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Decreasing malaria prevalence and its potential consequences for immunity in pregnant women

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Abstract

Background: As malaria control is intensified, pregnant women may be less exposed to malaria, thus affecting the acquisition of protective antibody.

Methods: Plasma samples were collected from Malawian and Papua New Guinean (PNG) pregnant women enrolled over seven year periods, during which malaria prevalence fell by over two thirds. Immunoglobulin G (IgG) levels to schizont extract, merozoite antigens and VAR2CSA-DBL5ε were measured by ELISA. Levels of IgG to variant surface antigens of infected erythrocytes (IEs) and merozoites, and levels of opsonising IgG to IEs, were measured by flow cytometry.

Results: In both settings, levels of antibodies in pregnant women to recombinant antigens and to intact IEs but not opsonising antibodies decreased over time. After controlling for coverage with insecticide-treated nets (ITNs) these differences disappeared in the Malawian cohort, whereas in the PNG cohort time period was independently associated with decrease in several antibody responses measured by ELISA.

Conclusion: The impact of falling parasite prevalence on anti-*P. falciparum* serological indicators in pregnant women varies by setting. Increased ITN coverage may affect development of antibodies to recombinant antigens, but levels of opsonising IgG remained stable over time. Opsonising IgG against placental-binding IEs may persist, thus offering longer-lasting protection against malaria during pregnancy.

Introduction

Plasmodium falciparum malaria remains entrenched in Sub-Saharan Africa and the Pacific Islands. Women in their first pregnancy are particularly susceptible to malaria [1, 2], attributed in part to the ability of *P. falciparum* infected erythrocytes (IEs) to sequester within the placenta [3, 4]. Placental-binding IEs express different variant surface antigens (VSA) from IEs causing malaria in childhood, so they are not recognised by existing malarial immunity [5, 6].

Malaria in pregnancy (MiP) increases the risk of maternal anaemia and low birth weight (LBW) delivery, which are important causes of maternal and infant mortality [7-9]. Antibodies to placental-binding IEs are associated with protection against MiP and are acquired in a gravidity-dependent manner [1, 6, 10]. Development of these antibodies is influenced by transmission intensity, HIV infection, and the use of malaria prevention strategies including insecticide-treated nets (ITN) [11-14].

As malaria exposure declines, immunity may decrease. This is indirectly supported by data showing that, as infection prevalence fell in Malawian pregnant women, parasite densities increased among infected women [15]. Taken together, if exposure to MiP falls, antibody to placental-binding IEs may be less developed, susceptibility to MiP may spread into later gravidities, and amongst those infected, the consequences may be more severe [16].

Malaria prevalence in pregnancy has declined substantially in Malawi and Papua New Guinea (PNG) and we hypothesized that this would be associated with reduced levels of pregnancy-specific malaria immunity, but not of immunity to non-pregnancy-associated antigens.

Materials and Methods

Ethics approval

Ethics approvals were obtained from the College of Medicine Research Ethics Committee, University of Malawi (P99/00/91R, P00/01/107), PNG Institute of Medical Research's Institutional Review Board (08.15), the PNG Medical Research Advisory Council (05.03, 10.50) and the Human Research Ethics Committee of Melbourne Health (2001.016, 2008.162). All participants provided informed written consent.

Study sites and participants

Plasma samples came from two cohorts. In Malawi, pregnant women were recruited at delivery between 1999 to 2006 from the maternity unit of Queen Elizabeth Central Hospital in Blantyre, as previously described [15]. Women were classified into early (1999-2000, parasite prevalence 25.2%) and late (2004-2006, parasite prevalence 6.2%) groups. In PNG, pregnant women were recruited at first antenatal visit (ANC) and followed to delivery at rural clinics in Madang Province between 2005 and 2012. Women were classified as early (2005-2007, parasite prevalence 18.0%) and late (2010-2012, parasite prevalence 3.1%) groups. In the latter period, women were randomised to receive three courses of intermittent preventive treatment (IPTp) with sulphadoxine-pyrimethamine (SP) and azithromycin (AZ) during pregnancy, or a single course of SP and chloroquine (CQ). ITN use was documented.

Samples were collected at delivery (and at first ANC visit in PNG), and malaria infection was defined as presence of parasites on concurrent peripheral or placental blood microscopy. Because current infection increases antibody responses [17], only samples from uninfected women were selected. We did not study primigravidae because, when uninfected, they have

very low levels of antibodies against placental-binding IEs [17]. All available samples from women in second to fourth pregnancy were used.

In the PNG cohort, paired enrolment and delivery samples were tested together, to determine whether declining exposure impairs the acquisition of antibody over the course of pregnancy. Samples were tested in duplicate and randomised to minimise testing samples from the same year together.

Parasite and cell cultures

The laboratory adapted *P. falciparum* lines CS2 (placental-binding) and E8B-ICAM (endothelial-binding), were cultured as described [18].

THP-1 monocyte-like cells [19] were cultured as described [20].

Assays of IgG to schizont extract, merozoite antigens and VAR2CSA-DBL5ε

Antibody responses to recombinant *P. falciparum* antigens were measured by enzyme-linked immunosorbent assay (ELISA) using established methods [21]. Microtitre plates were coated with 50 µL of individual targets diluted in phosphate buffered saline (PBS) at the following concentrations: schizont extract [22] from CS2 IEs 1/2000, MSP2 from FC27 0.5 µg/ml [21], MSP3 from 3D7 full ectodomain 2 µg/ml [23], PfRh2 (construct PfRh2-2030 from 3D7) 0.5 µg/ml [24] and VAR2CSA-DBL5ε from 3D7 0.5 µg/ml [25, 26]. Plasma was added in duplicate at 1/1000 dilution. A standard curve generated from serial dilution of our positive control (pooled from 44 pregnant women with high antibody responses) was used to convert OD into antibody level represented by arbitrary units, where positive control= 100 units.

Merozoite phagocytosis assay

Antibody opsonic phagocytosis of merozoites was performed using a recently developed assay (Osier, Feng, Beeson, submitted). Whole merozoites were obtained as described [27]. In brief, merozoites were stained with 10 µg/mL ethidium bromide (EtBr) (Sigma-Aldrich) for 30 minutes and washed thrice at 2200 x g for 5 minutes [27]. The cell density was determined using relative counting against CountBright™ Absolute Counting Beads (Invitrogen) as per manufacturer's protocol. The merozoites were resuspended at 5 x 10^7/ml in RPMI-HEPES and 30 µl of suspension were opsonised with 3.5 µl of plasma (1/250 dilution) in newborn calf serum (NCS) (GIBCO®) -coated 96-well U-bottom plates, for 1 hour in the dark. The cells were washed thrice, resuspended in 150 µl of THP-1 medium [RPMI 1640 supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin-Glutamine and 25 mM HEPES (GIBCO®)], and 50 µl of suspension was transferred in duplicate into fresh NCS-coated 96-well U-bottom plates, followed by adding 100 µl of THP-1 cells at 5 x10^5/ml (target:effector, 10:1). The cells were incubated in 5% humidified CO_2 at 37°C for 10 minutes. Phagocytosis was stopped by centrifugation at 4°C at 350 g for 5 minutes and washed thrice with FACS buffer. THP-1 cells were fixed in 2% paraformaldehyde (PFA) in PBS before acquisition with BD FACSCantoII flow cytometer (BD Biosciences). The background non-specific phagocytosis observed with the no plasma (negative) control was set to be less than 5% and the data are represented as percentage of THP-1 cells that have ingested free merozoites.

