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Resource and Technical Advance In-Press Preview **Infectious disease**

Controlled human malaria infection (CHMI) provides a highly informative means to investigate host-pathogen interactions and enable in vivo proof-of-concept efficacy testing of new drugs and vaccines. However, unlike *Plasmodium falciparum*, well-characterized *P. vivax* parasites that are safe and suitable for use in modern CHMI models are limited. Here, two healthy malaria-naïve UK adults with universal donor blood group were safely infected with a clone of *P. vivax* from Thailand by mosquito-bite CHMI. Parasitemia developed in both volunteers and, prior to treatment, each volunteer donated blood to produce a cryopreserved stabilate of infected red blood cells. Following stringent safety screening, the parasite stabilate from one of these donors ("PvW1") was thawed and used to inoculate six healthy malaria-naïve UK adults by blood-stage CHMI, at three different dilutions. Parasitemia developed in all volunteers, who were then successfully drug treated. PvW1 parasite DNA was isolated and sequenced to produce a high quality genome assembly by using a hybrid assembly method. We analysed leading vaccine candidate antigens and multigene families, including the Vivax interspersed repeat (VIR) genes of which we identified 1145 in the PvW1 genome. Our genomic analysis will guide future assessment of candidate vaccines and drugs, as well as experimental medicine studies.

Controlled human malaria infection with a clone of *Plasmodium vivax* with high quality genome assembly

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Abstract

Controlled human malaria infection (CHMI) provides a highly informative means to investigate host-pathogen interactions and enable *in vivo* proof-of-concept efficacy testing of new drugs and vaccines. However, unlike *Plasmodium falciparum*, well-characterized *P. vivax* parasites that are safe and suitable for use in modern CHMI models are limited. Here, two healthy malaria-naïve UK adults with universal donor blood group were safely infected with a clone of *P. vivax* from Thailand by mosquito-bite CHMI. Parasitemia developed in both volunteers and, prior to treatment, each volunteer donated blood to produce a cryopreserved stabilate of infected red blood cells. Following stringent safety screening, the parasite stabilate from one of these donors ("PvW1") was thawed and used to inoculate six healthy malaria-naïve UK adults by blood-stage CHMI, at three different dilutions. Parasitemia developed in all volunteers, who were then successfully drug treated. PvW1 parasite DNA was isolated and sequenced to produce a high quality genome assembly by using a hybrid assembly method. We analysed leading vaccine candidate antigens and multigene families, including the Vivax interspersed repeat (VIR) genes of which we identified 1145 in the PvW1 genome. Our genomic analysis will guide future assessment of candidate vaccines and drugs, as well as experimental medicine studies.

Introduction

The majority of human malaria is caused by two species of parasite – *Plasmodium falciparum* and *P. vivax*. Infection is initiated by an infected *Anopheles* mosquito bite, delivering sporozoites which rapidly migrate to and infect the liver. Asexual replication in the liver sees each infected cell produce thousands of merozoites. These rupture out into the blood and infect red blood cells (RBC), before undergoing exponential growth that leads to clinical symptoms and the associated morbidity and mortality. *P. vivax* is the predominant cause of malaria outside of Africa and is more geographically widespread than *P. falciparum*, with 2.5 billion people living at risk in Latin America, Oceania, Asia and the horn of Africa (1). Moreover, recent data demonstrate a significant burden of morbidity and associated mortality in young children and pregnant women (2), challenging the long-held dogma that this parasite is "benign" (3).

A number of factors also underlie the differing epidemiology of *P. vivax* and make it more difficult to control and eliminate than *P. falciparum* (4). Most notably earlier development of gametocytes leads to transmission prior to symptom onset, and its ability to form dormant liver-stage forms, termed "hypnozoites", causes waves of relapsing bood-stage parasitemia and sustained transmission (5). However, despite a clear global health need to develop an effective vaccine and improved antimalarial drugs, these efforts continue to lag behind those for *P. falciparum*. The reasons for this are numerous, but perhaps most significant is the fact that *P. vivax* has not been able to be adapted to long-term *in vitro* culture, despite extensive efforts. This has severely limited laboratory studies, as well as the development of modern controlled human malaria infection (CHMI) models, which rely on a well-defined isolate of *P. vivax*, and would enable *in vivo* efficacy testing of candidate vaccines and anti-malarial drugs in proof-of-concept clinical trials. This is in contrast to *P. falciparum* where *in vitro* culture and sophisticated genetic modification experiments are carried out all over the world, and CHMI can be initiated by the traditional mosquito-bite method, or by injection of cryopreserved sporozoites or an inoculum of blood-stage parasites (6). Most of these studies have been carried out in non-endemic settings, but CHMI trial capacity is now expanding across endemic countries in Africa, enabled by the use of cryopreserved sporozoites. In contrast, modern CHMI with *P. vivax* has been less utilized, with only a handful of studies reported (7).

For mosquito-bite *P. vivax* CHMI trials, most have taken place in Cali, Columbia (8-11) plus one at the Walter Reed Army Institute of Research (WRAIR), USA (12), with 108 volunteers challenged in total. Such trials necessitate production of infected mosquitoes in an endemic setting using fresh gametocytes from an infected patient. Shipment of the mosquitoes to non-endemic areas, and timing these activities with

recruitment of volunteers who may receive an intervention such as a vaccine, poses significant logistical challenges. Moreover, a different isolate of *P. vivax* is inevitably used for every trial which can hamper interpretation of the results and inter-study comparability. These studies also pose the risk of relapse, and thus require participants to be screened for glucose-6-phosphate dehydrogenase (G6PD) deficiency (to avoid hemolysis induced by primaquine treatment). They also require assessment of the volunteers' ability to metabolize primaquine, given relapsing infection occurred in two volunteers in the CHMI study at WRAIR despite primaquine treatment. Here, drug failure was subsequently linked to the volunteers' cytochrome P450 2D6 (CYP2D6) genotypes that were predicted to be poor or intermediate metabolizer phenotypes of the drug (13).

The use of the blood-stage CHMI model (14, 15) has several advantages over mosquito-bite CHMI, although it does not mimic the route of natural infection. Here a cryopreserved stabilate of infected RBC (iRBC) is produced from a donor volunteer, enabling subsequent direct blood-stage inoculation of other volunteers with small numbers of parasites. This model is more practical in non-endemic settings; enables access to the parasite's genetic data before CHMI; removes all risk of relapsing infection; and enables multiple studies with the same strain of parasite (for which a safety database can be established). In the case of *P. falciparum*, this model has also proved particularly suitable for estimating the blood-stage parasite multiplication rate (PMR) (16) and for enabling experimental transmission to mosquitoes (17), as compared to studies initiated by mosquito-bite. The blood-stage model is also advantageous because it extends the period of blood-stage infection, allowing for longer studies of the human immune response and also switching/selection of parasite variant surface antigens (18).

Two cryopreserved stabilates of blood-stage *P. vivax* have been reported to-date, both produced by the group at the QIMR Berghofer Medical Research Institute, Australia and obtained from returning travellers who donated infected blood prior to treatment. The first isolate, HMPBS-*Pv* from the Solomon Islands, was safely tested by blood-stage CHMI in eight volunteers (19, 20), however this necessitated recruitment of individuals with blood group A to match that of the donor. The second *P. vivax* isolate, HMP013-*Pv*, was from India and a blood group O+ donor. This has been tested in healthy adult volunteers and showed successful induction of gametocytemia and experimental transmission of *P. vivax* from humans to mosquitoes (21), and also enabled trials of candidate drugs and further methodology development (22, 23).

Here we take a significant step forward for *P. vivax* CHMI by establishing a well-characterized Thai clone of *P. vivax* suitable for both mosquito-bite and blood-stage CHMI. We elected to produce a

cryopreserved stabilate of iRBC from blood donated by healthy volunteers infected via mosquito-bite CHMI, as opposed to using a blood donation from a returning traveller. This provided numerous advantages in terms of logistical timing, and our ability to recruit in advance volunteers who passed a full health screen and who had universal donor blood group. In real-time we were able to select mosquitoes infected in Thailand with a single *P. vivax* genotype, thus avoiding production of a cryopreserved iRBC stabilate from a polyclonal infection. It also minimized the time from mosquito to blood bank (compared to infected returning travellers); this is important as it has previously been shown that mosquitoes reset parasite virulence and expression of variant surface antigens (24). Following production of the cryopreserved parasite stabilate, which we called “PvW1”, we demonstrated safety and infectivity by blood-stage CHMI in six healthy adults, and we also report a full genomic analysis of the new PvW1 clone.

Results

Source patient case-finding and preparation of infected mosquitoes

For infection of mosquitoes, source patients were recruited from a medical clinic in southern Thailand. Patient blood samples that tested positive for *P. vivax* and negative for filarial disease were fed to *Anopheles dirus* mosquitoes via a direct membrane feeding system in Thailand. Oocyst and sporozoite counts subsequently confirmed successful production of three independent batches of infected mosquitoes (**Figure S1A**). In parallel, and in real-time, source patient samples underwent additional and rigorous testing in the UK for blood-borne infections and mosquito-borne diseases other than malaria; all tests were negative. Nested PCR reported mono-infection with *P. vivax* (**Figure S1B,C**), thus confirming the diagnosis in Thailand, however, genotyping analysis suggested that only one blood sample (C05-001) contained a single *P. vivax* genotype (**Figure S1A**). Mosquitoes fed off this patient's blood were therefore selected and shipped from Thailand to the UK.

