Development and validation of serological markers for detecting recent *Plasmodium vivax* infection

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A major gap in the *Plasmodium vivax* elimination toolkit is the identification of individuals carrying clinically silent and undetectable liver-stage parasites, called hypnozoites. This study developed a panel of serological exposure markers capable of classifying individuals with recent *P. vivax* infections who have a high likelihood of harboring hypnozoites. We measured IgG antibody responses to 342 *P. vivax* proteins in longitudinal clinical cohorts conducted in Thailand and Brazil and identified candidate serological markers of exposure. Candidate markers were validated using samples from year-long observational cohorts conducted in Thailand, Brazil and the Solomon Islands and antibody responses to eight *P. vivax* proteins classified *P. vivax* infections in the previous 9 months with 80% sensitivity and specificity. Mathematical models demonstrate that a serological testing and treatment strategy could reduce *P. vivax* prevalence by 59–69%. These eight antibody responses can serve as a biomarker, identifying individuals who should be targeted with anti-hypnozoite therapy.

Elimination of malaria by 2030 is now the explicit goal of all malaria-endemic countries in the Asia-Pacific region and the Americas. While impressive progress toward this goal has been made, global funding for malaria control has remained unchanged since 2010 and progress has stalled in many parts of the world. New interventions and tools for better targeting of limited resources are urgently needed.

A major hurdle for elimination is the increasing proportion of malaria cases caused by *P. vivax* as malaria endemicity declines. *P. vivax* has unique biological features that make its control difficult, including high prevalence of low-density, asymptomatic infections¹ and a liver stage that can reactivate weeks to months after initial infection, resulting in relapses that cause morbidity and sustain transmission. These hypnozoites, undetectable with current diagnostics, can be responsible for >80% of all blood-stage infections.¹ Identifying and targeting individuals with hypnozoites is thus essential for accelerating and achieving malaria elimination. In addition, as endemicity decreases, malaria transmission becomes increasingly fragmented and highly seasonal rendering blanket approaches to malaria control and elimination inefficient. This requires new, innovative tools and approaches specifically designed to assist in targeting interventions to the changing malaria epidemiological landscape.

National malaria control programs rely almost exclusively on microscopy or rapid diagnostic tests for routine detection of malaria cases at health clinics and for surveillance using mass blood surveys. These tools have limited sensitivity for detecting individuals with low-density asymptomatic infections, making it difficult for control programs to efficiently identify areas of low and high *P. vivax* transmission and target their resources accordingly. Molecular techniques such as PCR have greater sensitivity but are rarely implemented by control programs owing to high cost and the need for specialized laboratories. All of these methods can only detect individuals with a current blood-stage infection, rendering
mass screening and treatment (MSAT) approaches ineffective for reducing Plasmodium vivax transmission because they fail to treat individuals who only carry hidden Plasmodium vivax liver-stage infections\(^3\) with no circulating blood-stage parasites. Mass drug administration (MDA) is predicted to be a highly effective control tool but only if it includes a drug that targets Plasmodium vivax hypnozoites\(^1\). Unfortunately, primaquine and tafenoquine, the only current drugs able to eliminate hypnozoites, have toxic side effects in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals\(^6\), limiting their acceptability for MDA campaigns, particularly in low-transmission areas where >90% recipients will have no direct benefit from the treatment. There is thus a diagnostic gap, with MSAT ineffective due to under-treatment and MDA unacceptable due to over-treatment. A strategy that efficiently targets individuals with current blood-stage infections and those with hypnozoites is required.

Blood-stage Plasmodium vivax infections induce robust IgG antibody responses to a broad range of Plasmodium vivax proteins, even following low-density asymptomatic infections\(^2\). These responses can be long lived, even after clearance of blood-stage infection\(^2\). Hence, antibodies are markers of past exposure as well as current infection\(^1\). Serological exposure markers (SEMs) have been used for surveillance of malaria and a number of other infectious diseases (leishmaniasis, influenza and trachoma) and antibody responses can be simply and cheaply measured in point-of-care tests\(^6\). SEMs may also be used for risk stratification and as guidance for targeted malaria control and elimination interventions\(^7\). For Plasmodium vivax there is an important additional, individual-level application for SEMs: to target treatment to people at risk of carrying clinically silent hypnozoites. While it is not possible to directly detect hypnozoites with current technology, all tropical and subtropical Plasmodium vivax strains cause a primary infection followed by a first relapse after no more than 6–9 months\(^8\). Therefore, any individual with a blood-stage infection within the previous 9 months, who has not received anti-liver-stage drugs, is likely to be a hypnozoite carrier. A carefully selected panel of proteins, inducing antibodies that signify exposure within the previous 9 months, could provide an accurate measure of current transmission and also highlight individuals likely to harbor hypnozoites who could be targeted for treatment with anti-hypnozoite drugs after serological testing and treatment (seroTAT).

Here, we screened 342 Plasmodium vivax proteins for their ability to detect recent Plasmodium vivax infections and validated their use in malaria-endemic regions of Thailand, Brazil and the Solomon Islands.

**Results**

**Antigen discovery phase.** Data on the magnitude and longevity of IgG responses to 342 Plasmodium vivax proteins following symptomatic Plasmodium vivax infections\(^8\) were used to identify suitable proteins for detecting recent exposure. IgG was measured in four longitudinal plasma samples collected over a 9-month period from individuals in Thailand (\(n=32\)) and Brazil (\(n=33\)) to enable estimation of antibody half-life\(^8\) (Supplementary Table 1).

A downselection pipeline was developed to identify candidate serological markers using these data (Fig. 1). Plasmodium vivax proteins were first prioritized, with selection of those that had similar estimated IgG half-lives in both antigen discovery cohorts (Thailand and Brazil) and were highly immunogenic at the time of infection (≥50% seropositive individuals) (Fig. 1a). Using these, 142 of the 342 Plasmodium vivax proteins were prioritized as having suitable characteristics for candidate SEMs.

