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Authors
Boudová, Sarah
Walldorf, Jenny A
Bailey, Jason A
et al.

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CLINICAL IMMUNOLOGY

Mother-Newborn Pairs in Malawi Have Similar Antibody Repertoires to Diverse Malaria Antigens

Sarah Boudová,[a] Jenny A. Walldorf,[a]* Jason A. Bailey,[a] Titus Divala,[b] Randy Mungwira,[b] Patricia Mawindo,[b] Jozelyn Pablo,[c] Algis Jasinskas,[c] Rie Nakajima,[c] Amed Ouattara,[a] Matthew Adams,[a] Philip L. Felgner,[c,d] Christopher V. Plowe,[a] Mark A. Travassos,[a] Miriam K. Laufer[a]

Division of Malaria Research, Institute for Global Health, University of Maryland School of Medicine, Baltimore, Maryland, USA[a]; Blantyre Malaria Project, Malawi College of Medicine, Blantyre, Malawi[b]; Division of Infectious Diseases, Department of Medicine, University of California, Irvine, California, USA[c]; Antigen Discovery, Irvine, California, USA[d]

ABSTRACT Maternal antibodies may play a role in protecting newborns against malaria disease. *Plasmodium falciparum* parasite surface antigens are diverse, and protection from infection requires allele-specific immunity. Although malaria-specific antibodies have been shown to cross the placenta, the extent to which antibodies that respond to the full repertoire of diverse antigens are transferred from the mother to the infant has not been explored. Understanding the breadth of maternal antibody responses and to what extent these antibodies are transferred to the child can inform vaccine design and evaluation. We probed plasma from cord blood and serum from mothers at delivery using a customized protein microarray that included variants of malaria vaccine target antigens to assess the intensity and breadth of seroreactivity to three malaria vaccine candidate antigens in mother-newborn pairs in Malawi. Among the 33 paired specimens that were assessed, mothers and newborns had similar intensity and repertoire of seroreactivity. Maternal antibody levels against vaccine candidate antigens were the strongest predictors of infant antibody levels. Placental malaria did not significantly impair transplacental antibody transfer. However, mothers with placental malaria had significantly higher antibody levels against these blood-stage antigens than mothers without placental malaria. The repertoire and levels of infant antibodies against a wide range of malaria vaccine candidate antigen variants closely mirror maternal levels in breadth and magnitude regardless of evidence of placental malaria. Vaccinating mothers with an effective malaria vaccine during pregnancy may induce high and potentially protective antibody repertoires in newborns.

KEYWORDS malaria, vaccine, pregnancy, infant, placental malaria, protein microarray, antigenic diversity, antibody repertoire, vaccines

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Address correspondence to Miriam K. Laufer, mlaufer@som.umaryland.edu.

* Present address: Jenny A. Walldorf, Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

S.B. and J.A.W. contributed equally to this article.

Malaria is a major cause of morbidity and mortality for children throughout sub-Saharan Africa. Children in high transmission areas are at high risk of severe malaria early in life until they develop a state of semi-immunity following repeated exposure to *Plasmodium falciparum* infection. Developing a malaria vaccine has become a global public health priority. The first malaria vaccine to reach phase 3 trials and licensure (RTS,S) has reduced efficacy in infants compared to children (1), suggesting that alternative approaches will be needed to protect this age group.

Maternal immunoglobulin G (IgG) antibodies are transferred from the maternal circulation across the placenta to the fetus during pregnancy and are critical for protection of the neonate from infectious disease during the first few months of life.

The presence of these antibodies may protect infants from clinical malaria (2). In an effort to protect newborns against infectious diseases, mothers are given vaccines during pregnancy to increase the infant antibody level through transplacental transfer. For example, maternal tetanus vaccination is estimated to have reduced neonatal tetanus mortality by >90% (3, 4). Maternal influenza vaccination is estimated to have reduced respiratory illness hospitalizations in newborns by 29% and laboratory-confirmed influenza among newborns by 63% (5, 6).

