IMMUNITY TO PARASITIC INFECTIONS IN PREGNANCY

EDITED BY: Justin Yai Alamou Doritchamou, Adrian John Frederick Luty and Elizabeth Helen Aitken

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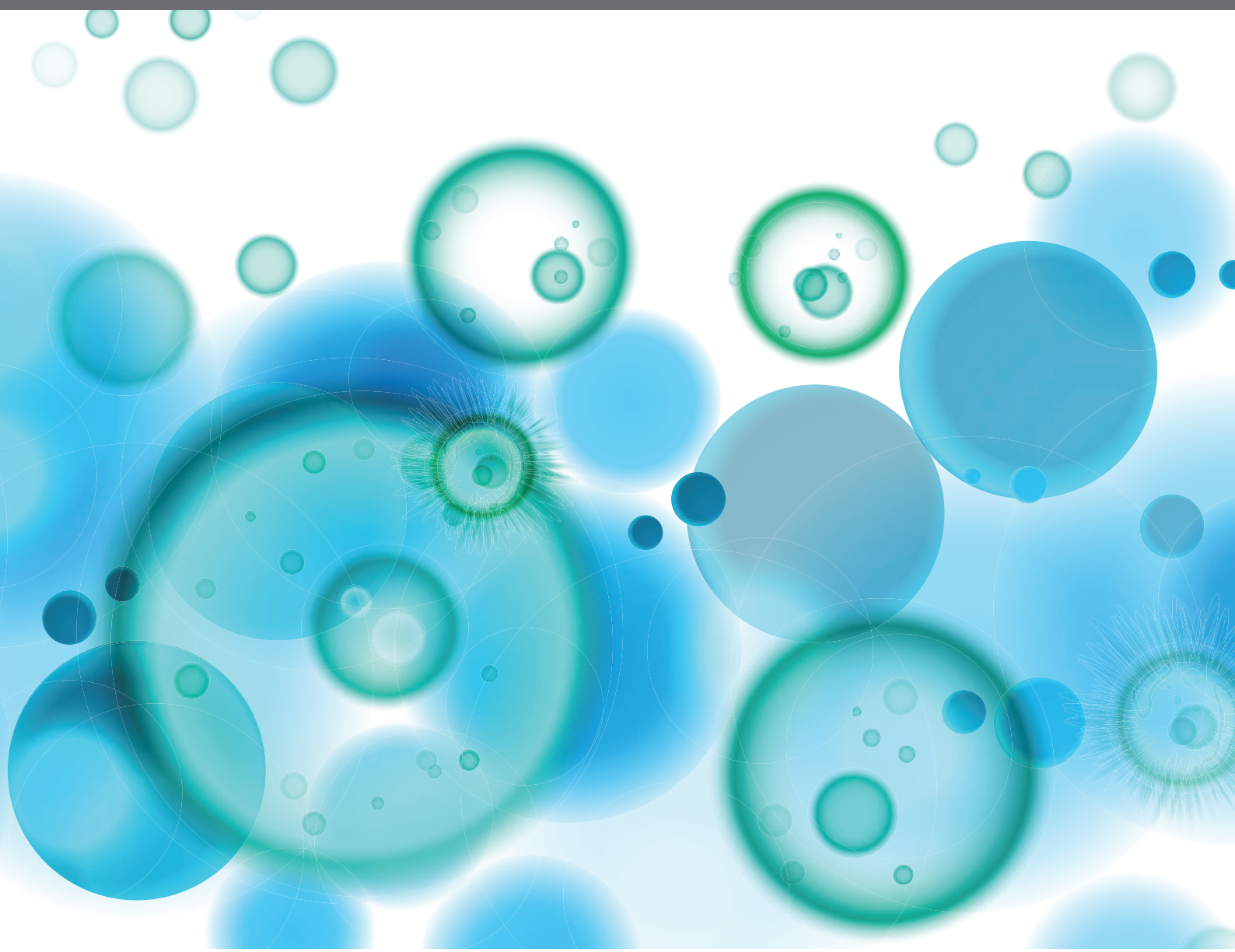

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IMMUNITY TO PARASITIC INFECTIONS IN PREGNANCY

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EDITORIAL
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Editorial: Immunity to Parasitic Infections in Pregnancy

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Keywords: pregnancy, parasitic infections, immunity, therapeutics, vaccine, malaria, toxoplasmosis, trypanosomiasis

Editorial on the Research Topic

Immunity to Parasitic Infections in Pregnancy

INTRODUCTION

Despite having a degree of pre-existing immunity to plasmodial and other parasitic infections, pregnancy may render women more susceptible to these pathogens than their non-pregnant counterparts due, at least in part, to pregnancy-related alterations in immune responsiveness. Parasitic infections during pregnancy represent a major risk to maternal and fetal health (1–3). The maternal immune system must establish a balance between immune tolerance of the semi-allogeneic fetus and appropriate immune responses to pathogens. The particular case of infection with *Plasmodium falciparum*, the cause of the syndrome known as placental malaria (PM), illustrates the pathological consequences of excessive inflammatory responses on fetal development and maternal health. Not all parasitic pathogens are as virulent, but protozoa such as *Toxoplasma gondii* or *Trypanosoma cruzi* also challenge the maternal immune system and affect susceptibility to infections in early life. Thus, improved understanding of host-parasite interactions and the underlying immune mechanisms involved in parasitic infections during pregnancy will help define more efficient and effective approaches to tackle the burden of these infections and improve maternal-fetal health.

Plasmodium and other human parasitic species rely largely on their well-developed ability to manipulate the immune system to their own advantage, allowing them to establish chronic infections. On the other hand, pregnant women can develop a protective immunity that relies on both humoral and cellular immune mechanisms. The six original research and six review articles, published in this Research Topic, provide insights into our understanding of the diverse mechanisms involved in generating immune response to toxoplasmosis, trypanosomiasis and plasmodial infections, in the context of pregnancy, their impact on pregnancy outcomes, and disease control strategies including vaccines.

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TOXOPLASMOSIS AND TRYPANOSOMIASIS DURING PREGNANCY

Chagas disease and toxoplasmosis, caused by *Trypanosoma cruzi* and *Toxoplasma gondii* (respectively), are two parasitic infections congenitally transmissible to the child, causing perinatal morbidity and mortality (2, 3). Medina et al. demonstrate these parasites induce a differential microRNA profile in human placental explants in an *ex-vivo* model. Furthermore, *in silico* analysis of the differentially expressed miRNAs reveals the predicted-targets regulated different cellular processes involved in cell development and immunity. Barros et al. review current advances and perspectives in the development of vaccines in congenital toxoplasmosis, highlighting the need for investigations to study the nature of protective immunity at the maternal-fetal interface; there has been no consensus on the optimal immune response required to protect both mother and fetus in congenital infection using live or subunit vaccination approaches.

MALARIA DURING PREGNANCY: PATHOGENESIS

In malaria infection during pregnancy, there has been a strong interest to understand how malaria infection and placental pathology may result in adverse outcomes. Sarr et al. examine granulocytes and markers of neutrophil activation in pregnant women with, and without, *P. falciparum* and HIV infections. They note that granulocyte counts in peripheral and placental blood are differentially affected by infection and that markers of activation are increased in those with PM. Kabyemela et al. investigate potential causes of fetal anemia in a region of high malaria transmission, and report that fetal anemia is not associated with the presence of *P. falciparum* infection at delivery; they identify other factors (including cytokines, α thalassemia, and soluble transferrin receptor), which have been previously implicated in the pathogenesis of anemia. Finally, Chua et al. review our current knowledge on the different pathological pathways leading to low birthweight of babies from mothers that experienced malaria during pregnancy. Current strategies to prevent and manage malaria in pregnancy as well as potential therapeutic interventions that may improve birth outcomes are also discussed.

MALARIA DURING PREGNANCY: IMMUNITY

Although common in pregnancy, maternal anemia can be exacerbated by malaria resulting in increased risk of maternal morbidity and mortality (1). Wiebe and Yanow review the possible roles played by antibodies to malarial variant surface antigens (VSA) on infected erythrocytes (IEs) in protecting mothers from maternal anemia, and discuss current knowledge gaps. It appears that pathways leading to anemia in PM are complicated and multifactorial, with several conflicting studies finding no-, mixed- or inverse-associations between anti-VSA antibodies and maternal anemia during pregnancy malaria.

Immune competence in the infant during malaria infection is also reviewed by Callaway et al. in order to translate our current understanding of fetal and neonatal immunology into safe and immunogenic vaccines that can be administered in early infancy. The authors highlight that the fetus is immunologically competent and can mount adaptive B and T cell responses to perinatal pathogens *in utero*, providing proof-of-concept that induction of protective immunity prior to birth may be possible.

VAR2CSA ANTIBODIES AND PLACENTAL MALARIA VACCINE DEVELOPMENT

P. falciparum parasites, which cause PM, express the protein VAR2CSA on the surface of IEs (4), and pregnant women with elevated levels of high avidity antibodies to VAR2CSA early in pregnancy had a reduced risk of PM at delivery (5). Vanda et al. demonstrate that high avidity antibodies to VAR2CSA are predominately restricted to the DBL5 domain of VAR2CSA, and their levels increase from first to second pregnancy (with affinity maturation of antibody taking place primarily during the second pregnancy), while positively influencing the baby's birthweight. Elevated levels of VAR2CSA-specific total IgG and cytophilic IgG3 during pregnancy are also associated with higher birth weights, as shown by Tornyigah et al. The authors evaluate the subclass of anti-VAR2CSA IgG responses in a cohort of pregnant Beninese women using the PAMVAC candidate vaccine antigen, and demonstrate that cytophilic IgG1 and IgG3 responses to VAR2CSA are the most frequent, whilst high levels of IgG4 are associated with reduced risk of placental infections. This study provides evidence that protection induced by VAR2CSA antibodies results from coordinated activities between both cytophilic and non-cytophilic antibodies.

Consistent with reports from Vanda et al. and Tornyigah et al., McLean et al. demonstrate that levels of VAR2CSA IgG are significantly associated with increased birth weight in a longitudinal study of women infected with malaria during pregnancy. In particular, the authors show that women who had been infected by mid-pregnancy at enrolment had higher levels of antibodies to VAR2CSA associated with a reduced risk of adverse outcomes, in contrast to women uninfected at enrolment. Findings from this study highlight that high levels of VAR2CSA antibodies early in pregnancy may lead to better pregnancy outcomes.

VAR2CSA is the leading vaccine candidate to prevent PM particularly in first-time mothers who are at greater risk of poor pregnancy outcomes due to PM. Tomlinson et al. critically review the host defense evasion mechanisms employed by VAR2CSA-expressing *P. falciparum* during PM. The review focuses on VAR2CSA roles in PM pathogenesis, including

cytoadhesion in the placenta as well as modulation of the placental microenvironment by pregnancy-specific IEs to escape recognition by protective antibodies.

After decades of preclinical development of PM vaccines, two VAR2CSA-based subunit vaccines have recently been tested in phase 1 trials (6, 7). In their review, Gamain et al. discuss recent advances in PM vaccine development, with a focus on recent clinical data, and outline the next clinical steps required to improve our understanding of vaccine-induced immunity and accelerate second generation of PM vaccine development.

CONCLUSION

In summary, the burden of parasitic diseases in pregnant women remains a major global health problem. The published articles in this Research Topic show the complexity of the relationship between infection, immunity, and clinical outcomes of parasitic infections in pregnancy. Although *P. falciparum* is a dominant pathogen in parasitic infections during pregnancy, this collection also notes the roles of other protozoa that can challenge the maternal-fetal immunity. These articles also provide a better understanding of the diverse mechanisms involved in generating immune responses to parasitic infections during pregnancy and their impact on pregnancy outcomes that will inform future therapeutic approaches, including vaccination, to protect the mother and fetus health.

AUTHOR CONTRIBUTIONS

JD and EA wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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ORIGINAL RESEARCH
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Trypanosoma cruzi and *Toxoplasma gondii* Induce a Differential MicroRNA Profile in Human Placental Explants

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Trypanosoma cruzi and *Toxoplasma gondii* are two parasites than can be transmitted from mother to child through the placenta. However, congenital transmission rates are low for *T. cruzi* and high for *T. gondii*. Infection success or failure depends on complex parasite-host interactions in which parasites can alter host gene expression by modulating non-coding RNAs such as miRNAs. As of yet, there are no reports on altered miRNA expression in placental tissue in response to either parasite. Therefore, we infected human placental explants *ex vivo* by cultivation with either *T. cruzi* or *T. gondii* for 2 h. We then analyzed the miRNA expression profiles of both types of infected tissue by miRNA sequencing and quantitative PCR, sequence-based miRNA target prediction, pathway functional enrichment, and upstream regulator analysis of differentially expressed genes targeted by differentially expressed miRNAs. Both parasites induced specific miRNA profiles. GO analysis revealed that the *in silico* predicted targets of the differentially expressed miRNAs regulated different cellular processes involved in development and immunity, and most of the identified KEGG pathways were related to chronic diseases and infection. Considering that the differentially expressed miRNAs identified here modulated crucial host cellular targets that participate in determining the success of infection, these miRNAs might explain the differing congenital transmission rates between the two parasites. Molecules of the different pathways that are regulated by miRNAs and modulated during infection, as well as the miRNAs themselves, may be potential targets for the therapeutic control of either congenital Chagas disease or toxoplasmosis.

Keywords: *Trypanosoma cruzi*, *Toxoplasma gondii*, human placental explants, miRNA profile, host gene expression

INTRODUCTION

More than one billion people worldwide are burdened by parasitic diseases (1). Of these, Chagas disease (American trypanosomiasis) and toxoplasmosis are caused by *Trypanosoma cruzi* (*T. cruzi*) and *Toxoplasma gondii* (*T. gondii*), respectively (2–4). Chagas disease is a devastating but neglected health problem in Latin America. Due to the extensive global migration of asymptomatic individuals, this infection has become an emerging disease in non-endemic countries. Congenital transmission is partially responsible for the progressive globalization of Chagas disease (5, 6). *T. gondii* is one of the most successful parasites on earth and is estimated to infect over one billion people worldwide (7). Importantly, both parasites can be congenitally transmitted and cause perinatal morbidity and mortality (2–4) but present different transmission rates. *T. cruzi* has a low transmission rate (1–12%) (6, 8) while *T. gondii* has a high transmission rate (22–72%) (3). Moreover, both parasites elicit a different local placental immune response that might be related to infection susceptibility (9, 10). Thus, *T. cruzi* and *T. gondi* infection is related to the expression and activation of different Toll-like receptors, which in turn mediate the secretion of different cytokines and chemokines in defense against both parasites in the placenta (9, 11).

However, the probability of congenital transmission depends on a variety of complex interactions between the pathogen and the host (4, 12). In particular, parasite factors, placental factors, and maternal and developing fetal immune systems determine infection occurrence (4, 13). In this context, both parasites display sophisticated strategies to avoid host defenses and virulence factors that increase the chance of establishing infection and long-term persistence. One of these strategies is the ability to modulate host cell gene expression (14–16) through small non-coding RNAs such as microRNAs (miRNAs) that repress mRNAs in a sequence-specific manner by either an mRNA degradation process or through mRNA translation inhibition (17–19). MiRNAs play a key role in fine tuning gene expression in multiple physiological and pathological conditions including *T. cruzi* (20) or *T. gondii* (21) infection. Interestingly, the largest miRNA cluster in humans is encoded in chromosome 19 (C19MC; 19q13.41) and is almost exclusively expressed in the placenta (22). Both C19MC-derived and non-C19MC-derived miRNAs have been associated with placental development pathologies such as pre-eclampsia and infection (23, 24). However, there is no report in the literature regarding altered miRNA expression in placental tissue in response to either parasite.

Here, we infected human placental explants (HPE) *ex vivo* by 2 h of incubation with either *T. cruzi* or *T. gondii*, then analyzed both miRNA expression profiles by miRNA sequencing and quantitative PCR of selected miRNAs. In addition, we used sequence-based miRNA target prediction and performed pathway functional enrichment and upstream regulator analysis of differentially expressed genes targeted by differentially expressed miRNAs (DEMs).

MATERIALS AND METHODS

Parasite Culture and Harvesting

For *T. cruzi*, Y strain (*T. cruzi* II) trypomastigotes were obtained from previously infected Vero cells (ATCC® CCL-81) grown in RPMI medium supplemented with 5% fetal bovine serum and 1% antibiotics (penicillin-streptomycin) at 37°C in a humid atmosphere with 5% CO_2. Parasites invaded the cells and replicated intracellularly as amastigotes. After 48–72 h, amastigotes transformed back to trypomastigotes and lysed the host cells. The infective trypomastigotes were separated from cellular debris by low speed centrifugation (500 × *g*) for 10 min. Parasites were isolated from the supernatant by centrifugation at 3500×*g* during 15 min, suspended in RPMI media (without fetal bovine serum, 1% (penicillin-streptomycin) (RPMI 1640®, Biological Industries Ltd.), and quantified in a Neubauer chamber (9).

For *T. gondii*, semi-confluent HFF cells were infected with RH tachyzoites at a multiplicity of infection of 3 to 5 parasites per cell. After 40 h, the infected cells were washed, then monolayers were scraped from the flasks and passed through 20-, 23-, and 25-gauge needles. Tachyzoites were purified from host cell debris with a 3.0 µm Isopore filter (Merck Millipore®) (25).

The laboratory has been certificated as a Biosafety level 2 laboratory by the Biosafety Committee ("Unidad de Prevención de Riesgo") of the "Facultad de Medicina, Universidad de Chile" (approval # 0403/2019).

HPE Infection

Human term placentas were obtained from 3 women with uncomplicated pregnancies with vaginal or caesarean delivery. Informed consent for experimental use of the placenta was given by each patient as stipulated by the Code of Ethics of the "Servicio de Salud Metropolitana Norte" (approval number 0010/2019). Exclusion criteria consisted of the following: major fetal abnormalities, placental tumor, intrauterine infection, obstetric pathology, positive serology for Chagas disease, and any other maternal disease. Donor patients were negative for anti-*T. gondii* IgG/IgM antibodies. The organs were collected in cold, sterile, saline-buffered solution (PBS) and processed no more than 30 min after delivery. The dissected explants (approximately 50 mg of tissue) were washed with sterile PBS to remove the blood and co-cultivated with *T. cruzi* trypomastigotes or *T. gondii* tachyzoites (10^5 parasites/ml) in serum free RPMI media. After 2 h of co-cultivation, explants were collected in RNAlater™ solution (ThermoFisher Scientific®), then stored at 4°C for 24 h and at -80°C for posterior RNA isolation (9). Three independent experiments were carried out in triplicates; HPEs from each placenta were infected with either *T. cruzi* or *T. gondii* parasites. The parasite load in the HPEs was confirmed by real-time PCR as described previously by us (9, 10, 26).

RNA Extraction

Total RNA was extracted from HPE by mechanical disruption in 1.3 ml of RNA-solv® reagent (Omega Bio-tek) and isolated using

an E.Z.N.A® total RNA kit I (Omega Bio-tek) according to manufacturer instructions. RNA was stored at -80°C until analysis. The concentration and quality of RNA was determined with a Qubit® RNA HS Assay kit and an IQ Assay kit (Invitrogen), respectively. Only RNA samples with an IQ ≥8 were further analyzed for quality with an Agilent 2100 Bioanalyzer System (Agilent Technologies, USA) using an RNA Nano 6000 Assay Kit. RNA samples with RNA integrity numbers >5.0 were used for miRNA profiling analysis (9).

Library Construction and Sequencing

Small RNA-Seq libraries were constructed with an Illumina TruSeq Small RNA library preparation kit according to manufacturer protocols. To assess the quality of the libraries, a DNA High Sensitivity Chip was used in an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The libraries were sequenced on an Illumina NextSeq 500 platform. For each condition, three independent biological replicates were sequenced and paired-end reads were generated.

Data Analysis

Raw read quality was evaluated using the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were analyzed with Trim Galore Cutadapt software (27) and low-quality reads were removed (phred value < 30) in order to obtain clean reads. Clean reads with a length range of 18–35 nucleotides were chosen to perform all subsequent analyses. The sofware STAR (28) was used to align all reads to the reference human genome sequence (Hg38). The read counts per coding sequence were determined using HTSeq-count (29). To evaluate replicates, we used Principal Component Analysis, Pearson correlation and standardized median correlation analyses and box plots. The program EdgeR was used for differential expression analysis (30). Differentially expressed genes were defined as genes with p-value <0.05. Target gene prediction performed by using miRDB, psRNA target, and TargetScan sofwares.

Enrichment Analyses

miRNA set enrichment analysis was performed using the TAM 2.0 tool (http://www.lirmed.com/tam2/). KEGG pathways and functional annotation of the predicted target genes (https://www.genome.jp/kegg/kegg2.html) were also analyzed to determine the biological processes, molecular functions, cellular components, and associations with disease.