A statistical model for estimating time since infection was used to test the ability of the 142 prioritized proteins, individually or in combination, to predict time since last infection (see Supplementary Methods and Supplementary Figs. 1–3). Figure 2a,b shows examples of antibody kinetics to five proteins in two representative individuals from Thailand and Brazil. Figure 2c,d shows the estimated time since last infection with uncertainty, for antibody responses measured to the five proteins in Fig. 2a,b at 6 months after infection, for the same two representative individuals. Increasing the number of proteins to at least five resulted in higher accuracy compared to using a single protein alone, with only small incremental improvements in the accuracy beyond 20 proteins (Supplementary Fig. 4). A simulated annealing algorithm was employed to determine optimal combinations of proteins that maximize the likelihood of correctly estimating the time since last infection (Fig. 2e). While some proteins had 100% probability of being included in a successful panel of a set size (Fig. 2e), there was redundancy in choice of additional proteins and hence all were ranked based on their probability of inclusion in a 20-protein panel (given the limited improvements beyond 20 proteins). The 24 highest ranked proteins were selected for further testing.

The wheat germ cell-free (WGCF) system was selected for expression of proteins owing to its eukaryotic nature and past success in expressing Plasmodium proteins\(^1\). Despite all proteins being expressed in this system previously as crude proteins\(^1\), not all could be produced at high yield and high purity and thus an additional round of downselection using ranking from the simulated annealing algorithm was performed to increase the number of proteins retained at this point in the pipeline. The algorithm was run on 104 of the 142 prioritized proteins, excluding the top 24 already selected (to provide more discriminatory power for selecting additional proteins) and 14 proteins known to have low yields or form aggregates from our previous work. An additional 31 proteins were selected in the second round using the same methodology, with 55 Plasmodium vivax proteins in total identified as suitable candidate SEMs. Of these, 40 were successfully produced at high yield and purity using the WGCF system (Supplementary Table 2).

An additional 20 proteins known to be highly immunogenic were added to these 40 proteins\(^2\). This included 4 proteins already within the downselected panel of 40 (different constructs or produced by a different laboratory and protein expression systems) and 3 distinct constructs of PVX_110810 (Duffy binding protein,
Validation phase. The 60 candidate SEMs were validated in larger geographically diverse cohorts with a known history of malaria infections in the preceding 12 months, using plasma samples from three year-long observational cohort studies in Thailand, Brazil and the Solomon Islands (see Methods and Extended Data Fig. 1). Individuals in these cohorts were assessed monthly for Plasmodium spp. by quantitative PCR (qPCR), with continuous concurrent parasitic case detection at local malaria clinics and hospitals. Plasma samples from the last visit of these cohorts (n = 829, 928 and 754 in Thailand, Brazil and the Solomon Islands, respectively) were used to measure IgG responses in a multiplex Luminex assay to the 60 P. vivax proteins. Overall, 158 of 2,511 individuals in the cohorts had a concurrent P. vivax infection, detected by qPCR, at the time the plasma was collected; IgG antibody levels were strongly associated with current infection status for each of the 60 proteins in the Thai cohort (odds ratio (OR) 2.1–7.7, P < 0.05) and most proteins in the Brazilian cohort (57 of 60, OR 1.5–7.1, P < 0.05; Supplementary Table 3). This association was not as clear for the pediatric Solomon Islands cohort, with IgG levels to only 29 out of 60 proteins significantly associated with current P. vivax infections (OR 1.7–6.3, P < 0.05). Overall, there was a pattern of decreasing IgG magnitude with increasing time since last P. vivax infection (Fig. 3a–h and Extended Data Fig. 2), with minimal reactivity in malaria-naïve negative control individuals from Bangkok, Rio de Janeiro and Melbourne.

Recent infections were defined as PCR-confirmed infections occurring within the past 9 months. Measurements of IgG antibodies to a number of individual proteins were able to classify individuals as infected with P. vivax within the past 9 months or not, with differing degrees of accuracy (Fig. 3i). Protein PVX_094255 (reticulocyte binding protein 2b, RBP2b) reached 75% sensitivity and 75% specificity when used alone. IgG levels to the 60 candidate SEMs were correlated (Fig. 3j) with different distributions evident between the three geographic regions (Fig. 3k). A linear discriminant analysis (LDA) classification algorithm was used to identify combinations of the 60 candidate SEMs that could accurately predict recent infections. Redundancy was found with multiple combinations of P. vivax proteins able to accurately predict recent infection when included in panels of up to eight proteins (Supplementary Fig. 5). The top eight most frequently identified proteins when used in combination were: PVX_094255 (RBP2b), PVX_087885 (rhoptry-associated membrane antigen (RAMA), putative), PVX_099980 (merozoite surface protein (MSP)1 (ref. 30)), PVX_096995 (tryptophan-rich antigen (Pv-fam-a), PvTRAg_2 (ref. 31)), PVX_097625 (MSP8 (ref. 32), putative), PVX_112670 (unspecified product previously...
Constructs (RBP2b161–1,454 and RBP2b1,986–2,653) were highly correlated with the highest individual AUC that work best in combination. Under the curve (AUC), note that it is not necessarily the proteins annotated as a tryptophan-rich antigen (PvTRAg_28 (ref. 1)), KMZ83376.1 (erythrocyte-binding protein II, EBPII 33) and PVX_097720 (MSP3.10 (ref. 34)) (see Fig. 3i for receiver operating characteristic (ROC) curves and Table 1 for protein details and Supplementary Fig. 6 for network analysis). Supplementary Table 2 shows the individual ranking of all 60 proteins by area under the curve (AUC), note that it is not necessarily the proteins with the highest individual AUC that work best in combination.

As shown in Table 1, for the top protein PVX_094255 (RBP2b), there were two separate constructs mapping to different regions of the protein (RBP2b1,41–1,454 and RBP2b1,986–2,653), expressed and purified using different methods. Antibody levels to these two constructs (RBP2b1,41–1,454 and RBP2b1,986–2,653) were highly correlated (Spearman = 0.72, P < 0.0001, all cohorts combined) and for this reason the construct that provided lower levels of classification accuracy (RBP2b1,986–2,653) was excluded from the top eight. RBP2b1,41–1,454 induced very low levels of antibody reactivity in the malaria-naive control panels. Across all candidate SEMs, a significant negative association was observed between the mean antibody levels detected in the malaria-naive negative control panels (n = 274 individuals, see Methods) and the AUC values of the 60 SEMs determined from their individual ROC curves (Spearman = −0.5, P < 0.0001, Extended Data Fig. 3). Removing data from these malaria-naive control participants did not cause any substantial reductions in classification accuracy for the top eight proteins (Supplementary Fig. 7).

