HIV-1 Infection and Antibodies to *Plasmodium falciparum* in Adults

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**Background.** Coinfection with human immunodeficiency virus (HIV) may increase susceptibility to malaria by compromising naturally acquired immunity.

**Methods.** In 339 adults (64% HIV infected), we measured antibodies to *Plasmodium falciparum* variant surface antigens (VSA) and antibodies that opsonise infected erythrocytes using parasite lines FCR3, E8B, and R29, and antibodies to merozoite antigens AMA-1 and MSP2. We determined the relationship between malaria antibodies, HIV infection, markers of immune compromise, and risk of incident parasitemia.

**Results.** HIV-infected adults had significantly lower mean levels of opsonizing antibody to all parasite lines (P < .0001), and lower levels of antibody to AMA-1 (P = .01) and MSP2 (P < .0001). Levels of immunoglobulin G (IgG) to VSA were not affected by HIV status. Opsonising antibody titres against some isolates were positively correlated with CD4 count. There were negative associations between human immunodeficiency virus type 1 (HIV-1) viral load and opsonizing antibodies to FCR3 (P = .04), and levels of IgG to AMA-1 (P ≤ .03) and MSP2-3D7 (P = .05). Lower opsonizing antibody levels on enrollment were seen in those who became parasitemic during follow-up, independent of HIV infection (P ≤ .04 for each line).

**Conclusions.** HIV-1 infection decreases opsonizing antibodies to VSA, and antibody to merozoite antigens. Opsonizing antibodies were associated with lack of parasitemia during follow up, suggesting a role in protection.

**Keywords.** *Plasmodium falciparum*; HIV; malaria; antibody; IgG; phagocytosis; merozoite antigens; variant surface antigens.

Coinfection with *Plasmodium falciparum* malaria and human immunodeficiency virus (HIV) is common in sub-Saharan Africa. Together these infections cause approximately 4 million deaths each year [1], and interactions between them are well recognised [2, 3]. By compromising host immunity, HIV infection increases susceptibility to malaria and reduces the efficacy of antimalarial drugs [4–7]. In pregnant women, HIV infection is associated with greater risk of severe anemia, decreased antimalarial antibody and increased density of placental parasitemia [8–11], and HIV-malaria coinfection leads to poor pregnancy outcomes such as low birth weight, preterm birth, and post-neonatal mortality [2, 12–14].

In nonpregnant adults, HIV infection is associated with increased risks of parasitemia and uncomplicated malaria, of severe malaria, and of treatment failure [3]; these risks increase with advancing immunosuppression. The effect on malaria immunity, and how this effect translates into increased susceptibility, is less well understood. In 1 study, HIV infection was associated with lower levels of antibody to apical membrane antigen-1 (AMA-1) but not to variant surface antigens (VSA) on infected erythrocytes (IE) [5, 7].

Anti-malarial antibody has an important role in malaria immunity. AMA-1 and merozoite surface proteins
(MSPs) have roles in erythrocyte invasion, and these antigens have been considered as vaccine candidates [15]. VSA s on the infected erythrocyte (IE) surface are involved in sequestration [16–18] and are believed to be major targets for immunity to blood stage infection.

In a longitudinal cohort study conducted before widespread availability of antiretroviral drugs, malaria was shown to increase HIV viral load and HIV infection predisposed to malaria parasitemia [19, 20]. Using samples from that study, we examined the humoral immune response to malaria in adults with and without HIV-1 infection. We hypothesised that HIV might have differential effects on different types of antibody to malaria blood stage antigens, and that adults lacking antibodies to one or more such targets were at increased risk of malaria infection during follow-up. We measured antibody levels to VSA on IE, levels of functional antibodies that opsonised IE for phagocytosis, and antibody levels to merozoite antigens, in adults followed prospectively for 6 months, and determined whether antibody levels correlated with measures of HIV-1-mediated immune compromise including viral load and CD4 T-cell counts. We examined whether antibody levels predicted risk of parasitemia during follow-up.

**METHODS**

**Ethics Statement**

Ethical clearance for this study was obtained from the University of Malawi College of Medicine Research Ethics Committee and the Melbourne Health Human Research Ethics Committee. Written informed consent for HIV testing and enrollment in this study for all the participants was obtained in the initial study [20].

