5 IMMUNOEPIDEMIOLOGY OF *P. VIVAX* PROTEIN LIBRARY

Generation of the recombinant protein library described in Chapter 4 gave us a unique opportunity to perform *P. vivax* immunoepidemiology studies in several *P. vivax*-endemic areas. In this chapter, I will describe those studies. Publication note: the results described in section 5.2.1.2 and portions of the introduction and discussion were slightly modified from a published manuscript (Hostetler et al., 2015). I drafted the text in these sections, which was edited by co-authors prior to publication. I am solely responsible for the work described in section 5.2.1, under the supervision of my PhD supervisors, Rick Fairhurst and Julian Rayner, except where noted in the text. The results in sections 5.2.2.1 and 5.2.2.2 were generated in close collaboration with Camila Franca and Ivo Mueller. Camila Franca performed all experiments in section 5.2.2.4. The results in 5.2.2 were recently published (Franca et al., 2016).

5.1 Introduction

The screening of our *P. vivax* recombinant proteins in several functional studies (Chapter 4) revealed several predicted and novel parasite protein-protein interactions, but did not investigate the potential significance of these proteins as vaccine candidates. Identifying naturally-acquired, clinically-protective associations with any of the proteins, while not confirming a causal relationship, could prioritize them for further functional studies and increase the pool of potential *P. vivax* vaccine candidates. Evaluating our panel of *P.*

vivax recombinant proteins, representing full-length ectodomains, in a series of immunoepidemiological studies would be a useful step in addressing this. In this chapter, I will outline studies in 3 *P. vivax*-endemic countries: Cambodia, the Solomon Islands (SI), and Papua New Guinea (PNG).

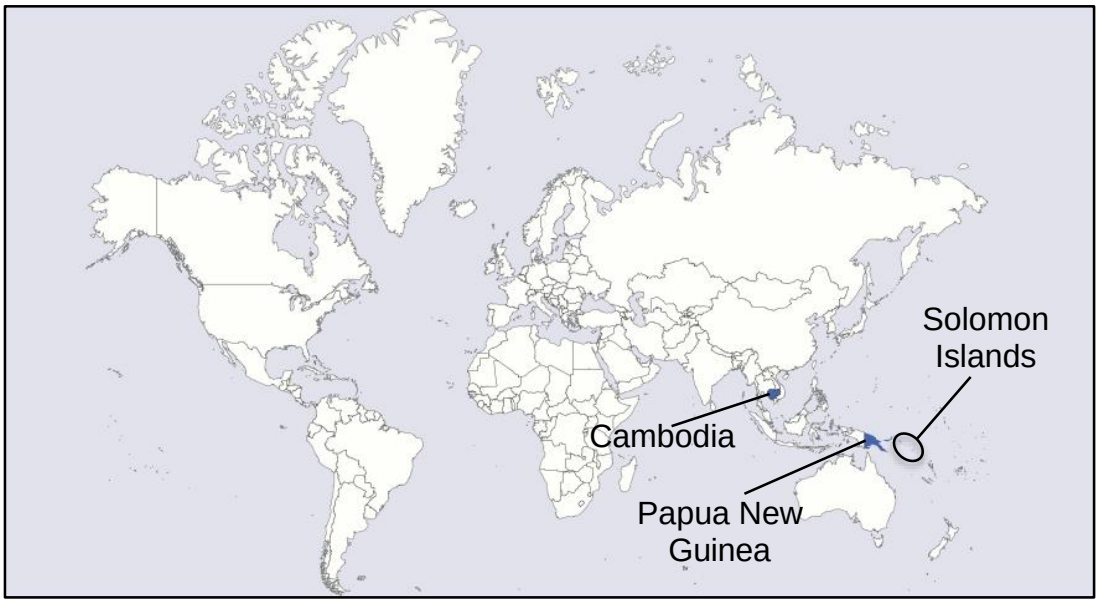

Figure 5.1: Immunoepidemiological study sites
Immunoepidemiological studies were designed using patient plasma collected in 3 *P. vivax*-endemic countries: Cambodia, Papua New Guinea, and the Solomon Islands. Map created at https://www.amcharts.com/.

Natural human immune responses to *P. vivax* during and after infection have been the subject of only limited study. While a few small-scale studies have produced full-length *P. vivax* recombinant proteins, even fewer have investigated whether immune IgG from *P. vivax*-exposed individuals recognize these proteins (Barbedo et al., 2007, Fowkes et al., 2012, Fraser et al., 1997, Garg et al., 2008, Lima-Junior et al., 2011, Lima-Junior et al., 2012, Michon et al., 1998, Oliveira et al., 2006, Rodrigues et al., 2005, Souza-Silva et al., 2010, Wickramarachchi et al., 2006, Woodberry et al., 2008, Xainli et al., 2003, Yildiz Zeyrek et al., 2011, Zeeshan et al., 2013, Ceravolo et al., 2009). The only 2 large-scale immunoreactivity screens did not exclusively use full-length proteins, and may therefore have missed critical epitopes (Lu et al., 2014, Chen et al., 2010).

It would first be important to evaluate the general IgG reactivity to the *P. vivax* recombinant protein library in clinical samples from *P. vivax*-infected patients. The NIH laboratory field site in Pursat Province, Cambodia (with Rick Fairhurst, LMVR/NIH and Socheat Duong, Cambodian National Center for Parasitology, Entomology, and Malaria

Control), had several ongoing *P. vivax* studies that could provide access to hundreds of plasma samples from *P. vivax*-infected patients. Cambodia is endemic for both *P. falciparum* and *P. vivax*. A recent publication by Maude et al. (2014) found that *P. falciparum* malaria transmission has greatly declined in recent years, with an estimated 81% decline in annual cases from 2009 to 2013 (Figure 5.2). This coincided with several intensified control measures including a scale-up of village malaria workers and insecticide-treated bed nets. In contrast, the number of *P. vivax* cases increased 490% from 2008 to 2011 and then declined 50% from 2011 to 2013. The annual parasite incidence (API) in Cambodia in 2013 was 4.6/1000, with *P. vivax* accounting for 67% of malaria cases (Maude et al., 2014). This finding underscores the challenge of eliminating *P. vivax*, which may become the predominant *Plasmodium* species disease burden in many areas of declining *P. falciparum* infections (Yekutiel, 1980).

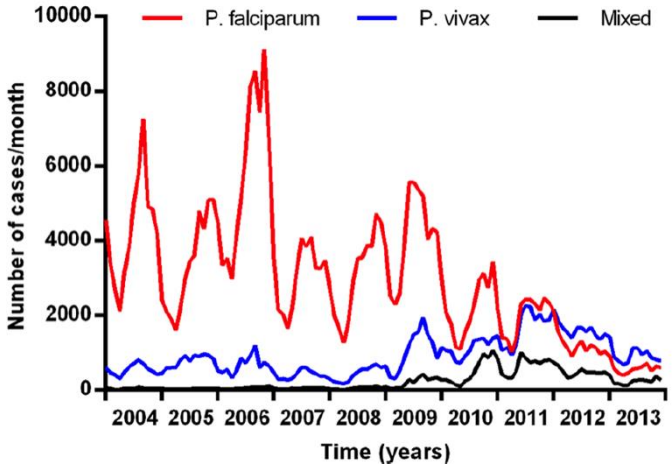

Figure 5.2: *Plasmodium* malaria cases in Cambodia from 2004 to 2014
Monthly malaria cases in Cambodia. *P. falciparum* cases declined while *P. vivax* cases increased from 2009 to 2013. Figure reprinted from (Maude et al., 2014) under the Creative Commons Attribution (CC BY) license.

Any IgG responses to the *P. vivax* recombinant protein library in Cambodian patient samples would suggest that the proteins are properly folded and further validate the use of the mammalian HEK293E system for expressing high-quality *P. vivax* recombinant proteins. Since the protein sequences were only based on the available *P. vivax* reference genome at the time (*P. vivax* Sal 1, originally from an El Salvadoran isolate), it was unknown whether IgG to proteins expressed by naturally-circulating Cambodian isolates would also react to our panel of proteins. The *msp3* genes, for example, are highly divergent among the *P. vivax* isolates sequenced to date (Neafsey et al., 2012, Rice et al.,

2014). The genetic divergence between *P. vivax* Sal 1 and naturally-circulating Cambodian isolates might lead to strain-specific IgG responses, thus limiting their potential usefulness in future immunoepidemiological studies and as vaccine candidates. We hypothesized that some reactivity would be detected, however, as 2 previous immunoreactivity screens, using *P. vivax* Sal 1-based proteins expressed in the wheat germ cell-free system and Korean isolates, detected several highly-immunogenic proteins (Lu et al., 2014, Chen et al., 2010).

The Cambodian patient samples would also facilitate the study of the development of the immune response following infection. In 2011, several hundred pairs of plasma samples were collected from Cambodian patients at the time of clinical presentation with *P. vivax* malaria ("acute") and 21-28 days later ("convalescent"). Evaluating whether IgG responses to *P. vivax* antigens are boosted or decline during the convalescent period may clarify the early phase of *P. vivax* immunity development. Proteins with a declining IgG response in convalescent plasma may also serve as useful markers for recent past exposure.

Evaluating IgG reactivity to our protein panel in symptomatic patients would be a useful first step for identifying targets of natural immunity. Evaluating IgG reactivity in a cross-sectional study of both infected and uninfected subjects would enable us to investigate whether IgG responses correlate with exposure. Screening a panel of proteins would facilitate more systematic comparisons between proteins, in contrast to previous studies that have used only 1 or several proteins. Understanding exposure is particularly important for malaria control, as *P. vivax* infections are often low-density, asymptomatic, or both, making surveillance challenging (Waltmann et al., 2015). As indicators of recent exposure, IgG responses may serve as a marker for circulating *P. vivax* malaria even when parasite-positive samples are uncommon. This could be very useful in distributing resources in areas nearing *P. vivax* elimination. A cross-sectional survey conducted by Ivo Mueller and colleagues in SI in 2012 provided an ideal patient population to investigate age-stratified associations between IgG responses in subjects with and without *P. vivax* infections (Waltmann et al., 2015).

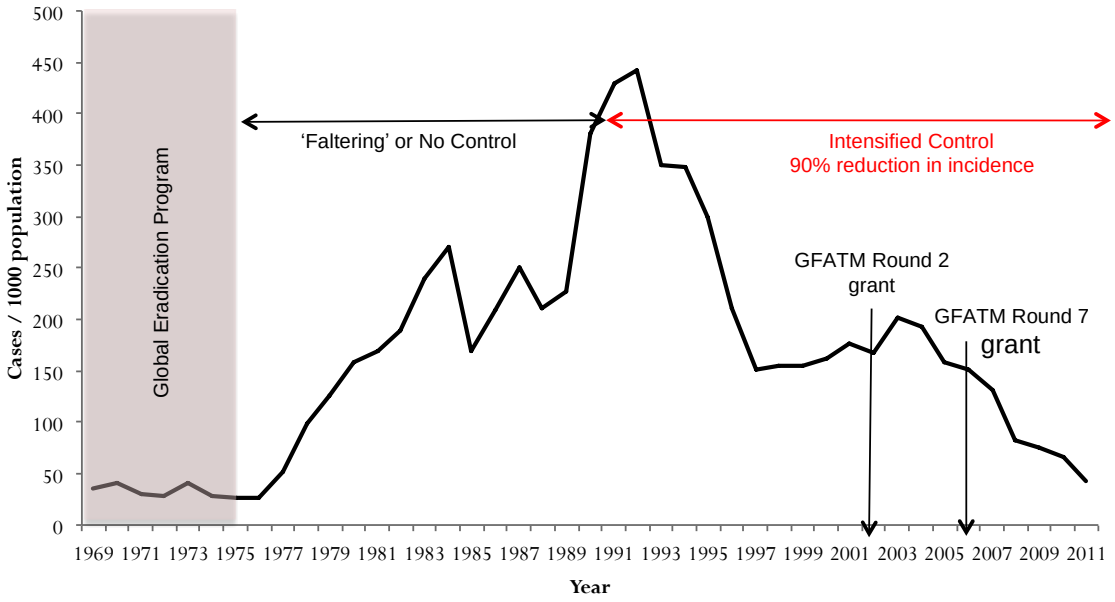

Figure 5.3: Malaria incidence in the Solomon Islands from 1969 to 2011
Malaria cases per 1000 people per year in SI. Malaria neared eradication during the Global Eradication Program in the early 1970's, followed by resurgence through 1992. Intensified control measures, including those funded by 2 Global Fund to Fight AIDS, Tuberculosis, and Malaria (GFATM) grants, reduced incidence by 90% by 2011. Image adapted from Andreea Waltmann; data from National Vector Borne Disease Control Program.