Classification performance of an eight-protein SEM panel. Figure 4a–d presents ROC curves for assessing the classification performance of the top panel of eight SEMs for identifying individuals with exposure to P. vivax within the previous 9 months. There are only incremental improvements in classification performance as the number of proteins is increased, with a plateau of 80% sensitivity and 80% specificity reached in all three geographic regions with five proteins. The algorithm correctly classifies more than 97% of malaria-naive negative controls, but classification performance was poor for individuals who had their last blood-stage P. vivax infection 9–12 months previously, with 40–66% of samples misclassified (Extended Data Fig. 4). Incorporating an individual’s age into the classification algorithm did not result in substantial improvements.
improvements in classification accuracy (Supplementary Fig. 9). The cohort studies were conducted in regions co-endemic for *P. vivax* and *P. falciparum*, although the total number of individuals experiencing a *P. falciparum* infection during the study period was low (n = 19, 31 and 22 for Thailand, Brazil and the Solomon Islands, respectively). No associations were observed between recent *P. falciparum* infections and antibody-level to the top eight proteins (Supplementary Fig. 10).

We assessed the potential performance of targeted treatment using a seroTAT approach (with 80% sensitivity and specificity) compared to both MDA and MSAT with PCR (Fig. 4e–l). In an MDA campaign, all hypnozoite carriers are targeted (except those ineligible due to G6PD deficiency), but >80% of the population receive unnecessary primaquine (Fig. 4h). In contrast, for MSAT with PCR, no individual was over-treated (it is assumed that an individual with blood-stage *P. vivax* is a likely hypnozoite carrier). However, only 20–40% of all likely hypnozoite carriers are targeted. Using seroTAT, at least 80% of individuals with hypnozoites are targeted (outperforming MSAT with PCR; Fig. 5h), with <20% of the population treated unnecessarily (substantially less than an MDA approach). We also assessed the potential performance of seroTAT using two alternate strategies: 50% sensitivity/98% specificity and 98% sensitivity/50% specificity. The high-specificity approach only slightly outperforms the high-sensitivity approach (Fig. 5d–f). PCR prevalence was based on monthly data from our 2013 to 2014 year-long observational cohort studies and was assumed to provide baseline *P. vivax* transmission levels for population-level treatment strategies beginning in 2020. The public health impact of three treatment strategies was simulated: MDA, MSAT with light microscopy and seroTAT with 80% sensitivity and specificity. Each strategy was modeled to be implemented at 80% population coverage for two rounds with a primaquine regimen assumed to clear all hypnozoites. Two yearly rounds of MDA resulted in an estimated 70–84% reduction in *P. vivax* PCR-detected prevalence across all three study sites, while MSAT with light microscopy was predicted to have substantially less impact with a 33–45% reduction. SeroTAT was not as effective as MDA but resulted in an estimated 59–69% reduction in PCR-detected prevalence.

### Discussion

New tools and strategies to directly target *P. vivax* are urgently needed if malaria elimination in the Asia-Pacific region and Americas is to be achieved by 2030 (ref. 37). *P. vivax* presents a unique challenge to elimination owing to the presence of undetectable hypnozoites that contribute to maintaining residual transmission. *P. vivax* relapses are expected to occur at a frequency of every 1–2 months21,22. Apart from temperate ‘hibernans’ strains that are now restricted to the Korean Peninsula and have no primary blood-stage infections26, virtually all individuals who carry hypnozoites will have had a *P. vivax* blood-stage infection within the previous 9 months20–22. Herein, a panel of candidate SEMs was identified and validated that allows detection of recent exposure to *P. vivax* within the previous 9 months. These SEMs represent a method that can indirectly identify likely hypnozoite carriers that could be targeted for treatment with liver-stage drugs.

We undertook a relatively unbiased approach to choosing the best markers by screening a large panel of 342 *P. vivax* proteins and strategically selecting those that can predict recent infection based on immunogenicity and antibody half-lives. The final panel, which has a sensitivity and specificity of 80% at identifying individuals with PCR-detectable blood-stage infection in the last 9 months in three geographically distinct regions, incorporates antibody responses to eight *P. vivax* proteins: PVX_094255 (RBP2b161–1,454), PVX_087885 (RAMA, putative), PVX_099980 (MSPA), PVX_096995 (Pv-fam-a), PVX_097625 (MSP8, putative), PVX_112670 (unspecified product), PVX_097720 (MSP3,10), PVX_097625 (MSP8, putative) and PVX_094255 (MSP8, putative), PVX_112670 (unspecified product), PVX_094255 (MSP8, putative)

Table 1 | Top *P. vivax* proteins identified in the validation phase and their individual performance

| Protein ID | Gene annotation | Protein length, aa | Construct, aa (size) | Expression system | Purification method | AUC |
|------------|----------------|-------------------|----------------------|------------------|-------------------|-----|
| PVX_094255 | RBP2b          | 2,806             | 161-1,454 (1,294)    | E. coli          | 2X affinity + size exclusion| 0.816 |
| PVX_094255A| RBP2b          | 2,806             | 1986-2,653 (667)     | WGCF             | One-step Ni column| 0.748 |
| PVX_087885B| RAMA, putative | 730               | 462-730 (269)        | WGCF             | One-step Ni column| 0.728 |
| PVX_099980 | MSPA1-19       | 1,751             | 1,622-1,729 (108)    | WGCF             | One-step Ni column| 0.713 |
| PVX_112670 | unspecified product | 335 | 34-end (302) | WGCF | One-step Ni column | 0.698 |
| PVX_096995 | Pv-fam-a       | 480               | 61-end (420)         | WGCF             | One-step Ni column| 0.689 |
| KMBZ3376 | PvEBPII        | 786               | 109-432 (324)        | E. coli          | Ni, ion exchange, gel filtration | 0.684 |
| PVX_097625 | MSPB, putative | 487               | 24-463 (440)         | WGCF             | One-step Ni column| 0.681 |
| PVX_097720 | MSP3,10        | 852               | 25-end (828)         | WGCF             | One-step Ni column| 0.670 |

*PlasmoDB release 36 (http://plasmodb.org/plasmo/)  GenBank AUCs from the single antigen classifier are shown; proteins are listed in order of best individual performance. Nine of 60 proteins are shown here; the top eight identified as best used in combination, plus the alternate construct of RBP2b. See Supplementary Table 2 for the extended and complete Table. References listed are for the protein production and purification method.
KMZ83376.1 (EBPII) and PVX_097720 (MSP3.10). Most of these \textit{P. vivax} proteins are not well described in the literature and only MSP1 and RAMA have previously been used, or suggested as, markers of exposure\textsuperscript{27,38–40}. Our strategy was thus successful in identifying new exposure markers.

