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Expressed *var* gene repertoire and variant surface antigen diversity in a shrinking *Plasmodium falciparum* population

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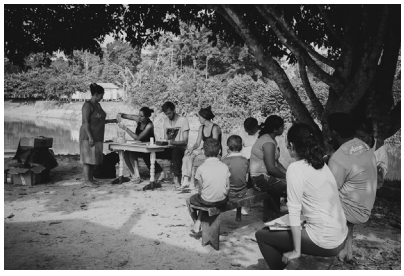
HIGHLIGHTS

- PfEMP-1 is the main variant surface antigen (VSA) expressed on infected erythrocytes.
- Parasites from the Amazon typically express a restricted PfEMP-1 repertoire.
- We hypothesized that Amazonians would rapidly acquire antibodies to local VSAs.
- Nevertheless, Amazonians displayed major gaps in their repertoire of anti-VSA antibodies.
- Variant-specific anti-VSA antibodies appear to develop slowly in low-endemicity settings.

GRAPHICAL ABSTRACT



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ABSTRACT

The *var* gene-encoded erythrocyte membrane protein-1 of *Plasmodium falciparum* (PfEMP-1) is the main variant surface antigen (VSA) expressed on infected erythrocytes. The rate at which antibody responses to VSA expressed by circulating parasites are acquired depends on the size of the local VSA repertoire and the frequency of exposure to new VSA. Because parasites from areas with declining malaria endemicity, such as the Amazon, typically express a restricted PfEMP-1 repertoire, we hypothesized that Amazonians would rapidly acquire antibodies to most locally circulating VSA. Consistent with our expectations, the analysis of 5878 sequence tags expressed by 10 local *P. falciparum* samples revealed little PfEMP-1 DBL1α domain diversity. Among the most commonly expressed DBL1α types, 45% were shared by two or more independent parasite lines. Nevertheless, Amazonians displayed major gaps in their repertoire of anti-VSA antibodies, although the breadth of anti-VSA antibody responses correlated positively with their cumulative exposure to malaria. We found little antibody cross-reactivity even when testing VSA from related parasites expressing the same dominant DBL1α types. We conclude that variant-specific

Abbreviations: bp, base pairs; CHO, Chinese hamster ovary; cDNA, complementary DNA; CIDR, cysteine-rich interdomain region; DBL1α, Duffy binding-like domain-α; DSID, distinct sequence identifiers; EPCR, endothelial protein C receptor; GST, glutathione S-transferase; KAHRP, knob-associated histidine-rich protein; MFI, geometric mean fluorescence intensity; PfEMP-1, erythrocyte membrane protein-1 of *Plasmodium falciparum*; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PoLV, positions of limited variability; RBC, red blood cells; TMB, tetramethylbenzidine; VSA, variant surface antigen.

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immunity to *P. falciparum* VSAs develops slowly despite the relatively restricted PfEMP-1 repertoire found in low-endemicity settings.

1. Introduction

Much is currently known about the development of clinical immunity to *Plasmodium falciparum* in holoendemic Africa. Infants usually experience their primary malaria attacks during the first year of life, while toddlers and juveniles have developed some tolerance against severe disease but still have a few clinical episodes. African adolescents and adults, in contrast, are clinically immune; they may present low-grade infections but typically remain free of malaria symptoms (Hviid, 2005; Doolan et al., 2009).

Whether clinical immunity develops under conditions of low malaria endemicity outside Africa has been a matter of debate. However, subclinical infections with very low parasite loads, most of them detected only by polymerase chain reaction (PCR), have been observed over the past decade in rural communities exposed to low-level *P. falciparum* transmission across the Amazon (Coura et al., 2006). Although both clinical and subclinical infections are experienced by all age groups, the proportion of infections that are asymptomatic increases with subjects' cumulative exposure to malaria, consistent with a gradual acquisition of clinical immunity (Alves et al., 2002; da Silva-Nunes et al., 2008; Ladeia-Andrade et al., 2009).

Antigenic variation in parasite's surface proteins, such as the erythrocyte membrane protein-1 of *Plasmodium falciparum* (PfEMP-1), is one of the main reasons why clinical immunity to malaria in Africa only develops after several repeated infections (Hviid, 2010). PfEMP-1 is the most frequently recognized protein among the variant surface antigens (VSA) exported to the surface of infected red blood cells as the parasite develops intracellularly (Chan et al., 2012). The parasite successively expresses antigenically diverse PfEMP-1 variants, in a mutually exclusive manner, to evade hosts' immune responses. A vast repertoire of PfEMP-1 variants, which are encoded by as many as 60 *var* genes present in each parasite's haploid genome, is generated by frequent recombination followed by immune-mediated selection in *P. falciparum* populations (Kirkman and Deitsch, 2014). Worldwide studies of PfEMP-1 diversity have mainly focused on its N-terminal Duffy binding-like domain-α (DBL1α), which is relatively conserved and mediates adhesion of parasitized red blood cells (RBC) to the vascular endothelium and uninfected RBC. These studies revealed that only 0.3% of the DBL1α sequences are shared by different isolates within the same area in Africa (Chen et al., 2011; Tessema et al., 2015), while Amazonian *P. falciparum* populations, in contrast, typically display a relatively restricted genomic repertoire of *var* genes, with 9.7% of the DBL1α sequences being shared among local isolates (Albrecht et al., 2006, 2010). The drastic decline of *P. falciparum* transmission in Brazil over the past two decades, leading to its near disappearance from most endemic sites (Ferreira and Castro, 2016), may have further limited *var* gene diversity in recent years.

Here we examined whether a limited repertoire of expressed *var* genes – as previously reported for Amazonian *P. falciparum* populations – translates into few antigenically diverse VSAs being exported to the surface of infected RBC. If so, we would expect that relatively few infections would be enough to elicit variant-specific antibodies that cover most of the VSA repertoire in local parasites. This would be consistent with the finding that clinical immunity to malaria in rural Amazonians may be detected after 5–8 years of exposure to relatively low levels of malaria transmission (Alves et al., 2002; da Silva-Nunes et al., 2008; Ladeia-Andrade et al., 2009). To test this hypothesis, we measured antibody responses of rural Amazonians to locally prevalent VSAs and found major gaps in the repertoire of variant-specific antibodies even in subjects with decades of malaria exposure. Nevertheless, allele sharing was commonly found in the DBL1α repertoire expressed by local *P. falciparum* isolates. We conclude that the repertoire of variant-specific antibodies to *P. falciparum* VSAs develops slowly despite the relatively limited variation of PfEMP-1 found in shrinking *P. falciparum* populations from areas with declining malaria endemicity such as the Amazon Basin.

2. Materials and methods

2.1. Parasite sampling sites

The *P. falciparum* isolates analyzed in this study (Table 1) were obtained between 2008 and 2009; 3 were collected in the farming settlement of Remansinho, in southern Amazonas state (9°40′S-9°43′S, 66°52′W-66°59′W), and one in the town of Acrelândia, in Acre state (9°43′S, 66°53′W). These sites are located 120 km apart in the Western Amazon Basin of Brazil, close to the border with Bolivia (Fig. 1), an area characterized by a humid equatorial climate and year-long malaria transmission. Ten sequential cross-sectional surveys revealed a sharp decline in overall malaria prevalence in Remansinho between March 2010 and October 2014; *P. falciparum,* which contributed about 10% of the local malaria burden between 2008 and 2009, has not been found in the area since April 2011 (Barbosa et al., 2014). Similarly, little residual malaria transmission remains in Acrelândia and surrounding rural areas (population, 16,613). Only 272 locally acquired infections (<10% of them due to *P. falciparum*) were confirmed by microscopy between 2009 and 2012 (Ministry of Health of Brazil, unpublished data). Since 2011, no *P. falciparum* infections have been diagnosed in Acrelândia, although *P. vivax* transmission persists.

2.2. Serosurvey

Plasma samples were collected in 2004 in the farming settlement of Granada (9°41′S-9°49′S, 67°05′W-67°07′W), situated in the eastern corner of Acre, 35 km northwest of the town of Acrelândia and 150 km southwest of Remansinho (Fig. 1). All households of Granada settlement were visited between March and April 2004, and 466 dwellers aged <1–90 years (98.5% of the 473 permanent residents in the area) were enrolled. An additional 43 individuals (mostly newcomers to the area) were enrolled between September and October 2004 (da Silva-Nunes et al., 2008). The 425 study participants aged five years or older were invited to contribute a 5-ml venous blood sample for plasma separation; of them, 306 subjects (72.0% of the eligible; age range, 5–90 years; median, 23 years) had samples available for antibody testing and constituted the population sample of the serosurvey. *P. falciparum* incidence rates in Granada ranged from 4.2 to 16.5 slide-confirmed infections/100 person-years at risk between 2004 and 2006 (da Silva et al., 2010), with no *P. falciparum* infections diagnosed in the area since 2010 (Ministry of Health of Brazil, unpublished data).