The possibility of maternal vaccination to protect infants from malaria infection poses two unique challenges. First, because an effective malaria vaccine for any population must overcome the extreme antigenic diversity of the parasites (7, 8), a maternal vaccine must generate a broad array of antibodies that traverse the placenta. In addition, some studies have suggested that placental malaria infection interferes with the transfer of antibodies across the placenta (9–11). If this is true, vaccines administered to mothers who live in the highest risk settings may have compromised efficacy.

We have a unique opportunity to evaluate the repertoire of antibodies to diverse parasite antigen in mother-newborn pairs using a diversity-reflecting protein microarray we developed to assess seroreactivity to a wide range of naturally occurring variants in vaccine candidate antigens simultaneously. We used specimens collected from a well-characterized cohort of women with and without placental malaria infection in a clinical trial in Malawi to study maternal-fetal transfer of antibodies targeting leading vaccine candidates and their naturally occurring variants. The microarray included variants of *Plasmodium falciparum* apical membrane antigen 1 (AMA1), the 19-kDa fragment of merozoite surface protein 1 ($MSP1_{19}$), and reticulocyte binding-like homologue proteins (RH5). We sought to assess the breadth and the intensity of seroreactivity for these vaccine candidate antigens in a cohort of Malawian mother-infant pairs at the time of delivery. We determined the extent to which antibody repertoire is transferred to newborns and identified the factors that are associated with antibody levels at birth which may provide protection from malaria infection and disease during the first 6 months of life.

RESULTS

Demographics. Among 33 mother-infant pairs, the mean gestational age was 39.4 weeks (range 37.1 to 41.9). Most mothers were primigravid (84.8%); the remainder were secundigravid. The average maternal age was 19.6 years (range, 15 to 25 years). Two-thirds (66.7%) of mothers reported sleeping under a bed net the previous night. This cohort included 11 women whose placentas had evidence of infection at delivery: seven with only hemozoin pigment detected, one with both hemozoin- and quantitative PCR (qPCR)-detected parasites, and three with only parasites detectable by qPCR. There were no significant demographic differences between women with and those without placental malaria. Among the 11 women with placental malaria, 9 had one peripheral infection detected during pregnancy. Among the 22 women without placental malaria, 3 had a peripheral infection detected during pregnancy. No women had more than one peripheral infection during pregnancy.

Seroreactivity of specimens. Mothers and neonates had similar antibody repertoires (Fig. 1A). Each neonate's cord blood plasma recognized on average 66% of the variants of AMA1, 63% of the variants of $MSP1_{19}$, and 11% of the variants of RH5 proteins on the array. Antibodies in each mother's serum recognized on average 64% of the AMA1 variants, 62% of the MSP1 variants, and 11% of the RH5 variants on the array. North American malaria-naive controls had significantly lower mean seroreactivity compared to mothers or infants ($P = 0.001$ or $P < 0.001$, respectively; Fig. 1B). However, neither mothers nor infants had significantly greater mean seroreactivity to RH5 proteins than malaria-naive North American controls ($P = 0.96$ and $P = 0.48$, respectively; Fig. 1A).

Correlation between maternal and neonatal antibody breadth and levels. Mother and infant mean seroreactivities were not significantly different against any