Individually, PVX_094255 (RBP2$_{m3-1445}$) was able to classify exposure with 75% sensitivity and specificity. This may be sufficient for sero-surveillance and community-level targeting of preventative measures such as intensified vector control or focal test and treat\textsuperscript{16}. Greater classification accuracy will be required for individual-level targeting for treatment with liver-stage drugs, for example in an elimination campaign using seroTAT, where the goal is to treat all individuals who have hypnozoites or blood-stage \textit{P. vivax} parasites. The final panel of eight proteins obtained 80% sensitivity and specificity across three geographic regions. Using antibody responses to these eight proteins, we show that individuals classified as seropositive at baseline in our year-long observational cohort studies subsequently develop PCR-detectable \textit{P. vivax} infections at a far higher rate than their seronegative counterparts. While in these cohorts we cannot distinguish whether these \textit{P. vivax} infections are due to relapses or new mosquito-bite-derived primary infections, these data clearly demonstrate the ability of our SEMs to identify individuals at risk for recurrent infections.

Many factors affect antimalarial antibody responses, most notably the time since last infection, intensity of infection and age. Antibody responses may also be associated with other factors such as human host or parasite genetic variation that are challenging to account for. Key properties of the antibody response data are the high level of individual variation and the high degree of correlation. Our analytic pipeline accounts for these data properties and the possibility that combinations of antibody responses may best identify recent infections. However, rather than past infections being identified by complex antibody signatures and sophisticated algorithms, a number of simple factors contributed to good classification performance: (1) \textit{P. vivax} proteins that can identify recent infections when used individually also do well in combination; (2) only small incremental improvements in classification performance occur as new \textit{P. vivax} proteins are added to combinations; (3)
are diminishing returns to algorithmic complexity: simple algorithms such as logistic regression capture most signals in the data, with more complex classifiers such as random forests providing only incremental improvements; (4) there is no single best combination of antigens, there is redundancy; (5) there was no substantial advantage to including an individual’s age once three or more antigens were included; and (6) algorithms and combinations of antigens that provided good classification in one region also performed well in another region.

Although the performance of the pilot marker panel may seem modest at 80% sensitivity and specificity, several factors need to be taken into account when evaluating the performance of the SEMs. First, given that individual P. vivax blood-stage infections can be relatively short (<4–6 weeks)\(^41\), some blood-stage infections may have been missed in the validation cohorts as they had only monthly active follow-up. In the analyses, such cases would be classified as false positive by the SEMs, when in reality the SEMs would have accurately detected these infections. Therefore, the real specificity of the test may well be higher. Similarly, a number of people (5–20%) with PCR-positive infections at the time of antibody measurement were missed. This may represent individuals with very recent infections who have not yet generated a notable rise in antibody titers\(^42\). These missed, concurrent infections could however be detected using an ultrasensitive antigen-capture test. A combination of antibody detection and antigen-capture assays would further increase the sensitivity of the diagnostic approach.

**Fig. 5 | Pilot application phase.** a–c, Kaplan–Meier analyses of time to first P. vivax blood-stage infection detected by PCR. Using measured antibody responses at the first time point, participants were classified as positive (blood-stage infection within the previous 9 months) or negative (n = 985 biologically independent samples in Thailand, n = 1,207 in Brazil; n = 754 in Solomon Islands). HRs were calculated using Cox proportional hazards and were statistically significant with \(P < 10^{-10}\) in all cases. Solid lines denote the proportion uninfected by PCR and shaded regions denote the 95% confidence intervals. d–f, Prediction from a mathematical model of P. vivax transmission of the effect of population-level treatment strategies with primaquine at 80% coverage. Solid lines denote model predictions and shaded regions denote 95% uncertainty intervals due to stochasticity. The model was calibrated to longitudinal data on PCR prevalence and the potential impact of two rounds of treatment in 2020 and 2021 were simulated. The percentage reduction in transmission was calculated as the estimated prevalence in June 2021 compared to June 2019. Red points denote measured PCR prevalence with 95% confidence intervals. Estimates of PCR prevalence are based on n = 12,829 measured samples in Thailand, n = 13,973 samples in Brazil and n = 8,276 samples in the Solomon Islands. LM, light microscopy.
limited cross-reactivity between human malaria species may not be
of the top eight proteins have cross-reactivity is unlikely given the relatively low sequence homol-
antibody cross-reactivity between
P. falciparum
carriers (Fig. 5)5,11. Testing and treatment with SEMs thus results in less over-treatment compared to MDA, while still targeting a
elimination.
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infec-
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and
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human malaria parasite.
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infections and antibody responses could also be influenced by other factors such as genetic diversity of
P. vivax
parasite proteins. Notably, despite the lower magnitude of antibody responses, the top eight SEMs could still accurately classify recent infections in the Solomon Islands.
To further improve classification performance, modifications of
P. vivax
protein constructs resulting in reduced background in malaria-naive control samples would be advantageous, as proteins with lower levels of antibody reactivity in the controls had greater classification performance. Addition of further purification steps, testing of other protein expression systems, design of smaller protein fragments, investigations of antigenic diversity and strain specificity of antibody responses, could all be considered. Further information could potentially be gained by looking at antibody subclasses and/ or IgM. While antibody subclass responses will likely correlate with total IgG, the relationship with IgM is expected to be weaker and acquisition and breadth of responses to malaria proteins has been shown to differ for IgG and IgM11. Such optimization and improvement of our assay may allow selection of a smaller panel of three to five proteins with comparable classification accuracy to our current eight-protein panel. This would reduce the cost of running our SEMs in their current format (Luminox assay) and make a simpler point-of-care test feasible.
The current level of SEM performance and the application in potential public health interventions such as seroTAT needs to be seen in the context of the alternatives. MDA (with anti-hypnozoite drugs) is a blanket approach that while effectively targeting all hypnozoite carriers, results in substantial over-treatment. In low-transmission settings, up to 90% of people treated do not carry hypnozoites (that is a specificity of only 6.5–23.3%; Fig. 5) and do not derive any direct benefit from a treatment that may carry risks. Therefore, few
P. vivax endemic countries are considering MDA with primaquine. Conversely MSAT interventions, even with a highly sensitive molecular test, miss 64.9–80.5% of hypnozoite carriers (Fig. 5)121. Testing and treatment with SEMs thus results in less over-treatment compared to MDA, while still targeting a higher proportion of likely hypnozoite carriers compared to MSAT.
Through modeling we show that implementation of seroTAT could result in a 59–69% reduction in
P. vivax prevalence in our cohort settings. While preliminary, these modeling results indicate that seroTAT has potential to be a highly promising new intervention to assist control programs in accelerating toward
P. vivax elimination.
Similar efforts to develop SEMs are currently underway for
P. falciparum
and efforts to develop SEMs for both species should be coordinated where possible. In addition, there is evidence of antibody cross-reactivity between
P. falciparum
and
P. vivax
. Five of the top eight proteins have
P. falciparum orthologs but extensive cross-reactivity is unlikely given the relatively low sequence homology (ranging 20.7–37.4%). Indeed, no association was detected between recent
P. falciparum infections and antibody levels to
P. vivax SEMs in our validation. This may not hold for
P. ovale,
P. malariae or
P. knowlesi, which are more closely related to
P. vivax
(protein sequence identity 13.7–83.4%). On a programmatic level, limited cross-reactivity between human malaria species may not be an issue in areas outside Africa as rates of
P. vivax relapse after
P. falciparum infections are very high, sometimes as high as following a
P. vivax infection. Given the marked difference in patterns of exposure to the zoonotic
P. knowlesi, cross-reactivity between
P. vivax
and
P. knowlesi would be substantially more problematic.
In summary, the panel of eight SEMs can accurately predict recent
P. vivax infection. As almost all
P. vivax relapses occur within 9 months of a previous blood-stage infection, these markers can indirectly identify individuals with the highest risk of harboring hypnozoites. With our markers able to identify individuals at risk of future recurrent infections, we demonstrate that this is indeed the case. We have shown that a carefully selected panel of SEMs can specifically detect recent exposure and could be used in a programmatic setting (surveillance as an intervention). Application of our SEMs for seroTAT holds promise for an effective elimination strategy using primaquine or tafenoquine to target dormant hypnozoites. Given the risk of hemolysis in G6PD-deficient individuals treated with primaquine or tafenoquine, our tool has the potential to ensure such elimination strategies are targeted and therefore a safer, more acceptable and more effective option in malaria-endemic communities.

