Antiphosphatidylserine Immunoglobulin M and Immunoglobulin G Antibodies Are Higher in Vivax Than Falciparum Malaria, and Associated With Early Anemia in Both Species

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Background. Anemia is a major complication of vivax malaria. Antiphosphatidylserine (PS) antibodies generated during falciparum malaria mediate phagocytosis of uninfected red blood cells 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 immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies in Malaysian patients with vivax, falciparum, knowlesi, and malariae malaria, and in healthy controls, and correlated antibody titres with hemoglobin. PS antibodies were also measured in volunteers experimentally infected with *Plasmodium vivax* and *Plasmodium falciparum*.

Results. PS IgM and IgG antibodies were elevated in patients with vivax, falciparum, knowlesi, and malariae malaria ($P < .0001$ 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 red blood cells found in malarial anemia from both species.

Keywords. phosphatidylserine antibodies; anemia; malaria; *Plasmodium vivax*; *Plasmodium falciparum*; *Plasmodium knowlesi*; *Plasmodium malariae*.

Anemia is a major complication of malaria from all *Plasmodium* species, causing significant morbidity and mortality not only in *Plasmodium falciparum* malaria, but also in *Plasmodium 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 autoantibodies 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 but that may become exposed as a result of a number of stimuli such as oxidative stress [9]. In *P. 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 *Plasmodium yoelii* [7, 14], PS is also externalized 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 hypothesized that autoantibodies directed against PS may contribute to malarial anemia [7]. In keeping with this, in a recent study involving the *P. yoelii* mouse model of malaria, infection was shown to lead to the generation of anti-PS

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immunoglobulin M (IgM) and immunoglobulin G (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 nonimmune humans with primary falciparum malaria total PS antibodies correlated with late postmalaria 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-naïve 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 the 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 hospitalized 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 3 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, not pregnant, and had no major comorbidities 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 polymerase chain reaction (PCR)-confirmed *P. vivax*, *P. falciparum*, and *Plasmodium knowlesi* were included from the tertiary-hospital cohort, whereas patients with PCR-confirmed *P. vivax*, *P. falciparum*, and *Plasmodium 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°C. Whole blood was also collected in ethylenediaminetetraacetic acid for measurement of RBC deformability (below). At QEH, blood was collected for PS antibodies on enrollment 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 enrollment, 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 during admission. At all sites, hemoglobin was measured daily during admission, and at follow-up visits. In a subset of patients, plasma cell-free hemoglobin was measured by enzyme-linked immunosorbent assay (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 enrollment in a subset of patients by laser-assisted optical rotational cell analyzer (LORCA Mechatronics), 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 approximately 2800 viable *P. falciparum* 3D7-infected RBCs, or approximately 1680 *P. vivax*-infected RBCs. Peripheral blood parasitemia was measured at least daily by quantitative PCR, 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°C .

Statistical Analysis

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 *t* test or Mann–Whitney tests were used for 2-group comparisons. Categorical variables were compared using χ^2 test. Associations between continuous variables were assessed using Spearman or Pearson correlation coefficients, depending on distribution. Partial correlation was used to evaluate associations between variables after adjusting for parasitemia, fever duration, and age, with nonnormally distributed

variables log-transformed to normality. Wilcoxon signed-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*. Baseline demographic and clinical characteristics are shown in Table 1. Overall, 375 (74%) patients were male, and median age was 26 years (interquartile range, 17–40 years). A total of 102 (20%) patients reported a previous episode of malaria. Anemia by World Health Organization criteria [28] was common, occurring on admission in 221 (44%) patients overall, and in

Table 1. Baseline Characteristics and Phosphatidylserine Immunoglobulin M and Immunoglobulin G Antibodies in Controls and Patients With Malaria

Characteristic	Controls (n = 50)	<i>Plasmodium falciparum</i> (n = 269)	<i>Plasmodium vivax</i> (n = 176)	<i>Plasmodium knowlesi</i> (n = 42)	<i>Plasmodium malariae</i> (n = 21)
Age, y, mean (range)	35 (14–69)	28 (7–78)	24 (2–79)	43 (17–75)	17 (5–54)
Male sex, No. (%)	34 (68)	202 (75)	127 (72)	30 (77)	16 (76)
Previous malaria	NA	34 (13)	51 (29)	14 (34)	51 (29)
Fever duration, d	...	5 (3–7)	5 (3–7)	5 (4–7)	6 (3–7)
Parasite count, parasites/ μL ...	...	10 011 (2902–32 472)	4000 (1775–9548)	4215 (2372–29 922)	1313 (162–2778)
Severe malaria, No. (%)	...	29 (11)	12 (7)	10 (26)	0 (0)
Hb on enrollment, 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 decline, 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 ^a on admission, No. (%)	...	97 (36)	89 (51)	21 (50)	14 (67)
Anemia ^a during follow-up, No. (%)	...	251 (93)	167 (95)	39 (93)	20 (95)
Admission Hb <10 g/dL, No. (%)	...	23 (9)	30 (17)	6 (14)	6 (29)
Nadir Hb <10 g/dL, No. (%)	...	56 (21)	61 (35)	13 (31)	9 (43)
CFHb, ng/mL	15 146 (9641–25 256)	34 309 (15 330–527 779) (n = 171)	32 498 (16 813–44 489) (n = 62)	20 042 (15 072–44 242) (n = 15)	...
RBC-D (at 1.7 Pa), EI	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), EI	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) (n = 32)	143 (49–2686) (n = 15)	81 (50–116) (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)

