Asymptomatic infection in individuals from the municipality of Barcelos (Brazilian Amazon) is not associated with the anti-*Plasmodium falciparum* glycosylphosphatidylinositol antibody response

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Anti-glycosylphosphatidylinositol (GPI) antibodies (Abs) may reflect and mediate, at least partially, anti-disease immunity in malaria by neutralising the toxic effect of parasitic GPI. Thus, we assessed the anti-GPI Ab response in asymptomatic individuals living in an area of the Brazilian Amazon that has a high level of malaria transmission. For comparative purposes, we also investigated the Ab response to a crude extract prepared from Plasmodium falciparum, *the merozoite surface protein (MSP)3 antigen of* P. falciparum *and the MSP 1 antigen of* Plasmodium vivax *(PvMSP1-$_{19}$) in these individuals and in Angolan patients with acute malaria. Our data suggest that the Ab response against* P. falciparum *GPI is not associated with* P. falciparum *asymptomatic infection in individuals who have been chronically exposed to malaria in the Brazilian Amazon. However, this Ab response could be related to ongoing parasitaemia (as was previously shown) in the Angolan patients. In addition, our data show that PvMSP1-$_{19}$ may be a good marker antigen to reflect previous exposure to* Plasmodium *in areas that have a high transmission rate of* P. vivax.

Key words: *Plasmodium falciparum* - *Plasmodium vivax* - glycosylphosphatidylinositol -
asymptomatic infection - Brazilian Amazon - Angola

Glycosylphosphatidylinositol (GPI), a glycolipid that is ubiquitously present in eukaryotic cells, has been implicated in the pathogenesis of many parasitic diseases (Debierre-Grockiego 2010). In malaria, the GPI structures of *Plasmodium falciparum* are conserved among isolates (Berhe et al. 1999). This molecule is released at the end of the schizogonic process and induces an important host immune response, which includes the production of pro-inflammatory cytokines, such as tumour necrosis factor-α, interleukin (IL)-1 and IL-6 and the augmented expression of cell adhesion molecules on macrophages and vascular endothelial cells (Schofield et al. 1996). The clinical presentation of the toxaemic syndrome that accompanies the septicaemia caused by Gram-negative bacteria (van Amersfoort et al. 2003) and the usual triad of clinical symptoms of malaria (fever, chills and sweating, which are commonly accompanied by headache) are similar and can result from the pro-inflammatory responses that are induced by lipopolysaccharide and *P. falciparum* GPI, respectively. These pro-inflammatory responses can also account for the development of complications, such as severe anaemia and the cerebral form of malaria. Indeed, the administration of *P. falciparum* GPI in mice induces malaria-like symptoms (Schofield et al. 1993) and immunisation with a non-toxic moiety of GPI that elicits an anti-GPI antibody (Ab) response can prevent malaria pathology and death caused by experimental *Plasmodium berghei* ANKA [because of the discovery of this murine African *Plasmodium* in Katanga (NK for New York-Katanga) and in Kasapa (ANKA for Antwerpen-Kasapa (Vincke & Bafort 1968)] infection in mice (Schofield et al. 2002).

Studies performed in areas of high malaria transmission in Africa and Indonesia have demonstrated the potential protective role of the anti-GPI response in malaria (Naik et al. 2000) and the association of the anti-GPI re-

sponse with age (Naik et al. 2000, Keenihan et al. 2003). According to these studies, anti-GPI Ab would reflect and mediate, at least partially, anti-disease immunity in malaria by neutralising the toxic effect of parasitic GPI (de Souza et al. 2010). However, studies by Boutlis et al. (2002) and Cissoko et al. (2006) found no relationship between anti-GPI Ab levels and clinical protection from the disease. Here, we investigate the anti-GPI Ab response in asymptomatic individuals living in Barcelos, an area of the Brazilian Amazon with high endemic levels of malaria. Twenty-eight asymptomatic individuals ranging from three-50 years of age (mean = 22) with either *P. falciparum* (n = 13) or *Plasmodium vivax* (n = 15) infections and 12 non-infected individuals ranging from three-66 years of age (mean = 16) with no declared previous history of malaria were recruited in Barcelos, which has an annual parasitic index (API) of 70.9 cases of malaria per 1,000 inhabitants. In a fieldwork conducted from 2004-2007 (Suárez-Mutis 2007), these asymptomatic individuals tested positive for the presence of *P. falciparum* and *P. vivax* DNA by polymerase chain reaction (PCR) (Snounou 1996). None of these asymptomatic individuals were positive when tested by microscopic examination at the time of blood collection or presented any symptoms during the 30 days preceding or the 30 days succeeding the PCR diagnosis; none of these individuals received anti-malarial treatment during this timeframe. All individuals reported previous malaria attacks (3-12; mean = 7.3) from both *P. falciparum* and *P. vivax*. Comparatively, we also studied 97 individuals ranging from 15-67 years of age (mean = 26) with non-complicated *P. falciparum* malaria that was diagnosed by microscopic examination (parasitaemia ranging from 500-3,000; mean = 1,320 parasites/µL). These individuals lived in Lubango, which is an area of intense malaria transmission in Angola (API = higher than 100 cases per 1,000 inhabitants, depending on the season) and were diagnosed and treated at the Central Lubango Hospital. Brazilian individuals living in the non-endemic southeast region that had never visited malaria-endemic areas were included in the study to establish a threshold (or cut-off) value for ELISA detection. The statistical analysis was performed using Epi Info® software v. 7 (Centers for Disease Control and Prevention). This study was approved by Brazilian (Fiocruz, state of Rio de Janeiro, CEP157/02) and Angolan Ethical Committees (National Malaria Control Program).

