The Duffy binding protein as a key target for a *Plasmodium vivax* vaccine: lessons from the Brazilian Amazon

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Plasmodium vivax *infects human erythrocytes through a major pathway that requires interaction between an apical parasite protein, the Duffy binding protein (PvDBP) and its receptor on reticulocytes, the Duffy antigen/ receptor for chemokines (DARC). The importance of the interaction between PvDBP (region II, DBPII) and DARC to* P. vivax *infection has motivated our malaria research group at Oswaldo Cruz Foundation (state of Minas Gerais, Brazil) to conduct a number of immunoepidemiological studies to characterise the naturally acquired immunity to PvDBP in populations living in the Amazon rainforest. In this review, we provide an update on the immunology and molecular epidemiology of PvDBP in the Brazilian Amazon - an area of markedly unstable malaria transmission - and compare it with data from other parts of Latin America, as well as Asia and Oceania.*

Key words: malaria - *Plasmodium vivax* - Duffy binding protein - naturally acquired antibodies - genetic variability

Plasmodium vivax malaria in the Brazilian Amazon - Malaria is still a major public health problem in Brazil, with 244,000 cases registered in 2012 (WHO 2013), with 99.9% of them distributed through the Amazon Basin, including the states of Pará, Rondônia, Amazonas (AM), Mato Grosso, Amapá, Acre and Roraima (saude.gov.br/sivep_malaria). In these areas, the spatial distribution of malaria is not homogenous and changes over time at individual, community, state and national scales (Tauil & Daniel-Ribeiro 1998, de Castro et al. 2006, 2007, Barbieri & Sawyer 2007, da Silva-Nunes et al. 2008, Oliveira-Ferreira et al. 2010). In the Brazilian Amazon, the instability of transmission is the dominant feature of malaria (Camargo et al. 1994), with exposed populations consisting mostly of migrants from malaria-free areas. In these individuals, the infection is generally accompanied by clinical symptoms of variable degrees of intensity. Nevertheless, during the past few years, epidemiologic studies carried out among individuals with long-term exposure to malaria in Brazil clearly show the existence of symptomless malaria infections (Camargo et al. 1999a, Alves et al. 2002, da Silva-Nunes et al. 2008, Ladeia-Andrade et al. 2009).

Of the five species of malaria parasites known to infect humans, three species occur in Brazil: *P. vivax, Plasmodium falciparum* and *Plasmodium malariae*. Until 1990, the prevalence of *P. falciparum* and *P. vivax* infections was similar with roughly 50% of each species (Marques et al. 1986, Tauil & Daniel-Ribeiro 1998, Loiola et al. 2002), while the prevalence of *P. malariae* was very low. After that time, the incidence of both *P. falciparum* and *P. vivax* has decreased, probably due to the intensification of malaria control measures, which included early diagnosis and treatment (Loiola et al. 2002). However, certain features of the biology of *P. vivax* give this species greater resilience than *P. falciparum*. Whereas *P. falciparum* parasites invade blood cells at various stages of development, *P. vivax* infects reticulocytes and the latter parasite species seems to be more transmissible at low parasite densities (Boyd & Kitchen 1937). Furthermore, *P. vivax* parasites are associated with the early appearance of infective sexual stages (gametocytes) in the blood and can remain in the liver as dormant hypnozoites responsible for relapses. These unique characteristics of the biology of *P. vivax* make its management and elimination particularly challenging. In fact, as control measures become more effective, the residual malaria burden is increasingly shifting towards *P. vivax* malaria (Mendis et al. 2001). Consequently, the number of *P. vivax* cases has increased over the years and this malaria parasite species is now responsible for roughly 80% of all malaria cases in the Brazilian Amazon Region (Camargo et al. 1999b, Ladeia-Andrade et al. 2009, WHO 2012). Although *P. vivax* malaria is often regarded as benign due to its low mortality, its morbidity is high, reducing the prosperity of affected populations. Of note, in the last few years, complicated *P. vivax* clinical malaria has been reported around the world (Tjitra et al. 2008, Anstey et al. 2012), including the Brazilian Amazon area (Alexandre et al. 2010, Lacerda et al. 2012).

