

# Inhibitory Properties of the Antibody Response to *Plasmodium vivax* Duffy Binding Protein in an Area with Unstable Malaria Transmission

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## Abstract

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The function of the *Plasmodium vivax* Duffy binding protein (DBP) during the erythrocyte invasion process is critical for successful parasite growth and pathogenesis in human infections. Although DBP is the subject of intensive malaria vaccine research, investigations on the functional properties of anti-DBP antibodies in the human population have been limited [*Infect Immun* 68 (2000) 3164]. In the present study, we examined the ability of sera from different populations of the Brazilian Amazon – an area of markedly unstable malaria transmission – to inhibit the erythrocyte-binding function of the DBP ligand domain (region II, DBP<sub>II</sub>). We found that long-term exposure to malaria in the Amazon area elicits DBP-specific antibodies that inhibit the binding of different DBP<sub>II</sub> variants to erythrocytes. Despite the great variability of inhibitory antibody responses observed among study participants, we observed a positive correlation between erythrocyte binding-inhibitory activity and enzyme-linked immunosorbent assay anti-DBP antibodies. Of importance, there was a non-significant tendency towards increased levels of anti-DBP antibodies among individuals with asymptomatic *P. vivax* infections.

## Introduction

*Plasmodium* merozoites initiate erythrocyte invasion through an active process mediated by parasite ligands that interact with erythrocyte receptors. A key step during host cell invasion is the irreversible commitment of the merozoite to the selected host cell by formation of a tight junction between the parasite and the erythrocyte [1, 2]. In the case of *P. vivax*, junction formation requires that Duffy binding protein (DBP) binds to its cognate receptor on erythrocytes, the Duffy blood group antigen [3–5] also known as Duffy antigen receptor for chemokines [6]. Individuals that lack the Duffy antigen on their erythrocytes are highly resistant to *P. vivax* infection [7]. Thus, DBP represents one of the most promising vaccine candidate antigens against *P. vivax* infection.

*Plasmodium vivax* DBP is a 140-kDa protein that belongs to a family of homologous Duffy binding-like erythrocyte binding proteins (DBL-EBP) located within the micronemes of *Plasmodium* merozoites [8]. The simi-

larity among DBL-EBPs is most prominent in two conserved Cys-rich domains, region II and VI. The functional binding domains of DBL-EBP lie in region II, which for *P. vivax* DBP is 330-aa, and the critical binding residues map to a central 170-amino-acid stretch that includes cysteines 5–8 [9–11]. DBP is likely to be exposed on the merozoite surface during invasion, enabling it to bind to its receptor and, thus, making it accessible to serum antibodies. Currently, the available data regarding humoral immune responses to DBP in the human population are limited, and studies have been essentially restricted to areas where malaria is highly endemic [12, 13, 14]. Recently, we demonstrated that DBP is naturally immunogenic in the Brazilian Amazon area, where malaria transmission rates are low-to-medium and clinical symptoms affect people of all ages [15]. In the Amazon, the proportions of DBP IgG-positive subjects increased with exposure to malaria, reaching a peak in those subjects with long-term exposure in the endemic area (>15 years). This observation was subsequently confirmed in malaria-endemic populations of Rondônia State,

Brazil, where specific antibodies to DBP were biased towards the cytophilic subclasses IgG1 and IgG3 [16].

Many field studies examining immunity to malaria have focused on measuring the concentrations of antibodies to vaccine candidate antigens, while less attention has been paid to complementary approaches defining the functional relevance of these antibodies [17]. For example, measuring antibodies to recombinant merozoite antigens by enzyme-linked immunosorbent assay (ELISA) is a simple, robust procedure widely used in population studies, but provides little qualitative information when used alone [18]. In the present study, we hypothesized that part of the humoral response to malaria infection in the Amazon area is mediated by antibodies that inhibit the interaction between DBP and its receptor on erythrocytes. To date, this inhibitory antibody response was demonstrated only in highly endemic regions of Papua New Guinea [12], which is representative of only some tropical regions where *P. vivax* is prevalent. To characterize these inhibitory antibodies in our study, we used an *in vitro* erythrocyte-binding assay, in which the putative ligand domain of DBP (region II, DBP<sub>II</sub>) was expressed on the surface of cultivated mammalian cells [12, 19]. To assess the potential effects of DBP polymorphisms, three different DBP<sub>II</sub> variants were used in the erythrocyte-binding assays.

