Anti-phosphatidylserine IgM and IgG antibodies are higher in vivax than falciparum malaria, and associated with early anemia in both species

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Summary

Anti-phosphatidylserine antibodies (PS-Abs) are elevated in Malaysian patients with vivax and falciparum malaria, and are highest in vivax malaria. In vivax and falciparum malaria PS-Abs correlate inversely with admission and nadir haemoglobin, suggesting PS-Abs contribute to anaemia from these species.

Abstract

Background: Anemia is a major complication of vivax malaria. Anti-phosphatidylserine (PS) antibodies generated during falciparum malaria mediate phagocytosis of uninfected red blood cells (RBCs) that expose PS, and have been linked to late malarial-anemia. However, their role in anemia from non-falciparum *Plasmodium* species is not known, nor their role in early anemia from falciparum malaria.

Methods: We measured PS-IgG and IgM antibodies in Malaysian patients with vivax, falciparum, knowlesi and malariae malaria, and in healthy controls, and correlated with hemoglobin. PS antibodies were also measured in volunteers experimentally infected with *P. vivax* and *P. falciparum*.

Results: PS-IgM and IgG were elevated in patients with vivax, falciparum, knowlesi and malariae malaria ($p<0.0001$ for all comparisons with controls), and were highest in vivax malaria. In vivax and falciparum malaria, PS-IgM and IgG on admission correlated inversely with admission and nadir hemoglobin, controlling for parasitemia and fever duration. PS-IgM and IgG were also increased in volunteers infected with blood-stage *P. vivax* and *P. falciparum*, and were higher in *P. vivax* infection.

Conclusions: PS antibodies are higher in vivax than falciparum malaria, correlate inversely with hemoglobin and may contribute to the early loss of uninfected-RBC found in malarial anemia from both species.

Key Words: Phosphatidylserine antibodies, anemia, malaria, Plasmodium vivax, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae .

Background

Anemia is a major complication of malaria from all *Plasmodium* species, causing significant morbidity and mortality not only in *P. falciparum* malaria, but also in *P. vivax* malaria [1-3]. Although occurring in part due to rupture of infected red blood cells (RBCs), as well as reduced erythropoiesis, the major contributor to malarial anemia is the loss of uninfected RBCs [4], particularly in vivax malaria [5]. Mechanisms leading to the loss of uninfected RBCs are incompletely understood, but likely include oxidative damage, as well as complement-mediated lysis [6]. In addition, recent evidence has emerged regarding the role of auto-antibodies directed against RBC components, including phosphatidylserine (PS) [7, 8].

Phosphatidylserine is a membrane phospholipid that is normally located on the internal leaflet of the RBC lipid bilayer, however may become exposed as a result of a number of stimuli such as oxidative stress [9]. In *Plasmodium falciparum*, PS becomes exposed on infected RBCs during parasite maturation [10-12], enhancing phagocytosis of these cells. In addition, in *P. falciparum in vitro* cultures [13], and in mice infected with *P. yoelii* [7, 14], PS is also externalised on uninfected RBCs. The mechanisms of PS exposure on uninfected RBCs remain unclear, but may relate to oxidative damage from parasite degradation with release of hemozoin or reactive oxygen species, or release and circulation of toxic heme [15, 16].

With exposure of PS on uninfected as well as infected RBCs, it has been hypothesised that autoantibodies directed against PS may contribute to malarial anemia [7]. In keeping with this, in a recent study involving the *P. yoelli* mouse model of malaria, infection was shown to lead to the generation of anti-PS IgM and IgG antibodies, with subsequent enhanced phagocytosis by macrophages of PS-expressing uninfected RBCs [7]. Furthermore, blocking of PS antibodies in infected mice led to faster recovery from anemia. PS IgM antibodies have been shown to be induced by *P. falciparum in vitro* [8], and in non-immune humans with primary falciparum malaria total PS antibodies correlated with late post-malarial anemia [7]. However, the role of PS IgG and IgM

antibodies in the anemia from non-falciparum *Plasmodium* species, particularly *P. vivax*, has not been defined, nor their role in early anemia in falciparum malaria.

In this study, we evaluated PS IgM and IgG in Malaysian patients with vivax, falciparum, knowlesi and malariae malaria, and in healthy controls, and evaluated associations with hemoglobin, intravascular hemolysis (as a cause of oxidative stress in severe malaria), and RBC deformability. To define antibody kinetics and magnitude in primary infection with *P. vivax* and *P. falciparum*, we also measured PS antibodies longitudinally in malaria-naive human volunteers experimentally infected with these species.

Methods

Ethics statement

The studies conducted in Malaysia were approved by the Ethics Committees of the Malaysian Ministry of Health, and Menzies School of Health Research, Darwin, Australia. The malaria volunteer infection studies were approved by the QIMR Berghofer Ethics Committee. Informed written consent was provided by all participating adults, and by the parent or guardian of any participant aged <18 years.

Malaysian study sites, patients and study procedures

Patients hospitalised with malaria were enrolled as part of concurrent observational studies [17, 18] and/or clinical trials [19-21] conducted at 4 study sites in Sabah, Malaysia, including a tertiary referral hospital (Queen Elizabeth Hospital; QEH) and three district hospitals (Kudat, Kota Marudu and Pitas District Hospitals) during 2010 – 2016. At the tertiary referral hospital site, patients were enrolled if they were within 18 hours of commencing antimalarial treatment, aged >12 years, non-pregnant, and had no major co-morbidities or concurrent illnesses. Inclusion criteria were the same at the district

hospitals, except that all patients were enrolled prior to commencing antimalarial treatment, and all ages were included. For the current study, patients with PCR-confirmed *P. vivax, P. falciparum* and *P. knowlesi* were included from the tertiary-hospital cohort, while patients with PCR-confirmed *P. vivax, P. falciparum* and *P. malariae* were included from the district-hospital cohort. Patients enrolled in the observational studies [17, 18] received treatment according to hospital guidelines at the time of the studies, including artemisinin-combination therapy (ACT) for uncomplicated falciparum, knowlesi, or malariae malaria, and ACT or chloroquine plus primaquine for uncomplicated vivax malaria. Patients with knowlesi, vivax or malariae malaria and enrolled in clinical trials received either ACT or chloroquine, according to the study protocols [19-21]. Patients with severe malaria received intravenous artesunate.

Healthy controls were visitors or relatives of malaria patients admitted to QEH, with no history of fever in the past 48 hours and with a blood film negative for malaria parasites.

Venous blood was collected in lithium heparin and centrifuged within 30 minutes, with plasma stored at -70^{o} C. Whole blood was also collected in EDTA for measurement of RBC deformability (below). At QEH, blood was collected for PS antibodies on enrolment and at days 14 and/or 28 in a subset of patients able to return for follow-up. At district hospitals, blood was collected for PS antibodies on enrolment, and on days 7 and 28 in patients with *P. vivax* and *P. malariae.* Follow-up PS antibody measurements were excluded from analysis if a patient had received a blood transfusion following during admission. At all sites haemoglobin was measured daily during admission, and at follow-up visits. In a subset of patients plasma cell-free hemoglobin was measured by ELISA according to the manufacturer's instructions (Bethyl Laboratories). Anti-PS IgG and IgM antibodies were detected by ELISA (Orgentec).

RBC deformability was measured on enrolment in a subset of patients by Laser Assisted Optical Rotational Cell Analyser (LORCA Mechatronics, Netherlands), as previously described [22]. RBC deformability was assessed at shear stresses of 1.7 Pa and 30 Pa. Shear stresses of 1.7 Pa are

encountered in the capillaries [23]. Shear stresses of 30 Pa provide information on cell geometry, in particular surface area to volume ratios [24], and approximate values encountered by RBCs passing through intercellular gaps in the splenic sinusoids [25].