RT-qPCR

RNA enriched in small RNAs was extracted from HPEs (approximately 50 mg of tissue) by mechanical disruption in 1 ml RNAzol® RT (Sigma-Aldrich) according to manufacturer instructions and stored at -80°C until analysis. The concentration of the isolated miRNAs was determined using a Qubit® Quant-iT™ microRNA Assay Kit (Molecular Probes). cDNA of mature miRNAs was synthesized with a MystiCq™ microRNA cDNA Synthesis Mix Kit (Sigma-Aldrich Merck) per manufacturer guidelines. The 25 µl RT-qPCR reaction contained 12.5 µl 2× MystiCq microRNA SYBR Green qPCR Ready Mix, 0.5 µl of 10 µM MystiCq Universal PCR Primer, 0.5 µl of 10 µM of each specific MystiCq microRNA qPCR Assay Primer (**Supplementary Table 1**), 10.5 µl nuclease-free water, and 1 µl cDNA. All RT-qPCR reactions were performed in triplicates. RT-qPCR was performed under the following cycling conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Gene expressions were calculated using the $2^{-\Delta\Delta CT}$ relative expression method and normalized to snRNA U6 (RNU6-1) expression levels (31).

RESULTS

T. cruzi and *T. gondii* Change the miRNA Expression Profile in HPE

The effects of *T. cruzi* and *T. gondii* on placental tissue were assayed in HPE after a 2 h challenge with 10^5 parasites/ml. Total miRNA extracted from infected and non-infected control HPE was analyzed by miRNA Seq. Key characteristics of the obtained sequencing data are summarized in **Table 1**. A total of 680 and 686 DEMs were identified in *T. cruzi* and *T. gondii* infected HPE, respectively. Only 14 DEMs with a minimum 1.5-fold change in expression and a 95% probability of being differentially expressed ($p \leq 0.05$) were identified in *T. cruzi* challenged samples (**Figure 1A**). In *T. gondii* challenged samples, the number of DEMs increased to 42 (**Figure 1B**). Comparison of *T. cruzi* infected HPE with non-infected control samples showed that five

TABLE 1 | Statistics of the small RNA sequences obtained in this study.

	M reads (millions)	M Aligned (millions)	% aligned	Mature microRNA reads	# Mature MicroRNAs	# Mature MicroRNA (single aligned)
S1 Control	12,96	11,68	90,12	3489287	922	732
S2 Control	15,17	13,87	91,43	4855933	938	760
S3 Control	15,58	13,16	84,50	3030989	843	714
S4 *T. cruzi*	14,35	12,60	87,77	3000672	907	712
S5 *T. cruzi*	14,76	12,94	87,69	3910944	907	737
S6 *T. cruzi*	16,13	13,15	81,55	3571933	914	720
S7 *T. gondii*	13,57	11,82	87,07	3160630	916	737
S8 *T. gondii*	14,40	12,79	88,77	4747358	962	757
S9 *T. gondii*	13,51	12,16	89,99	4148647	931	763

M reads, total of sequences in analysis after raw data processing (millions); M aligned, total of mapped sequences to the human genome (millions); % aligned, total of mapped sequences to the human genome (percentage); Mature microRNA reads, mapped reads counted as mature miRNAs; # Mature microRNAs, number of mature miRNAs; # Mature MicroRNA (single aligned), number of mature miRNAs associated to unique miRNA precursors in the reference.

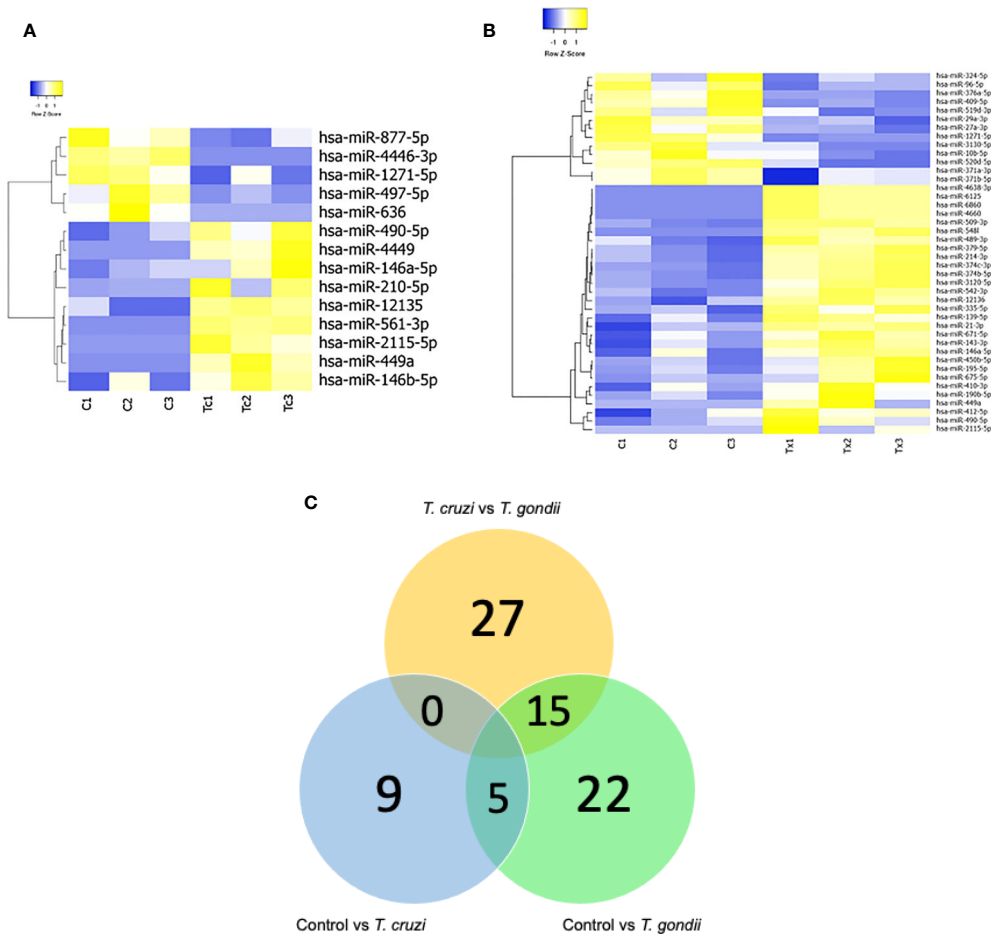

FIGURE 1 | Expression profiling of mature miRNAs in human placental explants (HPEs) following *T. cruzi* or *T. gondii* infection. **(A)** Heat-map of differentially expressed microRNAs (DEMs) in control vs. *T. cruzi* infected HPE and **(B)** control vs. *T. gondii* infected HPE. The filtered miRNA data were subjected to unsupervised hierarchical clustering analysis. The metric was set as the Euclidean distance. Control: C1, C2, C3; *T. cruzi:* Tc1, Tc2, Tc3; *T. gondii:* Tx1, Tx2, Tx3. **(C)** Venn diagram showing the number of commonly expressed and specifically expressed miRNAs between the infected HPE groups. Significant miRNAs for HPE with *T cruzi* infection vs. those with *T. gondii* infection are shown in the yellow circle. The blue circle represents the miRNAs that discriminate between the control (uninfected HPE) and *T. cruzi* infected HPE, while the green circle represents the miRNAs that distinguish the control (uninfected HPE) and *T. gondii* infected HPE.

miRNAs were downregulated and nine were upregulated. In *T. gondii* infected tissues, 13 miRNAs were downregulated and 29 were upregulated. The Venn diagram in **Figure 1C** shows the miRNAs that were differentially expressed in the presence of both parasites compared to non-infected control samples and in HPE infected with either parasite. The complete list of DEMs in response to *ex vivo T. cruzi* and *T. gondii* infection is shown in **Table 2**.

Functional Annotation and KEGG Pathway Enrichment Analysis of miRNA Target Genes

To better understand the roles of the miRNAs identified in HPE in response to *ex vivo* infection with both parasites, the target genes of the miRNAs were identified using miRDB, psRNA target, and TargetScan. GO and KEGG enrichment analyses used to identify the biological functions of the DEMs ($p < 0.05$) during *ex vivo T. cruzi* and *T. gondii* infection revealed 679 best scored target genes of the 14 miRNAs from *T. cruzi* vs. control, 1970 best scored target genes of the 42 miRNAs from *T. gondii* vs. control, and 2011 best scored target genes of the 42 miRNAs from *T. cruzi* vs. *T. gondi*. The target genes of the DEMs are shown in **Supplementary Table 2**. Among the significantly enriched GO terms in *T. cruzi* vs. control samples, DEMs were significantly enriched in regulation of NFκB pathways, chondrocyte development, cell death including apoptosis, peritoneal cavity homeostasis, angiogenesis, cell cycle, megakaryocyte differentiation, Toll-like receptor signaling pathway, and immune response including innate immunity (**Figure 2A**). Among the significantly enriched GO terms in *T. gondii* vs. control samples, DEMs were significantly enriched in cell proliferation, cell migration, osteoblast differentiation, oxidative stress, lipid metabolism, regulation of stem cells including embryonic stem cells, hepatotoxicity, DNA damage response, regulation of NFκB pathways, smooth muscle

TABLE 2 | The top differentially expressed miRNAs (P < 0.05) in HPE challenged with 10^5 *T. cruzi* trypomastigotes or *T. gondii* tachyzoites during 2 h.

	miRNAs	Fold Change	p-value	p-adjustment	Expression
Control vs *T. cruzi*	hsa-miR-490-5p	1,47644601	0,011939305	1	Up-regulated
	hsa-miR-497-5p	-0,95822045	0,021380502	1	Down-regulated
	hsa-miR-146a-5p	0,9111643	0,024398238	1	Up-regulated
	hsa-miR-12135	2,76826832	0,025125553	1	Up-regulated
	hsa-miR-210-5p	1,17471038	0,038576256	1	Up-regulated
	hsa-miR-146b-5p	0,70985057	0,041583753	1	Up-regulated
	hsa-miR-877-5p	-0,71572824	0,049301458	1	Down-regulated
	hsa-miR-1271-5p	-1,48497011	0,049553349	1	Down-regulated
T. cruzi (*treatment exclusive)	hsa-miR-636		0,003652701	1	Up-regulated
	hsa-miR-4449		0,005197851	1	Up-regulated
	hsa-miR-449a		0,009529598	1	Up-regulated
	hsa-miR-2115-5p		0,013101594	1	Up-regulated
	hsa-miR-561-3p		0,023021063	1	Up-regulated
	hsa-miR-4446-3p		0,041796581	1	Up-regulated
Control vs *T. gondii*	hsa-miR-12136	1,79576197	0,00017626	0,120736654	Up-regulated
	hsa-miR-335-5p	1,35817451	0,0007489	0,227732906	Up-regulated
	hsa-miR-10b-5p	-1,51399053	0,00099737	0,227732906	Down-regulated
	hsa-miR-1271-5p	-2,41452099	0,00206894	0,267855922	Down-regulated
	hsa-miR-409-5p	-2,51430487	0,00225422	0,267855922	Down-regulated
	hsa-miR-27a-3p	-0,97356696	0,00234618	0,267855922	Down-regulated
	hsa-miR-29a-3p	-0,92877383	0,00344997	0,337603687	Down-regulated
	hsa-miR-214-3p	0,9642214	0,00435186	0,372627686	Up-regulated
	hsa-miR-379-5p	1,46136088	0,00619518	0,453658878	Up-regulated
	hsa-miR-3120-5p	1,05085403	0,00662276	0,453658878	Up-regulated
	hsa-miR-376a-5p	-1,06317073	0,00928715	0,578335925	Down-regulated
	hsa-miR-542-3p	1,36466429	0,01456701	0,760274481	Up-regulated
	hsa-miR-195-5p	1,29637989	0,01520981	0,760274481	Up-regulated
	hsa-miR-3130-5p	-2,64645685	0,01847345	0,760274481	Down-regulated
	hsa-miR-519d-3p	-0,91993812	0,0188483	0,760274481	Down-regulated
	hsa-miR-490-5p	1,42743639	0,01886813	0,760274481	Up-regulated
	hsa-miR-450b-5p	1,11411811	0,02263153	0,763303449	Up-regulated
	hsa-miR-374b-5p	0,86524386	0,02512358	0,763303449	Up-regulated
	hsa-miR-374c-3p	0,87017312	0,02523843	0,763303449	Up-regulated
	hsa-miR-143-3p	0,78495077	0,02900217	0,763303449	Up-regulated
	hsa-miR-21-3p	0,79317052	0,0291757	0,763303449	Up-regulated
	hsa-miR-675-5p	1,1546043	0,03213608	0,763303449	Up-regulated
	hsa-miR-671-5p	1,76630085	0,03293839	0,763303449	Up-regulated
	hsa-miR-146a-5p	0,86261284	0,03359957	0,763303449	Up-regulated
	hsa-miR-489-3p	1,02467619	0,03372793	0,763303449	Up-regulated
	hsa-miR-96-5p	-2,45982286	0,03878464	0,77095716	Down-regulated
	hsa-miR-509-3p	0,86545472	0,03909277	0,77095716	Up-regulated
	hsa-miR-190b-5p	2,26556495	0,04749263	0,77095716	Up-regulated
	hsa-miR-371b-5p	-0,92943309	0,04875339	0,77095716	Down-regulated
	hsa-miR-520d-5p	-0,6667811	0,04979001	0,77095716	Down-regulated
	hsa-miR-371a-3p	-0,92563764	0,04993063	0,77095716	Down-regulated
	hsa-miR-412-5p	0,86533956	0,0514002	0,77095716	Up-regulated
	hsa-miR-324-5p	-1,01010797	0,05178532	0,77095716	Down-regulated
	hsa-miR-410-3p	0,63475365	0,05378966	0,77095716	Up-regulated
	hsa-miR-139-5p	0,66901369	0,0543406	0,77095716	Up-regulated
T. gondii (*treatment exclusive)	hsa-miR-548l		0,01756271	0,760274481	Up-regulated
	hsa-miR-449a		0,02617767	0,763303449	Up-regulated
	hsa-miR-6125		0,03454366	0,763303449	Up-regulated
	hsa-miR-4638-3p		0,03454366	0,763303449	Up-regulated
	hsa-miR-6860		0,03454366	0,763303449	Up-regulated
	hsa-miR-4660		0,03454366	0,763303449	Up-regulated
	hsa-miR-2115-5p		0,04354449	0,77095716	Up-regulated

**Treatment Exclusive: refers to miRNAs sequenced only in the T. cruzi or T. gondii infected condition (treatment), they are not expressed in control explants.*

proliferation, T-helper 17 cell differentiation, T-cell activation, and response to estrogen (**Figure 2B**).

In addition, we performed GO and KEGG analyses to identify different pathologies in which the *T. cruzi*- and *T. gondii*-induced DEMs were related. In *T. cruzi* vs. control samples, significantly enriched DEMs were related to metabolic syndrome, IgA-nephropathy, acute childhood lymphoblastic leukemia, atherosclerosis, oral lichen planus, human papilloma virus

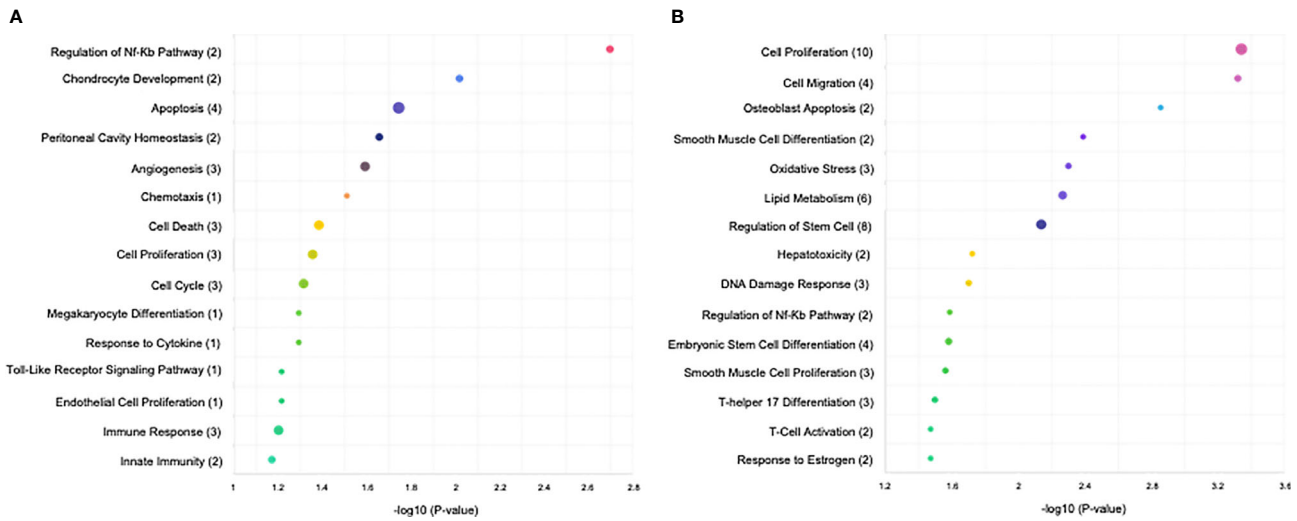

FIGURE 2 | KEGG analysis of the differentially expressed miRNAs ($P < 0.05$) revealed significant enrichment in immune-related and cell cycle pathways. The top 15 enriched pathways of the differentially expressed miRNAs are presented for **(A)** control vs. *T. cruzi* and **(B)** control vs. *T. gondii*. The sizes and colors of the circles represent the number of predicted gene targets of the differentially expressed miRNAs ($P < 0.05$) and the q-value, respectively.

infection, psoriasis, neuropsychiatric disorders, heart diseases, pancreatic carcinoma, Löfgren's syndrome, *Mycobacterium tuberculosis* infection, male infertility, and gastric carcinoma (**Figure 3A**). In *T. gondii* vs. control samples, the significantly enriched DEMs were related to ankylosing spondylitis, type 2 diabetes mellitus, hypertrophic cardiomyopathy, congenital heart disease, fetal alcohol syndrome, pulmonary hypertension, ulcerative colitis, cystic fibrosis, vascular diseases, human cytomegalovirus infection, muscular dystrophy, liver diseases, coxsackievirus infection, and diabetic retinopathy (**Figure 3B**).

Specific GO and KEGG enrichment analyses focused on the functions of DEMs in response to both parasites that were related to parasitic diseases and/or placenta pathology. These DEMs are listed in **Table 3**. In this context, we found that the significantly enriched DEMs were related to the regulation of apoptosis, wound healing, cardiomyocyte apoptosis, heart development, skeletal muscle cell differentiation, Toll-like receptor signaling pathway, innate immunity, epithelial to mesenchymal transition, chromatin remodeling, and nephrotoxicity (**Figure 4**). Moreover, we analyzed the significantly enriched DEMs related

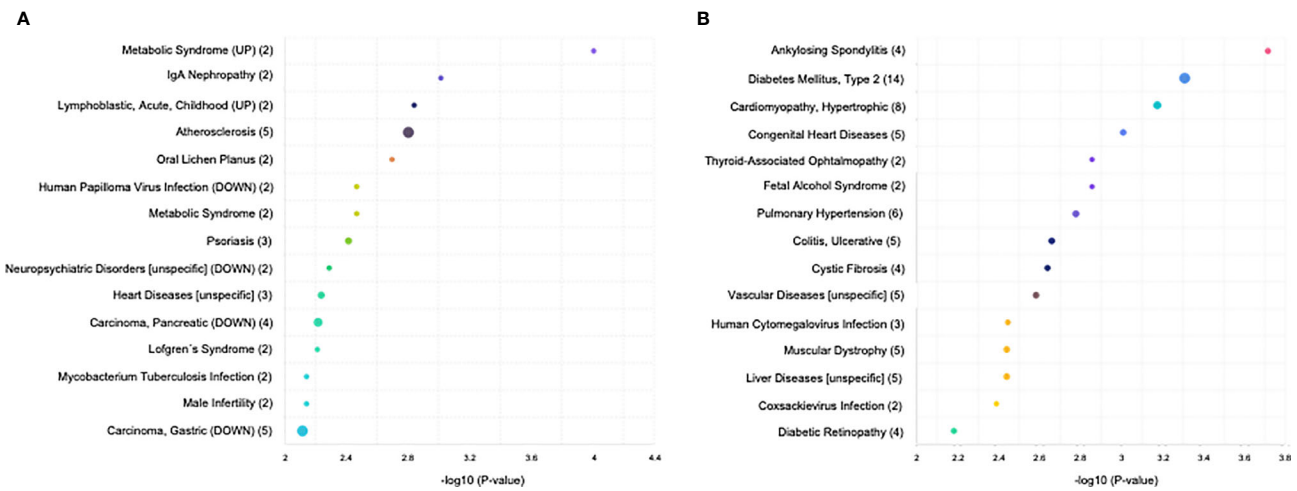

FIGURE 3 | KEGG analysis of the differentially expressed miRNAs ($P < 0.05$) revealed significant enrichment in cancer-related, infections, and inflammatory disease pathways. The top 15 enriched pathways of the differentially expressed miRNAs are presented for **(A)** control vs. *T. cruzi* and **(B)** control vs. *T. gondii*. The sizes and colors of the circles represent the number of predicted gene targets of the differentially expressed miRNAs ($P < 0.05$) and the q-value, respectively.