## Materials and methods

**Study areas and subjects.** We analysed subjects from three previously well-characterized areas of the Brazilian Amazon [15, 20] who had been exposed to different levels of malaria transmission (Table 1). The first group consisted of individuals living in Belém, the capital of the State of Pará. They had acquired a single episode of *P. vivax* malaria after short trips to islands located near the capital, where levels of malaria transmission are low and

unstable. The second group was composed of individuals who had lived for about 10 years in a small rural community of Mato Grosso (MT) State, Terra Nova do Norte (TNN), where malaria is endemic with intermittent transmission. These individuals reported a variable number of previous malaria episodes caused by *P. vivax* and/or *P. falciparum*. The third group, named the Apiacas group, consisted of migrant miners who had lived for approximately 17 years in several gold-mining areas of the Brazilian Amazon where *P. vivax* and *P. falciparum* malaria are endemic. At the time of blood collection, the three groups (Belém, TNN and Apiacas) consisted of asexual individuals, as assessed by microscopic examination.

A previous study on the prevalence of malaria in Apiacas (MT) has suggested that gold-miners develop partial resistance to clinical malaria [21]. At the time of the field survey, 10% of the gold-miner population (36 out of 369) had *P. vivax* parasites, as detected by microscopy or species-specific PCR [22], 19 of which were classified as asymptomatic and 17 as symptomatic. Asymptomatic malaria infection was defined as absence of classical malaria symptoms, such as fever, headache, muscle and/or joint pain, for at least 72 h after parasite detection. In the current study, we selected 15 of the 19 gold-miners with asymptomatic *P. vivax* infection and 10 of the 17 with symptomatic *P. vivax* infection.

**Human plasma.** Human plasma samples were collected from *P. vivax*-exposed residents of the Brazilian Amazon area, as described earlier [20, 23]. Initially, the DBP-erythrocyte binding assay relied on pooled samples. For each area, samples were pooled as following: (i) from individuals who had anti-DBP antibodies, as detected by ELISA (responders), and (ii) from individuals who had not developed anti-DBP antibodies (non-responders) (Table 1). The criterion for classifying individuals as responders or non-responders to DBP was based on the ELISA cutoff point, as described in the 'Antibody measurement' section; individuals whose plasma samples had an optical density  $\geq 0.1$  (OD value at 492 nm) were classified as responders, and those whose plasmas had an OD value  $< 0.1$  were classified as non-responders. For further experiments, individual samples from these Amazonian communities were used; these individuals were subsequently classified as high, low and non-responders, according to their ELISA antibody titre. Additional plasma samples were obtained from individuals infected with *P. vivax* that had developed asymptomatic ( $n = 15$ ) or symptomatic ( $n = 10$ ) malaria infection after a short follow-up period [21]. Negative control plasma samples were collected from Brazilian volunteers ( $n = 20$ ) with no previous malaria exposure.

The ethical and methodological aspects of this study were approved by the Ethical Committee of Research on Human beings from the Centro de Pesquisas René

**Table 1** Characteristics of the three communities studied in the Brazilian Amazon area.

Characteristics	Area		
	Belém	TNN	Apiacas
Antibodies anti-DBP, $n$ /total (%)	5/36 (14%)*	18/47 (38%)	24/37 (65%)
Age (mean $\pm$ SD) <sup>†</sup>	32 $\pm$ 19	40 $\pm$ 28	32 $\pm$ 8
Exposure to malaria transmission	Sporadic	Constant	Constant
Time of malaria exposure	<1 month	~10 years	~17 years
Number of past malaria episodes	1	1–10	>10
Previous malaria episodes by	Pv	Pv and/or Pf	Pv and/or Pf

Pv = *P. vivax*, Pf = *P. falciparum*.

\*As previously detected by ELISA [15].

<sup>†</sup>Differences not significant ( $F = 1.47$ ,  $P = 0.234$ ).

Rachou/FIOCRUZ (Report 002/2002 and Report 07/2006), according to the Resolution of the Brazilian Council on Health-CNS 196/96, and by the WHO Secretariat Committee for Research Involving Human Subjects (SCRIHS).

**Antibody measurement.** Specific DBP antibody reactivities were determined by an ELISA against a recombinant protein containing region II–IV of the DBP. Detailed construction of this GST fusion protein, which represents amino acids 177–815 of DBP has been described elsewhere [13, 15]. The ELISA for total IgG antibodies was performed as described previously [15] and the recombinant protein was used at a concentration of 5 µg/ml. All samples were initially diluted 1:100 and evaluated for total IgG using peroxidase-conjugated anti-human IgG antibodies (Sigma, St Louis, MO, USA). The threshold of positivity was an OD value of 0.1, which was based on the mean plus three standard deviations (SD) reactivity of sera from 30 non-exposed subjects.