Screening of healthy UK volunteers for blood donation

In parallel we enrolled two healthy UK adult volunteers into the VAC068 clinical trial (**Figure S2**). These volunteers were specifically screened to be universal blood donors (blood group O rhesus-negative), Duffy-blood group positive (7, 25); G6PD normal (26); and to have a CYP2D6 genotype predicted to be an extensive metabolizer phenotype (27) alongside satisfactory demonstration of primaquine metabolism following administration of a single test dose of drug (13) (**Table S1**, **Figure S3**). Each volunteer also underwent an extensive screen for blood-borne infections; all test results were negative, except both participants were IgG seropositive for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) (**Table S1**), indicating past infection. However, we did not exclude volunteers based on their serostatus for these two viruses.

Mosquito-bite CHMI – safety and parasite growth dynamics

For the C05-001 mosquito batch, the mean [range] number of oocysts per mosquito was 3 [0-6] at day 7 post-feeding and the median score for number of sporozoites observed in the salivary glands at day 14 post-feeding was +2 (defined as >10-100 sporozoites) (**Figure S1A**). This was relatively low but agreed to be sufficient for human transmission. Subsequently, the two healthy UK adult volunteers screened and consented to take part in VAC068 were each exposed to five "infectious bites" as defined post-skin

feeding by microscopic examination of each mosquito. To achieve this, volunteers 01-004 and 01-008 required 17 and 33 mosquitoes, respectively, to bite their arm.

Parasites were first reliably detected in the blood of both volunteers by qPCR at the evening clinic visit 8 days post-CHMI (dC+8.5), and parasitemia then steadily rose over time (**Figure 1A**, **Table S2**). Over the course of the CHMI period, the two volunteers experienced a range of solicited adverse events (AEs), with both reporting grade 3 fatigue and at least grade 2 anorexia, chills, feverishness, headache, malaise, nausea and sweats (**Figure 1B**). Both volunteers were admitted for blood donation when they met protocol-specified criteria defined by symptoms and or threshold levels of parasitemia as measured in genome copies (gc)/mL by qPCR. This occurred on the morning of dC+14 for both volunteers, who both crossed the 10,000 gc/mL threshold on dC+13.5 and developed fever on dC+14. Following admission to the clinical trials unit, a 250 mL blood sample was collected (at dC+14 for volunteer 01-008 and dC+14.5 for volunteer 01-004); both were positive by thick film microscopy and reported 16,717 or 31,010 gc/mL by qPCR, respectively. Prior to cryopreservation, these blood samples were then randomized and relabelled either “Donor 1” or “Donor 2” and are now referred to as such in the Results.

After blood donation, each volunteer was immediately treated with Riamet® followed by a 14-day course of primaquine; no supportive treatment or hospital admission was required for either volunteer. Monitoring by qPCR on days 1, 2, 4, 10 and 16 post-treatment showed a rapid decline in blood-stage parasitemia followed by negative readings for both volunteers (**Figure 1A**, **Table S2**). Most solicited symptoms increased in severity in the first 24 hours after starting anti-malarial treatment (**Figure S4A**). Objective fever also increased in the 24 hours post-treatment (**Figure 1C**), and one volunteer developed a grade 3 pyrexia (**Figure S4B**), but all symptoms had completely resolved within 5 days of starting treatment. Both volunteers also experienced some short-lived grade 1 or 2 AEs possibly related to the anti-malarial treatment (dizziness, insomnia and abdominal pain) (**Figure S4C**). Very few unsolicited AEs (at least possibly related to CHMI) were reported by either volunteer (**Table S3A**), and only one grade 3 unsolicited AE (migraine, not related to CHMI) was reported by 01-004 more than 2 months post-challenge, requiring attendance to their doctor and resolving within 48 hours (**Table S3B**). Lymphocyte and platelet counts dropped in both volunteers around the time of blood donation (platelets remained within the normal range but both developed a grade 2 lymphocytopenia), rising back to pre-challenge levels within 48 hours (**Figure 1D-E** and **Table S3C**). Volunteer 01-008 also developed a transient grade 1 anemia ~6 weeks post-challenge (123 g/L at dC+47) which may or may not have been related to CHMI, but this resolved within 3 months (131 g/L at dC+94).

Following completion of the study, the parasite multiplication rate (PMR) for both volunteers was calculated using a linear model fitted to $\log_{10}$-transformed qPCR data (28). These data showed comparable PMRs in both volunteers, with 10.7- and 11.5-fold growth per 48 hours (**Figure 1G**). We also analysed gametocytemia using a qRT-PCR assay to detect mature female gametocyte *pvs25* transcripts. Volunteer 01-004 showed only low levels at the final time-point pre-treatment (dC+14.5) whilst none were detected in volunteer 01-008 (**Figure 1H**).

Finally, with regard to longer-term safety monitoring, clinic visits at dC+45 and dC+90 gave rise to no safety concerns or indication of relapsing infection, and repeat serological tests for blood-borne infections at dC+90 all remained negative. Ongoing annual follow-up by email will continue for 5 years post-CHMI, however, as of time of writing (3 years post-primaquine treatment) no relapse of *P. vivax* has been diagnosed for either volunteer (see **Supplementary Text**).

Cryopreservation and *in vitro* testing of *P. vivax* infected blood

After blood donation, the leukodepleted blood from both volunteers in VAC068 was processed, and the RBC mixed with Glycerolyte 57 to form a stabilate prior to cryopreservation. In total, 190 vials were frozen for Donor 1 and 185 for Donor 2. In process testing by qPCR indicated minimal or no loss of parasites during filtration (95% and 105% recovery for Donor 1 and Donor 2, respectively). We next tested for parasite viability in both cryopreserved stabilates. Vials were thawed and cells used in a short-term *in vitro* parasite culture assay, given *P. vivax* cannot currently be cultured long-term *in vitro*. Parasite growth was detectable by qPCR and light microscopy through one initial growth cycle in samples collected from Donor 1, with normal progression of parasite morphology seen on Giemsa stained thick and thin blood films (**Figure 2**). However, no growth was discernible in samples obtained from Donor 2. We therefore undertook further QC testing on vials from Donor 1, with the material tested for sterility, mycoplasma and endotoxin; all tests were passed. Another screen for blood-borne infections was also conducted on the plasma derived directly from the blood donation; all tests were negative.

Finally, we also screened Donor 1 for the Kell blood group antigen because women of childbearing potential that receive a blood transfusion have a small additional risk of developing RBC alloantibodies that could cause problems during pregnancy. In particular, there is a potential risk of development of

hemolytic disease of the newborn in relation to Kell antigen incompatibility, i.e. if Kell-positive donor blood is transfused to a Kell-negative female recipient. However, testing of the donor's blood sample confirmed Kell antigen negativity, thereby allowing future universal administration of the cryopreserved *P. vivax* iRBC stabilate with respect to gender.

Blood-stage CHMI – PvW1 infectivity, parasite growth dynamics and safety

Given all safety and viability tests were passed for the cryopreserved stabilate of *P. vivax* iRBC from Donor 1, we named this clonal isolate "PvW1" and proceeded to test safety and infectivity by blood-stage CHMI. We therefore recruited six healthy, malaria-naïve UK adults into the VAC069A clinical trial, comprising three groups of two volunteers (**Figure S5**), and tested feasibility of infection at three different doses of PvW1 blood-stage inoculum. Five vials of the PvW1 cryopreserved stabilate were thawed and then combined to produce a single batch of blood-stage inoculum. Two volunteers receive a whole vial's worth of iRBC ("neat"), two volunteers received one fifth of the challenge dose via a 1:5 dilution, and the final two volunteers were inoculated with one twentieth of the dose via a 1:20 dilution. All six volunteers underwent blood-stage CHMI at the same time.

Blood-stage parasitemia was monitored as previously by qPCR, beginning one day after challenge (dC+1) (**Figure 3A**, **Table S4**). All six volunteers were successfully infected, with a median time to diagnosis of 15.25 days post-CHMI (range 12.5-16.5) (**Figure 3B**). The median (range) parasitemia at diagnosis across all six volunteers was 9,178 (3,779 – 17,795) gc/mL (**Figure 3C**). We also calculated the PMR as before using a linear model fitted to $\log_{10}$-transformed qPCR data (28). These data showed a median (range) of 5.7 (3.6 – 7.0)-fold growth per 48 hours across the six volunteers (**Figure 3D**), notably lower than that previously observed in the mosquito-bite CHMI study (**Figure 1F**). There was also no discernible difference in the PMRs across the three different challenge dose cohorts (**Figure 3D**); or across the three different Duffy blood group sero-phenotypes, all with median values between 5.3 – 5.9-fold growth per 48 hours (**Figure 3E**). We also analysed gametocytemia at the six time-points preceding diagnosis for each volunteer, and observed rising levels in all individuals (**Figure 3F**). This was in clear contrast to the observations post-mosquito bite CHMI (**Figure 1G**) and despite comparable (if not slightly lower) levels of overall blood-stage parasitemia as measured in gc/mL. Here, we also saw a strong positive correlation between the measured overall levels of parasitemia in gc/mL versus *pvs25* transcripts/µL (**Figure 3G**).