TABLE 3 | Sequenced miRNA related to placental and/or parasitic diseases in HPE challenged with 10^5 *T. cruzi* trypomastigotes or *T. gondii* tachyzoites during 2 h.

miRNAs	Control vs T. cruzi		Control vs T. gondii	
	Expression	p-value	Expression	p-value
hsa-miR-3074	Up-regulated	0,282778175	Down-regulated	0,906716427
hsa-miR-518e-5p	Down-regulated	0,736493282	Up-regulated	0,655921033
hsa-miR-127-3p	Down-regulated	0,454473692	Up-regulated	0,899001399
hsa-miR-512-3p	Up-regulated	0,893095693	Up-regulated	0,554888541
hsa-miR-516a-5p	Down-regulated	0,573579895	Down-regulated	0,875664943
hsa-miR-376a-3p	Up-regulated	0,26239456	Up-regulated	0,890873441
hsa-miR-523-5p	Down-regulated	0,736493282	Up-regulated	0,655921033
hsa-miR-517-5p	Down-regulated	0,356215385	Down-regulated	0,131367096
hsa-miR-523-3p	Up-regulated	0,558965508	Down-regulated	0,070624754
hsa-miR-519a-5p	Down-regulated	0,902087668	Up-regulated	0,683786458
hsa-miR-526a-5p	Down-regulated	0,878621126	Up-regulated	0,975623088
hsa-miR-519a-3p	Up-regulated	0,855200309	Down-regulated	0,216046946
hsa-miR-518e-3p	Up-regulated	0,878596629	Down-regulated	0,062749763
hsa-miR-520c-5p	Down-regulated	0,878525243	Up-regulated	0,981710662
hsa-miR-526a-3p	Down-regulated	0,648457618	Down-regulated	0,194744348
hsa-miR-29b-3p	Up-regulated	0,688264908	Down-regulated	0,461394462
hsa-miR-520c-3p	Down-regulated	0,492741665	Down-regulated	0,321148465
hsa-miR-133a-3p	Up-regulated	0,800691009	Down-regulated	0,140142195
hsa-miR-525-5p	Down-regulated	0,892625222	Down-regulated	0,624806979
hsa-miR-525-3p	Up-regulated	0,896175408	Down-regulated	0,075998808
hsa-miR-519c-5p	Down-regulated	0,736493282	Up-regulated	0,655921033
hsa-miR-518b	Up-regulated	0,991656949	Down-regulated	0,078353573
hsa-miR-519c-3p	Down-regulated	0,262008953	Down-regulated	0,786169155
hsa-miR-520e-5p	Down-regulated	0,485165729	Up-regulated	0,996288443
hsa-miR-520e-3p	Up-regulated	1	Up-regulated	0,818473073
hsa-miR-21-5p	Up-regulated	0,783933507	Up-regulated	0,129614377
hsa-miR-21-3p	Up-regulated	0,749764521	Up-regulated	0,029175701
hsa-miR-517a-3p	Up-regulated	0,596271375	Down-regulated	0,461347018
hsa-miR-519e-5p	Up-regulated	0,105129034	Up-regulated	0,757374294
hsa-miR-519e-3p	Up-regulated	0,385269175	Up-regulated	0,69099671
hsa-miR-518d-5p	Down-regulated	0,878525243	Up-regulated	0,981710662
hsa-miR-520g-5p	Up-regulated	0,892300156	Up-regulated	0,751979117
hsa-miR-518d-3p	Up-regulated	0,55414416	Down-regulated	0,942071295
hsa-miR-520b-5p	Down-regulated	0,421187791	Down-regulated	0,477328076
hsa-miR-520g-3p	Up-regulated	0,728189158	Down-regulated	0,352658846
hsa-miR-519a-2-5p	Down-regulated	0,421187791	Down-regulated	0,477328076
hsa-miR-520b-3p	Up-regulated	0,530509712	Down-regulated	0,954450249
hsa-miR-517c-3p	Up-regulated	0,760586655	Down-regulated	0,675509383
hsa-miR-524-5p	Down-regulated	0,490615316	Down-regulated	0,115381834
hsa-miR-210-5p	Up-regulated	0,038576256	Up-regulated	0,576691951
hsa-miR-204-5p	Up-regulated	0,62227226	Down-regulated	0,583793777
hsa-miR-524-3p	Down-regulated	0,907890609	Down-regulated	0,099098809
hsa-miR-519b-5p	Down-regulated	0,736493282	Up-regulated	0,655921033
hsa-miR-210-3p	Up-regulated	0,497217414	Down-regulated	0,843820092
hsa-miR-378a-5p	Down-regulated	0,915728534	Down-regulated	0,504825517
hsa-miR-526b-5p	Down-regulated	0,501400731	Down-regulated	0,534886926
hsa-miR-519b-3p	Up-regulated	0,996536077	Up-regulated	0,986254335
hsa-miR-518a-5p	Down-regulated	0,396658878	Down-regulated	0,099042516
hsa-miR-520d-5p	Down-regulated	0,163923317	Down-regulated	0,049790011
hsa-miR-526b-3p	Up-regulated	0,690372369	Down-regulated	0,356016985
hsa-miR-520d-3p	Down-regulated	0,368956264	Down-regulated	0,595399092
hsa-miR-30e-3p	Up-regulated	0,708643074	Down-regulated	0,606142557
hsa-miR-520h	Up-regulated	0,420508027	Down-regulated	0,48103803
hsa-miR-519d-5p	Down-regulated	0,699132481	Down-regulated	0,210250996
hsa-miR-515-5p	Down-regulated	0,451420159	Down-regulated	0,479490879
hsa-miR-519d-3p	Up-regulated	0,795099209	Down-regulated	0,018848296
hsa-miR-515-3p	Down-regulated	0,961837732	Down-regulated	0,056222245
hsa-miR-518c-5p	Down-regulated	0,433629597	Down-regulated	0,461615584
hsa-miR-155-5p	Up-regulated	0,089556012	Up-regulated	0,307144182
hsa-miR-518c-3p	Up-regulated	0,825417746	Down-regulated	0,393062915
hsa-miR-520a-5p	Up-regulated	0,829237058	Down-regulated	0,786085152

(Continued)

TABLE 3 | Continued

miRNAs	Control vs T. cruzi		Control vs T. gondii	
	Expression	p-value	Expression	p-value
hsa-miR-376a-5p	Down-regulated	0,101845272	Down-regulated	0,009287146
hsa-miR-520a-3p	Up-regulated	0,303331111	Down-regulated	0,733502488
hsa-miR-144-5p	Up-regulated	0,979441392	Down-regulated	0,781688613
hsa-miR-204-5p	Up-regulated	0,62227226	Down-regulated	0,583793777
hsa-miR-424-5p	Up-regulated	0,52797867	Down-regulated	0,318765339
hsa-miR-346	Down-regulated	0,996223653	Up-regulated	0,484560011

to transcription activation and found that the following transcription factors or its activators were modulated by them: MYOG, calcineurin, AP-1, TNFSF12, NFκB1, myogenin, MYOD, MYF5, MRF4, and TP53 (**Figure 5**).

Validation of miRNA Expression by RT-qPCR

Validation of miRNAs associated with parasite infection or pregnancy related pathologies was performed by selecting six miRNAs [miR-3074 (26), miR-127-3p (27, 28), miR-30e-3p (29), miR-512-3p (30), miR-515-5p (31), and miR-190b (32)] for confirmation by real time PCR to verify the DEM expression levels. Expression of miR-3074, miR-127-3p, and miR-30e-3p (**Figures 6A–C**) was analyzed in HPE in response to both parasites. miR-512-3p and miR-515-5p (**Figures 6D–E**) expression was determined in response to *T. cruzi* infection and miR-190b expression in response to *T. gondii* infection (**Figure 6F**). All selected miRNAs except for miR-30e-3p (**Figure 6C**) were differentially expressed. Thus, miR-3074 expression (**Figure 6A**) was significantly decreased (*T. cruzi*: 57.03 ± 19.99%, $p \leq 0.01$; *T. gondii*: 69.84 ± 24.67%, $p \leq 0.01$) with respect to the control but not the infected samples. Decreased miR-3074 expression was expected in the *T. gondii* infected samples but not in the *T. cruzi* infected samples. According to the miRNA Seq data, miR-3074 was upregulated in *T. cruzi* challenged samples. Similar results were observed for miR-127-3p (**Figigure 6B**). Expression of miR-127-3p was significantly decreased in HPE infected with either parasite (*T. cruzi*: 68.218 ± 16.41%, $p \leq 0.01$; *T. gondii*: 73.13 ± 22.45%, $p \leq 0.01$) compared to the control but not to infected samples; we expected an increase in miR-127-3p expression in the presence of *T. gondii* since in the miRNA Seq data this particular miRNA was increased (**Table 3**). RT-qPCR validation results for miR-512-3p, miR-515-5p, and miR-190b confirmed the miRNA Seq data. Thus, miR-512-3p expression increased (40.83 ± 22.53%, $p \leq 0.01$) (**Figure 6D**) and miR-515-5p expression decreased (21.44 ± 8.60%, $p \leq 0.01$) (**Figure 6E**) significantly in *T. cruzi* infected samples. In *T. gondii* infected HPE, miR-190b expression was significantly increased (59.02 ± 37.73%, $p \leq 0.01$) (**Figure 6F**).

DISCUSSION

Pathogens have evolved strategies to exploit resources from their hosts to maximize their own survival, replication, and dissemination. Thus, different kinds of pathogens (including

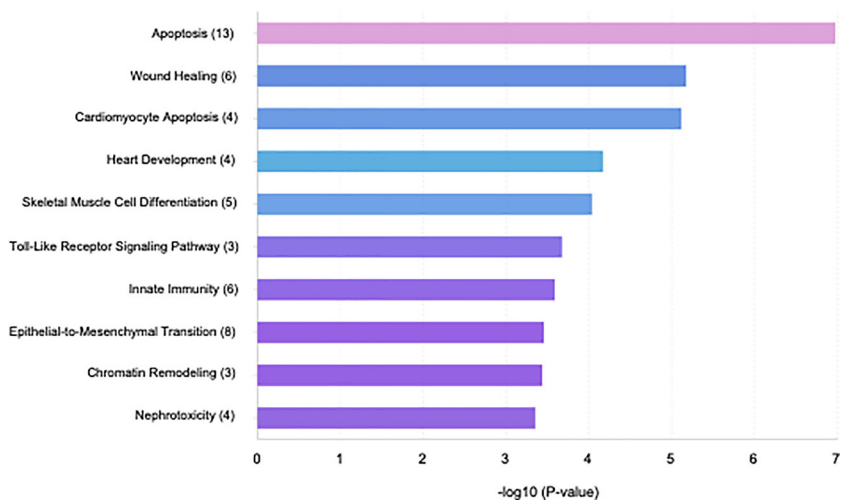

FIGURE 4 | Bar plot illustrating the top 10 significant miRNA-function associations of the sequenced miRNA related to placental and/or parasitic diseases in human placental explants infected with *T. cruzi* or *T. gondii*.

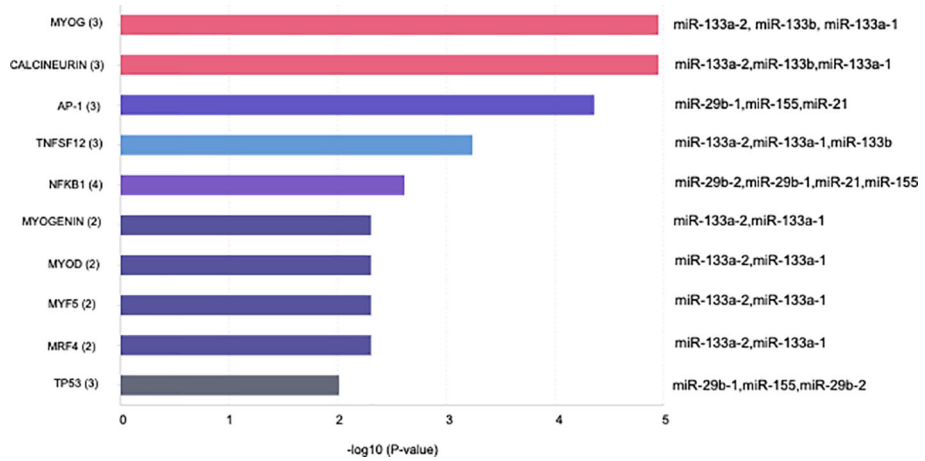

FIGURE 5 | Bar plot illustrating the top 10 significant miRNA transcription factor associations of the sequenced miRNA related to placental and/or parasitic diseases in human placental explants infected with *T. cruzi* or *T. gondii*.

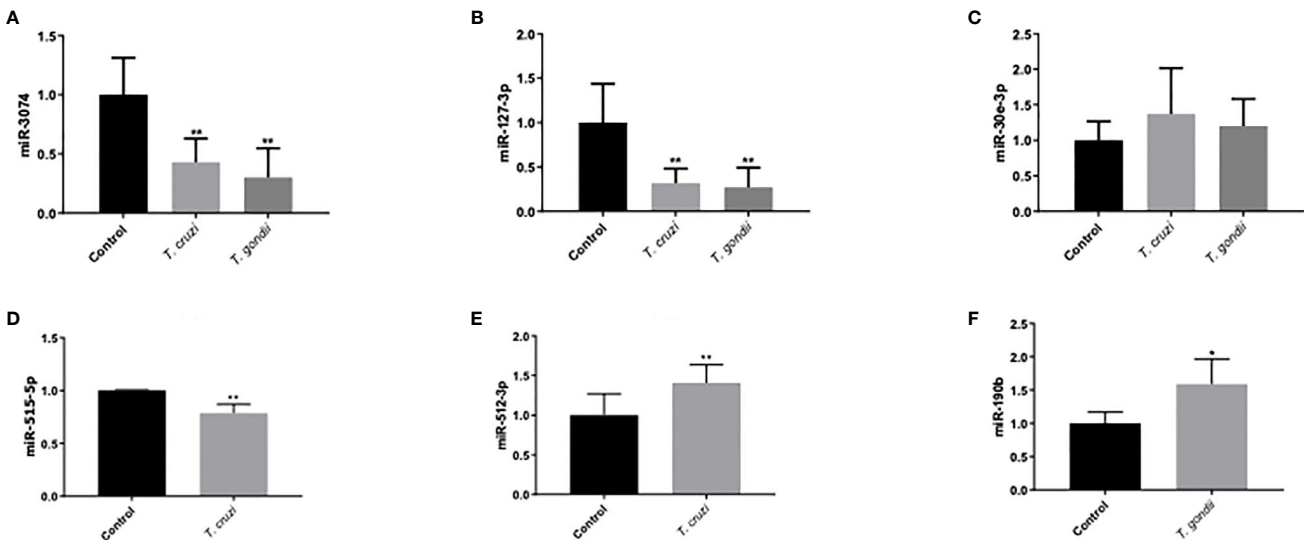

FIGURE 6 | Validation of miRNAs related to placental and/or parasitic diseases using RT-qPCR. Human placental explants were challenged with 10^5 *T. cruzi* trypomastigotes or *T. gondii* tachyzoites for 2 h. The presence of miRNA was determined by real-time PCR. *T. cruzi* and *T. gondii* decreased miR-3074 and miR-127-3p expression, while no change was observed in miR-30e-3p expression **(A–C)**. *T. cruzi* inhibited miR-515-5p expression and induced miR512-3p **(D, E)**. *T. gondii* induced miR-190b-5p **(F)**. All values are the mean ± S.D. and correspond to at least three independent experiments that were performed in triplicate. Data were normalized in terms of the control values and analyzed by Student's t-test or ANOVA. *$p \leq 0.05$; **$p \leq 0.01$.

parasites) have developed sophisticated mechanisms that include hijacking host cellular machinery to modulate host gene expression to inhibit defense responses (16, 32, 33). Both of the parasites studied here are able to manipulate host gene expression. For instance, during *T. cruzi* cell and tissue invasion, cell reprogramming affects cellular stress responses, host metabolism, and a significant number of transcription factors (16, 34). *T. gondii* also reprograms host cells, primarily targeting cell-specific transcription factors that regulate host defenses (i.e., NF-κB, interferon regulatory factor, and JAK/STAT) by regulating their intrinsic activities and expression levels (35). In addition, certain parasites including *T. cruzi* and *T. gondii* can alter host miRNA expression to favor both parasite clearance and infection (18, 19). Moreover, different strains of *T. gondii* can induce specific miRNAs in mice that have been proposed as biomarkers for early infection (19, 36).

Mature miRNAs regulate the expression of over 30% of fundamental genes; these are involved in key biological

processes including development, cellular proliferation and differentiation, apoptosis, metabolism, and immune response (18, 19, 37); all of these determine infection success or failure. Moreover, epigenetic and genetic defects in miRNAs and their processing machinery are a common hallmark of infection and diseases that include pregnancy-specific pathologies such as preeclampsia (17, 38).

Most of the transcriptomic studies as well as those analyzing miRNA profiles have focused on a single type of cell response (34) or on tissues or organs in animal models (39, 40); no studies have focused on human tissues. The present study is the first report on the miRNA profile of the human placenta in response to *T. cruzi* or *T. gondii* infection. We identified 680 and 686 DEMs, respectively, in *T. cruzi* and *T. gondii* infected samples. *T. cruzi* modulated only 14 DEMs with a minimum of a 1.5-fold change in expression and a 95% probability of being differentially expressed (**Figure 1A**). In contrast, the number of DEMs increased to 42 in *T. gondii* challenged HPE (**Figure 2B**). Our results showed that the DEMs identified here are related to the regulation of different fundamental cellular processes (**Figure 2**) as well as different pathologies (**Figure 3**). Moreover, fundamental cellular processes related to placenta pathologies and embryonic development are affected by the presence of both parasites (**Figures 4–5**). It is important to point out, that tissue response to infection is relevant during disease progression. The presence of the parasites leads to tissue damage as well as immune and regulatory/repair responses, which can lead to fibrosis and tissue dysfunction as observed in chagasic cardiomyopathy (41) or *Toxoplasma* induced encephalitis in immune-compromised individuals (42).

Three miRNAs, miR-21, miR-146a/b, and miR-210, were overrepresented in most of the ontology terms (**Table 2**, **Supplementary Tables 3–4**). Previous studies have implicated these miRNAs in immune and inflammatory response regulation *via* macrophage polarization controlled through transcription factor regulation in response to signals from the microenvironment (43, 44). Concordantly, in *T. cruzi*-infected mice, increased miR-21 expression in the heart has been correlated with a parasitemia peak at 30 days post-infection (39). In placenta, miR-21 has been associated with trophoblast differentiation and invasion and miR-21 dysregulation leads to placental pathology (45). MiR-146a is a negative feedback regulator in TLR-4 signaling that acts by repressing TRAF6 to inhibit NFκB transcription factor activation (46, 47). In macrophages, TRAF6 mediates the induction of the pro-inflammatory cytokine IL-12, which is essential to control *T. gondii* infection (48). TRAF6 activation is also required for vacuole-lysosome fusion, a fundamental step during *T. gondii* infection (49). Our results showed that in HPEs, *T. gondii* and *T. cruzi* infection increased miR-146a expression. Our previous studies showed that both parasites modulate placental immune response differentially through TLRs and NFκB pathways in HPEs (9, 10) Interestingly, the inhibition of these pathways increased the DNA loads of both parasites in HPEs (10). Increased *T. gondii* infection in placental tissue is also induced by TLR-4 inhibition (9). In addition, increased levels of miR-146a have been reported in the brains of mice infected with *T. gondii*, moreover, miR-146a ablation affects early parasite burden and improves survival (50). It was previously reported that miR-210 is induced by damage associated molecular patterns (51). In preeclamptic placentas, miR-210 is increased (52); in the present study, miR-210 was increased in HPE infected with *T. cruzi* but not with *T. gondii*. Expression of miR-210 can be directly regulated by the specific binding of NF-κB p50 to its putative promoter (53). In this context, it is important to mention that *T. cruzi*, but not *T. gondii*, infection of HPE activates both NF-κB signaling pathways (10). Therefore, the increased level of miR-210 might be a placental response to signal transduction pathway activation.