The presence of anti-GPI Ab in plasma samples was detected by ELISA (Naik et al. 2000) using *P. falciparum* GPI extracted from in vitro culture; the extraction of GPI was done according to the method described by Berhe et al. (1999) that maintains the GPI structure required for the recognition of the native molecule. Briefly, because late trophozoites (30-40 h) have been shown to have the largest GPI pool (Schmidt et al. 1998), GPI was extracted from 1 x 10^9 trophozoites in a one-step process that allowed the simultaneous extraction of all GPI intermediates and free GPI anchors. A mixture of chloroform:methanol (1:1, v:v) was added to the sediment so that the final proportion of solvents (which comprised the aqueous phase of the crude parasite GPI extract) was 10:10:3 (v:v:v) chloroform:methanol:water (C:M:W). The suspension was sonicated for 5 min and then centrifuged (18,000 *g* at 25ºC for 5 min). The supernatant was collected and the sediment was subsequently extracted two more times with 1 mL C:M:W (10:10:3, v:v:v). All of the supernatants were pooled and dried in a SpeedVac vacuum evaporator to obtain C:M:W erythrocytic extracts. To remove water-soluble contaminants, such as nucleotides and phosphate sugars and other lipids, such as phospho and sphingolipids, "Folch washing" was performed (Folch et al. 1957, Field & Menon 1992). The lipid extract was then resuspended in 1:1 (v:v) C:M and 1/5 volume of 4 mM $MgCl_2$ was added. The suspension was mixed and centrifuged (18,000 *g* at 25ºC for 5 min). The upper phase was removed and the organic phase was washed twice with 1/2 volume of an artificial upper phase (C:M:W:1 M $MgCl_2$, 6:96:94:0.336, v:v:v:v). The C:M extracts were vacuum-dried and submitted to a butanol:water phase partition. The dried C:M extracts were resuspended in 200 µL water-saturated n-butanol and then the same volume of water was added. After mixing, the extracts were centrifuged (18,000 *g* at 25ºC for 5 min) and the aqueous phase was removed. This process was repeated twice. The aqueous phases were then pooled and back-washed with 200 µL water-saturated n-butanol. Finally, the butanol phases were pooled, yielding the entire *P. falciparum* GPI content (Gerold et al. 1994), which was used as an antigen in this study.
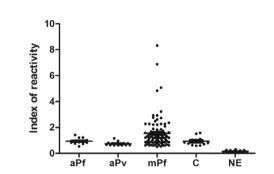
Ideally, after the collection of the butanol phases, the GPI extraction is followed by an additional purification step using metabolic [³H] glucosamine to label the GPI for high-performance liquid chromatography. However, [³H] glucosamine was not available at our institution due to environmental restrictions. [³H] glucosamine has a half-life of approximately 13 years and is potentially harmful to health and the environment. Because GPIs are, so far, the only known glycoconjugates present in *P. falciparum* parasites (Gerold et al. 1994, Schmidt et al. 1998, Berhe et al. 1999, Azzouz et al. 2008) and because the solvent specificity for GPI extraction (C:M:W) together with the subsequent washing step assured the elimination of eventual contaminants (Folch et al. 1957, Field & Menon 1992), we decided to use the *P. falciparum* GPI pool without an additional radioactivity purification step. To circumvent false-positive ELISA results, additional "GPI controls", obtained from non-parasitised erythrocytes (treated or not with saponin and submitted to the same extraction protocol), were utilised; only one saponin-treated and two non-saponin-treated non-parasitised erythrocyte samples out of 137 were found to be positive.