Malaria volunteer infection studies

Malaria volunteer infection studies were conducted at QIMR Berghofer, Australia, as previously described [26, 27]. In brief, healthy malaria-naïve volunteers were inoculated with ~2800 viable *P. falciparum* 3D7-infected RBCs, or ~1,680 *P. vivax*-infected RBCs. Peripheral blood parasitemia was measured at least daily by qPCR, and participants were treated with antimalarial drugs on day 8 (*P. falciparum*) or day 10 (*P. vivax*), when parasitemia had exceeded 5000 parasites/ml. Participants with *P. falciparum* and with samples available were enrolled in studies NCT02661373 (n=13), NCT02783833 (n=4), and NCT02573857 (n=6). Participants with *P. vivax* were enrolled in study NCT02573857 (n=8). PS IgG and IgM antibodies were measured from blood samples collected prior to infection, prior to antimalarial treatment, and at day 18 (*P. vivax*), or days 14 – 15 and day 20 (*P. falciparum*). Blood was collected in lithium heparin, with plasma frozen at -70^{o} C.

Statistics

Statistical analysis was performed with STATA software (version 14). For continuous variables intergroup differences were compared using analysis of variance or Kruskal-Wallis tests depending on distribution. Student's T-test or Mann-Whitney tests were used for two-group comparisons. Categorical variables were compared using χ^2 test. Associations between continuous variables were assessed using Spearman's or Pearson's correlation coefficients, depending on distribution. Partial correlation was used to evaluate associations between variables after adjusting for parasitemia, fever

duration and age, with non-normally distributed variables log-transformed to normality. Wilcoxon sign-rank test was used to compare baseline and follow-up measurements.

Results

Malaysian malaria patients

A total of 508 malaria patients and 50 controls were included. Malaria patients included 269 patients with *P. falciparum*, 176 with *P. vivax*, 42 with *P. knowlesi* and 21 with *P. malariae* malaria. Baseline demographic and clinical characteristics are shown in **Table 1**. Overall, 375 (74%) patients were male, and median age was 26 years (IQR 17 – 40 years). A total of 102 (20%) patients reported a previous episode of malaria. Anaemia by World Heath Organisation (WHO) criteria [28] was common, occuring on admission in 221 (44%) patients overall, and in >90% of patients with malaria from all species during follow-up (**Table 1**). Moderate anaemia (haemoglobin <10 g/dL) occurred in 65 (13%) patients overall on admission, and in 139 (27%) patients during follow-up (**Table 1**).

Phosphatidylserine IgM and IgG antibodies in Malaysian malaria patients

Phosphatidylserine IgM antibodies were increased on enrolment in patients with vivax, falciparum, knowlesi, and malariae malaria ($p<0.0001$ for all comparisons with controls; **Table 1, Figure 1**). PS IgM antibodies were highest in patients with vivax malaria ($p<0.0001$ for *P. vivax vs*. *P. falciparum*, and for *P. vivax vs*. *P. knowlesi*), and higher in falciparum compared to knowlesi malaria ($p=0.014$). PS IgG antibodies were also higher on enrolment in vivax, falciparum, knowlesi and malariae malaria ($p<0.0001$ for all comparisons with controls; **Figure 1)**, and were higher in vivax compared to falciparum ($p<0.0001$) and knowlesi ($p=0.0007$) malaria. For both IgM and IgG, the difference between *P. vivax* and *P. falciparum* remained significant after controlling for parasitemia, fever duration and

age ($p<0.0001$ for both comparisons). No significant differences were seen overall in PS IgM or IgG antibodies between patients who did, or did not, report having had a previous episode of malaria.

Clinical correlates of phosphatidylserine IgG and IgM antibodies in Malaysian malaria patients

In both vivax and falciparum malaria patients, PS IgM and IgG each correlated inversely with hemoglobin on enrolment, and with hemoglobin nadir ($p=0.001$ for correlation between PS-IgM and haemoglobin on enrolment in vivax malaria; $p<0.0001$ for all other correlations; **Table 2, Supplementary Figure 1)**. In vivax and falciparum malaria, there was a correlation between PS IgM and fever duration ($r=0.22$, $p=0.004$ and $r=0.30$, $p<0.0001$, respectively). In patients with vivax malaria, there was also an inverse association with PS IgM and IgG antibodies and age ($r=-0.21$, $p=0.005$ and $r=-0.23$, $p=0.002$, respectively). No correlation was observed with parasitemia in either species. In both falciparum and vivax malaria, the correlations between PS IgM and IgG antibodies and enrolment and nadir haemoglobin remained significant after controlling for fever duration, age, and parasitemia (**Supplementary Table 1**).

In falciparum malaria, there was a correlation between PS IgM and PS IgG and intravascular hemolysis, as measured by cell-free hemoglobin (PS IgM: $r=0.19$, $p=0.014$; PS IgG: $r=0.27$, $p=0.027$), and an inverse correlation between PS IgM and red blood cell (RBC) deformability ($r=-0.28$, $p=0.0008$, RBC Elongation Index at 1.7 Pascals; **Table 2**). Both associations with PS IgM remained significant after controlling for parasitemia.

In *P. knowlesi* and *P. malariae* malaria, there was no correlation between either PS IgM or IgG and admission or nadir hemoglobin. There was also no correlation between PS IgM or IgG and parasitemia in either species.

Longitudinal PS IgM and IgG antibody titres in Malaysian malaria patients

At the district hospital sites, in patients with vivax malaria PS IgM and IgG levels were higher at day 7 compared to enrolment (**Table 1, Supplementary Figure 2**), and for PS IgM this increase correlated significantly with the fractional fall in haemoglobin between day 0 and day 7 (r=0.31, p=0.006 for PS IgM, and r=0.22, p=0.059 for IgG. In patients with *P. malariae* there was no significant increase in PS IgM or IgG at day 7. In district malaria patients with *P. vivax* and *P. malariae* PS IgM and IgG had fallen by day 28, but levels remained above those of healthy controls (**Table 1, Supplementary Figure 2)**.

For the subset of tertiary-referral patients who had PS antibodies measured at day 14 following enrolment (*P. knowlesi*=33, *P. falciparum*=32, *P. vivax*=15), PS IgM and IgG were higher at day 14 compared to day 0, although this was only statistically significant for patients with *P. knowlesi* **(Table 1, Supplementary Figure 3).** No correlation was observed between day 14 PS IgM or PS IgG and day 14 hemoglobin. As with the district hospital patients, by day 28 PS IgG and IgM had returned to day 0 levels, but remained above the levels found in community controls (p<0.0001 for all comparisons).

Phosphatidylserine IgG and IgM antibodies in volunteers infected with *P. falciparum* and *P. vivax*

In participants infected with *P. falciparum* (n=23), there was a significant increase in PS IgM and IgG antibodies by day 20 (**Table 3, Figure 2**). For both PS IgM and IgG, the magnitude of this increase correlated with peak parasitemia (r=0.80, p<0.0001 for PS IgM, and r=0.59, p=0.003 for IgG). There was an inverse correlation between PS IgM at day 20 and hemoglobin measured at day 20 (or first available day 20 – 28; r=-0.41, p=0.054); this relationship was significant after controlling for peak parasitemia (r=-0.43, p=0.045). There was also a correlation between the increase in both PS IgM and IgG between day 0 and day 20 and the fractional fall in haemoglobin (r=0.49, p=0.018 for PS IgM, and r=0.43, p=0.042 for PS IgG); however, this was not significant after controlling for peak parasitemia.

In participants infected with *P. vivax* (n=8), PS IgM and IgG antibodies increased by day 18. Titres of both PS IgM and IgG were higher in participants infected with *P. vivax* than in participants with *P. falciparum* (**Table 3, Figure 3**). In participants with *P. vivax* there was no correlation between PS IgM or IgG and hemoglobin parameters, or parasitemia, although numbers were small.