In addition, several identified pathways, important, e.g. for chondrocyte development, megakaryocyte smooth and muscle cell differentiation, hepatotoxicity, and DNA damage response, are neither related to infection or with placental tissues (**Figure 3**). This can be explained be the fact, that miRNAs target multiple genes, while individual genes are targeted by multiple miRNAs. Moreover, the same miRNA regulates different genes in different tissues and organs (54, 55). Here, we chose to validate six miRNAs that were associated specifically with parasite infection and/or pregnancy related pathologies (**Figure 6**). Deregulation of miR-30e-3p has been reported in mice that were experimentally infected with *T. gondii* (40). This miRNA is also related to Chagas cardiomyopathy (39) and is upregulated in placentas with intrauterine growth restriction (56). Nonetheless, miR-30e-3p expression was unaffected by *T. cruzi* or *T. gondii* infection in HPE (**Figure 6C**). Increased miR-3074-5p expression has been described in placentas from recurrent miscarriages (57) and in livers from *T. gondii*-infected cats (42). However, miR-3074-5p expression was diminished in HPE infected either with *T. cruzi* or *T. gondii* (**Figure 6A**). The differences between our results and the reported data might be explained by differences in the studied organs (heart and liver *versus* placenta) and the complexity of the above mentioned placental pathologies. MiR-127 is a placenta-specific miRNA codified in the C14MC cluster (58) and its levels are decreased in placenta-related pathologies such as recurrent miscarriage and small-for-gestational age (59, 60); the downregulation of MiR-127 was also detected in babies infected congenitally with either parasite (3, 4). Concordantly, our results showed that HPE infection with either *T. cruzi* or *T. gondii* led to the decrease of this miRNA (**Figure 6B**). Moreover, a decreased expression of miR-127-3p in non-placental tissues has been reported during *T. gondii* infection in mice and cats (40, 42, 61), but there is no report regarding miR-127-3p expression in response to *T. cruzi* infection. Both miR-515-5p and miR-512-3p are placenta-specific miRNAs that are codified in the C19MC cluster (62). Decreased miR-515-5p expression is related to fetal growth restriction (63) and preeclampsia (64). Importantly, this miRNA inhibits human trophoblast differentiation by directly repressing the aromatase P450 (*CYP19A1*), frizzled 5 (*FZD5*), and glial cells missing 1 transcription factor (*GCM1*) genes (65). Trophoblast differentiation is part of the trophoblast epithelial turnover

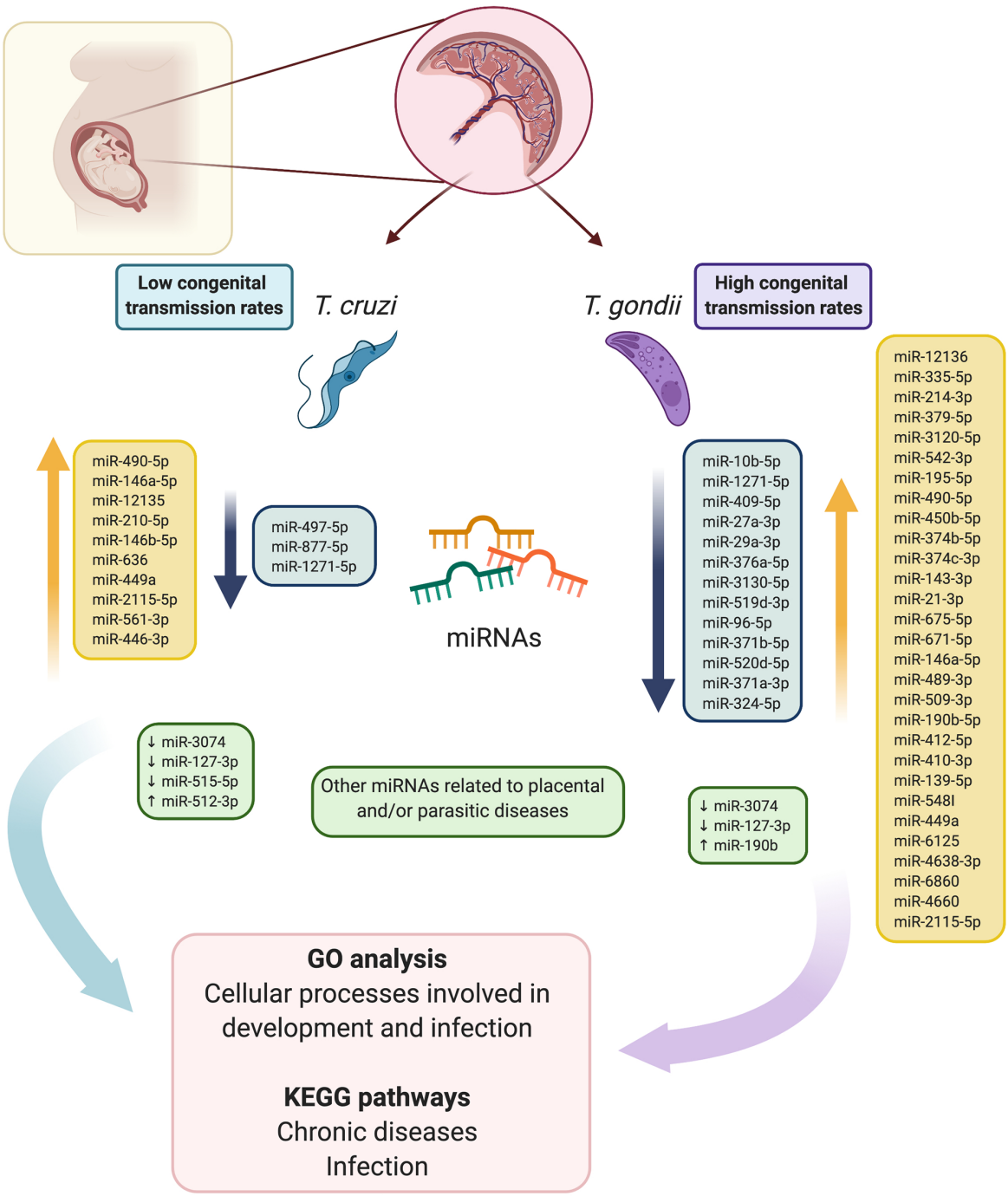

FIGURE 7 | Schematic summary of the comparative analysis of RNA sequencing-based miRNA profiles in HPEs in response to *ex vivo T. cruzi* and *T. gondii* infection. Fourteen, and 42 DEMs were identified in *T. cruzi* and *T. gondii* infected samples. In *T. cruzi* infected HPEs, 5 miRNAs were downregulated and 9 were upregulated. In *T. gondii* infected HPEs, 13 miRNAs were downregulated and 29 were upregulated. In addition, five miRNAs that are associated specifically with parasite infection and/or pregnancy-related pathologies were validated. GO analysis revealed that the predicted targets of the DEMs were different cellular processes involved in development and immunity, and most of the identified KEGG pathways were related to chronic diseases and infection. Considering that the DEMs identified herein modulate crucial host cellular targets potentially determining the success of infection, these miRNAs might explain the differences in the congenital transmission rates of the two parasites. This figure was created using BioRender.com.

and it has been proposed that this mechanism is part of an antiparasitic placental response against *T. cruzi* infection (11, 13, 66, 67). Therefore, our reported decrease of miR-515-5p expression during *ex vivo T. cruzi* infection of HPE (**Figure 6D**) might be at least partially responsible for the parasite-induced trophoblast differentiation. In contrast, miR-512-3p was upregulated in HPE in response to *T. cruzi* infection (**Figure 6E**). Interestingly, miR-512-3p confers resistance to vesicular stomatitis virus in non-placental recipient cells (68) and represses the caspase 8 inhibitor c-FLIP (cellular FLICE-like inhibitory protein); it consequently increases caspase 8 activity (69). Caspase 8 regulates trophoblast differentiation and apoptotic cell death and is activated by *T. cruzi* (66). Therefore, miR-512-3p upregulation might also be a protective placental response to *T. cruzi* infection, as it is to viral infection. The upregulation of miR-190b in HPE during *T. gondii* infection was observed in the RNAseq analysis (**Figure 1B**, **Table 3**), then validated by qPCR (**Figure 6F**). Upregulation of miR-190b promotes cell proliferation and migration and reduces cell apoptosis in different types of cancer (70, 71). Parasites modulate apoptotic responses in infected cells to avoid rapid clearance; *T. gondii* is particularly capable of blocking apoptosis by different mechanisms (33). In neurons, increased miR-190b expression also increases cell viability, suppresses autophagy, and significantly decreases the levels of pro-inflammatory TNF-α, IL-6, and IL-1β cytokines (72). In this context, we have shown that *T. gondii*, in contrast to *T. cruzi*, does not induce pro-inflammatory cytokines in HPE (9). Therefore, it is postulated that the lack of pro-inflammatory cytokine secretion in response to *T. gondii* in HPE might be related to an increase in miR-190b expression and that, together with the modulation of the apoptotic pathway, it could allow parasite persistence and infection in the placenta.

In conclusion, the present study provides a comparative analysis of RNA sequencing-based miRNA profiles in HPE in response to *ex vivo T. cruzi* and *T. gondii* infection (**Figure 7**). Our findings provide new insight into the capacity of both parasites to modulate host gene expression. GO analysis revealed that the predicted targets of the DEMs were different cellular processes involved in development and immunity, and most of the identified KEGG pathways were related to chronic diseases and infection. Considering that the DEMs identified herein modulate crucial host cellular targets that participate in determining the success of infection, these miRNAs might explain the differences in congenital transmission rates. Molecules of the different pathways that are regulated by miRNAs and modulated during infection, as well as the miRNAs themselves, may be potential targets for the therapeutic control of either congenital Chagas disease or toxoplasmosis.

DATA AVAILABILITY STATEMENT

The RNA-seq data reported in the present study have been submitted to the NCBI SRA database (http://www.ncbi.nlm.nih.gov/bioproject/656620; accession number PRJNA656620). All other data supporting the findings can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the "Servicio de Salud Metropolitano Norte" Santiago de Chile, Chile. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM and UK conceived of and planned experiments. LM, CC, MR-P, AL, and JG-M carried out experiments. JM and HP contributed to the interpretation of the results. UK and LM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.595250/full#supplementary-material

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REVIEW
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Do Antibodies to Malaria Surface Antigens Play a Role in Protecting Mothers From Maternal Anemia?

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Pregnancy-associated malaria (PAM) caused by *Plasmodium falciparum* can result in detrimental outcomes for both mother and infant, including low infant birth weight, preterm birth, maternal anemia, spontaneous abortion, and maternal and/or infant mortality. Maternal anemia is a particularly complex outcome, as the body must both maintain erythropoiesis and tolerance of the growing fetus, while directing a Th1 response against the parasite. Underlying the pathogenesis of PAM is the expression of variant surface antigens (VSA_{PAM}) on the surface of infected red blood cells (iRBC) that mediate sequestration of the iRBC in the placenta. Naturally acquired antibodies to VSA_{PAM} can block sequestration and activate opsonic phagocytosis, both associated with improved pregnancy outcomes. In this review, we ask whether VSA_{PAM} antibodies can also protect mothers against malarial anemia. Studies were identified where VSA_{PAM} antibody titres and/or function were associated with higher maternal hemoglobin levels, thus supporting additional protective mechanisms for these antibodies against PAM. Yet these associations were not widely observed, and many studies reported no association between protection from maternal anemia and VSA_{PAM} antibodies. We discuss the epidemiological, biological and technical factors that may explain some of the variability among these studies. We appraise the current evidence of these complex interactions between PAM-specific immunity and maternal anemia, propose potential mechanisms, and discuss knowledge gaps.

Keywords: pregnancy-associated malaria, anemia, inflammation, placenta, VAR2CSA antibodies, VSA_{PAM} antibodies

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INTRODUCTION

Pregnant women are especially vulnerable to malaria compared to non-pregnant adults in Africa (1) and 11 million pregnancies are at risk for complications from pregnancy-associated malaria (PAM) on this continent (2) in addition to those in lower transmission regions elsewhere (3). PAM is attributed to poor fetal outcomes including stillbirths (4), low birth weight infants (2) and premature births (5). PAM also significantly contributes to severe maternal anemia (6).

During a *Plasmodium falciparum* infection in high transmission settings, primigravid women have an especially high risk of poor pregnancy outcomes, and this risk decreases with gravidity [reviewed in (7)]. Pregnancy outcomes can affect both mother (maternal anemia, maternal morbidity and mortality) and child (low infant birth weight, pre-term birth, and spontaneous

abortion). After several instances of PAM, protection may be conferred by acquired immunity (8). Specifically, antibodies to pregnancy-specific variant surface antigens (VSA$_{PAM}$) expressed on the surface of the infected red blood cell (iRBC) are known to disrupt the adherence of iRBCs to chondroitin sulfate A (CSA) in the placenta and prevent parasite sequestration here (9, 10). One of the key targets of these maternal antibodies is VAR2CSA, a VSA expressed on the iRBC surface that directly binds to CSA (11). VAR2CSA antibodies are associated with improved pregnancy outcomes (12) and as a result, are the focus of vaccines to prevent PAM (13, 14).

While antibody-mediated immune mechanisms are associated with improved birth outcomes, particularly increased infant birth weight, we do not know whether these humoral mechanisms could protect women from developing anemia. By preventing sequestration of iRBCs in the placenta or opsonizing VSAs on the surface of iRBCs, VSA$_{PAM}$ antibodies may promote iRBC phagocytosis in the intervillous spaces (IVS) of the placenta or parasite killing in the spleen, lowering parasitemia and reducing the risk of maternal anemia. However, the development of anemia in PAM and its connection to humoral immunity is not well defined and potential underlying mechanisms of protection warrant investigation (15). Here, we first consider the causes of maternal anemia in PAM, and then examine the studies that investigated associations between anemia and VSA$_{PAM}$ antibodies.

MECHANISMS OF ANEMIA IN THE CONTEXT OF PAM

Maternal anemia is a common, but particularly complex consequence of PAM, typically defined as a hemoglobin level less than 11 g/dL, with mild and severe anemia for pregnant women defined at 10–10.9 g/dL and less than 7 g/dL, respectively (16). In a healthy pregnancy, the maternal vasculature must vasodilate approximately 5 weeks into pregnancy, with total blood volume and red cell mass significantly increased (17). An increase in plasma volume compared to red cell production results in physiological anemia during pregnancy that usually resolves by the third trimester (reviewed in (18, 19)). However, anemia is exacerbated by poor nutrition, iron deficiency, and maternal hypertension (reviewed in (20)).

If anemia develops due to malaria infection, this can result in maternal morbidity and mortality as the red cell population is compromised. Some mechanisms of anemia are shared between uncomplicated malaria and PAM, such as obligate hemolysis as the schizont ruptures, which triggers a pro-inflammatory response. In uncomplicated malaria, a profound loss of nRBCs contributes to anemia (21), as red cell deformability is reduced, which has been correlated with a lower hemoglobin concentration (22, 23) but whether this also occurs in the placenta is not known. This loss of non-infected RBCs (nRBCs) is yet to be observed in the PAM setting. Dysregulated erythropoiesis is also reported in uncomplicated malaria, often due to production of pro-inflammatory mediators including cytokines, nitric oxide, and lipoperoxides that can lead to bone marrow dysfunction [reviewed in (24, 25)].

In pregnancy, tolerance to the fetus must be maintained primarily with a T helper (Th) 2 response, through secretions of TGF-β, IL-4, and IL-6 (26–28). However, a *P. falciparum* infection triggers a Th1 response, increasing pro-inflammatory cytokine secretions including IL-1β, TNFα, and IFNγ. Moore et al. observed that intervillous blood mononuclear cells from multigravid women negative for placental malaria had the highest secretions of IFNγ, while cells from primigravid and secundigravid women positive for placental malaria had low IFNγ secretions (29). Thus, an increased production of IFNγ may help control a placental infection. However, pro-inflammatory mediators have also been associated with detrimental placental inflammation. Increased TNFα in primigravid women was associated with placental lesions, while increased IL-1β was associated with congenital malaria (30). This pro-inflammatory response was associated with severe maternal anemia (27). The increase in pro-inflammatory cytokines may also inhibit erythropoiesis in the bone marrow, leading to bone marrow dysfunction and contributing to overall maternal anemia (reviewed in (31), though this has not been shown specifically for PAM. In response to the pro-inflammatory state in the placenta, IL-10 is frequently elevated along with pro-inflammatory mediators in PAM (32, 33) in attempts to maintain a healthy pregnancy (reviewed in (34). Thus, IL-10 can be considered a marker for dangerous placental inflammation (29, 32, 35, 36) and levels of IL-10 correlated with maternal anemia (32, 33).

A source of these cytokines in the placenta are monocytes and macrophages (34, 37, 38), which often accumulate in the IVS during infection. Antibodies may fine-tune this response by directly targeting the parasite, blocking iRBC sequestration in the placenta and/or increasing opsonic phagocytosis by targeting iRBCs for engulfment. These potential mechanisms, as illustrated in **Figure 1**, may lower parasitemia in the placenta so that a prolonged inflammatory response is not required.

Further investigation is required to elucidate the mechanisms of anemia in PAM compared with uncomplicated malaria. Far less is published on anemia in PAM, and yet there may be significant differences due to changes in immune regulation that occur in pregnancy. For example, a Th1 response to combat a malaria infection could pose a greater risk in pregnancy compared to uncomplicated malaria as maintenance of a Th2 response is important for the health of the fetus. The investigation of the role of VSA$_{PAM}$ antibodies is further complicated by the multifactorial causes of anemia during PAM, which may alter the relationship between antibody titre/function and maternal anemia. Thus, a connection between VSA$_{PAM}$ antibodies and maternal anemia may be difficult to elucidate.

STUDIES OF VSA$_{PAM}$ ANTIBODIES AND MATERNAL ANEMIA

Women without prior exposure to PAM lack antibodies to the unique pregnancy-specific VSA that mediate sequestration of iRBCs to the placenta. Pregnant women can eventually acquire antibodies against these VSA and the levels of these antibodies increase with gravidity (8, 39). There is evidence for an

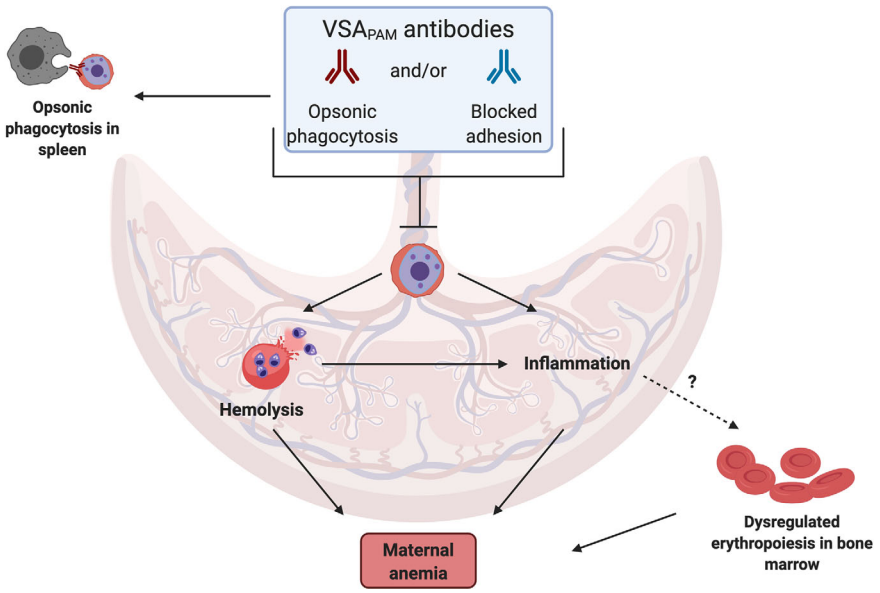

FIGURE 1 | Mechanisms by which VSA$_{PAM}$ antibodies could protect against anemia in PAM. VSA$_{PAM}$ antibodies that bind to the surface of iRBCs can promote iRBC clearance from the placenta by preventing sequestration and/or promoting opsonic phagocytosis in the IVS of the placenta or in the spleen. Both of these mechanisms would reduce placental parasitemia and could prevent several of the downstream mechanisms that lead to maternal anemia; one of these is iRBC hemolysis, where parasite material is released and detected by toll-like receptors present on cells in the IVS, activating a pro-inflammatory response. Reduced inflammation may in turn prevent dysregulated erythropoiesis in the bone marrow, further protecting against maternal anemia. (Created with BioRender.com).

association between VSA$_{PAM}$ antibodies and improved birth weight (40), but less is known whether these antibodies can also protect women from maternal anemia. Here, we discuss the limited studies on VSA$_{PAM}$ antibodies and anemia (**Table 1**).

Studies With No Correlation Between VSA$_{PAM}$ Antibodies and Maternal Anemia

In the first study to relate measures of VSA$_{PAM}$ antibodies to birth outcomes, plasma from Kenyan pregnant mothers was tested in the inhibition of binding assay (IBA) with placental isolates and correlated to infant birth weight, gestational age, and maternal hemoglobin at delivery (40). Plasma that blocked >35% of parasite binding to CSA was considered positive for blocking antibodies. Such antibodies were only observed in 1 of 47 primigravid women, while in secondigravid women, anti-adhesion activity was greater and associated with both increased infant birth weight and gestational age. However, there was no relationship between anti-adhesion antibodies and maternal hemoglobin levels (40).

This association was also observed in a study in Benin, where antibody titres were measured against the recombinant proteins DBL1-DBL2X, DBL5ϵ, and DBL6ϵ by ELISA, and functional activity was assessed by IBA (41). Increased anti-adhesion activity was associated with a decreased risk of placental infection at delivery and low birth weight, but there was no association between antibody titres or anti-adhesion activity and maternal anemia at delivery (41).

Opsonic phagocytosis of iRBCs is an important measure of antibody function. In primigravid women from Malawi, serum samples were collected in the late third trimester and tested for reactivity to CS2 parasites by flow cytometry and opsonic phagocytosis (42). No association was observed with maternal anemia or infant birth weight and VSA$_{PAM}$ antibodies. The study was continued with third trimester samples from secundigravid women (43). Again, no association was observed between maternal anemia and VSA$_{PAM}$ IgG or opsonic activity, but a positive correlation between opsonic phagocytosis and infant birth weight was observed.

Other studies compared hemoglobin measurements to IgG titres but not to antibody function. In Mozambique, serum samples collected at delivery were tested by ELISA for reactivity against a number of malarial antigens expressed in uncomplicated malaria and during PAM, including DBL5ϵ and DBL6ϵ, along with reactivity to two placental isolates (44). VSA$_{PAM}$ IgG against one isolate was associated with increased birth weight and gestational age of infants, but no association was found with maternal anemia. In Senegal, IgG was measured against the VAR2CSA subdomains DBL1X, DBL5ϵ, and DBL6ϵ by ELISA and compared with maternal anemia at enrollment and delivery in samples from primigravid, secondigravid, and multigravid women (45). IgG levels against DBL5ϵ and DBL6ϵ at enrollment and delivery were parity-dependent, and high IgG titres correlated with past malaria infection rather than acute PAM. In this study, there was no association between the levels of antibodies against these VAR2CSA domains and maternal anemia or birth weight at any measured time point. Similarly, in a study from Mozambique, the levels of antibody that stained the native VSA on CS2 parasites in flow cytometry did not associate with maternal anemia at delivery (46). Surprisingly, these

TABLE 1 | Summary of studies on VSA$_{PAM}$ antibodies and maternal anemia.