P. vivax malaria in SI is moderately endemic and but has a history of high transmission (Figure 5.3) (Pacific Malaria Initiative Survey Group on behalf of the Ministries of Health of and Solomon, 2010). Malaria incidence has dropped over a period of renewed control measures introduced in the early 1990's and Global Fund to Fight AIDS, Tuberculosis, and Malaria grants awarded in the 2000's. The 2012 cross-sectional survey enrolled 3501 residents of all ages in the Central Islands Province and assessed the prevalence of *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae.* In contrast to Cambodia, where both *P. falciparum* and *P. vivax* are present relatively equally (Maude et al., 2014), *P. falciparum* infections in SI were nearly eliminated, with only 5 qPCR-detected cases, all of which were co-infections with *P. vivax*. *P. vivax*, however, maintained a prevalence of 13.4% overall. The vast majority of *P. vivax* infections were submicroscopic (72.9%) and/or afebrile (84.5%), and 23.5% contained gametocytes (Waltmann et al., 2015). Profiling IgG responses in this population would clarify the effects of age and infection status in an area of declining *P. vivax* transmission.

We next wanted to investigate whether any IgG responses to our proteins correlate with protection against clinical *P. vivax* malaria. There are few studies evaluating protective IgG responses, and a review of immunoepidemiological studies of *P. vivax* malaria found that only 3 antigens [MSP1, MSP3.10 (MSP3α), and MSP9] were consistently associated with protection (Cutts et al., 2014), underscoring the need for a much broader set of antigens for study. The breadth of antibody response was shown to be important for protection from severe malaria in a Kenyan cohort study in 2014 using a panel of *P. falciparum* merozoite proteins expressed in the same system (Osier et al., 2014). Whether this observation holds true for *P. vivax* is unknown, as no studies evaluating protection from disease using a large panel of *P. vivax* proteins has been published. A longitudinal cohort study in PNG, led by Ivo Mueller, provided an ideal study population in which to address this research gap (Lin et al., 2010).

Four of the 5 *Plasmodium* species causing human malaria (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) are endemic to PNG. While Cambodia has low transmission and SI has moderate transmission, PNG has one of the highest *P. vivax* transmission rates in the world, with a 25% parasitemia prevalence recorded in 1995 (Genton et al., 1995). The cohort enrolled 264 PNG children aged 1-3 years, and followed them for 16 months. The median age of study participants was 1.7 years (IQR 1.3-2.5) and the PCR-detected prevalence of *P. vivax* infection at the beginning of the study was 55%. Studying naturally-acquired immunity (NAI) to *P. vivax* in very young children is essential since this immunity seems to develop faster than immunity to *P. falciparum* (Jeffery, 1966, Ciuca, 1934). This is supported by numerous studies in co-endemic areas that recorded the incidence of *P. vivax* peaking earlier than that of *P. falciparum* (Michon et al., 2007, Maitland et al., 1996, Phimpraphi et al., 2008, Mueller et al., 2009b, Lin et al., 2010).

5.1.1 Benefits of these studies

The development of NAI during *P. vivax* infection has been the subject of limited study, partially due to the difficulty in producing full-length *Plasmodium* proteins. The *P. vivax* recombinant protein library facilitates the systematic comparison of responses to a panel of proteins produced on the same platform. Our studies with Cambodian patient plasma aim to characterize the IgG reactivity to the proteins as a useful first step to prioritize them for further study. Our studies evaluating IgG responses from population studies in SI and PNG aim to address whether any of these responses is correlated with age,

infection status, and protection from disease. The results from each of these studies will give additional information about the potential utility of the protein library in global *P. vivax* immunoepidemiological studies and prioritize potential vaccine candidates for testing in functional studies.

5.1.2 Objectives

i. To evaluate IgG reactivity from *P. vivax*-exposed individuals to the library of *P. vivax* recombinant proteins

ii. To examine whether IgG responses correlate with *P. vivax* exposure or protection from clinical *P. vivax* malaria.

5.2 Results

5.2.1 Antibody responses to *P. vivax* recombinant proteins in Cambodia

5.2.1.1 Pilot reactivity screen using Cambodian patient plasma and a subset of *P. vivax* recombinant proteins

To test whether the *P. vivax* recombinant proteins were immunoreactive in Cambodian patient plasma and what plasma concentration was best to use, I serially diluted (1:200, 1:400, 1:800, 1:1600) plasma samples from 5 *P. vivax* malaria-exposed Cambodian individuals against 3 proteins (P12, P41, and MSP10). Negative controls were a pooled serum sample from American malaria-naïve individuals and the Cd4d3+d4 tag present in all proteins. Cambodian plasma pairs were selected based on prior ELISA data showing reactivity to recombinant *P. vivax* MSP1 (produced by Daria Nikolaeva in the Long laboratory), in order to maximize our chances of observing reactivity. The ELISA data suggested that all our proteins might be immunoreactive, with P12 showing the highest reactivity (Figure 5.4). A single plasma sample, CAM29, showed 5 times higher reactivity to the Cd4d3+d3 negative control than the other plasma samples. Higher dilutions reduced this response to that of negative controls, but correspondingly reduced the overall response to the recombinant proteins. A 1:600 dilution seemed optimal for observing IgG responses for the largest number of proteins versus plasma samples, while minimizing non-specific responses to the negative controls (the CD4d3+d4 tag alone and malaria-naïve control sera). This plasma dilution was then used in subsequent ELISAs.

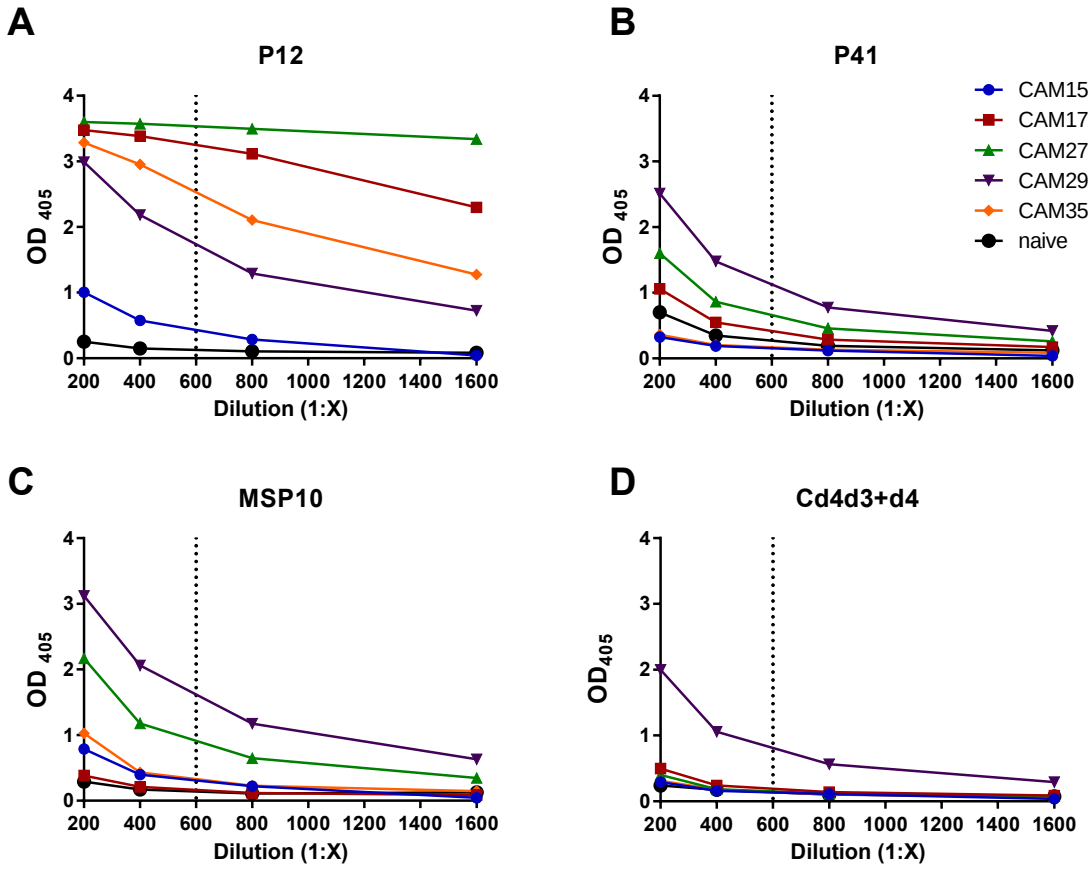

Figure 5.4: Testing Cambodian patient plasma against *P. vivax* recombinant proteins

(A-D) Serially diluted (1:200, 1:400, 1:800, 1:1600) plasma samples from 5 *P. vivax*-infected Cambodian patients (CAM15, CAM17, CAM27, CAM29, CAM35) and a pooled sera sample from American *P. vivax*-naïve individuals (negative control) were screened against 3 *P. vivax* recombinant proteins (P12, P41, MSP10) and a negative control protein (Cd4d3+d4) using ELISA with optical density (OD) measured at 405 nm. Plasma dilution 1:600 (dotted lines) minimized reactivity to negative controls while enabling detection of IgG reactivity to *P. vivax* proteins.

To expand the IgG reactivity screening, I used ELISAs to measure IgG responses in 42 *P. vivax*-infected Cambodian patient plasma samples to 6 full-length *P. vivax* recombinant protein ectodomains [P12, P12p, P41, MSP7 (PVX_082675), MSP10, Pv34]. I included 6 American malaria-naïve sera samples and Cd4+d3+d4 as negative controls. All antigens showed reactivity above Cd4d3+d4 when comparing the mean OD values for all tested samples; however, only those differences for P12, Pv34, P41, and MSP10 were statistically significant (Figure 5.5) (Mann-Whitney U test, $P<0.0001$-0.04).

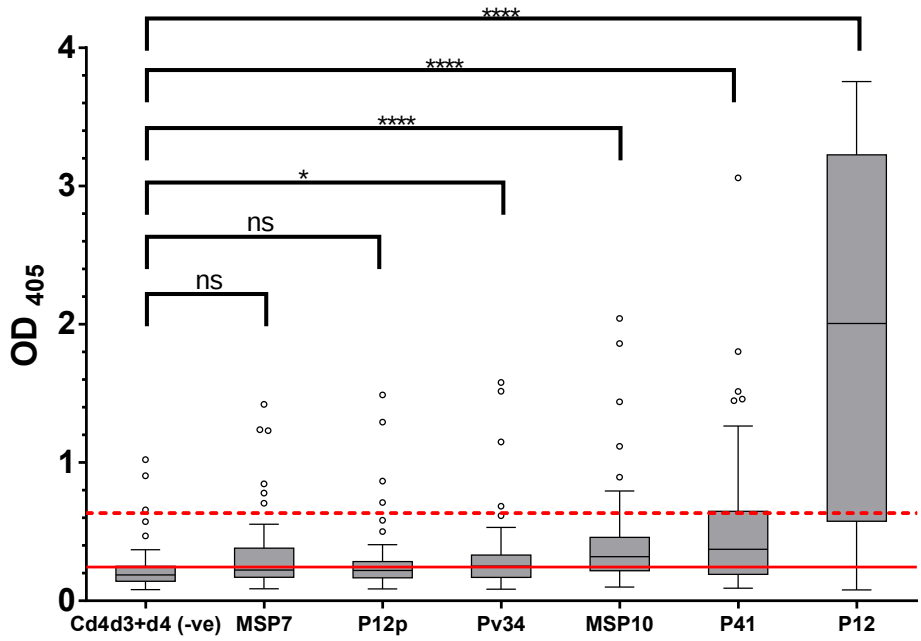

Figure 5.5: Cambodian patient plasma IgG reactivity to full-length *P. vivax* recombinant protein ectodomains

42 Cambodian *P. vivax*-infected plasma samples and 6 American *P. vivax*-naïve control sera were tested against 6 proteins (P12, P12p, Pv34, P41, MSP7.6, MSP10) and the rat CD4d3+d4 tag present in all proteins by ELISA; optical density (OD) was measured at 405 nm. Boxplots show median OD (horizontal bar), IQR (boxes), range (whiskers), and outliers (open circles). IgG responses to the rat Cd4d3+d4 tag alone are shown as mean (solid red line) and mean + 2 standard deviations (dashed red line). P12, Pv34, P41, and MSP10 showed significantly higher population responses than Cd4d3+d4 (*). ns=Not significant. (Mann-Whitney U test, P<0.0001-0.02; P values were deemed significant if <0.05).