With regards to safety, there were no serious adverse events (SAEs) in the VAC069A study and all volunteers completed treatment without complication. One volunteer withdrew at dC+28, with the remaining five completing clinical follow-up at dC+90 (**Figure S5**). The maximum severity of solicited AEs at any time during the CHMI period is shown for all six volunteers in **Figure 4A**, with four volunteers reporting grade 3 solicited AEs (most commonly feverishness) persisting for 24 hours and one for 48 hours (**Table S5A**). The proportion of volunteers reporting solicited AEs specifically pre-diagnosis, peri-diagnosis and post-treatment is shown in **Figure 4B**. Around the time of diagnosis, 33-50% of the volunteers reported mild-to-moderate symptoms; mainly fatigue, headache, myalgia, malaise, feverishness and chills. Symptoms peaked in severity in the first 24 hours after starting anti-malarial treatment with Riamet® or Malarone, with only one volunteer remaining asymptomatic (**Figure 4B**). Objective fever also increased in the 24 hours post-treatment, with 3/6 volunteers developing pyrexia, one of each grades 1-3 (**Figure 4C**, **Figure S6A**). Nevertheless, most symptoms had completely resolved within a few days of starting treatment and only one volunteer still had headache and fatigue at 6 days post-starting treatment (T+6) (**Figure 4B**). Three volunteers (50%) also experienced short-lived AEs possibly related to the anti-malarial drugs (50% moderate dizziness, 33% mild insomnia, cough and palpitations) (**Figure S6B**). Very few unsolicited AEs (at least possibly related to CHMI) were reported by any of the volunteers (**Table S5B**).

With regard to laboratory AEs (**Table S5C**), lymphocyte counts dropped significantly in 4/6 volunteers around the time of diagnosis or 1 day post-treatment (grade 3 lymphocytopenia in two volunteers), but all counts normalized within 6 days of starting treatment (**Figure 4D**). Two volunteers developed a short-lived grade 2 thrombocytopenia, again normalizing within 6 days of treatment (**Figure 4E**); whilst two volunteers also developed a mild-moderate anaemia post-diagnosis. With regards to the latter, one normalized within 28 days of challenge, the other persisted at grade 1 at dC+90 (102 g/L) and so was referred to their medical practitioner for ongoing monitoring as a precautionary measure (**Table S5C** and **Figure S6C**). The only notable change in blood chemistry was a transient grade 1-2 rise in the ALT in 4/6 volunteers, captured consistently at 6 days post-treatment (**Figure 4F**). All fully resolved to pre-challenge levels with no associated abnormalities in other indices of liver function (**Figure S6C**, **Table S5D**). Finally, we also confirmed CMV and EBV sero-status of all volunteers pre- and post-CHMI. All six volunteers were EBV sero-positive pre-CHMI and three were CMV sero-positive. Of the three CMV sero-negative volunteers, one withdrew consent and left the trial at C+28 and was therefore not re-tested, whilst the other two remained sero-negative when re-tested at C+90.

Antibody responses to blood-stage merozoite antigens post-CHMI

We next assessed for the induction of serum IgG antibody responses post-CHMI against two well-known blood-stage merozoite antigens – *P. vivax* merozoite surface protein 1 C-terminal 19 kDa region ($PvMSP1_{19}$) and *P. vivax* Duffy-binding protein region II (PvDBP_RII). All volunteers had detectable IgG against $PvMSP1_{19}$ post-CHMI, with similar results seen in the VAC068 mosquito-bite sporozoite CHMI study and the VAC069A blood-stage CHMI study (**Figure 5A**). However, there were no detectable responses post-CHMI against PvDBP_RII in any of the volunteers, in contrast to positive control samples from a cohort of healthy UK adult volunteers previously vaccinated with the PvDBP_RII antigen (29) which were included here for comparison (**Figure 5B**). We also assessed for the induction of serum IgG antibody responses post-CHMI against the well-known pre-erythrocytic antigen, *P. vivax* circumsporozoite protein (PvCSP). Responses were negative pre-CHMI in both VAC068 volunteers, with no evidence of seroconversion to PvCSP at dC+90 post-sporozoite CHMI (data not shown).

PvW1 genome assembly allows resolution of complex multi-gene families

Finally, we produced a genome assembly for PvW1 by using a hybrid assembly method which combined long PacBio reads with short Illumina reads. The PvW1 genome assembled into 14 scaffolds (the 14 *P. vivax* chromosomes), and is comparable in both assembly size and number of genes to the highest quality existing *P. vivax* assembly, PvP01 (30) (**Table 1**). The PvW1 assembly has fewer unassigned scaffolds than any other assembly, indicating the completeness of the assembled genome and the benefits of using a combination of long and short reads; note the PvP01, PvC01 and PvT01 were all assembled using Illumina data only (30), while the original reference, PvSalvador-1 (SalI), was created using capillary sequence data (31).

The high quality of the PvW1 assembly allowed us to identify 1145 Vivax interspersed repeat (VIR) genes within the genome, comparable in number to the PvP01 genome. Computational studies have shown that the VIR genes from different *P. vivax* isolates can be grouped into a number of clusters, and it is possible that genes within clusters may be performing a similar function (30, 32). Cluster analysis showed that the majority of the 1145 PvW1 VIR proteins could be clustered into groups with VIRs from the PvP01, PvT01, PvC01 and SalI strains (**Figure 6**), with no evidence that specific clusters are restricted to specific genomes or geographical regions. Of 206 vir clusters that had >5 genes, and therefore had the potential to include vir representatives from all five isolates, 98 were missing at least one isolate.

However, in 90 of those cases, the missing isolate was SalI. As shown in **Table 1**, the SalI genome, which was sequenced more than ten years ago using earlier genome sequencing technology, has significantly fewer vir genes, presumably because such genes are concentrated in subtelomeric regions which are largely unassembled in that genome. There were only 24 clusters that were missing at least one isolate from the more fully assembled genomes (PvW01, PvT01, PvC01 and PvP01); if the size of the clusters was increased to greater than 8 genes, that number of clusters missing at least one isolate dropped to 9. This emphasises that the vast majority of clusters appear to be present across genomes and geographic regions, raising the hypothesis that the clusters may have primarily emerged before the broad geographic dispersal of *P. vivax.*Similarly, we resolved other smaller but still highly polymorphic multigene families such as the merozoite surface protein 3 (MSP3) family. These proteins are expressed on the surface of the invasive merozoite and are known to be highly polymorphic both in sequence and gene number between isolates. We compared the organization of the MSP3 multigene family in PvW1 to *P. vivax* isolates: PvP01 (30) and SalI, India-7, North Korean, Mauritania-1 and Brazil-1 (33). Genes flanking the MSP3 cluster (PVX_097665 and PVX_097740) are syntenic across all isolates, as are MSP3.1, MSP3.2, MSP3.3, MSP3.G, MSP3.10 and MSP3.11. There is however clearly variability in the central region of the MSP3 region, with MSP3.4, MSP3.5, MSP3.6, MSP3.7, MSP3.8 and MSP3.9 all present in some isolates but not others (**Figure S7**). The arrangement of the PvW1 MSP3 cluster appears identical to that of PvP01.

PvW1 vaccine candidate and drug resistance associated genes

The quality of the PvW1 genome also makes it easy to obtain and analyze potential vaccine targets, which we did for three high profile candidates (34), comparing the PvW1 sequence with those from PvP01 and SalI. The sporozoite-stage target PvCSP is known to contain one of two major types of repeat called VK210 and VK247 (35, 36), with this heterogeneity an important factor for vaccine design. PvW1 contains VK210 repeats, the most prevalent form worldwide (**Figure S8A**). The sequence of the transmission-stage candidate Pvs25 is highly conserved between PvW1 and other genomes, apart from the commonly variable amino acids 130 and 131 within the third epidermal growth factor (EGF)-like domain (**Figure S8B**). Finally, we reviewed the PvDBP sequence given two vaccine candidates targeting region II are currently in early-phase clinical trials (29, 37). PvDBP in PvW1 has multiple polymorphisms with 10 in region II, including the DEK epitope (38), as compared to the SalI sequence used in the current clinical vaccines (29, 37). Like PvDBP from SalI, this gene in PvW1 also has a nine amino acid deletion

(downstream of region II) that is not present in PvP01 (**Figure S8C**). As well varying at a sequence level, PvDBP is also known to vary between isolates in copy number, with some isolates containing multiple copies (39, 40) now linked to evasion of humoral immunity (41). We therefore used Illumina read mapping across the PvW1 genome assembly to check for copy number variation of genes. Here, if regions of the genome are present in multiple copies then the read coverage over that region would be higher than the surrounding regions. There was no evidence for increased coverage at either PvDBP or its homologue PvDBP2 (also called *P. vivax* erythrocyte-binding protein, PvEBP), suggesting both are present at a single copy within the PvW1 genome (**Figure S9A-B**). We also looked at an uncharacterized gene on chromosome 14, homologous to PVX_101445 / PvP01_1468200, which has been shown to be duplicated in some isolates (42). This gene is also present in a single copy in PvW1 (**Figure S9D**).