There are no reports of *P. vivax* GPI extraction and asymptomatic *P. vivax*-infected individuals were also tested for *P. falciparum* GPI because they had previous *P. falciparum* infections. ELISA positivity was calculated using the index of reactivity (IR) ratio (the absorbance value of each sample divided by the cut-off value; the samples with a ratio of ≥ 1 were considered positive). The cut-off value for each reaction was calculated as the mean plus two times the standard deviation of a sera panel from 20 clinically healthy individuals who neither lived in nor visited malaria-endemic areas.

The frequency and reactivity of anti-GPI Ab in individuals from Barcelos with asymptomatic *P. falciparum* infection were not significantly different (p > 0.05) from those recorded in non-infected individuals from the same area without a previous history of malaria (Figure, Table). The same was true for *P. vivax* asymptomatic carriers. Because GPI levels decline with time after infection, we divided the individuals from Barcelos into three groups according to the time elapsed after their last malaria attack caused by *P. falciparum* infection (≤ 2 months, > 2 and ≤ 6 months and > 6 and ≤ 12 months) (n = 28). The anti-GPI Ab levels in these three groups were then analysed. Anti-GPI Ab levels ranged from 1.01-1.42 IR (mean = 1.2) and were independent of the time of infection. Therefore, this parameter does not seem to be relevant in determining the anti-GPI Ab level. Conversely, patients from Lubango with *P. falciparum* malaria had a significantly higher anti-GPI Ab frequency (p < 0.0001, analysis of variance) and reactivity (p < 0.0001, Student's *t* test) (Figure, Table). These contrasting data do not support the hypothesis that the anti-GPI Ab level reflects clinical immunity to malaria (Naik et al. 2000, de Souza et al. 2002, Keenihan et al. 2003), but are in accordance with the conclusions of Boutlis et al. (2002) and Cissoko et al. (2006). Although the anti-GPI response was not higher in asymptomatic infection, this response seemed to be related to age; positive individuals were older (33.8 ± 7.2) than negative individuals (18.6 ± 14.5) (p < 0.03, Student's *t* test), indicating a possible association with repeated and chronic infection, which accompany aging in endemic areas. Indeed, the anti-GPI response tended to correlate with the number of past episodes of malaria, even though this correlation did not reach statistical significance; positive individuals had more previous malaria attacks (8.8 ± 2.7) than the negative individuals (6.0 ± 3.2) (p < 0.08, Student's *t* test). Moreover, it has already been reported that the prevalence and concentration of anti-GPI Ab in hyperendemic areas of malaria increase with age (Naik et al. 2000, Boutlis et al. 2002, de Souza et al. 2002). Conversely, in the symptomatic malaria patients from Lubango, the GPI immune response was independent of age, but was positively correlated with parasitaemia (r = 0.398; p = 0.010, Spearman's rank correlation), which could have resulted

from a booster effect of parasitic antigens during acute infection. Similar observations have been described by de Souza et al. (2002) and Cissoko et al. (2006), who reported that in cases of clinical malaria, anti-GPI Ab can be augmented in a parasitaemia-dependent manner. Considering our results here, we hypothesise that in the patients with uncomplicated malaria from Lubango, GPI Abs could protect against the development of severe malaria because circulating anti-GPI Abs can neutralise the toxic effects of parasitic GPI (Naik et al. 2000).

To further characterise the previous exposure of the studied individuals to *Plasmodium*, we also evaluated the Ab response to a crude *P. falciparum* extract by ELISA [obtained according to Sanchez et al. (1993)] as well as to the recombinant merozoite surface proteins (MSP) of *P. falciparum* (*Pf*MSP3) (a kind gift from Pierre Druilhe from Vac-4-All) and *P. vivax* (*Pv*MSP1-$_{19}$) (produced by Arianni Rondelli Sanchez and Eduardo Milton Ramos-Sanchez at the Institute of Tropical Medicine, state of São Paulo, Brazil), as described elsewhere (Theisen et al. 2001, Cunha et al. 2001, respectively). The Ab levels to all antigens in the asymptomatic individuals were

Antibody (Ab) reactivity against *Plasmodium falciparum* glycosylphosphatidylinositol (GPI) in serum samples from asymptomatic *P. falciparum* (aPf) and *Plasmodium vivax* (aPv) infected and control (C) individuals from Barcelos (Brazilian Amazon), as well as from malarious *P. falciparum* (mPf) patients from Lubango (Angola). Samples were diluted at 1:100 and the Ab reactivity to *P. falciparum* GPI (IgG) was detected by ELISA. Individuals never exposed (NE) to malaria were used as negative controls.