**DBP<sub>II</sub>-pEGFP constructs.** Region II of DBP from a *P. vivax* laboratory reference clone Sal-1 (DBP<sub>II</sub>V1) was previously subcloned into the pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) along with a flanking signal sequence from the herpes simplex virus glycoprotein D1 [12, 19]. This plasmid allows for expression of a recombinant fusion protein to the N terminus of the enhanced-green fluorescent protein (GFP), which is used as a transfection marker. Additional GFP constructs with two DBP region II variants, PNG-7.18 (DBP<sub>II</sub>V2) and PNG-27.16 (DBP<sub>II</sub>V3) [24], were also used in the erythrocyte-binding assays. All DBP<sub>II</sub>-pEGFP plasmids were purified by use of an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA, USA).

**COS-7 cultures and erythrocyte-binding assays.** Recombinant plasmids were transfected into Green monkey kidney cells (COS-7, American Type Culture Collection, Manassas, VA, USA) by use of lipofectamine and PLUS-reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocols. Briefly, COS-7 cells seed in six-well culture plate ( $1.5 \times 10^5$  cells/well) were transfected with plasmid (0.5 µg/well)-liposome complexes (5% Plus-reagent and 3% lipofectamine) in Dulbecco's Modified Eagle Medium (DMEM, Sigma) without serum. After 6 h of exposure to the DNA-liposome complexes (37 °C, 5% CO<sub>2</sub>), the transfection medium was replaced with DMEM plus 10% foetal bovine serum (Gibco-BRL Life Technologies, Rockville, MS, USA). 24 h after transfection, the culture medium was replaced again and the efficiency of transfection was assessed using an inverted fluorescence microscope (Nikon TE2000E, Melville, NY, USA). Over 80% of the cells demonstrated green fluorescence, indicating that they were successfully transfected.

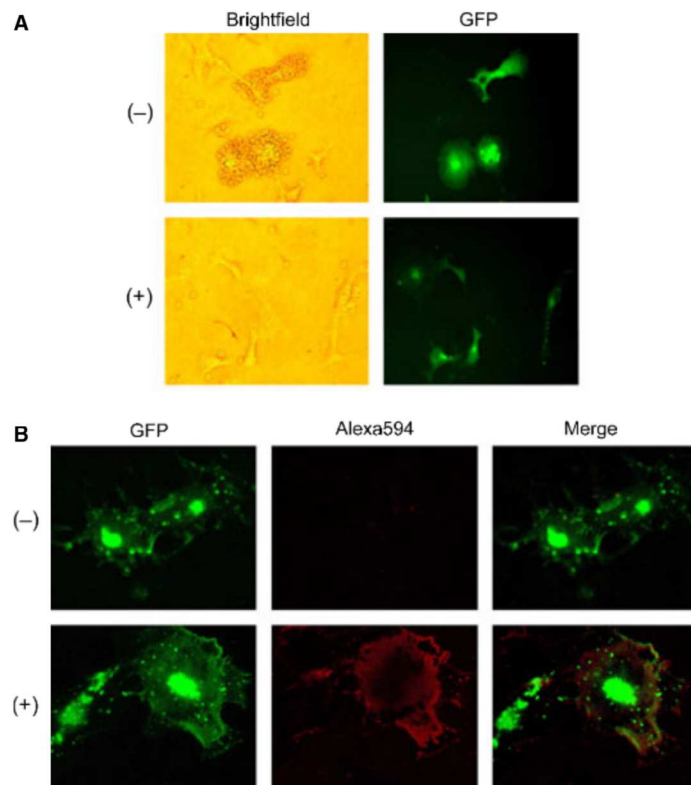
Forty-eight hours after transfection, the erythrocyte-binding assays were performed as previously described

[12]. For this, transfected COS-7 cells were incubated for 1 h at 37 °C in 5% CO<sub>2</sub> with various dilutions of the individual and/or pooled human plasmas in DMEM without serum. Human O<sup>+</sup> erythrocytes in a 10% suspension were added to each well (200 µl/well), and plates were incubated for 2 h at room temperature. Unbound erythrocytes were then removed by washing the wells three times with PBS. Binding was quantified by counting positive rosettes around GFP-positive cells within 20 fields at a magnification of  $\times 200$  (Fig. 1A). Rosettes were counted as positive when adherent erythrocytes covered more than 50% of the cell surface. To assess their inhibitory activity, all human plasmas were first tested (duplicate) at a 1:40 dilution. Serial dilutions were then tested for positive samples. Binding for each plasma dilution was compared to binding of transfected COS-7 cells incubated with negative control plasma at a 1:40 dilution (100% binding). The per cent inhibition was calculated as  $100 \times (R_c - R_t)/R_c$ , where  $R_c$  was the average of the number of rosettes in the control wells and  $R_t$  was the average of the number of rosettes in the test wells. The threshold of positivity was a 50% rosette-inhibitory activity at the 1:40 plasma dilution; this dilution was chosen because in preliminary experiments this dilution provided a wide range of inhibitory activity among different plasmas.