Online content
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Methods

Study design. The goal of the study was to identify and validate suitable candidate *P. vivax* proteins for use as serological markers of recent exposure to *P. vivax* infections. This study was conducted in three parts: (1) an antigen discovery phase, utilizing samples from two longitudinal cohorts that followed asymptomatic *P. vivax* malaria patients over 9 months; (2) a validation phase, utilizing samples from three year-long observational cohort studies; and (3) a pilot application phase to demonstrate the ability of SEMs to identify at-risk individuals. The sample size was predefined by availability of suitable plasma samples with matching epidemiological and molecular data.

Study populations: antigen discovery phase. Patients with confirmed *P. vivax* malaria (by qPCR, see below) were enrolled from local malaria clinics and hospitals in two sites in 2014: Tha Song Yang, Tak Province, Thailand and Manaus, Amazonas State, Brazil. After receiving antimalarial drug treatment according to respective national treatment guidelines and providing written informed consent and/or assent, blood samples were taken over a period of 9 months as previously described\(^1\) (Extended Data Fig. 1a and Supplementary Table 4). Presence or absence of *Plasmodium* spp. parasites during follow-up was determined by both microscopy and qPCR. Blood samples were collected at enrollment and week 1, then every 2 weeks for 6 months, then every month until the end of the study. A subset of enrolled participants who had no evidence of recurrent parasitemia during follow-up was selected for antibody measurements (n = 32 Thai patients and n = 33 Brazilian patients).

Study populations: validation phase. Year-long longitudinal observational cohort studies were conducted between 2013 and 2014 in three sites: Kanchanaburi and Ratchaburi provinces, Thailand\(^5\), Manaus, Brazil (W.M., manuscript in preparation) and the island of Ngella, Solomon Islands\(^6\) (Extended Data Fig. 1b and Supplementary Table 4). A total of 999 volunteers were enrolled from Thailand and sampled every month over the year-long cohort, with 14 active case detection visits performed in total. Overall, 829 volunteers attended the final visit. A total of 1,274 residents of all age groups were enrolled from Brazil and sampled every month over the year-long period, with 13 active case detection visits performed in total. Overall, 928 volunteers attended the final visit with plasma from 925 available. There were 1,111 children enrolled from the Solomon Islands, with 860 used for final analysis. Of children who attended the final visit, 29% (32 Thai patients who had inconsistent attendance or failed to meet other inclusion criteria). The children were sampled monthly, with 11 active case detection visits in total. Of the 860 children, 754 attended the final visit. For all three cohorts, at each visit volunteers completed a brief survey outlining their health over the previous month (to determine the possibility of missed malarial infections), in addition to travel history and bed net usage. A finger-prick blood sample was also taken and axillary temperature was recorded. Passive case detection was performed throughout the year-long period. All cohort participants provided individual consent for both participation in the study and the future use of samples for the study of antimalarial immune responses. In cases involving children, parental consent and (where appropriate) assent was obtained.

Study populations: pilot application phase. To assess the ability of our SEM to identify individuals at risk of future recurrent *P. vivax* infections, we utilized plasma samples from the first visit of the three year-long observational cohorts used in the validation phase (Extended Data Fig. 1b). Antibody measurements were made in 992 plasma samples from the Solomon Islands cohort, 1,207 samples from the Brazilian cohort and 655 samples from the Solomon Islands cohort.

Study populations: malaria-naïve control panels. Four panels of malaria-naïve control plasma samples were collected from individuals with no known recent exposure to malaria (Supplementary Table 4). Samples were as follows: 102 individuals from the Volunteer Blood Donor Registry in Melbourne, Australia; 100 individuals from the Australian Red Cross, Melbourne, Australia; 72 individuals from the Thai Red Cross, Bangkok, Thailand; and 96 individuals from the Rio de Janeiro State Blood Bank (RBB), Rio de Janeiro, Brazil, an area nonendemic for malaria since the 1960s. Standard Thai Red Cross and RBB screening procedures exclude individuals from donating blood if they have had a confirmed malaria infection within the previous 3 years or have traveled to malaria-endemic regions within the previous year.