Citation	Setting	Assays used	Time of serum/ plasma collection	Anemia-related outcomes
No association between VSA$_{PAM}$ antibodies and maternal anemia				
Duffy and Fried (40)	Kenya	IBA	Delivery	VSA$_{PAM}$ blocking antibodies were not associated with maternal hemoglobin at delivery
Ndam et al. (41)	Benin	ELISA, IBA	Delivery	VSA$_{PAM}$ blocking antibodies were not associated with maternal anemia at delivery
Ataide et al. (42)	Malawi	Flow, opsonic phagocytosis	Late third trimester	VSA$_{PAM}$ antibodies from primigravid women were not associated with maternal hemoglobin
Ataide et al. (43)	Malawi	Flow, opsonic phagocytosis	Late third trimester	VSA$_{PAM}$ antibodies from secundigravid women were not associated with maternal hemoglobin
Mayor et al. (44)	Mozambique	ELISA, Flow	Delivery	VSA$_{PAM}$ antibodies were not associated with maternal anemia
Ndam et al., 2006 (45)	Senegal	ELISA	First to second trimester and delivery	Seroreactive antibodies against VAR2CSA subdomains were not associated with maternal anemia
Serra-Casas et al. (46)	Mozambique	Flow	Delivery	VSA$_{PAM}$ antibodies were not associated with maternal anemia
Aitken et al. (47)	Malawi	Flow	28–34 weeks gestation	VSA$_{PAM}$ antibodies were not associated with maternal hemoglobin
Fried et al. (48)	Mali	Multiplex	Second trimester	Seroreactive antibodies against VAR2CSA subdomains were not correlated with a reduced risk of maternal anemia
Mixed association between VSA$_{PAM}$ antibodies and maternal anemia				
Lloyd et al. (49)	Cameroon	Multiplex	Delivery	Seroreactive antibodies against VAR2CSA were associated with lower hematocrit levels and increased prevalence of anemia in women negative for placental infection
Staalsoe et al. (50)	Kenya	Flow	Delivery	Levels of VSA$_{PAM}$ antibodies correlated with hemoglobin in women with chronic placental infection but not acute or past infection
Association between VSA$_{PAM}$ antibodies and maternal anemia				
Feng et al., (51)	Malawi	Opsonic phagocytosis	14–20 weeks gestation	Two-fold increase in opsonic phagocytosis index was associated with reduced odds of maternal anemia
Jaworowski et al. (52)	Malawi	Opsonic phagocytosis	Third trimester	VSA$_{PAM}$ antibodies from anemic women displayed significantly lower opsonizing activity compared to non-anemic women
Chandrasiri et al. (53)	Malawi	Flow, opsonic phagocytosis	Before 20 weeks gestation	10% increase in opsonic phagocytosis index was associated with maternal hemoglobin increase of 0.4 g/L at 36 weeks gestation
Sander et al. (54)	Cameroon	Detection of *var2csa* copy number	First, second, and third trimester	In Ngali II, increasing *var2csa* copy number, which correlated with antibodies against DBL4ε, was associated with higher maternal hemoglobin
Chandrasiri et al. (55)	Sudan	Flow, opsonic phagocytosis, cytokine profiles	Second and third trimester	DBL5ε antibodies and maternal hemoglobin were negatively correlated with pro-inflammatory cytokines; opsonizing antibodies were positively associated with hemoglobin
Gavina et al. (56)	Colombia	ELISA, IBA	First and second trimester	VSA$_{PAM}$ blocking antibodies were positively associated with maternal hemoglobin

antibodies did not correlate with other birth outcomes either, including preterm birth or low birth weight.

Two longitudinal studies reported VSA$_{PAM}$ antibody and hemoglobin levels throughout pregnancy, and both failed to identify a correlation between antibody levels at enrollment and pregnancy outcomes. The first study reported IgG titres in pregnant Malawi women from serum collected at various points in pregnancy including study enrollment, 28–34 gestational weeks, and 1–6 months postpartum (47). There was a non-significant downward trend in antibody levels against VSA-expressing parasites CS2 and HCS3-VSA from enrollment to one month postpartum, but IgG remained detectable 6 months after delivery in 72% of women. Despite the persistence of the VSA$_{PAM}$ antibodies, they were not associated with an increase in maternal hemoglobin levels. Fried et al. published findings from Mali with plasma samples collected at enrollment, 30–32 gestational weeks, and at delivery (48). A multiplex bead assay was used to determine seropositivity to a large number of VAR2CSA subdomains, including DBL2X, DBL4ε, and ID1-ID2a, but notably not the full-length protein. Increased antibody titres against either of the DBL domains were not significantly associated with any type of protection.

Mixed Association Between VSA$_{PAM}$ Antibodies and Maternal Anemia

In Cameroon, two groups of women, one positive and one negative for placental malaria, both had overall low antibody levels to full-length VAR2CSA at delivery (49). Among women positive for placental malaria, higher seropositivity against VAR2CSA was associated with a reduced risk of placental parasitemia and low birth weight infants, but not maternal anemia at delivery. Interestingly, in women negative for placental malaria, the presence of VAR2CSA antibodies was associated with lower hematocrit at delivery and increased prevalence of maternal anemia. This is hypothesized to be the product of a malaria infection that was cleared early in pregnancy, with the presence of antibodies marking this past infection. But, the maternal hematocrit and prevalence of

anemia are only slightly different between women with and without VAR2CSA antibodies, thus this effect may not be biologically relevant.

With samples from a large cohort of pregnant women from Kilifi, VSA$_{PAM}$ IgG levels were measured by flow cytometry against various *P. falciparum* placental isolates and compared to hemoglobin levels (57). Increased severity of maternal anemia was correlated with low IgG levels in chronic cases of PAM (placentas infected with parasites and hemozoin pigment present) and the level of VSA$_{PAM}$ antibodies in these women was a strong predictor of maternal hemoglobin levels. However, no correlation was observed in women with acute or past placental infection.

Studies That Inversely Correlate VSA$_{PAM}$ Antibodies to Maternal Anemia

The findings from six studies suggest that VSA$_{PAM}$ antibodies can reduce the severity of maternal anemia. In Malawi, two studies investigated the function of VSA$_{PAM}$ antibodies by testing for opsonic phagocytosis. The first study revealed that a two-fold increase in the phagocytosis index was associated with a significant, 70% reduction in the odds of developing maternal anemia at delivery (51). Another study investigated third trimester serum samples and found that VSA$_{PAM}$ IgG reactivity in flow correlated with activity in the opsonic phagocytosis assay ($r = 0.60$), but serum from anemic women displayed significantly lower opsonic activity (52). Notably, coinfection with HIV reduced opsonic activity, but did not affect inhibition of binding levels or total IgG against VSA$_{PAM}$ (52).

In a later study from Malawi, antibody titres and function correlated to maternal hemoglobin at 36 weeks gestation, infant birth weight, gestational age, infant length, and placental histology at delivery (53). There was a significant positive association between IgG reactivity against CS2 parasites and maternal hemoglobin levels; a 10% increase in reactivity in flow corresponded to a hemoglobin increase of 0.5 g/L. Similarly, a 10% increase in the phagocytosis index corresponded to a hemoglobin increase of 0.4 g/L. Collectively, these studies support the hypothesis that opsonic phagocytosis could be an important effector mechanism of protection from maternal anemia.

VSA$_{PAM}$ antibody-mediated protection from anemia may relate to the number of *var2csa* genes per *P. falciparum* genome in the parasites that infect pregnant women (54). Parasites with more than one *var2csa* gene are more common in PAM, compared to the non-pregnant population, and may provide a selective advantage over parasites with only one *var2csa* gene. In the Cameroonian village of Ngali II, increasing *var2csa* copy numbers in the parasites infecting pregnant women were associated with higher hemoglobin levels at delivery, but not with birth weight. However, in another region, Yaoundé, this relationship was reversed, with a negative correlation observed between *var2csa* copy number and birth weight. In Ngali II, IgG against DBL4ϵ, but not the full-length VAR2CSA, correlated positively with gene copy number. The authors hypothesized that parasites with multiple copies of *var2csa* may elicit immunity to specific DBL domains of VAR2CSA, and the protection observed might be due to a strong immune response that develops in women infected with these parasites.

Along with titre and functional assays, Chandrasiri et al. examined cytokine profiles of pregnant women from Sudan (55). Cytokine profiles were measured with a multiplex bead array that detected IL-1β, IL-6, IL-8, IL-10, IL-12, and TNFα. IFNγ was also measured by ELISA. Distinct cytokine profiles were observed in women with severe PAM compared to uninfected controls, with IFNγ, IL-6, and IL-10 significantly elevated. Parasite density was positively associated with levels of pro-inflammatory cytokines IL-6 and IL-8. Women with severe cases had low levels of IgG against VSA$_{PAM}$ tested in flow, compared to uninfected controls. However, antibodies against DBL5ϵ were significantly greater than controls and negatively correlated with IL-1β, IL-6, and IL-8. Hemoglobin levels in these women were also negatively associated with IL-1β, IL-6, IL-8, and TNFα. Consistent with the studies from Malawi, opsonizing activity was positively associated with maternal hemoglobin at study enrollment. This study revealed associations between cytokine levels and VSA$_{PAM}$ antibodies. An influx of pro-inflammatory cytokines could increase the risk of maternal anemia, but the presence of antibodies may have an effect on cytokine profiles.

Only one study from outside Africa identified a positive correlation between VSA$_{PAM}$ antibodies and hemoglobin levels. This study was based in Colombia and examined a cohort of women with submicroscopic PAM (56). While VAR2CSA-specific antibody levels did not correlate with hemoglobin levels, the activity of these sera in the IBA was significantly associated with higher maternal hemoglobin levels at delivery. Interestingly, an association between antibody titre or function was not seen with birth weight, which is more commonly observed than associations with maternal anemia.

TRENDS AND CAVEATS

The results summarized above are divided on whether VSA$_{PAM}$ antibodies contribute to protection from maternal anemia induced by PAM. These conclusions are consistent with a recent meta-analysis that examined associations between VSA$_{PAM}$ antibodies and poor pregnancy outcomes, and included many of the same studies (58). Overall, the meta-analysis suggested that VSA$_{PAM}$ antibodies are more likely to be markers of infection, instead of a source of protection against adverse outcomes, including anemia (58). However, additional analyses of the primary data provided by the authors revealed heterogeneous associations of VSA$_{PAM}$ antibodies with anemia depending on a number of variables, such as gravidity, study design and the type of assay used to characterize antibodies. Considering these data and the results from the studies described previously, we identified various factors that may explain the discrepancies between these study findings.

Study Setting

The level of malaria transmission in the study region influences the frequency of exposure to parasites and the diversity of strains.

In turn, this could impact the magnitude and breadth of antibodies to VSA. Lloyd et al. collected samples from Yaoundé, a low transmission area with an entomological inoculation rate of approximately one infectious bite per person per month (49). In this study, just over half of women with a placental infection had antibodies to VAR2CSA, and this frequency was only 26.9% among multigravid women. Without sufficient exposure during pregnancy, a correlation between antibodies and pregnancy outcomes may not be detected. However, the study site of Fried et al. had intense seasonal transmission but there was no association between VSA$_{PAM}$ antibodies and anemia, despite a positive correlation between infection history and antibody titres against several VAR2CSA subdomains (48). In this setting submicroscopic infections (SMI) were common, with a frequency between 19.8% and 25.4% in women who were blood smear negative at enrollment, and these infections were associated with an increase in VSA$_{PAM}$ antibody levels, particularly in multigravid women. SMI may boost antibody responses without contributing to poor pregnancy outcomes, thus an association between VSA$_{PAM}$ antibody titres and outcomes may not be observed. A similar finding was observed in Colombia, a low transmission setting, where history of SMI did not correlate directly with maternal hemoglobin measurements (56). Based on these few studies, it is unclear how transmission intensity impacts associations between VSA$_{PAM}$ antibodies and anemia.

In certain settings, the fraction of anemia attributable to malaria may be low, masking a protective effect of VSA$_{PAM}$ antibodies. In one of the cohorts from Malawi, 62% of women were anemic and the mean hemoglobin level was 10.6 g/dL, likely reflecting non-malarial causes of anemia (43). In Kenya, Staalsoe et al. also reported overall low hemoglobin values, with 15.6% of women having hemoglobin concentrations under 7 g/dL (57). Ndam et al. reported 62% of women were anemic at enrollment and 45% at delivery in a cohort from Benin, with only 16% and 12% of women testing positive for malaria infection at enrollment and delivery, respectively (41). Several factors besides malaria could influence maternal anemia, including micronutrient deficiency, coinfections, and quality of prenatal care [reviewed in (24, 25)]. Interestingly, in the cohort investigated by Chandrasiri et al. where there was a protective association between VSA$_{PAM}$ antibodies and maternal anemia, women were given supplements (including iron) and the median hemoglobin was 11 g/dL (53). Perhaps this supplementation controlled for malaria-independent causes of anemia, revealing an association between the VSA$_{PAM}$ antibodies and malaria-attributable anemia.

Study Design

Depending on the study design, samples are collected at various times during pregnancy and/or at delivery and this could affect the interpretation of study findings. For example, VSA$_{PAM}$ antibodies detected early in pregnancy may indicate a prior exposure to VSA$_{PAM}$ in a previous pregnancy; thus, a pregnant woman may begin pregnancy with some immunity that may be boosted during subsequent pregnancies. Staalsoe et al. found that by six months postpartum, titres of VSA$_{PAM}$ IgG decreased, but levels were boosted in women by the second trimester of their next pregnancy (59). Modeling studies estimate that VAR2CSA antibodies can persist over many years (60). Of those studies that reported an association between VSA$_{PAM}$ antibodies and maternal hemoglobin levels, 4 out of 6 assessed antibody titre and/or function measured at first to second trimester (51, 53, 55, 56). In contrast, most of the studies that did not find a protective association with maternal anemia collected samples late in pregnancy, in the third trimester and at delivery (40, 41, 44, 46, 49, 45). If these VSA$_{PAM}$ antibodies developed as a result of infection late in pregnancy, increased antibody levels may reflect recent boosting from infection and obscure a relationship between antibody and protection from anemia. Thus, VSA$_{PAM}$ antibodies detected later in pregnancy may be markers of infection, rather than a source of protection, as noted by Cutts et al. (58), and others.

The gravidity of women included in the study can also greatly impact the titre, persistence and quality of antibodies investigated. Antibodies against VSA expressed in PAM develop with each successive infection in pregnancy; in high transmission settings, multigravid women are more likely to have antibodies that correlate with better pregnancy outcomes than primigravid women, supporting that these antibodies are maintained into the next pregnancy. Fried et al. reported that VSA$_{PAM}$ antibodies from primigravid women are short-lived, compared to those from secundigravid and multigravid women (48). In the 2010 Ataide et al. study, only primigravid women were included and samples were collected in the late third trimester, with no protective association between VSA$_{PAM}$ antibodies and anemia observed (42). The same cohort was followed into the second pregnancy, and again there was no association between maternal hemoglobin and VSA$_{PAM}$ IgG or opsonic phagocytosis activity (43). Thus, protective qualities of VSA$_{PAM}$ antibodies might not fully develop until infections in multiple pregnancies have occurred. In Malawi, recognition of iRBCs by VSA$_{PAM}$ antibodies from secundigravid and multigravid women, but not primigravid women, was associated with a reduced odds of anemia (47, 58). In contrast to the related studies discussed above, this association was observed in samples collected in the third trimester of pregnancy.

Another important consideration is treatment history. IPTp with 1500/75 mg of sulphadoxine and pyrimethamine (SP) during antenatal care visits is recommended by WHO in most areas in sub-Saharan Africa to reduce placental malaria (61). This treatment has the potential to affect the acquisition of VSA$_{PAM}$ antibodies, as parasitemia is prevented or cleared to the point that plasma cells will not produce antibodies against VSA$_{PAM}$ targets. In Ndam et al. where no association was observed between VSA$_{PAM}$ antibodies and maternal anemia at delivery, women received two doses of SP during pregnancy (41). Aitken et al. reported lower levels of VSA$_{PAM}$ antibodies associated with IPTp treatment (47). Similar observations were made in other studies from Kenya (50) and Ghana (62), where reductions in antibody titres were associated with IPTp treatment (though pregnancy outcomes were not

reported). In contrast, Serra-Casas et al. failed to observe an effect of IPTp on VSA$_{PAM}$ antibody levels (46). Therefore, the effects of IPTp on VSA$_{PAM}$ antibodies in relation to anemia are unclear. But, it is unlikely that IPTp treatment contributes to the severity of maternal anemia itself, as a controlled or lowered parasitemia should benefit the health of the mother.

Finally, HIV is known to affect the humoral response in PAM, which was observed by a number of studies examined in this review (42–44, 46, 52). In the two studies by Ataide et al. VSA$_{PAM}$ phagocytic antibodies were reduced in HIV positive women compared to women who were HIV negative but this was only observed when antibody activity was measured using a functional assay; there was no change in the antibody reactivity to iRBCs by flow cytometry (42, 43). The presence of HIV in a cohort may therefore reduce VSA$_{PAM}$ antibody levels and/or function to a level too low to improve outcomes, particularly in lower transmission settings (49) or when women are treated with IPTp (46).

Assay Selection

It is necessary to assess antibody titre and particularly function to make comparisons with pregnancy outcomes. From the group of studies that did not find an association with maternal anemia, IBA was the primary functional assay used, with only two studies in this group that measured opsonic phagocytosis activity (42, 43). Inhibition of adhesion by VSA$_{PAM}$ antibodies is more commonly associated with higher infant birth weight due to *P. falciparum* infections during pregnancy (40). In only one study, from Colombia, adhesion-blocking activity was associated with increased maternal hemoglobin at delivery (56). Several of the studies point to opsonic phagocytic activity as a particularly important correlate of protection against maternal anemia. Chandrasiri et al. showed that only antibodies with opsonic phagocytic activity were associated with reduced odds of severe malaria, rather than antibody titre measured by ELISA (55). Similarly, Feng et al. observed a correlation between a reduced odds ratio of maternal anemia and VSA$_{PAM}$ antibody activity both by flow cytometry and opsonic phagocytosis, but the relationship between phagocytic activity was stronger than VSA$_{PAM}$ seroreactivity in flow (51). Interestingly, in one study, VSA$_{PAM}$ IgG measured by flow correlated more strongly with opsonic activity than anti-adhesion activity in the IBA (52).

Functional assays can also focus on specific IgG subclasses, rather than measuring total IgG by flow cytometry or ELISA. IgG1 and IgG3 are cytophilic subclasses that interact with Fc receptors on other immune cells, including monocytes and macrophages, and promote opsonic phagocytosis (63). These are the dominant subclasses in PAM, while IgG2 and IgG4 levels do not significantly change (64). Opsonic phagocytosis assays specifically measure these cytophilic subclasses, whereas testing for total IgG may not reveal a relationship with pregnancy outcomes. Future studies should characterize antibody populations using functional assays, along with assessments of titre and subclass, to test for associations with anemia.

Overall, there are many factors that can affect the relationship between maternal anemia and VSA$_{PAM}$ antibodies. Based on our interpretation of the studies described above, the discordance between the study findings may be attributed in part to the timing of sample collection and assay selection.

Potential Mechanisms of Antibody-Mediated Protection from Maternal Anemia in PAM

Though evidence for a specific role for VSA$_{PAM}$ antibodies in protection against maternal anemia is currently inconclusive, we can speculate on putative mechanisms of protection to help inform future research (**Figure 1**). We hypothesize that protective antibodies could directly target the iRBCs in the placental environment, either through opsonization or by physically blocking adhesion to CSA. Based on the group of studies that found an association with improved outcomes, VSA$_{PAM}$ antibodies that mediate opsonic phagocytosis of iRBCs may be more likely to be protective against maternal anemia than antibodies that inhibit parasite binding to the placenta, suggesting these antibodies may have distinct effector mechanisms. Antibodies with dual functions may also exist.

Antibody binding to iRBCs can lead to different downstream effects. Clearing the iRBC from the placenta either by preventing sequestration and/or recruiting immune cells would prevent the hemolysis associated with anemia and the associated inflammatory response. Erythropoiesis is also susceptible to a dysregulated pro-inflammatory response, a common cause of anemia in uncomplicated malaria and PAM [reviewed in (24)]. VSA$_{PAM}$ antibodies can potentially contribute to these responses by reducing cytokine dysregulation and targeting immune cells to iRBCs. As observed by Chandrasiri et al. pro-inflammatory cytokines IL-1β, IL-6, and IL-8 negatively correlated with antibody titres against DBL5ϵ in pregnant women with severe malaria (55). In this study, opsonizing antibody activity and cytokine profiles were not compared directly, but maternal hemoglobin levels increased with increasing levels of opsonizing antibodies (53). In another study, cytokine secretions were measured after opsonic phagocytosis of CS2 parasites had occurred, using serum from Malawian pregnant women. These results were compared to antibody-independent phagocytosis and a shift in the cytokine profile was observed, with an increase in production of IL-1β and TNFα when antibodies were present (65). Opsonized iRBCs activated the inflammasome and production of IL-1β by macrophages, but this was also observed with unopsonized iRBCs. Thus, the secretion of pro-inflammatory mediators likely occurs in the placenta with and without sufficient titres of VSA$_{PAM}$ antibodies. Perhaps the labeling of iRBCs with VSA$_{PAM}$ antibodies leads to Th1-like immune responses to appropriately respond to the parasite without leading to dysregulated inflammation and inhibition of erythropoiesis.