**Confocal fluorescence microscopy.** Under the same culture conditions as above, transfected COS-7 cells growing on glass coverslips (25 mm diameter; Fisher Scientific, Slangerup, Denmark) were placed into six-well culture plates and incubated either with plasma samples that inhibit or do not inhibit erythrocyte-binding to DBP<sub>II</sub> (1:80, 1 h at 37 °C). After washing the wells with PBS, cells were incubated (30 min at 4 °C) with goat anti-human IgG conjugated to Alexa Fluor<sup>®</sup> 594 dye (1:500; Molecular Probes, Invitrogen). Cells were then washed and coverslipped images were acquired with a LSM 510 Zeiss confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA). The signal from Alexa594-labelled conjugate is in the red region of the spectrum (absorption/emission maximum: 590/619 nm), and the signal from DBP<sub>II</sub>-GFP expressing COS-cells is in the green region (absorption/emission: 488/509 nm). The detector sensitivity and emission wavelength range were set independently for optimal special resolution of each fluorescence signal. Images were acquired using LSM 510 Zeiss software.

**Statistical analysis.** Statistical analyses were performed using the Epi-Info 2002 software (Centers for Disease Control and Prevention, Atlanta, GA, USA) and/or Mini-Tab statistical software (Minitab Inc., State College, PA, USA). Differences between two medians were compared by the non-parametric Wilcoxon rank sum test. The strength of the association between variables was analysed by the non-parametric Spearman rank correlation coefficient ( $r_s$ ). Statistical significance was defined as  $P < 0.05$ .

**Figure 1** Binding of human anti-DBP antibodies to the surface of COS cells expressing DBP<sub>II</sub>. (A) Erythrocyte-binding assay; *Upper panels* illustrate rosettes of erythrocytes on the surface of COS-7 cells transfected with DBP<sub>II</sub>-GFP after incubation with plasma, at 1:40 dilution, of a Brazilian resident characterized as a non-responder to DBP (-); *lower panels* illustrate a complete inhibition of erythrocyte binding (no-rosettes) by a human plasma characterized as an anti-DBP responder (+). Cells were observed using either brightfield or conventional epifluorescence microscopy (×200). (B) Localization by confocal microscopy of anti-DBP<sub>II</sub> IgG antibodies (red fluorescence) on the surface of COS cells expressing DBP<sub>II</sub>-GFP (green fluorescence). Cultures were incubated with plasma that either inhibited (+) or did not inhibit (-) erythrocyte-binding to DBP<sub>II</sub>, as described in (A), followed by incubation with Alexa594-labeled goat antibody to human IgG (red fluorescence). The last panels on the right illustrate overlapped images, which were acquired with a LSM 510 Zeiss confocal microscope (×630).



## Results

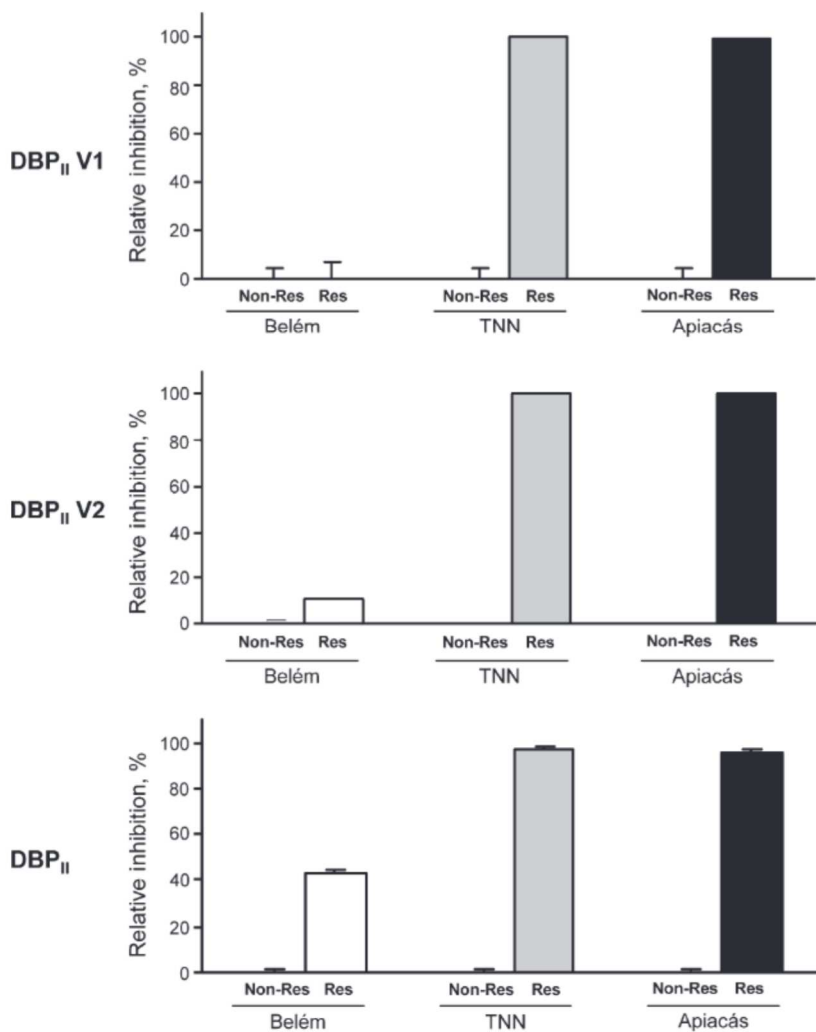
### Natural exposure to *P. vivax* in the Amazon area induces an inhibitory antibody response