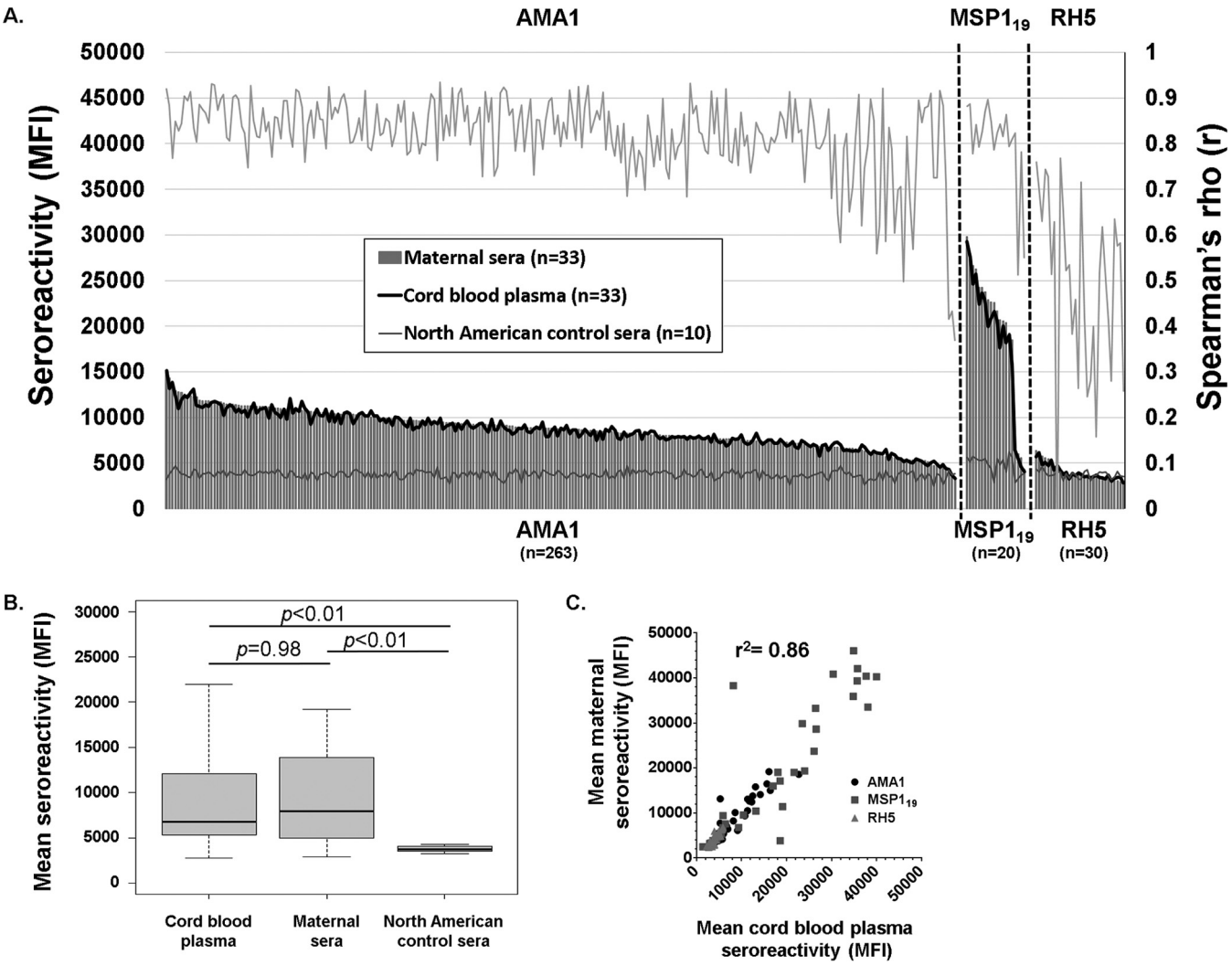

FIG 1 Seroreactivity to malaria vaccine candidate antigens in Malawian mothers and infants and in North American malaria-naive controls. (A) Mean seroreactivity and correlation of maternal serum ($n = 33$), infant cord plasma ($n = 33$), and North American control serum ($n = 10$) to 263 AMA1 variants, 20 $MSP1_{19}$ variants, and 15 RH5 variants printed in duplicate on the diversity-reflecting protein microarray. Antigen variants are arranged from highest to lowest mean maternal seroreactivity. Solid gray bars represent mean maternal seroreactivity, the black line depicts mean cord seroreactivity, and the gray line represents mean North American control seroreactivity. The secondary axis is the Spearman's rho correlation coefficient comparing seroreactivity to individual antigens in paired maternal cord blood samples. (B) Box-and-whisker plot displaying the distributions, medians, and interquartile ranges of mean seroreactivities to all antigens on the protein microarray by cohort. Mothers and infants had similar antibody profiles at birth ($P = 0.98$, Wilcoxon rank sum test). North American malaria-naive controls had significantly less seroreactivity to malaria antigens than Malawian mothers and newborns ($P < 0.01$, Wilcoxon rank sum tests). (C) Scatterplot showing the correlation of mean seroreactivity to malaria vaccine candidate antigens AMA1 (black circles), $MSP1_{19}$ (gray squares), and RH5 (light gray triangles) between paired mother and cord blood samples (Spearman's rho = 0.91, $P < 0.0001$). The slope of the line of best fit is 0.96 (95% confidence interval = 0.80 to 1.13), the overall goodness-of-fit is $r^2 = 0.86$, and the per-antigen-group Spearman's rho values are 0.93 for AMA1, 0.90 for $MSP1_{19}$, and 0.77 for RH5 ($P < 0.0001$ for all tests).