Finally, in the Amazon, local *P. vivax* populations are extremely genetically diverse and also show substantial genetic differentiation among populations (Ferreira et al. 2007, Rezende et al. 2009, 2010, Orjuela-Sanchez et al. 2010, Sousa et al. 2010). Beyond the complexity of parasite population, it has been proposed that asymptomatic parasite carriage and massive environmental

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changes - that affect vector abundance and behaviour - are major contributors to malaria transmission in the epidemiologically diverse areas across the Amazon Basin (da Silva-Nunes et al. 2012). It may explain why malaria has proved so difficult to control in the Amazon Basin, where transmission rates remain far below those recorded in tropical Africa.

The rationale behind P. vivax Duffy binding protein (PvDBP) *as a blood-stage vaccine candidate* - The invasion of red blood cells (RBCs) by *Plasmodium* merozoites - an essential event in the life cycle of all malaria parasites - is a highly complex, multistep process that is dependent on a cascade of specific molecular interactions (Gaur et al. 2004). Despite this complexity, time-lapse microscopy of live parasites demonstrates that parasite entry into RBCs is a rapid process that is completed, on average, within 30 s after primary contact of the merozoite (Gilson & Crabb 2009). This multistep invasion process requires coordinated activities of host cell attachment, reorientation placing the apical end of the parasite adjacent to the erythrocyte membrane and active penetration of the host cell. Central to this process is the establishment of a structure called a tight or moving junction, which forms a tight connection between the invading parasite and host cell membranes (Aikawa et al. 1978). For *P. vivax*, the formation of this irreversible junction is mediated by the PvDBP, a protein of approximately 140 kDa localised in merozoite apical organelles called micronemes (Wertheimer & Barnwell 1989, Adams et al. 1990, 1992, Fang et al. 1991). During invasion, PvDBP is secreted from the micronemes and binds to its cognate host receptor on the reticulocyte surface, the Duffy antigen/receptor for chemokines (DARC) (Wertheimer & Barnwell 1989, Adams et al. 1990). Although *P. vivax* can infect and cause disease in DARC-negative individuals (Ryan et al. 2006, Cavasini et al. 2007, Menard et al. 2013), this situation seems to be rare and/or occur only in specific areas; so far, no other alternative ligand for *P. vivax* binding to reticulocytes has been identified, which makes the PvDBP one of the most promising *P. vivax* vaccine targets.

PvDBP belongs to the Duffy binding-like erythrocyte-binding protein (DBL-EBP) family, which encompasses other micronemal proteins, such as the DBP of the simian malaria parasite *Plasmodium knowlesi* and the *P. falciparum* ligands EBA-175, EBA-181/JESEBL and EBA-140/BAEBL (Sim et al. 1990, Adams et al. 1992, 2001, Mayer et al. 2001, Gilberger et al. 2003). The members of the DBL-EBP family share a similar gene structure and this homology was used to define six extracellular regions (I-VI) followed by a type I transmembrane domain and a short cytoplasmic tail (Adams et al. 1992, 2001). Common to all EBPs are the two cysteine-rich domains (regions II and VI) in the ectodomain, with the erythrocyte ligand-binding domain lying within region II (DBPII) (Fig. 1). In *P. falciparum*, the EBPs and the reticulocyte-binding-like protein homologues play an important role in phenotypic variation, allowing different parasite isolates to utilise alternative erythrocyte invasion pathways (Orlandi et al. 1992, Rayner et al. 2000, Triglia et al. 2001, Duraisingh et al. 2003). Until recently, the gene that encodes PvDBP was described as single copy gene (Carlton et al. 2008). However, new whole genome sequences from field isolates provides evidence for a duplication of the *dbp* gene in *P. vivax* (Menard et al. 2013). Interestingly, the frequency of the *dbp* duplication was highest in geographical regions where the highest frequencies of *P. vivax*-infected Duffy-negative people were reported. These data suggest that PvDBP is rapidly evolving, possibly in response to constraints imposed by erythrocyte DARC-negativity in some human populations.