Ethical approvals. The Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand, approved the Thai antigen discovery study (MUTM 2014-0229) and the Thai year-long cohort study (MUTM 2013-027-01). The Ethics Review Board of the Fundação de Medicina Tropical Dr. Héctor Vieira Dourado (FMT-HVD) approved the Brazilian antigen discovery study (957.875/2014) and the Brazilian year-long cohort study (349.211/2013). The Brazilian year-long cohort study was also approved by the Brazilian National Committee of Ethics and by the Ethics Committee of the Hospital Clinic, University of Navarra, Spain (2012/306). The National Health Research Ethics Committee of the Solomon Islands Ministry of Health and Medical Services (HRC12/022) approved the Solomon Islands year-long cohort study. The Human Research Ethics Committee at the Walter and Eliza Hall Institute of Medical Research (WEHI) approved samples for use in Melbourne (14/02) and also approved use and collection of the control panel samples (14/02).

Procedures. Blood samples were collected by finger prick into EDTA tubes. Blood was separated into packed red cells and plasma at the field site. Packed red cells were stored at −20°C and plasma at −80°C before use in molecular and serological assays, respectively. *Plasmodium* spp. parasites were detected by qPCR as previously described\(^6\). The limit of detection of the molecular methods was 1–3 copy numbers\(^1\) (ref. 1).

Antibody measurements. The majority of *P. vivax* malaria proteins (SalI strain) were expressed using the WGCF system (CellFree Sciences) at either Ehime University or CellFree Sciences. For the antigen discovery phase, 342 *P. vivax* protein constructs were expressed at a small scale with validated and antibodies were measured using the AlphaScreen assay, as previously described\(^1\). A total of 307 of these proteins were selected as previously described\(^6\) and are known or expected to be immunogenic on the basis of protein features such as the presence of signal peptides, transmembrane domains or orthology with immunogenic *P. falciparum* proteins. An additional 35 proteins were selected to enrich the largely blood-stage expressed set of proteins with pre-erythrocytic and sexual stage proteins. Plasma samples from weeks zero (enrollment), 12, 24 and 36 in the subset of volunteers described above were used (n = 32 Thai patients and n = 33 Brazilian patients). Raw AlphaScreen data were converted to relative antibody units based on protein-specific standard curves, with final units ranging 0–400 (seropositivity was defined as a relative antibody unit > 20).

For the validation and pilot application phases, downselected proteins were produced at a high yield using dialysis-based refeeding WGCF methods and purified on an affinity matrix using a His-tag. The purified proteins were stored and shipped in the following buffer: 20 mM Na-phosphate (pH 7.5), 0.3 M NaCl, 500 mM imidazole and 10% (v/v) glycerol. Protein yields and purity were determined using SDS–PAGE followed by Coomassie brilliant blue staining using standard laboratory methods. An additional 20 *P. vivax* proteins known to be highly immunogenic were also included at the validation phase and were produced using previously described methods\(^7\) (Supplementary Table 2). All expressed proteins were based on the SalI strain unless otherwise stated in Supplementary Table 2. The *P. vivax* proteins were coupled to COOH microspheres as previously described\(^7\), with the optimal amount of protein to be coupled determined experimentally to achieve a log-linear standard curve with our positive control pool. Protein-specific IgG antibody levels were measured in a multiplexed Lumines assay as previously described\(^6\). Plasma samples from both the first and last visits of the three year-long observational cohort studies were used. Median fluorescent intensity values from Lumines-200 were converted to relative antibody units based on a standard curve generated with a positive control plasma pool from highly immune adults from Papua New Guinea\(^8\).

Statistical modeling: antigen discovery phase. The change in measured antibody responses following *P. vivax* infection in patients in the antigen discovery phase was analyzed using mixed-effects linear regression models, as previously described\(^8\). Estimated half-lives for 307 *P. vivax* proteins were previously reported\(^9\), with an average of 3.3 ± 0.6 months (σ\(^2\) = 1–3 copy numbers\(^1\) (ref. 1).

The linear regression model for the decay of antibody titers in equation (1) has three sources of variation: (1) variation in initial antibody response following infection; (2) between-individual variation in antibody decay rate; and (3) measurement error. Notably, all these sources of variation are described by normal distributions so their combined variation is also described by a normal distribution. Therefore, \(x_t = \log(A_t)\), the expected log antibody titer in individual \(i\) to protein \(j\) at time \(t\), can be described by the following linear model:

\[
\log(A_{ij}) \sim \log(a_{ij}) + r_j + r_{ij} + \varepsilon_{ij}
\]

where \(a_{ij}\) is the geometric mean titer at the time of infection; \(\log(a_{ij})\) is a random effect for the difference between participant \(i\)’s initial antibody titer and the population-level geometric mean titer; \(r_j\) is the average rate of decay of antibodies to protein \(j\) in the population; \(r_{ij}\) is a random effect for the difference between the decay rate of individual \(i\) with the population-level average; and \(\varepsilon_{ij} \sim N(0, \sigma_{\varepsilon})\) is the error term. It is assumed that the random effects for the antibody titers are normally distributed: \(\log(a_{ij}) \sim N(0, \sigma_a)\), and that the random effects for the variation in decay rates are also normally distributed: \(\varepsilon_{ij} \sim N(0, \sigma_{\varepsilon})\).

The linear regression model for the decay of antibody titers in equation (1) has three sources of variation: (1) variation in initial antibody response following infection; (2) between-individual variation in antibody decay rate; and (3) measurement error. Notably, all these sources of variation are described by normal distributions so their combined variation is also described by a normal distribution. Therefore, \(x_t = \log(A_t)\), the expected log antibody titer in individual \(i\) to protein \(j\) at time \(t\), can be described by the following linear model:

\[
P(x_{ij} | t) = \frac{1}{\sqrt{2\pi} \sigma_{\varepsilon}} e^{-\frac{1}{2} \left( \frac{\log(x_{ij} - \log(a_{ij}) - r_j - t r_{ij})}{\sigma_{\varepsilon}} \right)^2}
\]
The probability for the time since infection t given measured antibody titer χ can be calculated by inverting equation (2) using Bayes rule36, allowing estimation of the probability that the time since last infection was <9 months.

A simulated annealing algorithm was used to explore the high-dimensional space arising from all combinations of the 142 prioritized SEMs in the antigen discovery phase and to select optimal combinations of proteins37,46. Combinations of proteins were chosen to optimize the likelihood that sampled antibody responses were correctly classified as having occurred within 9 months of infection.