**Study Design**

The serum samples used in this study came from a cohort that has previously been described [19, 20]. Briefly, the study was conducted in Thyolo district, Malawi. Participants were enrolled before the malaria season and were followed until the end of the malaria season (from 1 October 2000 to 30 June 2001). Serum was available for 339 of 349 adults aged 18–70 years. Participants were recruited following testing for HIV-1 infection during follow-up. We measured antibody levels to VSA on IE, levels of functional antibodies that opsonised IE for phagocytosis, and antibody levels to merozoite antigens, in adults followed prospectively for 6 months, and determined whether antibody levels correlated with measures of HIV-1-mediated immune compromise including viral load and CD4 T-cell counts. We examined whether antibody levels predicted risk of parasitemia during follow-up.

**Phagocytosis Assay**

Phagocytosis was measured as described by Ataide and colleagues with minor modifications [22]. Trophozoite-stage IE were purified by density gradient centrifugation by layering on a gradient of 80%, 60%, and 40% Percoll (Amersham) in Roswell Park Memorial Institute Medium (RPMI) 1640-HEPES. Purified IE on the 60% layer were collected, were washed 3 times with RPMI-1640-HEPES, and stained with 10 µg/mL of ethidium bromide (EtBr, Bio-Rad Laboratories) for 30 minutes. Stained IE were washed 5 times with RPMI-1640-HEPES before being resuspended at 3.3 × 10⁷ cells/mL. In total, 3.3 µL of test serum, patient pooled serum (positive control for FCR3 and R29 with working concentration of 90 µg/mL, MP CAPPEL) that had been previously plated in 96-well U-bottom plate were incubated with 30 µL of IE suspension (1:10 dilution) for 1 hour. A no-serum control was used as negative control to establish appropriate gates for the flow cytometry. IE were incubated with 5 × 10⁴ uTHP-1
cells/well at 1:10 ratio for 40 minutes at 37°C in humidified 5% CO₂. Phagocytosis was stopped by centrifugation at 4°C at 350 g for 5 minutes). Unphagocytosed IE were lysed by adding FACS Lysing solution (prepared in 1:10 dilution in distilled water; BD Biosciences) for 10 minutes. Lysis was stopped by adding cold FACS Buffer solution (phosphate-buffered saline (PBS (−Ca²⁺−Mg²⁺)), 2% fetal bovine serum (FBS), and 0.02% NaNO₃). Cells were washed 3 times with FACS Buffer before the cells were fixed with cold 2% paraformaldehyde in PBS.

Cells were acquired in a CYAN hypercyt flow cytometer. THP-1 cells were gated based on forward (FSC) and side scatter (SSC) properties. No-serum controls were used to set up the gating of non-Fc-receptor-mediated phagocytosis, which was set to be <5%. The phagocytosed iRBC were recognized by EtBr fluorescence (FL2 channel). Sample duplicates were acquired for 55 seconds per well; 8000–15 000 THP-1 cells were acquired per well.

IntelliCyt HyperView (IntelliCyt Corp) was used to analyze the data and the opsonizing antibodies were measured by calculating the percentage of THP-1 cells that phagocytosed the IE relative to the percentage of the positive control.

**IgG to Variant Surface Antigen Assay**
Levels of IgG to VSA were measured as described with minor modifications [11]. Trophozoite-stage IE were harvested at 5%–8% parasitemia and washed 3 times with PBS/1% newborn calf serum (NCS, Gibco). IE were prepared at 0.2% hematocrit in PBS/1% NCS and incubated with patient serum or control at 1 in 20 dilution (final volume 50 µL) in 96-well U-bottom plates at room temperature for 30 minutes, in duplicate. Cells were washed as before and incubated for 30 minutes at room temperature with rabbit anti-human IgG (Dako; 1:100 in PBS/1% NCS). After washing three times as before, Alexafluor 488-conjugated donkey anti-rabbit IgG (1:500 in PBS/1% NCS, Invitrogen) and 10 µg/mL EtBr were added, and samples were incubated in the dark for 30 minutes at room temperature. Cells were washed three times, resuspended and fixed with 2% paraformaldehyde in PBS.