The PvDBP ligand domain (DBPII) is a 330 amino acid (aa) region characterised by 12 conserved cysteine residues (Chitnis & Miller 1994). The critical binding residues have been mapped to a central 170-aa stretch that includes cysteines 4-7 (Ranjan & Chitnis 1999, Singh et al. 2003, VanBuskirk et al. 2004b, Hans et al. 2005, Batchelor et al. 2011, Bolton & Garry 2011, Sampath et al. 2013). This is the minimal domain responsible for binding to DARC-positive human reticulocytes. In 2006, the structure of the *P. knowlesi* DBL domain was determined by X-ray crystallography and characterised as an all-helical, monomeric module containing 12 helices spread over three distinct subdomains (SD1-SD3) that are stabilised by intra-SD disulfide bridges (Singh et al. 2006). Essential and invariant residues required for recognition of DARC on human erythrocytes were identified within a region on SD2 (Singh et al. 2003, 2006, VanBuskirk et al. 2004b, Hans et al. 2005). Recently, the crystal structure of DBPII was elucidated and a model was proposed of receptor recognition through PvDBP dimerisation upon receptor binding, leading to the formation of a complex composed of two PvDBP and two DARC molecules (Batchelor et al. 2011). Despite the conserved nature of regions spanning the DARC-binding groove and dimer interface, many residues in DBPII are variable and these polymorphisms map to multiple non-functional regions of the protein (Tsuboi et al. 1994, Ampudia et al. 1996, Xainli et al. 2000, Kho et al. 2001, VanBuskirk et al. 2004b, Sousa et al. 2006, Gosi et al. 2008, Babaeekho et al. 2009, Batchelor et al. 2011, Premaratne

Fig. 1: schematic drawing of the *Plasmodium vivax* Duffy binding protein gene structure. Exons are represented as blocks and drawn to scale. Exon 1 encodes a peptide signal sequence, exon 3 encodes a transmembrane domain and exons 4 and 5 encode a cytoplasmic domain. Exon 2 encodes a large protein domain that contains six regions (Roman numerals) as defined by amino acid (aa) sequence identity to Duffy binding-like erythrocyte-binding protein of other *Plasmodium* species (Adams et al. 1992). The erythrocyte-binding domain lies in the 5' cysteine-rich region (region II) and the critical binding residues have been mapped to a 170-aa stretch between cysteines 4-7 (Ranjan & Chitnis 1999).

et al. 2011, Chenet et al. 2012, Ju et al. 2012, 2013). Some of these naturally-occurring polymorphisms flank critical residues and it is suggested that protective antibodies that target the functional regions in DBPII lead to disruption of dimerisation and/or prevention of receptor binding (Batchelor et al. 2011). This pattern of diversity is consistent with strong immune selection pressure on DBPII and suggests that allelic variation is an important mechanism of immune evasion (Tsuboi et al. 1994, Cole-Tobian & King 2003, Martinez et al. 2004, Sousa et al. 2010). Although PvDBP is a vital ligand for blood-stage infection, its use for vaccine development poses significant challenges. These include: (i) brief exposure of PvDBP to the host immune system, due to its micronemal location (Adams et al. 1990) and the rapid kinetics of parasite invasion (Dvorak et al. 1975, Gilson & Crabb 2009) and (ii) polymorphisms in PvDBP, which seem to be critical for the evasion of host immune response (VanBuskirk et al. 2004a), as further discussed below.

Naturally-acquired antibodies against PvDBP - The goal in developing PvDBP as a vaccine directed against asexual blood-stage of *P. vivax* is to elicit an antibody response that inhibits parasite adhesion to DARC-positive human reticulocytes and thereby abrogate merozoite invasion. Unfortunately, the available data on the functional properties of anti-PvDBP antibodies in human populations are still limited (Michon et al. 2000, Ceravolo et al. 2008, King et al. 2008, Souza-Silva et al. 2010, Chootong et al. 2012), partly due to constraints on performing in vitro functional assays in the absence of a continuous culture method for *P. vivax* blood-stages (Ntumngia et al. 2012). Consequently, many field studies of immunity to PvDBP have focused on measuring antibodies to recombinant antigens, but paid less attention to approaches that evaluate functionally important immune mechanisms.