A preliminary analysis was used to screen individuals in the Amazon area, which is an area with unstable malaria transmission, for exposure to *P. vivax* and to assess whether their acquired antibodies had the ability to inhibit DBP binding to erythrocytes. To measure inhibition of DBP–erythrocyte binding activity, assays were performed using transiently transfected COS-7 cells expressing DBP<sub>II</sub> variants as GFP fusion proteins (Fig. 1A). Confocal microscopy immunofluorescence analysis illustrates the specificity of the inhibitory human anti-DBP IgG, but not the non-inhibitory antibody, in transfected COS cells (Fig. 1B). Our results demonstrate that individuals with long-term exposure to malaria in the Amazon area (TNN and Apiacas) acquire inhibitory antibodies to the various DBP<sub>II</sub> variants (Fig. 2). In contrast, plasma samples from residents who had been sporadically exposed to *P. vivax* (<1 month) did not inhibit the ligand–receptor interaction. In this group, a low level of inhibition (<50%) was detected, but only for the DBP<sub>II</sub>V3 allele (Fig. 2). Taken together, these results suggest that an efficient inhibitory antibody response requires long-term exposure to malaria. No inhibition was observed using pooled plasmas at the same dilution from individuals

living in endemic areas (Belém, TNN or Apiacas) but who did not have anti-DBP antibodies (classified as non-responders), as detected by conventional serology.

### Anti-DBP antibody levels and inhibition of binding

We sought to determine whether the level of anti-DBP IgG antibodies, as detected by ELISA, correlated with the levels of binding-inhibitory antibodies to DBP<sub>II</sub>. For this, we analysed plasma samples from 17 individuals with long-term exposure to malaria in the Amazon area (TNN or Apiacas) who had been characterized as high or low responders by endpoint ELISA experiments (Fig. 3A). The efficacy of these samples to inhibit DBP–erythrocyte interactions was measured utilizing COS-7 cells expressing the DBP<sub>II</sub>V3 (Fig. 3B), and we observed a wide range of DBP–erythrocyte inhibition activity among the 17 selected individuals. Importantly, samples from the high-responder group showed significantly greater anti-DBP inhibition than samples from the low-responder group (Fig. 3C). This difference was estimated statistically as a moderate positive correlation between anti-DBP antibodies and inhibitory activity (Spearman's coefficient: 0.49;  $P < 0.05$ ) (Fig. 3D). Of great interest in our study was the fact that the levels of these inhibitory antibodies were relatively high in the Amazon area, with titres at 640–1280 in some individuals (Fig. 3B–C).



**Figure 2** Inhibition of erythrocyte binding to DBP<sub>II</sub> variants expressed in COS cells by plasma from individuals who had been sporadically (Belém) or continuously exposed to malaria (TNN and Apicás) in the Amazon area. COS-7 cells expressing each variant (DBP<sub>II</sub>V1, DBP<sub>II</sub>V2 or DBP<sub>II</sub>V3) were incubated with different pooled plasma samples, at 1:40 dilution, prior to the addition of human erythrocytes. Binding was quantified by counting rosettes around GFP positive cells within 20 fields at a magnification of  $\times 200$ ; per cent inhibition was determined relative to pooled plasma from unexposed Brazilian donors (negative control). Plasmas from Amazonian residents were designated as non-responders (Non-Res) or responders (Res) to DBP according to their previous ELISA positivity [15]. Bars correspond to the mean  $\pm$  standard deviation of two independent experiments.