Table 1
Characteristics of the *Plasmodium falciparum* samples from the Brazilian Amazon and the 3D7 strain, of putative African origin, used in this study.

Parasite line	Site (year) of collection	Donor's exposure to malaria (years)	Number of cycles of *in vitro* culture	Binding to endothelial receptors		
				kahrp gene	CD36	ICAM-1
38A.1	Remansinho (2008)	31	0	ND[a]	ND	ND
38A			21	Present	+	+
$38A^{CD36}$			51	Present	++	+
$38A^{ICAM}$			66	Present	+	++
94A	Remansinho (2009)	23	61	Absent	−	−
FMS.1	Acrelândia (2008)	26	0	ND	ND	ND
FMS			19	Present	+	+
FMS^{CD36}			44	Present	++	+
Pf03.1	Remansinho (2008)	12	0	ND	ND	ND
Pf03			19	Present	++	++
3D7	Netherlands (1979) (putative African origin)	0	12[b]	Present	ND	ND

[a] ND = not determined.
[b] This is the number of culture cycles in our laboratory. The total number of *in vitro* cycles of this parasite line before it arrived to our laboratory is unknown.

Because most (62.2%) subjects living in Granada were migrants from malaria-free areas, their ages do not necessarily correlate with exposure to malaria. Cumulative exposure to malaria was therefore estimated as the length (in years) of residence in malaria-endemic areas, either in Acre or elsewhere in Amazonia.

2.3. *In vitro culture and characterization of Plasmodium falciparum isolates*

Four field-collected isolates (38A, 94A, FMS, and Pf03) and the culture-adapted 3D7 strain were cultivated *in vitro* in O^+ RBC in RPMI-1640 medium (Trager and Jensen, 1976). All parasite lines were confirmed, by multilocus microsatellite genotyping (Orjuela-Sánchez et al., 2009), to comprise a single clone (Supplementary Methods online), consistent with previous reports of infrequent mixed-clone *P. falciparum* infections in this region (Anderson et al., 2000; Machado et al., 2004; Orjuela-Sánchez et al., 2009). Each field isolate had a unique genotype – *i.e.*, different microsatellite haplotypes were found, also consistent with a previous microsatellite analysis in the same region (Orjuela-Sánchez et al., 2009). Parasite lines labeled as 38A.1, FMS.1, and Pf03.1, which were analyzed for DBL1α transcription (Table 1), are aliquots of the original isolates prior to *in vitro* culture adaptation. The adhesive properties of field-collected parasites were characterized, essentially as described (Gölnitz et al., 2008), using transfected Chinese hamster ovary (CHO) cells expressing either CD36 (CHO-CD36), ICAM-1 (CHO-ICAM) or no human endothelial receptor (CHO-745) (Hasler et al., 1993). Specific binding was scored semi-quantitatively (Table 1) as -, + or ++ (Supplementary Methods online). We also used the panning strategy described in Supplementary Methods online, on CHO-CD36 and CHO-ICAM cells, to obtain culture-adapted parasite lines with enhanced binding to CD36 (originating the lines $38A^{CD36}$ and FMS^{CD36}) and ICAM-1 (originating the line $38A^{ICAM}$), used in both antibody-binding assays and DBL1α transcription studies (Table 1). Finally, we used real-time PCR to test whether field-collected isolates had the gene encoding the knob-associated histidine-rich protein (KAHRP), which is required for the generation of functional knobs on the RBC surface (Supplementary Methods online). We showed that the field-collected isolate 94A, which failed to bind CHO cells expressing either CD36 or ICAM-1, lacked the *kahrp* gene (Table 1).

2.4. *RNA and complementary DNA preparation*

To determine the major DBL1α domains transcribed by field-collected parasites and by the 3D7 strain, we purified RNA from each parasite line at the ring stage, using the TRIzol reagent (ThermoFischer Scientific, Carlsbad, CA). After RNA isolation, all samples were treated three times with RNase-free DNase (Fermentas, Burlington, Canada), according to the manufacturer's protocol, for removal of residual genomic DNA contaminating RNA preparations prior to their use in complementary DNA (cDNA) synthesis. cDNA was synthesized using the single-tube procedure of the Maxima First Strand cDNA synthesis kit (Fermentas), according to the manufacturer's instructions.

2.5. *DBL1α sequencing*

DBL1α target sequences were PCR-amplified from cDNA samples using the oligonucleotide primers described by Warimwe et al. (2009) with Illumina adapter sequences: tagDBLaAF9: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GCA CG(A/C) AGT TT(C/T) GC-3′ and tagDBLaBR: 5′-GTC TCG TGG GCT CGG AGAT GTG TAT AAG AGA CAG GCC CAT TC(G/C) TCG AAC CA-3’. PCR reactions were performed with Platinum Taq DNA Polymerase (ThermoFisher Scientific) and the conditions were adapted using the following program 94 °C for 2 min, 10 cycles of dissociation, annealing, and extension at 94 °C for 30 s, 42 °C for 30 s, and 72 °C for 30 s, respectively, then 30 cycles at 94 °C for 30 s and 69 °C for 90 s, followed by a final extension at 72 °C for 5 min. The 450-base pair (bp) amplified fragments were purified using Agencourt AMPure XP kits (Beckman Coulter, Brea, CA) according to the manufacturer's protocol. Indexes were added to each sample into the Illumina adapter region, through a second amplification step, using 15 μl of purified PCR product, 25 μl of GoTaq Master Mix Colorless, 5 μl of F index and 5 μl of R index from the Multiplex Sample Preparation Oligonucleotide kit (Illumina, San Diego, CA). Amplification was performed as following: 72 °C for 3 min, 95 °C for 30 s, then 16 cycles at 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. After DNA concentration was normalized to 2 nM, samples were pooled to a final concentration of 10 pM for sequencing using the MiSeq Nano Kit v.2 (Illumina). DNA sequencing was performed on the Illumina MiSeq platform using a paired-end 500 base pair (bp) run.

Sequences were analyzed using CLC Genomic Workbench 7.0. Merged reads were submitted to quality filters (0.01 of quality score and maximum number of ambiguities equal to 1). Each *P. falciparum* isolate generated 2500 to 5000 reads, which were translated and compared to PfEMP-1 sequences available in the NCBI database using BLASTX search. Only full-length DBL1α sequences (starting with a DIGDI motif and ending with PQFLR) associated with Evalue < 0.001 in BLASTX searches against PfEMP-1