Cd4d3+d4 had higher reactivity against Cambodian *P. vivax*-infected patient plasma compared to American *P. vivax*-naïve control sera, with 62% of patient samples showing reactivity greater than 2 standard deviations above the mean reactivity of the naïve controls. This reactivity to Cd4d3+d4 was not observed in screens using a panel of *P. falciparum* recombinant proteins produced in the same expression system against a cohort of Kenyan samples (Osier et al., 2014). This increased reactivity could be more visible in this ELISA because a 1:600 plasma dilution was used instead of the 1:1000 plasma dilution that was used in the *P. falciparum* ELISAs. The Cambodian plasma samples were selected based on reactivity to recombinant MSP1 (data provided by Daria Nikolaeva and Carole Long, LMVR/NIH). This may have biased the plasma set toward samples that were generally more reactive, even non-specifically; downstream ELISAs included samples based on patient history or chosen randomly (as described) in order to

minimize this potential bias. The Cd4d3+d4 responses were also subtracted to correct for background when setting a reactivity cut-off value.

When considering individual responses, at least some patient samples reacted to all antigens. The reactivity cut-off value was set at 2 standard deviations above the mean of the naïve sera controls after subtracting the Cd4d3+d4 response from each sample. Reactivity to P12 was highest in both magnitude and breadth. There was significant variation in reactivity with P41 and P12 reactive in 43% and 98% of samples, respectively (Table 5.1).

Table 5.1: Pilot Cambodian seropositivity summary

	***P. vivax* recombinant protein**					
	P12	MSP10	Pv34	MSP7.6	P41	P12p
Reactive plasma samples* n (%)	41(98)	29(69)	24(57)	24(57)	18(43)	21(50)

*Reactivity cut-off value set at 2 SD above mean of 6 American malaria-naïve controls after correcting for background responses by subtracting the Cd4d3+d4 values.

5.2.1.2 *P. vivax* recombinant proteins are immunoreactive and contain conformational epitopes.

To test whether the library of biotinylated *P. vivax* recombinant protein ectodomains were immunoreactive, and to establish whether they contained conformational epitopes, I screened them by ELISA against diluted (1:600) pooled plasma from 14 Cambodian patients with acute vivax malaria and pooled control sera from 5 American malaria-naïve individuals (Figure 5.6). Exposed IgG reacted more strongly than naïve IgG to all *P. vivax* proteins, and reacted only weakly to the rat Cd4d3+4 tag present in all expressed proteins. Five proteins (MSP5, P12, GAMA, CyRPA, PVX_081550) were particularly reactive; thus, ELISAs for these proteins were repeated using more-diluted (1:1000) plasma pools (Figure 5.6). Of the 34 proteins tested, 27 showed at least a 2-fold change in seroreactivity between the naïve IgG versus the exposed IgG for each protein, with 7 proteins (P92, PVX_084815, PVX_116775, RBP2-like, PVX_001015, PVX_110960, and PVX_110965) showing a lesser change. The majority of proteins showed at least a 3-fold change (MSP1, MSP3.1, MSP3.3, MSP3.10, MSP4, MSP5, MSP7.1, MSP10, P12, P12p, P38, P41, GAMA, ARP, CyRPA, DBP, DBP-RII, PVX_081550).

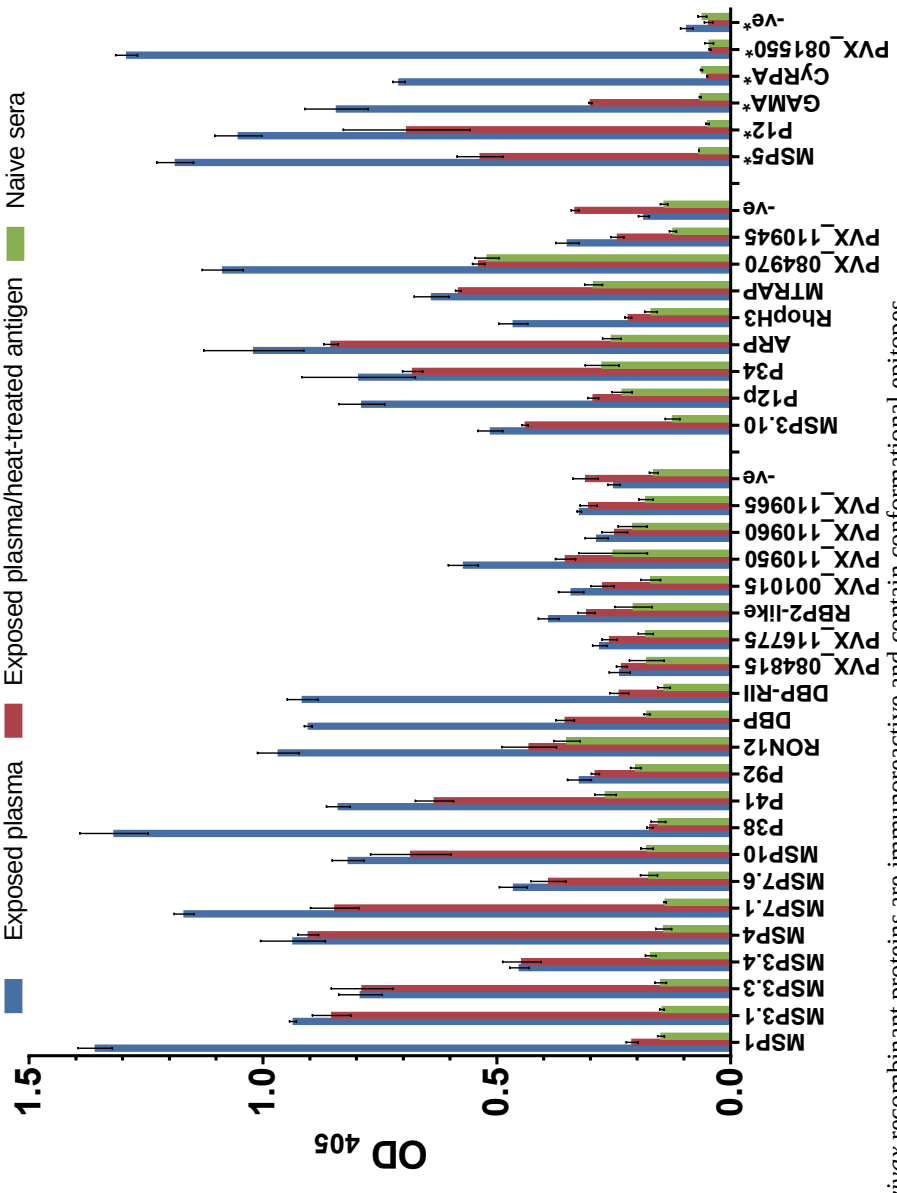

Figure 5.6: Multiple *P. vivax* recombinant proteins are immunoreactive and contain conformational epitopes

The immunoreactivity of 34 biotinylated *P. vivax* recombinant proteins was assessed using diluted (1:600) plasma pools from 14 Cambodian vivax malaria patients (blue bars) and 5 American malaria-naïve individuals (green bars). The immunoreactivity of heat-treated proteins was assessed in parallel using the Cambodian plasma pool (red bars); reduced responses indicate the presence of heat-labile conformational epitopes. The immunoreactivity of highly reactive proteins (*) was assessed using more-diluted (1:1000) plasma pools. Optical density (OD) at 405 nm was measured at various times, but only the mean value nearest to 1.0 for each antigen is shown. Negative control (–ve) was rat Cd4d3+d4 tag. Bar charts show mean ± SD; n=3. Figure reprinted from (Hostetler et al., 2015) under the Creative Commons Attribution (CC BY) license.

To test whether IgG responses were directed at conformational epitopes, I heat-treated all 34 proteins and screened them for seroreactivity in parallel with untreated proteins. Of the 34 proteins tested, 18 (MSP1, MSP7.1, P38, P41, RON12, DBP, DBP-RII, RBP2-like, PVX_110950, P12p, RhopH3, PVX_084970, PVX_110945, MSP5, P12, GAMA, CyRPA, PVX_081550) showed at least a 20% decrease in seroreactivity when heat treated (Figure 5.6), indicating they contained conformation-sensitive epitopes, and suggesting that the recombinant proteins were properly-folded. Twelve of these proteins (MSP1, P38, RON12, DBP, DBP-RII, P12p, RhopH3, PVX_084970, MSP5, GAMA, CyRPA, PVX_081550) showed at least a 50% reduction in seroreactivity when heat-treated (Figure 5.6). Naïve IgG showed appreciable reactivity to PVX_084970; screening this protein against individual plasma samples may resolve whether it nonspecifically reacts to IgG from all or only some of the 5 serum donors.

5.2.1.3 IgG response in *P. vivax* Cambodian acute and convalescent plasma

A study conducted in 2011 at the NIH Cambodian field site collected several hundred pairs of "acute" plasma samples when patients presented to the clinic with symptomatic *P. vivax* malaria and "convalescent" samples 21-28 days later. We were interested in understanding whether any IgG boosting occurred during this period, which might serve as a signal for the development of NAI. I first needed to create a standard pool of highly reactive plasma to include on each plate to account for plate-to-plate variations. I started with a subset of 11 available proteins (MSP1, MSP5, MSP10, P12, P41, ARP, CyRPA, DBP, GAMA, RBP2-like, PVX_081550) and screened them by ELISA to 48 Cambodian plasma samples (1:1000) and 24 American malaria-naïve adult plasma samples (1:1000). All proteins showed significantly higher reactivity compared to the Cd4d3+d4 tag (Mann-Whitney U tests, $P<0.0001$), and reactivity varied for each protein (Figure 5.7). Responses to Cd4d3+d4 were significantly lower than in the ELISA optimization screens. This is likely due to the use of a 1:1000 plasma dilution, which was selected based on the Cambodian reactivity screen using pooled plasma.

