidin pressure, the transcription of PD2 and D2_18 was reduced to background levels and the *bsd* locus was the dominantly transcribed *var* gene/promoter. Despite this, both lines exhibited an identical surface signal in flow cytometry with orthologous, convalescent serum from day 70 (Fig 7B). The data show that in contrast to the transgenic parasite line ΔE5E2 and ΔNF54-C2, the antibody recognition signal of ΔMOA D2 is not affected by PfEMP1 knock-down.

Surface antigens of NF54 E5 and MOA D2 are trypsin sensitive and located in membrane areas with and without knobs

To further characterize the targets of the MOA antibodies on infected erythrocytes, ΔE5E2 CD36+ and MOA D2 were trypsinised. Trypsinisation and thereby shearing of the proteins on the erythrocyte surface of ΔE5E2 CD36+, which initially had a strong flow cytometry signal

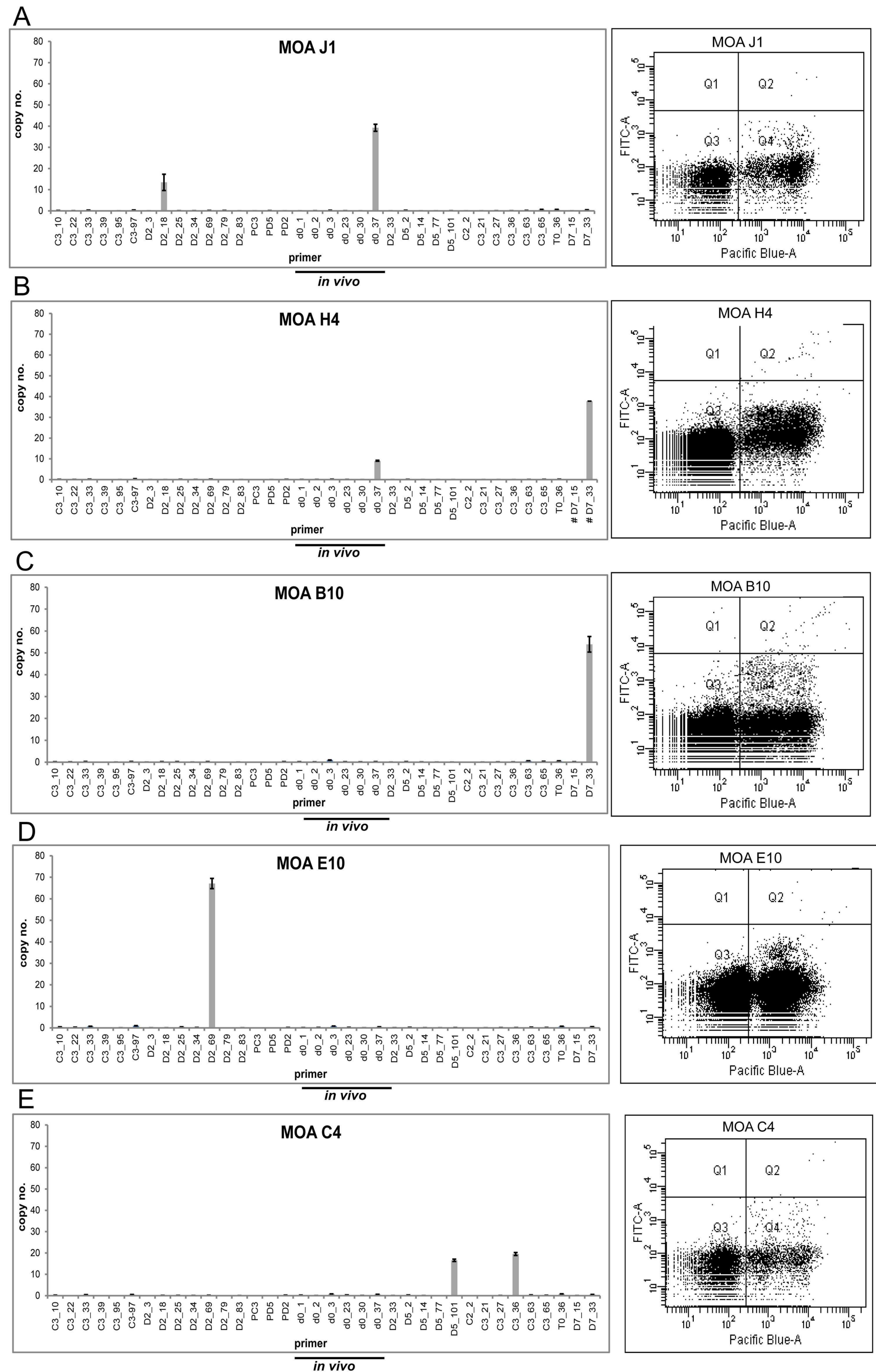

Fig 4. No correlation of FACS signal and switching or transcription strength at 35 generations after cloning. (A) Clone J 1 transcribes the *in vivo* transcript d0_37 and additionally DBL D2_18 but exhibits a low surface signal (MFI of 71). (B) and (C) Transcription of d0_37 and DBL D7_33 in Clone H4 is associated with a high surface signal (MFI of 223.67), but exclusive transcription of DBL D7_33 in clone B10 has a medium surface signal (MFI 109). (D) The exclusive transcription of D2_69 in clone E10 (at the highest individual transcription signal of all clones) is associated with low surface signal (MFI of 55.33). (E) Clone C4 Transcription of D5_101 and C3_36 at close to identical copy numbers. The clone has a medium MFI of 84.

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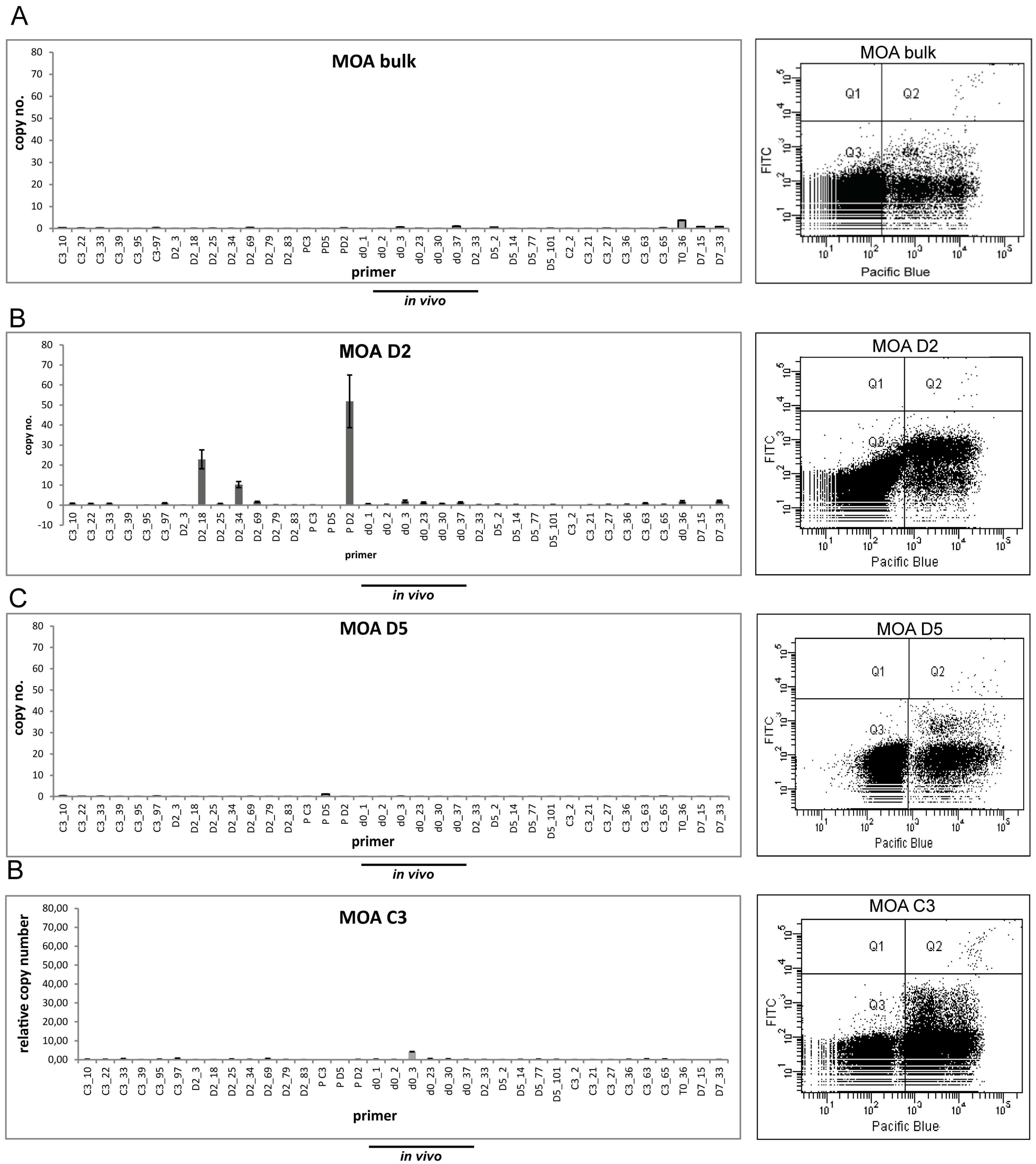

Fig 5. No correlation of FACS and *var* signals in MOA bulk and clones at >65 generations. (A) The MOA bulk culture exhibits low *var* gene transcription signals, but a high MFI (201). (B) Clone D2 exhibits the highest MFI (489) in the entire population and high *var* gene transcription signals for transcripts P_D2 and D2_18. (C) and (D) Clones D5 and C3 display low *var* gene transcription signals but show MFIs in the medium range (MFI of 63.67 and 139.67 respectively).

doi:10.1371/journal.pone.0166135.g005

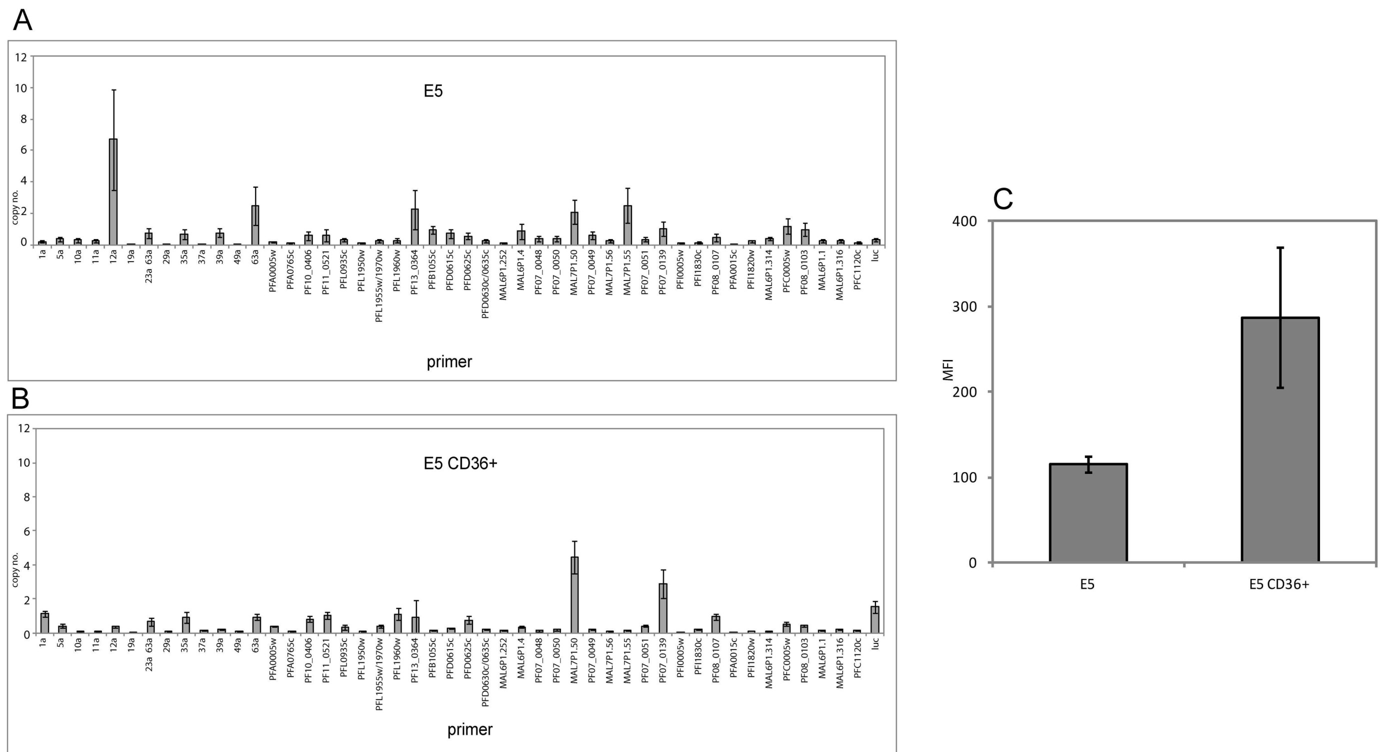

Fig 6. FACS signal with MOA day 70 serum of the laboratory strain E5 increases after CD36 selection. (A) *var* gene transcription profile of the laboratory clone E5 prior to CD36 receptor binding selection. Transcription was quantified with an E5-specific primer set (x-axis) (n = 3, standard errors are given). (B) E5 *var* gene transcription after 3 consecutive rounds of selection for binding to the CD36+ receptor. (C) Surface antigen recognition measured by flow cytometry with serum of MOA d70 shows a stronger signal for the CD36-selected E5 lab strain (standard errors given, n = 3).

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with MOA d70 serum, resulted in a reduction of the surface signal (one-tailed paired t-test $p = 0.05$) (Fig 8A). Trypsinisation of MOA D2 wild type resulted in highly significant signal reduction ($p = 0.003$). MOA antibodies thus clearly detected trypsin sensitive surface antigens in both strains. To test if the surface signal of MOA d70 serum was associated with knobs, electron microscopy and staining with gold-labelled anti-human IgG antibodies after incubation with serum of day 70 was performed. In ΔE5E2 CD36+ and in MOA D2 gold particles were identified on knobs and on membrane areas without knobs (Fig 8B). This demonstrates that the MOA serum targets knob-independent and knob-associated surface antigens.

Table 2. CD36 receptor binding can be increased in laboratory strains but not in culture adapted field isolates.

strain	Binding/ 50 C32 nuclei before selection	Binding/ 50 C32 nuclei after selection
NF54	11	164
E5	12	232
MOA D2	2	11
MOA D5	15	1
MOA C3	0,7	15

Binding of the trophozoites to the CD36 receptor at the surface of C32 melanoma cells was quantified after several consecutive rounds of panning. The numbers depict the amount of bound *Plasmodium* trophozoites per 50 C32 cell nuclei. Adhesion of the laboratory strains NF54 and E5 was increased, but binding of MOA D2, D5 and C3 was not influenced.