FUTURE DIRECTIONS

As the pathways leading to anemia in PAM are complicated and multifactorial, further research is critical to better understand any potential underlying immune mechanisms in response to

malaria infection that contribute to or protect women from anemia. Longitudinal studies should be continued in and outside of Africa to increase the body of knowledge available on infection, VSA$_{PAM}$ antibody levels, and pregnancy outcomes. Studies should be conducted in many diverse settings to reveal context-specific effects on the acquisition of PAM immunity and the prevalence of maternal anemia. Treatment practices and circulation of other pathogens in a particular area can also affect humoral immunity.

In those studies where VSA$_{PAM}$ antibodies are associated with higher hemoglobin levels in mothers, it will be exciting to unravel the underlying mechanisms of protection. We propose a basic model for how these antibodies that target placental iRBCs could block downstream pathogenic effects that lead to anemia, yet the mechanisms that directly contribute to this process need to be delineated. While the data support a role for these antibodies in blocking sequestration in the placenta and promoting opsonic phagocytosis, emerging evidence linking the innate and adaptive immune systems merit exploration. For example, data is emerging on the role of the complement system in uncomplicated malaria. The antibody-mediated activation of the classical complement pathway was observed in samples from children and was an indicator of protective immunity (66). In addition, NK cells may participate in the response to PAM. Long et al. showed that antibodies from VAR2CSA-immunized rabbits induced iRBC lysis *in vitro* (67). Further, human IgG pooled from plasma of multigravid women and incubated with parasites and NK cells inhibited parasite growth by approximately 50%. No connection to anemia was examined, but this is an interesting component of the PAM response that should be further investigated with respect to pregnancy outcomes.

This review highlights the complexity of the body's response to PAM and the large gaps in the current knowledge on immune mechanisms underlying protection. Pregnant women and young children remain at great risk of morbidity and mortality from malaria. Continued research in this area will add to our understanding of PAM and potentially reveal novel therapy and vaccine strategies to combat this disease.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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VAR2CSA-Mediated Host Defense Evasion of *Plasmodium falciparum* Infected Erythrocytes in Placental Malaria

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Over 30 million women living in *P. falciparum* endemic areas are at risk of developing malaria during pregnancy every year. Placental malaria is characterized by massive accumulation of infected erythrocytes in the intervillous space of the placenta, accompanied by infiltration of immune cells, particularly monocytes. The consequent local inflammation and the obstruction of the maternofetal exchanges can lead to severe clinical outcomes for both mother and child. Even if protection against the disease can gradually be acquired following successive pregnancies, the malaria parasite has developed a large panel of evasion mechanisms to escape from host defense mechanisms and manipulate the immune system to its advantage. Infected erythrocytes isolated from placentas of women suffering from placental malaria present a unique phenotype and express the pregnancy-specific variant VAR2CSA of the *Plasmodium falciparum* Erythrocyte Membrane Protein (PfEMP1) family at their surface. The polymorphic VAR2CSA protein is able to mediate the interaction of infected erythrocytes with a variety of host cells including placental syncytiotrophoblasts and leukocytes but also with components of the immune system such as non-specific IgM. This review summarizes the described VAR2CSA-mediated host defense evasion mechanisms employed by the parasite during placental malaria to ensure its survival and persistence.

Keywords: *Plasmodium falciparum*, placental malaria, VAR2CSA, PfEMP1, immune evasion, immuno-modulation, VAR2CSA polymorphism

INTRODUCTION

Nearly half the world's population, implicating 90 countries, lives in areas at risk of malaria transmission. In 2019, an estimated 11 million pregnant women were infected by *Plasmodium* in sub-Saharan Africa, where *P. falciparum* is the most prevalent parasite species, accounting for 99.7% of estimated malaria cases (1). *P. falciparum* infection contracted during pregnancy can lead to placental malaria (PM), a condition that could cause very serious clinical outcomes for both mother

and child, including maternal anemia (2, 3), hypertension (4, 5), stillbirth (6, 7) as well as low birth-weight infants, which affected over 800,000 children in 2019 (1).

PM may result in significant morphological and immunological changes in the placenta. Focal syncytial necrosis, loss of syncytial microvilli, and proliferation of cytotrophoblastic cells are frequently observed as well as thickening of trophoblastic basement membranes together with the apparition of syncytial knots (8–10). Acute infection is also characterized by the substantial presence of infected erythrocytes (IEs) in the intervillous spaces of the placenta (**Figure 1A**).

Several transcriptomic and proteomic studies revealed that parasitized red blood cells isolated from *P. falciparum*-infected pregnant women display specific signatures, over-expressing a variety of different genes (11–13) and proteins (14–16) as compared to non-pregnancy-specific parasites. They also present a unique adhesive phenotype, interacting with chondroitin sulfate A (CSA), a low-sulfated glycosaminoglycan (GAG), which is the major host receptor involved in the adhesion of IEs to syncytiotrophoblastic cells (17–21) (**Figure 1B**). Chondroitin sulfate-proteoglycans (CSPGs) are present in the intervillous space of the placenta during the entire second and third trimesters and possibly during the latter part of the first trimester (22).

To date, the pregnancy-specific variant of the *Plasmodium falciparum* erythrocyte membrane protein 1 family (PfEMP1) VAR2CSA has been identified as the sole parasite-derived protein interacting with placental CSA (23–28).

This review focuses on the roles played by VAR2CSA in PM pathogenesis and introduces the latest information on its involvement in host defense evasion mechanisms ranging from cytoadhesion in the placenta, modulation of the placental microenvironment to escape of pregnancy-specific IEs from recognition by protective antibodies.

VAR2CSA STRUCTURE AND CHONDROITIN SULFATE A (CSA)-BINDING

VAR2CSA is a large protein of 350 kDa, with an extracellular region of approximately 300 kDa, displayed at the surface of IEs on membrane protrusions called knobs (29). PfEMP1 clustering

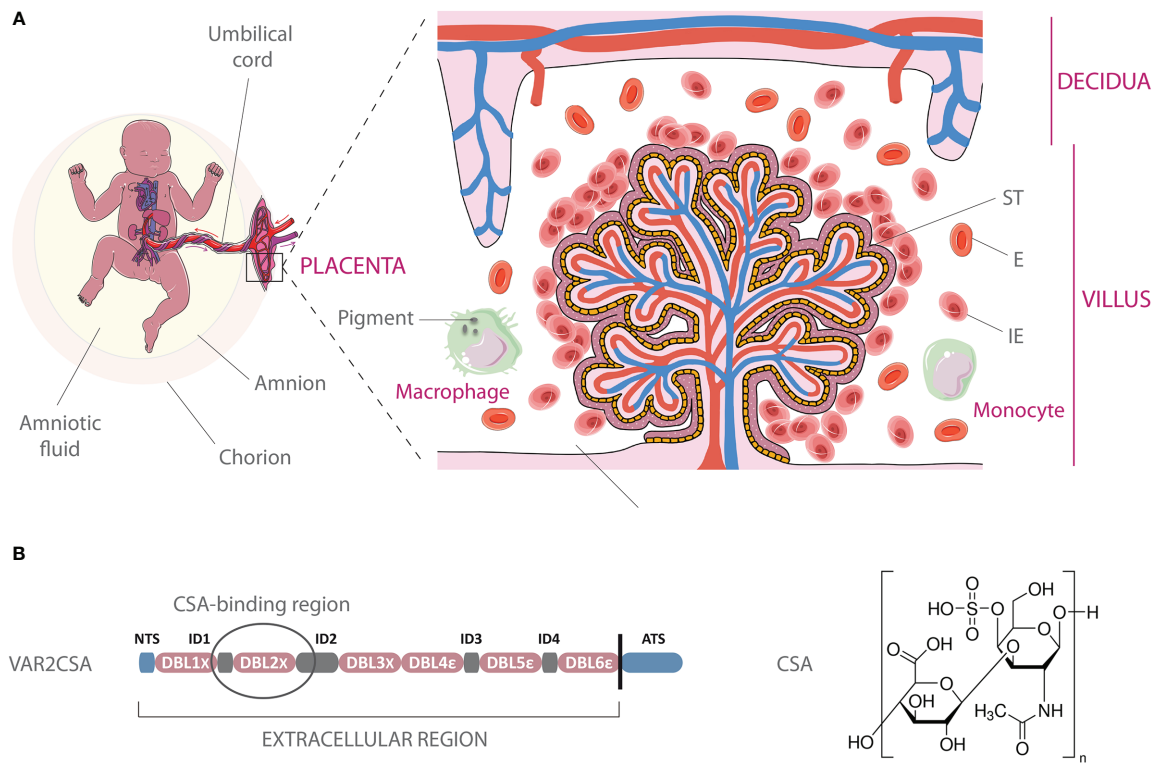

FIGURE 1 | Infected erythrocyte sequestration within the intervillous space of the placenta. **(A)** Schematic representation of infected erythrocytes (IE) adhering to the syncytiotrophoblastic lining of the fetal villus, with increased presence of macrophages and monocytes in the maternal blood. Parasite pigments (hemozoin) remain visible in macrophages following IEs phagocytosis. The natural transfer of gases and nutrients between maternal blood in the intervillous space and fetal blood circulating in the villi is impaired by IEs sequestration. E, Erythrocyte; ST, syncytiotrophoblast. **(B)** Architecture of the VAR2CSA protein and chemical structure of chondroitin-4-sulfate A. The circled region within VAR2CSA (ID1-ID2a) represents the CSA-binding region. The art pieces used in this figure were modified from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/).

on knob structures is thought to maximize cytoadhesion under flow conditions but also to act as an immune evasion mechanism, impairing antibody accessibility to key residues involved in CSA-binding (30, 31). Quantitative studies report an estimate of 3 to 80 VAR2CSA molecules per knob (32, 33). Knob density at the IEs surface has been shown to be linked to the PfEMP1 variant expressed by the parasite (34) and IEs stained by the monoclonal antibody PAM1.4 revealed that erythrocytes infected by the FCR3 parasite strains displayed more VAR2CSA clusters at the cell surface than erythrocytes infected by NF54 (35). Even if further studies are needed to precisely determine how these differences in PfEMP1 presentation impact antibody recognition, these observations highlight that *P. falciparum* is capable of complex variations at both intra- and inter-strain levels.

The cysteine-rich extracellular region of VAR2CSA has a complex architecture and is composed of six Duffy-Binding Like domains (DBLs), which are interspaced by four inter-domain regions (IDs) (**Figure 1B**). High-resolution structures have been obtained for the individual domains DBL3x, DBL6ϵ (36–40) as well as for the multidomain DBL3x-DBL4ϵ (41), providing a first step towards the definition of inter-domain interfaces and of the overall structure of the extracellular part of VAR2CSA. Low-resolution structures of the full-length extracellular part of VAR2CSA, obtained by small-angle X-ray scattering or single particle electron microscopy, reveal a compact organization of the protein maintained by specific inter-domain interactions (42–44). Nevertheless, the relative locations of the DBL domains within the overall structure of VAR2CSA significantly differ from one study to another (43, 44). In the recent work from Bewley et al., the VAR2CSA ectodomain low resolution structure appears as a duck-like shape with a packing of three tandem domains (DBL1x/DBL2x, DBL3x/DBL4ϵ, and DBL5ϵ/DBL6ϵ), which would form two pores, each theoretically susceptible to accommodate a 10-12-mer CSA. molecule (44). This model suggests that the higher-order structural organization of VAR2CSA is most likely allowing the formation of one, or maybe two, CSA-binding site(s), which comprise(s) several domains. The current definition of the boundaries of the core binding region, established using truncated fragments of recombinant VAR2CSA, localizes the high affinity CSA-binding site within the N-terminal part of the protein (45) between the ID1-ID2a section (46) even-though the accessory implication of other domains such as DBL4ϵ cannot be excluded (44). Additional VAR2CSA structural data at high resolution, ideally in complex with CSA, is still required to determine the precise determinants of CSA-binding, which might also include post-translational modifications (47).

VAR2CSA-MEDIATED INFECTED ERYTHROCYTES CYTOADHESION IN THE PLACENTA AND EVASION FROM SPLENIC FILTRATION

As parasites develop from ring stage to schizont stage within erythrocytes, the biomechanical properties of the host cells are subjected to considerable modifications, leading to decreased cellular deformability and loss of membrane elasticity [Reviewed in (48)]. Cytoadhesion of mature pregnancy-specific IEs to syncytiotrophoblasts leads to their sequestration in the intervillous spaces of the placenta. By sequestering in the placenta, biomechanically altered IEs avoid splenic retention at the level of the reticular mesh of the red pulp or during the challenging passage through the inter-endothelial slits of the organ (49–52). *P. falciparum* has therefore developed an efficient host defense evasion mechanism, which relies on a tight interaction between IEs and the syncytiotrophoblastic lining delimiting the intervillous spaces of the maternal portion of the placenta. As CSPGs are also present within the micro-vascular system, notably in the lungs and brain (53), the reason for exclusive placental sequestration of VAR2CSA-expressing IEs remains unclear. A body of work elucidated some comprehensive elements by demonstrating that the interaction of VAR2CSA with CSA is highly correlated with the degree of C-4 sulfation and the length of the CS chain (54–56), which may vary in different tissues. CSA density and wall shear stress also appear as two components influencing the IEs binding to CSA (57). CSA density on syncytiotrophoblasts and forces acting upon placental tissues could thus determine the selective cytoadhesion of IEs in the organ. If placental sequestration of IEs represents an effective immune evasion mechanism employed by *P. falciparum* to avoid its clearance by the spleen, this is not without harmful consequences for the women and the fetus. Sequestration is thought to be one of the prime mediators of biological alterations leading to placental insufficiency and subsequently to fetal growth restriction and poor birth outcomes [Reviewed in (58, 59)].

VAR2CSA-MEDIATED MODULATION OF THE PLACENTAL MICROENVIRONMENT

The placenta is a tightly controlled pro-inflammatory and anti-inflammatory environment, depending upon the stage of gestation. In healthy pregnancies, a pro-inflammatory milieu is required for fetal implantation, notably by promoting trophoblast invasion. A shift toward a type 2 cytokine/chemokine milieu gradually occurs during gestation favoring pregnancy maintenance and rapid fetal growth and development [reviewed in (60)]. *P. falciparum* infection during pregnancy can affect the placental environment, notably promoting inflammatory responses (61–65), some of which are associated with fetal growth retardation, low birth-weight babies, and in more extreme cases, poor pregnancy outcomes, such as preterm delivery and pregnancy loss (66–71). *P. falciparum* is thus able to upset the fine equilibrium between pro-inflammatory and anti-inflammatory responses, deregulating the immune system, with detrimental consequences for the human host.

Syncytiotrophoblast Activation

The syncytiotrophoblasts covering the placental villi are terminally differentiated cells, which result from the

syncytialization of underlying villous cytotrophoblasts. They exhibit high metabolic activity and are involved in many physiological processes such as the active transport of molecules, the diffusion of gases, and the synthesis and secretion of large amounts of hormones, including steroids [Reviewed in (72)]. Experiments performed using primary placental cells, as well as the widely used choriocarcinoma cell line BeWo, revealed that VAR2CSA-dependent binding of IEs to syncytiotrophoblasts induces a broad range of cellular responses, notably activating MAPK pathways (73, 74). Activation of syncytiotrophoblasts leads to the secretion of pro-inflammatory cytokines/chemokines such as macrophage inflammatory protein (MIP), the neutrophil chemotactic factor interleukin (IL) 8 and IL-6 (74, 75), but also to the production of soluble ICAM-1 (75), which may act as a protection mechanism to regulate the inflammatory response (76). The interaction of syncytiotrophoblasts with VAR2CSA-expressing IEs might therefore participate in the immunological shaping of the local environment, establishing a complex network of factors which could promote the migration of immune cells to the intervillous space (74), as well as the *in situ* modulation of their activity.

Macrophage and Monocyte Immunomodulation

Sections taken from healthy placenta at different time-points throughout normal pregnancy showed that nearly half of the decidual cells are of bone marrow origin, comprising 18–20% macrophages (77, 78). Polarization of decidual macrophages varies with gestational age, shifting from an M1 polarization during fetal implantation, towards a mixed M1/M2 profile which remains until mid-pregnancy (79). After the placental development is completed, decidual macrophages are predominantly of the M2 phenotype, contributing to a tolerant immune environment and to fetal immunoprotection (80, 81).

PM is characterized by a significant increase in the number of monocytes and macrophages in the intervillous space (8–10, 82, 83), which is notably associated with elevated expression of the β chemokines IL-8 and MIP-1 (84). *In vitro* co-incubation experiments, performed in absence of human plasma/serum, i.e. in absence of opsonic antibodies, showed that VAR2CSA-expressing IEs are able to modulate specific transcription factor activation in RAW-macrophages, as compared to erythrocytes infected with genetically modified parasites presenting a deficiency in the export of PfEMP1 at the cell surface (PfEMP1-null) (85). The decreased activation of NF-κB-, CREB-, and GAS/ISRE-binding factors is accompanied by reduced production of TNF and IL-10. Similar experiments using human primary monocytes also revealed that VAR2CSA-expressing IEs are able to alter the production profiles of other cytokines/chemokines, limiting the release of IL-1β, IL-6, IL-10, MCP-1, MIP-1α, and MIP-1β, as compared to cells infected withfimmu.2020.624126 PfEMP1-null parasites (85). Although the precise nature of the monocyte receptor(s) involved still remains to be elucidated, these observations highlight how *P. falciparum* could exploit the host cellular pathways to modulate the immune response.

Interestingly, a study performed in an area of low prevalence of malaria, revealed gravidity-dependent differences in the capacity of peripheral blood mononuclear cells (PBMCs) to produce cytokines and chemokines in response to pregnancy-specific IEs (86). Despite no differences in opsonic antibody levels, cellular immune responses differed between women in their second to fourth pregnancy (G2-4) and grand multigravida (G5-G7). Indeed, more IL-10, IL-1β, IL-6, tumor necrosis factor (TNF) but less CXCL-8, CCL-8, IFNγ, and CXCL-10 were detected in G2-4 compared to G5-7, highlighting the modulation of immune cell function occurring during PM (86).

VAR2CSA BINDING TO NON-SPECIFIC IGM AND DIVERSION OF THE IMMUNE RESPONSE

PM induces VAR2CSA-specific immunoglobulin Gs (IgGs) belonging to the IgG1 subclass, and to a lower extent the IgG3 subclass (87, 88), both highly potent at interacting with Fcγ receptors present at the surface of phagocytic cells. Concordantly, women living in areas where malaria is endemic naturally acquire specific antibodies that promote the phagocytosis of VAR2CSA-expressing IEs (89–91), thus participating in parasite clearance. Binding of non-specific IgM on the surface of IEs was first demonstrated on rosetting parasites (92–94) and subsequently on VAR2CSA-expressing red blood cells (95). Following these observations, the function of IgM binding to VAR2CSA has been uncertain for several years. In 2011, a study performed by Barfod et al. showed that non-specific IgM binding participates in the masking of protective epitopes on VAR2CSA, leading to IE evasion of macrophage-mediated opsonic phagocytosis (96). The same study revealed that non-specific IgM binding to VAR2CSA-expressing IEs did not interfere with their capacity to adhere to CSA and did not increase their susceptibility to undergo complement-mediated lysis (96). The extensive analysis of non-specific IgM binding to large panels of PfEMP1 members demonstrated that IgM binding is a common functional phenotype found in multiple PfEMP1 variants across various parasite strains, thus providing a better understanding of the underlying molecular mechanisms (97–99). Although the CSA-binding site of VAR2CSA resides within the N-terminal region of the protein (100, 101), the IgM interacting residues appear to be mainly located within the C-terminal section, at the level of the DBL5ϵ or DBL6ϵ domains in VAR2CSA variants carried by the 3D7 and FCR3/IT parasite strains, respectively (102, 103) as well as in DBLϵ and DBLζ domains near the C-terminus of other PfEMP1 variants (98, 99, 104, 105).

The PfEMP1 binding sites on IgM have been located within the μ region of the fragment crystallizable (Fcμ) of polymeric immunoglobulins (97), and more precisely in the Cμ4 domain for the DBL4β domain of PfEMP1-VAR1 of the TM284 strain (106). These observations, together with the additional definition of the architecture of the IgM/PfEMP1 complex (107), provide critical molecular elements which could explain how PfEMP1s

interfere with the binding of the complement component C1q to the adjacent Cμ3 domain, thus inhibiting complement-mediated lysis. Furthermore, these findings demonstrate how IgMs participate in PfEMP1 clustering on the cell surface, strengthening the interactions with host receptors (107–109). PfEMP1 binding to IgM has also been proposed as a non-exclusive molecular mechanism involved in the triggering of polyclonal B cell activation, a hall mark of malaria (110, 111). This activation would lead to hyper-gamma-immunoglobulinemia and the subsequent diversion of the specific humoral immune response towards antigens relevant for protection.