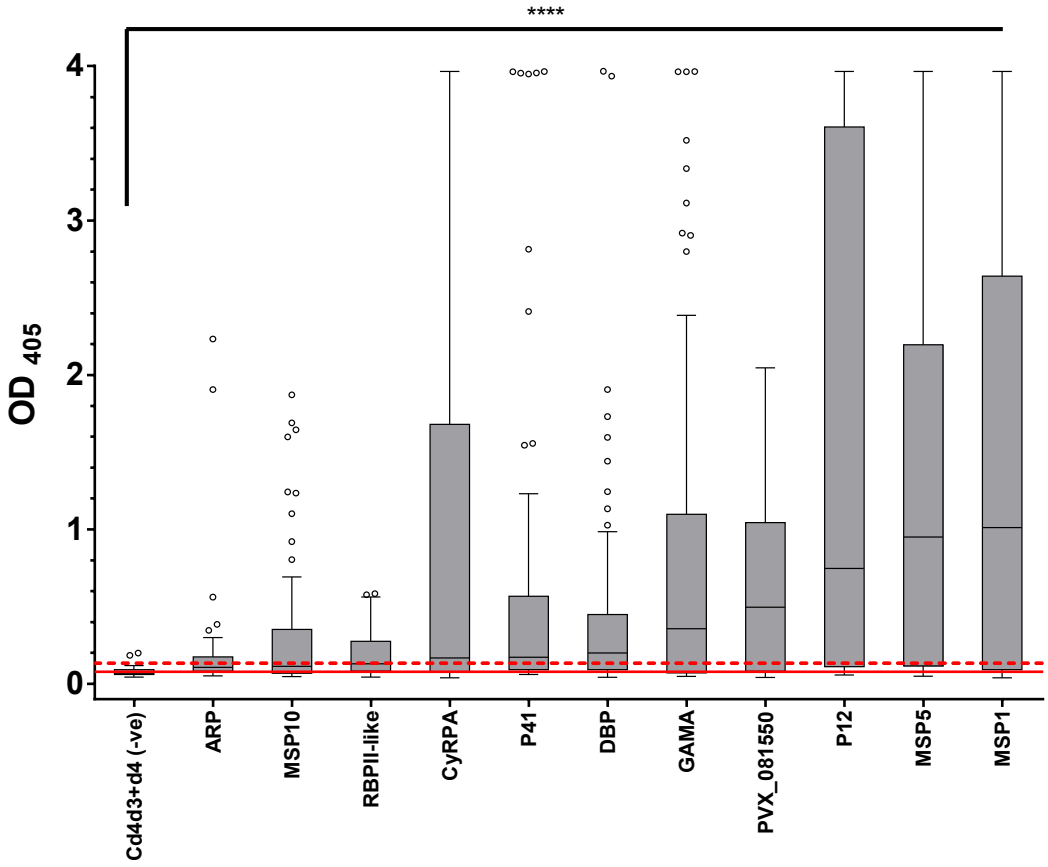

Figure 5.7: Reactivity in individual Cambodian plasma samples

48 Cambodian *P. vivax*-infected plasma samples and 24 American *P. vivax*-naïve controls were screened against 11 proteins (MSP1, MSP5, MSP10, P12, P41, ARP, CyRPA, DBP, GAMA, RBP2-like, PVX_081550) and rat CD4d3+d4 present in all proteins by ELISA. Optical densities (OD) at 405 nm are shown. Boxplots show median OD (horizontal bar), IQR (box) range (whiskers), and outliers (open circles). IgG response to Cd4d3+d4 tag alone are shown with mean (solid red line) and mean + 2 SD (dashed red line). All proteins showed significantly higher population responses than Cd4d3+d4 (*) (Mann-Whitney U tests, $P<0.0001$; P values were deemed significant if <0.05).

I calculated reactivity cut-off values for each protein as 2 SD above the mean of the American malaria-naïve adult plasma samples after correcting for background by subtracting the Cd4d3+d4 values. The reactivity ranged from 35% in ARP and MSP10 to 100% for MSP1 and GAMA. Over 90% of samples reacted to 5 proteins (MSP1, GAMA, MSP5, PVX_081550, P12). The results showed a similar relationship between P12, P41, and MSP10 compared to the initial ELISA optimization experiments, with P12 the most

reactive of these 3 proteins. These seropositivity results can subsequently be compared to those obtained at other study sites.

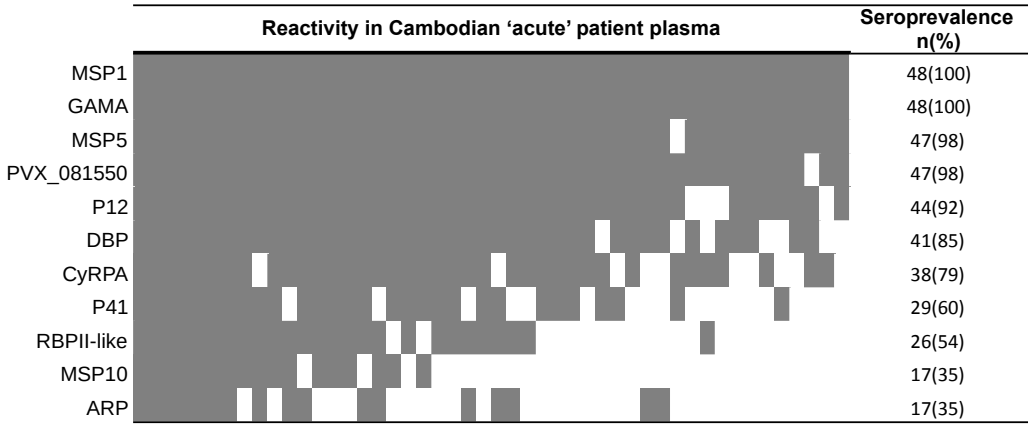

Figure 5.8: Seroprevalence in "acute" Cambodian plasma samples

Heatmap showing reactivity in 48 acute Cambodian plasma samples (columns) for 11 *P. vivax* recombinant proteins (rows; MSP1, MSP5, MSP10, P12, P41, ARP, CyRPA, DBP, GAMA, RBP2-like, PVX_081550). Gray boxes represent optical density (OD) values 2 SD above the mean of the American malaria-naïve adult plasma samples after correcting for background by subtracting the Cd4d3+d4 values.

From these results, I created a standard pool of high responders for each protein to control for plate-to-plate variation in subsequent ELISAs. I next screened 10 *P. vivax* recombinant proteins (MSP1, MSP5, MSP10, P12, P41, ARP, CyRPA, DBP, GAMA, PVX_081550) in duplicate wells against 18 pairs of acute and convalescent plasma samples. I had insufficient amounts of RBP2-like and therefore could not test this protein. All of the proteins showed mean reactivity significantly higher than the Cd4d3+d4 tag alone (Mann-Whitney *U* test, $P<0.0001$). The mean reactivity in convalescent plasma was 1.9 times higher in ARP, but was similar for other proteins, with acute mean OD values less than 25% higher or lower than convalescent mean OD values. There were no significant differences between acute and convalescent groups when comparing the unpaired data (Mann-Whitney *U* tests, $P=0.35$-0.95) (Figure 5.9). There were also no significant differences when analysing the plasma time points as pairs (Wilcoxon signed-rank test, $P=0.08$-0.97) (Figure 5.10). This result could indicate that more samples may be required to detect small changes in IgG in the month following infection. Alternatively, a 3-4 week separation in plasma collection time points may be too narrow to observe changes immediately following infection and recovery.

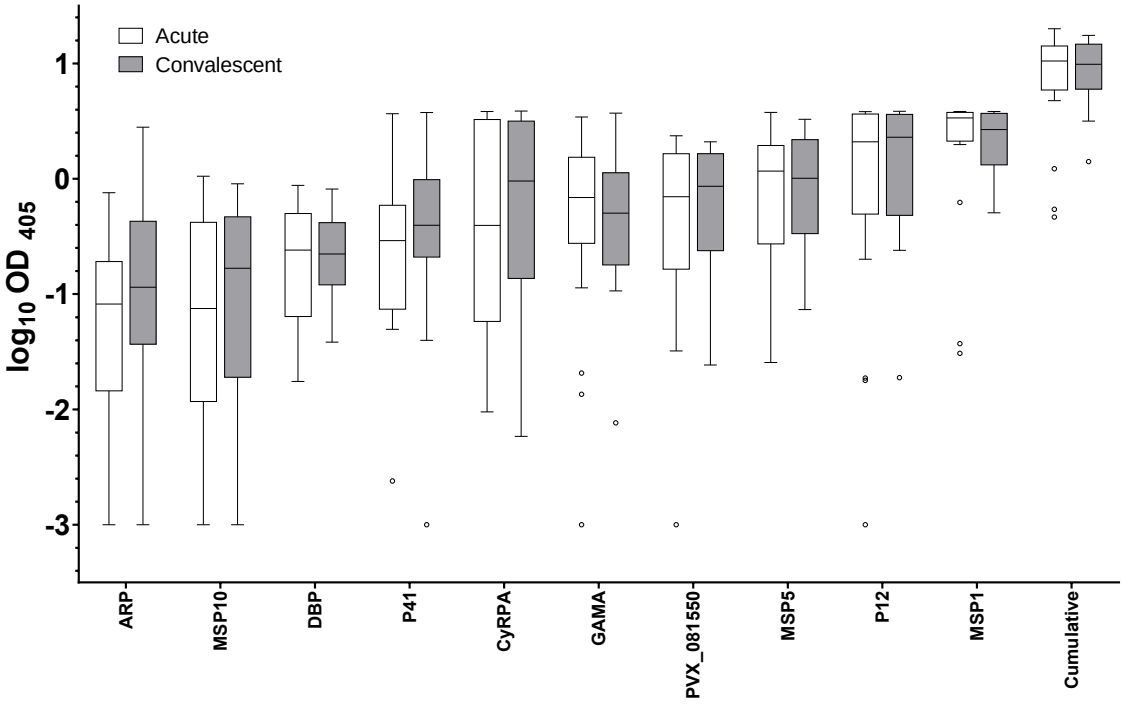

Figure 5.9: IgG responses in acute and convalescent Cambodian patient plasma samples (unpaired)

18 Cambodian paired "acute" and "convalescent" plasma samples were screened against 10 *P. vivax* recombinant proteins (ARP, MSP10, DBP, P41, CyRPA, GAMA, PVX_081550, MSP5, P12, MSP1) by ELISA. Log optical densities (OD) at 405 nm are shown. Boxplots show median (horizontal bar), IQR (box), range (whiskers), and outliers (open circles). No significant differences between plasma time points were detected (Mann-Whitney *U* tests, *P*=0.35-0.95).

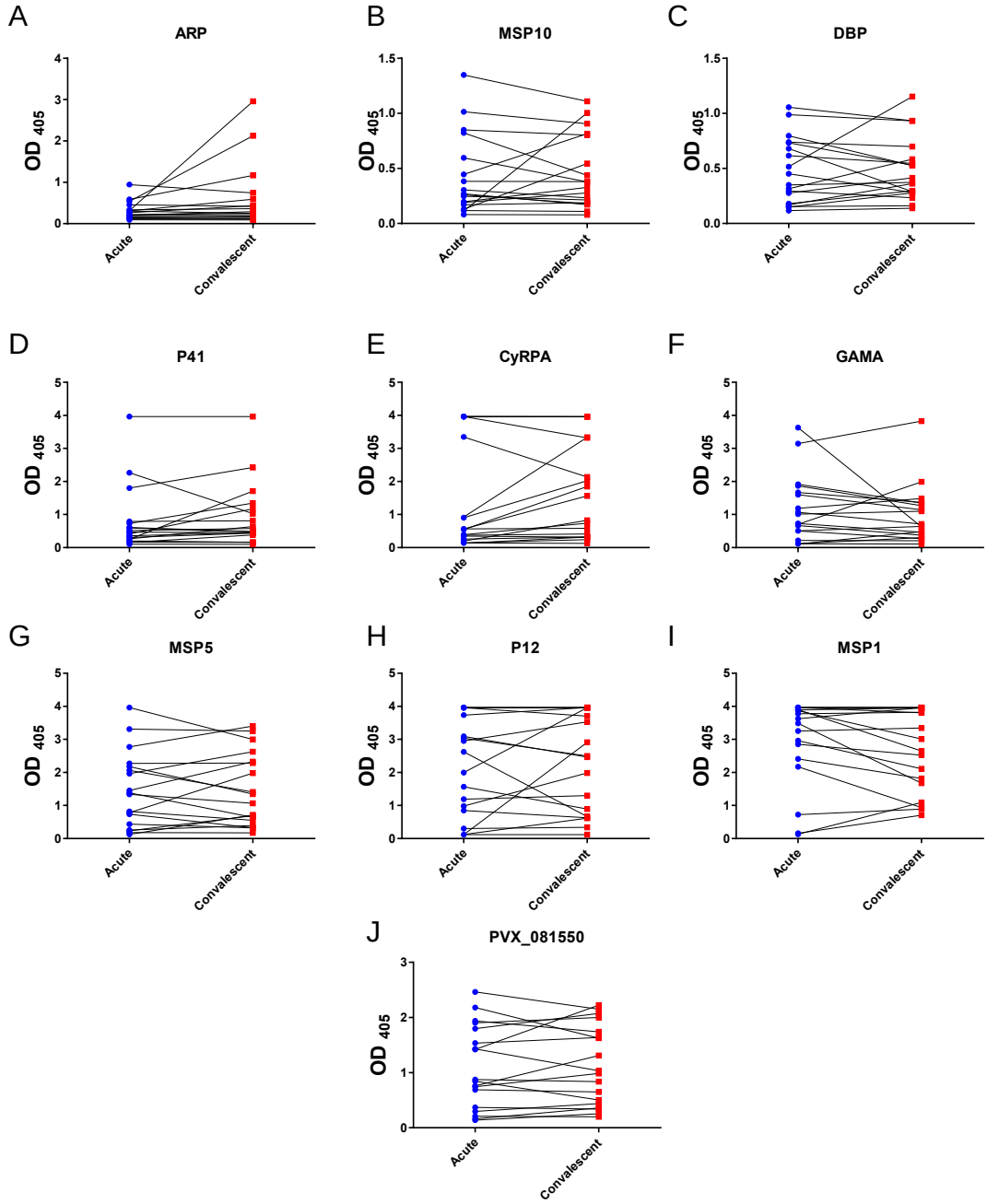

Figure 5.10: IgG responses in acute and convalescent Cambodian patient plasma samples (paired)

ELISA results measuring OD at 405 nm for 18 paired "acute" and "convalescent" plasma samples against 10 *P. vivax* recombinant proteins (ARP, MSP10, DBP, P41, CyRPA, GAMA, PVX_081550, MSP5, P12, MSP1) are shown. The lines connect plasma time points. No significant differences were detected (Wilcoxon signed-rank test, P=0.08-0.97).