Measuring IgG to Variant Surface Antigens

Total IgG levels against VSA expressed on CS2 and E8B-ICAM IEs was measured as described [28] with slight modifications. In brief, trophozoite-stage IEs at 4-8% parasitaemia were washed thrice with 1% NCS in PBS, resuspended at 0.2% haematocrit in PBS/NCS,

incubated for 30 minutes with test plasma (1/20 dilution, in duplicate) in NCS-coated 96-well U-bottom plates at RT. IEs were washed, incubated with rabbit anti-human IgG (1/100 dilution, Dako), followed by three washes. IEs were incubated in the dark with AlexaFluor 647 donkey anti-rabbit IgG (1/500 dilution, Invitrogen) containing 10 µg/ml EtBr. IEs were washed and resuspended in 2% PFA. The data are represented as relative geometric mean fluorescence intensity (MFI), the percentage of the MFI of a positive (hyperimmune pooled sera) control, after subtraction of the negative control, which was the average MFI of samples from 6 Melbournian donors.

Opsonic phagocytosis assay

We improved our previously established phagocytosis assay [20] to measure opsonic antibody responses to CS2 and E8B-ICAM IEs. In summary, trophozoite-stage IEs were purified by density gradient centrifugation [20], stained with 10 µg/mL EtBr, washed thrice and resuspended at 1.67 x 10^7/ml. Thirty µl of IEs were then opsonised with 3.3 µl (1/10 dilution) of plasma in NCS-coated 96-well U-bottom plates for 1 hour in the dark. IEs were washed thrice, resuspended in 50 µl of THP-1 medium, aliquoted in duplicate into NCS-coated 96-well U-bottom plates, THP-1 cells (25 µl at 5 x10^5/ml; target:effector, 10:1) were added. The cells were incubated at 37°C in 5% humidified CO_2 for 40 minutes. Phagocytosis was stopped and unphagocytosed IEs were lysed [20]. THP-1 cells were washed and fixed in 2% PFA [20]. Results were represented as percentage of THP-1 cells that ingested IEs. A sample caused phagocytosis when ingestion was >mean + 3SD of negative controls.

Assays for total IgG and opsonic IgG to IEs were acquired using HyperCyt® CyAn flow cytometer (Beckman Coulter), and in each assay discordant samples were re-run using our published rules [28].

Antibody responses in the PNG cohort

To assess whether antibody levels changed between enrolment and delivery, we used the antibody levels for each sample, and applied our published rules in determining discordant samples [28]. Samples with an adjusted mean variance of <20% and mean difference of <10% between enrolment and delivery antibody levels were categorised as no change in antibody responses, whereas samples with an adjusted mean variance of >20% and mean difference of >10% were classified as either decrease or increase in antibody responses.

Statistical analyses

Data obtained from assays were combined with clinical information on the study participants and analysed using Stata v11.2 (StataCorp). In some instances, analyses were done in GraphPad Prism v5 (GraphPad Software, Inc.).

The Mann-Whitney *U* test was performed on continuous non-parametric variables and categorical variables were assessed using χ^2 tests. Multiple linear regression models were performed to determine association between continuous and categorical variables. Potential confounders including gravidity, age, IPTp and ITN use and maternal characteristics were included.

Results

Study populations characteristics

In Malawi, samples from 184 pregnant women enrolled early (1999-2004) and 148 women enrolled late (2005-2006) were assayed for antibody responses against *P. falciparum* antigens (Table 1). At recruitment, women enrolled later were significantly heavier than the women enrolled earlier. The percentage of secundigravid women varied between 31.5% (early) and 46.6% (late, p = 0.02), bed net use increased from 19.0% (early) to 54.7% (late, p<0.0001),

and a higher percentage of women who enrolled later were taking more doses of SP, p = 0.005.

In the PNG cohort, samples from 131 pregnant women who were recruited early (2005-2007) and 281 women recruited late (2010-2012) were used, Table 2. At first ANC visit, women enrolled later were significantly older and had greater mid-upper arm circumference. Use of bed nets and malaria preventive drugs were significantly higher in women enrolled late (Table 2).

Antibody responses against schizont extract, merozoite antigens and VAR2CSA-DBL5ε

To determine malaria exposure, we assayed the antibody response to schizont extract. In both cohorts, women enrolled later exhibited significantly lower levels of antibody to schizont extract, indicative of reduced exposure. Prior studies have identified merozoite antigens, MSP2, MSP3 and PfRh2 [21, 23, 24] as targets of antibodies associated with immunity in non-pregnant individuals, and VAR2CSA-DBL5ε [29] as a highly immunogenic target associated with protection from low birth weight. Antibody responses to merozoite antigens and VAR2CSA-DBL5ε were significantly lower in later-enrolled women in both study population (Figure 1A and 2A). A similar significant decrease over time was observed in plasma samples collected at first ANC visit in the PNG cohort (data not shown).

Opsonic antibody responses against whole merozoites

The difference in antibody responses to each merozoite antigen observed in the PNG cohort led to the evaluation of the opsonic IgG response to whole merozoites. We restricted this analysis to delivery plasma from women in second and third pregnancies because the assays are resource-intensive. Although women recruited later had lower IgG antibody responses to

merozoite antigens, levels of opsonic IgG to whole merozoites did not vary over time (Z = 0.6; p=0.5) (Figure 2B).

Total antibodies levels against infected erythrocytes

In Malawian women, median levels of anti-VSA IgG antibody to E8B-ICAM declined over time (p<0.01), but responses against CS2 did not vary, Figure 1B. In PNG, using the same subset of women for opsonic IgG responses against whole merozoites, median levels of anti-VSA IgG antibodies to both E8B-ICAM and CS2 declined over time (p<0.01), Figure 2C.

Opsonic antibodies against infected erythrocytes

Levels of opsonic IgG responses against E8B-ICAM and CS2 did not vary significantly over time in either study population (Figure 1C and 2D). In Malawi, the proportion of women with opsonic IgG antibodies to CS2 [early: 85.2%; late 88.7%, p=0.3] or E8B-ICAM [early: 96.7%; late: 96.0%, p=0.7] did not differ between the two time periods. In PNG, similar results were observed for CS2 [early: 72.4%; late 63.0%, p=0.08] and E8B-ICAM [early: 90.8%; late: 94.1%, p=0.2]. To determine whether differences in antibody titre were obscured by performing assays at above-saturating concentrations, pooled plasma samples from each cohort and time period were titrated. No significant differences in the dilution curves generated were observed (data not shown).

Antibodies against *P. falciparum* antigens adjusting for confounders

The changes in antibody responses over time, adjusted for confounding and interaction variables, are presented in Tables 3 and 4. In the Malawian cohort, bed net use was associated with lower levels of IgG to schizont extract. The levels of IgG antibodies to MSP2, MSP3, VAR2CSA-DBL5ε and VSA-CS2 were significantly lower with increasing maternal weight.

Additionally, levels of opsonic IgG against CS2 increased with gravidity and women enrolled later who took a single dose of SP had significantly lower IgG antibody to MSP2, Table 3.