Anti-PvDBP IgG antibodies measured by conventional serology - Given the limitations of performing functional assays, ELISAs using different recombinant PvDBP proteins have been useful in evaluating the level of PvDBP IgG antibodies in different endemic populations. Using conventional serology, we demonstrated that PvDBP is naturally immunogenic in the Amazon area and that the proportions of PvDBP IgG-positive subjects increased with exposure to malaria, reaching a peak in those subjects with long-term exposure in the Amazon (Table) (Ceravolo et al. 2005). Of importance, this study provided an additional insight by demonstrating for the first time that cumulative exposure is a determinant that acts independently of host age in the generation of anti-PvDBP IgG response. In fact, we demonstrated that each additional year of exposure to malaria increased the probability of having anti-PvDBP IgG antibodies by 2% (Souza-Silva et al. 2010). While these previous studies included subjects who were migrants from malaria-free areas of Brazil - whose ages did not correlate with exposure to malaria - further studies were carried-out with native populations of the Amazon area (Kano et al. 2012). In this area, a well-established frontier settlement located in AM, a significant proportion of the community (50%) had acquired anti-PvDBP antibodies, with the subject's age being the only strong predictor of seropositivity to PvDBP. Together, these data reinforce the variety of malaria transmission patterns in communities from the Amazon area.

So far, few studies have investigated anti-PvDBP antibody response in Latin America and data are still restricted to endemic areas of Brazil and Colombia (Table). In general, the pattern of antibody response described in these studies corroborated our data that anti-PvDBP antibodies increase with exposure to *P. vivax*. In addition, PvDBP antibodies seem to be biased toward the cytophilic subclasses IgG1 and IgG3 (Tran et al. 2005, Maestre et al. 2010).

From our experience in the Amazon area, it has become evident that PvDBP has relatively low immunogenicity, similar to what has been described in other epidemiological contexts. For example, in the Colombian Pacific coastal region, an area of unstable malaria transmission and mainly composed of Afro-Colombian individuals, as well in the Caribbean Coast, less than 40% of the total number of patients sampled responded to PvDBP (Michon et al. 1998, Herrera et al. 2005, Maestre et al. 2010). This antibody response profile is quite different from those described in highly endemic areas for malaria, such as Papua New Guinea (PNG), where antibody responses to PvDBP seem to be much more common (60-80%) (Table) and reach a plateau at ages of 15 years and older (Xainli et al. 2003). In the latter endemic regions, the proportion of individuals developing T cell responses to PvDBP increased rapidly within the first four years of life such that by five-nine years of age 80% of children responded (Xainli et al. 2002). The cellular response against PvDBP has not yet been evaluated in Latin America.

DBPII binding inhibitory antibodies (BIAbs) - Currently, few reports have examined functional antibodies in malaria-exposed populations and most of them were carried-out in the highly endemic areas of PNG (Michon et al. 2000, King et al. 2008), which might not be representative of many *P. vivax* endemic regions. Consequently, our goal was to characterise the DBPII BIAbs response in individuals from an area of markedly unstable malaria transmission, as found in the Brazilian Amazon (Table). Our results indicate that long-term exposure to malaria in the Brazilian Amazon elicits DBP-specific antibodies that inhibit the binding of different DBPII variants to erythrocytes (Ceravolo et al. 2008, Souza-Silva et al. 2010). However, this inhibitory activity was detected only in one third of malaria-exposed subjects, with a moderate correlation between DBPII BIAbs and ELISA anti-PvDBP antibodies.