5.2.2 Antibody responses to *P. vivax* recombinant proteins in Solomon Islands and Papua New Guinea

The Cambodian plasma studies suggested the majority of our antigen library was immunoreactive, but these studies were not designed to investigate correlations with exposure or protection. Examining these correlations required cross-sectional or longitudinal cohort study designs, respectively. We addressed these questions in collaboration with Ivo Mueller's laboratory at WEHI. The Mueller laboratory conducted a collaborative cross-sectional survey in SI in 2011 (Waltmann et al., 2015) and a longitudinal cohort study in PNG in 2006 (Lin et al., 2010). These studies were ideal for pursuing a collaborative set of projects to examine antibody responses comprehensively to our library of *P. vivax* recombinant proteins. This culminated in 3 studies described below, with the relative laboratory contributions of each group noted:

SI reactivity screening: screened 34 recombinant *P. vivax* proteins against 48 plasma samples stratified by 2 age groups. Proteins were expressed by Jessica Hostetler (Rayner and Fairhurst laboratories), and ELISAs were performed by Jessica Hostetler and Camila Franca (Mueller laboratory).

SI comprehensive screening: expanded screening against a subset 12 proteins against 144 plasma samples stratified by 3 age groups and 3 infection statuses. Proteins were expressed by Jessica Hostetler, and ELISAs were performed by Jessica Hostetler and Camila Franca.

PNG cohort screening: selected 6 proteins from our SI screening results to study in high-throughput Luminex assays. Proteins were selected by Jessica Hostetler and Julian Rayner, expressed and purified by Sumana Sharma (Wright and Rayner laboratories). Camila Franca performed all Luminex plasma screening assays, analysed the results, and produced the figures.

The proteins used in each study are summarized in Table 5.2.

Table 5.2: *P. vivax* recombinant proteins used in SI and PNG screens

Group	Accession number	Name	Product	Screen		
				SIR *	SIC *	PNG **
Merozoite surface proteins (MSPs)	PVX_099980	MSP1	merozoite surface protein 1	X	X	
	PVX_097680	MSP3.3, MSP3β	merozoite surface protein 3	X	X	
	PVX_097720	MSP3.10, MSP3α	merozoite surface protein 3	X	X	
	PVX_097685	MSP3.4	merozoite surface protein 3	X		
	PVX_003775	MSP4	merozoite surface protein 4, putative	X		
	PVX_003770	MSP5	merozoite surface protein 5	X	X	
	PVX_082700	MSP7.1	merozoite surface protein 7 (MSP7)	X		
	PVX_082675	MSP7.6	merozoite surface protein 7 (MSP7)	X	X	
	PVX_082655	MSP7.9	merozoite surface protein 7 (MSP7), putative	X		
	PVX_114145	MSP10	merozoite surface protein 10, putative	X	X	
6-cysteine proteins	PVX_113775	P12	6-cysteine protein	X	X	X
	PVX_113780	P12p	6-cysteine protein (P12p)	X		
	PVX_097960	P38	6-cysteine protein (P38)	X		
	PVX_000995	P41	6-cysteine protein	X	X	X
	PVX_115165	P92	6-cysteine protein	X		
Merozoite proteins not in other families	PVX_001725	RON12	rhoptry neck protein 12, putative	X		
	PVX_088910	GAMA	GPI-anchored micronemal antigen, putative	X	X	X
	PVX_090075	Pv34, PV2	apical merozoite protein (Pf34)	X		
	PVX_090210	ARP	asparagine-rich protein	X	X	X
	PVX_090240	CyRPA	cysteine-rich protective antigen	X	X	X
	PVX_095055	RIPR	Rh5 interacting protein, putative	X	X	
	PVX_098712	RhopH3	high molecular weight rhoptry protein 3, putative	X		
	PVX_110810	DBP	Duffy receptor precursor	X		
		DBP-RII	Duffy binding protein reg. II	X		

	PVX_111290	MTRAP	merozoite TRAP-like protein, putative	X	
	PVX_081550		StAR-related lipid transfer protein, putative	X	X
	PVX_084815		conserved *Plasmodium* membrane protein, unknown function	X	
	PVX_084970		hypothetical protein, conserved	X	
	PVX_116775		hypothetical protein, conserved	X	
No known *P. falciparum* 3D7 homologs	PVX_101590	RBP2-like	reticulocyte-binding protein 2 (RBP2), like	X	
	PVX_001015		Pf52-like protein, putative	X	
	PVX_110950		hypothetical protein	X	
	PVX_110960		hypothetical protein	X	
	PVX_110965		hypothetical protein	X	

Abbreviations: SI-R=Solomon Islands reactivity screen; SI-C=Solomon Islands comprehensive screen; PNG=Papua New Guinea screen

* Biotinylated protein

** 6-His-tagged (purified) protein

5.2.2.1 Solomon Islands reactivity screening

I expressed 34 biotinylated *P. vivax* recombinant proteins (as in section 2.3.3), summarized in Table 5.2, and Camila Franca and I screened them against 48 plasma samples (1:500, based on data not shown from pilot sera screening) from a cross-sectional cohort study in SI. Plasma samples from infected and uninfected individuals were selected at random from adolescent and adult age-groups (ages 10-19 and 20-99 years) as they had a higher lifetime exposure to *P. vivax* and were more likely to have acquired immunity. Over 85% (29/34) of the proteins showed mean reactivity significantly higher than the Cd4d3+d4 tag alone (Figure 5.11) (Mann-Whitney *U* tests, $P<0.0001$-0.018). These initial results confirmed that the protein library was largely immunogenic in a second *P. vivax* endemic area. Reactivity was highly variable with a 12-fold difference in mean response from P92 to CyRPA. Mean responses were similar in adolescents and adults except for 7/34 proteins: PVX_110960, PVX_110965, MTRAP, ARP, GAMA, PVX_081550, and MSP1 ($P<0.0001$-0.042).

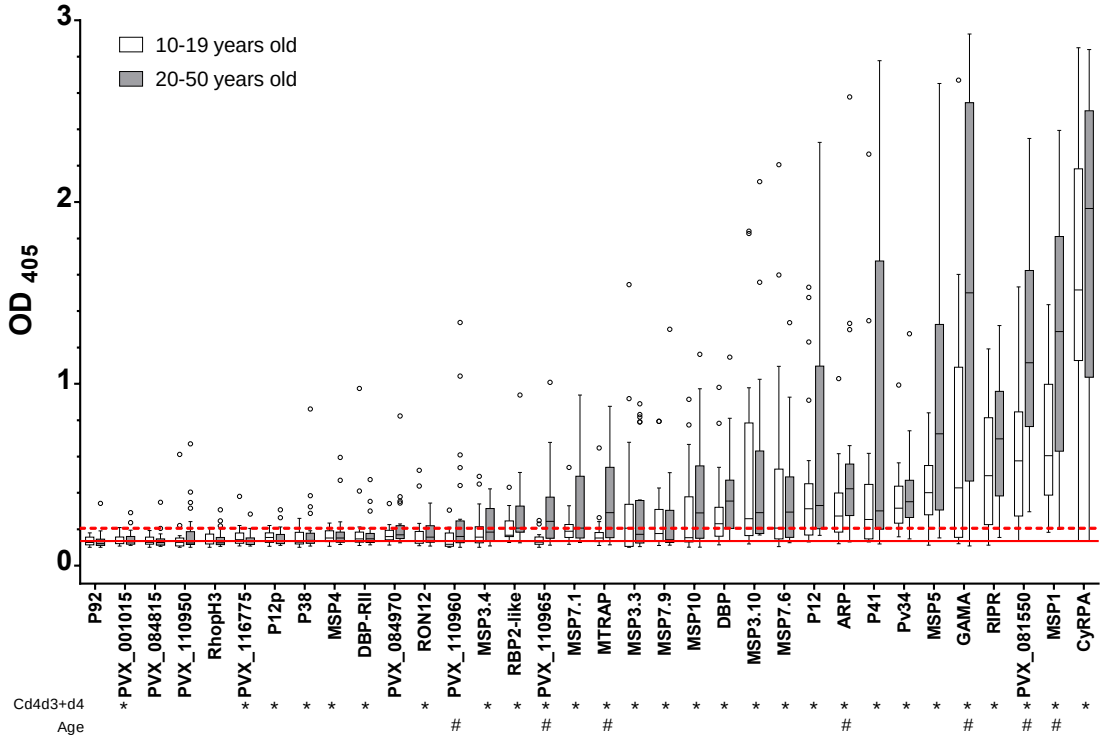

Figure 5.11: *P. vivax* recombinant proteins are immunoreactive in SI patient plasma samples

The total IgG response to 34 biotinylated *P. vivax* recombinant proteins was assessed using diluted (1:500) plasma from Solomon Islander plasma from adolescents (10-19 years, white boxes, n=22) and adults (20-50 years, gray boxes, n=24). Optical densities (OD) at 405 nm are shown. Boxplots show median OD (horizontal bar), IQR (box), range (whiskers), and outliers (open circles). IgG response to rat Cd4d3+d4 tag alone is shown as mean (solid red line) and mean + 2 standard deviations (dashed red line). 29/34 proteins showed significantly higher population responses than Cd4d3+d4 (*). 7/34 proteins showed significant differences between age group responses (¶) (Mann-Whitney U tests, $P<0.0001$-0.042; P values were deemed significant if <0.05).

5.2.2.2 Solomon Islands comprehensive screening

The protein reactivity varied widely in the SI reactivity screen, and we wanted to expand the screening for a subset of proteins. This was limited based on reagents, supplies, and time to screening 12 of the most highly immunogenic proteins (MSP1, MSP3.3, MSP3.10, MSP5, MSP7.6, MSP10, P12, P41, ARP, CyRPA, GAMA, RIPR) against 144 sera samples (including the 48 included in the reactivity screen) selected randomly for a 3x3 factorial design stratifying 3 age groups (5-9, 10-19, 20-80 years) and by 3 infection groups:

- Negative by PCR and light microscopy (LM) = Not infected
- Positive by PCR and negative by LM = PCR+
- Positive by PCR and positive by LM = PCR+ LM+

We first evaluated reactivity by defining positivity as responses more than 2 SD above the mean of the Australian malaria-naïve adult plasma samples after correcting for background by subtracting the Cd4d3+d4 values (Figure 5.12, Table 5.3).