VAR2CSA POLYMORPHISM

All the *P. falciparum* genomes sequenced to date reveal the presence of one or more *var2csa* gene copies (112–114). VAR2CSA is a highly polymorphic multidomain protein, usually consisting of six DBL domains; the first three DBL domains belong to the DBLx subtype and the three others to the DBLε subtype. The protein also contains a $CIDR_{PAM}$ domain (also referred to as ID2) between the DBL2x and DBL3x domains. A recent study has identified atypical extended or truncated VAR2CSA structures (115). Extended structures include one or two additional DBLε domains downstream of the conventional DBL1x-6ε domain structure (115). Within the conventional six DBL domain structure, DBL4ε is the most conserved DBL domain while DBL6ε is the most polymorphic DBL domain (112). *Var2csa* is present in all genomes of known *Laverania* sub-genus members (116). One of the closest *P. falciparum* relatives, the chimpanzee parasite *Plasmodium reichenowi*, possesses a *var2csa*-like gene which is annotated as a pseudogene and encodes a functional truncated protein (NTS-DBL1x-ID1-DBL2x-truncated ID2) (117).

Global sequence diversity and analysis of *var2csa* have been reported in different studies (118–121) and more recently for 1,249 sequences spanning 7 Kb of *var2csa* (NTS-DBL5ε) from various strains and field isolates (122). Although it was previously shown that the DBL6ε domain is the most polymorphic domain (112), this latest study, which does not include DBL6ε, demonstrates that the nucleotide diversity is higher towards the N-terminus of the protein and that the

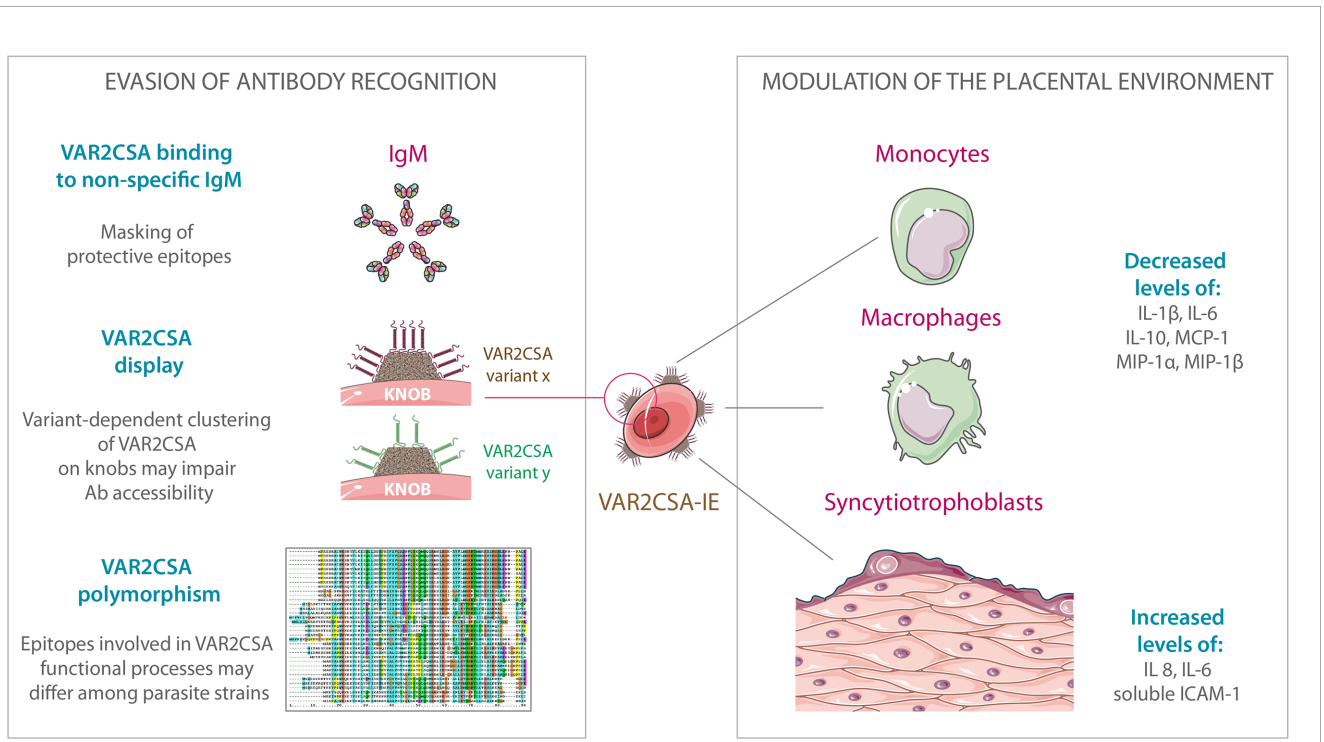

FIGURE 2 | Evasion of antibody recognition and modulation of the placental environment by VAR2CSA-expressing infected erythrocytes. IgM binding to VAR2CSA could mask protein epitopes recognized by anti-VAR2CSA IgGs and consequently alter opsonic phagocytosis of IEs. PfEMP1 clustering on knob structures may act as an immune evasion mechanism, impairing antibody accessibility to key residues involved in CSA-binding. Due to extensive polymorphism, epitopes involved in each VAR2CSA functional process may differ among parasite strains. Furthermore, multiplicity of *var2csa* genes within the parasite genome may also confer a greater capacity for antigenic variation and evasion of variant-specific immune responses. The presence of VAR2CSA on the IEs surface could lead to decreased production of IL-1β, IL-6, IL-10, MCP-1, MIP-1α, and MIP-1β by monocytes and macrophages. VAR2CSA-dependent binding of IEs to syncytiotrophoblasts is able to activate MAPK pathways and lead to increased secretion of IL-8, IL-6, and soluble ICAM-1. The art pieces used in this figure were modified from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/). The illustration of the protein sequence alignment is licensed under a Creative Commons Attribution, CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0/).

diversity is generally higher in African parasite populations than in South East Asian populations. While the DBL2x domain has the lowest nucleotide diversity (122), it possesses the highest density of insertions and deletions, with sequence length across samples ranging from 430 to 550 amino acids (122). In a population structure analysis performed on *var2csa* sequences from Benin and Malawi, five different clades of ID1-DBL2x (encoding for the CSA-binding region) were identified and the authors found an association between the 3D7-like clade and low birth-weight (120). Only four clades were identified, including a 3D7-like clade (clade 1) and an FCR3-like clade (clade 2) (120). Indeed, two of the previously identified clades could not be separated using this much larger dataset. Clades 1, 2, and 4 were present across all the *P. falciparum* malaria endemic areas and clade 1, which is associated with low birth-weight, is highly represented in the West African populations (41.7%), followed by East Africa (27.5%), South East Asia (23.5%), and South America (21.1%). However, clade 3 is exclusively found in African parasite populations but appears to represent less than 1% of the *var2csa* sequences.

A recent study, which used plasma obtained from Tanzanian and Malian women at the time of delivery, simultaneously examined the capability of antibodies to recognize native VAR2CSA expressed by either NF54 or FCR3, to inhibit the binding of IEs to CSA and to promote phagocytosis by THP1 cells. Plasma from Malian women reacted more strongly with VAR2CSA-expressing erythrocytes infected by the FCR3 parasite strain whereas Tanzanian plasma preferentially reacted with erythrocytes infected by NF54 (35). Analysis of antibody functionality showed that the balance between binding inhibition capability and opsonizing activity could be biased depending on the expressed VAR2CSA variant and on the geographical location (35), suggesting that epitopes involved in each functional process may differ among parasite strains and that parasite transmission in a given place could therefore shape antibody profiles. In addition, the multiplicity of *var2csa* genes within the parasite genome may also confer a greater capacity for antigenic variation and evasion of variant-specific immune responses (114).

CONCLUDING REMARKS

P. falciparum infection contracted during pregnancy elicits a broad range of immune responses, combining components of both the innate and the adaptive immunity, orchestrated by a complex network of pro- and anti-inflammatory cytokines (**Figure 2**). *P. falciparum* has developed the ability to manipulate the immune system to its advantage to ensure its survival and persistence within the human host.

Although the parasite is able to escape host defense processes and manipulate the induced immune response using a variety of mechanisms described herein, women living in malaria endemic areas can gradually acquire protective clinical immunity against PM, depending on the intensity of parasite transmission (123). In moderate malaria transmission. PM adverse clinical outcomes can be seen in women of all parity status (124), whereas protection appears to develop in a more marked parity-dependent manner in high transmission settings (125). Importantly, PM protection has been linked to the presence of antibodies targeting PM-specific variant surface antigens (126) and more specifically VAR2CSA (127–129). These observations led to the belief that a VAR2CSA-based vaccine against PM could potentially be achieved. However, the high degree of sequence diversity within VAR2CSA represents a major hurdle for vaccine design.

Following extensive preclinical evaluation, two recombinant vaccine candidates PRIMVAC and PAMVAC, comprising the CSA-binding region of VAR2CSA from the 3D7 (clade 1) and FCR3 (clade 2) strains respectively, have been assessed in Phase I clinical trials in Europe and Africa (ClinicalTrials.gov identifiers NCT02658253 and NCT02647489, respectively) (130–134). The identification of immunological correlates of protection against PM being complex, there is to date no clear surrogate allowing an easy evaluation of the protective effects of vaccines in early clinical trials (135). Exploratory analyses performed for the PRIMVAC and PAMVAC trials nevertheless revealed that vaccine-induced antibodies had a limited capability to cross-react with VAR2CSA originating from heterologous parasite strains (133, 134), highlighting the difficulty to compose with the high degree of polymorphisms of the protein when designing vaccines. Alternative vaccine approaches, using VAR2CSA in combination with other *P. falciparum* antigens, such as the circumsporozoite protein (CSP) (136), or virus-like particles (VLPs) to display VAR2CSA-derived antigens are also currently under investigation (137–139).

Improving our understanding on how *P. falciparum* escapes host defenses, modulates the immune system and on how natural immunity develops during PM despite VAR2CSA polymorphisms is therefore crucial to design efficient and effective immuno-therapeutic approaches but also to appropriately evaluate them.

AUTHOR CONTRIBUTIONS

AT, J-PS, BG, and AC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Vaccines in Congenital Toxoplasmosis: Advances and Perspectives

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Congenital toxoplasmosis has a high impact on human disease worldwide, inducing serious consequences from fetus to adulthood. Despite this, there are currently no human vaccines available to prevent this infection. Most vaccination studies against *Toxoplasma gondii* infection used animal models in which the infection was established by exogenous inoculation. Here, we review recent research on potential *T. gondii* vaccines using animal models in which infection was congenitally established. Endeavors in this field have so far revealed that live or subunit vaccines previously found to confer protection against extrinsically established infections can also protect, at least partially, from vertically transmitted infection. Nevertheless, there is no consensus on the more adequate immune response to protect the host and the fetus in congenital infection. Most of the vaccination studies rely on the assessment of maternal systemic immune responses, quantification of parasitic loads in the fetuses, and survival indexes and/or brain parasitic burden in the neonates. More research must be carried out not only to explore new vaccines but also to further study the nature of the elicited immune protection at the maternal-fetal interface. Particularly, the cellular and molecular effector mechanisms at the maternal-fetal interface induced by immunization remain poorly characterized. Deeper knowledge on the immune response at this specific location will certainly help to refine the vaccine-induced immunity and, consequently, to provide the most effective and safest protection against *T. gondii* vertical infection.

Keywords: pregnancy, toxoplasmosis, congenital, vaccination, maternal-fetal

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite and the etiologic agent of congenital toxoplasmosis. *T. gondii* is considered one of the most successful parasites worldwide, infecting over 30% of the human population, with high associated disease burden (1, 2). Seroprevalence varies greatly from region to region, ranging from approximately 30% in the American, European, and Asiatic regions, to more than 60% in the African continent (3, 4). The

disease is potentially dangerous in women who become infected during pregnancy, as it can lead to transplacental transmission of the parasite upon primary infection or re-infection with highly virulent strains (5, 6). The incidence of congenital toxoplasmosis varies according to the timing of infection during pregnancy. The transmission rate is greater in the final stages of pregnancy, but the severity of infection is greater in early gestation (7). In the more severe cases, hydrocephalus, chorioretinitis, and cerebral calcification may occur, according to the parasite's brain and ocular tropism (5). An association between congenital infection and the development of neurological and psychiatric disorders later in life, including schizophrenia, Alzheimer's disease, bipolar disease, and even suicidal tendencies has also been suggested (8, 9).

Innate and adaptive immunity determines protection against *T. gondii* infection. An effective immune response must control parasite growth while avoiding immunopathology. In both mice and humans, the IL-12- IFN-γ axis is the main immune mechanism responsible for parasite control. Protection mediated by IFN-γ produced by NK and Th1 cells induces the expression of immunity-related GTPase and/or guanylate-binding proteins, indoleamine-2,3-dioxygenase, and NO production. TNF-α has also been associated with host protection, as highlighted in patients with defective IFN-γ signaling (10). TGF-β, IL-6, and IL-23 promote the production of IL-17 that may also play a host protective role in toxoplasmosis by avoiding excessive IFN-γ-dependent inflammation (11). T cells producing IL-10, which include T regulatory cells and $Foxp3^{-}T\text{-}bet^{+}$ Th1 cells, can limit excessive inflammation driven by *T. gondii* (12). A regulatory role for IL-4, and IL-27 in minimizing host tissue injury due to exacerbated inflammation has also been shown (10, 13). A good vaccine candidate would thus induce not only IL-12 and IFN-γ, but also counterbalancing cytokines such as IL-4, IL-10, and IL-27 (14).

It is important to note that there are no licensed human vaccines able to prevent toxoplasmosis (15). The lack of effective treatment makes the development of a vaccine against congenital toxoplasmosis one of the main objectives in the management of this disease. Here, recent findings in vaccination approaches to congenital toxoplasmosis using animal models of vertical *T. gondii* infection will be reviewed and the possible implications in the quest for a vaccine protecting from congenital toxoplasmosis will be discussed.

IN VIVO MODELS TO STUDY CONGENITAL INFECTION

In the study of toxoplasmosis, animal models are used to better understand the disease pathology and the immunological mechanisms induced by infection, as well as to assess the effectiveness of experimental vaccination. However, no single animal model has, so far, been able to mimic all clinical symptoms and signs developed by humans in response to *T. gondii* infection (16). For congenital toxoplasmosis, the murine model is commonly used as it allows a short pregnancy period and mimics features of human congenital toxoplasmosis, namely the co-localization of inflammatory cells and necrosis at the maternal-fetal interface after primo-infection during pregnancy (17). Further, primo-infection confers resistance to maternal fetal transmission throughout later infections (16). However, the immune response to *T. gondii* in mice and humans presents distinct features and this should be adequately considered (18, 19).

Most vaccination studies in congenital infection reviewed here used Kunming, BALB/c, Swiss OF1, and CBA/J mice. No study was found using the C57BL/6 mouse strain. This might be due to excessive susceptibility to disease exhibited by *T. gondii* infected C57BL/6 mice. In contrast, BALB/c mice present higher resistance to infection, more closely resembling humans, rendering this strain more suitable for vaccination studies in congenital infection models (20–22). In murine congenital toxoplasmosis, C57BL/6 mice exhibited higher abortion rate compared to BALB/c due to exacerbated proinflammatory cytokines such as TNF-α (23). Mouse strain differences in major histocompatibility complex haplotypes (*e.g.*, $H\text{-}2^{b}$, $H\text{-}2^{d}$, $H\text{-}2^{k}$ for C57BL/6, BALB/c, and CBA/J, respectively), and therefore, antigenic presentation, could explain different susceptibility to *T. gondii* infection and induced immunopathology (20).

Other animal models can be used such as sheep, rats, guinea pig, or hamster (24–28). Rats and sheep are widely used in studies addressing drug effectiveness to *T. gondii* (18). Rats have placental development and hemochorial placentation identical to humans (29). In sheep, congenital toxoplasmosis is very similar to what occurs in humans (15). Thus, sheep is an adequate animal model to study congenital toxoplasmosis, not only because it shares important aspects with the human infection, but also because it directly contributes to the study of new disease control measures in livestock, also severely affected by *T. gondii* (15). There are well established models of toxoplasmosis in pregnant sheep that provide a starting point for the preparation and testing of new vaccines (15).

VACCINES IN VERTICAL INFECTION

This section aims to describe the vaccines already tested in congenital infection models, and to reveal the gap of analysis concerning immune cells and related mechanisms induced by immunization at the level of maternal-fetal interface as wells as in the neonates. Literature research was performed through a PubMed search, using the query "[(*Toxoplasma gondii*) AND (vaccine)] AND (congenital toxoplasmosis [title/abstract])". The results presented pertain to studies using pregnant mice where protection and immune responses of pregnant mice and their offspring was evaluated.

Live-Attenuated Vaccines

Live-attenuated vaccines have been the most studied in the context of congenital transmission (**Table 1**). These vaccines consist of parasites with reduced virulence but are nevertheless capable of inducing an immune response (36). Alternatively,

TABLE 1 | Live-attenuated vaccines tested in vertical *Toxoplasma gondii* infection models.

Strain/animal	Immunogen/strain/ delivery	Day of mating post-immunization	ChallengePregnancy day/route/#parasite form/Strain	Dam sacrifice	Maternal parameters	Progeny parameters	Highlighted results	References
Swiss OF1/ mice*	20 tachyzoites Δ*mic1-3*/ RH/ ip	Day 60	Day 11/oral/ 45 cysts/ 76K strain	-Day 17 of pregnancy (exp#1) -1 month after delivery (exp#2)	-Toxoplasma specific IgG levels -*Ex-vivo* splenocyte specific cytokine production (exp#1) -Brain cyst load (exp#2)	-Fetus parasite load (exp#1) -Survival of neonates and mean weight of pups at day 11 after birth -Brain cyst load at day 35 after birth (exp#2).	-Potent maternal humoral and Th1 responses. -Decreased fetus parasite load and, maternal and neonate brain parasite load. -100% Neonate survival and higher pups weight.	(30)
Bizet, Romanov, and Solognot/ ewes**	10^5(exp#1), $2x10^6$ (exp#2), and 10^5 (exp#3) tachyzoites Δ*mic1-3*/ RH/ sc (exp#1, #2) and ip (exp#3)	Day 60	~ Day 75 (mid gestation)/ oral/ 400 sporulated oocysts (exp#1, #2), 100 sporulated oocysts (exp#3)/ PRU strain	~ Day 135 of pregnancy (exp#1, #3)	-Seroconversion after immunization and before pregnancy (exp#1, #2, #3) -Evaluation of infection clinical signs (febrile response) (exp#1, #2, #3) -Brain cyst load (exp#1, 3)	-Number of non-viable and viable lambs. -Evaluation of abortion. -Mean weights of viable lambs. -Brain cyst load in lambs.	-Maternal seroconversion and lower febrile response. -Protection against *T. gondii* abortion and higher rate of viable lambs. -Decreased brain parasite load in vaccinated ewes and in the lambs.	(31)
Kunming/mice	$5x10^4$ tachyzoites Δ*gra17*/ RH/ ip	Day 70	-Day 12/oral/ 10 Cysts/Pru strain (exp#1) -Day 18/ip/ 200 tachyzoites/ RH strain (exp#2)	-Day 18 of pregnancy (exp#1) -Day 30 after delivery (exp#2)	-*Ex-vivo* splenocyte specific cytokine production (exp#1) -Brain cyst load (exp#2)	-Litter size and survival of delivered pups at birth and 5 days after birth (exp#2). -Body weight 35 days after birth (exp#1) and 5 days after birth (exp#2). -Brain cyst burden in pups at 35 days after birth.	-Higher maternal IFN-γ, IL-12, and IL-10 productions. -Decreased maternal brain parasite load and no clinical signs in immunized dams. -Protection against *T. gondii* abortion. -Higher litter sizes of viable neonates, survival rate and body weight. -Decreased pup brain parasite load.	(32)
Kunming/mice	10^6 tachyzoites Δ*gra17*Δ*npt1*/ RH/ ip	Day 60	Day 5/oral/ 10 cysts/ Pru strain	Day 30 after delivery	Brain cyst load.	-Litter size and survival rates of delivered pups at birth and 30 days after birth - Body weight 30 days after birth. -Brain cysts burden in pups at 30 after birth.	-Decreased maternal brain parasite load. -No abortions in immunized mice. -Increased litter size, pups survival rate, and body weight.	(33)
Kunming/mice	500 tachyzoites Δ*cdpk2*/ Pru/ ip	Day 70	Day 12/oral/ 10 cysts /Pru strain	-Day 18 of pregnancy (exp#1) -Day 5 after delivery (exp#2)	-*Ex-vivo* splenocyte specific cytokine (exp#1). -Brain cyst load (exp#2).	-Litter size and survival of delivered pups at birth and 35 after birth. - Body weight 35 days after birth. - Brain cysts burden in pups at 35 after birth.	-Higher maternal IFN-γ, IL-2, IL-12, and IL-10 production. - Decreased maternal brain parasite load. - Higher litter sizes of viable neonates, survival rate, and body weight. -Decreased pup brain parasite load.	(34)

(Continued)

TABLE 1 | Continued

Strain/animal	Immunogen/strain/delivery	Day of mating post-immunization	Challenge Pregnancy day/route/#parasite form/Strain	Dam sacrifice	Maternal parameters	Progeny parameters	Highlighted results	References
Kunming/mice	10^6 tachyzoites Δ*tkl1*/RH/ip	Day 60	Day 5/oral/10 oocysts/Pru strain	Day 30 after delivery	Brain cyst load	Evaluation of abortion.	-Healthy dams and decreased maternal brain parasite load. - Protection against *T. gondii* abortion. - Higher litter sizes of viable neonates.	(35)

**Mice: pregnancy length of 21 days; ** ewes: pregnancy length of 152 days. MIC1-3KO: micronemal protein 1 and 3 double deletion mutant; ΔGRA17: dense granule protein 17 deletion mutant; Δgra17Δnpt1/: dense granule protein 17 (gra17) and novel putative transporter (npt1) double deletion mutant; ΔCDPK2: calcium-dependent protein kinase 2 (cdpk2) deletion mutant; Δtkl1: tyrosine kinase-like 1 (tkl1) deletion mutant. RH strain (type I); 76K and PRU strains: type II: ip: intraperitoneal; sc: subcutaneous. exp#1: experimental design 1; exp#2: experimental design 2.*

attenuated virulent strains can also be used, albeit, in this case, the attenuation must be complete to ensure that the vaccine will not cause the disease (37). These vaccines present several advantages, such as using whole parasites, meaning that multiple antigens are available simultaneously. Live vaccines also do not usually require repeated immunizations or the use of adjuvants. However, a concern in using this type of vaccination is the possibility of reversing the parasite to a virulent state causing infection (36). Moreover, live vaccines are not recommended to be used in immunocompromised hosts. Toxovax®, the only licensed vaccine for toxoplasmosis, administered to avoid abortion in sheep, is a live-attenuated vaccine, using the strain S48 tachyzoites, originally isolated from an aborted lamb in New Zealand (14). This vaccine is not licensed for humans due to the possibility of parasite reversion to its virulent form (38). Moreover, it has a short shelf life and does not lead to full parasite elimination (14).