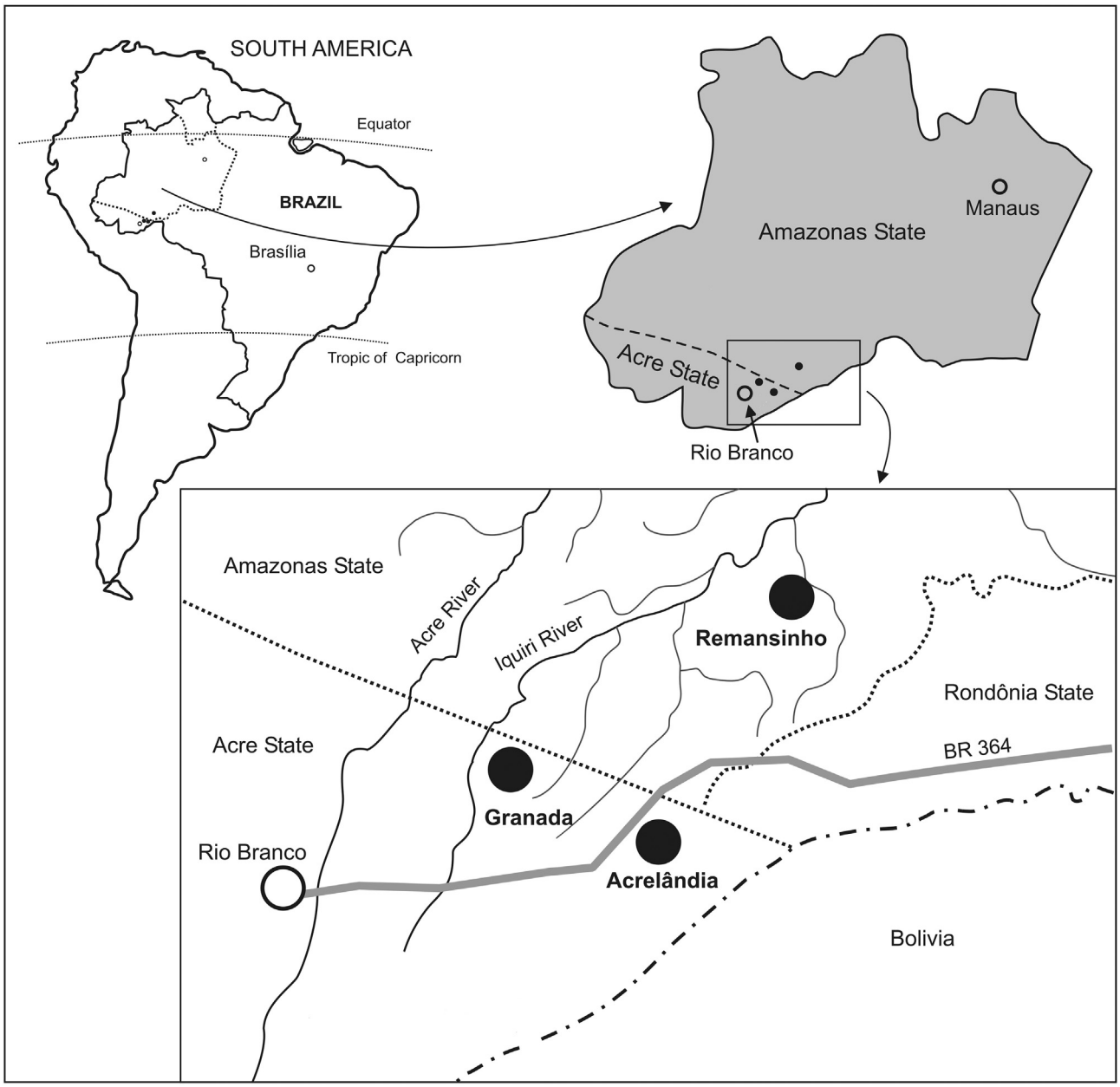

Fig. 1. Map of northwestern Brazil, showing the two field sites in the Acre state, namely the farming settlement of Granada and the town of Acrelândia (35 km southeast of Granada) and the one in Amazonas state, the farming settlement of Remansinho (150 km east of Granada). BR 364 is the paved highway that connects Rio Branco (capital of Acre) to the central and southeast regions of Brazil.

were further analyzed. Those with >95% identity were grouped into the same DBL1α type. The best matches of DBL1α types obtained in PSI-BLAST searches were used as sequence identifiers throughout this paper (Table 2). DNA sequences obtained during this study were deposited into GenBank database (accession number, SRP070478). The resulting full-length DBL1α sequences were analyzed for their distinct sequence identifiers (DSID) using the approach described by Bull et al. (2007). The DSID classification into 6 groups is based on the number of cysteine residues (either 2 or 4) and the analysis of four semi-conserved tetrapeptides, termed positions of limited variability (PoLV), in the amplified DBL1α region.

2.6. Antibody recognition of variant surface antigens (VSA)

Levels of anti-VSA plasma IgG antibodies were determined by flow cytometry essentially as described (Staalsoe et al., 1999). Aliquots of 5×10^6 infected RCB from synchronous *in vitro* cultures at 2% parasitemia were labeled with ethidium bromide (100 μg/ml in phosphate-buffered saline [PBS]) and incubated for 30 min, in triplicate, with 2 μl of human plasma at 1:10 dilution. Although all parasite samples were derived from single-clone infections (see above), not necessarily a single VSA was expressed by each sample. Next, samples were washed twice in PBS with 1% fetal calf serum and incubated with goat, anti-human IgG conjugated with Alexa Fluor 488 (1:600 dilution) for 30 min. A hyperimmune immunoglobulin pool (final concentration, 2.5 mg/100 ml) from malaria-

Table 2
Common *var* gene DBL1α domains (comprising ≥ 5% sequence reads obtained in each isolate) expressed by *Plasmodium falciparum* samples from the Brazilian Amazon and the 3D7 strain, of African origin.

Parasite sample	Number of reads	(%)	Best PSI-BLAST match				
			GenBank no.	% Identity	DBL1α DSID group[i]	Origin	Reference[j]
38A.1	1023/1092	(93.7)	DQ265601	100	2	Rondônia, Brazil	[1]
38A	819/891	(91.9)	DQ265476[a]	100	5	Rondônia, Brazil	[1]
$38A^{CD36}$	560/705	(79.4)	DQ265476[a]	100	5	Rondônia, Brazil	[1]
	55/705	(7.8)	KE123603	99	4	Amazonas, Brazil (strain 7G8)	
$38A^{ICAM}$	196/336	(58.3)	AF416584[b]	100	4	Mato Grosso, Brazil	[2]
	76/336	(22.6)	KE123603	99	4	Amazonas, Brazil (strain 7G8)	
	21/336	(6.3)	DQ265476[a]	100	5	Rondônia, Brazil	[1]
94A	21/89	(23.6)	HQ732950[c]	99	5	Kilifi, Kenya	[3]
	10/89	(11.2)	FJ935896[d]	100	6	Rondônia, Brazil	[5]
	8/89	(9.0)	FJ935945[e]	100	2	Rondônia, Brazil	[5]
	6/89	(6.7)	XM_961212	100	4	Africa (strain 3D7)	
	5/89	(5.6)	KP220334[f]	99	4	Mugil, Papua New Guinea	[4]
	5/89	(5.6)	KP220334[f]	91	4	Mugil, Papua New Guinea	[4]
	5/89	(5.6)	XM_001351477	83	4	Africa (strain 3D7)	
FMS.1	326/447	(72.9)	FJ935865	100	4	Rondônia, Brazil	[5]
	76/447	(17.0)	FJ935829[g]	100	5	Rondônia, Brazil	[5]
FMS	185/481	(38.5)	DQ265565[h]	100	5	Rondônia, Brazil	[5]
	110/481	(22.9)	AF416584[b]	100	4	Mato Grosso, Brazil	[2]
	57/481	(11.9)	FJ935865	100	4	Rondônia, Brazil	[5]
	30/481	(6.2)	FJ935941	99	2	Rondônia, Brazil	[5]
FMS^{CD36}	56/119	(47.1)	DQ265494	100	5	Rondônia, Brazil	[1]
	32/119	(26.9)	FJ935941	99	2	Rondônia, Brazil	[5]
	11/119	(9.2)	DQ265565[h]	100	5	Rondônia, Brazil	[5]
	10/119	(8.4)	FJ935865	100	4	Rondônia, Brazil	[5]
Pf03.1	114/548	(20.8)	FJ935941	99	2	Rondônia, Brazil	[5]
	95/548	(17.3)	DQ265493	100	5	Rondônia, Brazil	[1]
	94/548	(17.2)	CH672280	98	5	Honduras (strain HB3)	
	61/548	(11.1)	AJ536718	99	3	Amazonas, Venezuela	[6]
	50/548	(9.1)	FJ935829[g]	100	5	Rondônia, Brazil	[5]
	37/548	(6.8)	DQ265494	100	5	Rondônia, Brazil	[1]
	35/548	(6.4)	KE123579	100	5	Amazonas, Brazil (strain 7G8)	
Pf03	203/625	(32.5)	DQ265476[a]	100	5	Rondônia, Brazil	[1]
	102/625	(16.3)	KE123603	98	4	Amazonas, Brazil (strain 7G8)	
	92/625	(14.7)	DQ265565[h]	100	5	Rondônia, Brazil	[5]
	78/625	(12.5)	FJ935829[g]	100	5	Rondônia, Brazil	[5]
3D7	462/545	(84.8)	FJ935896[d]	100	6	Rondônia, Brazil	[5]

Underlined GenBank accession numbers indicate sequences shared by two or more parasite lines.
[a] DQ265476 is identical to KE123602 (Brazil).
[b] AF41658 is identical to HQ732437 and HE654544 (Kenya), AJ536715 (Venezuela), KE123588, and KE123689 (Brazil).
[c] HQ732950 is identical to XM_001351481 (isolate 3D7) and KE123746 (isolate NF54).
[d] FJ935896 is identical to AF133878 and XM_002585441 (strain 3D7), KE123852 (strain NF54), AY462812 and KP220535 (Papua New Guinea).
[e] FJ935945 is identical to AF133853 and XM_960904 (strain 3D7) and KE123786 (strain NF54).
[f] KP220334 is identical to XM_001351401 (isolate 3D7).
[g] FJ935829 is identical to HQ733483 (Senegal).
[h] DQ265565 is identical to KE123609 (Brazil).
[i] DSID = distinct sequence identifier (Bull et al., 2007).
[j] References: [1] Albrecht et al., 2006; [2] Kirchgatter and del Portillo, 2002; [3] Chen et al., 2011; [4] Tessema et al., 2015; [5] Albrecht et al., 2010; [6] Tami et al., 2003.