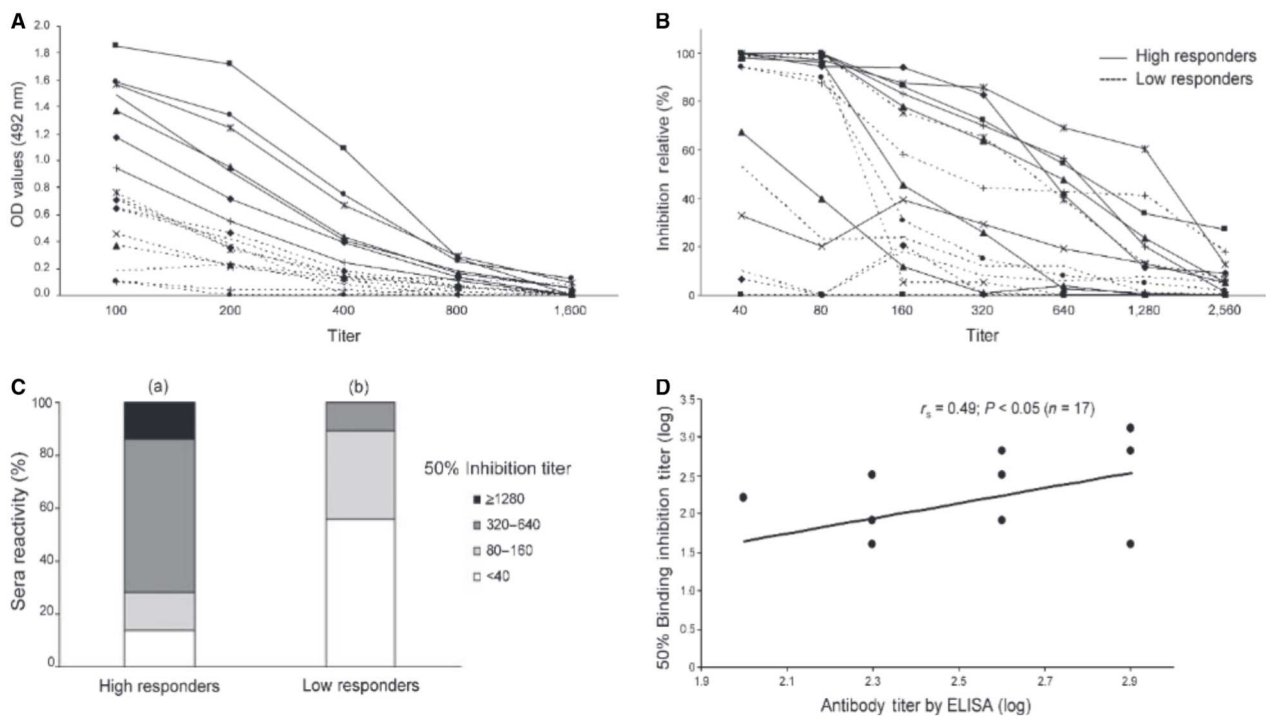
#### Asymptomatic *P. vivax* infection and inhibition of binding

To determine whether there is a relationship between the prevalence of anti-DBP and clinical illness, we analysed plasma samples from acutely infected *P. vivax* individuals who had ( $n = 10$ ) or had not ( $n = 15$ ) developed clinical malaria after a short follow-up period in the Apicás area. ELISA results demonstrated that the frequency of anti-DBP antibodies was similar between symptomatic (7/10; 70%) and asymptomatic (9/15; 60%) infections, but there was a tendency towards increased levels of anti-DBP antibodies among asymptomatic individuals (Fig. 4A). Plasmas from those individuals who were ELISA positive for anti-DBP antibodies (seven symptomatic and nine asymptomatic infections) were analysed for the efficacy of these antibodies to inhibit erythrocyte–DBP<sub>II</sub> binding. Despite the variability of individual responses, there was a non-significant tendency towards increased levels of inhibition among asymptomatic infections (Fig. 4B).

#### Discussion

The goal in developing DBP as a vaccine against blood-stages of *P. vivax* is to elicit an antibody response that inhibits the adhesion of this parasite ligand to its cognate erythrocyte receptor and thereby abrogate merozoite invasion. Unfortunately, due to the methodological constraints on performing functional assays with the limiting amount of test sera typically obtained from field studies [18], few reports have examined functional antibodies in the malaria-exposed population. To date, a single study carried out in highly endemic areas of Papua New Guinea demonstrated that individuals naturally exposed to malaria acquire antibodies that inhibit DBP binding to erythrocytes [12].