Statistical modeling: validation phase. Individuals in the validation phase were classified into two categories depending on whether they had PCR-detectable blood-stage P. vivax infections within the 9 months before measurement of antibody responses (Supplementary Table 4). In theory it is possible to provide quantitative estimates of the time since last infection; however, in practice, a more useful outcome is whether an individual has been infected within some previous time period. Nine months was selected due to the biology of P. vivax relapses (highest incidence of relapse within the first 9 months following mosquito-bite infection29) and because this threshold fell within a time period for which data were available (the three longitudinal cohorts had follow-up data for 12 months).

Initial classification performance of antibody responses to the 60 downselected SEMs to classify recent P. vivax infections was assessed using LDA. There were 1,770 ways of choosing 2 out of 60 proteins, 34,220 ways of choosing 3 proteins, 487,635 ways of choosing 4 proteins and 5,461,512 ways of choosing 5 proteins. All combinations of up to four proteins were exhaustively evaluated to optimize classification performance for three sensitivity and specificity targets (80% for both; 50% sensitivity and 98% specificity; and 98% sensitivity and 50% specificity). To investigate combinations of five proteins, the 100 best combinations of four proteins were identified for each target. All possible remaining proteins were added to each of these 300 combinations and the classification performance of all the combinations of five proteins was assessed. A similar procedure was implemented to investigate classification performance of combinations of up to eight proteins. Combinations beyond eight proteins were not tested, given the diminishing returns in classification performance.

After determining the highest ranking SEMs using the LDA classifier, the top eight candidates were further assessed using a range of other classification algorithms, including logistic regression, quadratic discriminant analysis, decision trees and random forests46. Decision trees were implemented using the rpart R package and random forests were implemented using the randomForest R package. Two new classification algorithms incorporating statistical models of antibody decay over time were also developed (Supplementary Information). These algorithms were combined into a composite algorithm so that the optimal algorithm and selection of proteins was selected for each target of sensitivity and specificity. All classification algorithms were cross-validated using 1,000 randomly sampled, disjoint training and testing subsets (Extended Data Fig. 5 and Supplementary Fig. 11).

Statistical modeling: pilot application phase. Antibody levels to the top eight proteins were measured in samples from the first time point in the longitudinal cohorts used in the validation phase. At the start of longitudinal follow-up, individuals were classified as being exposed to blood-stage P. vivax infection within the previous 9 months using measured antibody levels and the composite classification algorithm. To test the hypothesis that infection in the previous 9 months is associated with increased risk of future blood-stage infection (possibly relapses), the time to first PCR-positive P. vivax infection was analyzed using Cox proportional hazards to estimate the HR.

The potential public health impact of seroTAT strategies with primaquine was assessed using a mathematical model of P. vivax transmission32, using sensitivity and specificity targets achieved in the validation phase. Two annual rounds of seroTAT were modeled in the Thai, Brazilian and Solomon Islands populations at 80% coverage (Fig. 5 and Supplementary Fig. 12).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data and code availability

All data and code for reproducing this analysis are available online at https://github.com/MWhite-InstitutPasteur/Pvivax_sero_dx.

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Author contributions

R.J.L., M.T.W., T.T. and I.M. designed the study. R.J.L., M.T.W. and I.M. conducted the cohort studies. E.T., M.M., J.B. and Z.S.-J.L. performed antibody measurements. S.T., T.J.L., C.S.N.L.-W. and P. M.T.W. conducted data management and analysis. M.T.W. and T.O. performed modeling. R.J.L., M.T.W. and I.M. wrote the draft of the report. L.J.R., C.P., D.L.D., X.C.D. and I.J.G. provided expert advice. All authors contributed to data interpretation and revision of the report.

Competing interests

FIND contributed to early funding of this work and had a role in data interpretation, writing of the report and the decision to submit. No other funders of this study had any role in study design, data collection, data analysis, data interpretation, writing of the report or the decision to submit. R.J.L., M.W., T.T. and I.M. are inventors on patent PCT/US17/67926 on a system, method, apparatus and diagnostic test for P. vivax.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-020-0884-1. Supplementary information is available for this paper at https://doi.org/10.1038/s41591-020-0841-4. Correspondence and requests for materials should be addressed to I.M. Peer review information Alison Farrell is the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team. Reprints and permissions information is available at https://www.nature.com/reprints.
Extended Data Fig. 1 | Study design and follow-up schedule. a, Thai and Brazilian patients were enrolled following a clinical episode of *P. vivax* and treated according to the relevant National Guidelines, with directly observed treatment (DOT) to ensure compliance and reduced risk of relapse. Volunteers were followed for nine months after enrollment, with finger-prick blood samples collected at enrollment and week 1, then every two weeks for six months, then every month. Antibody levels were measured in a subset of 32 Thai and 33 Brazilian volunteers who were confirmed to be free of blood-stage *Plasmodium* parasites by analysing all samples by light microscopy and qPCR. b, 999 participants from Thailand, 1274 participants from Brazil, and 860 participants from the Solomon Islands were followed longitudinally for 12 months with active surveillance visits every month. For the analysis in the validation phase antibody levels were measured in plasma samples from the last visit. For the analysis in the application phase, antibody levels were measured in plasma samples from the first visit.
Extended Data Fig. 2 | Measured antibody responses to 60 proteins on the Luminex® platform, stratified by geographical location and time since last PCR detected infection. Antibody responses to 60 antigens measured in $n = 2,281$ biologically independent samples on the Luminex® platform, stratified by geographical location and time since last PCR detected infection. Boxplots denote median and interquartile ranges (IQR) of the data, with whiskers denoting the median $\pm 1.5^\text{IQR}$. 
Extended Data Fig. 3 | Association between background reactivity in non-malaria exposed controls and ranking of candidate SEMs by area under the curve (AUC). Mean relative antibody units (RAU) detected in malaria-naïve control panels from Melbourne, Australia (n=202), Bangkok, Thailand (n=72) and Rio de Janeiro Brazil (n = 96) compared to the AUC of the 60 candidate P.vivax proteins generated during the validation phase. WGCF expressed proteins are in black and E. coli or Baculovirus expressed proteins are in blue. RBP2b161-1454 (E. coli) is in red and RBP2b1986-2653 is in orange.
Extended Data Fig. 4 | Breakdown of the classification of the Random Forests algorithm with target sensitivity and specificity of 80%. The size of each rectangle is proportional to the number of samples in each category (See Supplementary Table 4 of accompanying manuscript). The coloured area represents the proportion correctly classified, and the shaded area represents the proportion mis-classified.
Extended Data Fig. 5 | Receiver operating characteristic (ROC) curve for the composite classification algorithm. All curves presented are the median of 1000 repeat cross-validations.
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- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- [ ] Estimates of effect sizes (e.g. Cohen’s $d$, Pearson’s $r$), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- **Data collection** For these studies archival sample and datasets for used. Management of additional antibody data were done using MSExcel (Version 14.5.5), R (Version 3.6.1) and Stata (Version 12.1)
- **Data analysis** All data analyses were done using R (Version 3.6.1) and Stata (Version 12.1)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All databases used in the analyses are available on GitHub: https://github.com/MWhite-InstitutPasteur/Pvivax_sero_dx