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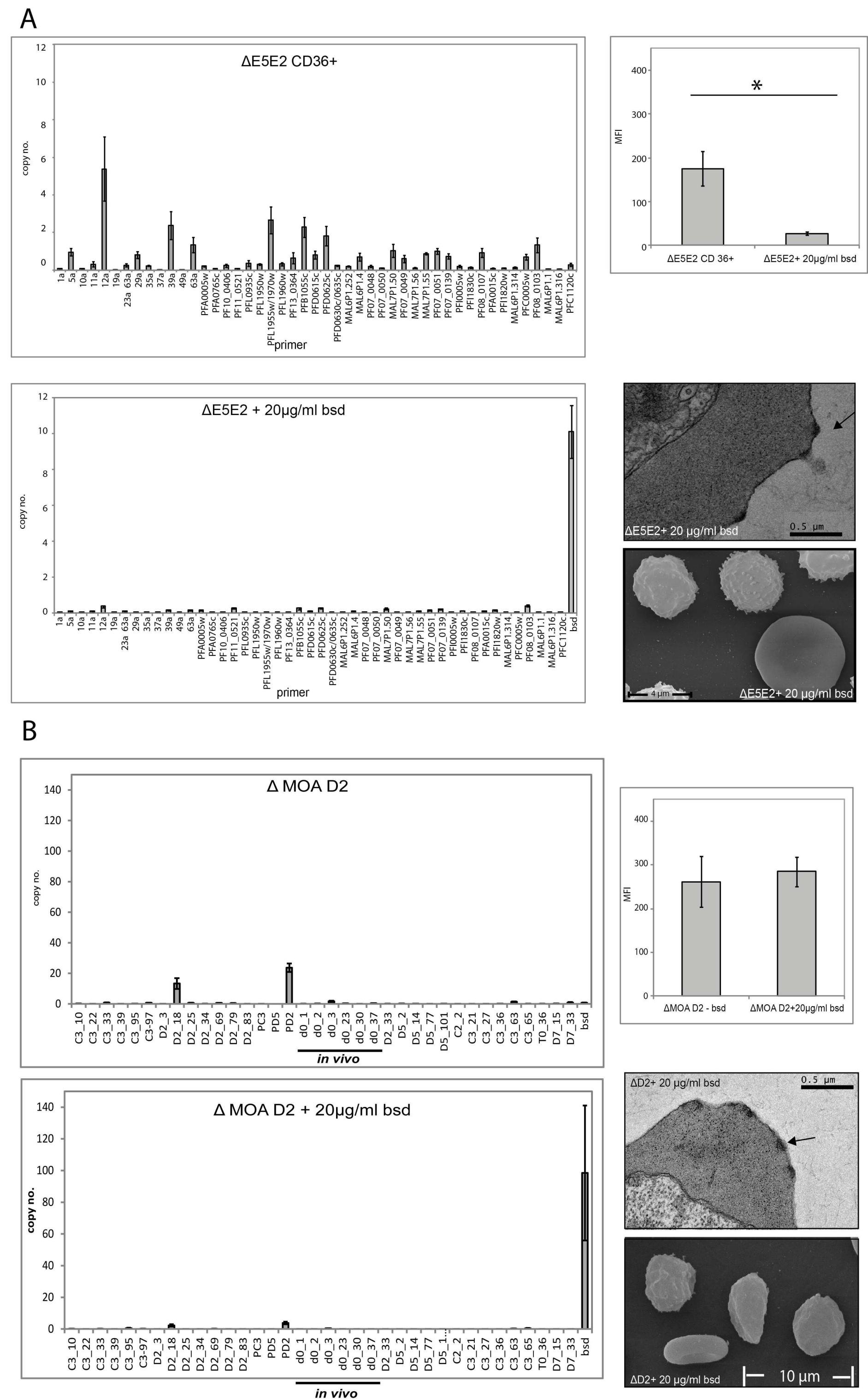

Fig 7. *var* knock-down removes the FACS signal in NF 54 E5 but not in MOA D2. (A) *var* gene transcription profiles of the transgenic ΔE5E2 strain. The upper panel shows the *var* gene transcription profile of the ΔE5E2 cell line after CD36 receptor binding selection without blasticidin pressure. The lower panel depicts the same cell line after *var* gene knock-down. The corresponding flow cytometry signals (serum of day 70) are shown in the upper bar graph on the right. The mean fluorescence (MFI) is significantly reduced in the knock-down cell line (standard errors are given, $p = 0.02$, $n = 4$). The transmission electron microscopy (TEM)

picture and the scanning electron microscopy picture (SEM) show that the E5 *var* knock-down cell line has intact knobs. (B) *var* gene transcription profile of transgenic MOA ΔD2 cell line without (upper graph) and with (lower graph) blasticidin pressure. Flow cytometry with serum of day 70 does not reveal any difference in their surface recognition signals of the two cell lines (standard errors are given, n = 3). The TEM and SEM pictures of MOA ΔD2 also clearly identify knobs in the erythrocyte surface membrane.

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Discussion

In semi-immune individuals living in high transmission areas, parasites are not eradicated completely from the host, but do survive at submicroscopic levels in the bloodstream.

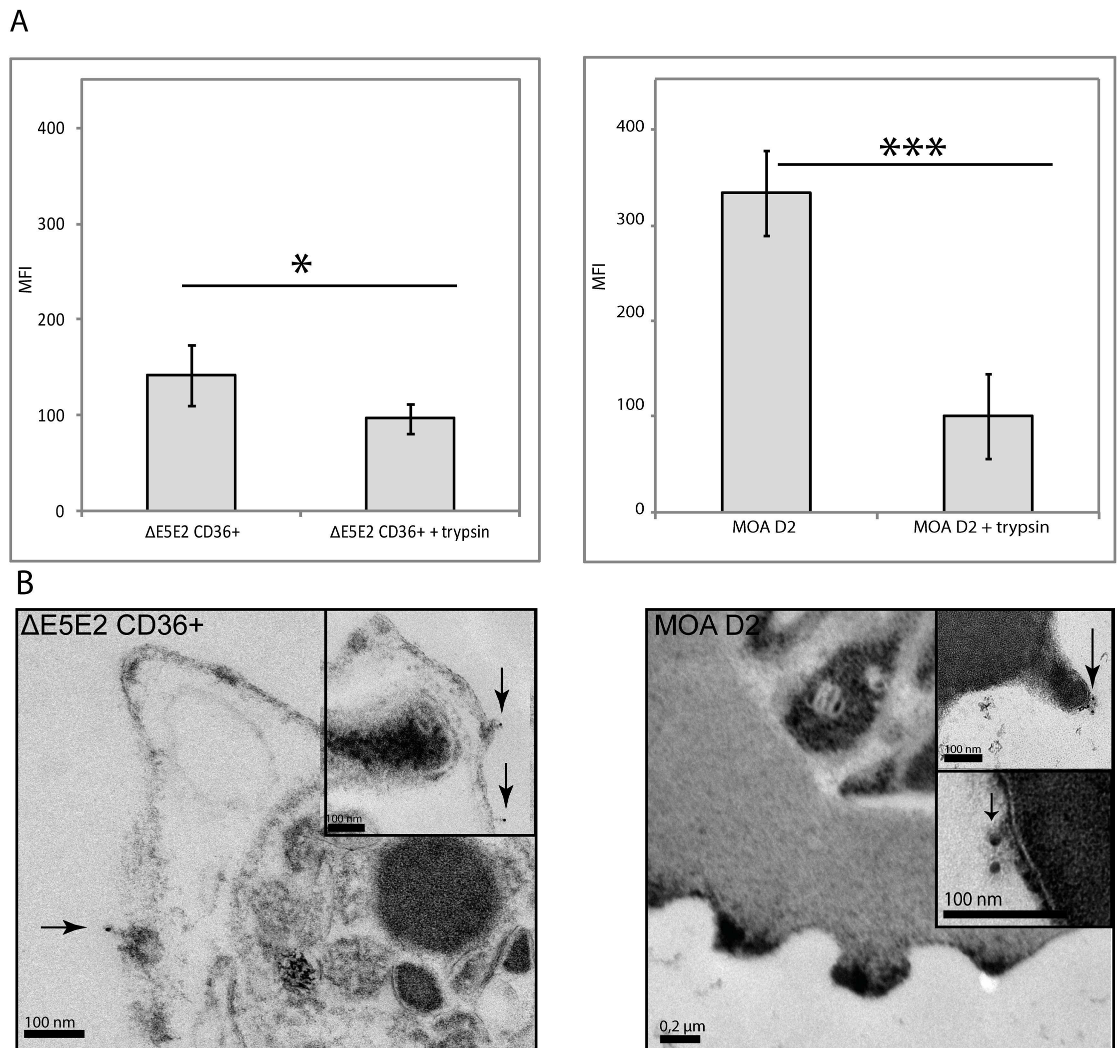

Fig 8. The MOA D2 surface recognition signal is trypsin sensitive. (A) Surface recognition signal with day 70 sera before and after trypsinisation of CD36-selected ΔE5E2 (left panel) and of MOA D2 (right panel). Both ΔE5E2 and MOA D2 were incubated with MOA serum of day 70 and labelled with a secondary FITC antibody for detection in flow cytometry. Trypsinisation resulted in a significant decrease of the antibody recognition signal (standard errors are given, $p = 0.05$ for ΔE5E2 (n = 4) and $p = 0.003$ (n = 3) for D2) in both cell lines. (B) Immuno-TEM after MOA day 70 serum labelling on CD36 selected ΔE5E2 parasites and MOA D2 parasites. A secondary, 12 nm gold-labelled anti-human IgG antibody was added. In ΔE5E2 and MOA D2, gold particles (marked with arrows) could be detected on membrane areas with knobs as well as on the membrane areas without knobs.

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Consequently, *P. falciparum* faces increasing immunologic pressure during chronic infections [4,54,55]. Here we try to address the determinants of *P. falciparum* persistence during a chronic asymptomatic infection of a semi-immune Gabonese individual. We show that the same individual parasite clones survive for at least four weeks in the host, although at drastically reduced parasitemia levels.

Historic serum transfer experiments have shown that transfusion of sera from a semi-immune adult will clear the infection of a child with malaria or a non-immune adult individual with malaria [2,3,36]. The decrease of parasitemia without any therapeutic intervention to sub-microscopic levels we observed in our patient is clearly consistent with these results. This raised the question how the parasites were able to escape the immune response, rendering long-term persistence possible. To characterize the surface antigens of the MOA isolates, convalescent MOA serum (day 70) was employed. This ensured enough time for the generation of anti-parasitic antibodies against the day 7 parasites investigated in this work. MOA serum FACS analysis with CD36 selected NF54 clones and NF54 PfEMP1 knock-down parasites clearly showed strong strain transcending anti-PfEMP1 reactivity, confirming that PfEMP1 is a major VSA target of the humoral immune response. FACS analysis of the isogenic MOA parasite clones showed a variable surface recognition signal, clearly supporting the role of VSAs in prolonged parasite persistence. Surprisingly, the surface recognition signal did not correlate with transcription of specific *var* genes.

Assessment of *var* gene transcription in NF54 clones is facilitated by the availability of the entire genome sequence [13]. In contrast, assessment of *var* gene transcription in a field isolate is complicated by the seemingly endless genetic diversity of this gene family [43]. To enable *var* gene specific transcription profiling in the MOA parasite, we developed a MOA specific primer set covering 36 of the presumed 60 MOA *var* genes. It was therefore possible that the observed dissociation of *var* gene transcription and surface signal in the MOA clones might have been simply a consequence of the inability of the primer set to detect specific *var* gene transcripts. Several aspects make this highly unlikely. *In vivo var* gene transcription analysis by cDNA DBL cloning identified d0_37 as the dominant *var* transcript. After tissue culture adaptation of the MOA bulk parasite, qRT PCR analysis with a newly designed MOA strain specific primer set identified d0_37, T0_36 and D7_15 as the dominant transcripts. Thus d0_37 was detected as the dominant transcript *in vivo* and *in vitro* by two different methods. After limiting dilution five MOA clones transcribed d0_37, five transcribed T0_36 and two transcribed D7_15 as the dominant transcripts. All other dominant transcripts were only detected once in the population of 19 clones. The transcript distribution among the MOA clones therefore clearly reflected the transcription pattern of the MOA bulk culture. Furthermore, the maximum transcription signal of the clones transcribing d0_37 and T0_36 was very high (60 and 42 RCN respectively) strongly suggesting that these were indeed the dominant transcripts. To verify the qRT PCR results of the MOA clones, a second method to identify the domimant *var* transcript (cDNA PCR with conserved DBL primers followed by Sanger sequencing) was performed. This confirmed the identity of the qRT PCR transcript in 15 of 16 clones (the exception being the clone E1).

The surface signal of iRBCs with sera from semi-immune individuals has recently been employed by several investigations as a marker for PfEMP1 expression in NF54 parasites. The surface reactivity of sera from Kenyan children against CD 36 selected 3D7 wild type parasites increased with age and was consistently higher than against 3D7 *var* knock down parasites [40]. Similarly, transgenic parasites devoid of the SBP1, a transport protein involved in the trafficking of PfEMP1 to the surface, were shown to display a decrease in surface reactivity with sera from children and adults from Kenya, Malawi and Papua New Guinea [56]. Consistent with this, CD 36 receptor binding selection of the NF54 clones in this work was associated

with transcriptional switches among the *var* gene family and this resulted in an increase in surface signal with the MOA serum.

In addition recent data from controlled human infections show that PfEMP1 expression is changed by intrahost replication. Intravenous inoculation of asexual NF 54 cell bank parasites increased the ability of NF54 to bind to CD 36 receptors. Scanning electron micrographs indicated that NF54 host passage had resulted in an increase of knobs in the membrane of the infected erythrocyte [57]. However, the relative importance of epigenetic reprogramming could not be assessed because asexual parasites were used for inoculation of the human volunteers. The importance of epigenetic reprogramming and host passage was investigated in parasites from the Tübingen controlled human malaria infection trial 1 [58]. NF54 passage through mosquito and human host resulted in a complete shift in *var* gene transcription compared to the premosquito [59] culture. Subsequent *in vitro* tissue culture growth showed that cultures from intradermally and intravenously infected individuals transcribed the same *var* genes but that the transcription signals were higher in parasites recovered after intradermal sporozoite inoculation [32]. Parasites from intradermal sporozoites infections showed higher surface signals after incubation with the MOA serum than parasites from intravenous infections. This indicates that the surface signal of the MOA serum is correlated with the transcription strength of individual *var* genes in the NF54 genetic background. Consistent with this, the decrease in the *var* gene transcription signal of the NF54 PfEMP1 knock-down clones delta C2 and delta E5 resulted in a decrease of the MOA serum surface signal. However, in the MOA PfEMP1 knock down clone, a decrease in the *var* transcription signal was not associated with a decrease in the surface signal.

Immuno-electron microscopy of parasites incubated with the MOA serum showed that in the MOA clone D2 and in the NF54 clone E5 gold particles bound to membrane areas with and without knobs. The surface signal of the MOA clone D2 and the NF54 clone E5 was removed by trypsinisation, confirming the surface localisation of these antigens in both genetic backgrounds. In contrast, *var* gene transcription knock down only affected the surface signal of the NF54 clone. Collectively the data suggest that the VSA surface signal of parasites from a chronic infection is predominately composed of trypsin-sensitive non-PfEMP1 VSAs, while in NF54 parasites the majority of the VSA surface signal is due to trypsin-sensitive PfEMP1 VSAs.

Of the *P. falciparum* VSAs, STEVOR, RIFIN, SURFIN and PfEMP1 have all been shown to be sensitive to trypsinisation, but the importance of the non-PfEMP1 VSAs for antigenic variation and as surface antigens has been as issue of scientific debate. Recently, it has been shown that the largest non-PFEMP1 VSA families STEVOR and RIFIN are displayed on the surface of the infected erythrocyte and that they mediate adhesion between infected erythrocytes, a phenomenon referred to as rosetting [60–62] by binding to glycophorin C and blood group A. Importantly, blood group A is also expressed on endothelial cells, suggesting a mechanism by which RIFINs could mediate cytoadhesion [62]. The importance of non-PfEMP1 VSA as antigens has recently been strongly supported by a study characterizing cross reactive antibodies from 2 semi-immune Kenyan individuals [63]. The authors identified highly reactive components of semi-immune sera through agglutination assays with multiple *P. falciparum* strains and subsequently characterized the antibodies with broadest reactivity by generating immortalized B Cells from the donors. Western blot analysis of 3D7 parasites enriched for binding to monoclonal cross reactive antibodies identified RIFIN proteins as the primary target of the antibodies.

Is there any seroepidemiologic evidence supporting the hypothesis that PfEMP1 may not be the only VSA expressed on the surface of infected red blood cells? Previous investigations in Gabon have indeed shown that the antibody repertoire against RIFINs expands with increasing age and that it represents a larger fraction of the total adult anti-VSA reactivity then anti-PfEMP1 antibodies [64,65]. A study from Papua New Guinea investigated the age dependent

acquisition of antibodies against recombinant DBLs from naturally circulating parasite populations. Antibody reactivity against the recombinant DBL proteins peaked at age 3–4, but subsequently plateaued. Analysis of antibody reactivity against VSA in the same populations showed that it continued to increase with age throughout adulthood, suggesting that VSAs are not exclusively composed of PfEMP1 [66]. Previous investigations employing sera and parasites from children and adults have shown that strain transcending VSA recognition develops within the first years of life [5,6,37]. Seroepidemiological studies based on the recombinant DBL-tags of the 3D7 *var* gene repertoire demonstrated that children develop anti-PfEMP1 immunity in a sequential fashion against UpsA, UpsB and UpsC *var* gene products during childhood [39]. These results have been further supported by a study assessing surface reactivity from Kenyan individuals with complicated and uncomplicated malaria against wild type and *var* knock down parasites of the NF54 clone 3D7 [40]. Surface reactivity against 3D7 wild type parasites was consistently higher than in 3D7 *var* knock down parasites and increased with age. However, sera from semi-immune individuals still exhibited IgG antibody binding in the 3D7 *var* knocks down parasites, suggesting that non-PfEMP1 VSAs were also targeted by the antibodies. A recent analysis of transcription data from field isolates suggests that anti-PfEMP1 antibodies not only select against specific PfEMP1 variants, but also against the amount of PfEMP1 on the surface of iRBCs in chronic infections [67]. The trypsin sensitive non-PfEMP1 VSA surface reactivity observed in the MOA clones is clearly consistent with these observations.