Drug resistance is not as well characterized in *P. vivax* as in *P. falciparum,* but several genes and polymorphisms have been associated with resistance in field studies. We therefore examined the sequences of four genes within the PvW1 genome that have been associated with drug resistance: dihydrofolate reductase (*PvDHFR*), dihydropteroate synthetase (*PvDHPS*), chloroquine resistance transporter (*PvCRT*) and multidrug resistance transporter 1 (*PvMDR1*). The PvW1 *PvDHFR* gene encodes a protein with the quadruple mutation F57L/S58R/T61M/S117T that has been linked to pyrimethamine resistance (43), whereas *PvDHPS* showed no mutations previously associated with sulfadoxine resistance (44). The molecular basis of *P. vivax* chloroquine resistance is less clear, although there is some evidence that mutations in *PvCRT* (K10 insertion) and *PvMDR1* (Y976F mutation) may be involved (45-47). Neither of these mutations are present in the PvW1 *PvCRT* and *PvMDR1* genes. It is important to note that both Riamet® (a combination of artemether and lumefantrine) and Malarone (a combination of atovaquone with proguanil) antimalarials were used with 100% treatment success rates in the VAC068 and VAC069A studies (both volunteers in VAC068 and 5/6 volunteers in VAC069A received Riamet®, 1/6 received Malarone), and none of the polymorphisms identified have been associated with resistance to either of these drugs.

Discussion

Here we undertook CHMI model development for *P. vivax* and established a new PvW1 clonal isolate from Thailand. Our methodology elected to focus on a mosquito-bite CHMI protocol to provide the initial source of blood-stage parasites for the cryopreserved stabilate. The main advantages here (over parasites donated by returning travellers) included the ability to control the parasite source, the recruitment of suitable healthy volunteers (especially with regard to health screening and universal donor blood group) and logistical timing. We also created the blood stabilate as close as possible to the mosquito-stage, with only ~3 cycles of replication from the liver (given it is known that mosquitoes reset parasite virulence (24)). If parasites had been cryopreserved from returning travellers or chronically-infected adults in an endemic setting they would have been selected over many rounds of asexual replication *in vivo* before creating the stabilate. This diminishes the criticism that blood-stage CHMI is not the natural route of infection. Furthermore, as many as 80% of *P. vivax* blood-stage infections are caused by relapsing parasites, which means that in the unique context of relapsing *P. vivax*, a challenge with recently emerged blood-stages is in many ways closer to most "natural challenges" than mosquito bite-delivered sporozoites. That being said, it is important to acknowledge that blood-stage CHMI is only useful to measure interventions against the blood-stage of infection rather than sporozoites or hypnozoite establishment.

Notably our real-time assessment of parasite genotypes in the infected mosquitoes in Thailand identified only one clonal infection out of three tested. In future it will likely be necessary to screen more infected patient samples if parasite clones with specific genotypes are desired. It is also probable that this clonal infection resulted from a single relapsing hypnozoite in the patient, given natural infections are frequently polyclonal, arising from primary infections with multiple genotypes and meiotic siblings produced in the mosquito and/or multiple heterologous hypnozoites relapsing at a similar time (48-50).

The VAC068 mosquito-bite trial demonstrated feasibility and safety of this CHMI model for the first time at a European site, albeit in only two healthy adult UK volunteers. Both were successfully infected, with parasites first detectable by qPCR on dC+8.5 and the first wave of blood-stage parasitemia peaking around dC+9. This is largely consistent with data from humanized mouse models suggesting that the complete maturation of *P. vivax* liver stages and exo-erythrocytic merozoite release occurs between days 9 and day 10 post-sporozoite infection (51). Growth of blood-stage parasitemia was subsequently

similar in the two volunteers, with both meeting criteria to donate blood on dC+14, prior to radical cure treatment with Riamet® followed by primaquine. Both volunteers were screened to have CYP2D6 genotypes predicted to be extensive metabolizer phenotypes of primaquine, and as of ~3 years' long-term follow-up, no relapse of infection has been documented.

Cryopreservation of the iRBC stabilate was performed successfully, however, given *P. vivax* cannot be cultured long-term *in vitro* it proved challenging to confirm parasite viability following thaw of the frozen stabilate, especially given the relatively low level of parasitemia achieved by CHMI in non-immune adults. However, given the stabilate from Donor 1 showed demonstrable growth *in vitro* using a short-term culture assay, we elected to proceed with this material for onward testing. Poor parasite recovery from Donor 2 could be associated with the predominant lifecycle stage at the time of cryopreservation; here microscopy records indicated the presence of more schizonts and a smaller proportion of early ring-stage trophozoites in comparison to Donor 1. Previous evidence suggests that the late asexual intra-erythrocytic parasites are not viable after cryopreservation with glycerolyte (52), and this may have led to the poor recovery of live parasites in Donor 2's stabilate.

Previous reports of blood-stage CHMI using *P. vivax* have used one vial of cryopreserved stabilate to infect one volunteer (19-21), in contrast to similar studies with the stabilate of 3D7 clone *P. falciparum* whereby a single vial is diluted and routinely used to infect ~20-30 volunteers (16, 53). Thawing many vials to undertake CHMI in larger cohorts of volunteers, e.g. for vaccine efficacy trials, brings many practical difficulties and, in turn, more rapidly depletes the bank of cryopreserved stabilate which is a finite resource. Conserving vials and building up a long-term safety database of the challenge agent for future use across many clinical studies is also preferable. Consequently, we assessed three different doses of the PvW1 blood-stage inoculum in the VAC069A study, with two volunteers receiving each dose. All six volunteers were successfully diagnosed at similar levels of blood-stage parasitemia within 12-16 days. Importantly, these data suggest that blood-stage CHMI trials in larger volunteer cohorts are now practical and feasible, whilst preserving the bank of PvW1 parasites for the long-term.

The AE profiles of both the mosquito-bite and blood-stage CHMI with PvW1 were highly comparable to previous reports of both models in malaria-naïve/non-immune adults using other isolates of *P. vivax* at the Colombian (8-10), American (54) or Australian sites (19-23). No SAEs occurred in either trial and all drug treatments were successful. Symptoms consistent with malaria were experienced and peaked post-treatment prior to resolving within a few days. We also observed transient thrombocytopenia and lymphocytopenia, as well as rises in ALT six days post-treatment, consistent with the reports of other

sites undertaking *P. vivax* CHMI (9, 10, 55) and with no apparent impact on volunteer safety. We also observed consistent sero-conversion to $PvMSP1_{19}$ post-CHMI in all volunteers, as reported in the Colombian CHMI trials (10, 56), but no detectable responses to PvDBP_RII or PvCSP. These data for the merozoite antigens are in line with our similar studies of *P. falciparum* CHMI, with sero-conversion of malaria-naïve adults observed to immuno-dominant merozoite surface proteins following primary acute malaria exposure, but not to more transiently exposed RBC invasion ligands (57, 58).

Following mosquito-bite CHMI we observed ~10-fold growth in blood-stage parasitemia per 48 hours, consistent with other reports for *P. vivax* (20), as well as our experience with *P. falciparum* (16, 53). Interestingly, however, the average PMR was lower (~5.5-fold growth per 48 hours) following blood-stage CHMI with the same parasite. There was no obvious effect of challenge dose or Duffy blood group sero-phenotype on the PMR, the latter consistent with our observations *in vitro* using *P. knowlesi* parasites transgenic for PvDBP (59). However, Duffy blood group sero-phenotype has been linked to susceptibility of *P. vivax* clinical malaria following natural infection (60). Consequently CHMI studies in larger numbers of volunteers will be required to more stringently assess for any relationships between blood group antigens and the observed PMR, and to more accurately establish the natural variability in the PMR observed in malaria-naïve adults. A second striking difference between the two CHMI models was the apparent minimal gametocytemia following mosquito-bite CHMI, in contrast to blood-stage CHMI. In the latter, the *pvs25* transcripts (a marker of mature female gametocytes) were reliably detected in all six volunteers, reaching comparable levels to those reported in other *P. vivax* blood-stage CHMI studies (19, 20). Notably, poor transmission to mosquitoes was reported in another *P. vivax* mosquito-bite CHMI trial, consistent with our data here (61). Interestingly, a more recent study comparing the same two CHMI models with *P. falciparum* reported the same finding (17). Why blood-stage CHMI appears to lead to much greater gametocytemia than mosquito-bite CHMI, despite reaching comparable levels of overall parasitemia by the time of diagnosis, remains to be determined. However, this might reflect the greater number of asexual growth cycles since liver egress, or a longer time to diagnosis allowing for an extended window for conversion of asexual parasites.

Finally, we proceeded to undertake a genomic analysis of the new *P. vivax* PvW1 clone. The need to drug treat volunteer infections at relatively low parasitemia limited the amount of PvW1 parasite DNA that could be isolated for sequencing. Nevertheless, a very high quality genome assembly for PvW1 was created by using a hybrid assembly method which combined long PacBio reads with short Illumina reads. The PacBio library was created using low-input PacBio technology developed to create a genome

assembly from a single mosquito (62), and is to our knowledge the first time that this has been applied to *Plasmodium* parasites. Our goal is that the PvW1 clone will become a valuable tool for vaccine discovery, drug testing and assessment of *P. vivax in vivo* immuno-biology. Accurate assessment of both the sequence and copy number of vaccine candidate antigens within the PvW1 genome will thus be critical in designing future vaccine immunogens and interpreting CHMI efficacy studies. The high quality of the PvW1 assembly allowed us to easily report on leading vaccine candidate antigens, analyze genes and polymorphisms associated with drug resistance in field studies, and resolve 1145 VIR genes as well as the smaller polymorphic PvMSP3 multigene family. Although the function of the highly variable subtelomeric multigene VIR family is not well defined, related genes are found in high numbers in most *Plasmodium* species which infect humans, monkeys and rodents, and some are thought to be involved in immune evasion, including by directly binding to and down-regulating natural killer (NK) cell ligands (63). Our cluster analysis will now enable comparison of gene function within and between clusters, and should help in the future elucidation of the function of the VIR gene family.