Despite significant epidemiological and host/parasite genetic differences between the Amazon Basin and PNG, the relatively low frequency of DBPII BIAbs described among long-term residents of the Amazon area (~30%) was also found in PNG (Table). In fact, in the latter highly endemic area, less than 10% of immune children had acquired a high level of DBPII BIAbs (King et al. 2008). It is very intriguing that in the Amazon and PNG,

TABLE

Summary of previous studies reporting naturally-acquired IgG antibody responses to *Plasmodium vivax* Duffy binding protein (PvDBP)[a]

Region [city/state (n)]	Time of malaria exposure [months (m) or years (y)]	Frequency of responders - ELISA (%)	Recombinant PvDBP[b]	Subjects with inhibitory antibodies[c] (%)	References
Latin America (low transmission)					
Brazilian Amazon					
Belém/Pará (36)	< 1 m	14	II-IV	0	Ceravolo et al. (2005, 2008)
Terra Nova do Norte/Mato Grosso (47)	10 y	38	-	12/87 (14)	
Apiacás/Mato Grosso (37)	17 y	65	-		
Buritis/Rondônia (30)	7 y	43	II	NA	Tran et al. (2005)
Colina/Rondônia (87)	21 y	70	-	NA	
Ribeirinha/Rondônia (177)	28 y	70	-	NA	
Several areas/Pará (220)	-	44.5	II	NA	Barbedo et al. (2007)
Granada/Acre (≈360)	14 y	9-18.6	II-IV	14-16/50 (28-32)	Souza-Silva et al. (2010)
Rio Pardo/Amazonas (432)	19 y	49.5	II-IV	NA	Kano et al. (2012)
Colombia					
Pacific Coast/Valle (92)	-	40.2	II-IV	NA	Michon et al. (1998)
Pacific Coast/Chocó and Valle (214)	-	≈8-12	II	NA	Herrera et al. (2005)
Caribbean Coast/Antioquia (185)	> 5 y	9	II	NA	Maestre et al. (2010)
Western Pacific					
Papua New Guinea (high transmission)					
Madang (100)	-	60	II-IV	NA	Fraser et al. (1997)
Liksul/Madang (551)	-	80	II-IV and II	NA	Xainli et al. (2003)
Mugil and Megiar/Madang (206)	-	72	II	58/206 (28)	King et al. (2008), Cole-Tobian et al. (2009)

a: previous studies not included here: Ceravolo et al. (2009), performed with samples from a small outbreak outside endemic area of Brazil, and Chootong et al. (2012), which only analysed acutely infected patients; *b*: region of the recombinant PvDBP used in the studies [the PvDBP Sal-1 variant was used in all studies; other variants were used by Ceravolo et al. (2008) and King et al. (2008)]; *c*: > 50% inhibitory activity; NA: not available.

the DBPII BIAbs response was remarkably stable over time (King et al. 2008, Souza-Silva et al. 2010). These findings imply that although the majority of people naturally-exposed to *P. vivax* do not develop antibodies that inhibit the DBPII-DARC interaction, once they are acquired, these inhibitory antibodies seem to be stable under continuous exposure to malaria transmission.

A plausible explanation for the low immunogenicity of PvDBP is the fact that this protein is localised in the micronemes until the beginning of the process of erythrocyte invasion by merozoites (Adams et al. 1990). As a consequence of its brief exposure, the host immune system seems to have little opportunity to produces an efficient antibody response. However, the "just-in-time" hypothesis of PvDBP exposure (Singh et al. 2006) does not completely explain the large proportion of individuals who remain unresponsive to PvDBP after prolonged exposure to malaria. The reasons for this are not clear, but may relate to the complexity of the immune response, in terms of genetic diversity of both the parasite and human populations. With regard to the genetic diversity of the parasite, several studies now indicate the existence of strain-specificity in the natural immune response against PvDBP (Ceravolo et al. 2009, Cole-Tobian et al. 2009, Chootong et al. 2012). In a study conducted during a malaria outbreak outside of the Brazilian endemic area, we demonstrated that the majority of responders had developed inhibitory antibodies against the homologous DBPII sequence identified in the outbreak isolate (Ceravolo et al. 2009). These findings provided the first clear evidence that naturally-acquired inhibitory antibodies to DBPII are biased towards a specific allele in individuals with no previous exposure to malaria infection (Ceravolo et al. 2009). In Thailand, an area of low unstable transmission of *P. vivax*, the inhibitory antibody responses against DBPII also correlated with homologous protection (Chootong et al. 2012). Similarly, a profile of strain-specific inhibitory activity was frequently observed among asymptomatic children from PNG (Cole-Tobian et al. 2009).