Our results raise a few fundamental questions with respect to *var* gene expression in different *P. falciparum* isolates. Differences in the strength of transcription signals between laboratory strains and field isolates suggest that wild type *var* promoters from field isolates may have a strong epigenetic imprint that contributes to the strong transcription activity and to the observed low switching propensity. We have shown before that in transgenic parasites the transcription activity of a subtelomeric recombinant *var* gene promoter can be increased markedly by the strength of applied drug selection [30]. At the same time this is associated with a lower switching propensity, suggesting that epigenetic modifications can influence the transcription activity and switching propensity of a recombinant *var* promoter. In a recent investigation of NF54 parasites recovered from controlled human infections [58], we have shown that longer in-host replication results in parasite populations with strong *var* gene transcription signals and low switching propensities [32].

In the MOA patient, semi-immunity likely exerted strong selective pressures on parasite replication and consequently parasites transcribing *var* genes with low off rates for these loci might have been at a selective advantage. The low switching propensity observed in the *in vitro* MOA parasites is clearly consistent with strong selective pressure favouring parasite populations transcribing specific *var* genes.

Recombination has been shown to occur amongst different members of the *var* gene families and it has been proposed that this is a mechanism to generate chronic infections [48]. We tried to address this question by designing gene specific primers for MOA DBL *var* sequences [30,68] a method that we have previously employed to confirm recombination events with the DBL alpha region [47]. We found no evidence for the generation of new chimeric sequences at different time points of the *in vivo* infection. Because our analysis is limited to the DBL alpha region, it does of course not exclude recombination in other regions of the *var* genes. We noted the absence of one *var* DBL in a subpopulation of MOA clones, which is consistent with previous reports of larger recombination during *in vitro* growth [48,49]. However, it is important to note that so far molecular epidemiologic investigations of *var* gene diversity have been performed mostly on the DBL alpha region [69,70]. The relative importance of recombination events for chronic infections therefore is currently not clear.

How could the presence of PfEMP1 in the membrane of the infected RBCs in a long term infection be reduced, despite a very strong *var* gene transcription signal? Here it is important to realize that although the MOA serum clearly exhibited broad anti-PfEMP1 reactivity, it is still possible that individual PfEMP1 variants were not detected by it. Indeed it has been described that IgM masking can prevent the development of PfEMP1 variant specific IgG antibody responses [71]. To definitively assess the relative contribution of the different VSAs to the surface signal of the MOA clones, investigations with VSA family specific monoclonal antibodies are necessary. The transmission electron microscopic experiments performed in this work detected no obvious difference with respect to knobs in the membrane of RBCs infected with NF54 or MOA clones, suggesting that knob architecture *per se* was not different in these strains. However, alterations in proteins that mediate PfEMP1 transport such as MAHRP1 could equally result in the observed phenotypes [72].

The data in this manuscript suggest that the VSAs responsible for the surface signal of parasites from a chronic infection differ from VSAs responsible for the surface signals in CD36 selected laboratory parasites. This is consistent with a previously proposed model that differentiates between VSAs expressed on parasites from individuals with severe malaria (VSA_{SM}) and VSA expressed on parasites from individuals with uncomplicated malaria (VSA_{UM}) [73,74]. According to this model, expression of VSA_{SM} may confer a growth advantage in children, generating high parasitemias and possibly a strong humoral immune response. During infections of semi-immune individuals this restricts the growth of parasites expressing VSA_{SM}, resulting in growth of parasites expressing VSA_{UM} that in turn replicate in a less efficient manner [75,76]. There is strong evidence that UpsA *var* genes may be part of VSA_{SM} because they mediate disease associated phenotypes such as binding to endothelial protein C receptor [77–81]. Studies assessing *var* gene transcription in children with uncomplicated malaria have consistently shown preferred transcription of Ups C and Ups B genes [82,83]. Given the importance of trypsin-sensitive non-PfEMP1 VSAs in the MOA parasites, we hypothesize that RIFIN and STEVOR proteins may be part of the VSA_{UM} expressed on the surface of infected red blood cells during chronic infections.

Understanding semi-immunity to blood stage parasites is critical for the development of a malaria vaccine. The work presented in this manuscript suggests that phenotypic and genetic analyses in culture adapted field isolates with serum from the individual patients can provide new insights that cannot be gathered from work with laboratory strains alone. Furthermore, controlled human infections of semi-immune individuals with NF54 parasites can provide new insights into the role of semi-immunity for VSA expression. Over the last years, PfEMP1 has emerged as a candidate for a vaccine targeting the blood stages of *P.falciparum*. The role of non-PfEMP1 VSAs for semi-immunity will have to be determined in future investigations.

Material and Methods

Ethics statement

The antigenic diversity study was approved by the local ethics committee in Lambaréné, Gabon: CERMEL (Centre de Recherche Médicale de Lambaréné), Lambaréné, Gabon. Written informed consent has been provided by all volunteers. The study followed the principal of the Declaration of Helsinki in the 6th revision as well as Good Clinical Practice (ICH-GCP).

Study protocol of the antigenic diversity study

All samples of the MOA individual investigated in this study were obtained as part of the antigenic diversity study in Lambaréné in Gabon from June 2006 until May 2007 [30,84]. This study included semi-immune asymptomatically *P. falciparum* infected adults in an area with

holoendemic malaria transmission. The study was approved by the local ethics committee. Inclusion criteria were: age of 18 years or older, microscopic evidence of *P. falciparum* parasitemia and absence of symptoms. Exclusion criteria were: age less than 18 years, pregnancy, symptoms or signs of malaria. The participants were followed for a total of 70 days and stayed untreated throughout the study period as long as they were asymptomatic. Blood and filter paper blood spots for PCR analysis were obtained twice per week for parasitemia assessment. Blood was drawn once per week for storage of serum, RNA in Trizol, RBC pellets and glycerol cryopreservation of malaria parasites. Parasitemia was assessed every three to four days by thick blood smear according to the Lambaréné method [85]. Extraction from filter papers was performed at the medical research unit of the Albert Schweitzer Hospital. DNA and all other specimens were transported to the Institute of Tropical Medicine at the University of Tuebingen, Germany. A total of 1017 individuals were screened and 36 individuals with microscopic parasitemias were identified. Based on *mspII* allele typing two clonal infections were identified. The asymptomatic clonal chronic infection of the 18 year-old male individual "MOA", was investigated further in this work. He became symptomatic with a new *P. ovale* infection on day 42 and was treated according to local guidelines. Blood draws were continued until day 70 of the investigation.

Parasite lines and culture

The field isolate MOA bulk represents cryopreserved *P. falciparum* parasites obtained from the MOA individual at day 7 of the 70 day sampling period. *In vitro* culture adaptation was performed in Tuebingen. Parasites were cultivated as reported elsewhere [30]. The parasites were kept in tissue culture, using 0+ blood from the local blood bank, for 25 generations prior to cloning by limiting dilution. Two limiting dilution experiments of MOA bulk were performed. The first one generated the 3 clones C3,D2 and D5 [30]. For the current manuscript a second limiting dilution experiment of the original MOA bulk culture generated 16 new clones (G3, E8,G9,B5,C4,E10,H4,G2, D11,F11,A1,H6,E1,B10,C8,J1). This resulted in a total of 19 MOA clones. At the time point of clonal *var* transcription analysis, the 16 MOA clones generated in the second limiting dilution experiment had been in clonal culture for approximately 35 generations (time since limiting dilution). The clones D2, D5 and C3 had been in continuous tissue culture for $>$ 65 generations (time since limiting dilution). E5 is a sibling parasite of 3D7 that shares 36 *var* genes with 3D7, but also carries 13 different *var* genes [47]. NF54-C2 is isogenic with 3D7 and was isolated from a bulk NF54 culture by limiting dilution [31].

DNA extraction and genotyping

DNA was extracted from filter paper blood spots, red blood cell pellets of the MOA individual and *in vitro* cultured parasites with the QIAamp DNA blood Midi Kit (Qiagen) according to the protocols provided by the manufacturer. *msp1* and *msp2* allele genotyping was conducted by standard nested PCR employing primers and conditions published elsewhere [86].

Characterization of *var* gene DBL sequences from DNA and cDNA

The *var* gene repertoires of the individual MOA D2, D5 and C3 clones were determined with a PCR cloning approach using the universal *var* primers αAF (`5'-GCACG(A/C)AGTTTTGC-3'`) and αBR (`5'-GCCCATTC(G/C)TCGAACCA-3'`) on genomic DNA [46]. PCR products were cloned in *E. coli* (TOPO TA cloning kit pCR 2.1-TOPO Vector, Invitrogen) according to standard procedures and DNA was extracted using the Qiagen Miniprep kit. Plasmid sequences were analysed and aligned with BioEdit software (Carlsbad, California). The same method was used to analyse the *var* repertoire in genomic DNA from blood samples of the

MOA individual. We used blood samples of day 0, day 7 and day 28 of the study. Cloning of *var* sequences obtained by universal *var* primers was also performed on copy DNA to identify the active *var* gene in RNA from tissue culture adapted field isolates (D2, D5 and C3 [30]) and *in vivo* RNA obtained from blood samples. In 9 of the 16 clones isolated in the second limiting dilution experiment (C8, G9, E1, B10, D11, F11, H6, A1, G2) the dominant *var* transcript was characterized by cDNA PCR with the universal *var* primers αAF and αBR followed by PCR fragment sequencing (see below). Fragment specific primer pairs were designed for all sequences (S2 Table). In this publication, differing from Enderes et al. [30], we named DBL MOA D5-D5 as PD5, MOA D2-D2 DBL as PD2, MOA C3-C3 as PC3. All primer pairs were validated by serial dilution of genomic DNA, PCR fragments were analyzed by Sanger sequencing prior to use by quantitative Real-Time PCR (see below).

RNA extraction and cDNA synthesis

A Sorbitol synchronisation of 20 ml parasite cultures, RNA extraction and cDNA synthesis was done as described in [30]. Possible DNA contamination in the cDNA was tested by the evaluation of proper splicing of the gene PFD1155w as described before [31].

Analysis of *var* gene *in vivo* transcription analysis by DBL cloning

To analyse *var* transcription *in vivo*, Trizol-conserved RNA from the MOA individual was analysed. cDNA was synthesized by two different approaches: one employing random primers and the other using the universal *var* primers αAF and αBR. The cDNA was analysed by PCR cloning as described above. Transcript quantification was done by counting the number of times that the individual sequences were obtained in the two cloning experiments. In both cloning experiments d0_37 was the most frequent transcript.

In vitro analysis of *var* gene transcription by quantitative Real-Time PCR and cDNA PCR fragment sequencing

DBL specific MOA primer pairs for all the 36 DBLs of the culture adapted MOA parasites were designed and subsequently validated on serial dilutions of MOA clone genomic DNA by Real-Time PCR followed by DNA sequencing of the PCR product as described previously [30]. The 16 MOA clones generated in the second limiting dilution experiment underwent a first transcription analysis with 33 primer pairs obtained from the DBL cloning experiments with the clones D2,D5 and C3 as well as the 6 *in vivo* transcripts. This primer set detected a dominant transcript in 7 (H4, G3, E8, B5, J1, E10 and C4) of the 16 clones. However in 9 clones (C8,G9,E1,B10,D11,F11,H6,A1,G2) no dominant transcript was detected. In these 9 clones cDNA PCR with the universal primer αAF and αBR followed by PCR fragment sequencing was employed to characterize the dominant transcript. This detected the *in vivo* DNA sequences (D0_36, D7_15 and D7_33) as the dominant transcripts in 9 clones. We subsequently designed DBL specific primers (T0_36, D7_15 and D7_33) and quantified the transcription signal by qRT PCR. To assess the possible presence of *var* transcripts not covered by the 36 gene specific primers we also performed PCR with the degenerate DBL primers on the clones in which a dominant transcript was detected with the initial 33 primer set. Specific *var* gene primer correction factors to correct for primer efficiency relative to the amplification of the housekeeping gene arginyl-tRNA synthetase (PFL0900 c) were assigned for all primers and ΔCt was calculated accordingly (User bulletin 2, Applied Biosystems). *var* gene transcription of NF54 was assessed as described previously [21,22,30]. Determination of the E5-specific *var* sequences was described previously [47]. Gene-specific primers were designed for 13 sequences (S2 Table) and validated on serial dilution of E5 genomic DNA. Transcription of

bsd (blasticidin S deaminase) in PfEMP1 knock-down strains was assessed with the primers *bsd* fwd 5′-TTGTCTCAAGAAGAATCCAC-3′ and *bsd* rev 5′-TCCCCCAGTAAAATGATA TAC-3′ [22,30].

Generation of transgenic *var* knock-down parasites

Plasmid pV_CBB/IDH [22] was utilized to generate transgenic *var* knock-down parasites in the MOA clone (MOA D2) as well as in NF54-C2 and E5. Here, expression of the transgene *bsd* is driven by an UpsC promoter that is paired with an intron promoter driving human *dhfr* (dihydrofolate reductase) gene. The knob-positive NF54-C2 clone was transfected with the plasmid pV_CBB/IDH. Transfection was conducted as described in Deitsch et al. [87]. Plasmids were propagated in *E. coli* and isolated with the Plasmid Maxi kit (Qiagen). Uninfected erythrocytes were loaded with plasmid DNA by electroporation and double sorbitol synchronised parasites were added. Subsequently, parasites were cultured for 4 generations in 2.5% hematocrit of loaded erythrocytes. Due to the high multiplication rate, the cultures were diluted every growth cycle with newly plasmid loaded erythrocytes. On day 9 of the experiment, the drug WR 99210 (5nM) was added to ring stage parasites of the transfectants in the MOA genetic backgrounds. Transfectants in the E5 genetic background were immediately selected with blasticidin because E5 already carries a *hdhfr* expression cassette [21]. Shut down of MOA D2 *var* expression in stably transfected parasites was induced by application of 20μg/ml blasticidin (final concentration). Episomally transfected ΔMOA D2 carrying the pV_CBB/IDH plasmid was selected with increasing blasticidin pressure starting at 1μg/ml up to 20μg/ml. Transcription of blasticidin S deaminase and absence of transcription of endogenous *var* genes was quantified by Real-Time PCR on cDNA as described above.

CD36 receptor binding selection

Human melanoma C32 (ATCC®, CRL-1585™) cells were cultured in DMEM (PAA Laboratories) supplemented with 10% FBS (PAA), 1% non essential amino acid solution (Sigma-Aldrich), 0,05 mg/ml Gentamicin (PAA), and 2 mM L-Glutamine (PAA). To quantify binding of parasitized red blood cells to the C32 cells microscopically, a cover slip was inserted into the C32 culture flasks. Trophozoite enrichment was performed with MACS columns (Miltenyi Biotec). Comparable (~$10x10^7$) numbers of parasites were diluted in binding medium (450ml H_20, 2.98g HEPES, 5.2g RPMI without glutamine and NaHCO3, 10% ml human serum 0^+), transferred into the C32 cell culture bottle ($25cm^2$) with ~80% confluency and incubated at 5% CO_2 for 2h at 37˚C. Unbound trophozoites were removed by washing with binding medium 3 times. The cover slip was removed from the culture flask with a forceps, fixed in 2% glutaraldehyde, stained with 5% Giemsa stain (Merck). The number of bound trophozoites per 50 C32 nuclei was determined by light microscopy. Subsequently, parasite medium and uninfected red blood cells (uRBCs) were added and the culture was incubated overnight in the parasite incubator. The following day the parasites were recovered in ring stage.