In the present study, our goal was to characterize the anti-DBP inhibitory antibody response in individuals from an area of markedly unstable malaria transmission. The transmission of malaria in the Amazon area is not homogeneous, similar to most of Latin America (where

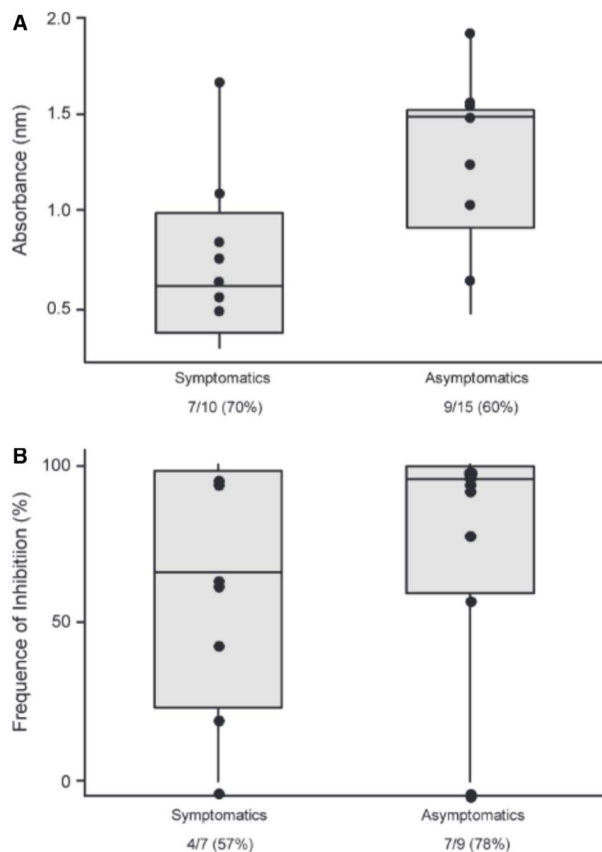


**Figure 3** Comparison between anti-DBP antibodies detected by ELISA and rosette-inhibitory activity. (A) After titration anti-DBP IgG antibodies in ELISA, individuals continuously exposed to malaria in the Amazon area (TNN and Apiacas) were classified as high ( $n = 8$ ) or low ( $n = 9$ ) responders. IgG antibody responses are expressed as optical density (OD values at 492 nm). (B) Titration of the rosette-inhibitory activity in the same plasma samples classified in (A). For this, COS cells expressing the DBP<sub>II</sub>V3 variant were incubated with different plasma dilutions of these individuals and the per cent inhibition was determined as described in Fig.1. (C) Fifty per cent binding inhibition titres were determined from curves obtained in (B) plotting the percentage of inhibition at different plasma dilutions. Different letters on the top of (C) indicate significantly different medians ( $P < 0.05$ ), as determined by the Wilcoxon Rank sum test. (D) Scatter plot showing the correlation between rosette-inhibitory activity and total anti-DBP IgG (Spearman's  $r_s = 0.49$ ). For each assay, titres were defined as the reciprocal of the plasma dilution, and log-transformed titres were used for statistical analysis.

about 1 million cases are reported per year) [25]. Vivax malaria in the Amazon basin tends to be concentrated in areas with uncontrolled establishment of rural and mining settlements, being associated with poorly maintained dwellings and favorable transmission conditions [26, 27]. In general, the exposed populations consist of migrants who are mostly from malaria-free areas. Under such a pattern of transmission, the infection is generally accompanied by clinical symptoms that vary from mild to severe. Recently, we and others have demonstrated that antibodies to DBP, as measured by ELISA, are prevalent in the Amazon area [15, 16, 28].

By using an *in vitro* assay to quantify inhibition of DBP–erythrocyte binding [12, 19], we demonstrated that natural exposure to *P. vivax* in the Amazon area induces anti-DBP antibodies that inhibit DBP–erythrocyte interactions. An efficient inhibitory antibody response was only detected in plasmas from individuals having a long-term exposure to malaria in that area (>10 years). Plasma samples from individuals sporadically exposed to *P. vivax* (Belém) could not inhibit DBP–erythrocyte binding. These results suggest that DBP is normally a poor

immunogen when presented through the ‘natural’ exposure of an infection and that chronic exposure is needed to induce a more effective inhibitory antibody response that recognizes different DBP<sub>II</sub> variants. This finding is in accordance with our previous work in the Amazon area, in which we clearly demonstrated that conventional serological responses to DBP correlate with the time of exposure to malaria transmission [15]. Although our current data suggest that an efficient inhibitory antibody response can be developed only in individuals demonstrating a long-term exposure to malaria, a longitudinal study is required to properly address this question, especially to evaluate the effect of further malaria episodes in the inhibitory antibody responses. Alternatively, it is possible that the people sporadically exposed to malaria in the Belém area were infected with different variants of DBP<sub>II</sub> that are antigenically distinct from the three alleles used in the present study. Recent findings with rabbit immune-sera support the idea that distinct strain-specific antibody response profiles can be a general phenomenon that restricts the functional immune response against DBP in an allele-specific manner [24]. These



**Figure 4** ELISA anti-DBP IgG antibodies (A) and rosette-inhibitory activity (B) among 25 individuals who were grouped based on the presence (symptomatic) or absence (asymptomatic) of clinical malaria. Initially, samples were assayed for the presence of conventional anti-DBP antibodies by ELISA (at 1:100 dilution) (A), and positive samples were evaluated for the presence of inhibitory antibody responses (1:80 dilution) (B). For each specific assay, the lines in the box-plots represent medians. Values on the bottom of figures represent the overall frequency of the response for each assay.

results point towards strain specificity in the natural immune response against DBP, which has also been suggested to another microneme protein and vaccine candidate, the apical membrane antigen 1 (AMA1) [29]. As expected, these factors can have consequences for the current efforts on vaccine development. However, the success of vaccines such as DBP will depend on how these immune obstacles can be overtaken, including strategies for improving vaccine efficacy.