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [X] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Discovery studies: Thailand n=32, Brazil n=33, Solomon Island n=860  
Valuation studies: Thailand n= 999, Brazil n= 1274, Solomon Island n=860  
Negative controls: VBDR (Australian volunteers) n = 102, Australian Red Cross n = 100, Thai Red Cross n = 72, Rio State Blood Bank n = 96. |
| --- | --- |
| Data exclusions | Discovery studies: Only participants with complete follow-up and no recurrent P. vivax infections were included.  
Validation studies: All participants with available plasma samples from final visit and sufficiently data on previous history of infections were included. |
| Replication | Discovery studies were independently conducted in two separate geographical areas: Thailand and Brazil  
Validation studies were independently conducted in three separate geographical areas: Thailand, Brazil and Solomon Islands  
Findings were successfully replicated across several studies and provided comparable results. Findings were also robust to cross-validation with training of algorithms on data from one region and testing on data from different regions |
| Randomization | All our studies were observational in nature and no interventions or groupings were applied. As a consequence, no randomisation was required and consequently none was done |
| Blinding | All our studies were observational in nature and no intervention or grouping were applied. Thus, no formal blinding was required. Nevertheless, the laboratory team doing all of the antibody measurement did not know which individuals had malaria exposure and which did not. In addition, all antibody measurements by Luminex were done on 96 well plates with samples allocated to plates without knowledge of exposure status. Therefore although not formally blinded, the laboratory team were effectively blinded while conducting the laboratory assessments. Likewise, during the initial QC (i.e. checking bead counts) and data curation (i.e. standard curve conversions) the team were effectively blinded as they only had study IDs and no knowledge of past malaria exposures |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
| --- | --- |
| n/a | Involved in the study |
| □ | Antibodies |
| □ | Eukaryotic cell lines |
| □ | Palaeontology |
| □ | Animals and other organisms |
| □ | Human research participants |
| □ | Clinical data |
| □ | Involved in the study |
| □ | ChiP-seq |
| □ | Flow cytometry |
| □ | MRI-based neuroimaging |

Antibodies

| Antibodies used | The secondary antibody, PE-conjugated anti-human IgG Fc, was purchased from Jackson ImmunoResearch Laboratories Inc. (product code 709-116-098, 1 mg/ml and used at a 1:100 dilution) |
| Validation | Only commercially available secondary antibodies were used |

Human research participants

| Policy information about studies involving human research participants |
| --- |
| Population characteristics | Discovery studies: Thailand: age range 7-71 yrs, 44% female; Brazil: age range 16-56, 21% female  
Validation studies: Thailand age range 1-78 yrs, 55% female, Brazil: age range 0 - 103, 51% female; Solomon Islands: 0.5-12.7 yrs |
Recruitment

Discovery studies: Only participants in the Thai discovery study were recruited with the intention of later using samples for studies of antibody kinetic studies. There, 61 patients presenting at a primary health facility in Tha Song Yang district were recruited. The only selection criteria were that patients were permanent local residents and could be followed-up for both directly observed treatment and 9 months follow-up. Participants from the Brazilian studies, were post-hoc ‘selected’ from another study assessing the efficacy of CQ-PQ for the treatment of *P. vivax* infections. From both studies only samples from participants that were confirmed to parasite free during the entire 9 months follow-up were included in the discovery studies. This selection is not expected to have any impact on study results.

Discovery studies: No recruitment of participants were done for any of the validations studies. We used archival samples and data from year-long longitudinal observational cohort studies that were conducted over 2013-2014 in three sites: Kanchanaburi/Ratchaburi provinces, Thailand; Manaus, Brazil (Monteiro et al., in preparation), and the island of Ngella, Solomon Islands25 (Fig 1B, Table 1). A total of 999 volunteers were enrolled from Thailand and sampled every month over the yearlong cohort, with 14 active case detection visits performed in total. 829 volunteers attended the final visit. A total of 1,274 residents of all age groups were enrolled from Brazil and sampled every month over the year-long period, with 13 active case detection visits performed in total. 928 volunteers attended the final visit with plasma from 925 available. 1,111 children were enrolled from the Solomon Islands, with 860 used for final analysis of the cohort (after exclusion of children who withdrew, had inconsistent attendance, or failed to meet other inclusion criteria). The children were sampled monthly, with 11 active case detection visits in total. Of the 860 children, 754 attended the final visit.

Ethics oversight

The Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand, approved the Thai antigen discovery study (MUTM 2014-025-01 and 02) and the Thai yearlong cohort study (MUTM 2013-027-01). The Ethics Review Board of the Fundação de Medicina Tropical Dr Heitor Vieira Dourado (FMT-HVD) approved the Brazilian antigen discovery study (957.875/2014) and the Brazilian yearlong cohort study (349.211/2013). The Brazilian yearlong cohort study was also approved by the Brazilian National Committee of Ethics (CONEP) and by the Ethics Committee of the Hospital Clinic, Barcelona, Spain (2012/7906). The National Health Research and Ethics Committee of the Solomon Islands Ministry of Health and Medical Services (HRC12/022) approved the Solomon Islands yearlong cohort study. The Human Research Ethics Committee (HREC) at the Walter and Eliza Hall Institute of Medical Research (WEHI) approved samples for use in Melbourne (#14/02), and also approved use and collection of the control panel samples (#14/02).

Note that full information on the approval of the study protocol must also be provided in the manuscript.