Discussion

Anti-PS IgM and IgG antibodies were increased on presentation in Malaysian patients with each of the major *Plasmodium* species infecting humans, with both being higher in vivax malaria than in falciparum malaria. Furthermore, in both vivax and falciparum malaria, IgM and IgG PS antibody titres were inversely correlated with admission and nadir hemoglobin, with these correlations being independent of parasitemia, fever duration and age. Findings suggest that anti-PS antibodies contribute to malarial anemia from *P. vivax* as well as *P. falciparum*, even relatively early in the disease process. These findings are supported by data from the malaria volunteer infection studies, where antibody titres increased to a greater extent following inoculation with *P. vivax* than *P. falciparum*, and in the larger cohort with falciparum malaria, correlated inversely with hemoglobin independent of parasitemia.

Our findings are consistent with an early study demonstrating increased antiphospholipid antibodies in patients with falciparum and vivax malaria [29], and another demonstrating a correlation between total PS antibodies and late anemia in non-immune patients with primary *P. falciparum* malaria [7]. PS antibodies are thought to contribute to malarial anemia by binding to infected and uninfected RBCs exposing PS, and enhancing the phagocytosis of uninfected RBCs [7]. In mice with *P. yoelli*, PS antibodies have been shown to be produced by atypical CD11c+ T-bet+ B cells, with expansion of these cells correlating directly with parasitemia and inversely with RBC density, suggesting a role in anemia [8]. The expansion of T-bet+ B cells was shown to occur through TLR9 and IFNg signalling, with PS

antibodies increasing shortly after the expansion of these cells. Similarly, exposure of peripheral blood mononuclear cells from healthy naïve donors to *P. falciparum in vitro* induced expansion of T-bet+ B cells and production of PS antibodies [8]. Taken together, these data suggest that production of PS antibodies by T-bet+ B cells is stimulated directly by parasite DNA, rather than as a result of PS exposure. This may explain the finding in our study that PS IgG and IgM were already elevated on presentation in Malaysian patients with malaria, and correlated with early anemia. This early increase in antibody titres was also seen in the malaria volunteer infection studies, where PS antibodies increased by day 18 (*P. vivax*) or day 20 (*P. falciparum*), despite the low number of parasites inoculated (well below the number of merozoites released during schizont rupture following mosquito infection), and with peak parasitemias well below those seen in the clinical studies.

In this study we found that PS IgM and IgG were significantly higher in vivax compared to falciparum malaria. *P. vivax* has a lower pyrogenic threshold than *P. falciparum* and is associated with a greater inflammatory response than that seen in *P. falciparum* infections with a similar or greater peripheral parasitemia [30-32]. It has been postulated that this may relate to the greater GC content of the *P. vivax* genome, with greater stimulation of TLR9 by CpG motifs within *P. vivax* hemozoin [33]. As TLR9 has also been shown to mediate the expansion of T-bet +B cells that produce PS antibodies [8], this greater GC content of *P. vivax* may also account for the higher titres of PS antibodies observed in this study. As PS antibodies are thought to mediate phagocytosis of uninfected RBCs, the higher antibody titres in vivax compared to falciparum malaria may explain, in part, the finding that anemia is a common complication of vivax malaria despite relatively low parasitemias, with the relative loss of uninfected RBCs to circulating iRBCs greater in vivax compared to falciparum malaria [4, 34].

In this study we did not find a correlation between PS IgM and IgG antibody titres and haemoglobin in patients with *P. malariae*, despite that fact that PS antibody titres in *P. malariae* were comparable to those of *P. vivax*, and that anaemia was at least as prevalent in patients with *P. malariae* as with the other species. Although this may have been because of small numbers of patients with *P. malariae*, it

may also be that other factors may play a relatively greater role in anaemia from *P. malariae*. Given the morbidity associated with anaemia from *P. malariae* [35], further studies are required to investigate mechanisms of anaemia from this species.

A notable finding of our study was the higher titres of PS IgM compared to IgG in both clinical studies and volunteer infection studies. IgM antibodies have recently been shown to be rapidly induced in falciparum malaria, in both volunteer infection studies and in children and adults with clinical malaria in endemic areas [36]. In volunteer infection studies, antigen-specific IgM responses were shown to be more prevalent than IgG responses. Furthermore, IgM blocked merozoite invasion of RBCs, and was associated with a significantly reduced risk of clinical malaria in a longitudinal cohort of Papuan children [36]. These results suggest that parasite specific IgM is an important functional antibody response targeting blood-stage malaria parasites. However, the results of the current study suggest that in addition to this contribution to malaria immunity, IgM antibodies may also be associated with pathogenic mechanisms of malarial anemia.

Our study found a correlation between PS antibodies and CFHb in patients with *P. falciparum* malaria. CFHb is released during intravascular hemolysis, and is readily oxidised to heme which induces lipid peroxidation in RBCs [37, 38]. Oxidative stress is a major stimulant of externalisation of PS in RBCs [39], and the correlation in our study between CFHb and PS antibodies suggests that in addition to direct stimulation of T-bet+ B cells by *Plasmodium* DNA, hemolysis-induced exposure of PS on uninfected RBCs may also be a driver of PS antibody production.

We also demonstrated an inverse correlation between PS IgM and RBC deformability in falciparum malaria. This is consistent with a previous study demonstrating that coating of RBCs with purified anti-RBC IgG antibodies from patients with *P. vivax*-associated anemia increased the rigidity of RBC membranes [40]. Reduced RBC deformability has been shown to be associated with anemia in both falciparum [41] and knowlesi [42] malaria, with enhanced phagocytosis and increased splenic clearance of the more rigid RBCs both potentially contributing. It is thus possible that PS antibodies

298 mediate anemia not only directly through phagocytosis of PS-exposing cells [7], but also through
299 increasing the rigidity of infected and uninfected RBCs.

300 Our study has several limitations. It is possible that the early and marked increase in PS antibodies
301 observed in Malaysian patients represents an epiphenomenon. However, the inverse relationships
302 between PS antibodies and hemoglobin were independent of possible confounders such as
303 parasitemia and duration of illness. Furthermore, the relationships between PS antibodies and
304 hemoglobin were strongest for the two species (*P. vivax* and *P. falciparum)* with the highest antibody
305 titres, and absent in *P. knowlesi*, where antibody responses were significantly lower. Moreover, PS
306 IgM was also inversely associated with hemoglobin, independent of parasitemia, in the volunteer
307 infection studies.

308 In conclusion, PS IgM and IgG antibodies are increased in falciparum, vivax, knowlesi and malariae
309 malaria. PS antibody responses were higher in *P. vivax* than *P. falciparum* infection, in both clinical
310 disease and experimental human challenge. Both PS IgM and IgG correlated with early anemia in
311 malaria from both species, suggesting that PS antibodies may contribute to the early loss of
312 uninfected-RBC found in malarial anemia in both vivax and falciparum malaria.

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Conflict of Interests

All authors report no conflicts of interest.

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References

1. Douglas NM, Lampah DA, Kenangalem E, et al. Major burden of severe anemia from non-falciparum malaria species in Southern Papua: a hospital-based surveillance study. PLoS Med **2013**; 10:e1001575.

2. Douglas NM, Pontororing GJ, Lampah DA, et al. Mortality attributable to *Plasmodium vivax* malaria: a clinical audit from Papua, Indonesia. BMC Med **2014**; 12:217.

3. Grigg MJ, William T, Barber BE, et al. Age-related clinical spectrum of *Plasmodium knowlesi* malaria and predictors of severity. Clin Infect Dis **2018**; 67:350-9.

4. Jakeman G, Saul A, Hogarth W, Collins W. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. Parasitology **1999**; 119:127-33.

5. Douglas N, Anstey N, Buffet P, et al. The anaemia of *Plasmodium vivax* malaria. Malar J **2012**; 11:135.

6. Oyong DA, Kenangalem E, Poespoprodjo JR, et al. Loss of complement regulatory proteins on uninfected erythrocytes in vivax and falciparum malaria anemia. J Clin Invest Insight **2018**; 3:124854

7. Fernandez-Arias C, Rivera-Correa J, Gallego-Delgado J, et al. Anti-self phosphatidylserine antibodies recognize uninfected erythrocytes promoting malarial anemia. Cell Host Microbe **2016**; 19:194-203.