Data are presented as median (interquartile range) unless otherwise indicated. For PS IgG and IgM antibodies at baseline, $P < .0001$ for healthy controls compared to all *Plasmodium* species. Abbreviations: CFHb, cell-free hemoglobin; EI, elongation index; Hb, hemoglobin; IgG, immunoglobulin G; IgM, immunoglobulin M; NA, not available; RBC-D, red blood cell deformability; PS, phosphatidylserine; SD, standard deviation.

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

>90% of patients with malaria from all species during follow-up (Table 1). Moderate anemia (hemoglobin <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 enrollment in patients with vivax, falciparum, knowlesi, and malariae malaria ($P < .0001$ for all comparisons with controls; Table 1, Figure 1). PS IgM antibodies were highest in patients with vivax malaria ($P < .0001$ for *P. vivax* vs *P. falciparum*, and for *P. vivax* vs *P. knowlesi*), and higher in falciparum compared to knowlesi malaria ($P = .014$). PS IgG antibodies were also higher on enrollment in vivax, falciparum, knowlesi, and malariae malaria ($P < .0001$ for all comparisons with controls; Figure 1), and were higher in vivax compared to falciparum ($P < .0001$) and knowlesi ($P = .0007$) malaria. For both IgM and IgG, the difference between *P. vivax* and *P. falciparum* remained significant after controlling for parasitemia, fever duration, and age

($P < .0001$ 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 enrollment, and with hemoglobin nadir ($P = .001$ for correlation between PS-IgM and hemoglobin on enrollment in vivax malaria; $P < .0001$ for all other correlations; Table 2, Supplementary Figure 1). In vivax and falciparum malaria, there was a correlation between PS IgM and fever duration ($r = 0.22$, $P = .004$ and $r = 0.30$, $P < .0001$, respectively). In patients with vivax malaria, there was also an inverse association with PS IgM and IgG antibodies and age ($r = -0.21$, $P = .005$ and $r = -0.23$, $P = .002$, 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 enrollment and nadir hemoglobin 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: $r = 0.19$, $P = .014$; PS IgG: $r = 0.27$, $P = .027$), and an inverse correlation between PS IgM and RBC deformability ($r = -0.28$, $P = .0008$, RBC elongation index at 1.7 Pa; 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 Titers 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 enrollment (Table 1, Supplementary Figure 2), and for PS IgM this increase correlated significantly with the fractional fall in hemoglobin between day 0 and day 7 ($r = 0.31$, $P = .006$ for PS IgM, and $r = 0.22$, $P = .059$ 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 enrollment (*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

Figure 1. Phosphatidylserine (PS) immunoglobulin M (IgM; A) and immunoglobulin G (IgG; B) antibodies in healthy controls and in patients hospitalized with falciparum (Pf), vivax (Pv), knowlesi (Pk), and malariae (Pm) malaria. PS IgM and IgG antibodies were lower in controls than in patients with malaria from any *Plasmodium* species ($P < .0001$ 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* ($P = .014$).

Table 2. Clinical Correlates of Phosphatidylserine Immunoglobulin M and Immunoglobulin G Antibodies in Patients With Malaria

Clinical Correlate	<i>Plasmodium falciparum</i> (n = 269)				<i>Plasmodium vivax</i> (n = 176)			
	PS IgM		PS IgG		PS IgM		PS IgG	
	Correlation	PValue	Correlation	PValue	Correlation	PValue	Correlation	PValue
Hb on enrollment	−0.27	< .0001 ^a	−0.26	< .0001 ^a	−0.30	.001	−0.31	< .0001 ^a
Hb nadir	−0.28	< .0001 ^a	−0.29	< .0001 ^a	−0.34	< .0001	−0.35	< .0001 ^a
Parasite count	−0.00	.943	0.01	.824	0.10	.170	0.10	.192
Fever duration	0.30	< .0001	0.11	.078	0.22	.004	0.15	.051
Age	0.11	.067	−0.06	.358	−0.21	.005	−0.23	.002
CFHb ^b	0.19	.014	0.27	.027	0.04	.779	−0.07	.601
RBC-D ^c (at 1.7 Pa)	−0.28	.008	−0.13	.227	0.22	.282	−0.04	.859
RBC-D ^c (at 30 Pa)	−0.21	.050	−0.18	.085	−0.05	.828	−0.28	.176

Abbreviations: CFHb, cell-free hemoglobin; Hb, hemoglobin; IgG, immunoglobulin G; IgM, immunoglobulin M; PS, phosphatidylserine; RBC-D, red blood cell deformability.