In conclusion, we have developed a mosquito-transmitted stabilate using a new clonal field isolate of *P. vivax* and combined new methodologies for parasite isolation and ultra-low input PacBio sequencing to assemble a reference-quality genome for CHMI. This has i) revealed polymorphisms in leading drug and vaccine targets that can now be functionally tested *in vivo* with PvW1 and ii) used a hybrid PacBio/Illumina genome assembly technique to identify 1145 unique VIR genes. This will allow for *in vivo* switching and selection of multi-gene families to be measured in *P. vivax* in the same way as has been done for *P. falciparum* (18). This has allowed us to open up many new research avenues, and in the first instance, we have used this model to investigate myeloid cell activation, systemic inflammation, and the fate and function of human T cells during a first-in-life *P. vivax* infection (64). The PvW1 parasite should prove to be an invaluable resource for the wider malaria community.

Methods

Detailed methods are provided in **Supplementary Methods**. In brief:

Study design. VAC068 was a clinical study to assess the safety of controlled human *P. vivax* malaria infection through experimental sporozoite inoculation (by mosquito-bite) of healthy malaria-naïve UK adults. The study was conducted in the UK at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), University of Oxford (recruitment, follow-up post-CHMI, admission for blood donation and treatment) and at the Sir Alexander Fleming Building (Infection and Immunity section) Imperial College of Science, Technology and Medicine, London (sporozoite challenge of volunteers, delivered by mosquito bite in the designated category 3 suite). Concurrent primary objectives of the trial were to assess the immune response to primary *P. vivax* infection and to assess gametocytemia following infection. Secondary objectives were to obtain up to 250 mL of blood from each infected volunteer and produce a cryopreserved stabilate of iRBC for future use in blood-stage *P. vivax* CHMI studies. VAC068 volunteers were admitted to the CCVTM in Oxford according to a clinical / diagnostic algorithm. Following admission, a 250 mL blood sample was collected using aseptic technique, via a whole blood donation kit containing an in-line leukodepletion filter (Leuokotrap WB, Haemonetics Corp), at room temperature. Antimalarial treatment (60-hour course of artemether/lumefantrine, Riamet®) was started immediately after blood donation, followed by a 14-day course of primaquine, 30 mg once daily. Follow-up was out to 5 years to monitor for any signs of relapse. The VAC069A study assessed the safety and infectivity of blood-stage *P. vivax* CHMI in healthy malaria-naïve UK adults, through experimental inoculation with the cryopreserved PvW1 infected erythrocytes collected from Donor 1 in VAC068, at three different doses. The PvW1 blood-stage inoculum was thawed and prepared under strict aseptic conditions as previously described for *P. falciparum* (16), with some modifications. All six volunteers were challenged (two receiving each dose dilution) and followed up at the CCVTM, Oxford, UK. Diagnostic criteria were based on thick blood film microscopy results and qPCR in the presence or absence of symptoms. Treatment was completed with either a 60-hour course of Riamet® or a 48-hour course of Malarone and volunteers followed up for 90 days. Full details of diagnostic criteria and follow-up schedules for both studies, case-finding in Thailand and preparation of infected mosquitoes for transfer to the UK, are described in **Supplementary Methods**.

Participants. Healthy, malaria-naive males and non-pregnant females aged 18-50 were invited to participate in the study. Two and six volunteers were enrolled for each respective trial in total. A full list of inclusion and exclusion criteria and specific considerations for screening of healthy UK adult volunteers for the VAC068 study are reported in **Supplementary Methods**.

Safety analysis. Data on both solicited AEs occurring during and after the CHMI period (that may have related to CHMI or antimalarial treatment) as well as any unsolicited AEs, were collected at clinic visits, from dC+1 up until the end of primaquine antimalarial treatment (VAC068) and until 6 days post-initiation of Riamet®/Malarone treatment (VAC069A). Volunteers were given a card on which to document the end date of any outstanding malaria symptoms on-going between completing anti-malarial therapy and their next clinic visit. Data on SAEs were collected throughout the entire study period. Details on assignment of severity grading and causality are provided in the **Supplementary Methods**.

Total parasite quantification. Quantitative PCR (qPCR) was used to monitor total *P. vivax* blood-stage parasitemia in volunteers' blood in real-time. The assay targets the 18S ribosomal RNA (rRNA) gene and was adapted from previously published methodology (19, 53).

Thick blood film microscopy. Collection of blood, preparation of thick films and slide reading for VAC068 volunteers were performed according to Jenner Institute Standard Operating Procedure (SOP) ML009. Briefly, slides were prepared using Field's stain A and then Field's stain B. 200 fields at high power (1000x) were read. Visualization of two or more parasites in 200 high power fields constituted a positive result.

Cryopreservation and *in vitro* testing of *P. vivax* infected blood (VAC068). After blood donation, the leukodepleted blood from both volunteers was maintained at ~37 ^{o}C and transported immediately to the Jenner Institute Laboratories, University of Oxford. Here, RBC were separated from plasma by centrifugation before mixing the RBC with Glycerolyte 57 (Fenwal 4A7833) at 1:2 volume ratio. All procedures were conducted according to SOPs under stringent Quality Assurance (QA) oversight and guidance from a Qualified Person (QP) at the University of Oxford. The RBC-Glycerolyte mixture was finally aliquoted at 1.5 mL per cryovial, transferred into CoolCells (Corning 432009) and placed at -80 ^{o}C within 2 h 30 min of blood donation to freeze overnight; the following day the frozen cryovials were transferred to long-term storage in liquid nitrogen. A final screen for blood-borne infections was

conducted on the plasma, derived directly from the blood donation (separated from the RBC prior to cryopreservation), in line with testing procedures performed by the UK NHS Blood Transfusion service. RNA PCR for HIV-1 and hepatitis C, DNA PCR for hepatitis B, EBV CMV, and serology for HIV-2, HTLV-1, HTLV-2, and Treponema pallidum was performed on thawed plasma samples at University Hospitals Birmingham NHS Foundation Trust, UK (Public Health England, Birmingham Laboratory). Separately, screening of a blood sample from Donor 1 for the Kell blood group antigen was performed by Oxford University Hospitals NHS Trust Haematology Laboratory, UK. The cryopreserved stabilate from Donor 1 was also tested for sterility by direct inoculation and mycoplasma by specific culture. Finally, endotoxin was quantified by kinetic chromogenic limulus amoebocyte lysate assay. These assays were conducted by a Contract Research Organization: SGS Vitrology, Glasgow, UK or SGS Vitrology's contracted services at Moredun Scientific, Penicuik, Scotland, UK. The tests were non-regulatory standard and performed for information only.

Gametocyte quantification. *P. vivax* gametocytemia was determined by one-step quantitative reverse transcription PCR (qRT-PCR) targeting the messenger RNA marker of female mature gametocytes, pvs25. For RNA extraction samples were processed within 4 h of blood sampling (Qiagen) followed by one-step RT-PCR using Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs).

Modelling of PMR – VAC068 and VAC069A. A qPCR-derived PMR was modelled based on previously described methodology (28, 53, 65).

Anti-PvDBP_RII standardized ELISA. ELISAs to quantify circulating PvDBP_RII-specific total IgG responses were performed using standardized methodology, similar to that previously described (29). Day C-1 and dC+90 serum or plasma samples from the VAC068 and VAC069A volunteers were tested, alongside samples from 8 healthy UK adults previously vaccinated in the VAC051 Phase Ia trial of a candidate PvDBP_RII vaccine (Group 2C) (29).

Anti-PvMSP1$_{19}$ ELISA. Anti-PvMSP1$_{19}$-specific total IgG responses were measured in VAC068 volunteer serum or plasma via indirect ELISA (same test samples as for the PvDBP_RII ELISA).

Illumina and long read sequencing. DNA was extracted from blood taken from the VAC068 volunteers at 11 and 14 days post-CHMI using the Qiagen blood DNA midi kit and sequenced with Illumina HiSeq X10 with 150 bp paired end reads. (See **Supplementary Methods** for details on preparation of schizonts,

high molecular weight DNA extraction and Shearing and PacBio library construction and sequencing, and VIR gene analysis).

Statistics. Unless otherwise stated, data were analyzed using GraphPad Prism version 9.1.1 for Windows (GraphPad Software Inc.). All tests used were 2-tailed and are described in the text. A value of $P<0.05$ was considered significant.

Study approvals. The VAC068 and VAC069 trials were registered on ClinicalTrials.gov (NCT03377296 and NCT03797989, respectively) and were conducted according to the principles of the current revision of the Declaration of Helsinki 2008 and in full conformity with the ICH guidelines for Good Clinical Practice (GCP). All volunteers signed written consent forms, and consent was checked to ensure volunteers were willing to proceed prior to CHMI. The VAC068 study received ethical approval from the UK NHS Research Ethics Service (Oxfordshire Research Ethics Committee A, Ref 17/SC/0389). The VAC069 study received ethical approval from the UK NHS Research Ethics Service (South Central – Hampshire A Research Ethics Committee), Ref 18/SC/0577.