8. Rivera-Correa J, Guthmiller J, Vijay R, et al. Plasmodium DNA-mediated TLR9 activation of T-bet+ B cells contributes to autoimmune anaemia during malaria. Nat Commun **2017**; 8:1282.

9. Lang E, Qadri SM, Lang F. Killing me softly–suicidal erythrocyte death. Int J Biochem Cell Biol **2012**; 44:1236-43.

10. Maguire P, Prudhomme J, Sherman I. Alterations in erythrocyte membrane phospholipid organization due to the intracellular growth of the human malaria parasite, *Plasmodium falciparum*. Parasitol **1991**; 102:179-86.

11. Sherman IW, Prudhomme J, Tait JF. Altered membrane phospholipid asymmetry in *Plasmodium falciparum*-infected erythrocytes. Parasitol Today **1997**; 13:242-3.

12. Pattanapanyasat K, Sratongno P, Chimma P, Chitjamnongchai S, Polsrila K, Chotivanich K. Febrile temperature but not proinflammatory cytokines promotes phosphatidylserine expression on *Plasmodium falciparum* malaria-infected red blood cells during parasite maturation. Cytometry Part A **2010**; 77:515-23.

13. Engelbrecht D, Coetzer TL. *Plasmodium falciparum* exhibits markers of regulated cell death at high population density in vitro. Parasitol Int **2016**; 65:715-27.

14. Totino PR, Magalhães AD, Silva LA, Banic DM, Daniel-Ribeiro CT, de Fátima Ferreira-da-Cruz M. Apoptosis of non-parasitized red blood cells in malaria: a putative mechanism involved in the pathogenesis of anaemia. Malar J **2010**; 9:350.

15. Föller M, Huber SM, Lang F. Erythrocyte programmed cell death. IUBMB Life **2008**; 60:661-8.

16. Matthews K, Duffy SP, Myrand-Lapierre M-E, et al. Microfluidic analysis of red blood cell deformability as a means to assess hemin-induced oxidative stress resulting from *Plasmodium falciparum* intraerythrocytic parasitism. Integr Biol **2017**; 9:519-28.

17. Barber BE, Grigg MJ, Piera KA, et al. Effects of aging on parasite biomass, inflammation, endothelial activation and microvascular dysfunction in *Plasmodium knowlesi* and *P. falciparum* malaria. J Infect Dis **2017**; 215:1908–17.

18. Barber BE, William T, Grigg MJ, et al. A prospective comparative study of knowlesi, falciparum and vivax malaria in Sabah, Malaysia: high proportion with severe disease from *Plasmodium knowlesi* and *P. vivax* but no mortality with early referral and artesunate therapy. Clin Infect Dis **2013**; 56:383-97.

19. Grigg MJ, William T, Menon J, et al. Efficacy of artesunate-mefloquine against high-grade chloroquine-resistant *Plasmodium vivax* malaria in Malaysia: an open-label randomised controlled trial. Clin Infect Dis **2016**; 62:1403-11.

20. Grigg MJ, William T, Menon J, et al. Artesunate–mefloquine versus chloroquine for treatment of uncomplicated *Plasmodium knowlesi* malaria in Malaysia (ACT KNOW): an open-label, randomised controlled trial. Lancet Infect Dis **2015**; 16:180-8.

21. Grigg MJ, T W, Barber BE, et al. Artemether-lumefantrine versus chloroquine for the treatment of uncomplicated *Plasmodium knowlesi* malaria (CAN KNOW): an open-label randomized controlled trial. Clin Infect Dis **2018**; 66:229-36.

22. Hardeman M, Goedhart P, Dobbe J, Lettinga K. Laser-assisted optical rotational cell analyser (LORCA); I. A new instrument for measurement of various structural hemorheological parameters. Clin Hemorheol Microcirc **1994**; 14:605-18.

23. Chien S. Physiological and pathophysiological significance of hemorheology. In: Chien S, Dormandy J, Ernst E, Matrai A, editors. Clinical Hemorheology. Dordrecht: Springer; 1986. p. 125-64.

24. Bessis M, Mohandas N, Feo C. Automated ektacytometry: a new method of measuring red cell deformability and red cell indices. Blood Cells **1980**; 6:315-7.

25. Chen L-T, Weiss L. The role of the sinus wall in the passage of erythrocytes through the spleen. Blood **1973**; 41:529-37.

26. McCarthy JS, Griffin PM, Sekuloski S, et al. Experimentally induced blood-stage *Plasmodium vivax* infection in healthy volunteers. J Infect Dis **2013**; 208:1688-94.

27. McCarthy JS, Sekuloski S, Griffin PM, et al. A pilot randomised trial of induced blood-stage *Plasmodium falciparum* infections in healthy volunteers for testing efficacy of new antimalarial drugs. PLoS ONE **2011**; 6:e21914.

28. World Health Organization. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity 2011 [15th Jan 2019]. Available from: http://www.who.int/vmnis/indicators/haemoglobin.pdf.

29. Facer C, Agiostratidou G. High levels of anti-phospholipid antibodies in uncomplicated and severe *Plasmodium falciparum* and in *P. vivax* malaria. Clin Exp Immunol **1994**; 95:304-9.

30. Yeo TW, Lampah DA, Tjitra E, et al. Greater endothelial activation, Weibel-Palade body release and host inflammatory response to *Plasmodium vivax*, compared with *Plasmodium falciparum*: a prospective study in Papua, Indonesia. J Infect Dis **2010**; 202:109-12.

31. Hemmer CJ, Holst FGE, Kern P, Chiwakata CB, Dietrich M, Reisinger EC. Stronger host response per parasitized erythrocyte in *Plasmodium vivax* or ovale than in *Plasmodium falciparum* malaria. Trop Med Int Health **2006**; 11:817-23.

32. Barber BE, William T, Grigg MJ, et al. Parasite biomass-related inflammation, endothelial activation, microvascular dysfunction and disease severity in vivax malaria. PLoS Pathog **2015**; 11:e1004558.

33. Anstey NM, Russell B, Yeo TW, Price RN. The pathophysiology of vivax malaria. Trends Parasitol **2009**; 25:220-7.

34. Collins WE, Jeffery GM, Roberts JM. A retrospective examination of anemia during infection of humans with *Plasmodium vivax*. Am J Trop Med Hyg **2003**; 68:410-2.

35. Langford S, Douglas NM, Lampah DA, et al. *Plasmodium malariae* infection associated with a high burden of anemia: a hospital-based surveillance study. PLoS Negl Trop Dis **2015**; 9:e0004195.

36. Boyle MJ, Chan J-A, Handayuni I, et al. IgM in human immunity to *Plasmodium falciparum* malaria. Sci Adv **2019**, In Press

37. Flynn T, Allen D, Johnson G, White J. Oxidant damage of the lipids and proteins of the erythrocyte membranes in unstable hemoglobin disease. Evidence for the role of lipid peroxidation. J Clin Invest **1983**; 71:1215.

38. Davies K, Goldberg A. Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. J Biol Chem **1987**; 262:8220-6.

39. Eda S, Sherman I. Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. Cell Physiol Biochem **2002**; 12:373-84.

40. Mourão LC, da Silva Roma PM, Aboobacar JdSS, et al. Anti-erythrocyte antibodies may contribute to anaemia in *Plasmodium vivax* malaria by decreasing red blood cell deformability and increasing erythrophagocytosis. Malar J **2016**; 15:397.

41. Dondorp A, Angus B, Chotivanich K, et al. Red blood cell deformability as a predictor of anemia in severe falciparum malaria. Am J Trop Med Hyg **1999**; 60:733-7.

42. Barber BE, Russell B, Grigg MJ, et al. Reduced red blood cell deformability in *Plasmodium knowlesi* malaria. Blood Advances **2017**; 2:433-43.