exposed African donors (Gysin et al., 1996; kindly supplied by Prof. L. Pereira da Silva) and plasma from 12 individuals living in malaria-free Sγo Paulo and reporting no previous exposure to malaria served as positive and negative controls, respectively, in each assay. A minimum of 15,000 events were recorded for each parasite-plasma combination, using a Guava easyCyte HT flow cytometer (Merck Millipore, Darmstadt, Germany), and analyzed with Guava ExpressPro 8.1 software. Parasitized RBC and uninfected RBC were gated according to the ethidium bromide fluorescence, and for each sample the geometric mean fluorescence intensity (MFI) was recorded as a measure of the amount of anti-VSA IgG present with specificity for that particular parasite isolate. Nonspecific labeling was evaluated by analysis of ethidium bromide-negative RBC. Plasma samples were tested against each parasite isolate in a single assay to minimize any inter-assay variations arising from antigenic variation of the isolate during its cultivation. A positive anti-VSA IgG response was defined by a MFI value above the mean plus 3 standard deviations of negative control samples. To allow for comparisons of antibody reactivity in different assays, MFI values for each parasite-plasma combination were expressed as a fraction of the MFI obtained, for the same parasite in the same assay, with the hyperimmune immunoglobulin pool. For example, a plasma sample whose MFI corresponded to half of the value obtained with the hyperimmune pool was assigned a relative MFI of 50%. Relative MFI of positive samples were stratified into tertiles for further analyses.

2.7. Production of PfD0020c recombinant antigen

Recent studies have shown that *P. falciparum* isolates from severe malaria patients preferentially express PfEMP-1 variants that are able to bind the endothelial protein C receptor (EPCR) in brain capillaries (Lavstsen et al., 2012; Avril et al., 2012; Claessens et al., 2012; Turner et al., 2013). We thus expressed an EPCR-binding cysteine-rich interdomain region (CIDR) derived from the 3D7 strain (PfD0020c) to assess the acquisition of antibodies to this

major adhesive domain under low-level malaria transmission. For expression as a recombinant protein fused to the C-terminus of *Schistosoma japonicum* glutathione S-transferase (GST), DNA coding for the CIDR domain of PfD0020c was amplified using the primers ggatccCCCGATTGTGTAGTTGAATGTG and ACAGGGGTTTGGTGTGGGG. PCR products (792 bp long) were cloned into pGEM T easy vector (Promega, Madison, WI), sequenced and subcloned into the expression vector pGEX-2T (GE Healthcare, Pittsburgh, PA) using the introduced BamH1 site and the flanking EcoR1 site from the pGEM T easy vector. Recombinant proteins were expressed in transformed *Escherichia coli,* strain BL31 DE3 pLys RIL (Stratagene, La Jolla, CA), and purified by affinity chromatography using Glutathion Sepharose 4B (GE Healthcare). GST was purified from *E. coli* transformed with the pGEX2T vector lacking the *PfD0020c* gene insert for use as a control antigen in serology. The purity of the recombinant protein was assessed by SDS-PAGE; its recognition by control sera from malaria-exposed subjects was confirmed by Western blotting.

2.8. Antibody recognition of PfD0020c recombinant antigen

High-binding 96-well microplates (Costar, Cambridge, MA) were coated with 200 ng/well of GST-PfD0020c fusion protein, dissolved in 50 μl of 0.1 M carbonate-bicarbonate buffer (pH 9.6), overnight at 4 °C. After blocking with 4% low-fat milk in PBS/Tween 20 (0.05%), plasma samples (50 μl/well) were tested at 1:200 dilution in 1% low-fat milk-PBS/Tween 20. After 1-h incubation at 37 °C, antibody binding to solid-phase antigen was detected with peroxidase-conjugated goat immunoglobulin anti-human IgG (Kierkegaard & Perry, Gaithersburg, MD) at a 1:4000 dilution. Peroxidase activity was detected using tetramethylbenzidine (TMB) substrate solution (Thermo Scientific Pierce). Absorbance values were measured at 450 and 595 nm after stopping the reaction with 50 μl of 1 M HCl. Net absorbance values were obtained by subtracting absorbance readings with GST run on the same microplate. A cut-off value, corresponding to the average net absorbance for samples from 10 malaria-naïve blood donors plus 3 standard deviations, was calculated as 0.15. Net absorbances of positive samples were stratified into terciles for further analyses.

2.9. Data analysis

A database with antibody response data was created with SPSS 17.0 (SPSS Inc., Chicago, IL). Correlations were assessed with ρ coefficients obtained with Spearman nonparametric correlation models. Statistical significance was defined at the 5% level.

2.10. Ethics statement

Study protocols have been approved by the Institutional Review Board of the Institute of Biomedical Sciences of the University of São Paulo (928/CEP). Written informed consent was obtained from all study participants or their parents or guardians.

3. Results

3.1. Overlapping repertoire of DBL1α types expressed by Amazonian isolates of Plasmodium falciparum

We analyzed 5878 DBL1α sequence tags expressed by the 3D7 strain and 10 parasite lines from the Brazilian Amazon. These included 3 field isolates analyzed prior to *in vitro* culture (38A.1, FMS.1, and Pf03.1), 4 field isolates after short-term culture adaptation (38A, 94A, FMS, and Pf03) and 3 lines (38A^{CD36}, 38A^{ICAM}, and FMS^{CD36}) selected *in vitro* for enhanced binding to selected endothelial receptors. Between 89 and 1092 transcripts were analyzed per parasite line. The number of different DBL1α types expressed per isolate ranged between 9 and 33, with an average of 21.1 per isolate; 95 (43.0%) of the DBL1α types were singletons. Supplementary Tables 1–11 (available online) list all transcripts found in each parasite line; Table 2 shows only the most common transcripts, *i.e.* those found in >5% of the reads obtained from each parasite. These common transcripts comprised 20 different DBL1α types; most of them had been previously described in other South American isolates of *P. falciparum.* Sixteen were classified into DSID groups 4, 5 or 6 DBL1α transcripts (Bull et al., 2007), which typically comprise DBL1α types associated with uncomplicated malaria, 1 into DSID group 3, and 3 into DSID group 2, which appears to comprise rosette-forming parasites (Bull et al., 2005). Nine common transcripts were shared by at least two independent parasite lines; 38A, FMS, and Pf03 isolates and derived 38A^{CD36}, 38A^{ICAM}, and FMS^{CD36} lines shared one or more common DBL1α sequences with other(s) unrelated parasite line(s) (Table 2). Interestingly, all of these isolates bind both CD36 and ICAM-1 (Table 1).

We found clear examples of transcriptional switching during *in vitro* culture. Accordingly, the dominant transcripts of 38A.1 and 38A isolates were of different DBL1α types (Table 2); DQ265601 (dominant transcript in 38A.1, 93.7% of all reads) represented only 1.1% of the reads from 38A, while DQ265476 (dominant transcript in 38A [91.9% of all reads] and 38A^{CD36} [79.4%], also found in 38A^{ICAM} [6.3%]) was much less frequent (4.6%) in 38A.1. Similarly, FJ935865, the dominant transcript (72.9% of the reads) in FMS.1, was much less common in FMS (11.9% of the reads), while the most common transcript in FMS (DQ265565 [38.5% of the reads], also found in Pf03 [14.7%]) was not found in FMS.1 (Table 2).

Selection for enhanced binding to either CD36 or ICAM-1 was also sometimes associated with dramatic changes in transcription patterns. For example, although 38A and 38A^{CD36} had the same dominant transcript (DQ265476), 38A^{ICAM} predominantly expressed AF416584 (58.3% of the reads), which was rare in 38A.1 (1 in 1092 reads), 38A (2 copies in 891 reads), and 38A^{CD36} (6 copies in 705 reads). This difference in transcript dominance may be associated with the selection for a specific binding phenotype (*i.e.*, parasites expressing AF416584 might display enhanced binding to ICAM-1) or merely result from random transcriptional switching during prolonged *in vitro* culture.