Even though the current data clearly demonstrated strain-specific immunity, we and others have also described strain-transcendent inhibitory responses to DBPII (King et al. 2008, Souza-Silva et al. 2010). In the Brazilian Amazon area, only individuals with long-term exposure to malaria (roughly 20 years) acquired DBPII BIAbs against different DBPII variants (Ceravolo et al. 2008). Similarly, only 9% of asymptomatic children residing in a *P. vivax* hyperendemic area had acquired a significant anti-PvDBP inhibitory antibody response that transcended strain-specificity (King et al. 2008). These findings highlight the complexity of the immune responses to DBPII, which includes both a strain-specific and strain-transcending component (Cole-Tobian et al. 2009).

Besides PvDBP allelic variation, recent evidence suggests that host genetic polymorphisms might also affect humoral immunity against PvDBP (Maestre et al. 2010, King et al. 2011). The most relevant finding was that DARC allelic polymorphisms are thought to affect the ability of anti-PvDBP antibodies to block parasite invasion (King et al. 2011). DARC is encoded by two codominant allele groups *FY*A* and *FY*B*, which differ by a single point mutation. Of particular interest is that we recently demonstrated that DBPII inhibitory antibody responses were approximately 50% lower in *FY*A/FY*A* and *FY*B/FY*B* homozygous individuals when compared with individuals heterozygous for *FY*A* or *FY*B* alleles, suggesting a gene-dosage effect (Souza-Silva et al. 2014). Due to the relevance of these findings for vaccines now in development, it would be pertinent to investigate whether such an association exists in other *P. vivax* malaria endemic countries. In this context, it would be relevant to determine if PvDBP non-responsiveness could be major histocompatibility complex-linked. So far, only a single study investigated the association between human leukocyte antigen (HLA) class II and PvDBP antibodies (Storti-Melo et al. 2012). Although that study was unable to demonstrate an association between HLA type and PvDBP antibodies, the low number of individuals studied (PvDBP IgG sera, n = 48) precludes any solid conclusions about the highly polymorphic HLA class II and PvDBP antibodies. In this context, follow-up studies are currently in progress in the Amazon area (LH Carvalho, unpublished observations).

Genetic diversity of PvDBP in the Amazon area - Analysis of the genetic variability of *P. vivax* isolates from the field revealed that the PvDBP binding domain (region II, DBPII) is highly polymorphic, similar to most blood-stage malaria vaccine candidates, which may facilitate parasite escape from host immune detection. Based on field-studies, it seems clear that this extensive diversity might hamper vaccine development, since variable residues could alter immune recognition of the protein (Ceravolo et al. 2009, Cole-Tobian et al. 2009). Thus, for development of PvDBP-based vaccine it is important to assess the levels of DBPII genetic diversity among *P. vivax* field isolates. The first data on the variability of PvDBP were obtained for isolates of the parasite from PNG and Colombia (Tsuboi et al. 1994, Ampudia et al. 1996). Further analyses showed that most of the variable residues lie within a 170-aa region of DBPII and, at that time, there was no evidence that variable residues could interfere with erythrocyte receptor recognition (Xainli et al. 2000). Later, the same authors confirmed the influence of DBPII polymorphism as a mechanism of immune evasion (VanBuskirk et al. 2004a). Aiming to contribute to efforts towards vaccine development, we initially investigated in the Brazilian Amazon those eight single nucleotide polymorphisms previously suggested to be involved in the immune recognition of PvDBP (VanBuskirk et al. 2004a). Brazilian *P. vivax* isolates showed variability in almost all residues and a strong association of residues at positions 417, 424 and 437, suggesting a synergistic functional effect of these aas, possibly constituting a discontinuous epitope in DBPII (Sousa et al. 2006) (Fig. 2). The synergistic effect of the duo 417 and 424, including residue 419 were reinforced by the findings that they are part of a block of high linkage disequilibrium and show a clear signature of positive natural selection among Brazilian isolates (Sousa et al. 2010). In accordance, these three residues were found to be part of

an *in silico* DBPII predicted epitope that is recognised by DBPII BIAbs present in sera from PNG (Chootong et al. 2010, Sousa et al. 2010).