Flow cytometry analysis

Parasites were synchronised with MACS (Miltenyi Biotec, order no: 130-042-901). The schizont pellet was mixed with uRBCs to obtain a parasitemia between 5–15%. 12μl of parasite pellet were stained with Hoechst 33342 (0.01mg/ml) for 30min at 37˚C under tissue culture conditions. After washing three times with 1x phosphate buffered saline (PBS) the pellet was incubated with MOA serum (1:10 in PBS) of day 70 of the study. The infected red blood cells (iRBCs) were washed three times and the pellet was stained with goat anti-human IgG (Southern Biotech) (1:200 in PBS) for 30min. After washing,the pellet was incubated with rabbit anti-

goat fluorescein isothiocyanate (FITC) conjugated IgG (1:50 in PBS) (Southern Biotech) for 30 min. After washing and dilution with 1 ml PBS the cells were measured in a FACS CantoII (BD). FACS (Fluorescence-activated cell sorting) was performed as described elsewhere [88,89]. All experiments were conducted at least in triplicates with uRBCs as control. Mean fluorescence intensity (MFI) for each tube was defined as: MFI = MFI_{iRBCs}–MFI_{uRBCs}. The CD 36 selected ΔE5E2 parasites and the blasticidin selected ΔE5E2 were used as positive and negative controls in all FACS experiments with the MOA clones.

Trypsinisation of erythrocyte surface proteins

Trypsinisation of *P. falciparum* trophozoites following MACS separation was done as described elsewhere [90].

Electron microscopy

Immune labeling was done using trophozoites purified from a 10ml- *P. falciparum* culture with MACS® (Miltenyi Biotec) and washed with 1x PBS. The cells were pelleted in a low-binding Eppendorf tube (Biozym) and and incubated with MOA serum of day 70 for 30 minutes, followed by 2–3 washing steps using SafeSeal Vertex tips (Biozym) with 1x PBS. Goat anti human IgG (Southern Biotech) was diluted 1:200 in 1x PBS prior to incubation with the pellet for 30 minutes. Then the mixture was washed 2–3 times with 1xPBS and the cells were stained with Gold-AffiniPure Donkey Anti-Goat IgG 12 nm Gold (dianova, 1:20) for 30 minutes before 2–3 washing steps. The cell pellet was chemically fixed (4% formaldehyde, 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.4), cryo-protected with 30% glycerol in water and cryo-fixed by high-pressure-freezing in a Balzers HPM-010. For subsequent freeze substitution, embedding, sectioning and contrasting and we followed the protocol described in Moussian et al. [91]. The sections were examined with a Tecnai G2 Spirit (FEI, Eindhoven, Netherlands) operated at 120kV transmission electron microscope that was equipped with a Gatan Ultrascan 4000 CCD-camera (Gatan, Pleasanton, CA, USA). Micrographs were recorded with the manufacturer's software at 4k x 4k resolution. For analysis by scanning electron microscopy cells were fixed with 0.5% glutaraldehyde, 2% paraformaldehyde mounted on polylysin-coated coverslips followed by another fixation step with 2.5% glutaraldehyde. The erythrocytes were postfixed with 1% osmium tetroxid in water, dehydrated in a graduated series of ethanol followed by critical-point-drying with CO2 in a Polaron critical-point-dryer. Finally the cells were sputter coated with a 6nm layer of platinum (Bal-Tec MED 010) and examined with a Hitachi S-800 field emission scanning electron microscope at an accelerating voltage of 15 KV. Knob quantification of the D2 and D5 clones was performed by two independent investigators (EB and MF) in a blinded fashion. TEM cuts of 8–10 iRBCs per clone were examined and the number of knobs counted.

Statistical analysis

The heat map was generated in R [92] using the package *lattice* and *latticeExtra*, and post-edited in GIMP 2.8.14. The individual MOA clones and the MOA bulk were ordered by size according to the strength of their flow cytometry signal (FACS). *var* gene expression signals for each of the 36 loci were expressed as percentage of total *var* signal of the individual clone. The strength of the expression signal of the individual *var* gene was colored in accordance with their magnitude using user-defined steps for color graduation in the R heat map. Two data points are missing (D7_15 and D7_33 for MOA F11). T-tests to calculate the statistical significance of data were analyzed with Microsoft Excel.

Supporting Information

S1 Fig. *in vitro var* gene transcription analysis of MOA parasites. Long term transcription profiling with gene specific primers for day 0 *var* transcripts in culture adapted MOA bulk parasites for a total of 150 generations of continued growth. Transcript d0_37 was the most abundant transcript *in vitro*. (A), (B) and (C) display *var* transcription after 30, 90 and 150 generations of *in vitro* growth.
(TIF)

S2 Fig. MOA Clones (35 generations after cloning) transcribing T0_36 display surface signals from high to low. (A) Clone G2 exhibits the strongest transcription signal and has a medium MFI of 69.67. (B), (C) and (D): The clones C8, A1 and H6 transcribe T0_36 at identical strength, yet the surface signal for clone C8 and A1 is high (MFI of 243 and 258.33) and low for clone H6 (MFI of 48.67, lowest surface signal in the entire population). (E) Clone F 11 transcribes T0_36 at high levels yet has medium surface signal (MFI of 81.67).
(TIF)

S3 Fig. MOA clones (35 generations after cloning) show low transcription signals but high to low FACS signals. (A) The clone E1 displays the lowest transcription signal yet has a high surface recognition signal (MFI of 161.67). (B) and (D) The clones D11 and G9 both transcribe DBL D7_35 at low levels yet have medium and low surface reactivity respectively (MFI of 62 and 53.75).
(TIF)

S4 Fig. MOA serum surface recognition of the laboratory strain NF54-C2 is increased after CD36 receptor binding. (A) The copy number is shown at the y-axis. The adhesion phenotype (bound trophozoites per 50 C32 cell nuclei) is depicted on the right and also the flow cytometry dot plot, where iRBCs (right lower corner) are not recognized by the antibodies of MOA day 70 serum. (B) Panning for CD36 binding resulted in a strong adhesion phenotype and an upward shift of the infected erythrocyte population in flow cytometry with MOA day 70 serum. Binding of the trophozoites (dark dots) to a C32 cell is demonstrated in the light microscopy picture below.
(TIF)

S5 Fig. PfEMP1 knock-down in NF54 clone C2 is efficient and can be reversed by CD36 binding. (A) Removal of blasticidin and selection for CD36 binding evokes *var* gene activation and cytoadhesion and yields a positive signal in flow cytometry. (B) Knock-down of PfEMP1 efficiency shown by transcription profiling. There is no adhesion to the CD36 receptor (right graph) and iRBCs are not recognized by MOA day70 serum (dot plot). (C) Western Blot demonstrating efficient PfEMP1 knock-down. Uninfected erythrocytes served as control. Cell lysates were generated using Triton X-100, run on a tris acetate gel, blotted on a nitrocellulose membrane and stained with the PfEMP1-specific antibody α-ATS. α-ATS detects PfEMP1 (marked with an asterisk) in CD36-selected ΔE5E2, but not in its PfEMP1-knock down cell line ΔE5E2+20μg/ml bsd. The transfected ΔNF54+20μg/ml clone also does not have a band for PfEMP1. Cross-reaction with the cytoskeletal protein spectrin is seen at ~250kDa in all samples. The columns were rearranged for clarity (dotted line).
(TIF)

S1 Table. DBL specific primers for amplification of DBLs from DNA of days 0, 7 and 28. Sequence D0H21 was identified by DBL cloning on all three days and was used as a control for primer design. All chimeric DBL sequences and the corresponding primers can be supplied by

the authors' upon request.
(DOCX)

S2 Table. *var* gene specific PCR primer set for MOA culture adapted field isolates and *in vivo var* transcripts. Forward and reverse sequences for the 36 *var* loci were designed based on the corresponding DBLα sequences. MOA clone names are indicated by capital letters. Day0 *in vivo* MOA transcripts are indicated by the prefix d0. DBL T0_36 is the same as DBL D7_87 (KC887630) and C3_42 (KC887685) in the NCBI database.
(DOCX)

S3 Table. Transcription strength of the dominant DBLs in all 19 MOA clones and the MOA bulk culture.
(DOCX)

S4 Table. Gene specific PCR primer pairs for E5 *var* sequences. Primer pairs were designed based on the hypervariable regions of the E5 specific DBLs.
(DOCX)

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Identification of a conserved *var* gene in different *Plasmodium falciparum* strains

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Abstract

Background: *Plasmodium falciparum* (*P. falciparum*) escapes the human immune system by alternate expression of Variant Surface Antigens (VSAs). The most important VSA is a polymorphic protein family collectively referred to as *Plasmodium falciparum* erythrocyte protein 1 (PfEMP1), which is encoded by the multicopy *var* gene family. Mutually exclusive expression of an individual *var* gene per parasite and frequent recombination events among *var* genes provide the basis for antigenic variation. So far, only the *var* gene responsible for placental malaria, *var2csa*, was found to be highly conserved among all *P. falciparum* strains. In previous investigations of *var* gene transcription of a field isolate from Gabon, we identified a *var* gene with a Duffy binding like domain (DBL) that is identical to PF3D7_0617400 on chromosome 6 in NF54 3D7 (Enderes et al 2011). Here we characterize the phenotype of laboratory and field isolate parasites expressing PF3D7_0617400 and investigate its genetic conservation in a panel of field isolates from different African countries.

Methods: Targeted Sanger sequencing was employed to characterize exon 1 of PF3D7_0617400. Transcriptional analysis, CD36-receptor binding, indirect immunofluorescence with PF3D7_0617400-antibodies and quantification of surface reactivity against semi-immune sera were used to characterize an NF54 clone and a field isolate clone transcribing 3D7_0617400. Nine additional field isolates from different African countries were screened for PF3D7_0617400. *var2csa* and 57 microsatellites markers were used to characterize genetic diversity within the *var* gene family and the non-coding regions of the *P. falciparum* genome.

Results: PF3D7_0617400 was transcribed in the NF54 A3 clone and the MOA C3 clone. Immunofluorescence confirmed expression of the corresponding PfEMP1. However, CD36 binding assays and surface reactivity to semi-immune sera differed markedly in the two clones.

PF3D7_0617400 could be detected in 2 field isolates while *var2csa* was amplified from all field isolates. Sequence analysis revealed that PF3D7_0617400 was virtually identical except for an approximately 200 bp region in both field isolates. In contrast, *var2csa* was in parts highly conserved (H_e=0) but in other parts highly variable (H_e=0.86). The average genetic diversity of non-coding regions was high (H_e=0.8) across all investigated field isolates.

Conclusions: Individual *var* genes exhibit less genetic diversity than the non-coding *P. falciparum* genome suggesting that purifying selection may limit overall genetic diversity of some PfEMP1-variants.

Keywords (3-10)

Malaria, *var* genes, genetic diversity, microsatellites, recombination, VSA, PfEMP1, RIFIN, STEVOR

Background

The most virulent form of malaria is caused by *Plasmodium falciparum* (*P. falciparum*)[1]. The virulence of *P. falciparum* is a consequence of adhesion of infected red blood cells (iRBCs) to different host endothelial receptors [2]. This process is mediated by a polymorphic protein family collectively referred to as *P. falciparum* membrane protein 1 (PfEMP1) [2–4]. Expression of PfEMP1 variants that exhibit binding to chondroitinsulfate A [5,6] in the placenta or to the EPCR [7] receptor has been associated with the development of placental malaria and cerebral malaria in children, respectively. PfEMP1 is encoded by the *var* gene family [8] , a multicopy gene family with approximately 59-60 different genes per parasite genome [9]. Only one *var* gene is expressed in an individual parasite at a time, a process referred to as mutually exclusive expression [10]. Switching between the active *var* locus generates antigenic variation leading to immune escape of the parasites [11].

var genes can be grouped according to their distribution across the 14 *P. falciparum* chromosomes into subtelomeric or central and this position correlates with their promoter type [12]. 36 of the 59 *var* genes in the 3D7 clone of *P. falciparum* are located in subtelomeric areas and have UPS A, UPS B type or UPS B/A promoters, while 23 are located in central *var* clusters and have UPS C or UPS B/C promoters. An individual *var* gene typically consists of exon 1 and exon 2 that encode for the extracellular and intracellular parts of the PfEMP1 protein, respectively. Exon 1 is hypervariable and consists of an N-terminal segment, followed by a variable number of domain cassettes (DCs) that are composed of several Duffy Binding Like- (DBL) and Cysteine Rich Interdomain Region- (CIDR) domains [9]. DBL and CIDR domains are assembled of conserved sequence blocks that share similarities in all parasite strains as well as hypervariable sequence blocks [13]. The 60 *var* genes of an individual parasite are completely distinct from each other and different *P. falciparum* strains carry almost completely different *var* gene repertories [14]. Indeed frequent recombination events generate new *var* gene variants during meiosis and mitosis and it has been suggested that mitotic recombination generates new PfEMP1-variants during chronic infections [15,16]. The contribution of recombination to antigenic diversity has recently been further supported by evidence showing that binding phenotypes

such as CD36 and EPCR binding are primarily mediated by the tertiary structures of the CIDR domain and by hydrophobicity, characteristics that do not require an exact amino acid motif, thus allowing a high degree of sequence variation without affecting the function of the respective PfEMP1 domains [17] .

Considering the high variability of the *var* gene family, it is remarkable that there is one *var* gene that is conserved among all *P. falciparum* strains. This gene, *var2csa*, [5] encodes the PfEMP1 VAR2CSA that binds to chondroitinsulfate A (CSA) in the human placenta and is primarily responsible for placental malaria during the first pregnancy [6]. Because of this unique binding phenotype there appears to be a positive selection pressure to maintain this *var* gene. Selection and expansion of parasite populations expressing VAR2CSA is dependent on the presence of the human placenta, ensuring that VAR2CSA will not be "seen" by the immune system until the first pregnancy.

During *var* gene transcription analysis of a field isolate from Gabon (MOA), we have previously discovered a DBL with 100% sequence identity to the DBL of PF3D7_0617400 of the 3D7 reference genome [18]. Here we report the presence of the entire exon 1 of PF3D7_061740 in 2 out of 10 field isolates, suggesting a selective advantage for parasites carrying this *var* gene. As a proof of principle we also compare the genetic diversity of *var2csa* and 57 microsatellite markers across this population of field isolates. This revealed that some areas of *var2csa* are highly conserved, while others are highly variable. Together the data suggest that some *var* genes are under purifying selection that limits their genetic diversity

Results:

Identification of the 3D7 *var* gene PF3D7_0617400 in a field isolate from Gabon

The *P. falciparum* field isolate MOA was obtained from an asymptomatically infected Gabonese individual [18–20]. After tissue culture adaption, several clones were generated by limiting dilution and the transcribed *var* genes were determined by cDNA DBL cloning. In the

MOA clone C3 [18], the transcribed DBL (MOA-C3) was found to be 100% identical with the DBL of the 3D7 *var* gene PF3D7_0617400 (previously annotated as MAL6P1.252 /PFF0845c). To determine the extent of sequence homology, a primer set (S1 Table) spanning the promoter region and the entire exon one (basapair (bp) 0-6271) of PF3D7_0617400 was designed based on the 3D7 genome sequence. All PCR fragments obtained from MOA C3 genomic DNA were characterized by Sanger sequencing and comparison of the assembled MOA C3 sequence with the 3D7 reference sequence showed that the sequences were 99-100% identical (Fig1). The only difference consisted of a small 194 bp insertion (Fig S1) between bp 3722 and 3871 of the MOA C3 gene (3721 and 3914 of the 3D7 reference sequence).

MOA C3 and NF54 A3 both express PF3D7_0617400 but have different cytoadhesion and surface recognition signal phenotypes

To determine if the entire open reading frame (ORF) of PF3D7_0617400 was transcribed in MOA-C3, cDNA was analyzed by qualitative and quantitative PCR (Fig. 2 a and b). PCR transcripts were obtained with 6 primer pairs along the ORF of PF3D7_0617400 and qPCR with DBL specific primers showed that the transcriptional signal for PF3D7_0617400 was in the range of a dominantly transcribed *var* gene in MOA-C3. During previous investigations the NF54 clone A3 was generated that transcribed PF3D7_0617400 in a very stable fashion [21]. NF54 A3 was therefore re-thawed and brought into tissue culture. Consistent with the previously reported low off rate of PF3D7_0617400, it was still the dominantly transcribed *var* gene in this re-grown culture (Fig 2 c) [5].