The KAHRP-negative 94A isolate was exceptional in several ways. First, few DBL1α transcript sequences were recovered, with no clear dominance; the most common sequence accounted for only 23.6% of the 89 transcripts characterized in 94A (Table 2). Second, all common sequences in 94A were similar, if not identical, to DBL1α types previously found in the 3D7 strain. Because strict precautions have been adopted to prevent and detect carry-over contamination with PCR products during amplification and sequencing experiments (including use of reagent-only, template-negative controls, aerosol-resistant pipette tips and sterile distilled water in all solutions), we consider unlikely that 94A cDNA samples have been contaminated with 3D7-derived amplicons. In fact, finding 3D7-like DBL1α transcripts in *P. falciparum* isolates collected outside Africa is not entirely surprising. Accordingly, two 3D7-like transcripts found in 94A, FJ935896 (also expressed by the 3D7 strain maintained in our laboratory) and FJ935945 (which together account for nearly 20% DBL1α tags from 94A), were recovered in a previous study of field-collected parasites from the nearby state of Rondônia, Brazil (Albrecht et al., 2010); FJ935896 has additionally been found in two parasite lines from Papua New Guinea (Tessema et al., 2015).

3.2. Exposure-dependent acquisition of antibodies to VSA

We evaluated naturally acquired IgG antibody responses to VSAs expressed by 8 parasite samples in plasmas from 288 (38A isolate) or 306 (all other parasites) malaria-exposed rural Amazonians. To this end, antibody binding to RBC parasitized with the field-collected isolates 38A (and derived lines $38A^{CD36}$ and $38A^{ICAM}$), 94A, FMS (and derived line FMS^{CD36}) and Pf03, all of them with unique multilocus microsatellite haplotypes, as well as the laboratory-adapted 3D7 strain, was measured by flow cytometry. Most (54.1%) plasma samples recognized one or more VSAs (range, 1 to 6); none reacted against VSAs expressed by all parasite samples. The breadth of anti-VSA antibody responses (*i.e.*, number of variants recognized by each subject) correlated positively with the number of years living in malaria-endemic Amazonia, a proxy of subjects' cumulative exposure to malaria (Spearman's rank correlation test, $\rho = 0.243$ and $P < 0.001$).

The proportions of antibody-positive samples ranged between 0.7% (Pf03) and 27.4% ($38A^{ICAM}$); interestingly, Pf03 and $38A^{ICAM}$ shared one DBL1α transcript (KE123603; see Table 2) and both are strong ICAM-1 binders (Table 1). Moreover, 38A and $38A^{CD36}$ shared the same dominant DBL1α transcript (DQ265476), but were recognized by rather different proportions of subjects (19.4% and 4.6%, respectively). Whether naturally acquired antibodies target antigenically diverse VSAs (instead of the dominant DBL1α type) remains to be determined. Alternatively, some highly expressed DBL1α tags may have been missed by the amplification strategy (*e.g.*, due to primer biases). Antibodies to FMS, 94A, and 3D7 VSA were found in similar proportions of subjects (10.1%, 9.5%, and 10.5%, respectively), despite their differences in origin (Brazil *vs.* Africa) and knob structure (*kahrp* gene was deleted in 94A). The dominant DBL1α transcript of $38A^{ICAM}$ (AF416584) is the second most common transcript in FMS, despite the 2.7-fold difference in the frequency of antibody recognition (27.4% vs. 10.1%). Finally, 24.6% of the samples had antibodies to the strong CD36 binder FMS^{CD36}, but few of them recognized the two other CD36 high-binders ($38A^{CD36}$ and Pf03). We thus conclude that the adhesive properties and expressed DBL1α repertoires of parasites are not associated with patterns of antibody recognition of their VSA.

Moreover, we found much less antibody cross-recognition of VSAs than we would expect from the limited *var* gene repertoire expressed by local *P. falciparum* isolates. We explored the specificity of VSA antibody recognition by comparing pairwise correlation coefficients of relative MFI values obtained by flow cytometry (Table 3). The highest correlation coefficients (≥0.400) were for antibodies to unrelated pairs of parasite lines (3D7-Pf03 and 3D7-$38A^{ICAM}$), while correlations between levels of antibodies to related parasite lines (38A- $38A^{CD36}$-$38A^{ICAM}$; FMS-FMS^{CD36}) were somewhat weaker. We next explored whether pairwise correlations were driven by the subjects with the highest cumulative exposure to malaria. To this end, we grouped subjects according to the number of years they have been living in malaria-endemic areas (0–10 years, 11–18 years and over 18 years, as in Fig. 2) and calculated correlation coefficients separately within each group. This supplementary analysis did not reveal increased correlation coefficients in the group with over 18 years of exposure to malaria (Supplementary Tables 12–14).

As expected, levels of antibodies to most VSAs (measured as relative MFI) correlated weakly but positively with the number of years of malaria exposure (Spearman's rank correlation test, ρ ranging from 0.130 to 0.237, *P* values between 0.023 and < 0.001), except for 3D7 ($\rho = 0.064$, $P = 0.265$) and the poorly recognized Pf03 VSA ($\rho = 0.084$, $P = 0.142$). Interestingly, no correlation was found between age and levels of antibodies to any of the VSAs tested. However, major gaps in the repertoire of anti-VSA antibodies of rural Amazonians remained despite several decades of continuous exposure to low-level *P. falciparum* transmission (Fig. 2). Although our study subjects in Granada have developed some degree of clinical immunity after 5–8 years of continuous exposure to malaria (da Silva-Nunes et al., 2008), few of them have antibodies to most VSA expressed by local parasites. In sharp contrast, children exposed to moderate to high malaria endemicity in Africa quickly develop a vast anti-VSA antibody repertoire (*e.g.*, Vestergaard et al., 2008).

3.3. Antibody recognition of PfD0020c

We also examined the exposure-dependent acquisition of IgG antibodies to PfD0020c, a subtype α1.1 PfEMP-1 CDIR domain that is predicted to bind EPCR. Eighty-four (29.8%) of the 291 plasma samples analyzed had anti-PfD0020c antibodies detected by ELISA, similar to the antibody positivity rate (24%) recently described in a large sample of malaria-exposed villagers in northern Tanzania (Turner et al., 2015). Levels of anti-PfD0020c antibodies (measured as net absorbance values) correlated weakly but positively with the subjects' number of years of malaria exposure (Spearman's rank correlation test, $\rho = 0.148$, $P = 0.013$); 37.2% of the study participants with >18 years of malaria exposure had detectable antibodies to PfD0020c (Fig. 2, lower panel). It remains to be determined whether naturally acquired anti-PfD0020c antibodies can protect against EPCR binding-associated complications of *P. falciparum* infection, such as cerebral malaria, which nowadays are exceedingly rare in Amazonian populations.

4. Discussion

Clinical episodes of *P. falciparum* malaria are typically caused by parasites expressing VSAs to which the patient had no preexisting variant-specific antibodies; parasites therefore explore gaps in the anti-VSA antibody repertoire of their hosts (Bull and Marsh, 2002). These gaps are gradually filled as hosts are exposed to new VSA types and become gradually able to recognize all locally circulating

Table 3
Spearman's ρ correlation coefficients (above diagonal) and corresponding *P* values (below diagonal) of levels of IgG antibodies in rural Amazonians to variant surface antigens expressed by *Plasmodium falciparum* samples from the Brazilian Amazon and the 3D7 strain, of putative African origin.