Author Contributions

- Conceived and performed the experiments: AMM, YT, SES, JRB, AK, DQ, CMN, NJE, TAR, FRL, WR, KJE, J-SC, TDO, AJR, FB, GML, IDP, AM, MZ, MM, RLR, MB, CHM, JCS, NR, CK, NM, CS, TP, KM, DJR, AMB, PJS, JS, SB, JCR, SJD.
- Analyzed the data: AMM, YT, SES, JRB, AK, NJE, KJE, J-SC, EA, TDO, AJR, JCS, MMH, BK, JS, SB, JCR, SJD.
- Project Management: AML, FLN, KJJ, IJT.
- Wrote the paper: AMM, AK, SES, JRB, JCR, SJD.

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613

614 Conflict of Interest Statement

615 The authors declare no conflicts of interest.

616

617 Data and Materials Availability

618 Requests for materials should be addressed to the corresponding authors.

619

620 The genome assembly and annotation for PvW1 are available from the European Nucleotide Archive
621 under project accession PRJEB45464.

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Figures and Legends

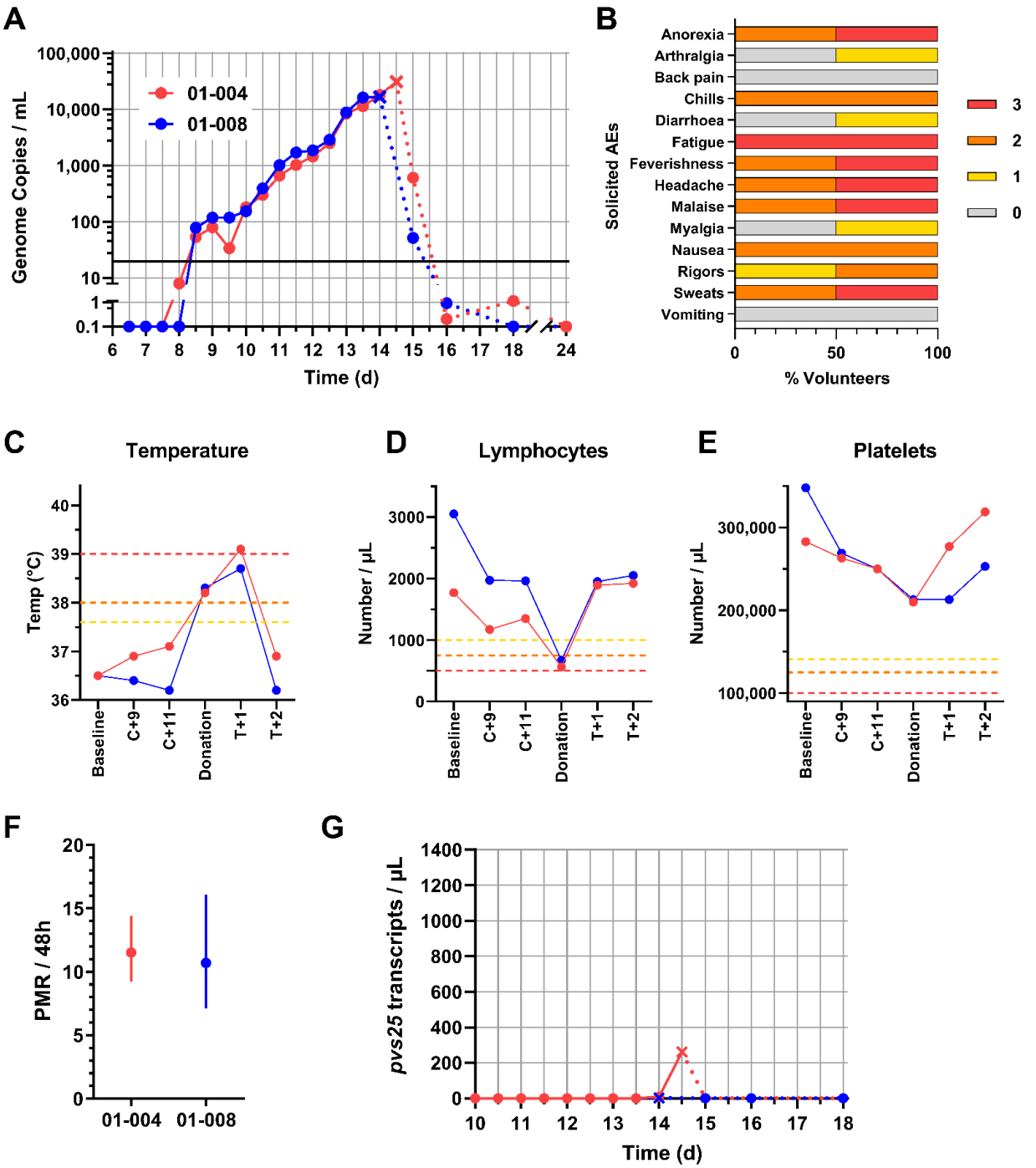


Figure 1. Safety and parasite growth dynamics of *P. vivax* sporozoite CHMI.

(**A**) qPCR data for the VAC068 trial (n=2). Parasitemia measured in genome copies / mL is shown over time for each volunteer. CHMI was initiated by mosquito bite on day 0. Cross symbols indicate the time-point of blood donation followed by anti-malarial treatment. Solid lines show qPCR readouts pre-

805 treatment, and dotted lines post-treatment. Solid black line indicates 20 gc/mL (the minimum level to
806 meet positive reporting criteria); samples below this are shown for information only. (**B**) The solicited
807 systemic adverse events (AEs) recorded during the CHMI period (from 1 day up until 45 days post-
808 challenge) are shown as the maximum severity reported by each volunteer and as a percentage of the
809 volunteers reporting each individual AE (n=2). Color-coding refers to AE grading: 0 = none; 1 = mild; 2 =
810 moderate; 3 = severe. (**C**) Volunteer temperature (maximum self-recorded by volunteer or measured in
811 clinic) at the indicated time-points: baseline pre-CHMI; 9 and 11 days post-CHMI (C+9, C+11); time of
812 blood donation; and 1 and 2 days post-treatment (T+1, T+2). AE grading cut-offs are indicated by the
813 dotted lines (yellow = grade 1; orange = grade 2; red = grade 3). (**D**) Lymphocyte and (**E**) platelet counts
814 plotted as for panel C. (**F**) The PMR per 48 h was modelled from the qPCR data up until the time-point of
815 blood donation/treatment; PMR ± 95% CI is shown for each volunteer. (**H**) Gametocytemia was assessed
816 over time by qRT-PCR for *pvs25* transcripts; symbols and lines as per panel A.

817

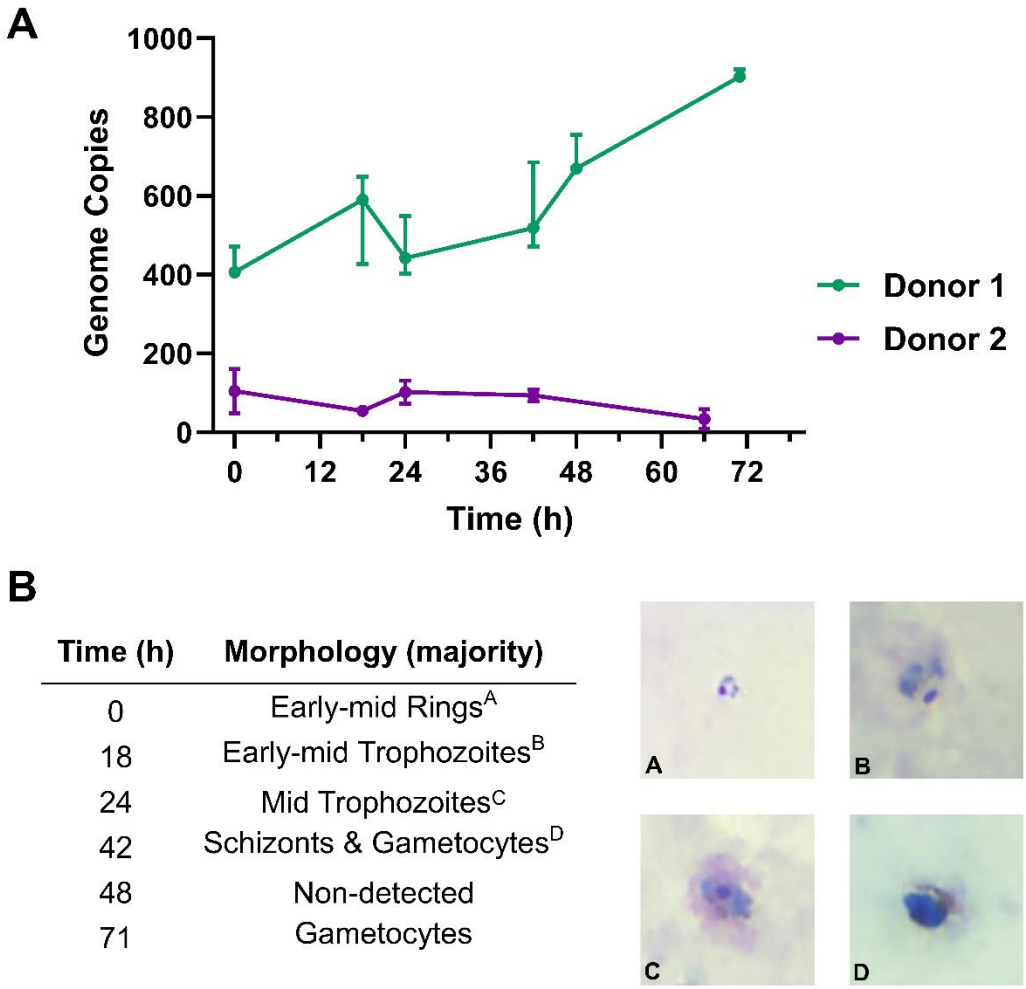


Figure 2. Test of cryopreserved parasite viability by short-term *in vitro* culture assay.