In an attempt to determine whether the level of serological responses correlates with the anti-DBP inhibitory activity, we analysed plasma samples from 17 individuals chronically exposed to malaria who had been classified by conventional serology (ELISA) as high or low responders. As determined by titration experiments, a positive correlation was observed between the anti-DBP antibody level and the ability to inhibit erythrocyte binding. This is consistent with the results of the Papua New Guinea study [12], in which the titre of naturally acquired

antibodies against DBP region II correlated with their rosette-inhibitory activity. However, it is of relevance that our study clearly showed a wide range of inhibitory antibody responses among the study participants. In fact, there were a number of samples with low anti-DBP antibodies and high rosette-inhibitory activity and vice versa. Although it is possible that this individual variability in the inhibitory immune response may be a characteristic of unstable malaria transmission areas, this conclusion remains uncertain as pooled samples were used in the PNG study [12], which may have masked individual variability in the inhibitory antibody response. The variability in anti-DBP inhibitory activity observed here is consistent with the results of Ohas et al. [30] using region II of *P. falciparum* EBA-175; these authors demonstrated a wide range of functional antibody responses among lifelong residents of a malaria holoendemic area in western Kenya.

Despite a wide range in the anti-DBP inhibitory activity among study participants, the titres of inhibitory antibodies in the Amazon area were relatively high in some individuals whose inhibitory activity could still be detected at a 1280 titre. Given the scarcity of data from the human population, this profile of inhibitory immune responses to DBP<sub>II</sub> can only be compared with those results obtained from experimental models. In mice, studies of the immunogenicity of a recombinant DPB<sub>II</sub> formulated with human compatible adjuvant demonstrated that maximum inhibitory activity was obtained with immune sera diluted up to 1:1800 [31]. So far, vaccination of monkeys with a formulation of DPB<sub>II</sub> suitable for human use (montanide ISA-720) did not induce significant levels of inhibitory antibody response; the 50% inhibition titres ranged from 1:16 to 1:366 [32]. Therefore, it is very intriguing that in the Amazon and PNG a strong inhibitory immune response to DBP is acquired under natural conditions.

The occurrence of asymptomatic malaria in Latin America is unusual, and only recently has been documented in detail among Amazonians living in remote riverine settlements [33, 34]. Considering the vastness of the Amazon region and the remoteness of some of its riverine settlements, the plasma samples available from a small group of asymptomatic *P. vivax*-infected individuals, previously identified in the Apiacas area [21], offered an exceptional opportunity to investigate the relationship between malaria symptoms and the functional immune response to DBP<sub>II</sub> *in vitro*. Although the percentage of responders was similar between groups, there was a trend in the levels of conventional as well as inhibitory antibodies to be higher in the asymptomatic group than in the symptomatic group; however, these differences did not reach the level of significance. Unfortunately, the size of our sample could not be increased in the Apiacas area; geographical access and political conflicts – which seems

to be common in the Brazilian Amazon area – hamper these studies in the endemic Apiacas area. A long-term prospective study is needed to determine the protective nature of the inhibitory anti-DBP<sub>II</sub> antibodies in terms of anti-disease immunity.

Taken together, our results demonstrate that individuals from unstable malaria transmission areas acquire anti-DBP antibodies that inhibit *in vitro* binding of different DBP<sub>II</sub> variants to erythrocytes. It is not yet clear whether this finding correlates with increased levels of protection. We believe that this first study on the profile of immune response to DBP<sub>II</sub> variants in the population of the Amazon area will contribute to current efforts on vaccine development, and may facilitate future clinical trials in areas of unstable malaria transmission.

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### Authors' contributions

I. P. Ceravolo and F. A. Souza-Silva were involved in all stages of this study. E. M. Braga and A. U. Krettli were involved in the initial design of the field study. C. J. F. Fontes and J. M. Souza participated in the coordination of the field work. C. A. Brito and J. H. Adams helped to compose the manuscript and gave substantial constructive advice during the project. A. P. Madureira performed statistical analysis and contributed to the interpretation of data. L. H. Carvalho accepts direct responsibility for the conception, design and coordination of the study. All authors read and approved the manuscript.

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