To assess the binding phenotype of PF3D7_0617400, CD36 binding assays with the clones NF54 A3 and MOA C3 were conducted on human melanoma cells expressing CD36. NF54 A3 exhibited CD36 binding even without prior selection and the CD36 binding capacity increased strongly after 2 additional rounds of panning (Fig 3 a). In contrast, MOA C3 did not exhibit significant CD36 binding prior to selection and even after 4 rounds of panning CD36 binding increased only marginally (Fig 3 b). NF54 A3 transcriptional analysis after CD36

selection showed that PF3D7_0617400 continued to be transcribed as the dominant *var* gene, showing that it mediated the phenotype (Fig S2 a). Similarly, transcriptional quantification of PF3D7_0617400 in MOA C3 before and after CD36 biding selection revealed that it continued to be transcribed at the level of a dominant *var* gene and indicating that CD36 selection had not resulted in the transcription of different *var* genes (Fig S2 b). To quantify overall PfEMP1 expression in CD36 selected NF54 A3 and MOA C3 clones, FACS analysis with serum from the semi-immune MOA [19] patient was conducted. CD36 selected NF54 A3 parasites displayed a strong FACS signal clearly showing that the serum detected the expressed PfEMP1 on the surface of the infected red blood cells (Fig3 c). In contrast, CD36 selected MOA C3 parasites only displayed a low FACS signal with MOA serum.

To determine if the corresponding PfEMP1 was indeed expressed in both strains, immunofluorescence assays with an antibody against the CIDR α 2.1 of PF3D7_0617400 were conducted [17]. An immunofluorescence signal was detected in MOA C3 (Fig 4a) and in the NF54 A3 infected erythrocytes (Fig 4b) [19] suggesting that both strains indeed expressed the same PfEMP1 [19]. Together the data suggested that although both strains expressed the same *var* gene, there was a difference in PfEMP1 display between the NF54 and MOA parasites.

Comparative analysis of *var* gene and microsatellite diversity in field isolates from West, Central and East Africa

To generate a first estimate of the degree of PF3D7_0617400 conservation in *P. falciparum* strains, a panel of 9 additional freshly culture adapted field isolates from the Central Africa (Congo, Cameroon), West Africa (Gambia, Ghana and Togo) and East Africa (Kenya and Sudan) (Table 1) were evaluated for the presence of the PF3D7_0617400 *var* locus. In addition, the highly conserved *var2csa* gene and panel of 57 microsatellites were characterized in each field isolate. All field isolates were obtained from travelers who developed symptomatic *P. falciparum* infections after returning from Africa. After successful tissue culture adaptation, DNA of the field isolates was screened by targeted PCR. Initial

screening for a 3 kb fragment of PF3D7_0617400 detected the gene in a field isolate originating from Togo (5798) (Fig 5a). Subsequent PCR with primers spanning the entire exon 1 (Table S1) amplified identical fragments in 5798 and 3D7 and targeted Sanger sequencing of PCR fragments showed that the exon 1 sequence of PF3D7_0617400 of the field isolate 5798 was 99-100% identical with the 3D7 reference sequence. As in MOA C3, the only exception was an insertion of 194bp ranging from 3722 to 3871bp (3721 and 3914 of the 3D7 reference sequence) (Fig 5b). Overall the PF3D7_0617400 *var* gene was thus detected in 2 of 10 field isolates. Next PCR for the highly conserved DBL2x and DBL 3x of *var2csa* was performed (Primers 10F and 75 R [5]). An approximately 1700bp fragment of *var2csa* was amplified in all 10 field isolates (Fig. 5 c), although the length of the amplification product differed in the different strains. Sanger sequencing of the *var2csa* PCR fragments confirmed that all sequences were indeed *var2csa* sequences but also demonstrate considerable sequence diversity in some parts of the *var2csa* sequence (GenBank submission in process).

To compare the genetic diversity of PF3D7_0617400 and *var2csa* to the genetic diversity of the non-coding genome, fragment length analysis of microsatellites was performed. A total of 57 microsatellites (MS) distributed across the 14 *P. falciparum* chromosomes (three to four MS per chromosome) (Fig 6 a, S3 Table) were typed for each field isolate. Fragment length diversity was high across all microsatellites with an expected heterozygosity (H_e) ranging between 0.53 and 0.98 with an average H_e across all microsatellites of 0.8 (Fig 6 b) (Table S2). The marker BM51 on chromosome 7 exhibited the lowest H_e (0.3), suggesting that this marker may be particularly conserved. Interestingly the MS 9B12 and B5M77 in the area of the chloroquine resistance sweep [22] on chromosome 7 exhibited high genetic diversity, suggesting that most of the parasites carried the sensitive *pfcrt* allele. Consistent with this only one field isolate carried the same B5 M77 allele as the chloroquine resistant MOA strain. Interestingly 3 Kenyan (3324, 5420, 12480) isolates exhibited a high proportion of shared alleles.

Table 1: Field isolate overview: Isolate numbers and country of origin

Isolate No.	Country of origin
5259	Kongo
12295	Kamerun
5420	Kenia
12480	Kenia
5798	Togo
3256	Togo, Ghana
3324	Kenia
6022	Gambia
6210	Sudan
MOA C3	Gabun

H_e comparison of microsatellites and *var2csa*

The expected heterozygosity (He) of *var2csa* in the field isolates was calculated for two fragments, a highly conserved (bp 3402-3558) (156 bp) (fragment I) as well as a variable region (bp 2664-2805) (141 bp) (fragment II). Fragment I had the same length and sequence in all 10 isolates and the H_e was 0. Fragment II differed in length and sequence in all field isolates and the H_e of 0.86. Sequence analysis of this fragment revealed deletions, insertion as well as single base pair substitutions as the most common differences (Table S3). Dot plot comparison of the 57 MS H_es and the two *var2csa* fragments H_es showed that fragment II was as diverse as the majority of the MS whereas fragment I was highly conserved (Fig. 7).

Together the data indicated that some *var* gene sequences exhibit less genetic diversity than the non-coding genome.

Discussion

In *P. falciparum* chronic infections are mediated by alternate expression of variant surface antigens (VSA). The most important VSA is PfEMP1, which is encoded by the *var* gene family, but recent evidence also supports a role for non-PfEMP1 VSA such as the RIFIN and STEVOR families in chronic infections [19] . PfEMP1 is under constant pressure by the immune system and there is evidence that frequent *var* gene recombination creates a seemingly endless diversity of PfEMP1 proteins [13–15,23].

In this work, we identify a conserved 3D7 *var* gene PF3D7_0617400 (previously annotated as MAL6P1.252 /PFF0845c) in 2 field isolates from Gabon in Central- and Togo in West Africa. Sequence identity between the field isolates and the 3D7 reference gene was almost complete except for a small insertion of approximately 200 bp in both field isolates. NF54 parasites transcribing PF3D7_0617400 bound efficiently to CD36 receptors on CHO cells, suggesting that the expressed PfEMP1 exhibits the promiscuous CD36 binding phenotype. This is in line with recent data by Hsieh et al showing that the recombinant CIDR of PF3D7_0617400 binds to the CD36 receptor via a hydrophobic pocket and that it belongs to the CIDRα2 family [17].

Although the hydrophobic pocket is present in virtually all CD36 binding CIDRα2-6, the sequence similarity in the hydrophobic pocket is generally very low, as is the overall sequence similarity across the global population of CIDRa2-6 [24]. This raises the question why in PF3D7_0617400 the CIDR as well as the remainder of exon 1 are almost completely identical among the field isolates and the 3D7 reference gene. Metwally et al [25] recently conducted a comprehensive cytoadhesion analysis of the 3D7 laboratory strains on CHO-745 cells and on CHO-745 cells expressing recombinant CD36, ICAM 1, P-selectin, E-selectin, CD9 and CD151 [25]. 3D7 parasites showed strong up regulation of

PF3D7_0617400 transcription after selection on all cell types. The strongest up regulation was seen after binding selection on CHO wild type cells with PF3D7_0617400 being the only significantly upregulated *var* gene. Together this data suggest that PF3D7_0617400 is able to bind to a yet unidentified receptor on CHO cells as well as to CD36, ICAM, P-selectin, E-selectin, CD9 and CD15. Synergistic binding to multiple receptors has [26] recently been shown to confer more efficient binding of infective erythrocytes providing a potential explanation why PF3D7_0617400 might confer a selective advantage.

The field isolate clone MOA C3 was originally isolated from an individual with a chronic submicroscopic infection [18,19]. This field isolate also expressed PF3D7_0617400 yet it was not possible to increase the low binding capacity of the strain to CHO cells expressing recombinant CD36. Consistent with this, surface reactivity against sera with anti-CD36 binding PfEMP1 reactivity [19] showed a higher surface reactivity of the NF54A 4 strain compared with the MOA C3 strain, despite the detection of the corresponding PfEMP1 by IFA in both cell lines. Together the data suggests a difference in PfEMP1 display between the two cell lines. This provides a possible explanation for the recent observation that VSA on MOA parasites consist primarily of non-PfEMP1 VSAs [19]. One limitation of the IFA experiments is that they were conducted at a later time point then the CD36 selection and MFI measurements and thus a proportion of the cells may have already switched to an alternate *var* locus. However prolonged tissue culture and switching to an alternate *var* locus did not result in a decrease in surface reactivity of the MOA C3 clone [19]. Thus together the data support a difference of PfEMP1 presentation between NF54 parasites and the recently culture adapted MOA parasites from a chronic asymptomatic infection.

The detection of 2 virtually identical PF3D7_0617400 alleles in two field isolates from two different African countries suggested the possibility of purifying selection that may limit the genetic diversity of this particular *var* locus. As an estimate of the genetic diversity of the non-coding genome of *P. falciparum* 57 MS were characterized by fragment length analysis. The ability of this method to type the respective chromosomal areas has recently been

compared with PacBio whole genome sequencing on two sibling parasites of the NF54 laboratory strain (Bruske et al in preparation)[21] [27,28]. At 53 MS positions typing results were identical between the two methods. 4 MS (ebp, hrp2, Ta 40 and C12M30) were not able to distinguish between 3D7 and non-3D7 alleles in chromosomal areas that were characterized as non-3D7 by whole genome sequencing, suggesting that in sibling parasites these markers are not diverse enough. The 10 field isolates investigated in this work originated from 8 different African countries and no subcloning experiments were performed prior to the MS analysis. In this population the 4 markers exhibited high H_e (ebp: 0.75, hrp2: 0.8, Ta 40: 0.94 and C12M20/30: 0.82) suggesting that they are able to detect allele differences in a consistent manner and can be used for future investigations in field isolates. The average H_e=0.8 for all 57 MS alleles was high and in the range of previously reported values for MS (0. 76-0.8) [29], reflecting the high genetic diversity of the non-coding *P. falciparum* genome sequence. Two isolates originated from individuals of the same family who had developed malaria after returning from Kenya. Interestingly these two isolates had identical alleles in 30% of the MS markers suggesting that the local parasite populations may exhibit significant genetic conservation. Bruske et al in prep have recently shown that in NF54 sibling parasites approximately 57 *var* genes were inherited without alterations in their respective chromosomal context. If this is equally true for other *P. falciparum* strains a significant degree of *var* gene conservation between closely genetically related isolates would be expected. Further characterisation of the *var* gene family in these Kenyan isolates could address the question if the *var* gene repertoire is more conserved in local subpopulations as has been recently suggested by others [30,31].

Because PF3D7_0617400 was not detected in all field isolates, the conserved *var2csa* gene was characterized in order to quantify genetic diversity of a *var* gene in the investigated population of field isolates. The VAR2CSA protein consists of DBL 1-6 and is predicted to have a globular conformation [32]. The DBL3 has been shown to exhibit CSA binding activity and is composed of conserved and highly variable sequence blocks [33–35]. Previous analyses of conserved regions have focused on comparing diversity among *var2csa* from

different isolates. In this work the H_e of conserved and non-conserved *var2csa* areas were determined and compared with the H_e of the 57 MS. This revealed that the H_e (0.86) of the variable sequence block corresponded to the average H_e (0.8) observed for the MS genetic diversity. By contrast the H_e (0) of the conserved *var2csa* stretch showed complete conservation across the entire parasite population. Together these data clearly support previous evidence that some parts of *var2csa* are under purifying selection [33], leading to high conservation, whereas others are as diverse as the noncoding part of the *P. falciparum* genome [34,35].

In the 3D7 parasite line, PF3D7_0617400 is located in the central area of chromosome 6 and belongs to the UpsC subclass of *var* genes and this promoter type was conserved in both field isolates. UpsC *var* genes have been shown to be preferentially transcribed during long term *in vitro* culture [18,21] and during chronic asymptomatic infections [36,37]. Recombination has therefore been postulated to be of critical importance for group C *var* genes [38] and for chronic infections in general [23]. Several investigations have recently shown that *var* gene expression is significantly lower during chronic infections [39] and that VSA expression in tissue adapted parasites from chronic infections shows preferred expression of non-PfEMP1 VSAs [19,40]. It is therefore tempting to speculate that expression of a PfEMP1 variant that is able to bind to multiple receptors may provide a selective advantage during chronic infections. Given that only 2 of the 10 isolates investigated in this work carried PF3D7_0617400 it would be expected that other conserved Ups C *var* genes also bind to multiple receptors. Large population based *var* gene investigations are necessary to investigate if there are other Ups C PfEMP1 variants with the same degree of conservation as PF3D7_0617400.

Despite the seemingly limitless diversity of the *var* gene family, strain-transcending immunity against PfEMP1 develops in endemic populations [41] and parasitemia levels in adult residents of endemic areas are often submicroscopic [19], clearly showing that the immune system exerts diversifying selection onto variant surface antigen families. The data reported

here suggests that individual PfEMP1 binding phenotypes may exert purifying selection on individual members of the *var* gene family.

Material and Methods

Parasite lines and cultures:

Parasite stocks in glycerolite of the NF54 A3 [21] laboratory strain, the culture adapted Gabonese MOA C3 [18] and Δ MOA D2 [19] field isolates were thawed by slowly adding 5 drops of 12% NaCl solution and 5ml of 1.6% NaCl solution. The stocks were then spun down, the supernatant was discarded and the parasites were used to inoculate an *in vitro* culture.

Freshly culture adapted field isolates were generated from diagnostic specimens submitted for routine malaria diagnosis to the laboratory of the outpatient clinic of the Institute of Tropical Medicine in Tuebingen, Germany. After conducting routine thick and thin blood smears, the remainder of the EDTA blood tube was centrifuged, the serum was separated and 500µl erythrocyte pellet was used to inoculate 5 ml culture. After successful culture adaptation, parasites were expanded into a 20 ml culture and cryopreserved stocks as well as cell pellets were stored for future investigations.

All *P. falciparum* isolates were cultivated at 5% hematocrit of 0^+ erythrocytes from a local cell bank. RPMI 1640 medium was completed with 10% Albumax (Gibco), 25mM Hepes Buffer, 2mM L-Glutamine and 0.05mg/ml gentamcin (all PAA Laboratories). Parasites were incubated at 37°C in 90% nitrogen, 5% oxygen and 5% carbon dioxide.

DNA extraction:

Cell pellets for DNA extraction were stored at -20°C. After thawing of the pellet, DNA was extracted using the QIAmp® DNA Blood Midi Kit (Quiagen, Cat. No. 51185) following the manufacturer's protocol. DNA content was measured by Nanodrop® 1000 3.7.1 (Nanodrop Technologies).

PCR and agarose gel electrophoresis:

For qualitative PF3D7_0617400 PCR, we designed a specific primer set, spanning exon 1 from promoter until the intron (S1 Table). The promoter region of the PF3D7_0627400 was characterized with Ups C primers designed by Rottmann et al [36] and evaluated as described in Enderes et al. Overlapping fragments beginning at the promoter spanning for the entire exon 1 were generated. PCR with the *var2csa* specific primers 10F and 75 R [5]) was employed to amplify an approximately 1700bp fragment of *var2csa*.

5µl genomic DNA were mixed with 31 µl H2O, 0.4µl dNTPs, 3µl MgCl, 5µl buffer, 2.5µl forward primer, 2.5µl reverse primer, and 0.3µl Taq-polymerase to a final volume of 50µl per reaction. Standard PCR conditions were 94°C for 3min, 40x (94°C for 10sec., 54°C for 30 sec., 72°C for 30sec.), 72°C for 3min. Annealing and elongation temperatures were adjusted according to primer melting temperature and fragment length (P3, P5, P6). Individual primer specific PCR conditions can be obtained from the author upon request.

PCR fragments were separated using gel electrophoresis with a 1% agarose gel.

Sorbitol synchronization, RNA extraction and cDNA synthesis:

20 ml parasite cultures were used for RNA extraction. The culture was pelleted, washed several times with 1x PBS and the erythrocytes were lysed with 0.02% saponine. The pellet was then washed 3 times with 1x PBS and resolved in 750µl of Trizol® LS Reagent (Invitrogen). The samples were stored at -20°C until further processing.