(**A**) Test vials of cryopreserved parasites from Donor 1 and Donor 2 were thawed and cells used in a short-term *in vitro* parasite culture assay. *P. vivax* parasite growth was monitored by qPCR in 20 µL samples of RBC extracted at the indicated time-points. Median and range of triplicate readings are shown in genome copies measured per 20 µL sample. (**B**) Parasite morphology was monitored at the same time-points over the first growth cycle by light microscopy of Giemsa-stained thick and thin blood films Representative images are shown from Donor 1, and the predominant morphology observed is reported.

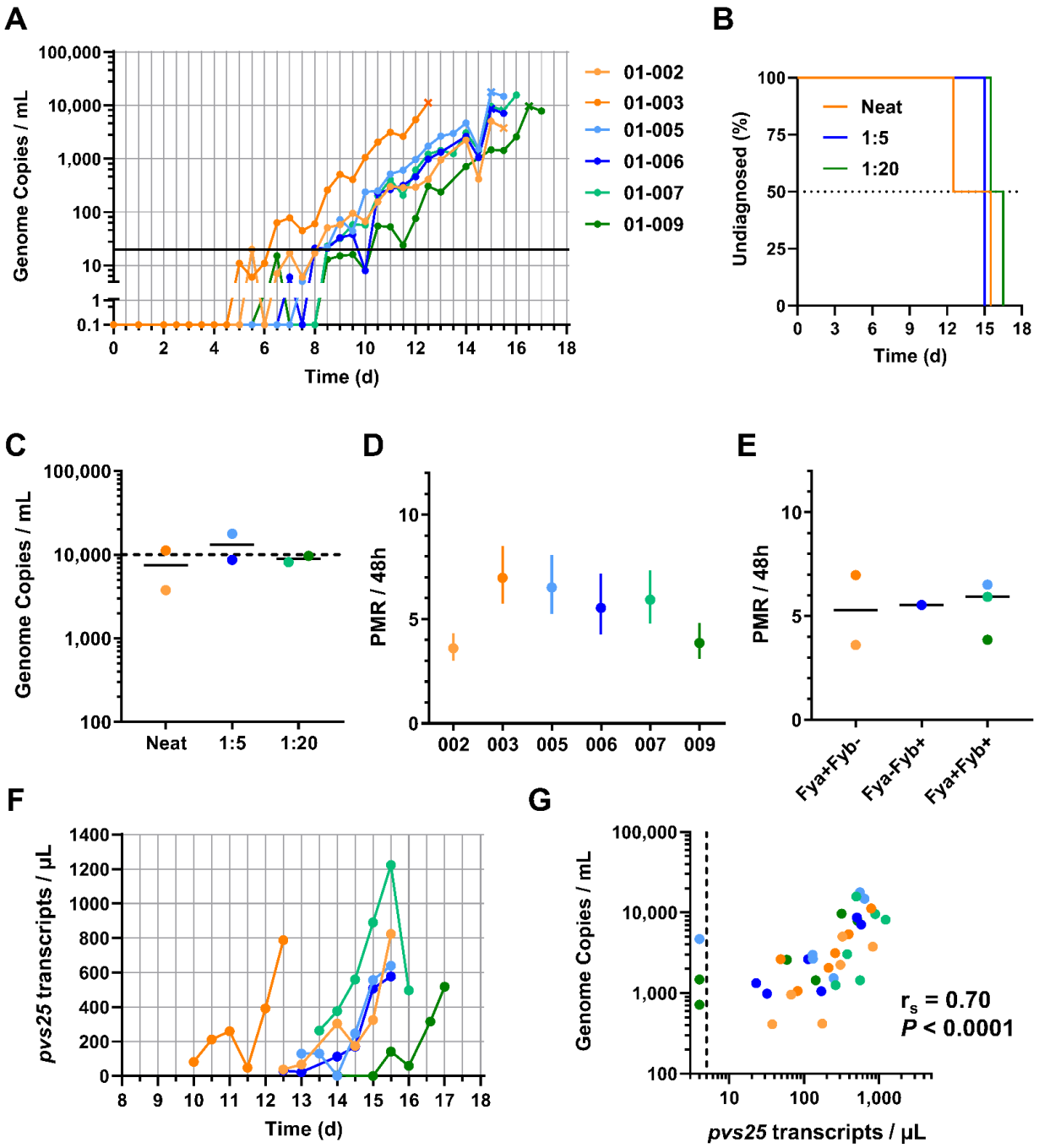


Figure 3. Parasite growth dynamics of *P. vivax* PvW1 clone blood-stage CHMI.

(**A**) qPCR data for the VAC069A trial (n=6). Parasitemia measured in genome copies (gc) / mL is shown over time for each volunteer. CHMI was initiated by blood-stage inoculation on day 0. Cross symbols indicate the time-point of diagnosis. Orange = neat inoculum dose; blue = 1:5 and green = 1:20 dilution of the neat inoculum dose. Solid black line indicates 20 gc/mL (the minimum level to meet positive reporting criteria); samples below this are shown for information only. (**B**) Kaplan-Meier plot of time to

diagnosis in days for the VAC069A study (n=2/group). (**C**) Parasitemia measured in gc/mL at the time-point of diagnosis. Individual data and median are indicated for each dose group. Volunteers were diagnosed when they reached a threshold of 10,000 gc/mL OR if they had symptoms of malaria with a parasitemia >5,000 gc/mL. (**D**) The PMR per 48 h was modelled from the qPCR data up until the time-point of diagnosis; PMR ± 95% CI is shown for each volunteer. (**E**) Individual and median PMR are shown with volunteers grouped according to their Duffy blood group antigen (Fy) serological phenotype. (**F**) Gametocytemia was assessed over time by qRT-PCR for *pvs25* transcripts; colored lines as per panel A. (**G**) Correlation of total parasitemia measured in gc/mL versus *pvs25* transcripts/µL. Spearman's rank correlation coefficient and *P* value are shown, n=36.

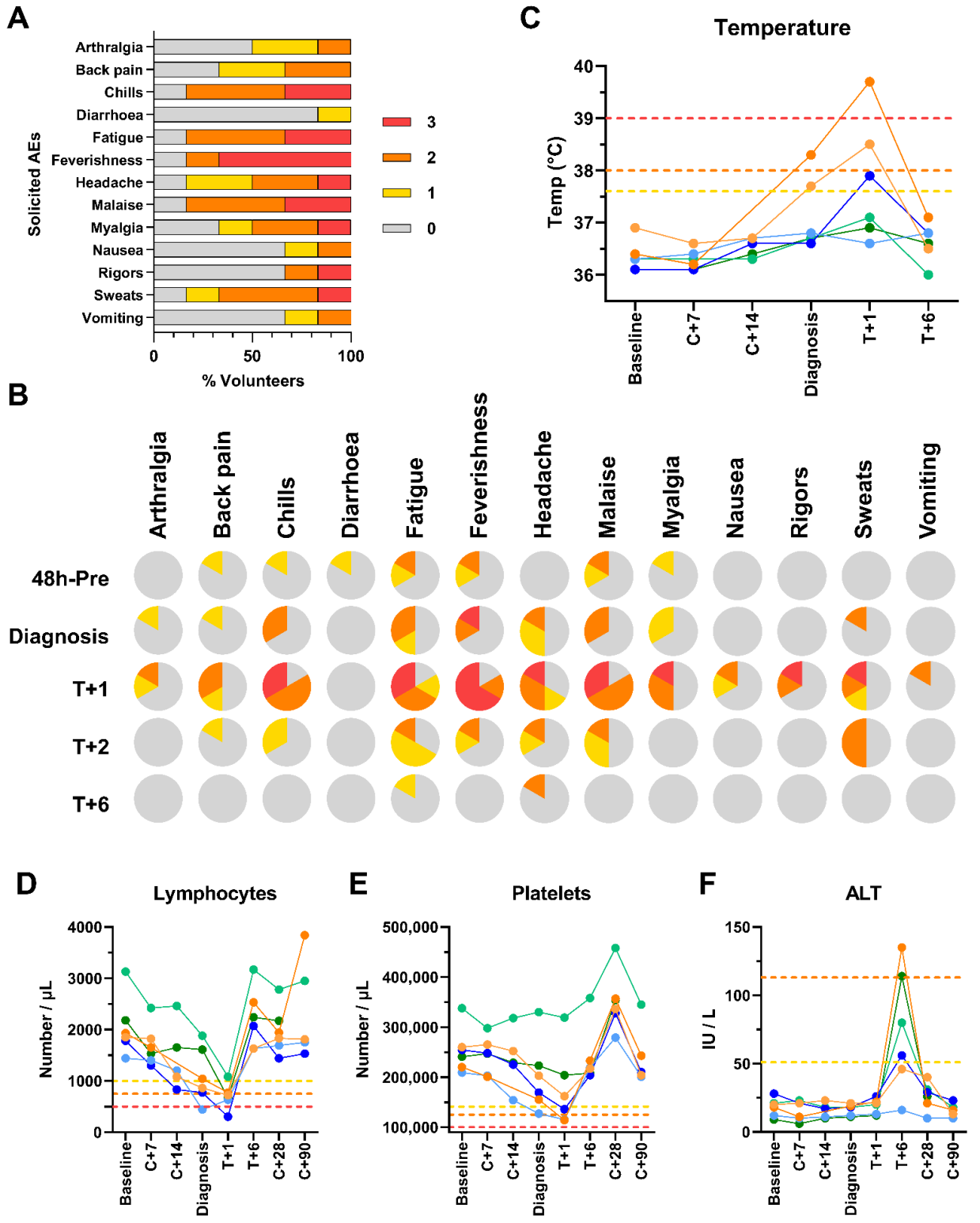


Figure 4. Safety analysis of *P. vivax* PvW1 clone blood-stage CHMI.