Parasite line	38A	$38A^{CD36}$	$38A^{ICAM}$	94A	FMS	FMS^{CD36}	Pf03	3D7
38A		0.200	0.294	0.319	0.308	0.232	0.269	0.260
$38A^{CD36}$	0.001		0.379	0.280	0.274	0.290	0.221	0.279
$38A^{ICAM}$	<0.0001	<0.0001		0.218	0.176	0.252	0.280	0.403
94A	<0.0001	<0.0001	<0.0001		0.385	0.382	0.346	0.327
FMS	<0.0001	<0.0001	0.002	<0.0001		0.304	0.266	0.221
FMS^{CD36}	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		0.215	0.232
Pf03	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		0.410
3D7	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

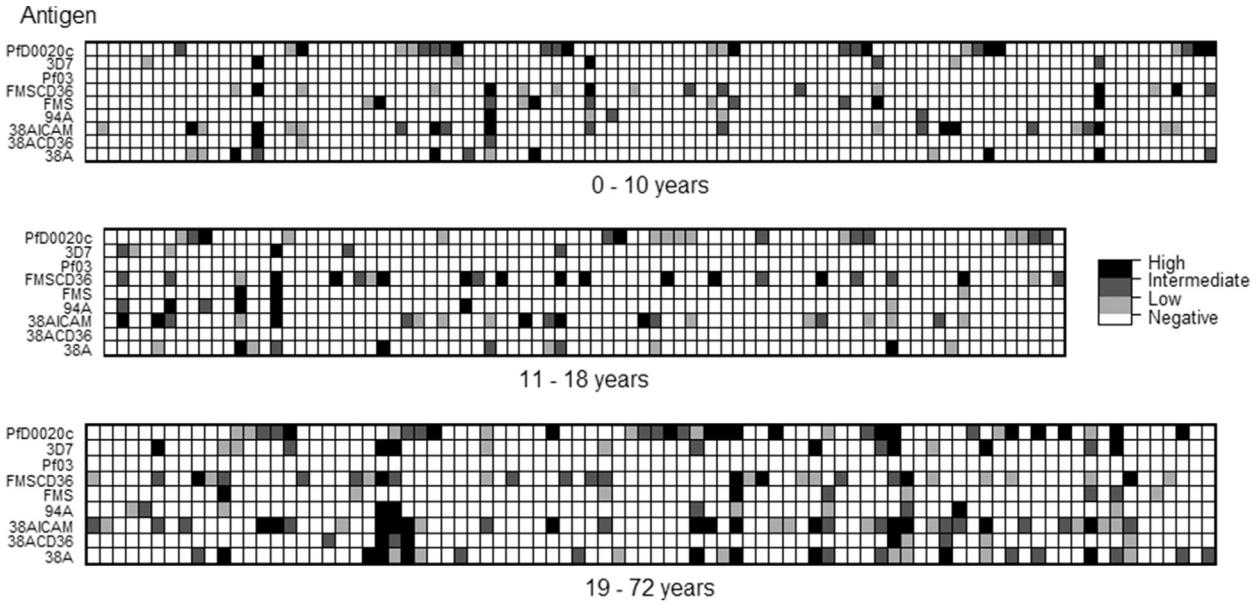

Fig. 2. Repertoire of anti-VSA IgG antibodies in rural Amazonians by cumulative exposure to malaria (years living in malaria-endemic sites). Squares represent individual plasma donors, who were tested against a panel of 7 *P. falciparum* isolates from Brazil (38A and derived lines $38A^{CD36}$ and $38A^{ICAM}$; 94A; FMS and derived line FMS^{CD36}; and Pf03) and one laboratory-adapted strain from Africa (3D7). Antibody responses to a fusion protein representing PfD0020c, an EPCR-binding CIDR domain of PfEMP-1 derived from the 3D7 strain, are also represented. Only subjects tested against all antigens ($n = 269$) are represented; therefore, the two subjects found to have Pf03-binding antibodies (of 306 tested against this antigen) are not shown here because they were not tested against the complete antigen panel. White squares indicate antibody-negative samples, whose relative MFI or absorbance fell below the cut-off value; antibody-positive samples were stratified into terciles, according to their relative MFI or absorbance values, to define low, intermediate and high responders (indicated as light grey, dark grey and black squares, respectively).

parasite variants. The rate at which variant-specific antibodies are acquired in different endemic settings depends on the size of the VSA repertoire expressed by local parasites and the frequency of exposure to new VSAs.

Analyses of microsatellites (Anderson et al., 2000; Machado et al., 2004) and single-nucleotide polymorphisms (Neafsey et al., 2008; Tanabe et al., 2010) have repeatedly shown that *P. falciparum* populations from the Amazon Basin of Brazil display little genetic diversity. Accordingly, our sequence data, although limited to expressed sequence tags from a few field-collected isolates, further suggest that Amazonian isolates of *P. falciparum* express a limited repertoire of DBL1α types. This repertoire has been previously shown to be often shared among local isolates collected in different parts of the Amazon (Kirchgatter and del Portillo, 2002; Albrecht et al., 2006, 2010) and to remain stable over time (Albrecht et al., 2010). Because *var* gene-encoded PfEMP-1 variants are the dominant antigenic component of VSAs (Chan et al., 2012), we would expect that a restricted, overlapping *var* gene repertoire would translate into little antigenic diversity in VSAs of local parasites. In fact, genetically identical *P. falciparum* isolates from Senegal have recently been shown to express similar *var* gene sequence types and display antigenically similar VSA repertoires, which are recognized at similar degrees by IgG antibodies from the same locally exposed subjects (Bei et al., 2015).

We hypothesized that rural Amazonians exposed to a genetically homogeneous parasite population would acquire, over a few years, a highly cross-reactive antibody repertoire that recognizes the vast majority of locally circulating VSA types. These naturally acquired variant-specific antibodies might confer some degree of clinical immunity, which is observed after only 5–8 years of exposure to hypo- or mesoendemic malaria transmission in the Amazon (da Silva-Nunes et al., 2008; Ladeia-Andrade et al., 2009). Consistent with this hypothesis, cross-reactive anti-VSA antibodies have been described in areas of low malaria endemicity in Sudan (Giha et al., 1999a) and India (Chattopadhyay et al., 2003). Moreover, given that *var* gene repertoires appear to vary little across time and space in the Amazon (Albrecht et al., 2006, 2010), the distance between the sites of parasite collection and plasma collection (4–5 years in time, 135 km in space) in our study would not severely affect the observed patterns of VSA recognition by antibodies.

However, contrary to our expectations, we found relatively little cross-reactivity in anti-VSA antibody responses of rural Amazonians, even when comparing VSA from related parasite lines expressing the same dominant DBL1α types (*e.g.*, 38A and $38A^{CD36}$). Although the breadth of anti-VSA antibody responses correlated positively with the subjects' cumulative exposure to malaria, no individual had antibodies to all VSA tested; major gaps in the repertoire of anti-VSA antibodies persisted despite decades of exposure to low-level *P. falciparum* transmission. This is in sharp contrast with the VSA-specific antibody repertoire observed in African children and adolescents, which comprises not only VSAs from locally circulating parasites (*e.g.*, Bull and Marsh, 2002; Ofori et al., 2002; Vestergaard et al., 2008) but also those expressed by parasites from distant endemic settings (Aguiar et al., 1992; Vestergaard et al., 2008).

Pf03 was particularly poorly recognized by rural Amazonians, although all common DBL1α types expressed by Pf03 were shared by other local parasites. Interestingly, parasites with a similar endothelial binding phenotype (*e.g.*, $38A^{CD36}$, FMS^{CD36} and Pf03, all of them strongly binding CD36) had VSA recognized by different proportions of study subjects, suggesting that their CD36-binding domains are likely to vary. Somewhat surprisingly, similar proportions of Tanzanian children (Turner et al., 2015) and rural Amazonians had antibodies to the recombinant protein PfD0020c, representing an EPCR-binding domain of PfEMP-1 expressed by the 3D7 strain that is commonly expressed in severe malaria parasites.

Studies in Africa have described some VSAs that are often recognized by exposed individuals ("common" VSAs), whereas others ("rare" VSAs) are recognized by only few of them. Common

VSA, typically found in parasites from nonimmune children, are thought to be more conserved and elicit more cross-reactive antibodies than rare VSAs, which in turn predominate in semi-immune hosts (Bull et al., 2000; Nielsen et al., 2002). Parasites expressing common VSAs predominate in naïve hosts and often lead to severe malaria, while parasites expressing the rare and immunologically most divergent variants explore the few remaining gaps in the anti-VSA antibody repertoire of semi-immune hosts (Bull et al., 2000; Nielsen et al., 2002; van Noort et al., 2010). One of the potential limitations of our study is that we used parasites from *P. falciparum*-infected adult subjects, with 12–26 years of exposure to malaria, to measure anti-VSA antibodies. Under the hypothesis of immune-mediated selection for rare VSAs in semi-immune hosts, we might have biased our sample towards parasites expressing relatively uncommon, highly divergent VSA types. However, we consider unlikely that acquired immunity among *P. falciparum* isolate donors would be strong enough to severely constrain the VSA repertoire expressed by infecting parasites, favoring the emergence of rare VSAs.

Since anti-VSA antibodies are relatively short-lived (Giha et al., 1999b), we might have underestimated the proportion of responding subjects by using a cross-sectional study design. In other words, we may have missed transient variant-specific anti-VSA antibody responses elicited by past *P. falciparum* infections. Carefully designed longitudinal cohort studies, with frequent parasite and plasma sampling, are thus required to investigate how anti-VSA antibody responses develop and are maintained in Amazonian communities exposed to a sharply declining *P. falciparum* transmission – a pattern likely to be observed in several other regions approaching malaria elimination.