Cells were acquired in a CYAN hypercyt flow cytometer. Red blood cells (RBC) were gated on FSC and SSC properties and IE were gated according to EtBr fluorescence (FL2 channel). The geometric mean fluorescence intensity (MFI) of Alexafluor 488 (FL1 channel) generated by the gated IE population was used as a relative measure of human IgG binding to iRBC for each serum sample. The positive control was a pool of serum with known high antibody recognition to the specific parasite lines, and negative controls were from 9 unexposed Australian donors.

The data were analyzed in Intellicyt Hyperview Analysis, and sample MFI was calculated relative to the MFI of positive and negative controls.

**ELISAs to Measure IgG to Merozoite Antigens**
Microtiter plates were coated with recombinant proteins AMA1 and MSP2 (3D7 and FC27 types) at 2 µg/mL in PBS (kindly provided by Prof. R. Anders) with a final volume of 100 µL per well and were left overnight at 4°C. After washing 3 times with PBS containing 0.05% of Tween (PBS/Tween), plates were blocked with 5% skim milk powder in PBS (Blotto) for 1 hour at room temperature. Plates were washed 5 times with PBS/Tween, and samples were diluted 1/1000 in Blotto before being added to plates (final volume of 50 µL, in duplicate) and left to incubate for 2 hours at room temperature. Plates were again washed 3 times in PBS/Tween, and horseradish peroxidase-conjugated sheep anti-human IgG (1:2000, 50 µL per well; Silenus) was added. Plates were incubated for 2 hours at room temperature and washed 3 times with PBS/Tween and twice with deionized water. Peroxidase substrate was added, and color was developed. Plates were read at 415 nm on a Biorad plate reader, and optical density (OD) results were obtained.

**Statistical Analysis**
Results were analyzed using Stata version 11.0 (Stata Corporation, College Station, Texas). Non-normally distributed variables such as level of phagocytosis and level of IgG to VSA and to merozoite antigens were analyzed using Mann–Whitney rank sum tests. P values <.05 were considered to be significant.

**RESULTS**

**Characteristics of Study Population**
Of 339 adults enrolled in this study, 216 (64%) were HIV-1 infected (Table 1). Compared to uninfected adults, HIV-1 infected adults had a lower mean hemoglobin concentration (P < .0001); no other significant differences were observed between groups.

**Table 1. Characteristics of Study Population Based on HIV-1 Status**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non HIV-1 Infected Adults (n = 123)</th>
<th>HIV-1 Infected Adults (n = 216)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of women, N (%)</td>
<td>56 (45.5)</td>
<td>88 (40.7)</td>
<td>.39</td>
</tr>
<tr>
<td>Mean weight, kg (SD)</td>
<td>55.5 (9.7)</td>
<td>54 (7.4)</td>
<td>.11</td>
</tr>
<tr>
<td>Mean age, years (SD)</td>
<td>31.9 (10.2)</td>
<td>32.7 (8.8)</td>
<td>.29</td>
</tr>
<tr>
<td>Mean hemoglobin, g/dL (SD)</td>
<td>13.9 (1.7)</td>
<td>12.6 (1.9)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Parasitemia episodes during follow-up, N (%)</td>
<td>48 (39)</td>
<td>100 (46.5)</td>
<td>.18</td>
</tr>
<tr>
<td>Mean log viral load HIV-1 RNA copies/μL (SD)</td>
<td>N/A</td>
<td>4.8 (0.7)</td>
<td></td>
</tr>
<tr>
<td>Median CD4 cell count at baseline, cells/mL(IQR)</td>
<td>N/A</td>
<td>347 (233–498.5)</td>
<td></td>
</tr>
</tbody>
</table>

P value based on Student t-test, χ², or Wilcoxon rank–sum as appropriate.

Abbreviations: HIV-1, human immunodeficiency virus type 1; IgG, immunoglobulin G; IQR, interquartile range; SD, standard deviation.
Effect of HIV-1 Serostatus on Antibody Level and Function

HIV-1 infected adults had significantly lower mean levels of opsonizing antibody against each of the 3 parasite lines tested, FCR3 (P < .0001), E8B-ICAM (P < .0001), and R29 (P < .0001; Figure 1A).