Subsequently, we compared the DBPII diversity among *P. vivax* isolates from different countries worldwide, including samples from the Brazilian Amazon. In general, high levels of haplotype diversity were observed among isolates independent of the malaria endemicity (Nóbrega de Sousa et al. 2011). These data on the nucleotide diversity of DBPII also provided evidence that recombination plays an important role in determining the haplotype structure of DBPII (Martinez et al. 2004, Sousa et al. 2010). In addition, it was possible to demonstrate that natural selection acts differentially across the DBPII sequence, with neutrally evolving codons as well as codons evolving under diversifying selection (Cole-Tobian & King 2003, Martinez et al. 2004, Sousa et al. 2010). Of importance, positive natural selection preferentially acts on epitopes in DBPII, which also have greater nucleotide diversity (Cole-Tobian & King 2003, Sousa et al. 2010, Ju et al. 2012, 2013). This is in agreement with the hypothesis that immune selection is the major evolutionary force that drives the generation of new PvDBP variants. In accordance with these findings, field-studies carried-out in different malaria endemic areas showed that naturally-acquired inhibitory antibodies to DBPII are biased towards a specific allele (Ceravolo et al. 2009, Cole-Tobian et al. 2009). This is relevant because current vaccine development is based only on the DBPII haplotype of the *P. vivax* laboratory-adapted strain Sal-1 (Yazdani et al. 2004, Arevalo-Herrera et al. 2005, Moreno et al. 2008). However, this haplotype has been found at only a low frequency in most Amazon regions and, of note, it seems to be largely restricted to some geographical areas of the world (Nóbrega de Sousa et al. 2011).

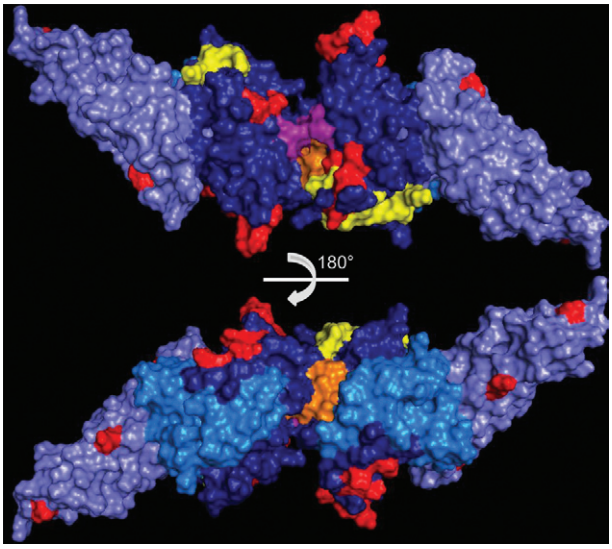

Fig. 2: Duffy binding protein (DBPII) dimeric structure. Subdomain (SD1) (sky blue), SD2 (dark blue) and SD3 (light blue). Critical binding residues are coloured yellow (Asn291, Tyr295, Asn296, Lys297, Phe299 and Val365, Lys366, Lys367, Arg368, Leu369, Phe373, Ile374, Ile376) (VanBuskirk et al. 2004b, Hans et al. 2005, Bolton & Garry 2011, Sampath et al. 2013). Residues that form the putative sulfotyrosine-binding pocket at the dimer interface are coloured purple (Lys273, Arg274 and Gln356). Residues that make contact creating the dimeric architecture are coloured orange (Phe267, Leu270, Ile277, Tyr278, Val282, Tyr363 and Arg274, Glu249) (Batchelor et al. 2011). Polymorphic residues in Brazilian isolates are coloured red (N305N, R308S, L333F, K371E, N375D, R378R, G384D, E385K, K386N, H390R, S398T, T404R, N417K, I419M, L424I, W437R, I464I, Q486E, I503K) (Sousa et al. 2006, 2010). Images modelled in PyMol on the DBPII dimer structure (Batchelor et al. 2011).