antigen individually after correction for multiple comparisons (Fig. 1A) or combined ($P = 0.98$; Fig. 1B). Maternal and neonatal mean antibody levels were highly correlated overall (Spearman's rho = 0.91, $P < 0.001$), and by antigen group (Spearman's rho = 0.93 AMA1, 0.90 $MSP1_{19}$, and 0.77 RH5 [$P < 0.001$ for all tests]; Fig. 1C).

To identify the factors associated with newborn antibody level, we modeled the average seroreactivity to all malaria antigens on the array. In the unadjusted linear regression model, increased mean maternal antibody level and placental malaria were associated with increased neonatal antibody levels ($P < 0.001$ and $P = 0.004$, respectively). In a multivariable linear regression model (including maternal antibody level, maternal age, placental malaria, gravidity, and gestational age at delivery) increased maternal antibody level was the only variable that was significantly correlated with increased infant antibody levels ($P < 0.001$).

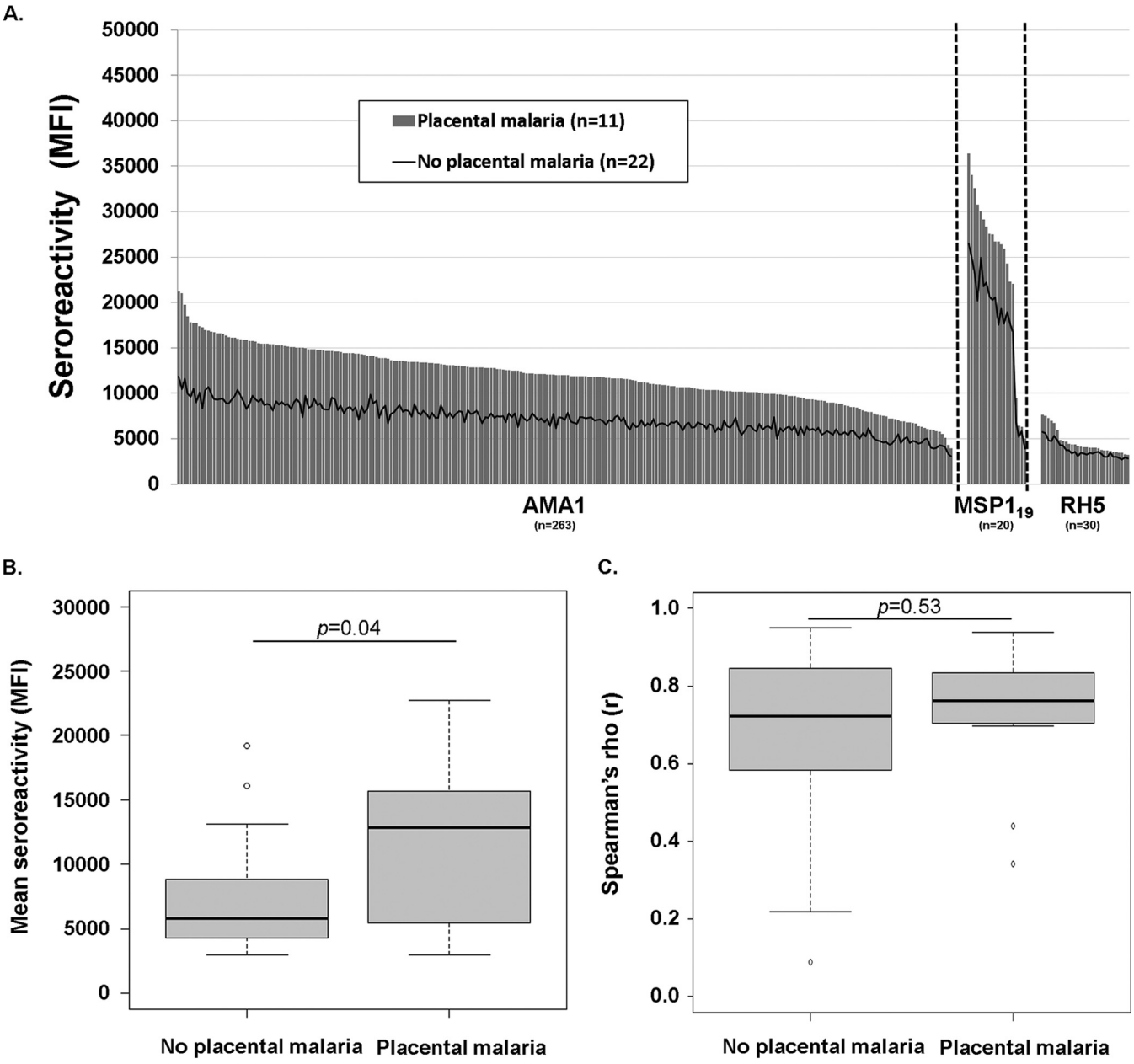

FIG 2 Mean seroreactivity to vaccine candidate antigen variants among mothers with and without placental malaria. (A) Bar-and-line plot of the mean seroreactivities of mothers with (n = 11) or without (n = 22) placental malaria to 263 AMA1 variants, 20 $MSP1_{19}$ variants, and 15 RH5 variants printed in duplicate on the diversity-reflecting protein microarray. Placental malaria was defined as women with histologic or molecular evidence of placental malaria. Antigen variants are arranged from highest to lowest mean seroreactivity for mothers with placental malaria. Solid gray bars represent the mean seroreactivity from mothers with placental malaria; the black line represents the mean seroreactivity from mothers without placental malaria. (B) Box-and-whisker plot depicting medians and interquartile ranges of mean maternal seroreactivities to AMA1, $MSP1_{19}$, and RH5 antigens by placental malaria status (*, P = 0.037 [Wilcoxon rank sum test]). (C) Box-and-whisker plot showing the medians and interquartile ranges of Spearman's rho correlation of mean all-antigen seroreactivities between paired maternal and cord blood samples in women with (n = 11) and without (n = 22) placental malaria.