Antibody levels to VSA were not different between HIV-infected adults and noninfected adults for parasite line FCR3 (P = .25), E8B-ICAM (P = .23), or R29 (P = .49; Figure 1A).

HIV-1 infected adults had significantly lower mean levels of IgG to merozoite antigens than noninfected adults. These differences were observed for responses to AMA-1 and to both 3D7 and FC27 MSP2 types (AMA-1: P = .01; MSP2-FC27: P < .0001; MSP2-3D7: P < .0001; Figure 1).

Effect of HIV-1 Severity on Antibody Level and Function

The relationship between HIV-related immune suppression (as measured by HIV-1 RNA concentration and CD4 T-cell count at enrollment), and antimalarial antibody levels was examined. CD4 counts were categorized as high (≥400 cells/mL), intermediate (200–399 cells/mL), and low (<200 cells/mL) [20]. There were positive associations between CD4 cell counts and amounts of opsonizing antibody to FCR3 and E8B-ICAM. Significantly decreased levels of IgG to VSA with decreasing CD4 count were seen in E8B-ICAM and R29 but not FCR3. A similar decreasing trend with lower CD4 cell count was observed in levels of IgG to AMA-1, MSP2-3D7, and MSP2-FC27, but this was not significant (Table 2).

HIV-1 viral load was categorized as low (<10 000 copies/mL), moderate (10 000–99 999 copies/mL) and high (≥100 000 copies/mL) [20]. There was a negative association between the concentration of HIV-1 and functional antibodies to FCR3 but not E8B and R29. In contrast, IgG to VSA showed a borderline significant positive correlation for FCR3 but not E8B and R29. With increasing HIV-1 concentration there was a significant decrease in mean levels of IgG to AMA-1 and MSP2-3D7 but not MSP2-FC27 (Table 2).

Relationship Between Antibodies at Enrollment and Malaria Infection During Follow-up

Among adults without malaria on enrollment, those who had parasitemia on follow-up had lower mean opsonizing antibody levels on enrollment than those who did not develop parasitemia, for all parasite lines (FR3: P = .01; E8B-ICAM: P = .01; R29: P = .04). After adjusting for HIV status, levels of opsonizing antibody to FCR3 (P = .03) and E8B (P = .03) but not R29 (P = .21) remained significantly associated with lack of parasitemia. By contrast, levels of IgG to VSA and to merozoite antigens were not associated with parasitemia on follow-up after adjusting for HIV status (Figure 2).

DISCUSSION

HIV-infected adults are more vulnerable to malaria parasitemia, and to symptomatic and severe malaria, and each of these risks increases with the severity of HIV-related...
immunocompromise [5, 20, 23]. However, little is known regarding the effect of HIV infection on malaria immunity in nonpregnant adults. To understand how HIV affects immunity to malaria, we measured parameters of humoral immunity in both HIV-infected and uninfected Malawian adults. These parameters included levels of antibodies that opsonized IE, levels of IgG to VSA of 3 parasite lines, and levels of IgG to 3 merozoite antigens.

The cohort we studied is a unique resource of adults of known HIV status followed prospectively for malaria infection. Predating the widespread availability of antiretroviral treatment (ART) in Malawi, the cohort allows us to observe the relationships between HIV, malaria, and malaria immunity in a large number of ART treatment-naive adults, with appropriate HIV negative controls. Antiretroviral drugs reconstitute the individual’s immune system, although this may not eliminate malaria risk [24], and protease inhibitors and cotrimoxazole prophylaxis both have direct antimalarial effects [25, 26], meaning that studies such as the present one cannot now be performed.

HIV-infected adults had significantly lower levels of opsonizing antibodies to the 3 parasite lines tested. This finding is consistent with previous studies showing an association between HIV and decreased opsonizing antibodies to pregnancy-associated IE in pregnant women [9, 10, 22, 27]. We have previously demonstrated that in vitro HIV infection of macrophages substantially decreases their ability to phagocytose opsonizes IE [28]. Reduced levels of opsonizing antibodies and of phagocytosis in HIV-infected adults suggest incompetency in clearing parasites, and this could explain the association between HIV-1 positivity and increased risk of parasitemia seen in this cohort [20].