For RNA extraction, 0.2 ml chloroform were added to 750µl of thawed Trizol-lysate, shaken vigorously for 15sec and left at room temperature for 10min. A 15min centrifugation step at 12,000g at room temperature followed for phase separation. RNA was extracted from the aqueous phase with the PureLink™ RNA Mini Kit (ambion by Life Technologies) according to the manufacturer's protocol. RNA content was measured by Nanodrop® 1000 3.7.1 (Nanodrop Technologies). All samples were treated with DNAse I® (Invitrogen) according to the manufacturer´s protocol to remove any remaining DNA. cDNA was synthesized with

random primers and Superscript II Reverse Transcriptase® (Invitrogen) according to the manufacturer´s protocol. cDNA was tested for absence of DNA contamination by evaluation of proper splicing of the gene PFD1155w by PCR with the primer 5’ GCAGGGAAAGGTTTTTCAAG 3’ and the reverse primer 5’ AAAGCTGAATCTTGGCCCGTT 3’ as described elsewhere [21].

Quantitative real time PCR:

For quantitative RT-PCR reactions of the NF54 A3 strain, we employed the primer set of Salanti et al [5] with the modifications described in Frank 2006, Dzikowski 2006 and Enderes 2011[10,18,27]. For quantitative RT-PCR of the MOA field isolate the MOA primers C3_C3, D2_D2 and D5_D5 were used [18]. All reactions included five housekeeping genes as controls: seryl-tRNA synthetase (PF3D7_0717700), fructose biphsphate aldolase (PF3D7_1444800), actin (PF3D7_1246200), arginyl-tRNA synthetase (PF3D7_1218600) and glutaminyl-tRNA synthetase (PF3D7_1331700)[27]. Reactions were performed at a final primer concentration of 0.25µM using SensiMix SYBR No-ROX Kit (Bioline, QT650-05) in 20µl reactions, measured in Corbett Research Rotorgene 3000 (95°C for 3min/95°C for 15sec, 54°C for 30sec, 68°C for 30sec, 40 cycles/68°C for 1min).

The same threshold was used for all analysis. *var* gene copy numbers were determined relatively to PF3D7_1218600, using the ΔΔCT method [5].

Sequencing:

For PCR fragment-sequencing, the PCR product was purified from an agarose gel or the PCR solution, using the Quiagen PCR purification kit according to the manufacturer’s protocol. Sequencing-PCR was performed at a final reaction volume of 10µl per reaction, containing 1µ BigDye, 2 µl reaction-buffer, 2.5µl forward or reverse primer, purified water and purified PCR product according to DNA concentration. Sequencing PCR conditions were: 94°C for 10 sec, 50°C for 5 sec, 60°C for 4 min, back cycle to beginning : 25 x. After PCR, all

samples were cleaned up by sephadex agarose column centrifugation. Sanger sequencing was performed in the Applied Biosystems ABI Prism 3130xl Genetic Analyzer.

Fragment analysis:

57 microsatellites distributed over the 14 chromosomes of *P. falciparum* were analyzed by multiplex fragment analysis (Table S3). DNA of all freshly culture adapted field isolates, the MOA C3 strain as well as the NF54 A3 were analyzed. Microsatellite PCR was carried out as described in Bruske et al in preparation. PCR products were all diluted 1:200 with water. A master mix was prepared with 5µl H2O and 5µl Formamide (Hi-Di Life Technologies) and 0,1µl Standard (LIZ 500 Life Technologies). Per singleplex reaction 10µl master mix and 1µl diluted PCR product were used. For multiplex fragment analysis 10µl master mix and 1µl diluted PCR product of each PCR reaction were used. The mix was heated at 95°C for 3 min and immediately chilled on ice for a few minutes before fragment analysis. PCR fragment size was determined by capillary gel electrophoresis in 96-well plates (Biozym Scientific GmbH) with the Applied Biosystems ABI Prism 3130xl Genetic Analyzer and data were evaluated using GeneMapper v 4.1.Allele analysis of the 57 MS across the 10 field isolates revealed the presence of a single reaction product for the majority of PCR microsatellite reactions. If more than 1 amplification product/peak was observed the highest peak was arbitrarily chosen as the representative allele for the respective marker.

Calculation of expected heterozygosity:

The expected heterozygosity (H_e) was calculated using the formula H_e= [n/(n-1)][1-Σpi2], where n is the total number of alleles at a distinct locus and reflects the proportion of the individual alleles as described previously i.e. by Anderson et al [29]. A new allele was defined as in Bruske et al. in preparation as a difference of > 3 bp between PCR fragments. The same criterion was applied for allele definition of the *var2csa* fragments used for the H_e calculation of the *var2csa* gene.

Assembling of sequences:

Sequences were assembled and aligned using SeqScape software by Applied Biosystems.

Comparing sequences:

Sequences were compared using the ACT software by artemis [42]

Antibody preparation and Immunofluorescence assay (IFA) :

Antibodies against the CIDR α 2.1 of PF3D7_0617400 were generated at the Statens Seruminstitut, Cohenhagen, Denmark (TL). Recombinant CIDR α 2.1 domain of PF3D7_0617400/MAL6P1.252 was produced as previously described [17]. Mice were immunized with the recombinant CIDR α 2.1 domain and blood of immunized mice was sent to the Institute of Tropical Medicine, Tuebingen, Germany. Mouse sera were depleted from RBCs by filtering through a MN 615 ¼ filter. IgG was purified using Protein G Spin Columns from Thermo Scientific according to the manufacturer's protocol.

For IFA, very thin blood smears of parasite cultures were fixated in -20°C cold 100% methanol for 5min and then stored at -20°C until further use. IFA was performed following a modified protocol of Bachmann et al 2012 [43].

After a 5 min rehydration step in 1xPBS, slides were incubated for 1-2h with mouse anti CIDR α 2.1 PF3D7_0617400 (diluted 1:50 in 1xPBS/1%BSA). The slides where then washed 3x with 1xPBS and incubated for 1h with Alexa488 coupled mouse sera IgG α PF3D7_0617400 mouse secondary antibody (not diluted, 0,44mg/ml). After another 3x washing with 1xPBS, slides were stained with Hoechst 33342 (diluted 1:1000) for 30min. Slides were mounted over night with MOWIOL-488 and viewed through 100x oil immersion lens at a fluorescent microscope.

CD36 receptor binding selection

Human melanoma C32 (ATCC1, CRL-1585™) cells were used to select CD36 binding infected red blood cells (iRBCs) as described in Bruske et al. 2016. [19]

Flow cytometry analysis

Flow cytometry analysis with serum of the MOA individual was performed as described in Bruske et al. 2016 [19].

Figure Legends:

Fig1: Screenshot of ACT program comparing the assembled sequence of PF3D7_0617400 of the field isolate clone MOA C3 with the reference strain 3D7. The upper part of the figure depicts the 3D7 reference sequence. The lower part shows the MOA C3 sequence. Blue and yellow = sequence identical. White: missing sequence (insertion). For orientation the sequence next to the insertion that differs between MOAC3 and 3D7 is coloured in yellow. The additional small white areas represent homopolymer runs. Visual examination of direct alignment of the two exon 1 sequences revealed no differences in these areas (data not shown).

Fig2: a) Gel electrophoresis after PCR with 6 specific PF3D7_0617400 primers on MOA C3 cDNA reveals transcription of the entire exon 1 sequence. b) qRT-PCR with P_C3 and two other MOA DBLs (encoding for the dominant *var* genes in the other clones generated in the same limiting dilution experiment) [18] shows that P-C3 is transcribed as the dominant *var* gene in MOA-C3. c) qRT-PCR with NF54 specific primer set on cDNA of NF54 A3 [21] shows continued dominant transcription of PF3D7_0617400. Transcription is quantified in relative copy numbers of the housekeeping gene arginyl tRNA sythetase in MOA C3 and NFA3.

Fig3: CD36 binding capacity of a) NF45 A3 before CD36 selection and after one to three rounds of selection and b) MOA C3 before CD36 selection as well as after one to four rounds of selection. c) Mean fluorescence intensity (MFI) of FACS analysis with semi-immune sera from the MOA individual [19] on NF54 A3 and MOA C3 after

CD36 selection shows a strong signal on NF45 A3 parasites and a weak signal on MOA C3 parasites

Fig4: Immunofluorescence with a specific antibody against the CIDR of PF3D7_0617400 shows expression in MOA C3 and NF54 A3 but not in the field isolate Δ MOA D2: A) MOA C3, B) NF54 A3, C) Δ MOA D2 (propagated without blasticidin pressure). The first images displays light microscopy, the second image identifies parasitized RBCs by Hoechst DNA staining (blue) and the third image displays IFA with mouse anti-CIDR-PF3D7_0617400 antibody and GFP. Note the anti-CIDR antibody signal in MOA C3 and NF54 A3. The D2 DBL expressed in Δ MOA D2 [19] is not detected by the antibody.

Fig5: a) Gel electrophoresis of PCR fragments of PF3D7_0617400 on all field isolates and controls. PCR fragments of PF3D7_0617400 (primer pair P0F + P0.1R) amplified on two field isolated (5798, MOA C3) and on the NF54 clones 3D7 and E5 (positive controls). NC = negative control. The gel was digitally rearranged to match the gel shown in Fig.5c. b) ACT program comparative analysis of exon 1 of PF3D7_0617400 from the field isolate 5798 and the 3D7 reference genome. The colouring scheme is as in Figure 1. C) Gel electrophoresis of PCR fragments of *var2csa* on all field isolates and controls. PCR fragments of *var2csa* amplified by one primer pair (10F and 75R, [5]) on all field isolates and on the NF54 clones 3D7 and E5 (positive controls). NC = negative control.

Fig 6: Microsatellite position and MS genetic diversity a): 14 chromosomes of *P. falciparum* with positions of *var* genes indicated in red and positions of microsatellites indicated in green. Microsatellites analysis was performed as described in Bruske et al. in prep. b): observed H_e of microsatellite loci on all 14 chromosomes of *P. falciparum* across all field isolates. 0=no diversity, 1= max. diversity.

Fig 7: Scatter plot of expected heterozygosity H_e of MS, *var2csa*, PF3D7_0617400 Scatter plot showing all H_e of the 57 microsatellites versus the two H_e of fragment (I) and (II) of *var2csa.* Note that the H_e of the *var2csa* fragment I is 0 while the H_e of fragment II (diamond) is as 0,8 and in the same range as the H_e of the MS markers.

Ethics approval

Ethical approval for characterization of the long-term chronic infection of MOA was obtained from the local ethics committee in Lambarene as previously described [18,19]. Field isolates obtained in Tuebingen were generated in the process of routine laboratory diagnosis and thus didn't require individual ethical approval.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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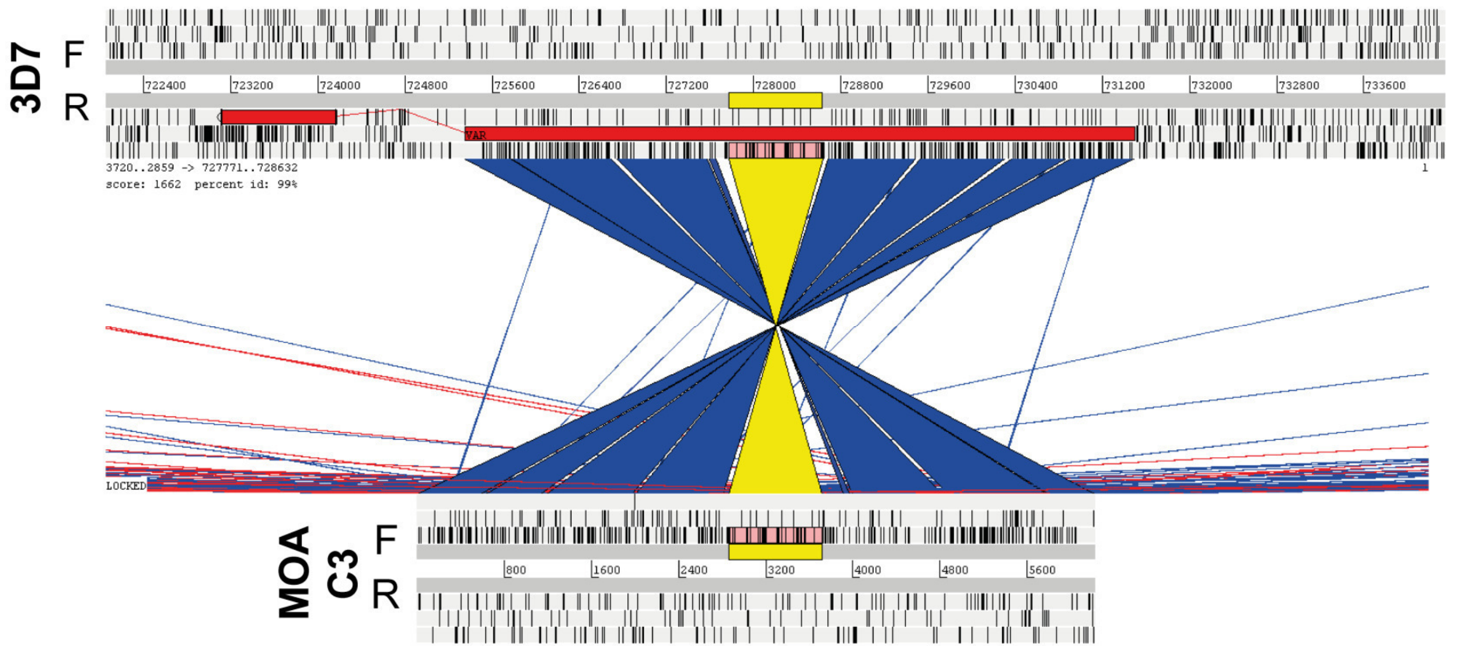