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All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

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References

Aguiar, J.C., Albrecht, G.R., Cegielski, P., Greenwood, B.M., Jensen, J.B., Lallinger, G., Martinez, A., McGregor, I.A., Minjas, J.N., Neequaye, J., Patarroyo, M.E., Sherwood, J.A., Howard, R.J., 1992. Agglutination of *Plasmodium falciparum*-infected erythrocytes from east and west African isolates by human sera from distant geographic regions. Am. J. Trop. Med. Hyg. 47, 621–632.

Albrecht, L., Castiñeiras, C., Carvalho, B.O., Ladeia-Andrade, S., Santos da Silva, N., Hoffmann, E.H., dalla Martha, R.C., Costa, F.T., Wunderlich, G., 2010. The South American *Plasmodium falciparum var* gene repertoire is limited, highly shared and possibly lacks several antigenic types. Gene 453, 37–44. http://dx.doi.org/10.1016/j.gene.2010.01.001.

Albrecht, L., Merino, E.F., Hoffmann, E.H., Ferreira, M.U., de Mattos Ferreira, R.G., Osakabe, A.L., dalla Martha, R.C., Ramharter, M., Durham, A.M., Ferreira, J.E., del Portillo, H.A., Wunderlich, G., 2006. Extense variant gene family repertoire overlap in Western Amazon *Plasmodium falciparum* isolates. Mol. Biochem. Parasitol. 150, 157–165.

Alves, F.P., Durlacher, R.R., Menezes, M.J., Krieger, H., Silva, L.H., Camargo, E.P., 2002. High prevalence of asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections in native Amazonian populations. Am. J. Trop. Med. Hyg. 66, 641–648.

Anderson, T.J., Haubold, B., Williams, J.T., Estrada-Franco, J.G., Richardson, L., Mollinedo, R., Bockarie, M., Mokili, J., Mharakurwa, S., French, N., Whitworth, J., Velez, I.D., Brockman, A.H., Nosten, F., Ferreira, M.U., Day, K.P., 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol. Biol. Evol. 17, 1467–1482.

Avril, M., Tripathi, A.K., Brazier, A.J., Andisi, C., Janes, J.H., Soma, V.L., Sullivan Jr., D.J., Bull, P.C., Stins, M.F., Smith, J.D., 2012. A restricted subset of *var* genes mediates adherence of *Plasmodium falciparum*-infected erythrocytes to brain endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 109, E1782–E1790. http://dx.doi.org/10.1073/pnas.1120534109.

Barbosa, S., Gozze, A.B., Lima, N.F., Batista, C.L., Bastos, M.S., Nicolete, V.C., Fontoura, P.S., Gonçalves, R.M., Viana, S.A., Menezes, M.J., Scopel, K.K., Cavasini, C.E., Malafronte, R.S., da Silva-Nunes, M., Vinetz, J.M., Castro, M.C., Ferreira, M.U., 2014. Epidemiology of disappearing *Plasmodium vivax* malaria: a case study in rural Amazonia. PLoS Negl. Trop. Dis. 8, e3109. http://dx.doi.org/10.1371/journal.pntd.0003109.

Bei, A.K., Diouf, A., Miura, K., Larremore, D.B., Ribacke, U., Tullo, G., Moss, E.L., Neafsey, D.E., Daniels, R.F., Zeituni, A.E., Nosamiefan, I., Volkman, S.K., Ahouidi, A.D., Ndiaye, D., Dieye, T., Mboup, S., Buckee, C.O., Long, C.A., Wirth, D.F., 2015. Immune characterization of *Plasmodium falciparum* parasites with a shared genetic signature in a region of decreasing transmission. Infect. Immun. 83, 276–285. http://dx.doi.org/10.1128/IAI.01979-14.

Bull, P.C., Berriman, M., Kyes, S., Quail, M.A., Hall, N., Kortok, M.M., Marsh, K., Newbold, C.I., 2005. *Plasmodium falciparum* variant surface antigen expression patterns during malaria. PLoS Pathog. 1, e26.

Bull, P.C., Kortok, M., Kai, O., Ndungu, F., Ross, A., Lowe, B.S., Newbold, C.I., Marsh, K., 2000. *Plasmodium falciparum*-infected erythrocytes: agglutination by diverse Kenyan plasma is associated with severe disease and young host age. J. Infect. Dis. 182, 252–259.

Bull, P.C., Kyes, S., Buckee, C.O., Montgomery, J., Kortok, M.M., Newbold, C.I., Marsh, K., 2007. An approach to classifying sequence tags sampled from *Plasmodium falciparum var* genes. Mol. Biochem. Parasitol. 154, 98–102.

Bull, P.C., Marsh, K., 2002. The role of antibodies to *Plasmodium falciparum*-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. Trends Microbiol. 10, 55–58.

Chan, J.A., Howell, K.B., Reiling, L., Ataide, R., Mackintosh, C.L., Fowkes, F.J., Petter, M., Chesson, J.M., Langer, C., Warimwe, G.M., Duffy, M.F., Rogerson, S.J., Bull, P.C., Cowman, A.F., Marsh, K., Beeson, J.G., 2012. Targets of antibodies against *Plasmodium falciparum*-infected erythrocytes in malaria immunity. J. Clin. Invest. 122, 3227–3238. http://dx.doi.org/10.1172/JCI62182.

Chen, D.S., Barry, A.E., Leliwa-Sytek, A., Smith, T.A., Peterson, I., Brown, S.M., Migot-Nabias, F., Deloron, P., Kortok, M.M., Marsh, K., Daily, J.P., Ndiaye, D., Sarr, O., Mboup, S., Day, K.P., 2011. A molecular epidemiological study of *var* gene diversity to characterize the reservoir of *Plasmodium falciparum* in humans in Africa. PLoS One 6, e16629. http://dx.doi.org/10.1371/journal.pone.0016629.

Chattopadhyay, R., Sharma, A., Srivastava, V.K., Pati, S.S., Sharma, S.K., Das, B.S., Chitnis, C.E., 2003. *Plasmodium falciparum* infection elicits both variant-specific and cross-reactive antibodies against parasite surface antigen variants. Infect. Immun. 71, 597–604.

Claessens, A., Adams, Y., Ghumra, A., Lindergard, G., Buchan, C.C., Andisi, C., Bull, P.C., Mok, S., Gupta, A.P., Wang, C.W., Turner, L., Arman, M., Raza, A., Bozdech, Z., Rowe, J.A., 2012. A subset of group A-like *var* genes encodes the malaria parasite ligands for binding to human brain endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 109, E1772–E1781. http://dx.doi.org/10.1073/pnas.1120461109.

Coura, J.R., Suárez-Mutis, M., Ladeia-Andrade, S., 2006. A new challenge for malaria control in Brazil: asymptomatic *Plasmodium* infection - a review. Mem. Inst. Oswaldo Cruz 101, 229–237.

da Silva, N.S., da Silva-Nunes, M., Malafronte, R.S., Menezes, M.J., D'Arcadia, R.R., Komatsu, N.T., Scopel, K.K., Braga, E.M., Cavasini, C.E., Cordeiro, J.A., Ferreira, M.U., 2010. Epidemiology and control of frontier malaria in Brazil: lessons from community-based studies in rural Amazonia. Trans. R. Soc. Trop. Med. Hyg. 104, 343–350. http://dx.doi.org/10.1016/j.trstmh.2009.12.010.

da Silva-Nunes, M., Codeço, C.T., Malafronte, R.S., da Silva, N.S., Juncansen, C., Muniz, P.T., Ferreira, M.U., 2008. Malaria on the Amazonian frontier: transmission dynamics, risk factors, spatial distribution, and prospects for control. Am. J. Trop. Med. Hyg. 79, 624–635.

Doolan, D.L., Dobaño, C., Baird, J.K., 2009. Acquired immunity to malaria. Clin. Microbiol. Rev. 22, 13–36. http://dx.doi.org/10.1128/CMR.00025-08.

Ferreira, M.U., Castro, M.C., 2016. Challenges for malaria elimination in Brazil. Mal. J. 15, 284. http://dx.doi.org/10.1186/s12936-016-1335-1.