Immunity against *P. falciparum* malaria requires, in part, acquisition of a broad repertoire of antibodies to *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a family of variant proteins expressed at the IE surface [29, 30]. We compared the level of antibodies to 3 parasite lines, one that forms rosettes and that is associated with severe malaria [31, 32], and 2 that are representative of those that might cause uncomplicated malaria in older children and adults [33, 34, 35]. Fewer HIV-infected than HIV-negative adults had high levels of opsonizing antibodies to these lines (Figure 2), which suggests a reduced capability to produce a repertoire of functional antibodies against different variants of proteins expressed on IE, potentially contributing to reduced parasite clearance.

In this cohort, levels of antibody to VSA, measured as antibody binding to the IE surface, were not affected by HIV infection. We made similar observations in another study of nonpregnant adults [7]. Unlike opsonizing antibodies, these antibody responses appear to be unchanged by HIV infection, perhaps because HIV infection has limited effects on established antibody responses, such as those associated with life-long exposure to malaria. By contrast, HIV-infected pregnant women of all gravidades have considerably impaired production of IgG to pregnancy-specific VSA [11]. Expression of this unique member of the PfEMP1 family is restricted to pregnant women, in whom it mediates sequestration of IE in the placenta [36]; thus women in their first pregnancy are encountering this antigen for the first time. HIV infection may have a greater impact on development of antibody responses to newly encountered antigens than on maintenance of IgG antibody responses to VSA in individuals with previously established antibody responses.

Our findings showed that levels of IgG antibody to merozoite antigens were on average significantly lower in HIV-infected than uninfected adults. Reduced antibody levels to AMA-1 in HIV-infected adults have also been observed in adults, children, and pregnant women; however, responses to MSP2 were minimally affected [7, 11, 37]. Reasons why HIV-infected adults had lower levels of IgG to merozoite antigens but not to IE are not known, but the persistence of antibody in the absence of boosting might provide some insights. In African immigrants with no or limited ongoing malaria exposure, antibody responses to

### Table 2. Antimalarial Antibody Based on Markers of HIV Severity Within HIV Infected Adults

<table>
<thead>
<tr>
<th>Variables</th>
<th>n (%)</th>
<th>Opsonic Antibodies</th>
<th>IgG Levels to VSA</th>
<th>IgG Levels to Merozoite Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FCR3</td>
<td>EBB</td>
<td>R29</td>
</tr>
<tr>
<td>CD4 count (c/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;400</td>
<td>74 (40.7)</td>
<td>.02 (−2.4)</td>
<td>.3 (−1.1)</td>
<td>.8 (0.20)</td>
</tr>
<tr>
<td>200–399</td>
<td>76 (41.8)</td>
<td>.09 (−1.7)</td>
<td>.001 (−3.2)</td>
<td>.96 (0.05)</td>
</tr>
<tr>
<td>≥10000</td>
<td>78 (41.9)</td>
<td>.08 (−1.8)</td>
<td>.39 (−0.9)</td>
<td>.87 (1.6)</td>
</tr>
</tbody>
</table>

P value base on Wilcoxon rank-sum test. Dotted lines represent the reference groups.

Abbreviations: HIV-1, human immunodeficiency virus type 1; IgG, immunoglobulin G; VSA, variant surface antigens.
merozoite antigens and to IE show a broadly similar decrease over time [38], whereas in pregnant Thai women the half-life of antibody to merozoite antigens was shorter than that for antibody to the VSA, VAR2CSA [39]. If responses to the merozoite antigens tested have shorter half-lives in our Malawian population, the effect of HIV infection on these responses would be manifest sooner.

The differential effect of HIV on opsonizing antibody levels, compared to total IgG to VSA, is unlikely to be due to assay characteristics. Opsonizing assays are performed at or above saturating concentrations [22], whereas for antibody to VSA a 50% reduction in antibody titer is associated with a similar decrease in measured antibody levels (W Hasang, unpublished). Thus, opsonizing antibodies would be less, not more, susceptible to minor decreases in antibody level. The difference is likely to be biologically relevant, as opsonizing antibody has been more strongly associated with protection from complications of malaria in several studies [9, 10, 40]. Cytophilic IgG3 antibodies that opsonize IE and promote phagocytosis via Fc receptors [41, 42] predominated in healthy Gabonese adults [43], and IgG1 and IgG3 antibodies were dominant in children with malaria infection [44]. HIV decreases these IgG1 and IgG3 responses to pregnancy associated IE [27], and IgG3 has the shortest half-life of antibody subclasses and so may be most affected by decreases in antibody production. Whether HIV differentially affects IgG subclass responses to VSA or other malaria antigens is unknown, but levels of IgG1 or IgG3 antibodies specific to H1N1 influenza virus do not appear to be affected by HIV infection [45, 46]. There could be more subtle differences in functional activity of antibodies to IE produced by HIV infected adults, and further research that includes antibody subclass is required to confirm this finding.