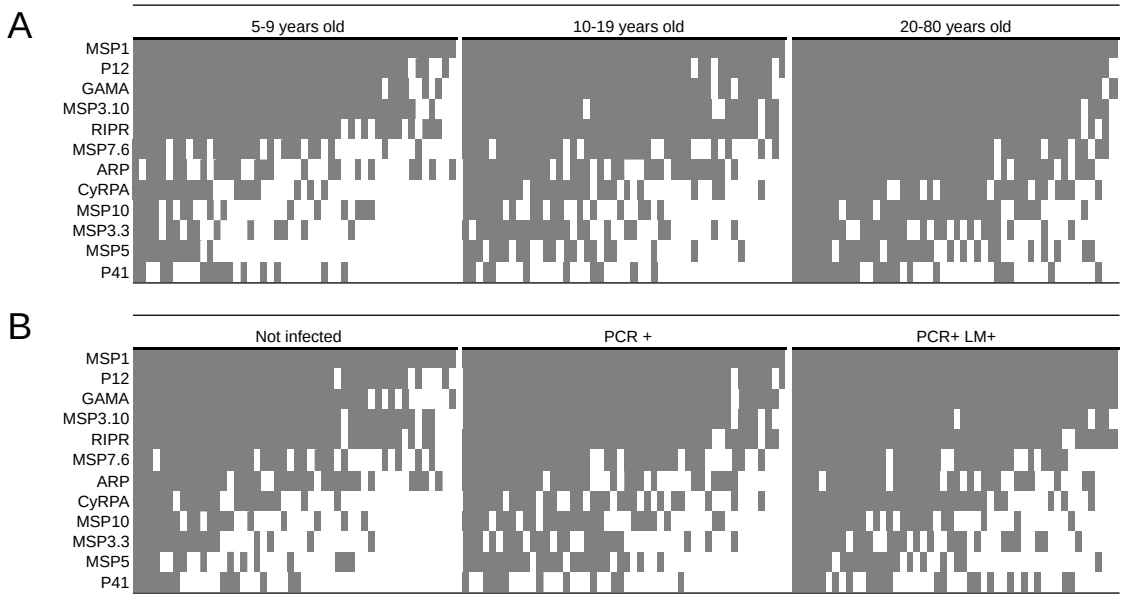

Figure 5.12: Seroreactivity in SI

Heatmaps of seropositivity, with columns representing individual plasma samples and rows representing proteins. Gray boxes represent optical density (OD) values 2 SD above the mean of the Australian malaria-naïve adult plasma samples after correcting for background by subtracting the Cd4d3+d4 values. (A) Samples grouped by age categories (5-9 years, n=48; 10-19 years, n=48; and 20-80 years, n=48). (B) Samples grouped by infection status: Not infected (n=48), samples positive by PCR (PCR+, n=48), and samples positive by both PCR and light microscopy (PCR+ LM+, n=48).

Table 5.3: Seroreactivity in SI comprehensive screen

	Age (years), n (%)			Infection status, n (%)			Total
	5-9	10-19	20-80	NI	PCR+	PCR+ LM +	
MSP1	48(100)	48(100)	48(100)	48(100)	48(100)	48(100)	144(100)
P12	44(92)	45(94)	47(98)	42(88)	46(96)	48(100)	136(94)
GAMA	42(88)	44(92)	47(98)	39(81)	46(96)	48(100)	133(92)
MSP3.10	43(90)	43(90)	46(96)	43(90)	44(92)	45(94)	132(92)
RIPR	41(85)	46(96)	45(94)	42(88)	44(92)	46(96)	132(92)
MSP7.6	25(52)	33(69)	43(90)	31(65)	35(73)	35(73)	101(70)
ARP	23(48)	32(67)	41(85)	35(73)	29(60)	32(67)	96(67)
CyRPA	19(40)	27(56)	36(75)	21(44)	27(56)	34(71)	82(57)
MSP10	15(31)	17(35)	34(71)	18(38)	25(52)	23(48)	66(46)
MSP3.3	14(29)	24(50)	26(54)	17(35)	21(44)	26(54)	64(44)
MSP5	11(23)	19(40)	29(60)	14(29)	24(50)	21(44)	59(41)
P41	14(29)	10(21)	21(44)	13(27)	11(23)	21(44)	45(31)

Table 5.4: Seroreactivity in SI and Cambodian parasitemic plasma samples

	SI (PCR+ LM +)	Cambodia (acute)
MSP1	48(100)	48(100)
P12	48(100)	44(92)
GAMA	48(100)	48(100)
PVX_081550		47(98)
MSP3.10	45(94)	
RIPR	46(96)	
DBP		41(85)
MSP7.6	35(73)	
ARP	32(67)	17(35)
CyRPA	34(71)	38(79)
MSP10	23(48)	17(35)
MSP3.3	26(54)	
RBPII-like		26(54)
MSP5	21(44)	47(98)
P41	21(44)	29(60)

Similar to Cambodia, the overall seropositivity varied widely from 31% for P41 to 100% for MSP1 (Table 5.3). Five of the 12 proteins were recognized in over 90% of samples (MSP1, P12, GAMA, MSP3.10, RIPR). The seropositivity values in the Cambodian survey of acute plasma (positive by LM) and the higher-density infections in SI (PCR+ LM+) were similar (within 16%) for 6/8 proteins in common (MSP1, P12, GAMA, CyRPA, MSP10, P41) (Table 5.4). ARP and MSP5 seropositivity varied more widely with a 32% and 54% difference between the datasets, respectively. The PCR+ LM+ group was primarily asymptomatic in contrast to the acutely febrile Cambodian group, which may contribute to the observed differences.

We next compared the age and infection groups by the breadth of response in each plasma sample. Histograms and density plots for the number of antigens recognized by each age and infection category show a shift in distribution toward greater numbers of antigens recognized in the older age groups and higher parasite densities (Figure 5.13), with mean numbers of antigens recognized as follows: 7.06 in children (5-9 years), 8.08 in adolescents (10-19 years), and 9.65 in adults (20-80 years). The shift between the children and adults was statistically significant (negative binomial regression, $P<0.001$). The shift between the different infection groups was less pronounced with mean numbers of antigens recognized as follows: 7.56 in uninfected individuals (Not infected), 8.33 in individuals positive by PCR only (PCR+), and 8.90 in individuals positive by both PCR and light microscopy (PCR+ LM+). The shift in numbers of antigens recognized in uninfected and higher-density infection (PCR+ LM+) samples was also statistically significant (negative binomial regression, $P=0.005$). These results indicated that the breadth of response increased both with age and infection density; shifts were widest between age groups than between infection groups.

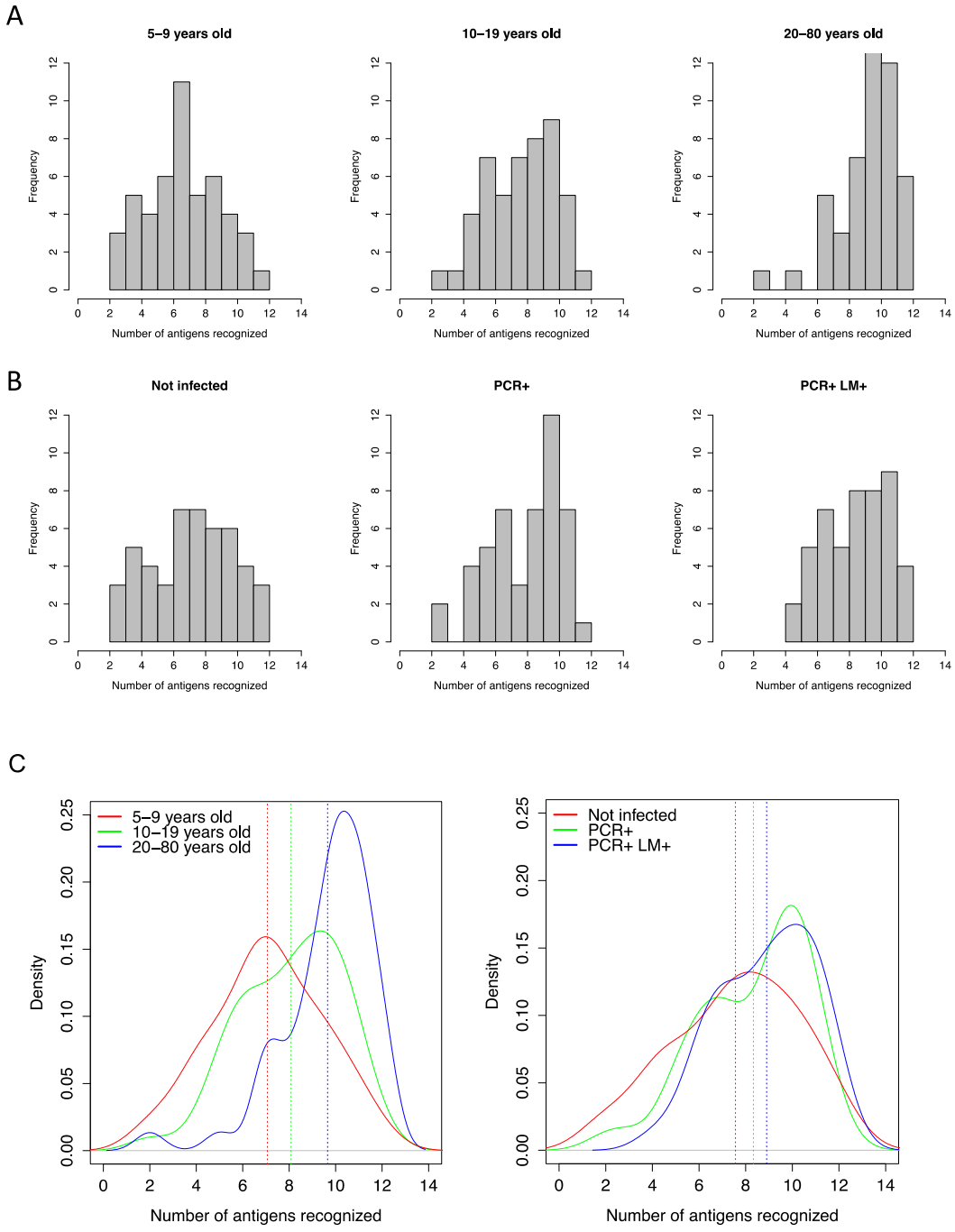

Figure 5.13: Breadth of antigens recognized in SI

(A-B) Histograms of the frequency of number of antigens recognized in SI plasma samples by age group (A) and infection status (B). (C) Density plots of number of antigens recognized by age group (left panel) and infection status (right panel), with mean numbers of antigens recognized by each group denoted by dotted lines. Significant shifts between children (red line, left panel) and adults (blue line, left panel) (P<0.001) and uninfected (red line, right panel) and PCR+ LM+ (blue line, right panel) (P=0.005) were observed. PCR+ = positive by PCR; PCR+LM+ = positive by both PCR and light microscopy. (Negative binomial regression, P values were deemed significant if <0.05).