(**A**) The solicited systemic adverse events (AEs) recorded during the CHMI period (from 1 day up until 90 days post-challenge) are shown as the maximum severity reported by each volunteer and as a

849 percentage of the volunteers reporting each individual AE (n=6). Color-coding refers to AE grading: 0 =
850 none; 1 = mild; 2 = moderate; 3 = severe. (**B**) The solicited systemic AEs recorded at the indicated time-
851 points during the CHMI period are shown as the maximum severity reported by each volunteer and as a
852 percentage of the volunteers reporting each individual AE (n=6). Color-coding as per panel A. 48h-pre =
853 the 48 hour period prior to *P. vivax* diagnosis; Diagnosis = time-point of diagnosis; +1, +2 and +6 days
854 post-treatment (T). (**C**) Volunteer temperature (maximum self-recorded by volunteer or measured in
855 clinic) at the indicated time-points: baseline pre-CHMI; 7 and 14 days post-CHMI (C+7, C+14); time of
856 diagnosis; and 1 and 6 days post-treatment (T+1, T+6). AE grading cut-offs are indicated by the dotted
857 lines (yellow = grade 1; orange = grade 2; red = grade 3). (**D**) Lymphocyte and (**E**) platelet counts, and (**F**)
858 alanine aminotransferase (ALT) measurements, all plotted as for panel C but also including C+28 and
859 C+90 time-points.

860

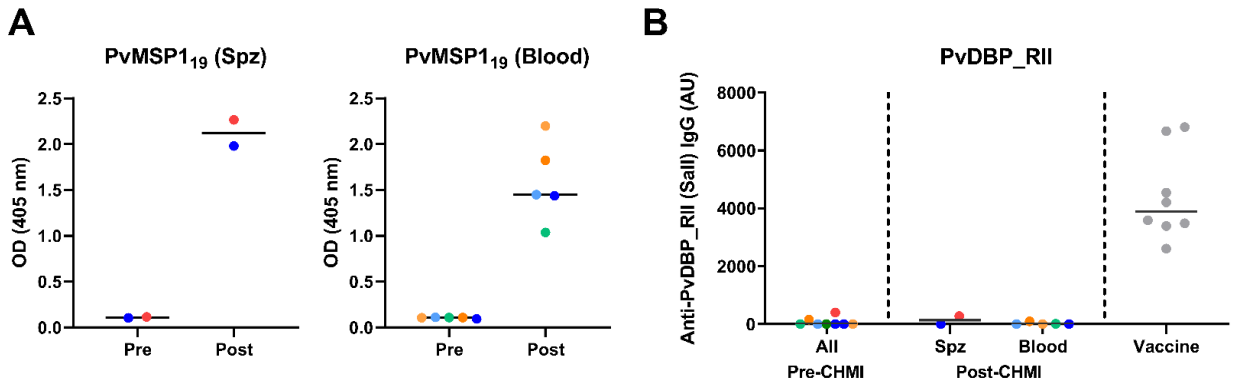
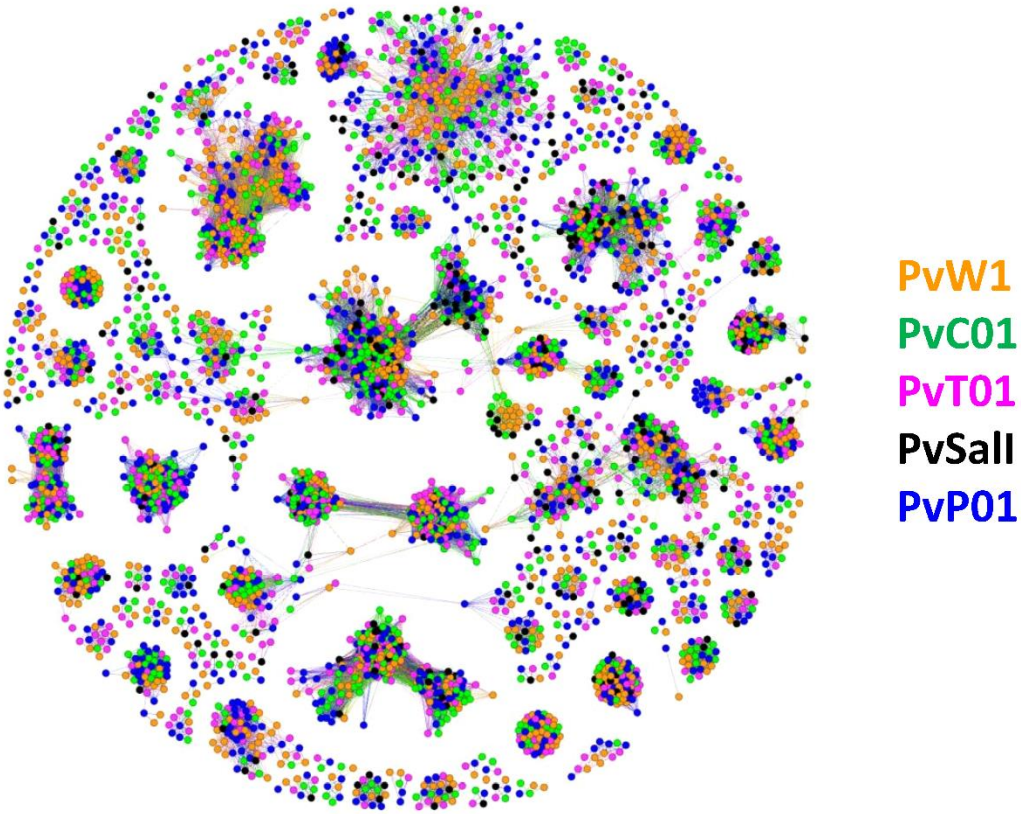


Figure 5. Induction of serum antibody responses to merozoite antigens during CHMI.

(**A**) Serum anti-PvMSP1$_{19}$ IgG ELISA was conducted on samples from the VAC068 mosquito-bite / sporozoite (spz) CHMI study (n=2) and the VAC069A blood-stage CHMI study (n=5, because one volunteer withdrew at dC+28). Optical density (OD) 405nm data are shown for sera tested at a 1:100 dilution from the pre-CHMI (dC-1) and 90 days post-CHMI (dC+90) time-points. Samples color-coded as per previous figures. (**B**) Serum anti-PvDBP_RII (SalI allele) IgG as measured by standardized ELISA, reporting in arbitrary units (AU). Same samples tested as in panel A. Vaccine = positive control samples (n=8) from a previous Phase Ia clinical trial of a PvDBP_RII vaccine (29). Individual data and median are shown.



872

873 **Figure 6. Cluster analysis of the PvW1 vivax interspersed repeat (VIR) proteins.**

874 Cluster analysis of the 1145 predicted VIR proteins encoded by the PvW1 genome compared to those of
875 other *P. vivax* isolates (30, 31). Each spot represents a VIR protein from either PvW1 (orange), PvC01
876 (green), PvT01 (pink), PvSall (black) and PvP01 (blue). Relatedness between the proteins is represented
877 by distance, therefore more closely related proteins cluster together. Most of the clusters contain
878 proteins from several isolates suggesting that the clusters are not restricted to specific genomes or
879 geographical distribution.

880 ## Table

881

Genome Features	PvW1	PvP01	PvC01	PvT01	SalI
Nuclear genome					
Assembly size (Mb)	28.9	29	30.2	28.9	26.8
G + C content (%)	39.9	39.8	39.2	39.7	42.3
No. scaffolds assigned to chrom.	14	14	14	14	30
No. unassigned scaffolds	3	226	529	359	2745
No. genes	6583	6642	6690	6464	5433
No. pir (VIR) genes	1145	1212	1061	867	346
Mitochondrial genome					
Assembly size (bp)	5994	5989	-	-	5990
G + C content (%)	30.5	30.5	-	-	30.5
Apicoplast genome					
Assembly size (kb)	34.5	29.6	27.6	6.6	5.1
G + C content (%)	14.4	13.3	12.7	19.7	17.1
No. genes	54	30	3	0	0

882

883 **Table 1. Comparison of genome assembly statistics between PvW1 and other *P. vivax***
884 **assemblies.**

885 PvW1 genome assembly statistics were compared with the best available existing assemblies: PvP01,
886 PvC01, PvT01 and SalI (30, 31). pir = *P. vivax Plasmodium* interspersed repeat, also known as VIR.