In the PNG cohort, later time period was associated with significantly lower levels of IgG antibodies to schizont extract, MSP2 and MSP3 in the adjusted analyses. Bed net use was associated with lower levels of IgG to schizont extract and MSP2. There was an important interaction between enrolment period and bed net use, such that women who enrolled later and who used a bed net had particularly low levels of IgG to MSP2, Table 4.

Changes in antibody responses between first antenatal visit and delivery

We compared differences in antibody responses between first ANC visit and delivery in the PNG cohort women enrolled early or late. There was significantly more change between enrolment and delivery in the earlier period than later period for antibody responses to schizont extract, MSP2, MSP3, PfRh2 and VAR2CSA-DBL5ε. Interestingly, women enrolled in the later period had an increased dynamism of opsonising antibody to CS2, which was largely confined to women in the SP and AZ treatment arm. In contrast, significantly more women administered with SP and CQ had increases in their levels of opsonising antibodies to E8B-ICAM than women on SP and AZ, Table 5.

Discussion

As malaria control intensifies, it is important to understand the impact on the development and maintenance of naturally acquired immunity to malaria in pregnancy. Examining a range of antibody responses in two different settings, there was evidence of lower levels of humoral immunity in the more recent cohorts of pregnant women since intensification of malaria control interventions, particularly in PNG. However, our results suggest that any decline in immunity with reduction in malaria transmission was modest and not evident across all

measures of humoral immunity, and established immunity may be maintained over our time frame. Although levels of some malaria antibodies measured by ELISA decreased, opsonising antibodies to intact parasites or IEs uniformly did not. Both settings experienced similar decreases in parasite prevalence, associated with increases in use of ITN and IPTp. Constant exposure may be more crucial in maintaining the levels of antibodies to specific antigenic determinants, such as the recombinant antigens used in our ELISA assays, than to intact merozoites or IEs [17, 25]. In PNG women, a decline over time in total IgG levels to IEs may be due to reduced malaria exposure, suggesting that repeated exposure may be required to maintain antibodies against malaria [30, 31]. On the other hand, the development of opsonising antibodies may be a better surrogate for protective immunity in areas with lower transmission.

Antibody responses to merozoite antigens are short-lived in young children [32, 33], but were better sustained in pregnant women [25] and their prevalence is influenced by transmission intensity [34-36]. In the PNG cohort, a decrease in merozoite antibodies by ELISA contrasted with no change in the levels of opsonic IgG against whole merozoites. This is the first assessment of changes in these functional opsonising antibodies over time, and suggests that opsonic IgG to merozoites may be more stable than antibodies to individual merozoite antigens. Given that opsonic IgG to merozoites was recently correlated with protection from clinical malaria in children [37], persistence of these responses may be critical for maintenance of functional protective immunity in the face of declining transmission.

Of clinical importance for the development of a vaccine targeting pregnancy-specific VSA [38], levels of IgG against placental-binding IEs declined in the PNG cohort only, while levels of opsonic IgG to placental-binding IEs did not change in either cohort. The stability of the latter may be important given that opsonic IgG has been associated with protection against maternal anaemia [39] and LBW [40].

Adhesion-inhibiting antibodies are also associated with protection from complications of MiP [41]. We did not measure ability of plasma to inhibit adhesion of IEs, but antibodies to VAR2CSA-DBL5ε may inhibit adhesion of IEs to placental cells (although results are conflicting [26, 42]). Pregnant women have a wide repertoire of antibodies against the VAR2CSA-DBL5ε domain, and these appear to be cross-reactive against placental isolates [16, 43]. Given that levels of IgG against VAR2CSA-DBL5ε were lower in the later time-points in both cohorts, this may indicate reduced ability to block placental sequestration of IEs at the population level. IgG against VAR2CSA-DBL5ε has been suggested to have a long half-life [25], and consistent with this, individuals' responses to this antigen rarely decreased between enrolment and delivery. The lower levels seen in the late than early populations may instead reflect reduced malaria exposure resulting in slower acquisition of anti-VAR2CSA-DBL5ε. Prospective studies over the course of a women's reproductive life of development of antibodies that opsonise IEs or merozoites, or that block IEs adhesion, and their relationship to protection from infection, would be of great value [1].

In the Malawi cohort, time period was not significantly associated with antibody levels after adjusting for confounders. Instead, ITN and maternal weight were independently associated with decline in levels of IgG against one or more antigens. Decreased exposure from increased coverage of ITN may be driving the decline in antibody responses. The direct relationship between maternal weight and antibody was surprising. Women enrolled later were on average heavier, perhaps reflecting robust economic growth [44] and declining HIV prevalence in Malawi [45]. Maternal weight may be a proxy for other indicators of decreased parasite exposure and improved maternal health, including access to health care services. Declining HIV prevalence could mitigate the decrease in IgG to placental-binding IEs, which

is decreased by HIV infection [14]. As expected, levels of opsonic IgG to CS2 showed gravidity dependence [1, 28]. Despite the lower force of infection, the gravidity dependent acquisition of opsonic IgG to CS2 suggests that opsonic antibody to placental-binding IEs may be carried forward to subsequent pregnancies.

In the PNG cohort, a significant association between antibody levels to some antigens and time period remained after adjusting for confounders. Similarly, ITN use was associated with lower levels of antibodies, and for MSP2 antibodies there was evidence of an important interaction between time period and ITNs use. Both IPTp and ITN have previously been shown to decrease development of pregnancy-specific immunity (but not other measures of malaria immunity) [11-13]. Women who use ITN from early pregnancy may be protected against malaria during early fetal development but higher ITN usage in the later time period may have reduced malaria exposure and led to a decline in antibody responses.

Antibody levels vary over a malaria season [34] or a single pregnancy [25, 28]. In PNG, among women who were negative for parasitemia at antenatal booking and at delivery, a high proportion of those enrolled early experienced changes in antibody levels to merozoite antigens and VAR2CSA-DBL5ε, whereas more responses were unchanged in the later period, consistent with a lower force of infection. By contrast, levels of opsonic IgG to CS2 became more variable with time. When we compared changes in antibody responses by treatment arm, responses measured by ELISA rarely changed, and this did not differ by intervention arm, but levels of opsonic IgG to E8B-ICAM increased more frequently in women receiving a single course of SP with CQ. By contrast, women receiving more intensive IPTp with SP and AZ more often had declining levels of opsonic IgG against placental-binding IEs [11], probably reflecting decreased exposure to malaria.

Pregnant women infected with HIV have reduced levels of antibodies to *P. falciparum* antigens [14, 44]. HIV is uncommon in pregnant women in PNG and we lacked information regarding HIV status in Malawian women, so we could not address this factor. Past malaria on placental histology is associated with higher levels of antibodies [46]. We did not examine this, but in the PNG cohort, where we have samples at ANC booking and delivery, the decline in antibody responses between time periods was similar, making it unlikely that past infection confounded our findings. Future studies should address these questions.

In conclusion, the impact of falling parasite prevalence on anti-*P. falciparum* serological indicators in pregnant women differed between settings. There were limited changes over time in Malawian women after adjusting for other important co-factors, but in PNG, decreases in several antibody responses remained significant after adjustment. For antibodies to IEs, findings varied depending on the assay used. Parasite prevalence in PNG and Malawi was quite similar in both early and late periods. ITN use was associated with lower malarial-antibodies and might increase women's susceptibility in subsequent pregnancies. Whether malaria transmission has declined overall in these two populations is currently unknown, but differences in local transmission might explain differences between the sites. Continued monitoring of immunity of susceptible populations, including assays that measure antibody to intact cells, will be important as malaria control intensifies.