TABLE

Frequency of antibodies (Abs) detected by ELISA against glycosylphosphatidylinositol (GPI), the crude extract (*Pf*Ag) and the recombinant protein merozoite surface proteins (MSP) of *Plasmodium falciparum* (*Pf*MSP3) and the recombinant protein of *Plasmodium vivax* (*Pv*MSP1-$_{19}$)

Groups	Frequency of Abs (%)			
	GPI	*Pf*Ag	*Pf*MSP3	*Pv*MSP1-$_{19}$
Asymptomatics (Barcelos)	18	96	46	86
Non-infected controls (Barcelos)	42	50	42	17
Malaria patients (Lubango)	65	70	44	2

significantly related to age (crude antigen: r = 0.523, p = 0.004; PfMSP3: r = 0.386, p = 0.04 and PvMSP1-$_{19}$: r = 0.496, p = 0.007, Spearman's rank correlation), indicating that, in a scenario of supposed premunition to malaria in areas of intense disease transmission (including those in the Brazilian Amazon), antiparasitic responses can be associated with age and the consequent cumulative exposure to infection. This idea has been classically reported for the hyperendemic areas of Africa (Soe et al. 2001, Dodoo et al. 2008). In Brazilian endemic areas, it has also been shown that the Ab response to PvMSP1-$_{19}$ increases with age and/or time of exposure to malaria (Braga et al. 2002, Nogueira et al. 2006). Additionally, the Ab response has even been linked to the absence of clinical symptoms (Nogueira et al. 2006). However, in the African patients with acute *P. falciparum* malaria, the Abs against the *P. falciparum* extract and PfMSP3 were associated with parasitaemia, supporting our data obtained for GPI that suggest a rather non-protective immune response linked to acute infection.

An interesting issue raised in our study was the observation of a high prevalence of Ab to *P. falciparum* blood-stage antigens, including PfMSP3, among the control individuals from Barcelos (Table). This was the first time it was studied in a Brazilian endemic area. These individuals presenting Ab to MSP3, but not Ab to GPI never reported previous malaria episodes nor were found to be infected with plasmodia by PCR analysis. The similar positivity of anti-PfMSP3 Ab in both Brazilians who had been exposed to infection, but did not have a previous history of malaria infection and Angolans who presented with malaria with positive thick blood smears is quite surprising and could be explained in a number of ways: (i) the Brazilian individuals could have had atypical malaria attacks that were not recognised as classical manifestations of the disease, (ii) PfMSP3 could have cross-reacted with molecules from other microorganisms that are present in the region or (iii) the anti-PfMSP3 Ab response might not have accurately reflected earlier exposure of the African individuals to the parasite.

In this study, the anti-PvMSP1-$_{19}$ Ab response seems to adequately reflect previous experience with *P. vivax*. These observations are in agreement with a study conducted in another malarious Brazilian region (Cotijuba, Belém, state of Pará) with exclusive transmission of *P. vivax*, in which 50% of individuals with no past history of the disease presented Abs against the blood stages of *P. vivax*, as measured by indirect fluorescent antibody test, while only approximately 6% of these individuals presented Abs against PvMSP1-$_{19}$, as measured by ELISA (Soares et al. 1999). In addition to the low prevalence of anti-PvMSP1-$_{19}$ Ab (17%) recorded in the control subjects from Barcelos, we observed a very low prevalence (2%) of these Abs among African patients living in areas where *P. falciparum* is highly prevalent. In contrast, these Abs were present in 86% of the asymptomatic infected subjects from Barcelos, where *P. vivax* is the major malaria-causing species. These data suggest that the assessment of positivity to Abs reacting with PvMSP1-$_{19}$ could be valuable to define exposure to *P. vivax* in endemic areas.

In summary, the data presented here, which for the first time address the anti-*P. falciparum* GPI immune response in Brazil, indicate that the Ab response against *P. falciparum* GPI is not associated with asymptomatic infection in individuals who have been chronically exposed to malaria in the Brazilian Amazon. These data are in agreement with data from other African endemic areas (de Souza et al. 2002, Cissoko et al. 2006). Supplementary studies with a higher number of asymptomatic individuals comparatively to patients with acute malaria from the same origin will increase the understanding of the relationship between anti-GPI Ab responses and asymptomatic infection.

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