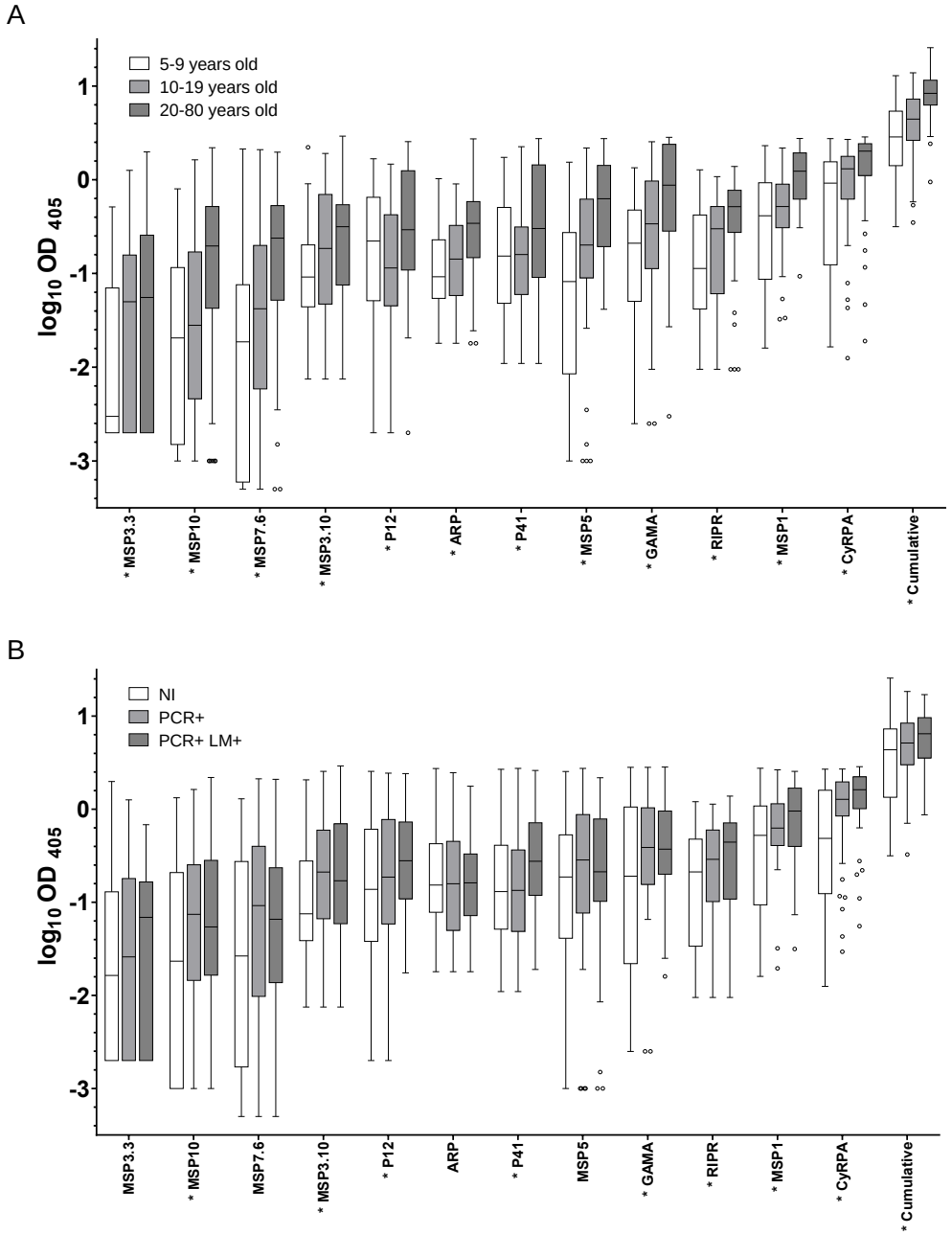

Figure 5.14: Age- and infection-associated increases in IgG in SI

SI plasma samples from 3 age categories (5-9 years, n=48; 10-20 years, n=48; 20-80 years, n=48) with 3 infection statuses (Not infected, NI, n=48; PCR positive, PCR+, n=48; PCR and light microscopy positive, PCR+LM+, n=48) and Australian malaria-naïve sera were screened against 12 *P. vivax* recombinant proteins (MSP3.3, MSP10, MSP7.6, MSP3.10, P12, ARP, P41, MSP5, GAMA, RIPR, MSP1, CyRPA) by ELISA. Log optical densities (OD) at 405 nm are shown. Boxplots show median (horizontal bar), IQR (box), range (whiskers), and outliers (open circles). (A) Age-associated increases in IgG response between children (5-9 years) and adults (20-80 years) were observed for all proteins (*, ANOVA, P<0.001-0.027). (B) Infection-associated increases in IgG responses were observed for a subset of proteins (*, ANOVA P<0.001-0.039). P values were deemed significant if <0.05.

We compared the individual responses between the children and adults (Figure 5.14A). Increases in IgG response were strongly associated with age for the cumulative response (combining the IgG response for all proteins) as well as for each individual protein (ANOVA, $P<0.001$ to 0.027). All *P* values are reported in Table 5.5.

Table 5.5: *P* values from ANOVA for SI comprehensive screen

	Children (5-9 years) Adults (20-80 years)	**Not infected**	
		PCR+	PCR+ LM+
MSP3.3	0.027	0.581	0.309
MSP10	0.001	0.116	0.039
MSP7.6	<0.001	0.108	0.141
MSP3.10	0.006	0.013	0.032
P12	0.009	0.228	0.020
ARP	<0.001	0.698	0.613
P41	0.012	0.483	0.035
MSP5	<0.001	0.111	0.094
GAMA	<0.001	0.015	0.015
RIPR	0.001	0.175	0.019
MSP1	<0.001	0.344	0.020
CyRPA	<0.001	0.022	<0.001
Cumulative response	<0.001	0.002	<0.001

When comparing the samples by infection status, the cumulative IgG response also increased from non-infected individuals to both low-density infections (PCR+) ($P=0.002$) and high-density infections (PCR+LM+) ($P<0.001$) (Figure 5.14B, Table 5.5). At the individual protein level, however, only a subset of proteins showed significant associations. In comparing the non-infected group to the low-density PCR+ group, 3 proteins showed significant differences: CyRPA ($P=0.022$), GAMA ($P=0.015$), and MSP3.10 ($P=0.013$). Four additional proteins showed significant increases in antibody levels between non-infected and higher-density infections (PCR+LM+): CyRPA ($P<0.001$), GAMA ($P=0.015$), MSP3.10 ($P=0.032$), P12 ($P=0.020$), P41 ($P=0.035$), MSP1 ($P=0.020$), MSP10 ($P=0.039$), and RIPR ($P=0.019$). Overall, the *P. vivax* recombinant proteins tested are markers of cumulative exposure, showing significant increases in IgG responses with age. Several proteins are also markers of current infection, showing increased IgG responses in individuals with detectable parasites.

5.2.2.3 SI Comprehensive screen multivariate analysis

The SI sample set was analysed by Camila Franca and Ivo Mueller in a multivariate analysis including all available variables to determine if additional associations with antibody responses could be made. Additional variables included region of collection, clinical symptoms, socioeconomic indicators, usage of ITNs, etc. Full details are published in S2 Table in (Franca et al., 2016):

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4868274/bin/pntd.0004639.s004.xlsx.

Age- and infection-associated increases in IgG responses remained significant (multivariate ANOVA, $P<0.001$-0.031), for all comparisons except for 2 individual protein responses within the infection status comparisons; MSP3.10 IgG increases in low-density infections were no longer significant and MSP5 IgG increases in high-density infections were only significantly higher in adults ($P=0.022$). ITN usage in previous years was the only other variable with significant associations; ITN usage associated with reduced IgG levels for 3 proteins: RIPR ($P=0.030$), MSP1 ($P=0.024$), and MSP3.3 ($P=0.015$). This is potentially due to decreased exposure in these households, which may indicate that IgG responses to these proteins declines in the absence of continued exposure.

5.2.2.4 Correlations with protection in PNG longitudinal cohort study

Our studies in SI demonstrated the utility of screening a panel of proteins, and we wanted to expand the study to investigate whether IgG responses to the proteins correlated with protection from clinical disease. A longitudinal cohort study in PNG led by Ivo Mueller (Lin et al., 2010) provided an ideal sample collection to address this question. Based on the results from the SI plasma screens, we prioritized 6 proteins to be screened in a high-throughput Luminex screening assay. Camila Franca conjugated His-purified *P. vivax* recombinant proteins (P12, P41, CyRPA, ARP, GAMA, PVX_081550) to Luminex beads in order to screen 230 PNG plasma samples, the collaborative results of which were published along with the SI results (Franca et al., 2016). Enrolled children, aged 1-3 years, provided blood samples every 2 weeks for 16 months, and any detected parasites were genotyped to estimate the number of genetically-distinct *P. vivax* infections acquired over time (termed the molecular force of blood-stage infection, $_{mol}$FOB).

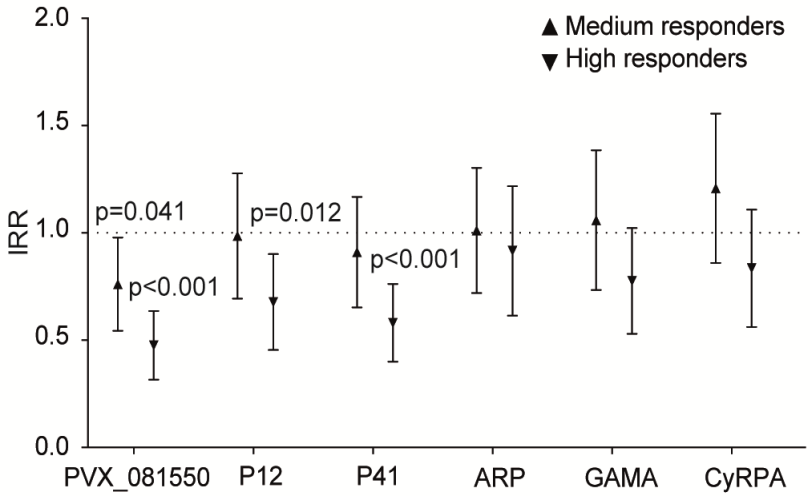

Figure 5.15: High IgG responses to PVX_081550, P12, and P41 are associated with reduced incidence of clinical disease

Incidence rate ratios and 95% confidence intervals, adjusted for exposure ($_{mol}$FOB), age, season, and village of residency are shown. Clinical malaria was defined as axillary temperature ≥37.5^{o}C or history of fever in the preceding 48 hours with a current *P. vivax* parasitemia >500 parasites/µl. (Negative binomial GEE models). Figure produced by Camila Franca and reprinted from (Franca et al., 2016) under the Creative Commons Attribution (CC BY) license.

Table 5.6: Association between levels of IgG to *P. vivax* merozoite proteins and protection against clinical malaria in PNG children*

Antigen	uIRR	95%CI		*P* value	aIRR*	95%CI		*P* value
PVX_081550 M	0.76	0.54	1.05	0.10	0.74	0.55	0.99	0.041
PVX_081550 H	0.41	0.29	0.60	<0.001	0.46	0.33	0.64	<0.001
ARP M	0.93	0.66	1.32	0.68	0.98	0.73	1.32	0.91
ARP H	1.00	0.69	1.46	0.98	0.88	0.63	1.23	0.47
GAMA M	1.12	0.80	1.57	0.51	1.03	0.75	1.40	0.87
GAMA H	0.82	0.55	1.23	0.34	0.75	0.54	1.04	0.08
P41 M	0.96	0.68	1.36	0.83	0.89	0.67	1.18	0.41
P41 H	0.63	0.43	0.93	0.019	0.56	0.41	0.77	<0.001
P12 M	1.05	0.75	1.47	0.79	0.96	0.71	1.29	0.77
P12 H	0.69	0.47	1.02	0.06	0.65	0.47	0.91	0.012
CyRPA M	1.06	0.76	1.46	0.74	1.17	0.88	1.57	0.28
CyRPA H	0.88	0.59	1.30	0.52	0.81	0.58	1.12	0.20

* With parasite density >500/µL of blood; table by Camila Franca and reprinted from (Franca et al., 2016) under the Creative Commons Attribution (CC BY) license.

The study produced several important results. The data show age-associated increases in IgG response for ARP, CyRPA, and PVX_081550 (r=0.15-0.25; *P*=0.001-0.027), indicating that these increases were generalizable to 2 different populations living in areas of moderate and high transmission. Infection was associated with increased IgG levels to CyRPA (*P*<0.001), P12 (P<0.001), P41 (P=0.001), and PVX_081550 (P=0.001), which was also observed in SI (except for PVX_081550, which was not included in the SI comprehensive screen).

Children in the study experienced an average of 1.11 malaria episodes during the follow-up period with *P. vivax* >500 parasites/µl/year at risk. After adjusting for confounders, high IgG responses to 2 proteins, P12 and P41, and both medium and high IgG responses to PVX_081550 were strongly associated with a reduced incidence of clinical disease (IRR 0.46-0.74: *P* <0.001-0.041) (Figure 5.15, Table 5.6). IgG responses to P12, P41, and PVX_081550 were significantly correlated (r=0.34-0.66; *P*<0.001). This may indicate that IgG responses against the 3 proteins are co-acquired. After performing a multivariate analysis, high levels to PVX_081550 remained significantly associated with protection from clinical disease (IRR_H 0.54; *P*=0.001). This result indicates PVX_081550 may be a target of NAI, a marker of a person's immune status, or both.