Footnote:

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Figure 1. Levels of immunoglobulin G (IgG) antibody in pregnant Malawian women to *Plasmodium falciparum* antigens over time. White bars - Pregnant women enrolled between 1999 – 2000, grey bars - Pregnant women enrolled between 2004 - 2006. **(A)** Levels of IgG antibody to schizont extract, PfRh2, MSP2 and VAR2CSA-DBL5, [N=175 (early); N=145 (late)]. Antibody levels are standardized using positive and negative control, and are presented as arbitrary units. **(B)** Levels of IgG antibody to endothelial-binding and placental-binding VSA, [N=141 (early); N=124 (late)]. Data are presented as mean fluorescence intensity relative to the positive and negative controls. **(C)** Levels of opsonic IgG antibody to VSA of both endothelial-binding and placental-binding IEs, [N=172 (early); N=139 (late)]. Data are presented as percentage of THP-1 cells that have ingested IEs (percentage phagocytosis). Mann-Whitney U test; * $p < 0.05$, ** $p < 0.01$. Error bars show 95% CI.

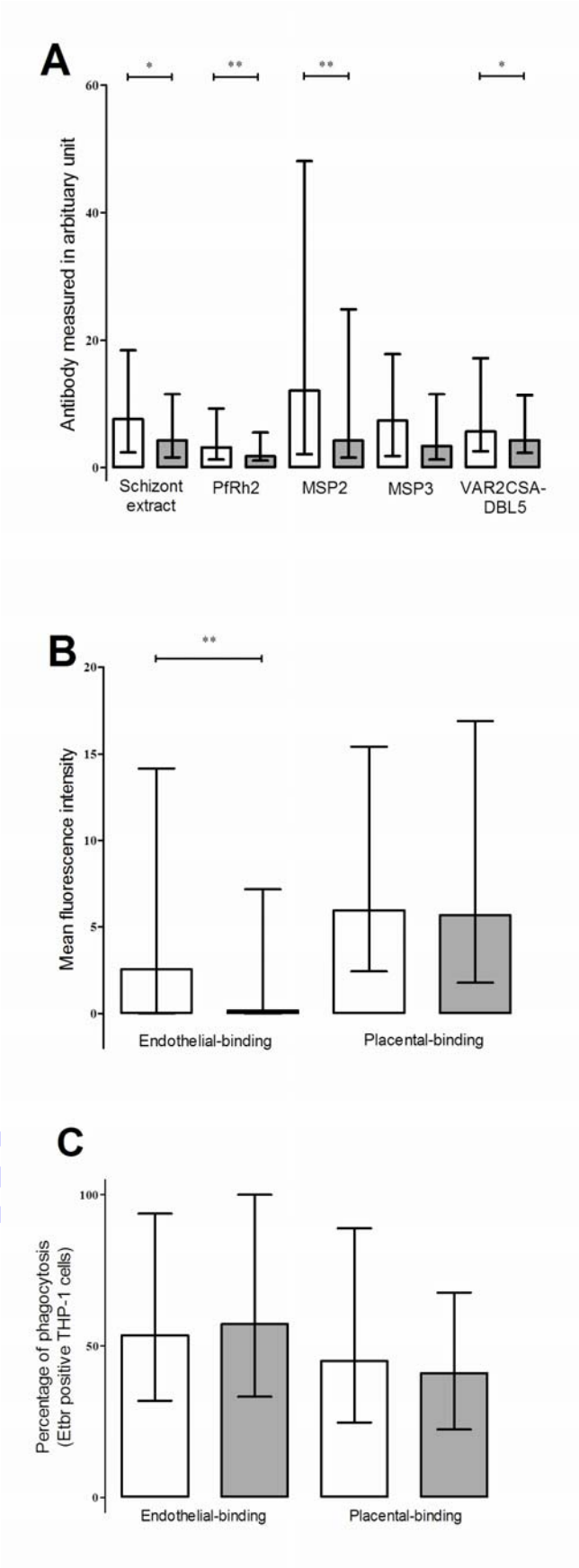
Figure 2. Levels of immunoglobulin G (IgG) in pregnant PNG women to *Plasmodium falciparum* antigens over time. White bars - Pregnant women enrolled between 2005 - 2007, grey bars - Pregnant women enrolled between 2010 - 2012. **(A)** Levels of IgG antibody to schizont extract, PfRh2, MSP2, MSP3 and VAR2CSA-DBL5, [N=118 (early); N=265 (late)]. Antibody levels are standardized using positive and negative control, and are presented as arbitrary units. **(B)** Levels of opsonic IgG antibody to whole merozoites [N=85 (early); N=95 (late)]. Data are presented as percentage of THP-1 cells that have ingested whole merozoite (percentage phagocytosis). **(C)** Levels of IgG antibody to endothelial-binding and placental-binding VSA (median level of IgG to placental-binding IEs is 0 for gray bar), [N=85 (early); N=95 (late)]. **(D)** Levels of opsonic IgG antibody to VSA of both non-pregnancy and pregnancy specific, [N=119 (early); N=263 (late)]. Data are presented as percentage of THP-1 cells that have ingested IEs (percentage phagocytosis). Mann-Whitney U test; *** $p < 0.0001$. Error bars show 95% CI.