Giha, H.A., Staalsoe, T., Dodoo, D., Elhassan, I.M., Roper, C., Satti, G.M., Arnot, D.E., Theander, T.G., Hviid, L., 1999a. Overlapping antigenic repertoires of variant

antigens expressed on the surface of erythrocytes infected by *Plasmodium falciparum*. Parasitology 119, 7–17.
Giha, H.A., Staalsoe, T., Dodoo, D., Elhassan, I.M., Roper, C., Satti, G.M., Arnot, D.E., Theander, T.G., Hviid, L., 1999b. Nine-year longitudinal study of antibodies to variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes. Infect. Immun. 67, 4092–4098.
Gölnitz, U., Albrecht, L., Wunderlich, G., 2008. *Var* transcription profiling of *Plasmodium falciparum* 3D7: assignment of cytoadherent phenotypes to dominant transcripts. Mal. J. 7, 14. http://dx.doi.org/10.1186/1475-2875-7-14.
Gysin, J., Moisson, P., Pereira da Silva, L., Druilhe, P., 1996. Antibodies from immune African donors with a protective effect in *Plasmodium falciparum* human infection are also able to control asexual blood forms of the parasite in *Saimiri* monkeys. Res. Immunol. 147, 397–401.
Hasler, T., Albrecht, G.R., Van Schravendijk, M.R., Aguiar, J.C., Morehead, K.E., Pasloske, B.L., Ma, C., Barnwell, J.W., Greenwood, B., Howard, R.J., 1993. An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. Am. J. Trop. Med. Hyg. 48, 332–347.
Hviid, L., 2005. Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. Acta Trop. 95, 270–275.
Hviid, L., 2010. The role of *Plasmodium falciparum* variant surface antigens in protective immunity and vaccine development. Hum. Vaccin 6, 84–89.
Kirchgatter, K., del Portillo, H.A., 2002. Association of severe noncerebral Plasmodium falciparum malaria in Brazil with expressed PfEMP1 DBL1 alpha sequences lacking cysteine residues, cysteine residues. Mol. Med. 8, 16–23.
Kirkman, L.A., Deitsch, K.W., 2014. Recombination and diversification of the variant antigen encoding genes in the malaria parasite *Plasmodium falciparum*. Microbiol. Spectr. 2, 6. http://dx.doi.org/10.1128/microbiolspec.MDNA3-0022-2014.
Ladeia-Andrade, S., Ferreira, M.U., de Carvalho, M.E., Curado, I., Coura, J.R., 2009. Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. Am. J. Trop. Med. Hyg. 80, 452–459.
Lavstsen, T., Turner, L., Saguti, F., Magistrado, P., Rask, T.S., Jespersen, J.S., Wang, C.W., Berger, S.S., Baraka, V., Marquard, A.M., Seguin-Orlando, A., Willerslev, E., Gilbert, M.T., Lusingu, J., Theander, T.G., 2012. *Plasmodium falciparum* erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. Proc. Natl. Acad. Sci. U. S. A. 109, E1791–E1800. http://dx.doi.org/10.1073/pnas.1120455109.
Machado, R.L., Póvoa, M.M., Calvosa, V.S., Ferreira, M.U., Rossit, A.R., dos Santos, E.J., Conway, D.J., 2004. Genetic structure of *Plasmodium falciparum* populations in the Brazilian Amazon region. J. Infect. Dis. 190, 1547–1555.
Neafsey, D.E., Schaffner, S.F., Volkman, S.K., Park, D., Montgomery, P., Milner Jr., D.A., Lukens, A., Rosen, D., Daniels, R., Houde, N., Cortese, J.F., Tyndall, E., Gates, C., Stange-Thomann, N., Sarr, O., Ndiaye, D., Ndir, O., Mboup, S., Ferreira, M.U., Moraes, S.L., Dash, A.P., Chitnis, C.E., Wiegand, R.C., Hartl, D.L., Birren, B.W., Lander, E.S., Sabeti, P.C., Wirth, D.F., 2008. Genome-wide SNP genotyping highlights the role of natural selection in *Plasmodium falciparum* population divergence. Genome Biol. 9, R171. http://dx.doi.org/10.1186/gb-2008-9-12-r171.
Nielsen, M.A., Staalsoe, T., Kurtzhals, J.A.L., Goka, B.Q., Dodoo, D., Alifrangis, M., Theander, T.G., Akanmori, B.D., Hviid, L., 2002. *Plasmodium falciparum* variant surface antigen expression varies between isolates causing severe and non-severe malaria and is modified by acquired immunity. J. Immunol. 168, 3444–3450.
Ofori, M.F., Dodoo, D., Staalsoe, T., Kurtzhals, J.A., Koram, K., Theander, T.G., Akanmori, B.D., Hviid, L., 2002. Malaria-induced acquisition of antibodies to *Plasmodium falciparum* variant surface antigens. Infect. Immun. 70, 2982–2988.
Orjuela-Sánchez, P., da Silva-Nunes, M., da Silva, N.S., Scopel, K.K., Gonçalves, R.M., Malafronte, R.S., Ferreira, M.U., 2009. Population dynamics of genetically diverse *Plasmodium falciparum* lineages: community-based prospective study in rural Amazonia. Parasitology 136, 1097–1105.
Staalsoe, T., Giha, H.A., Dodoo, D., Theander, T.G., Hviid, L., 1999. Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. Cytometry 35, 329–336.
Tami, A., Ord, R., Targett, G.A., Sutherland, C.J., 2003. Sympatric *Plasmodium falciparum*isolates from Venezuela have structured var gene repertoires. Malar J. 2, 7.
Tessema, S.K., Monk, S.L., Schultz, M.B., Tavul, L., Reeder, J.C., Siba, P.M., Mueller, I., Barry, A.E., 2015. Phylogeography of *var* gene repertoires reveals fine-scale geospatial clustering of *Plasmodium falciparum* populations in a highly endemic area. Mol. Ecol. 24, 484–497. http://dx.doi.org/10.1111/mec.13033.
Tanabe, K., Mita, T., Jombart, T., Eriksson, A., Horibe, S., Palacpac, N., Ranford-Cartwright, L., Sawai, H., Sakihama, N., Ohmae, H., Nakamura, M., Ferreira, M.U., Escalante, A.A., Prugnolle, F., Björkman, A., Färnert, A., Kaneko, A., Horii, T., Manica, A., Kishino, H., Balloux, F., 2010. *Plasmodium falciparum* accompanied the human expansion out of Africa. Curr. Biol. 20, 1283–1289. http://dx.doi.org/10.1016/j.cub.2010.05.053.
Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.
Turner, L., Lavstsen, T., Berger, S.S., Wang, C.W., Petersen, J.E., Avril, M., Brazier, A.J., Freeth, J., Jespersen, J.S., Nielsen, M.A., Magistrado, P., Lusingu, J., Smith, J.D., Higgins, M.K., Theander, T.G., 2013. Severe malaria is associated with parasite binding to endothelial protein C receptor. Nature 498, 502–505. http://dx.doi.org/10.1038/nature12216.
Turner, L., Lavstsen, T., Mmbando, B.P., Wang, C.W., Magistrado, P.A., Vestergaard, L.S., Ishengoma, D.S., Minja, D.T., Lusingu, J.P., Theander, T.G., 2015. IgG antibodies to endothelial protein C receptor-binding cysteine-rich interdomain region domains of *Plasmodium falciparum* erythrocyte membrane protein 1 are acquired early in life in individuals exposed to malaria. Infect. Immun. 83, 3096–3103. http://dx.doi.org/10.1128/IAI.00271-15.
van Noort, S.P., Nunes, M.C., Weedall, G.D., Hviid, L., Gomes, M.G., 2010. Immune selection and within-host competition can structure the repertoire of variant surface antigens in *Plasmodium falciparum*—a mathematical model. PLoS One 5, e9778. http://dx.doi.org/10.1371/journal.pone.0009778.
Vestergaard, L.S., Lusingu, J.P., Nielsen, M.A., Mmbando, B.P., Dodoo, D., Akanmori, B.D., Alifrangis, M., Bygbjerg, I.C., Lemnge, M.M., Staalsoe, T., Hviid, L., Theander, T.G., 2008. Differences in human antibody reactivity to *Plasmodium falciparum* variant surface antigens are dependent on age and malaria transmission intensity in northeastern Tanzania. Infect. Immun. 76, 2706–2714. http://dx.doi.org/10.1128/IAI.01401-06.
Warimwe, G.M., Keane, T.M., Fegan, G., Musyoki, J.N., Newton, C.R., Pain, A., Berriman, M., Marsh, K., Bull, P.C., 2009. *Plasmodium falciparum var* gene expression is modified by host immunity. Proc. Natl. Acad. Sci. U. S. A. 106, 21801–21806. http://dx.doi.org/10.1073/pnas.0907590106.