5.3 Discussion

In this chapter, I presented results demonstrating that the *P. vivax* recombinant protein library is immunoreactive and properly folded. I also presented immunoepidemiological studies from 3 *P. vivax*-endemic areas (Cambodia, SI, and PNG) aimed at furthering our understanding of immune responses during and after *P. vivax* infection.

5.3.1 Key findings in Cambodian plasma screens

Pilot ELISAs that screened 42 individual plasma samples from *P. vivax*-infected patients against 6 proteins suggested that our proteins were immunoreactive, though with varying breadth and levels; P12 showed the greatest initial response with 98% of samples reactive above 2 SD above the mean of American *P. vivax*-naïve controls.

However, limited conclusions could be drawn from this, as the plasma set used was pre-selected based on reactivity to recombinant *P. vivax* MSP1 (produced in the same mammalian expression system, but with a different plasmid backbone), which may not

represent the immunoreactivity of the *P. vivax*-exposed population. This may also have selected for plasma samples that were also non-specifically reactive, as several plasma showed reactivity to Cd4d3+d4 tag more than 5x higher than the reactivity in the American *P. vivax*-naïve controls. Overall, the pilot results established that our proteins were immunoreactive and that a 1:600 plasma dilution was optimal for screening plasma samples against the full *P. vivax* recombinant protein library.

In order to fully explore whether our library proteins were properly folded, contained conformational epitopes, and were targets for naturally-acquired humoral immunity, I screened 34 recombinant *P. vivax* proteins against pooled plasma from 14 Cambodian patients with acute vivax malaria. Of the 34 proteins screened, 27 showed at least a 2-fold change in IgG reactivity between naïve sera and *P. vivax*-exposed plasma samples. Our results aligned well with a *P. vivax* seroreactivity screen in Korean patients (Chen et al., 2010), which detected IgG reactivity to 18 full-length proteins and protein fragments, all produced in the wheat germ cell-free system. Of these 18 proteins, 7 (MSP1, MSP3.3, MSP10, P12, P41, ARP, PVX_081550) were represented in our library and all 7 showed at least a 3-fold change in IgG reactivity between naïve sera and *P. vivax*-exposed plasma samples in our Cambodian screen. This establishes that these antigens are common targets of immunoreactivity across different transmission regions.

Conformational epitopes were present in 18/34 antigens, as indicated by >20% reduction in IgG reactivity after heat treatment. IgG reactivity to 12 of these 18 antigens was predominantly conformation-specific, as indicated by >50% reduction in IgG reactivity after heat treatment. These findings suggest that our library proteins are properly folded. The tertiary structure of MSP3 family proteins is known to be recalcitrant to heat denaturation, so the limited change in immunoreactivity following heat treatment of these antigens does not necessarily indicate a lack of tertiary conformation. In addition to MSP3 proteins, several other proteins also show an absent or weak heat-sensitive response, including those with higher overall responses, such as MSP4 and MSP10, and several with weak overall responses, such as P92 and PVX_110965. The lack of heat-sensitive responses may be due to 1 of 3 factors, each of which might be applicable for a given protein. First, the protein may not denature or remain denatured under the heat treatment we used (80^{o}C for 10 min), which is likely the case for MSP3, which is known to be highly stable. Follow-up experiments testing other techniques, such as chemical denaturation or more extreme heating conditions, could clarify this point. Second, most of

the primary antibody response may target linear epitopes within the protein that are not affected by denaturation. At least 2 of the proteins where responses were not affected by heat treatment, MSP4 and ARP, contain regions of low complexity that could fit with such a model. Third, the lack of a change in response could indicate an issue with protein quality. Although all of these proteins are visible by Western blotting (Figure 4.6), P92 and PVX_001015 are very faint. Additional experiments are needed to fully explore these possibilities, though the fact that the majority of antigens displayed a heat-sensitive response suggests that most library proteins are properly folded. As well as suggesting that the full-length ectodomains contain folded epitopes, these data also indicate that humans naturally acquire IgG responses to multiple *P. vivax* proteins, thus supporting their further exploration as candidate vaccine antigens.

The Cambodian reactivity screen using individual plasma against a subset of 11 proteins demonstrated that reactivity to the *P. vivax* proteins varied widely from 35% for ARP and MSP10 to 100% for MSP1 and GAMA. This variation is similarly observed in *P. falciparum* where IgG responses to merozoite antigens can vary substantially between individuals (Osier et al., 2014). P12 was reactive in 92% of Cambodian samples; *P. falciparum* P12 has shown seroreactivity in 96% of 286 Kenyan individuals (Osier et al., 2014). IgG responses to P12 may be a useful marker of infection with *P. vivax*, *P. falciparum*, or both in broader epidemiological investigations. Recombinant *P. vivax* MSP1 and GAMA were recognized in 100% of Cambodian samples, but recombinant P12 (61 kDa) is smaller than GAMA (103 kDa) and MSP1 (215kDa), has high expression in the HEK293E cell system and is stable for longer periods at 4^{o}C, potentially making it easier to use in future sero-surveillance studies.

5.3.2 Key findings in SI and PNG populations

The SI reactivity screening demonstrated that the *P. vivax* recombinant protein library was largely immunogenic in a second *P. vivax*-endemic country. Over 75% of proteins were recognized in each country with 29/34 proteins recognized in SI compared to 27/34 in Cambodia. This represents the largest number to date of immunogenic responses to a *P. vivax* recombinant protein library, and supports the utility of the *P. vivax* recombinant library in future worldwide immunoepidemiological studies.

We performed an expanded screen for 12 highly immunogenic proteins identified in the SI reactivity screen (MSP3.3, MSP10, MSP7.6, MSP3.10, P12, ARP, P41, MSP5,

GAMA, RIPR, MSP1, CyRPA). Several proteins were immunogenic in over 90% of samples (MSP1, P12, GAMA, MSP3.10, RIPR). Reactivity in over 90% of samples was similarly found for MSP1, GAMA, and P12 in the Cambodian reactivity screen (section 5.2.1.3), indicating any of these 3 proteins may be useful as markers of *P. vivax* exposure. The comprehensive screen also confirmed that IgG increased both with age (12/12 proteins tested) and current infection (7/12 proteins tested). IgG increased more strongly with age than with infection, indicating that cumulative past exposure to *P. vivax* malaria was a stronger driver of immune response than current infection. These results, along with the fact that low-density asymptomatic parasitemias are common in SI indicate that long-lasting stable antibody responses persist even in an area of rapidly declining transmission.

The Cambodian and SI screens enabled us to prioritize 6 proteins (P12, P41, PVX_081550, ARP, GAMA, CyRPA) for screening against a longitudinal cohort of young children in a high *P. vivax* transmission setting in PNG. The PNG study found correlations with protection from disease for 3 proteins (P12, P41, PVX_081550), with a similar reduction in risk of disease as what was found for high antibody titers to *P. vivax* MSP3.10 and MSP9 (Stanisic et al., 2013). As with *P. falciparum* P12 and P41, *P. vivax* P12 and P41 form a heterodimer, though with a much higher affinity than with *P. falciparum* (discussed in detail in Chapter 4). The function of the complex, however, is not known. Antibodies directed individually against *P. falciparum* P12 and P41 did not inhibit parasite invasion or growth *in vitro* (Taechalertpaisarn et al., 2012). Functional work in *P. vivax* field isolates will be needed to establish whether the same is true for this species. *P. falciparum* P41 was associated with clinical protection in Kenya and PNG (Richards et al., 2013, Osier et al., 2014).

The strongest association with protection was for PVX_081550, for which relatively little is known. It was recently characterized as a StAR-related lipid transfer protein in *P. falciparum* (van Ooij et al., 2013). The protein localizes to the parasitophorous vacuole (PV) with some additional localization evidence in the apical region of merozoites. van Ooij et al. hypothesize the protein is involved with PV formation in newly-invaded erythrocytes (van Ooij et al., 2013). The protein was highly immunogenic in the Cambodian and SI screens, and this finding is also supported by a *P. falciparum* screen (Fan et al., 2013). Whether IgG responses directed against the protein actually inhibit invasion or are a by-product of exposure to the immune system after schizont rupture are

unknown. In the latter case, responses to the protein would serve as a useful marker of an individual's immune status. Both the *P. falciparum* and *P. vivax* orthologs appear to be polymorphic with nonsynonymous/synonymous SNP ratios of 1.9-2.3 (plasmodb.org), but whether this indicates any selective pressure is also not known. Follow-up studies involving both species are needed to determine the protein's function and potential as a vaccine candidate.

5.3.3 Limitations and future work

No differences were detected between acute and convalescent plasma samples, but additional experiments are needed to determine if this result is conclusive. A screening of a subset of proteins in more samples will clarify if small IgG changes are occurring in this period, and plasma pairs with the widest separation in dates (28 days) could be prioritized in order to maximize the possibility of observing changes.

The reactivity cut-offs for the SI comprehensive screening were set at 2SD above the mean of multiple readings from a pool of *P. vivax*-naïve Australian sera. While multiple readings were useful for obtaining an accurate measurement of the pool, the standard practice is to measure responses to individual control sera when calculating a cut-off. This potentially lowers the reactivity threshold for the assay, as individual sera may have had more variable responses. The fact that the Cambodian reactivity data, which included 24 individual malaria-naïve US sera samples, shows similar (within 16%) reactivity for most antigens (MSP5 and ARP as the exceptions), suggests that the threshold set using pooled sera was not a significant underestimation of background reactivity in this case. Ideally, all future screenings will include individual *P. vivax*-naïve sera controls.

Strong age-associated IgG increases in SI were detected even as *P. vivax* malaria continues to decline in this study site. It would be useful to follow up the 2011 cross-sectional study in several years to determine whether IgG responses continue to persist without continued exposure, or with greatly reduced exposure. Also, the screening of recombinant *P. vivax* proteins in the SI patient plasma used nearly the full library, with 34/39 proteins screened. The PNG plasma screen, however, included only 6 proteins, as the Luminex screening method required purified proteins. Ideally, this screen would have included additional proteins, but we were limited by both manpower and shared laboratory facilities to producing purified proteins in sufficient quantities for only a

subset. Correlations with protection were found in this study, and repeating the screen with additional purified proteins may expand this list.

The immunoepidemiological screens supported high immunogenicity for several proteins and protection from disease for 3 proteins in particular. From these data, we can prioritize a list of proteins for further functional studies. Such studies would include generating polyclonal antibodies against a subset of the recombinant *P. vivax* proteins for use both in immunofluorescence microscopy studies to establish protein locations within the merozoite (i.e., surface, apical, microneme, etc.), and *P. vivax ex vivo* reticulocyte invasion assays (Russell et al., 2011) to test whether they are able to block invasion. Antibodies could be tested both individually and in combination; such studies are planned to proceed later in 2016.

5.4 Conclusion

To characterize naturally-acquired immune responses in *P. vivax* malaria, we screened the *P. vivax* recombinant protein library against plasma collections from 3 *P. vivax*-endemic countries: Cambodia (low transmission), SI (moderate transmission), and PNG (high transmission). The results from Cambodia indicated that nearly all of the recombinant *P. vivax* full-length ectodomains are immunogenic, over half contain conformational epitopes, and humans naturally acquire IgG responses to multiple *P. vivax* proteins. SI screens supported nearly the entire library as being immunogenic in a second transmission setting, and found strong age-associated and less pronounced infection-associated increases in IgG, indicating that past-cumulative exposure is a stronger driver of IgG responses compared to recent exposure. The PNG screens found that responses to 3 proteins correlated with protection from disease, including a hypothetical protein for which little is known. The overall results support the utility of using a panel of proteins to make systematic comparisons to prioritize candidates for vaccine studies, and identify clear candidates for subsequent functional study.