References

1. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. Nature **1998**; 395:851-2.
2. Rijken MJ, McGready R, Boel ME, et al. Malaria in pregnancy in the Asia-Pacific region. Lancet Infect Dis **2012**; 12:75-88.
3. Beeson JG, Reeder JC, Rogerson SJ, Brown GV. Parasite adhesion and immune evasion in placental malaria. Trends in Parasitology **2001**; 17:331-7.
4. Fried M, Duffy PE. Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. Science **1996**; 272:1502-4.
5. Ricke CH, Staalsoe T, Koram K, et al. Plasma Antibodies from Malaria-Exposed Pregnant Women Recognize Variant Surface Antigens on Plasmodium falciparum-Infected Erythrocytes in a Parity-Dependent Manner and Block Parasite Adhesion to Chondroitin Sulfate A. The Journal of Immunology **2000**; 165:3309-16.
6. Beeson JG, Brown GV, Molyneux ME, Mhango C, Dzinjalamala F, Rogerson SJ. Plasmodium falciparum isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J Infect Dis **1999**; 180:464-72.
7. Rogerson SJ, Pollina E, Getachew A, Tadesse E, Lema VM, Molyneux ME. Placental monocyte infiltrates in response to Plasmodium falciparum malaria infection and their association with adverse pregnancy outcomes. Am J Trop Med Hyg **2003**; 68:115-9.
8. Brabin BJ. An analysis of malaria in pregnancy in Africa. Bull World Health Organ **1983**; 61:1005-16.
9. Desai M, ter Kuile FO, Nosten F, et al. Epidemiology and burden of malaria in pregnancy. Lancet Infect Dis **2007**; 7:93-104.
10. Staalsoe T, Shulman CE, Bulmer JN, Kawuondo K, Marsh K, Hviid L. Variant surface antigen-specific IgG and protection against clinical consequences of pregnancy-associated Plasmodium falciparum malaria. The Lancet **2004**; 363:283-9.
11. Aitken EH, Mbewe B, Luntamo M, et al. Antibody to P. falciparum in pregnancy varies with intermittent preventive treatment regime and bed net use. PLoS One **2012**; 7:e29874.
12. Staalsoe T, Shulman CE, Dorman EK, Kawuondo K, Marsh K, Hviid L. Intermittent preventive sulfadoxine-pyrimethamine treatment of primigravidae reduces levels of plasma immunoglobulin G, which protects against pregnancy-associated Plasmodium falciparum malaria. Infect Immun **2004**; 72:5027-30.
13. Staalsoe T, Megnekou R, Fievét N, et al. Acquisition and Decay of Antibodies to Pregnancy-Associated Variant Antigens on the Surface of Plasmodium falciparum-Infected Erythrocytes That Protect against Placental Parasitemia. The Journal of Infectious Diseases **2001**; 184:618-26.
14. Mount AM, Mwapasa V, Elliott SR, et al. Impairment of humoral immunity to Plasmodium falciparum malaria in pregnancy by HIV infection. The Lancet **2004**; 363:1860-7.
15. Feng G, Simpson JA, Chaluluka E, Molyneux ME, Rogerson SJ. Decreasing Burden of Malaria in Pregnancy in Malawian Women and Its Relationship to Use of Intermittent Preventive Therapy or Bed Nets. PLoS One **2010**; 5:e12012.
16. Gnidehou S, Jessen L, Gangnard S, et al. Insight into antigenic diversity of VAR2CSA-DBL5epsilon domain from multiple Plasmodium falciparum placental isolates. PLoS One **2010**; 5:e13105.
17. Beeson JG, Mann EJ, Elliott SR, et al. Antibodies to variant surface antigens of Plasmodium falciparum-infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. J Infect Dis **2004**; 189:540-51.

18. Chandrasiri UP, Randall LM, Saad AA, Bashir AM, Rogerson SJ, Adam I. Low antibody levels to pregnancy-specific malaria antigens and heightened cytokine responses associated with severe malaria in pregnancy. J Infect Dis **2013**.
19. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer **1980**; 26:171-6.
20. Ataíde R, Hasang W, Wilson DW, et al. Using an Improved Phagocytosis Assay to Evaluate the Effect of HIV on Specific Antibodies to Pregnancy-Associated Malaria. PLoS One **2010**; 5:e10807.
21. Stanisic DI, Richards JS, McCallum FJ, et al. Immunoglobulin G subclass-specific responses against Plasmodium falciparum merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. Infect Immun **2009**; 77:1165-74.
22. Persson KE, McCallum FJ, Reiling L, et al. Variation in use of erythrocyte invasion pathways by Plasmodium falciparum mediates evasion of human inhibitory antibodies. J Clin Invest **2008**; 118:342-51.
23. Osier FH, Fegan G, Polley SD, et al. Breadth and magnitude of antibody responses to multiple Plasmodium falciparum merozoite antigens are associated with protection from clinical malaria. Infect Immun **2008**; 76:2240-8.
24. Reiling L, Richards JS, Fowkes FJ, et al. Evidence that the erythrocyte invasion ligand PfRh2 is a target of protective immunity against Plasmodium falciparum malaria. J Immunol **2010**; 185:6157-67.
25. Fowkes FJ, McGready R, Cross NJ, et al. New insights into acquisition, boosting, and longevity of immunity to malaria in pregnant women. J Infect Dis **2012**; 206:1612-21.
26. Avril M, Cartwright MM, Hathaway MJ, et al. Immunization with VAR2CSA-DBL5 Recombinant Protein Elicits Broadly Cross-Reactive Antibodies to Placental Plasmodium falciparum-Infected Erythrocytes. Infection and Immunity **2010**; 78:2248-56.
27. Boyle MJ, Wilson DW, Richards JS, et al. Isolation of viable Plasmodium falciparum merozoites to define erythrocyte invasion events and advance vaccine and drug development. Proceedings of the National Academy of Sciences **2010**; 107:14378-83.
28. Aitken EH, Mbewe B, Luntamo M, et al. Antibodies to chondroitin sulfate A-binding infected erythrocytes: dynamics and protection during pregnancy in women receiving intermittent preventive treatment. J Infect Dis **2010**; 201:1316-25.
29. Salanti A, Dahlback M, Turner L, et al. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med **2004**; 200:1197-203.
30. Moncunill G, Mayor A, Jimenez A, et al. High antibody responses against Plasmodium falciparum in immigrants after extended periods of interrupted exposure to malaria. PLoS One **2013**; 8:e73624.
31. Vestergaard LS, Lusingu JP, Nielsen MA, et al. Differences in human antibody reactivity to Plasmodium falciparum variant surface antigens are dependent on age and malaria transmission intensity in northeastern Tanzania. Infect Immun **2008**; 76:2706-14.
32. Kinyanjui S, Conway D, Lanar D, Marsh K. IgG antibody responses to Plasmodium falciparum merozoite antigens in Kenyan children have a short half-life. Malaria Journal **2007**; 6:82.
33. Akpogheneta OJ, Duah NO, Tetteh KK, et al. Duration of naturally acquired antibody responses to blood-stage Plasmodium falciparum is age dependent and antigen specific. Infect Immun **2008**; 76:1748-55.
34. Crompton PD, Kayala MA, Traore B, et al. A prospective analysis of the Ab response to Plasmodium falciparum before and after a malaria season by protein microarray. Proc Natl Acad Sci U S A **2010**; 107:6958-63.
35. Nebie I, Tiono AB, Diallo DA, et al. Do antibody responses to malaria vaccine candidates influenced by the level of malaria transmission protect from malaria? Trop Med Int Health **2008**; 13:229-37.
36. Fonjungo PN, Elhassan IM, Cavanagh DR, et al. A longitudinal study of human antibody responses to Plasmodium falciparum rhoptry-associated protein 1 in a region of seasonal and unstable malaria transmission. Infect Immun **1999**; 67:2975-85.

37. Hill DL, Eriksson EM, Li Wai Suen CS, et al. Opsonising Antibodies to P. falciparum Merozoites Associated with Immunity to Clinical Malaria. PLoS One **2013**; 8:e74627.
38. Hviid L. The case for PfEMP1-based vaccines to protect pregnant women against Plasmodium falciparum malaria. Expert Review of Vaccines **2011**; 10:1405-14.
39. Feng G, Aitken E, Yosaatmadja F, et al. Antibodies to variant surface antigens of Plasmodium falciparum-infected erythrocytes are associated with protection from treatment failure and the development of anemia in pregnancy. J Infect Dis **2009**; 200:299-306.
40. Ataíde R, Mwapasa V, Molyneux ME, Meshnick SR, Rogerson SJ. Antibodies That Induce Phagocytosis of Malaria Infected Erythrocytes: Effect of HIV Infection and Correlation with Clinical Outcomes. PLoS ONE **2011**; 6:e22491.
41. Duffy PE, Fried M. Antibodies That Inhibit Plasmodium falciparum Adhesion to Chondroitin Sulfate A Are Associated with Increased Birth Weight and the Gestational Age of Newborns. Infect Immun **2003**; 71:6620-3.
42. Fernandez P, Petres S, Mecheri S, Gysin J, Scherf A. Strain-transcendent immune response to recombinant Var2CSA DBL5-epsilon domain block P. falciparum adhesion to placenta-derived BeWo cells under flow conditions. PLoS One **2010**; 5:e12558.
43. Magistrado P, Salanti A, Tuikue Ndam NG, et al. VAR2CSA expression on the surface of placenta-derived Plasmodium falciparum-infected erythrocytes. J Infect Dis **2008**; 198:1071-4.
44. M Vandemoortele and K Bird. Improved economic conditions in Malawi: Progress from a low base. Available at: http://www.odi.org.uk/publications/5480-malawi-economic-conditions-poverty-development-progress. Accessed 20 April 2014.
45. Malawi Government. 2012 Global AIDS response progress report: Malawi country report for 2010 and 2011. Available at: http://www.unaids.org/en/dataanalysis/knowyourresponse/countryprogressreports/2012countries/ce_MW_Narrative_Report%5B1%5D.pdf. Accessed 20 April 2014.
46. Mayor A, Kumar U, Bardaji A, et al. Improved pregnancy outcomes in women exposed to malaria with high antibody levels against Plasmodium falciparum. J Infect Dis **2013**; 207:1664-74.