Table 1. Baseline characteristics and phosphatidylserine IgM and IgG antibodies in controls and malaria patients

	Controls (n=50)	***P. falciparum* (n=269)**	***P. vivax* (n=176)**	***P. knowlesi* (n=42)**	***P. malariae* (n=21)**
Age, years (mean, range)	35 (14 - 69)	28 (7 - 78)	24 (2 - 79)	43 (17 - 75)	17 (5 – 54)
Male sex, n (%)	34 (68)	202 (75)	127 (72)	30 (77)	16 (76)
Previous malaria	NA	34 (13)	51 (29)	14 (34)	51 (29)
Fever duration, days		5 (3 - 7)	5 (3 - 7)	5 (4 - 7)	6 (3 – 7)
Parasite count, parasites/uL		10, 011 (2902 – 32,472)	4000 (1775 – 9548)	4215 (2372 - 29,922)	1313 (162 – 2778)
Severe malaria, n (%)		29 (11)	12 (7)	10 (26)	0 (0)
Hb on enrolment, g/dL, mean (SD)		12.9 (2.0)	12.2 (2.1)	12.4 (2.2)	11.6 (2.2)
Hb nadir, g/dL, mean (SD)		11.3 (1.8)	10.8 (1.9)	11.2 (2.3)	10.5 (1.9)
Hb fall, g/dL		1.4 (0.8 - 2.2)	1.3 (0.7 – 2.1)	1.2 (0.2 - 2.0)	1.2 (0.9)
Anemia* on admission (%)		97 (36)	89 (51)	21 (50)	14 (67)
Anemia* during follow-up (%)		251 (93)	167 (95)	39 (93)	20 (95)
Admission Hb <10 g/dL		23 (9)	30 (17)	6 (14)	6 (29)
Nadir Hb <10 g/dL		56 (21)	61 (35)	13 (31)	9 (43)
CFHb, ng/mL	15,146 (9641 – 25,256)	34,309 (15,330 – 52,7779) n=171	32,498 (16,813 – 44,489) n=62	20,042 (15,072 – 44,242) n=15	
RBC-D (at 1.7 Pa), Elongation index	0.197 (0.178 – 0.227) n=7	0.182 (0.163 - 0.198) n=90	0.196 (0.160 - 0.214) n=25	0.168 (0.147 - 0.190) n=11	
RBC-D (at 30 Pa), Elongation Index	0.587 (0.520 – 0.590) n=7	0.518 (0.480 - 0.557) n=90	0.543 (0.518 - 0.572) n=25	0.492 (0.448 - 0.550) n=11	
PS IgM day 0, U/ml	27 (19 - 41)	85 (55 - 134)	93 (62 - 181)	60 (41 - 108)	91 (58 – 146)
PS IgM, day 7, U/ml					103 (42 – 142) n=15
PS IgM, day 14, U/ml		130 (80 - 183)	143 (49 - 2686)	81 (50 - 116)	

		n=32	n=15	n=33	
PS IgM, day 28, U/ml		83 (66 - 131) n=69	84 (51 - 124) n=22	62 (36 - 92) n=22	55 (39 – 63) n=14
PS IgG, day 0, U/ml	17 (14 - 21)	49 (35 - 72)	59 (39 - 92)	44 (21 - 85)	63 (41 – 77)
PS IgG, day 7, U/ml					58 (20 – 85) n=15
PS IgG, day 14, U/ml		94 (64 - 112) n=32	62 (37 - 251) n=15	68 (30 - 93) n=33	
PS IgG, day 28, U/ml		43 (19 - 63) n=69	49 (22 - 120) n=22	46 (26 - 70) n=22	38 (20 – 47) n=14

NA = not available; Hb = haemoglobin; CFHb = cell free haemoglobin; RBC-D = red blood cell deformability; Pa = Pascals; PS = phosphatidylserine. Numbers are median (IQR) unless otherwise stated. For PS IgG and IgM antibodies at baseline: $p<0.0001$ for healthy controls (HCs) compared to all Plasmodium species.

* Self-reported

[#] Anemia based on World Health Organization 2011 hemoglobin measurement criteria [26]: age 6–59 months (≤10.0 g/dL), 5–11 years (<11.5 g/dL), 12–14 years (<12.0 g/dL), nonpregnant women ≥15 years (<12.0 g/dL), pregnant women (<11.0 g/dL), men ≥15 years (<13.0 g/dL).

Table 2. Clinical correlates of PS-IgM and IgG antibodies in malaria patients

	***P. falciparum* (n=269)**				***P. vivax* (n=176)**			
	PS IgM		**PS IgG**		**PS IgM**		**PS IgG**	
	Correlation	P value	Correlation	P value	Correlation	P value	Correlation	P value
Hb on enrolment	-0.27	<0.0001^	-0.26	<0.0001^	-0.30	0.001^	-0.31	<0.0001^
Hb nadir	-0.28	<0.0001^	-0.29	<0.0001^	-0.34	<0.0001^	-0.35	<0.0001^
Parasite count	-0.00	0.943	0.01	0.824	0.10	0.170	0.10	0.192
Fever duration	0.30	<0.0001	0.11	0.078	0.22	0.004	0.15	0.051
Age	0.11	0.067	-0.06	0.358	-0.21	0.005	-0.23	0.002
CFHb	0.19	0.014	0.27	0.027	0.04	0.779	-0.07	0.601
RBC-D (at 1.7 Pa)	-0.28	0.008	-0.13	0.227	0.22	0.282	-0.04	0.859
RBC-D (at 30 Pa)	-0.21	0.050	-0.18	0.085	-0.05	0.828	-0.28	0.176

PS = phosphatidylserine; Hb = haemoglobin; CFHb = cell free haemoglobin; RBC-D = red blood cell deformability; Pa = pascals. RBC-D was measured in 90 patients with falciparum malaria, and 25 patients with vivax malaria; CFHb was measured in 133 patients with falciparum malaria and 57 with vivax malaria.

^ Remained significant after controlling for parasitemia, fever duration and age (Supplementary Table 1)

Table 3. Anti-phosphatidylserine IgM and IgG antibodies in volunteers with experimental malaria infection

	***P. falciparum* (n=23)**	***P. vivax* (n=8)**	**P value**
Peak parasitemia (parasites/ml)	36,074 (8351 – 142,519)	219,136 (112,091 – 308,113)	0.008
Baseline Hb, g/dL, mean (SD)	14.8 (9.2)	14.5 (7.3)	0.456
Hb day 18 (Pv) or day 20-28 (Pf), g/dL, mean (SD)	13.5 (9.2)	13.7 (7.9)	0.786
Hb fall, g/dL	1.0 (0.6 – 1.5)	1.0 (0.7 – 1.2)	0.651
PS IgM, day 0. U/ml	21 (15 – 36)	35 (20 – 70)	0.124
PS IgG, day 0, U/ml	15 (6 – 24)	20 (10 – 25)	0.329
PS IgM, day 18 (Pv) or day 20 (Pf), U/ml	33 (27 – 52)	71 (56 – 121)	0.012
PS IgG, day 18 (Pv) or day 20 (Pf), U/ml	17 (3 – 24)	24 (20 – 42)	0.026

Hb = hemoglobin; Pf = P. *falciparum*; Pv = *P. vivax*; PS = phosphatidylserine. Numbers are median (IQR) unless otherwise stated. For volunteers with falciparum malaria, "Hb day 20 – 28" refers to hemoglobin measured on day 20, or the first available up to 28.

Figure Legends

Figure 1. Phosphatidylserine IgM (A) and IgG (B) antibodies in healthy controls and in patients hospitalised with falciparum, vivax, knowlesi, and malariae malaria. PS = phosphatidylserine; Pf = *P. falciparum*; Pv = *P. vivax*; Pk = *P. knowlesi*; Pm = *P. malariae*. PS IgM and IgG antibodies were lower in than in patients with malaria from any *Plasmodium* species ($p<0.0001$ for all comparisons). PS-IgM and IgG antibodies were higher in *P. vivax* compared to both *P. falciparum* and *P. knowlesi*. PS-IgM antibodies were higher in *P. falciparum* compared to *P. knowlesi* ($p=0.014$).