Fig 1

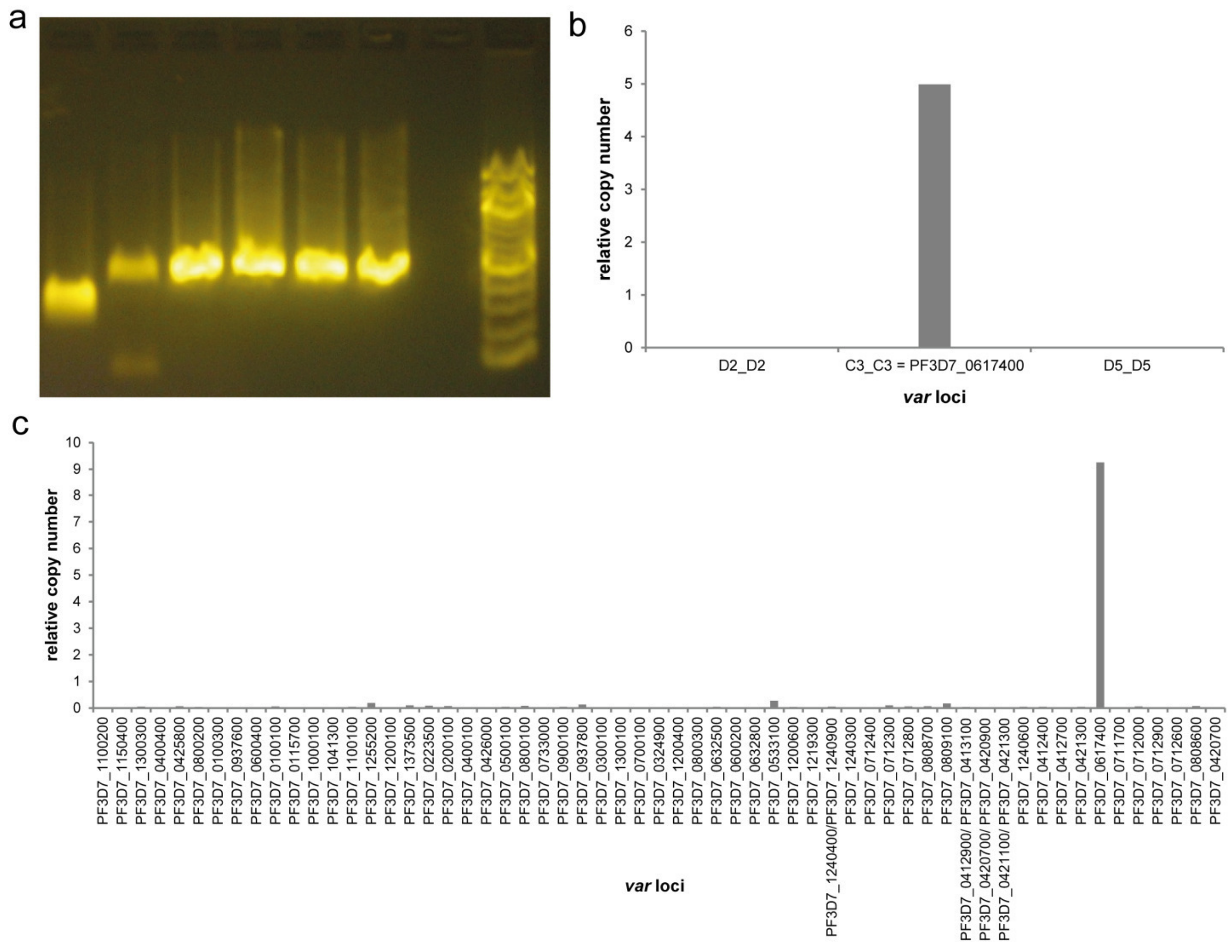


Fig 2

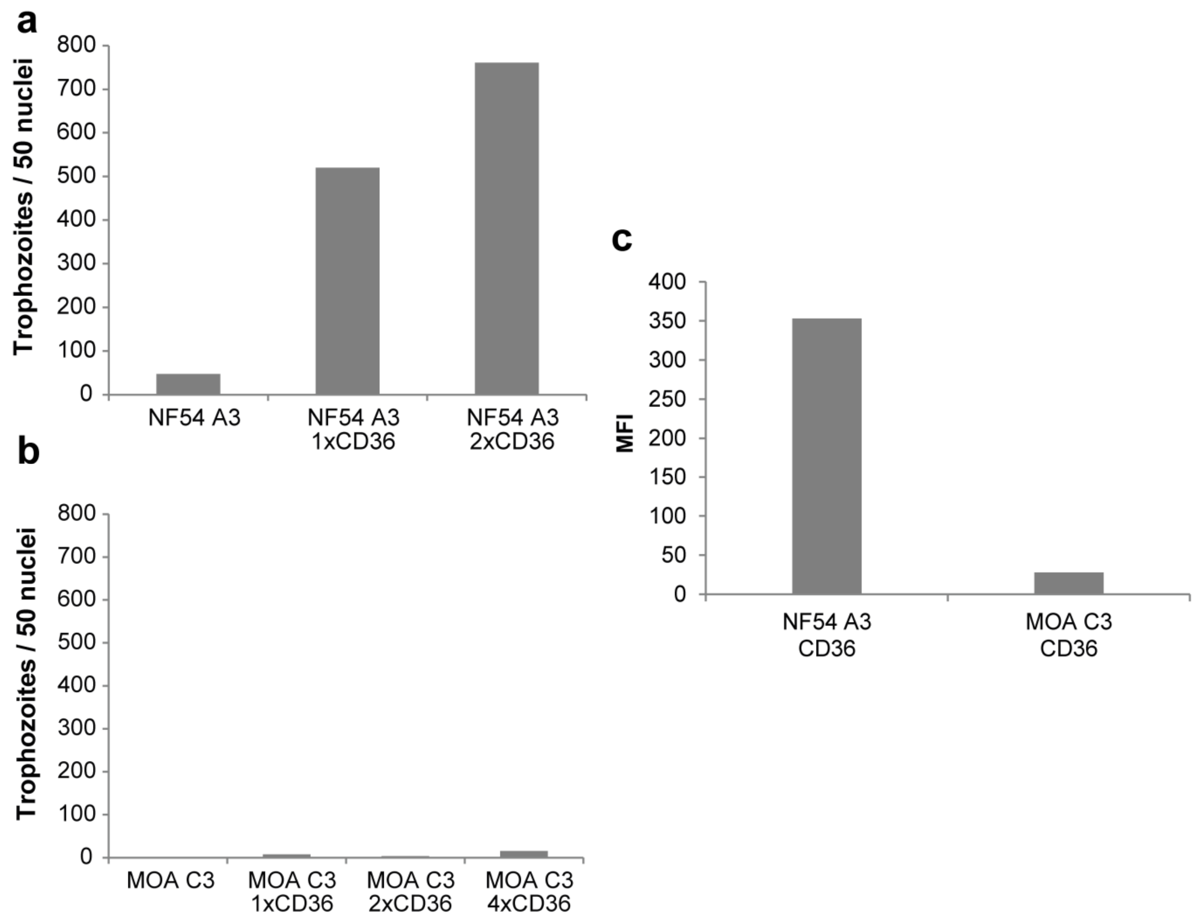


Fig. 3

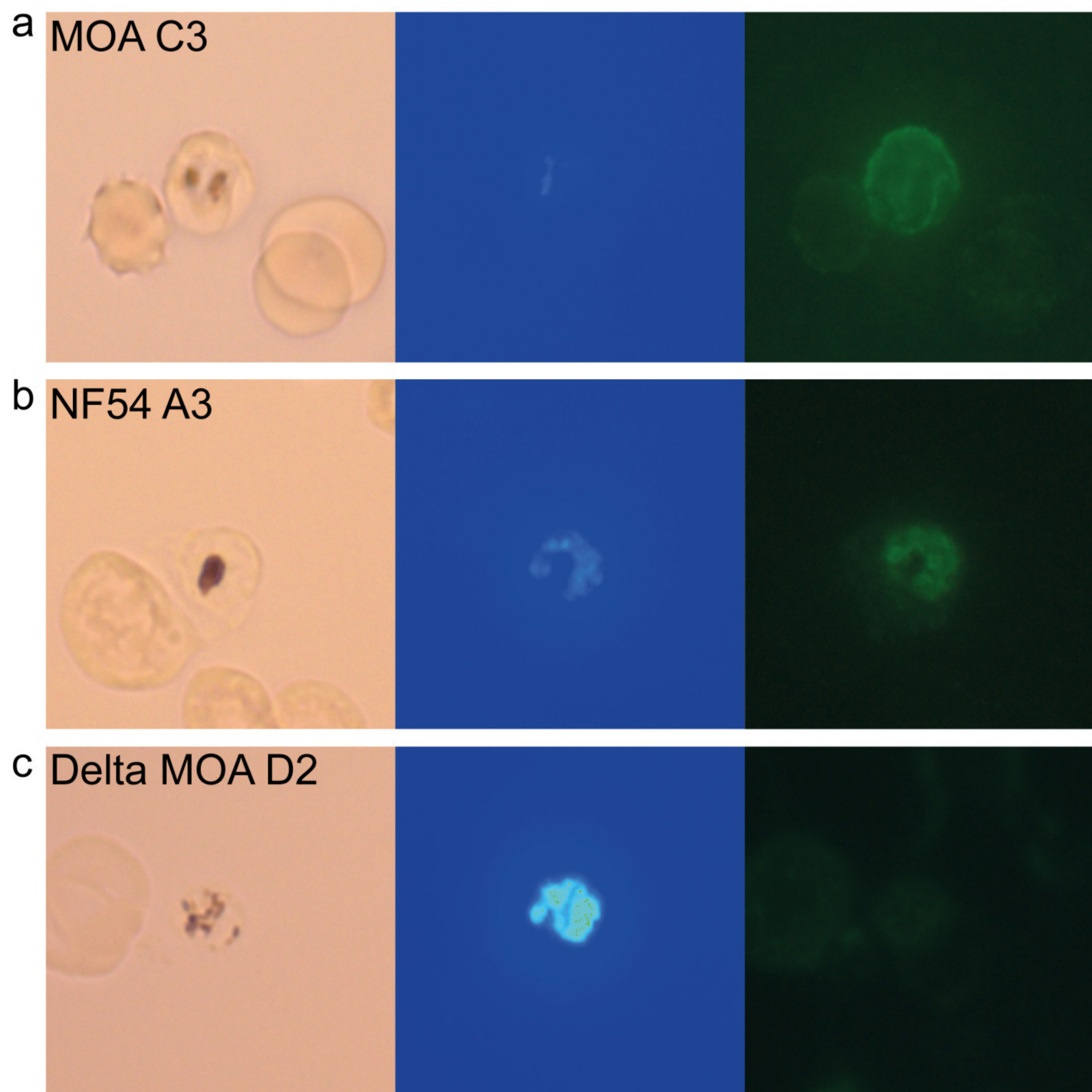


Fig. 4

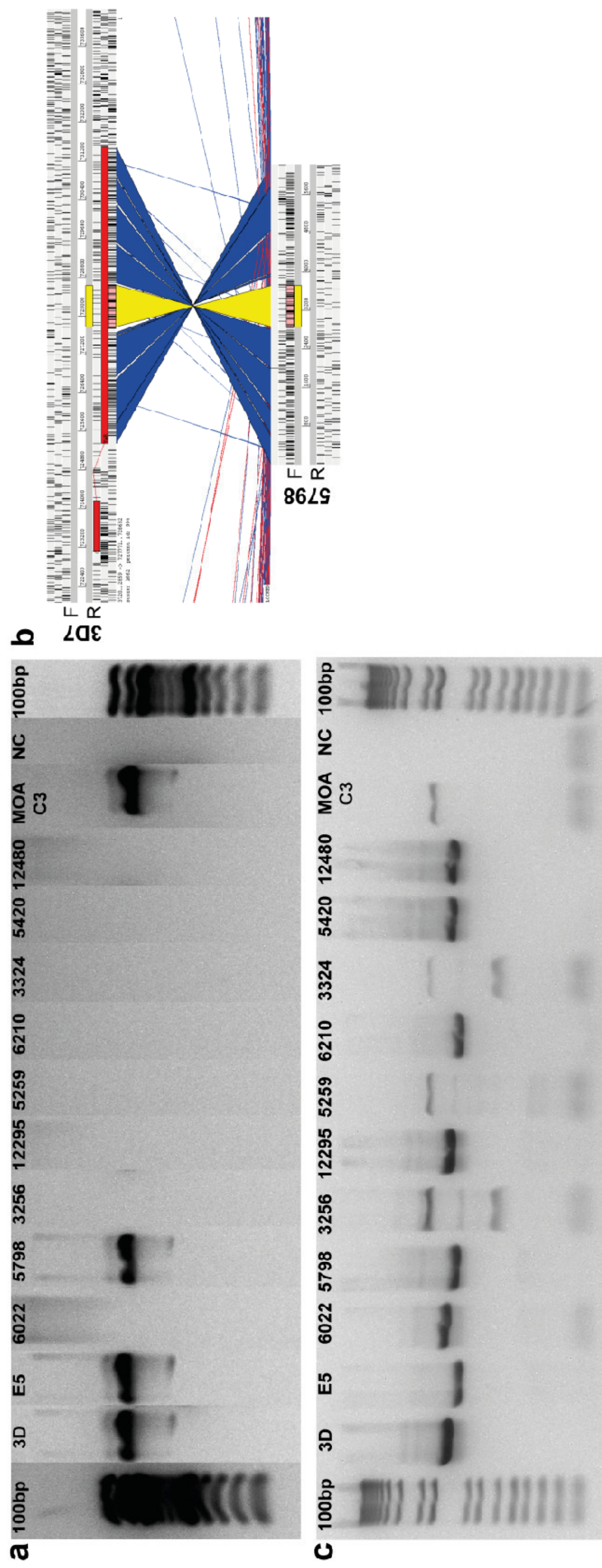


Fig. 5

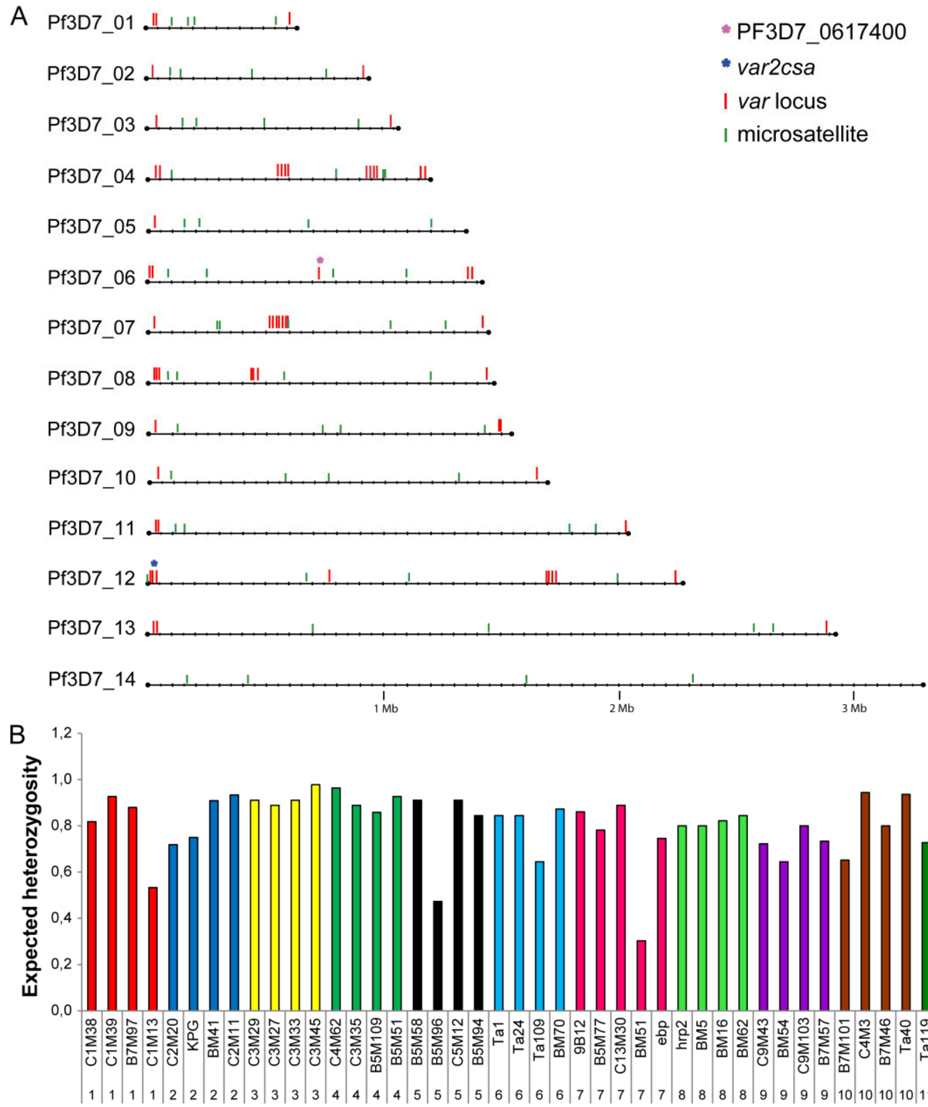


Fig. 6

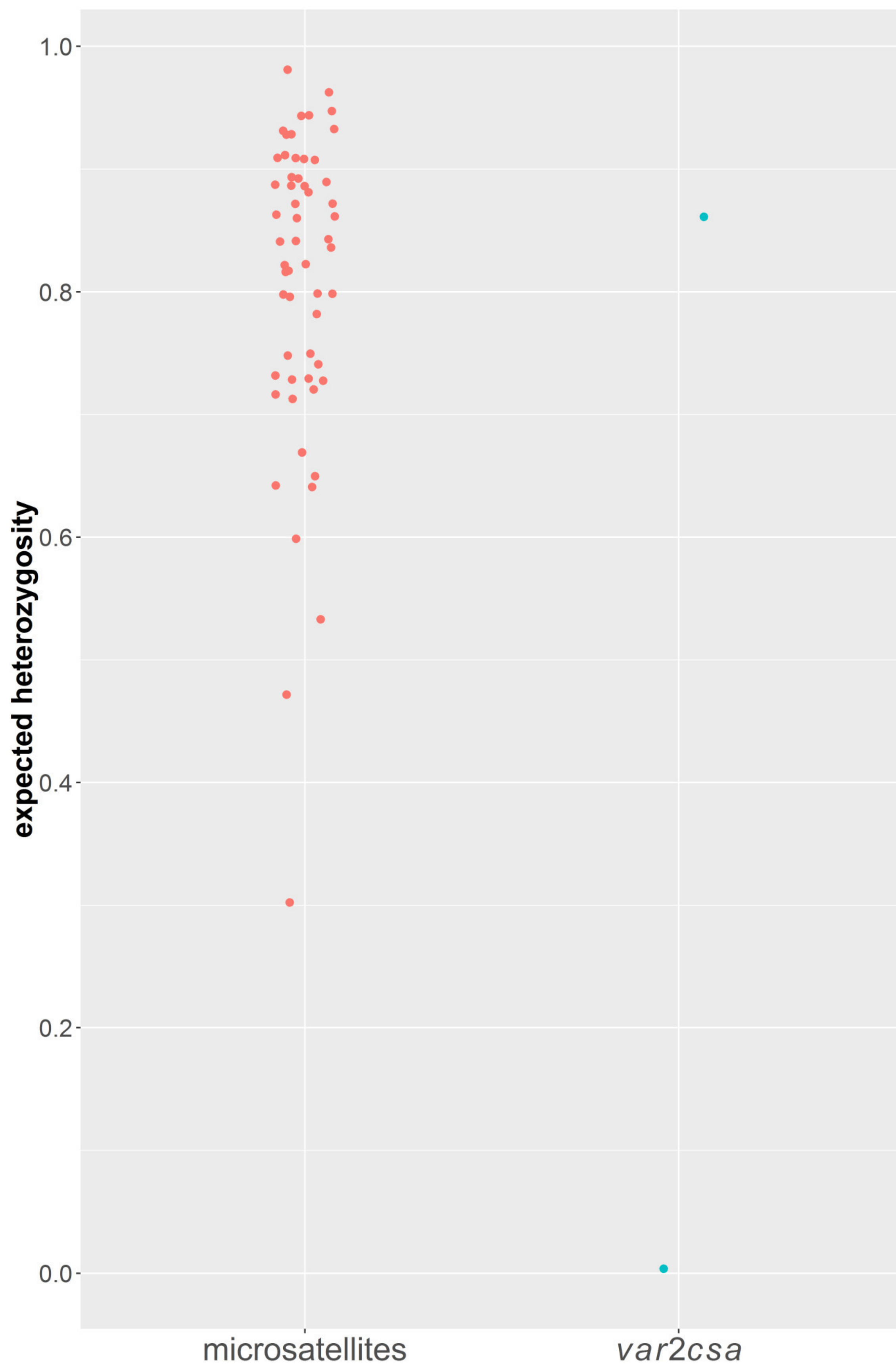


Fig. 7

Figure S1: insertion between basepairs (bp) 3722 and 3871 of the MOA C3 gene (3721 and 3914 of the 3D7 reference sequence).