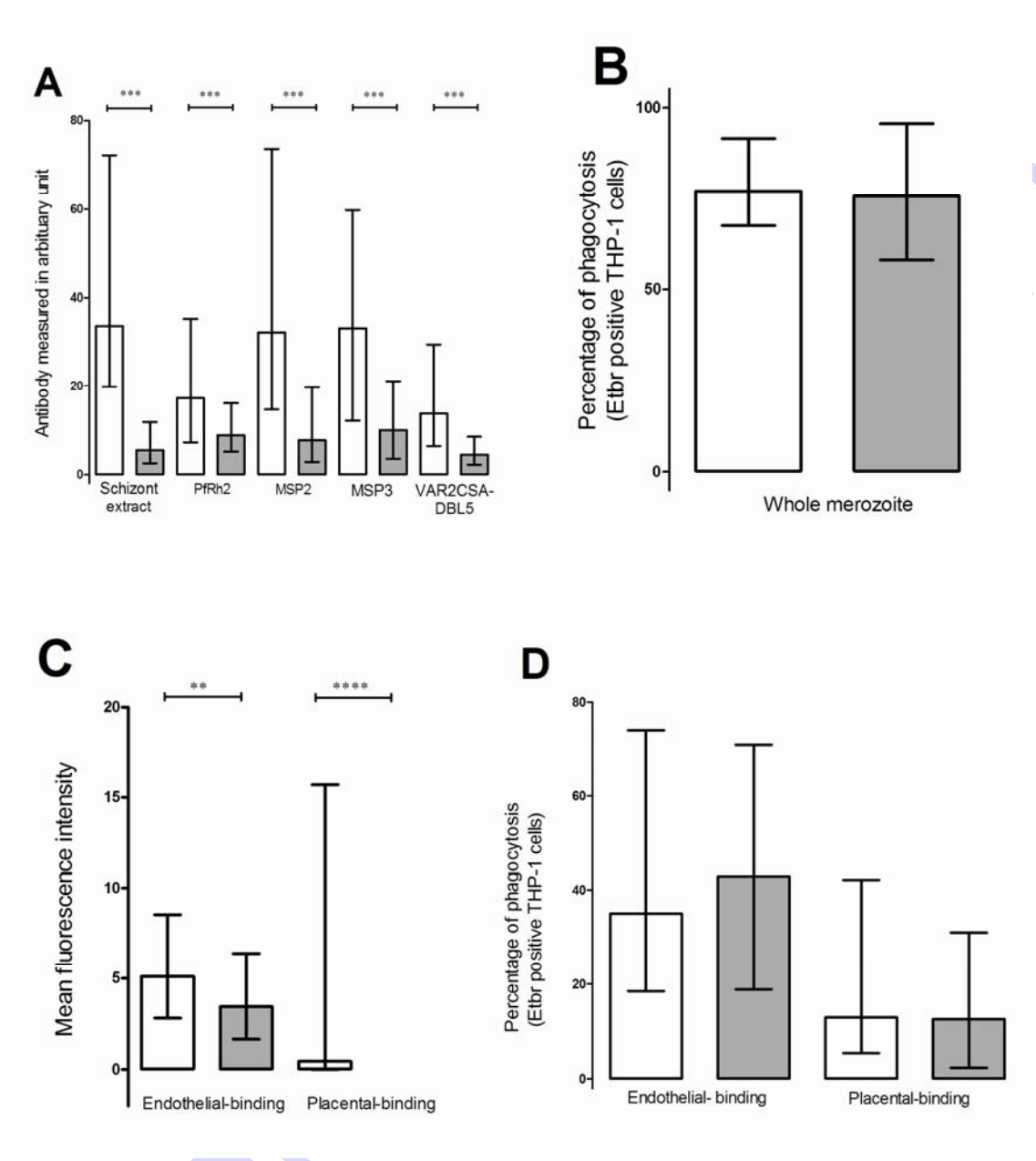


Table 1.

Study population characteristics of Malawian women.

Characteristic	Early year (1999-2000) (n=184)	Late year (2004-2006) (n=148)	P-value
Age, years	25.0 (21.0-27.0)	24.0 (21.0-26.0)	0.2
Maternal weight, kg	**55.0 (51.0-60.0)**	**57.0 (53.0-65.0)**	**0.02**
Maternal BMI	23.0 (21.2-25.8)	23.7(22.2-25.6)	0.5
Gravidity			**0.02**
Gravida 2, n (%)	**58 (31.5)**	**69(46.6)**	
Gravida 3, n (%)	**89 (48.4)**	**54(36.5)**	
Gravida 4, n (%)	**37 (20.1)**	**25(16.9)**	
Bed net user, n (%)	**35 (19.0)**	**81 (54.7)**	**<0.0001**
Received SP[a]			**0.005**
0, n (%)	**20 (10.9)**	**11 (7.4)**	
1, n (%)	**84 (45.7)**	**55 (37.2)**	
2, n (%)	**63 (34.2)**	**56 (37.8)**	
3+, n (%)	**15 (1.8)**	**35 (17.6)**	
Delivery mean haemoglobin, ± SD, g/dl	11.9 (1.7)	11.8 (1.8)	0.5
Birth weight, kg	3.1 (2.8-3.3)	3.1 (2.9-3.4)	0.8
Parasite prevalence[b]	25.2%	6.8%	

NOTE. Data represented as median and interquartile range, unless otherwise indicated, P-values are also shown. BMI, body mass index; SP, Sulphadoxine-pyrimethamine; SD, standard deviation. Significant associations ($p<0.05$) highlighted in bold.

[a] Doses of SP received during the course of pregnancy.

[b] Prevalence of placental malaria at each time point identified by light microscopy of placental blood smear (over-all incidence of placental malaria at each time point).

Table 2.

Study population characteristics of Papua New Guinean women.