Figure 2. Phosphatidylserine (PS) IgM and IgG antibodies in participants experimentally infected with *P. falciparum* (A and B) and *P. vivax* (C and D). A total of 23 participants in 3 study cohorts were infected with *P. falciparum* and included in the analysis; PS antibodies were measured at baseline, day 7 – 8 (prior to treatment), day 14 – 15, and day 20. Eight participants in one study cohort were infected with *P. vivax*, with PS antibodies measured at baseline and at days 8, 7, 10 and 18. P values represent difference between baseline and day 20 (*P. falciparum*), or day 18 (*P. vivax*), by Wilcoxon sign-rank test. Data are presented as median and interquartile range.

Figure 3. Phosphatidylserine IgM (A) and IgG (B) antibodies at day 20 in participants experimentally infected with *P. falciparum* and at day 18 in participants experimentally infected with *P. vivax*. Data are presented as median and interquartile range.

Footnote Page.

1. Conflicts of Interest

All authors report no conflicts of interest.

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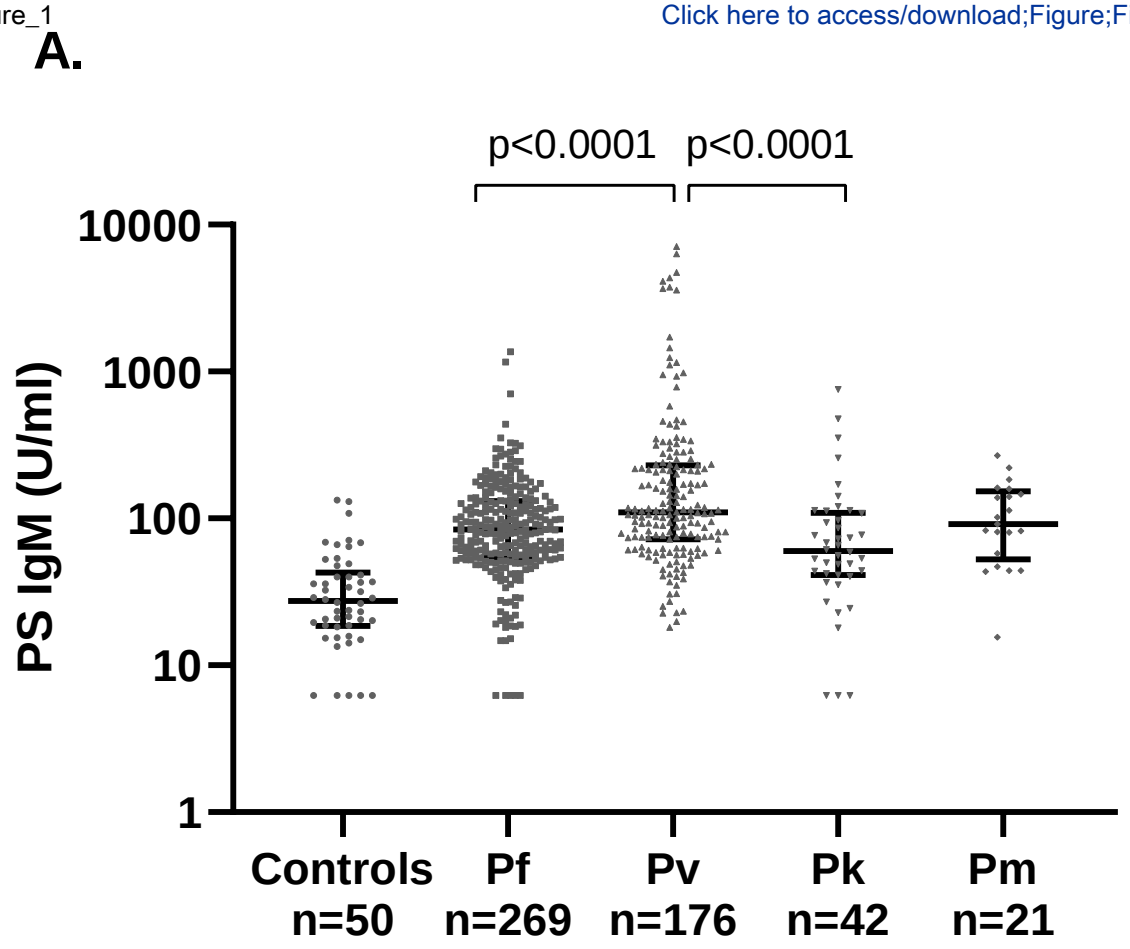