In HIV-positive adults, opsonizing antibodies and VSA antibodies were generally decreased with lower CD4 count, and opsonizing antibodies were also negatively correlated with HIV-1 viral load, suggesting that functional antibody to malaria antigens is particularly affected by the degree of immunosuppression. Weaker associations were seen between antibodies to merozoite antigens and indices of immune suppression. In the cohort, there were moderate but inconsistent associations between CD4 count or viral load and risk of parasitemia, and a stronger relationship between low CD4 count and symptomatic malaria [20]. Taken together, these findings suggest that decreases in opsonizing antibodies, which showed the strongest correlation with increased immune suppression, could be responsible, at least in part, for the increased predisposition to parasitemia and clinical malaria seen in the most immunocompromised participants. Although we did not see a direct relationship between HIV infection and increased episodes of malaria, in the complete study cohort HIV-infected adults had a higher incidence of parasitemia than controls [20]. This suggests that, in this Malawian community, poor antibody responses in HIV-infected adults may be contributing to their increased risk of parasitemia.

**Figure 2.** Relationship of antimalaria antibody levels at enrollment and parasitemia during follow-up. Serum was collected at enrollment, participants were followed prospectively for 6 months, and incidence rate of parasitemia was recorded. A, Levels of opsonizing antibodies to parasite lines FCR3, EBB-ICAM, and R29 were significantly lower in adults who became parasitemic on follow-up. After adjusting for HIV status, levels of opsonizing antibody to FCR3 (P = .03) and EBB (P = .03) but not R29 (P = .21) remained significantly associated with protection. Levels of IgG to variant surface antigens of FCR3, EBB-ICAM, and R29 and (C) IgG to AMA-1 and MSP2 (FC27 and 3D7) did not differ between individuals who did and did not develop parasitemia. P value by Mann–Whitney rank sum test. Boxes represent median (center line) and interquartile ranges. Whiskers represent 95% CI. Dots represent outliers. Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; IgG, immunoglobulin G.
Levels of opsonizing antibody (and not antibodies to VSA or merozoite antigens) were associated with lower risk of infection with malaria during follow-up, and this was independent of HIV status. This finding suggests that opsonizing antibodies directed against IE that are commonly recognised in adults have a role in protection against parasitemia. Clinical malaria was uncommon in this cohort, and it would be of interest to determine whether opsonizing antibodies are particularly protective against symptomatic disease in appropriate cohorts, to add to published observations of associations with protection against anemia, low birth weight, and treatment failure [9, 10, 40]. Taken together, these studies suggest that high levels of opsonizing antibodies could be a marker for protection against parasite infection. Opsonizing antibodies could contribute to protection by enhancing clearance of IE by blood monocytes or tissue macrophages. It has also been shown that antibodies of the cytophilic subclasses IgG1 and IgG3 were dominant among healthy adults, children, and pregnant women [42–44].

HIV-infected adults who are constantly exposed to malaria have altered humoral immunity to *P. falciparum*, with lower levels of opsonizing antibodies to IE. Low levels of opsonizing antibodies potentially explain the increase of parasitemia seen in HIV-infected adults and were associated with decreased risk of malaria infection during follow-up in this study. Measuring opsonizing antibodies to IE expressing locally prevalent surface antigens may be a useful correlate of the risk of malaria reinfection.

**Notes**

**Acknowledgments.** We thank all the participants who took part in the study. Prof. R. Anders is thanked for providing recombinant AMA1 and MSP2 (3D7 and FC27) proteins.

**Financial support.** The laboratory work was supported by the National Health and Medical Research Council of Australia.

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

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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45. 2014 JID 210 (1 November) Hasang et al