Characteristic	Early year (2005-2007) (n=131)	Late year (2010-2012) (n=281)	P-value
Age, years	**26 (24-29)**	**27 (24-30)**	**0.02**
Maternal weight, kg	53.0 (50.0-57.0)	53.0 (48.0-59.0)	0.2
Mid-upper arm circumference, cm	**22.0 (21.0-23.0)**	**23.0 (22.0-25.0)**	**<0.0001**
Gravidity			0.7
Gravida 2, n (%)	44 (33.6)	95 (33.8)	
Gravida 3, n (%)	52 (39.7)	96 (34.2)	
Gravida 4, n (%)	35 (26.7)	80 (32.0)	
Bed net user, n (%)	**80 (61.0)**	**264 (94.0)**	**<0.0001**
Received SP[a]			**<0.0001**
No, n (%)	**7 (5.3)**	**0 (0)**	
Yes, n (%)	**123 (94.7)**	**277 (100)**	
Delivery mean haemoglobin, ± SD, g/dl	**9.2 (1.6)**	**10.0 (1.6)**	**<0.0001**
Birth weight, kg	**2.6 (2.9-3.7)**	**3.1 (2.8-3.4)**	**0.001**
Parasite prevalence[b]	18.0%	3.1%	

NOTE. Data represented as median and interquartile range, unless otherwise indicated, P-values are also shown. SP, Sulphadoxine-pyrimethamine; SD, standard deviation. Significant associations ($p<0.05$) highlighted in bold.

[a] Received SP during the course of pregnancy.

[b] Prevalence of placental malaria at each time point identified by light microscopy of placental blood smear (over-all incidence of placental malaria at each time point).

Variables	IgG schizont extract		IgG RH2A9		IgG MSP2		IgG MSP3		IgG VAR2CSA-DBL5	
	Coeff (95%CI)	p	Coeff (95% CI)	p	Coeff (95% CI)	P	Coeff (95%CI)	p	Coeff (95%CI)	p
Early and Late enrolment	-7.6 (-27.3, 12.1)	0.4	-7.1 (-23.5, 9.3)	0.4	12.8 (-4.3, 29.8)	0.1	0.6 (-17.7, 19.0)	0.9	2.6 (-23.9, 29.1)	0.8
Maternal weight	-0.3 (-0.6, 0.1)	0.1	-0.02 (-0.3, 0.3)	0.9	**-0.4 (-0.7, -0.2)**	**0.003**	**-0.3 (-0.6, -0.02)**	**0.04**	**-0.6 (-1.0, -0.2)**	**0.007**
Gravida	-1.7 (-5.7, 2.2)	0.4	-1.3 (-4.7, 2.0)	0.4	-1.6 (-5.7, 11.9)	0.4	2.5 (-1.2, 6.3)	0.2	2.1 (-3.3, 7.6)	0.4
Bed net use	**-12.3 (-22.3, -2.2)**	**0.02**	-6.2 (-14.8,2.3)	0.2	-4.7 (-13.6, 4.1)	0.3	-6.4 (-1.2, 6.3)	0.2	-13.3 (-26.5, 0.4)	0.06
SP dosage	1.0 (-8.9, 11.0)	0.8	0.6 (-7.9, 9.0)	0.9	3.1 (-5.7, 11.9)	0.5	0.9 (-8.5, 10.4)	0.2	-1.2 (-9.5, 7.2)	0.8
Late enrolment and bed net use	10.1 (-2.6, 22.9)	0.1	6.6 (-4.2, 17.4)	0.2	1.3 (-10.0, 12.5)	0.8	6.0 (-6.1, 18.1)	0.3	14.9 (-2.2, 32.0)	0.09
Late enrolment and SP dosage										
1	2.7 (-18.1, 23.4)	0.8	2.6 (-14.6, 19.8)	0.8	**-20.4 (-38.3, -2.5)**	**0.03**	-5.0 (-24.3, 14.3)	0.6	-19.8 (-47.6, 8.1)	0.2
2	4.1 (-17.1, 25.4)	0.7	3.8 (-13.8, 21.4)	0.7	-15.5 (-33.8, 2.8)	0.1	-2.2 (-21.9, 17.5)	0.8	-4.5 (-33.1, 24.1)	0.8
3	8.3 (-18.7, 35.2)	0.5	5.3 (-17.3, 27.9)	0.6	1.5 (-22.0, 25.0)	0.9	0.7 (-19.5, 31.1)	0.7	-0.6 (-35.9, 34.6)	1.0

Variables	IgG VSA-E8B-ICAM		IgG VSA-CS2		Opsonic IgG E8B-ICAM		Opsonic IgG CS2	
	Coeff (95%CI)	p	Coeff (95%CI)	P	Coeff (95%CI)	p	Coeff (95%CI)	p
Early and Late enrolment	-8.0 (-31.7, 15.8)	0.5	4.1 (-13.6, 21.8)	0.6	-1.0 (-29.3, 27.2)	0.9	-3.9 (-31.0, 23.1)	0.8
Maternal weight	-0.2 (-0.6, 0.2)	0.3	**-0.3 (-0.6, -0.06)**	**0.02**	-0.1 (-0.6, 0.3)	0.5	-0.3 (-0.7, 0.2)	0.3
Gravida	3.7 (-0.8, 8.2)	0.1	-3.1 (-6.7, 0.5)	0.09	-0.3 (-5.9, 5.4)	0.9	**6.7 (1.4, 12.3)**	**0.01**
Bed net use	-4.2 (-15.4, 7.0)	0.5	-6.6 (-15.8, 2.5)	0.2	-12.3 (-26.6, 2.0)	0.1	-12.6 (-26.2, 1.0)	0.07
SP dosage	7.1 (-0.3, 14.6)	0.06	-1.0 (-6.7, 4.7)	0.7	0.5 (-12.9, 13.9)	0.9	3.1 (-6.7, 12.9)	0.5
Late enrolment and bed net use	5.3 (-9.1, 19.6)	0.5	10.7 (-0.8, 22.2)	0.07	9.2 (-8.9, 27.3)	0.3	15.3 (-2.1, 32.6)	0.09
Late enrolment and SP dosage								
1	0.7 (-23.8, 25.1)	1.0	-10.8 (-29.2, 7.6)	0.2	-2.8 (-32.6, 27.0)	0.9	-15.7 (-43.9, 12.6)	0.3
2	16.5 (-8.6, 41.6)	0.2	-0.2 (-19.0, 18.6)	1.0	10.4 (-20.1, 40.8)	0.5	2.6 (-26.2, 31.5)	0.9
3	12.5 (-18.6, 43.6)	0.4	7.3 (-15.2, 29.8)	0.5	8.7 (-29.4, 46.9)	0.7	34.4 (-2.5, 71.2)	0.07

NOTE. Data represented as coefficients and 95% confidence interval (Multiple linear regression models), P-values are also shown. SP, Sulphadoxine-pyrimethamine. A positive coefficient implies an increase of antibody levels. A negative coefficient implies a decrease of antibody levels. Significant associations ($p<0.05$) highlighted in bold.

Table 3: Associations between time of enrolment, maternal characteristics, malaria prevention strategies, and antibody responses to *P. falciparum* antigens in Malawian women.