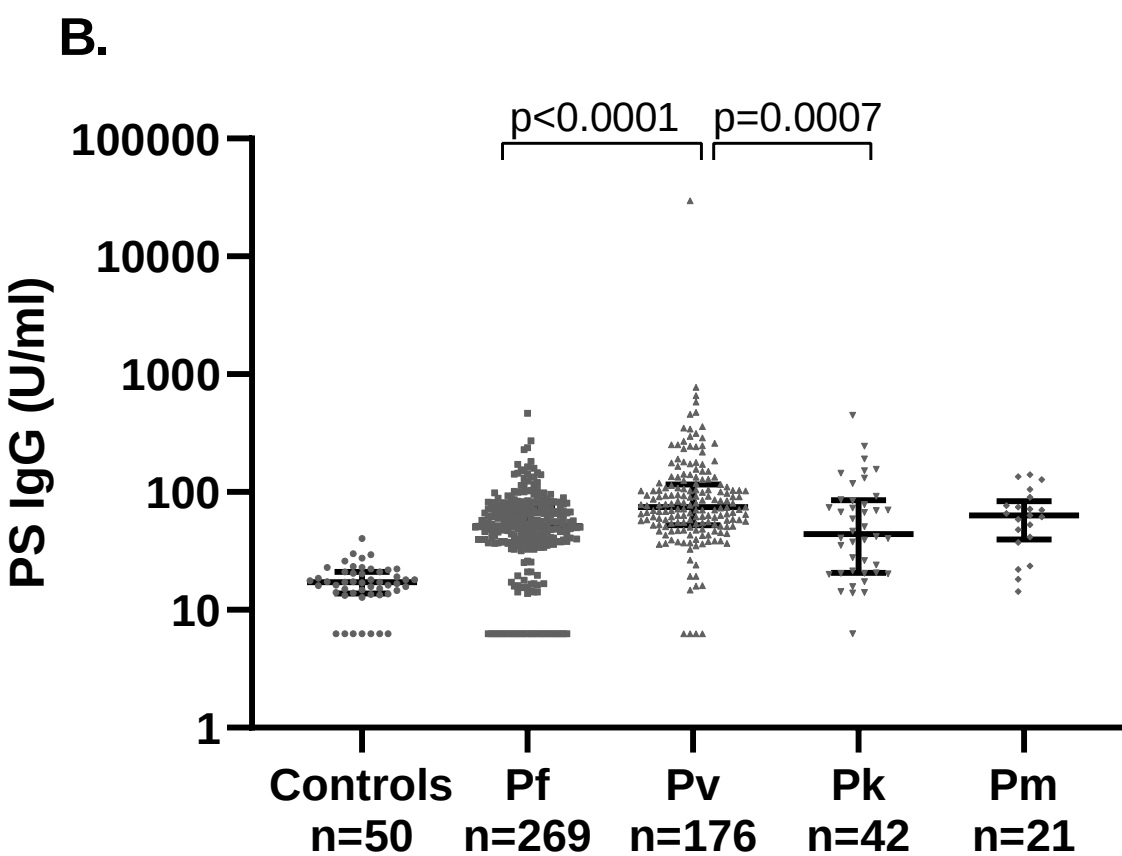

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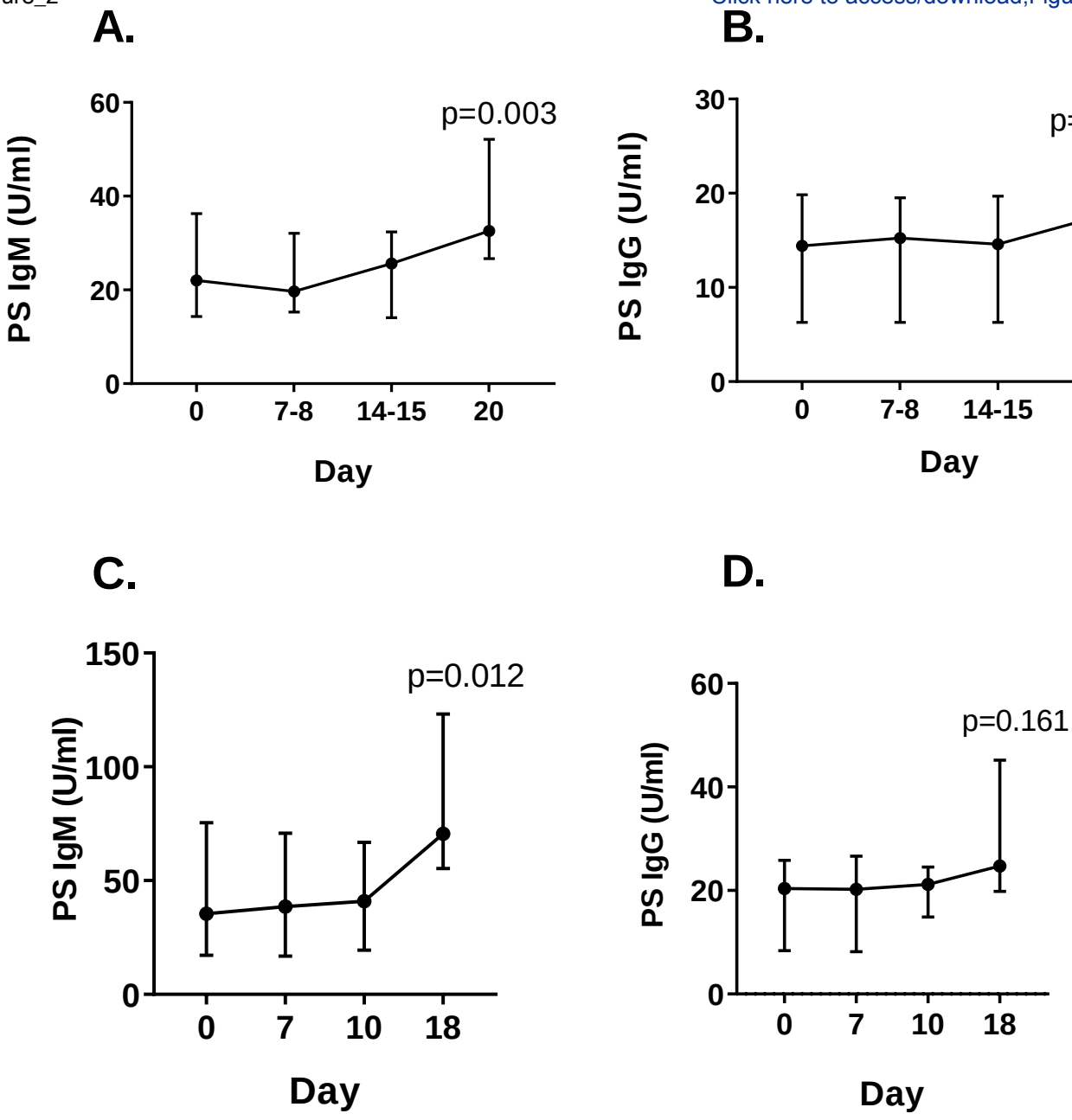

Figure_3

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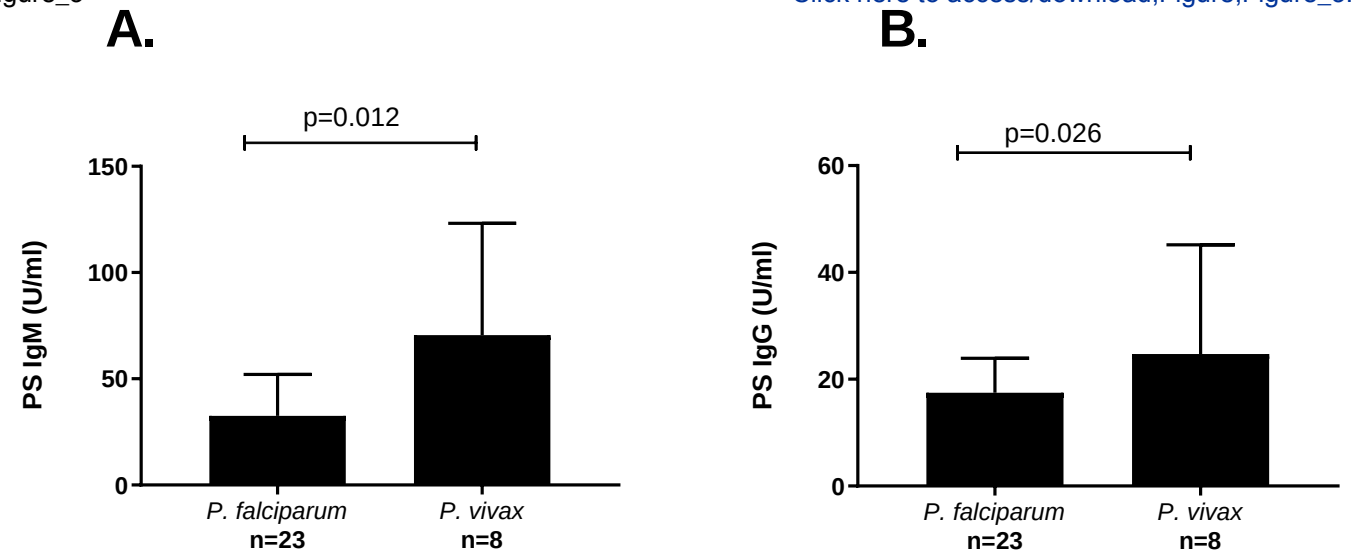
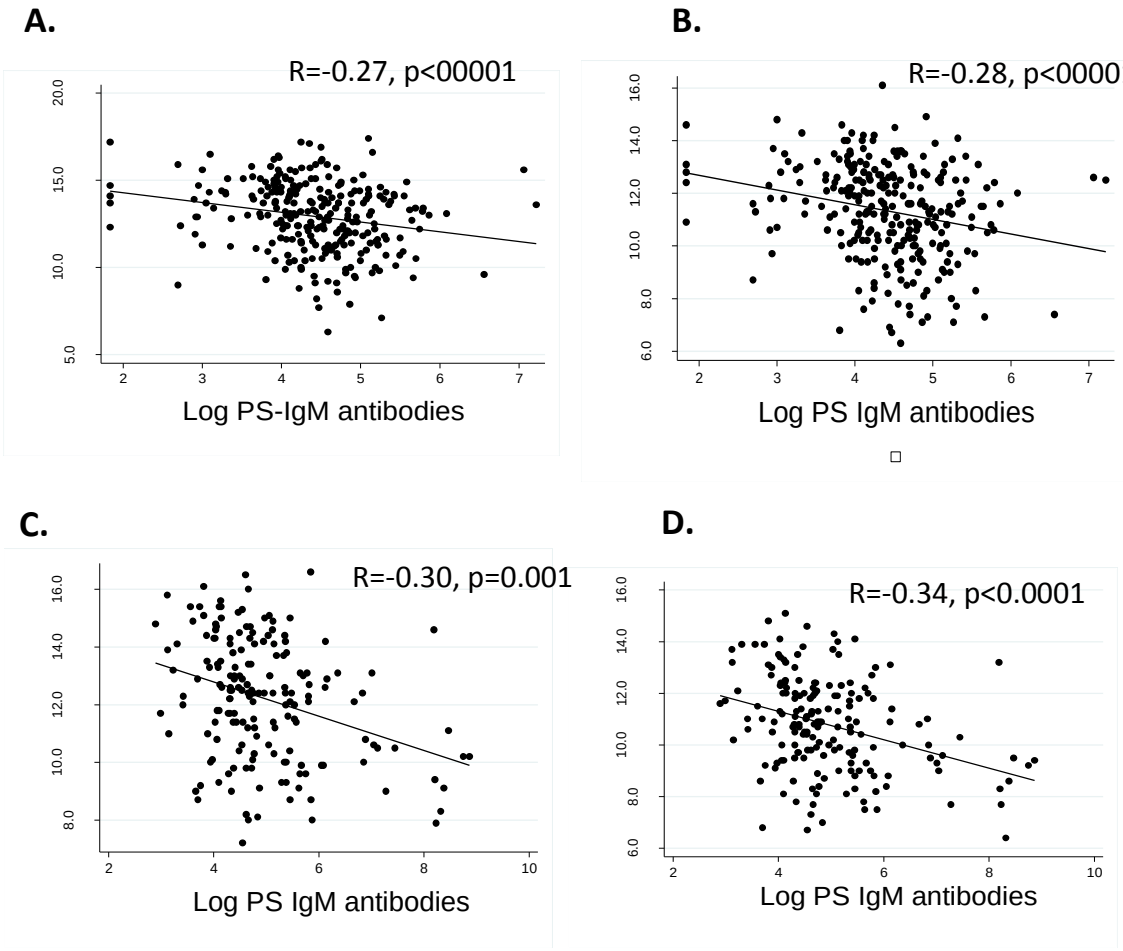