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

^bCFHb was measured in 133 patients with falciparum malaria and 57 with vivax malaria.

^cRBC-D was measured in 90 patients with falciparum malaria, and 25 patients with vivax malaria.

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 ($P < .0001$ 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 ($r = 0.80$, $P < .0001$ for PS IgM, and $r = 0.59$, $P = .003$ 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; $r = -0.41$, $P = .054$); this relationship was significant after controlling for peak parasitemia ($r = -0.43$, $P = .045$). 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 hemoglobin ($r = 0.49$, $P = .018$ for PS IgM, and $r = 0.43$, $P = .042$ 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. Titers 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 titers 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 titers increased to a greater extent following inoculation

Table 3. Antiphosphatidylserine Immunoglobulin M and Immunoglobulin G Antibodies in Volunteers With Experimental Malaria Infection

Parameters	<i>Plasmodium falciparum</i> (n = 23)	<i>Plasmodium vivax</i> (n = 8)	PValue
Peak parasitemia, parasites/mL	36 074 (8351–142 519)	219 136 (112 091–308 113)	.008
Baseline Hb, g/dL, mean (SD)	14.8 (9.2)	14.5 (7.3)	.456
Hb day 18 (Pv) or day 20–28 ^a (Pf), g/dL, mean (SD)	13.5 (9.2)	13.7 (7.9)	.786
Hb decline, g/dL	1.0 (0.6–1.5)	1.0 (0.7–1.2)	.651
PS IgM, day 0, U/mL	21 (15–36)	35 (20–70)	.124
PS IgG, day 0, U/mL	15 (6–24)	20 (10–25)	.329
PS IgM, day 18 (Pv) or day 20 (Pf), U/mL	33 (27–52)	71 (56–121)	.012
PS IgG, day 18 (Pv) or day 20 (Pf), U/mL	17 (3–24)	24 (20–42)	.026

Data are presented as median (interquartile range) unless otherwise indicated.

Abbreviations: Hb, hemoglobin; IgG, immunoglobulin G; IgM, immunoglobulin M; Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*; PS, phosphatidylserine; SD, standard deviation.

^aFor volunteers with falciparum malaria, “Hb day 20–28” refers to hemoglobin measured on day 20, or the first available up to day 28.

Figure 2. Phosphatidylserine (PS) immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies in participants experimentally infected with *Plasmodium falciparum* (A and B) and *Plasmodium 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, days 7–8 (prior to treatment), days 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 signed-rank test. Data are presented as median and interquartile range.

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 nonimmune 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 Toll-like receptor 9 (TLR9)

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

and interferon- γ signaling, with PS antibodies increasing shortly after the expansion of these cells. Similarly, exposure of peripheral blood mononuclear cells from healthy naive 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 titers 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. *Plasmodium 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 titers of PS antibodies observed in this study. As PS antibodies are thought to mediate phagocytosis of uninfected RBCs, the higher antibody titers 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 infected RBCs greater in vivax compared to falciparum malaria [4, 34].

In this study we did not find a correlation between PS IgM and IgG antibody titers and hemoglobin in patients with *P. malariae*, despite that fact that PS antibody titers in *P. malariae* were comparable to those of *P. vivax*, and that anemia was at least as prevalent in patients with *P. malariae* as with the other species. Although this may have been because of the small number of patients with *P. malariae*, it may also be that other factors may play a relatively greater role in anemia from *P. malariae*. Given the morbidity associated with anemia from *P. malariae* [35], further studies are required to investigate mechanisms of anemia from this species.

A notable finding of our study was the higher titers 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 cell-free hemoglobin (CFHb) in patients with *P. falciparum* malaria. CFHb is released during intravascular hemolysis and is readily oxidized to heme, which induces lipid peroxidation in RBCs [37, 38]. Oxidative stress is a major stimulant of externalization 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 mediate anemia not only directly through phagocytosis of PS-exposing cells [7], but also through increasing the rigidity of infected and uninfected RBCs.

Our study has several limitations. It is possible that the early and marked increase in PS antibodies observed in Malaysian patients represents an epiphenomenon. However, the inverse relationships between PS antibodies and hemoglobin were independent of possible confounders such as parasitemia and duration of illness. Furthermore, the relationships between PS antibodies and hemoglobin were strongest for the 2 species (*P. vivax* and *P. falciparum*) with the highest antibody titers, and absent in *P. knowlesi*, where antibody responses were significantly lower. Moreover, PS IgM was also inversely associated with hemoglobin, independent of parasitemia, in the volunteer infection studies.

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

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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