Variables	IgG-schizont extract		IgG-RH2A9		IgG-MSP2		IgG-MSP3		IgG-VAR2CSA DBL5	
	Coeff (95%CI)	p	Coeff (95%CI)	p	Coeff (95%CI)	p	Coeff (95%CI)	p	Coeff (95%CI)	p
Early and Late enrolment	**-49.8 (-70.6, -29.1)**	**<0.0001**	2.0 (-19.6, 23.6)	0.9	**-49.6 (-74.4, -24.9)**	**<0.0001**	**-26.4 (-5.4, 0.5)**	**0.05**	-26.4 (-56.0, 3.3)	0.08
Age	0.2 (-0.4, 0.7)	0.5	0.2 (-0.4, 0.8)	0.5	0.2 (-0.4, 0.9)	0.5	-0.1 (-0.8, 0.5)	0.7	0.1 (-0.6, 0.9)	0.7
Mid-upper arm circumference	0.3 (-0.8, 1.3)	0.5	-0.1 (-1.2, 0.9)	0.8	-0.01 (-1.2, 1.2)	1.0	0.2 (-1.0, 1.4)	0.7	-0.5 (-1.8, 0.8)	0.5
Bed net use	**-10.0 (-18.7, -1.1)**	**0.03**	-4.8 (-13.9, 4.4)	0.3	**-19.1 (-29.6, -8.6)**	**<0.0001**	0.4 (-10.0, 10.8)	0.9	-6.0 (-17.5, 5.6)	0.3
SP use	15.0 (-3.3, 33.3)	0.1	0.1 (-19.0, 19.1)	1.0	16.4 (-5.5, 38.2)	0.1	1.1 (-20.5, 22.8)	0.9	11.8 (-17.5, 5.5)	0.3
Late enrolment and bed net use	18.3 (-3.3, 39.8)	0.09	-10.9 (-33.3, 11.5)	0.3	**28.1 (2.4, 53.7)**	**0.03**	3.5 (-24.3, 31.3)	0.8	11.3 (-11.9, 35.6)	0.5

Variables	Opsonic IgG Whole merozoite		IgG VSA-E8B-ICAM		IgG VSA-CS2		Opsonic IgG E8B-ICAM		Opsonic IgG CS2	
	Coeff (95%CI)	p	Coeff (95%CI)	p	Coeff (95%CI)	p	Coeff (95%CI)	p	Coeff (95%CI)	p
Early and Late enrolment	-5.5 (-14.0, 3.1)	0.2	-3.1 (-7.4, 1.3)	0.2	-11.6 (-54.0, 30.8)	0.6	9.9 (-22.7, 42.5)	0.6	-8.8 (-37.6, 20.0)	0.5
Age	-0.2 (-1.1, 0.7)	0.7	-0.02 (-0.4, 0.8)	0.9	-0.2 (-0.9, 0.5)	0.6	-0.1 (-0.9, 0.7)	0.8	-0.1 (-0.8, 0.6)	0.8
Mid-upper arm circumference	0.9 (-0.9, 2.6)	0.3	0.2 (-0.4, 0.8)	0.4	0.1 (-1.3, 1.5)	0.9	-1.0 (-2.4, 0.5)	0.2	0.2 (-1.1, 1.4)	0.8
Bed net use	0.4 (-10.5, 11.3)	0.9	**3.4 (-0.4, 0.8)**	**0.05**	4.3 (-3.6, 12.2)	0.3	-3.7 (-16.3, 8.8)	0.6	2.7 (-8.4, 13.8)	0.6
SP use	6.8 (-10.5,11.3)	0.5	0.6 (-0.35, 4.8)	0.8	-0.2 (-12.3, 8.5)	0.7	-5.4 (-16.3, 8.8)	0.7	-0.02 (-23.1, 23.0)	1.0
Late enrolment and bed net use					-2.8 (-44.2, 38.6)	0.9	-7.2 (-40.8, 26.3)	0.7	4.3 (-25.3, 34.0)	0.8

NOTE. Data represented as coefficients and 95% confidence interval (Multiple linear regression models), P-values are also shown. SP, Sulphadoxine-pyrimethamine. A positive coefficient implies an increase of antibody levels. A negative coefficient implies a decrease of antibody levels. Significant associations ($p<0.05$) highlighted in bold.

Table 4: Associations between time of enrolment, maternal characteristics, malaria prevention strategies, and antibody responses to *P. falciparum* antigens in Papua New Guinean women.

Variable	Early year (2005-2007) (n=81)	Late year (2010-2012) (n=242)	P-value	Late year SP+AZ	Late year SP+CQ	P-value

IgG to schizont extract			**<0.0001**			0.6
Decrease (N) %	**27 (33.3)**	**30 (12.2)**		15 (11.7)	15 (12.8)	
No change (N) %	**39 (48.1)**	**202 (82.4)**		108 (84.4)	94 (80.3)	
Increase (N) %	**15 (18.5)**	**13 (5.3)**		5 (3.9)	8 (6.8)	
IgG to MSP2			**0.005**			0.7
Decrease (N) %	**19 (23.5)**	**40 (16.1)**		19 (14.5)	21 (17.9)	
No change (N) %	**51 (63.0)**	**196 (79.0)**		106 (80.9)	90 (76.9)	
Increase (N) %	**11 (13.5)**	**12 (4.8)**		6 (4.6)	6 (5.1)	
IgG to MSP3			**<0.0001**			0.5
Decrease (N) %	**21 (26.3)**	**51 (21.1)**		23 (18.4)	28 (23.9)	
No change (N) %	**37 (46.3)**	**168 (69.4)**		91 (72.8)	77 (65.8)	
Increase (N) %	**22 (27.5)**	**23 (9.5)**		11 (8.8)	12 (10.3)	
IgG to RH2A9			**0.003**			0.2
Decrease (N) %	**23 (28.4)**	**39 (15.8)**		16 (12.2)	23 (19.8)	
No change (N) %	**45 (55.6)**	**187 (75.7)**		102 (77.9)	85 (73.3)	
Increase (N) %	**13 (16.0)**	**21 (8.5)**		13 (9.9)	8 (6.9)	
IgG to DBL5			**<0.0001**			0.7
Decrease (N) %	**12 (18.8)**	**25 (10.4)**		14 (11.0)	11 (10.0)	
No change (N) %	**37 (53.4)**	**201 (84.5)**		108 (85.0)	93 (83.8)	
Increase (N) %	**15 (21.7)**	**12 (5.0)**		5 (4.0)	7 (6.3)	
Opsonic IgG to E8B-ICAM			0.07			**<0.0001**
Decrease (N) %	11(14.7)	50 (20.7)		**29 (22.8)**	**21 (18.4)**	
No change (N) %	46 (61.3)	111 (45.9)		**71 (55.9)**	**40 (35.1)**	
Increase (N) %	18 (24.0)	80 (33.4)		**27 (21.3)**	**53 (46.5)**	
Opsonic IgG to CS2			**0.03**			**0.05**
Decrease (N) %	**10 (12.7)**	**50 (22.9)**		**33 (28.2)**	**17 (16.8)**	
No change (N) %	**59 (74.7)**	**126 (57.8)**		**59 (50.4)**	**67 (66.3)**	
Increase (N) %	**10 (12.7)**	**42 (19.3)**		**25 (21.4)**	**17 (16.8)**	

NOTE. Data represented as numbers and percentage, P-values are also shown. SP, Sulphadoxine-pyrimethamine, AZ, azithromycin, CQ, chloroquine. Significant associations ($p<0.05$) highlighted in bold.

Table 5: Antibody responses against *P. falciparum* antigens in the Papua New Guinean cohort during the course of pregnancy.