Placental malaria increases maternal antibody levels. Mothers with placental malaria had significantly higher mean antibody levels against the vaccine candidate antigens compared to mothers without placental malaria (P = 0.037; Fig. 2A and 2B). Placental malaria was not associated with a decrease in antibody transfer to infants (P = 0.53; Fig. 2C). To determine the primary driving force behind maternal antibody levels, we used a multivariable linear regression model, including maternal age, placental malaria, and gravidity revealing that only placental malaria was significantly associated with maternal antibody levels (P = 0.008).

DISCUSSION

Newborns and mothers in this cohort from Malawi possessed similar levels of antibodies against blood-stage malaria vaccine candidate antigens. Maternal antibody level was the greatest predictor of neonatal antibody level, and placental malaria did not interfere with the repertoire or magnitude of antibodies transferred across the placenta in our multivariate model.

This study is the first to employ a high-throughput protein microarray to assess the maternal and infant repertoire of serological responses to malaria vaccine target antigen variants. This method allowed us to analyze infant seroreactivity to a large, diverse group of potential vaccine antigens and thoroughly characterize the efficiency of transplacental antibody transfer. Correlation between maternal and neonatal antibody responses to malarial antigens has been studied for whole parasite extract or for individual variants of liver-stage antigen, MSP, AMA, or glutamate-rich protein (12–14), whereas the protein microarray allows standardized methodology across a broad repertoire of multiple antigens and their variants. It is thought that a robust, strain-specific immune response develops after repeated exposure to specific variants. The variability in seroreactivity to AMA1, RH5, and $MSP1_{19}$ variants may reflect the relative abundance of each variant circulating in the population.