Recently, crystallographic and functional studies allowed the identification of the structural regions of PvDBP targeted by inhibitory antibodies (Chootong et al. 2010, Batchelor et al. 2011, Sampath et al. 2013). The findings suggest that some epitopes recognised by BIAbs lie close to the predicted DARC interaction site, suggesting that cause disruption of PvDBP dimerisation and/or prevention of PvDBP receptor binding (Batchelor et al. 2011, Sampath et al. 2013). Despite cumulative knowledge about DBPII structure, there is limited understanding about the molecular basis for protection and immune evasion. Research to date has underlined the importance of the SD2 of DBPII for DARC binding and immune selection (Singh et al. 2006, Batchelor et al. 2011, Sampath et al. 2013). Nevertheless, Siddiqui et al. (2012) showed that the SD3 is also important for binding to DARC. Furthermore, inhibitory murine monoclonal antibodies mapped to SD3 recognise epitopes that are strain transcendent (Siddiqui et al. 2012). Since this SD is relatively conserved it could form the basis of a strain-transcending vaccine against *P. vivax*.

Aiming towards universal, strain-transcending anti-PvDBP immunity, the current strategies of vaccine development have been focusing on immune responses against more conserved DBPII epitopes. An interesting approach was based on the hypothesis that the polymorphic residues, which are not functionally important for erythrocyte-binding, but flank the receptor binding motif of DBPII, comprise variant epitopes that tend to divert the immune response away from more conserved epitopes (Ntumngia & Adams 2012). In this respect, these authors demonstrated that immunisation with a synthetic antigen lacking the immunodominant DBPII variant epitopes enabled the development of immune responses towards the more conserved neutralising epitopes, which are the potential targets of a strain-transcending immunity. Another promising vaccine approach should be immunisation with a multiple component vaccine representing the major DBPII haplotypes (Ntumngia et al. 2013). In this context, a preliminary analysis of the worldwide DBPII sequences allowed us to determine that seven haplotypes should be the minimum number of haplotypes to be included in a DBP-based vaccine of broad coverage (Nóbrega de Sousa et al. 2011).

Concluding remarks - PvDBP 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. While measuring antibodies to recombinant PvDBP by ELISA is a simple and robust procedure widely used in human population studies, it pro-

vides little functional information when used alone. As a consequence, field-studies on PvDBP immune response should include assays to evaluate the functional proprieties of anti-PvDBP antibodies. Cross-sectional and cohort studies carried-out in the Amazon area provide evidence that the majority of people naturally-exposed to *P. vivax* do not develop antibodies that inhibit the DBPII-DARC interaction. Nevertheless, once they are acquired, these inhibitory antibodies seem to be stable under continuous exposure to malaria transmission. These results are intriguing and seem to be a common phenomenon in other endemic areas, including those highly endemic areas of PNG, where most individuals have developed clinical immunity to malaria (King et al. 2008). The results presented here also provide strong evidence that DARC interaction site and epitopes on PvDBP have sufficient overlap for antibodies to disrupt dimerisation and/or inhibit binding (Batchelor et al. 2011, Sampath et al. 2013) and provide support for the role of allelic diversity in anti-PvDBP immune responses. Of note, while our findings point to allelic variation eliciting a strain-specific immunity, individuals with long-term exposure in the Amazon area acquired DBPII antibodies that inhibit in vitro binding of different DBPII variants to erythrocytes. Future challenges include understanding why only few malaria exposed-individuals develop an immune response able to inhibit DBPII-DARC interaction and to establish whether PvDBP inhibitory immune response predicts partial protection from infection and/or disease, as suggested by others (King et al. 2008). We hope that these findings from unstable malaria transmission areas contribute to current efforts towards vaccine development and may facilitate future clinical trials in areas of unstable malaria transmission.

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