Supplementary Table 1. Associations between PS antibodies and haemoglobin in malaria patients: multivariate analyses

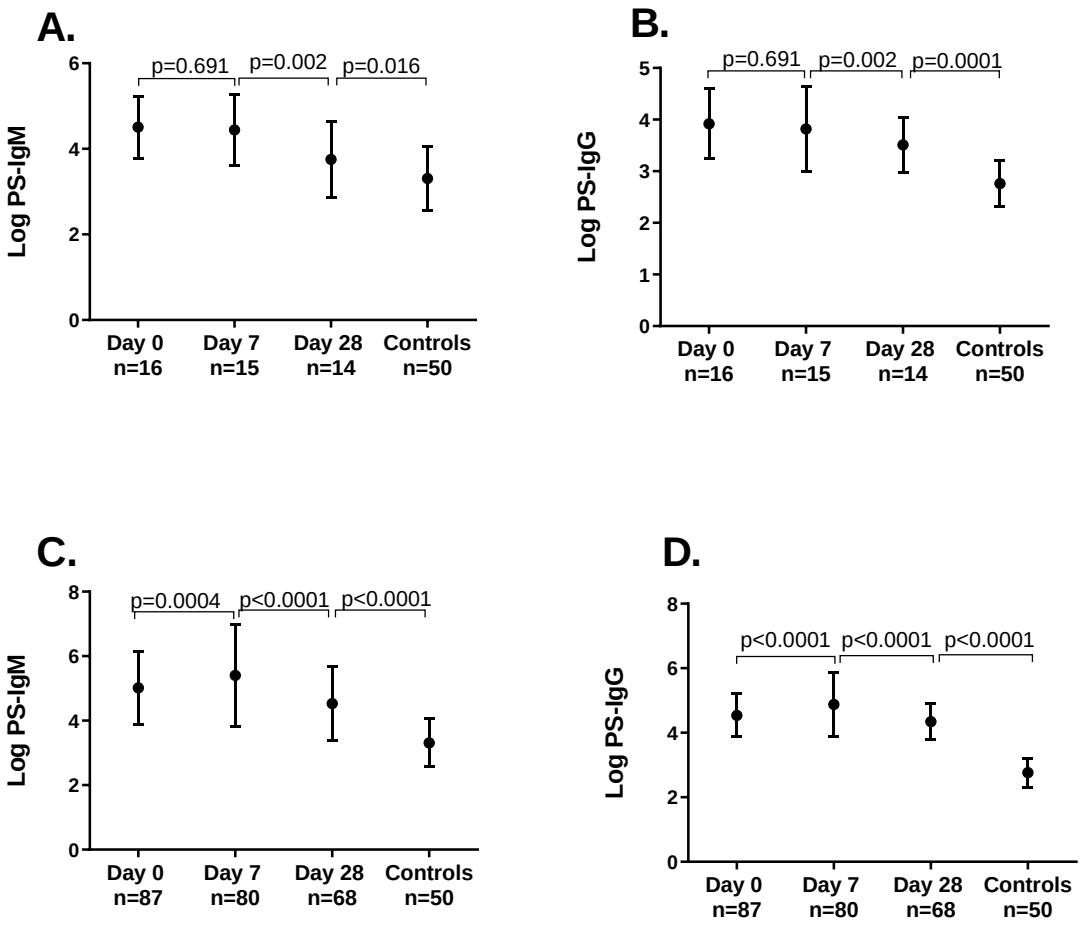
	P. falciparum		*P. vivax*	
	Partial correlation coefficient	P value	Partial correlation coefficient	P value
Model 1: PS-IgM and haemoglobin on admission				
Log PS-IgM (U/ml)	-0.16	0.010	-0.23	0.003
Log parasitemia	0.06	0.306	0.04	0.606
Log fever duration (days)	-0.23	0.0002	-0.22	0.005
Age (years)	0.08	0.177	0.30	0.0001
Model 2: PS-IgM and haemoglobin nadir				
Log PS-IgM (U/ml)	-0.22	0.0006	-0.23	0.002
Log parasitemia	-0.15	0.015	-0.13	0.083
Log fever duration (days)	-0.07	0.425	-0.25	0.001
Age (years)	0.09	0.135	0.28	0.0002
Model 3: PS-IgG and haemoglobin on admission				
Log PS-IgG (U/ml)	-0.22	0.0004	-0.16	0.040
Log parasitemia	0.08	0.222	0.03	0.677
Log fever duration (days)	-0.24	0.0001	-0.23	0.002
Age (years)	0.07	0.288	0.33	<0.0001
Model 4: PS-IgG and haemoglobin nadir				
Log PS-IgG (U/ml)	-0.24	0.0001	-0.19	0.014
Log parasitemia	-0.14	0.024	-0.13	0.080
Log fever duration (days)	-0.17	0.005	-0.26	0.0005
Age (years)	0.07	0.259	0.30	0.0001

PS = phosphatidylserine

Supplementary Figure 1. Correlations between phosphatidylserine IgM antibodies and enrolment haemoglobin and haemoglobin nadir in patients with *P. falciparum* (A and B) and *P. vivax* (C and D) malaria. Hb = haemoglobin; PS = phosphatidylserine.



Supplementary Figure 2. Phosphatidylserine IgM and IgG antibodies at Day 0, 7 and 28, in district hospital patients with *P. malariae* (A and B), and *P. vivax* (C and D). Error bars represent mean (SD) of log-transformed values. P values are calculated by Wilcoxon sign-rank test (for longitudinal measurements), or Wilcoxon rank-sum test for day 28 measurements vs controls.



Supplementary Figure 3. Phosphatidylserine IgM and PS IgG antibodies at Day 0, 14 and 28, in tertiary-referral hospital patients with *P. falciparum* (A and B), *P. vivax* (C and D), and *P. knowlesi* (E and F). Error bars represent mean and SD of log-transformed values. P values are calculated by Wilcoxon sign-rank test (for longitudinal measurements), or Wilcoxon rank-sum test for day 28 measurements vs controls.