ATAAGTGGTGACAAAATAGCCACAGATAGTGAAGCCAAAGGTCGTCAACGACGTGAAGC
ACCTGGTGAAGCCCCTAGTGGTGCCGTTACAGCCACTAGTGATAAGGACGGCGCCATA
TGTGTACCATCGCGTAGGAGAAAACTATACCTACACAAAGTAGGCGACGGCGAGGACA
TCACCGACGACAAGTCATTACGTGATTGGTTTGTGAAGTCGGCCGCCGTC

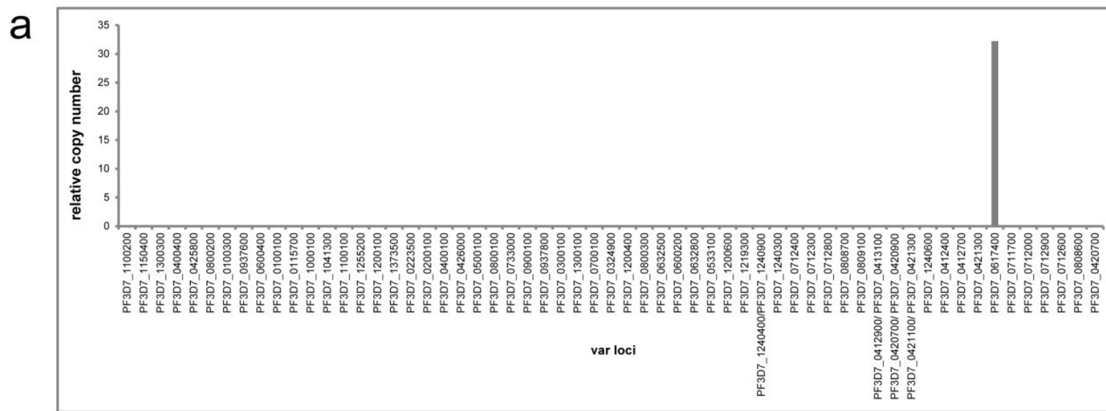
Primer	Forward 5'3'	position bp (start)	Reverse 5'3'	position bp (end)
UPS C1 [1]	CACATCGATTACATTTTAGCGTTT		TGTGGTAATATCATGTAATGG	
P0	TGCCAAGGACCTTTTGGAA	41	GGTAATATTAATAACACAGAT	419
P39 [2]	ATTTGTCGCACATGAAGGAA	376	TACAGCATTGGC	524,0
Primer 0.0	TCTGTTTCTTGGTTATACC	566	GGTGATTCTTCAGATT	703,0
P0.1	CAAGATCCTGCAAAATCTCA	660	CTTTAGAGAAATGTCGTGGA	975
P1	GATGAATGGAGCGAAGAA	903	AAAAATTCTAGTTTTTCGTTA	1130
Primer 1.1	TAAAAGAAAGAGAACCTAC	1159	TCGAAACATTTTCTCATAC	1376
P2	GCGAACCTTGCCCTTGGTGT	1384	CATTCTATAGAATCATCTAACA	1733
P1.1.1	GCATATTAATCATGATATTTGG	1571	CGACGAAGAAGAAACGGATA	2197
Primer 2.1	GTATACGGGGTTGTAAAAAG	1780	GCTTATGGAATAGAAAAATC	1976
P3	CACGGAATTATAAGAGATAC	2013	CCATGACATGGTCCTTGCGGA	2441
P2.1.1	GAAACCCATAATGATGAAAA	2100	CAGATTTCATAAAAGAGAAG	2691
Primer 3.1	GAAACCTGGGAACCAGAT	2482	GATTGGTGGGAGGCAAACA	2953
P3.1.1	GACAGAACACTTAATGGTAT	2583	CAAGTTGTAAGAAAAAGGGT	3148
P4	GAGCTATGACGTGTGCAACA	2968	ACTTCGCATTCACCTACACG	3467
P4.1.1	CTCAAAGATTACGTTGGATG	3040	CCACGAAAAATTCCCCAATT	3712
Primer 4.1	CGAAGAAGCATGTGCTTGT	3536	GATTGCTTTGTGAAAAGTGC	3863
P5.1.1	GCACCAGAAAAGAAGAAAG	3570	CAAATGTTCTACACATTGG	4065
P5	TTGAGACGTTTTTCCTATGGCAT	3868	GTCGCCGCGCGATAGGGCGCCA	4242
P6.1.1	CAAAAGGAAAAGAAACCACA	3903	GTGTCACACTTAAAGAAGAT	4440
Primer 5.1	TGGTGGGAACAACATGGTAA	4262	GCGTGTGATCGAACAAATAC	4667
P7.1.1	GAATGATTTGCGCTTTAACC	4276	CAATTTTGCGGAACACCAG	4867
P6	GCTTCTGCTGATTTAGAAGGT	4680	GCCGTAATCTTTCCTGTTGTGCAA	5081
P8.1.1	CAAGAAAAGGCATATGGTG	4770	GTCGTAATGTATGTGGTTATA	5245
Primer 6.1	GAAGTTGTTATGCGTGTTAG	5112	TGGCTATGGAGAAAATTAC	5514
P7 F	CACATTGGGTAGATAATTTT	5320	CAATCCACAAGAAAAATGTGA	5758
P8F	CCAACTTTAGATGCTTTCG	5580	CAAAAGGCAACCGAAAAAAC	6040
P9F	CCAGAAATTTGTAAAGATGT	6020	GTTTCGTGTATATGTATGTG	6271

Table S1: Primer set for PFF0845c Exon1 with basepair positions

1. Rottmann M, Lavstsen T, Mugasa JP, Kaestli M, Jensen ATR, Müller D, et al. Differential expression of var gene groups is associated with morbidity caused by Plasmodium falciparum infection in Tanzanian children. Infect Immun. 2006;74:3904–11.

2. Salanti A, Staalsoe T, Lavstsen T, Jensen ATR, Sowa MPK, Arnot DE, et al. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. Mol Microbiol. 2003;49:179–91.

a



b

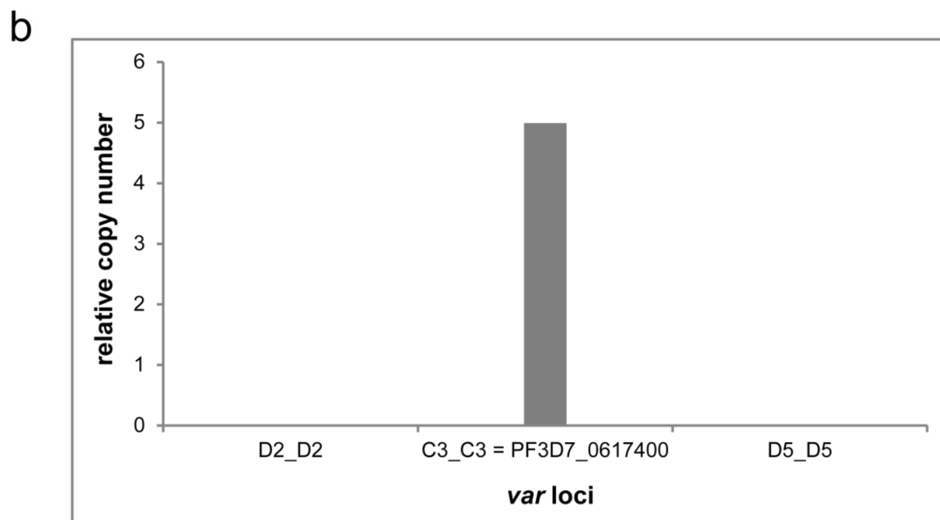


Fig. S2

Chromosome	MS	5798 Togo	6022 Gambia	3256 Togo/Ghana	12259 Cameroun	5259 Kongo	6210 Sudan	3324 Kenya	MOA Gabon	5420 Kenya	12480 Kenya	**He**
1	C1M38	158	173	163	165	165	162	162	162	171	171	**0,82**
1	C1M39	122	172	170	170	113	216 / 293	137	148	183	135	**0,93**
1	B7M97	200 / 194	200	190	185	218	193	191	185	196 / 193	196	**0,88**
1	C1M13	136	134	130	134	130	130	136	128	128	128	**0,53**
2	C2M20	123	137	137	119	121	141	135	123	121	121	**0,72**
2	KPG	163	167	151	no peak	167	no peak	163	167	165	149	**0,75**
2	BM41	155 / 161	151	157	177	154	171	146	154	173	157	**0,91**
2	C2M11	133	123	99	129	131	129	139	116	110	121	**0,93**
3	C3M29	184	185	157	174	165	160	170	181	173	184	**0,91**
3	C3M27	139	158	164	148	166	145	154	148	141	150	**0,89**
3	C3M33	115	134	137	115	101	119	111	117	95	95	**0,91**
3	C3M45	137	156	85	no peak	150 /167	114	177	144	144	131	**0,98**
4	C4M62	241 / 226	190	149	253	241	221	236	224	223	257	**0,96**
4	C3M35	207	186	191	194	183	200	188	206	191	191	**0,89**
4	B5M109	176 / 121	176	159	139	123	176	176	120	134 /159	159/ 139	**0,86**
4	B5M51	221 / 243	237	214	224	249	224	220	214	227	227	**0,93**
5	B5M58	191	194	193	202	163	204	167	202	209	205	**0,91**
5	B5M96	172 / 177	168	177	173	177	177	178	177	177	177	**0,47**
5	C5M12	151/ 170	157	177	178	179	161	162	168	171	151	**0,91**
5	B5M94	142	138	138	149	179	147	147	157	142	142	**0,84**
6	Ta1	176	179	173	178	202	170	176	194	173	176	**0,84**
6	Ta24	187	193	194	198	203	198	211	187	187	198	**0,84**
6	Ta109	168	177	177	174	177	177	174	186	177	177	**0,64**
6	BM70	173	193	193	183 / 206	189	197	175	191	175	189	**0,87**
7	9B12	167 / 171	160	163	160	no DNA	165	169		165	158	**0,86**
7	B5M77	141	141	150	150	143	143	131	154	143	145 / 154	**0,78**
7	C13M30	187	203	193	193	202	197	201	207	223	185	**0,89**
7	BM51	140	140	140	140	139	140	140	140	134 /140	134 / 140	**0,30**
7	ebp	134	136	134	134	139	138	141	136	138	147 / 149	**0,75**
8	hrp2	167	167	178	182	175	189	173	173	175	169	**0,80**
8	BM5	134	136	124	133	138	158	138	136	136	139	**0,80**
8	BM16	147	157	153	147	142	142	149	144	157	144	**0,82**
8	BM62	166	170	164	162	158	168	164	168	158	160	**0,84**
9	C9M43	130 /138	126	124	128	128	130	132	134	132	128	**0,72**
9	BM54	165	158	164	167	167	164	163	185	164	164	**0,64**

9	C9M103	149	149	145	153	136	138	149	145	144 / 152	145	**0,80**
9	B7M57	219	227	220	220	219	223	229	223	223	229	**0,73**
10	B7M101	204	204	204	204	196	229	204 / 185	204	229	185/ 204	**0,65**
10	C4M3	173	178	150	171	129	163	190	181	no peak	183	**0,94**
10	B7M46	187	150	169	161	160	178	169	161	170	160	**0,80**
10	Ta40	212/ 125	191	203	215	218	231	216	215	191 / 218	209/ 222	**0,94**
11	Ta119	228 / 235	235	250	250	250	235	238	251	250	250/ 219	**0,73**
11	C12M110	157	no peak	157	no peak	129	157	no peak	no Peak	154	156	**0,60**
11	Resa2	101	99	91	101	102	96	113	105	97	105	**0,89**
11	Ta117	169 / 175	180	168	180	175	186	175	181	180	192 / 175	**0,74**
12	C12M30	114	176/ 123	209	176	no peak	210	115	no Peak	no peak	176	**0,82**
12	Ta48	270	298	313	256	270	256	262	276	290/ 303	276/ 313	**0,95**
12	Ta34	105	116	144	139	105	106	133	135	126	111/ 151	**0,93**
12	Ta121	155 / 167	152	157	158	167	161	155	158	158	161	**0,89**
13	C14M35	181 / 204	166	197	181	166 /163	166	166	189	180	174	**0,86**
13	ta60	191	202	202	199	202	190	193	194	199	190	**0,82**
13	C1M70	161	165	162	165	164	164	167	164	170	168	**0,73**
13	B8M6	125 / 134	128	122	119	122	119	131	122	128	119	**0,87**
14	C14M59	153	162	145	147	147 /155	162	147	160	151	151	**0,73**
14	RHO1	195	218	216	214	194	194	203	199	201	208	**0,89**
14	Ta88	217	222	216	216	217/ 220	216	229	213	220	217	**0,67**
14	Pf9607	111	114	114	111	117	111	111	114	114	117	**0,71**

Table S2: 57 microsatellites (MS) on 14 chromosomes of *P. falciparum* (vertical) and corresponding fragment lengths in base pair on the 10 field isolates (horizontal). No peak = no MS allele detected. Two fragment lengths = two MS alleles detected.

Frag (bp)	Ref (bp)	6022	5798	3256	12259	6210	12480	MoaC3	3D7
84	2664	G	G	G		G			A
114	2694	A	A		A		A	A	C
119	2699				A		A	A	T
120	2700				T			T	A
126	2706	T	T	A	A		A	A	C
128	2708	A	A	A	A		A	A	C
129	2709	T	T						G
135	2715					T		T	A
141	2721			G	A	G		G	T
147-155	2727-2735		del 9b						
153	2733							T	C
162	2742					Ins 9b	Ins 18b		
166	2746			C					A
169-171	2749-2751				del ACA				ACA
172	2752	A				A	A	A	G
177	2757	T		T	T	T	T	T	A
181	2761	G		G	G	G	G	G	A
185	2765					T	T	T	A
187	2767					C	C	C	G
189	2769			T					A
191	2771	A		A	A	A	A	A	C
193	2773			A				A	G
195	2775			G				A	T
197-199	2777-2779							CTC	AAT
206	2786	C	C	C	C	C		C	T
211+212	2791+2792	TC		TC	TC	TC		TC	CA
214	2794						A		T
217	2797				G				A
222-225	2802-2805				GCTT				TGCC

S3 Table: var2csa sequence differences in the variable fragment on the analysed field isolates. First column (frag): Position of base on the var2csa sequence of the field isolate. Second column (ref): Position of base on var2csa reference sequence NF54 3D7. Columns 3-10: Basepair sequenced for field isolates.