In our study, infant seroreactivity to any given antigen was nearly identical to mean maternal seroreactivity for that antigen, regardless of placental malaria infection during pregnancy. Overall, given the consistency of these patterns across multiple variants of the malaria antigens tested, we would anticipate that the same holds true for other blood-stage antigens. We observed low antibody levels in both mothers and infants against all variants of RH5. This is not surprising given that RH5 is only transiently surface-exposed during cell attachment and entry and unlikely to elicit natural immunity (15). The overall low seroreactivity could explain lower, but still statistically significant, correlation between the mother-infant paired blood samples, compared to the other antigen groups.

For all antigen variants, mothers with placental malaria had higher mean seroreactivity than mothers without placental malaria. This likely reflects an increase in antibody production due to a recent malaria infection, and in univariate analysis placental malaria was associated with increased antibody levels in infants. Malaria during pregnancy can boost antibody levels against multiple antigenic variants, providing a broad antibody repertoire to the child when the antibodies are transferred across the placenta. This raises the possibility that maternal vaccination with even a single antigen variant could protect children against multiple malaria strains by boosting overall maternal anti-malarial antibodies—suggesting a possible new role for the RTS,S vaccine which has had limited efficacy when administered to infants. Although maternally transferred antibodies may simply be a marker of malaria exposure rather than a source of protection against severe malaria in infancy (16), our data support the possibility that maternal vaccination could protect infants.

There are a few limitations to this study that may affect generalizability. Due to our sample size and the capacity of our customized protein microarray, we could not examine the effects of HIV, preterm birth or hypergammaglobulinemia on transplacental antibody transfer. These conditions have previously been shown to limit the transfer of maternal antibodies (11, 17). While this may result in differences in total levels of antibodies transferred, we believe this would not change how well the infant and maternal antibody repertoires mirror each other. We also observed few cases of active placental malaria at the time of delivery. Most cases of placental malaria were cleared by the time of delivery, and only low concentrations of hemozoin pigment were detected. As a result, there was minimal placental inflammation and no observed reduction of transplacental transfer of antibodies, as has previously been observed (10, 11, 17). Thus, our conclusions may not be applicable in cases of severe or chronic placental malaria. With our current microarray platform we were unable to probe for differences in antibody subclasses being transferred. This will be an important outcome

for future studies of transplacental antibody transfer along with functional studies of the antibodies. Finally, women in our study were closely monitored and received high quality antenatal care, which may have resulted in fewer cases of malaria than women receiving standard antenatal care. However, with the widespread availability of intermittent preventive treatment of malaria during pregnancy, the lower incidence of placental malaria seen in our population may be more typical of pregnancy-associated malaria in African countries approaching elimination, as well as in lower transmission settings such as Southeast Asia and South America. These findings may be important as malaria approaches elimination is these regions.

In summary, our data suggest it is possible that interventions targeting the mother may impact infant health. We observed that in an area where malaria is endemic, mothers and their newborns had similar antibody levels across families of vaccine candidate antigens and the breadth of seroreactivity to diverse antigens was efficiently transferred from mother to infant. Our results have implications for malaria vaccine administration. Given the high efficiency of transplacental transfer of antibodies to diverse vaccine candidate antigen variants, these data support examining the functionality of antibodies transferred and the possibility of administration of malaria vaccines to pregnant women to protect infants in early life, until they can be effectively vaccinated. However, care should be taken as it is also possible that maternal vaccination may interfere with vaccine efficacy in the infant. Future studies will need to consider all of these possible outcomes.

MATERIALS AND METHODS

Population. Study samples were obtained from women enrolled in a randomized controlled trial of intermittent preventive treatment during pregnancy (IPT) in the periurban region of Blantyre, Malawi, where malaria is endemic (ClinicalTrials.gov identifier NCT01443130). Trial participants were women in their first or second pregnancy selected from the control arm receiving sulfadoxine-pyrimethamine IPT, which is the standard of care for pregnant women in sub-Saharan Africa. Participants were also confirmed to be HIV negative as part of the trial protocol. Because prematurity has been associated with decreased efficiency of transplacental antibody transfer (9, 10), deliveries that occurred prior to 37 weeks were excluded from this analysis. All mother-infant pairs with evidence of placental malaria and available specimens that met these criteria were included in this study. In addition, mother-infant pairs who met these criteria with no placental malaria were randomly selected from among 55 trial participants with available paired maternal delivery and cord specimens. A minimum number of paired samples was selected to ensure power to test statistical concordance between maternal and cord seroreactivity. The availability of specimens was limited based on the number of enrolled trial participants who had delivered by the time this analysis was designed. Ten North American malaria-naive controls were included for comparison with Malawian pairs.

Study procedures. At the time of enrollment in the study, demographic data, including gravidity and maternal age, were recorded. Study team members performed ultrasounds in the second trimester to determine gestational age. Women were monitored from 20 to 26 weeks gestation through delivery through monthly routine visits and evaluations when they were sick. When participants had symptoms suggestive of malaria, they were tested by microscopy and treated if malaria infection was detected. Fingerprick filter papers were collected for later molecular detection of malaria using 18S rRNA target for real-time qPCR (18).

Plasma and serum samples were collected at the time of delivery from the cord blood and from the participants, respectively. Peripheral plasma samples were collected from the cord blood from the umbilical cord at delivery, and serum samples were collected from the mother by finger prick using a Sarstedt microvette CB300 (clotting activator/serum, 16.440.100). Samples were frozen and shipped to the University of Maryland laboratory for analysis.

The placenta was collected for gross inspection and a full-thickness biopsy for histological examination. A filter paper blood spot was collected for molecular detection of malaria. Placental malaria was defined as the presence of malaria parasites or hemozoin pigment in the placenta or parasite DNA detected by qPCR.

Ethical approval was obtained from the University of Malawi College of Medicine's Research Ethics Committee and the University of Maryland Baltimore Institutional Review Board. Written informed consent was obtained from all participants before conducting any study-related activities. Participants had the option to withdraw from the study at any time. All data were recorded and analyzed anonymously.

Protein microarrays. Protein microarrays were populated with field-isolated, sequence-verified variants of AMA1, RH5, and $MSP1_{19}$ obtained from clinical infections collected during a phase 2 randomized control trial in Mali (18). This diversity-reflecting approach resulted in 263 AMA1, 20 $MSP1_{19}$, and 15 RH5 variants (printed in duplicate), as well as positive and negative controls. Protein microarrays were generated, probed, and analyzed as previously described (19, 20). Seroreactivity was defined as the median fluorescent intensity of each individual probe captured by the GenePix 4000B microarray scanner (Molecular Devices).

Statistical analysis. All statistical analyses were performed using R statistical environment (http://www.R-project.org) and GraphPad Prism (v6.01). For comparisons of mean seroreactivity to antigens and antigen variants, we used nonparametric Wilcoxon rank sum tests to accommodate skewness of the distribution of seroreactivity data. We considered any *P* value below the 0.05 alpha level to be statistically significant. Spearman's rho statistic was used to estimate the correlation of maternal to infant antibody titers. Linear regression of the correlation between mother-infant pairs was used to determine the slope and goodness of fit.

Generalized linear models were designed to measure the association of study variables controlling for covariates with the average median fluorescent intensity of all antigenic variants. We measured variance inflation factors to assess multicollinearity of study variables in the final model. One mother-infant pair was an outlier as determined by a statistically significant Student *t* test residual after Bonferroni correction and was removed from the final sample set. The removal of this sample did not change the direction of the associations or the conclusions drawn from the model.

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P.L.F. holds patents related to technology applied in this study and has stock positions with Antigen Discovery. All other authors report no potential conflicts.

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