Live-attenuated vaccines may be produced by gene targeted approaches. Such is the case of a modified RH strain lacking two genes, respectively encoding micronemal protein 1 (MIC1), which associates to MICs 4 and 6, rendering them active, and MIC3, a micronemal protein necessary for host cell invasion and MIC8 function. The double deletion of these genes (Δ*mic1-3*) resulted in the loss of function of these five proteins. Female Swiss OF1 mice immunized with Δ*mic1-3* strain exhibited higher levels of IFN-γ and IL-2 and a smaller number of brain cysts compared to non-immunized mice when infected with *T. gondii*. Also, all pups born from immunized animals survived compared to 64% of non-immunized mice. Moreover, 55% of the pups born from immunized mice did not present brain cysts and those with brain cysts exhibited a 91% reduction of cyst burden (30). This vaccine was also tested in Bizet, Romanov, and Solognot ewes, using the same experimental design but adapted to the ewes' pregnancy length. It showed to be effective by both subcutaneous (sc) and intraperitoneal (ip) routes, inducing protection against abortion, a higher rate of viable lambs and a decrease of brain parasite cysts in the lambs born from vaccinated ewes (31). Wang *et al*. attempted immunization before pregnancy with a live attenuated vaccine, using tachyzoites of the RH strain with a deletion of the dense granule protein 17 (*GRA17*) gene (Δ*gra17*) that had previously shown to protect mice from lethal infection (32). The deletion entailed a defective parasitophorous vacuole (PV) and decreased intravacuolar tachyzoite proliferation, due to interference with protein transport across the PV membrane. Ip immunization with Δ*gra17* strain elicited the production of Th1-type response cytokines, IL-12, and IFN-γ, as well as of IL-10 in Kunming mice (32). Thereafter, the same authors have tested this vaccine against vertical transmission using both acute and chronic infection models. No maternal clinical signs of infection and abortion were found and the litter sizes of viable neonates in immunized and RH inoculated dams were higher. In both models of infection, pups presented a higher survival rate. Maternal spleen *T. gondii*-induced cytokine production was evaluated at day 18 of pregnancy, 6 days after infection with Pru strain. Higher levels of Th1-type cytokines IFN-γ, IL-12, and IL-2, and of IL-10 were detected. Significantly lower parasite

burden was found in the brain of immunized dams. Further, partial protection was observed, concerning brain parasite load in the progeny of these animals (32). Recently, a live-attenuated vaccine of the RH strain was engineered to harbor a deletion of *Gra17* and of novel putative transporter 1 (*NPT1*) gene, encoding a selective arginine transporter (33). The virulence of this strain (RHΔ*gra17*Δ*npt1*) was completely attenuated *in vivo*. The brain cyst burden of immunized dams was significantly lower and no abortions were observed compared with non-immunized infected mice. All pups born from immunized infected mice had about 15 times fewer brain cysts than non-immunized infected mice pups (33).

A live-attenuated vaccine using tachyzoites of the Pru strain with a deletion of the calcium-dependent protein kinase 2 (*CDPK2*) gene (Δ*cdpk2*) was also developed by Wang *et al.* (34). CDPKs harbored by *T. gondii* are required for cell invasion and gliding motility and are important virulence factors. Specifically, CDPK2 prevents accumulation of amylopectin to toxic levels in the cell, that would cause the parasite to be morphologically defective and unable to form cysts. Therefore, these parasites were incapable of establishing chronic infection since they were not able to form tissue cysts. Dam brain cyst burden was, in average, 43 times lower than that of non-immunized challenged dams. Splenocytes from immunized dams produced higher levels of IFN-γ, IL-2, IL-12, and IL-10 compared with non-immunized mice when stimulated *in vitro* with soluble tachyzoite antigen. These results indicated that this vaccination approach led to a balanced pro- and counter-inflammatory maternal response, useful to control infection but also to avoid potentially harmful excessive inflammation. Pups from non-immunized infected mice harbored in average 919 ± 339 brain cysts, whereas only 41.4% of the pups from immunized infected mice harbored cysts, averaging 60 ± 33 cyst per brain (34). Recently, Wang *et al.* created a live-attenuated vaccine from the *T. gondii* RH strain with a deletion of the tyrosine kinase-like 1 (*TKL1*) gene (RHΔ*tkl1*) (35). Vaccinated Kunming mice remained without clinical signs of infection and showed significant decrease in brain cyst burden 30 days after delivery. No abortions occurred and litter size was unaltered in immunized mice when infected, while all non-immunized infected mice suffered abortions. A decreased brain cyst load was observed in the pups from immunized infected dams indicating reduced vertical transmission (35).

Recombinant Protein Vaccines

Recombinant *T. gondii* surface antigen 1 (rSAG1) protein (39) was assessed in two models of congenital infection (**Table 2**). Haumont *et al.* immunized Dunkin-Hartley guinea pigs subcutaneously (sc) with rSAG1 three times at 3-week intervals and intradermally challenged 3 weeks after breeding (25). Vaccination induced protection against maternal-fetal transmission as assessed by the brain parasite load in the live pups. However, the SAG1-specific IgG levels in newborn pups did not correlate with protection, while cellular responses were not evaluated (25). In another study, BALB/c and CBA/J mice ($H2^d$ and $H2^k$ background, respectively) were sc immunized twice with rSAG1 (40). A reduction of 50% of maternal-fetal transmission in BALB/c, but not in CBA/J mice, was observed. Protection found in immunized BALB/c mice correlated with a maternal increase in rSAG1-specific IgG1 and a decrease in rSAG1-specific IgG2a. IFN-γ and IL-10 levels were increased in serum and in supernatants of *T. gondii* lysate antigen (TLA)-stimulated splenocytes obtained from vaccinated animals. In contrast, the immunized CBA/J mice showed no protection and significantly increased serum IL-10 and IL-4 levels. Further, no differences were observed concerning serum IFN-γ or IFN-γ levels in the supernatants of TLA *ex-vivo* stimulated spleen cells from both rSAG1-vaccinated or control CBA/J animals. These observations suggest that the Th1-/Th2-type responses induced by the immunization used were affected by the host genetic background, such as the major histocompatibility complex, leading to the different outcomes after immunization (40).

DNA Vaccines

DNA vaccines are among the most promising in *T. gondii* research. These vaccines have numerous advantages such as ease of development, low-cost production, stable storage, and shipping. More than 50 vaccine variants have been experimentally produced and tested and have shown positive results in their protective capacity using exogenous infection models (36, 37). Vaccination with a DNA plasmid encoding SAG1, which previously showed to protect BALB/c mice against infection with the avirulent Beverly type-2 strain, upon intra-muscular immunization with PltPASAG1 plasmid, was tested in a congenital infection model. However, maternal-fetal transmission was not reduced as compared to sham-immunized control mice (41)

This observation led to the conclusion that different immune mechanisms could mediate protection in adult-acquired infection and congenital parasite transmission (41). Another approach was performed by Mevelec *et al.* combining DNA plasmids encoding SAG1, *T. gondii* dense granule antigen 4 (GRA4) and murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (42). The survival rates of pups from immunized infected dams was significantly higher compared to non-immunized infected dams. These results indicate that DNA plasmid multiantigen vaccine works better than a single antigen vaccine (42). Further studies are however necessary to highlight the suitability and efficacy of this type of vaccines in vertical infection.

Exosome Vaccines

Exosomes are nano-sized vesicles released by most eukaryotic cells (14). These vesicles can contain a wide variety of molecules, such as proteins, lipids, and nucleic acids, able to activate cellular and humoral responses altering the outcome of parasite infections (45).They can transfer mRNA, miRNA, and proteins between cells, representing a communication path between cells, necessary for immune homeostasis (14, 45). Studies showed that immunization with exosomes released from *T. gondii*-pulsed dendritic cells (DCs) induced protection against congenital toxoplasmosis, associated with IFN-γ and IL-10 responses in

TABLE 2 | Non-live vaccines in vertical *Toxoplasma gondii* infection models.

Strain/ animal	Immunogen/delivery	Day of mating post-immunization	ChallengePregnancy day/route/#parasite form/strain	Dam sacrifice	Maternal parameters	Progeny parameters	Highlighted results	References
Dunkin-Hartley/ guinea pigs*	Recombinant SAG1/ sc	Day 21	21 Days after mating/ id/ $5x10^5$ tachyzoites/ C56 strain	NS	-Specific IgG antibodies at the time of delivery	-Number of litters negative, partially positive, and positive. -Number of stillborn and viable pups and *T. gondii* positive. -Pups brain infectious status 2 days after birth. -Specific neonate IgG antibodies.	-Protection against maternal-fetal transmission (66–86%) -No correlation with specific IgG levels in the newborn pups.	(25)
BALB/c and CBA/J/mice	Recombinant SAG1/ sc	Day 60	Day 12 of pregnancy/ oral/ 10 cysts/ Me49 strain	Day 19 of pregnancy	-Maternal SAG1 IgG1 and IgG2a -*Ex-vivo* splenocyte specific cytokine production	-Fetus parasite load	-Protection against maternal-fetal transmission in BALB/c mice (50%) -Increased maternal IFN-γ and IL-10 production. Higher maternal rSAG1 specific IgG1 levels in BALB/c mice. - No protection in CBA/J mice associated with a Th2 response.	(40)
BALB/c/mice	SAG1-DNA plasmid/ im	Day 14	Day 12 of pregnancy/ oral/ 10 cystes/ Beverley strain	NS	Uterus examination (resorptions) or still births (at delivery)	-Survival and specific IgG in pups 12 weeks after birth -Number of survival pups at day 12 after birth	-No protection against maternal fetal transmission (no differences in pups's survival and specific IgG levels).	(41)
Swiss OF1/ mice	pSAG1mut+pGRA4+ pGMCSF DNA plasmids/ im	Day 15	Day 7-10 of pregnancy/oral/ 70 cysts/ 76K strain	NS	NE	-Survival (days 1–30 after birth) - Weight evaluation (days 8, 15, and 30 after birth). - Brain cyst burden (day 30 after birth)	-Higher survival rates but no differences in pups's body weight. -No protection against parasite vertical transmission (equal brain cyst burden).	(42)
CBA/J/mice	Exosomes derived from DC pulsed with *T. gondii* total antigen sc	Day 14	Day13 of pregnancy/ oral/ 25 cysts/ 76K strain	2 months after infection	Brain cyst load	-Survival (days 1–42 after birth) -Weight evaluation every 4 days for 42 days. -Brain cyst burden (6 weeks after birth) -Specific IgG isotypes and IgA response and *ex-vivo* spleen and lymph node specific cytokine production (6 weeks after birth).	- Decreased maternal brain parasite load. - Higher survival rate and pups body weight. - IgG2a, IgG2b, IL-2, IFN-γ, IL-4, and IL-10 responses in the pups. - Decreased pup brain parasite load.	(43)

(Continued)

TABLE 2 | Continued

Strain/ animal	Immunogen/delivery	Day of mating post-immunization	ChallengePregnancy day/route/#parasite form/strain	Dam sacrifice	Maternal parameters	Progeny parameters	Highlighted results	References
CBA/J/mice	Di-palmityol phosphatidyl glycerol-loaded nanoparticles (DGNP) containing total *T. gondii* extract/ in	Day 60	Day 11 of pregnancy/ oral/ 15 cysts/ 76K strain	Day 17 of pregnancy (exp#1) Day 60 after delivery (exp#2) (6 days post-infection the dams were sacrificed and fetus were collected for parasite load quantification)	- *Ex-vivo* cytokine secretion in placentas (Th1H1: IFN-γ and TNF-α, Th2 (IL4), Treg: TGF-β and IL-10, Th17: IL17A, and anti-inflammatory: IL6) (exo#1). -Brain cyst load evaluated 60 days after delivery (chronic infection) (exp#2).	- Parasites load n fetal tissues by real-time PCR (exp#1). - Evaluation of fetal resorption (exp#1). -Litter size and pups weight (exp#2). -Evaluation of protection in offspring against chronic infection (exp#2). -Average weight of each litter at 7, 14, and 21 days—after birth (exp#2). Ophthalmologic clinical signs (exp#2).	-Decreased maternal brain cyst load. -Reduction of IFN-γ and increase IL-6 and IL-10 placental cytokine levels favoring pregnancy maintenance -Protection against vertical transmission (fetus parasite load) or offspring (brain cyst load) - Higher mean weight and protection against ocular toxoplasmosis in the offspring.	(44)

**Guinea pigs: pregnancy length of 9 weeks. C56 strain: type III; Me49 and 76K strain: type II. sc, subcutaneous; im, intramuscular; in, intranasal; NS, no sacrifice; NE, not evaluated. exp#1: experimental design 1; exp#2: experimental design 2.*

the pups (43). This vaccine, when administered before pregnancy, provided strong fetus protection against infection. Low cyst burden was observed in both immunized dams and pups delivered from immunized dams (43). This was the first description, so far, studying immune responses in the offspring from vaccinated females.

Nanoparticle-Based Vaccines

Advances in research have revealed the use of nanoparticles (NP) as antigen delivery systems, thus setting the basis for a new type of vaccines. This antigen delivery system avoids antigen degradation and increases bloodstream life span, internalization, and presentation by antigen-presenting cells, such as DCs (14). Di-palmitoyl phosphatidyl glycerol-loaded nanoparticles (DGNP) loaded with *T. gondii* total antigen extract were shown to deliver parasitic antigens to mucosa after intranasal immunization, inducing a specific Th1/Th17 response *in vivo* (46). Further work has tested the safety and efficiency of DGNP in congenital toxoplasmosis (44). Placental levels of cytokine production were analyzed, which revealed no signs of inflammation exacerbation in immunized mice, even though, there was an increase in IFN-γ concentration. IL-10 and IL-6 levels were significantly raised. Survival of offspring and dams was 100% and mean litter size and pup weight was not diminished in infected immunized mice. The parasite burden on the fetus was 86% reduced from females immunized with DGNT/TE compared to controls (44).

CONCLUSION

Most of the research work that addresses vaccination using vertical *T. gondii* infection models assessed the immune response to vaccination, determining the IgG isotypes and cytokines produced in response to parasite antigen stimulation. Typically, production of IFN-γ, IL-2, IL-12, IL-10, and, occasionally, IL-4 was found elevated in response to vaccination in non-pregnant mice until 60–70 days post-vaccination. This might explain why this time-point has been referred in the achievement of pregnant animals, since the balance between these cytokines is essential in successful pregnancy as reported in almost all the studies referred here.

Most reports gather data from acute, chronic, and congenital infection experiments. However, the immune status during pregnancy is altered (7), therefore, the data from non-pregnant mice must be carefully discussed and not directly extrapolated to pregnancy due its specific immune status.

In two studies using live attenuated vaccines, mixed maternal Th1-/Th2-type responses were induced, being discussed as Th1 crucial against *T. gondii* congenital infection and Th2 essential for pregnancy maintenance (32, 34). Currently, a balance between several subsets of T cells must be considered such as Th1, Th2, Th9, Th17, Th22, and follicular Th cells (Tfh) for a successful human pregnancy (47). Indeed, these T cell subsets contribute to the immune response occurring at the maternal-fetal interface, known to be important not only in protecting against infection and controlling inflammatory response, but also contributing to

immune homeostasis, implantation, decidualization, maternal immune tolerance and acceptance of the fetus, and parturition (47).

A heterogeneity in the experimental designs was found in the studies reported. The difficulty of performing research work with congenital infection models is very high and may explain the scarcity of research in this area. It would be useful to choose a standard experimental design in the study of vaccines, using congenital models able to get valid and robust results. Murine models are the most chosen because they offer simplified logistics, including the facility to monitor physiologic parameters and the short length of pregnancy. Further, mice allow experiments with higher animal numbers, availability of immunological reagents and genetically modified hosts. Mice were largely validated as an adequate model to study congenital toxoplasmosis, by reproducing many features of human infection (21).

None of the vaccines described so far managed to fully protect against *T. gondii* vertical transmission, even if providing multi-antigenic stimulus. The lack of more effective vaccination approaches may be a consequence of the scarce knowledge on the host protective immune molecular and cellular mechanisms operating at the maternal-fetal interface, specifically at decidua and placenta. Indeed, only one work testing nanoparticles containing *T. gondii* total extracts, analyzed the cytokine profile in placentas from vaccinated dams (44). This study found that a reduction in IFN-γ and an increase in IL-6 and IL-10 production was associated with protection against vertical transmission and ocular toxoplasmosis in the offspring (44). Determining the type of immune response at maternal-fetal interface that can correlate with protection will be useful to refine vaccination approaches, by selecting adjuvants that could adequately polarize T cell responses, thereby leading to protection against congenital infection. On the other hand, it would be also important to understand the immune response developed in the pups born from vaccinated dams and infected during pregnancy. To our knowledge, only one study analyzed the immune response in surviving pups born from vaccinated dams, having found a mixed Th1- and Th2-type response associated with a high survival rate, high weight mean, and low cyst brain burden (43). The development of adaptive fetal immune responses are observed in neonates exposed to an infection environment *in uterus* but not necessarily infected themselves (48). Thus, it will be worthy to get new insights into how T cell responses and related mediators operate in fetuses and in neonates. Novel vaccine design formulations and delivery systems can also be improved, concerning parasite antigen determinants and elicited immune mechanisms involved in protection against *T. gondii* vertical infection (36).

AUTHOR CONTRIBUTIONS

MBa, DT, and MBo wrote the initial manuscript. All authors contributed to the article and approved the submitted version.

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Malaria and Early Life Immunity: Competence in Context

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Childhood vaccines have been the cornerstone tool of public health over the past century. A major barrier to neonatal vaccination is the "immaturity" of the infant immune system and the inefficiency of conventional vaccine approaches at inducing immunity at birth. While much of the literature on fetal and neonatal immunity has focused on the early life propensity toward immune tolerance, recent studies indicate that the fetus is more immunologically capable than previously thought, and can, in some circumstances, mount adaptive B and T cell responses to perinatal pathogens *in utero*. Although significant hurdles remain before these findings can be translated into vaccines and other protective strategies, they should lend optimism to the prospect that neonatal and even fetal vaccination is achievable. Next steps toward this goal should include efforts to define the conditions for optimal stimulation of infant immune responses, including antigen timing, dose, and route of delivery, as well as antigen presentation pathways and co-stimulatory requirements. A better understanding of these factors will enable optimal deployment of vaccines against malaria and other pathogens to protect infants during their period of greatest vulnerability.

Keywords: fetal immunity, neonatal immunity, malaria, plasmodium, neonatal vaccination

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NEITHER MAN NOR MOUSE: IMMUNE COMPETENCE IN THE INFANT

The human infant has long been considered immunologically immature, or deficient, when judged against the yardstick of the adult immune system. Indeed, the fetus and newborn are vulnerable to severe disease and high morbidity from numerous pathogens that cause only mild disease in older hosts. It had previously been believed, based largely on extrapolation from mouse models of immune development, that the tolerogenic intrauterine environment is incompatible with the priming of fetal T cells or results in marked polarization of CD4 T cells toward a Th2 response over a Th1 response. However, it is now appreciated that human fetal immune development differs quite markedly from that of the mouse. Neonatal mice are profoundly immunodeficient—indeed, migration of murine T cells from the thymus begins only after birth. In contrast, during human gestation, T cells appear in the fetal liver at week 10 and T cell zones can be seen in the spleen during week 18 (1). The recent development of single-cell analysis tools has led to a remarkable burst of progress in the study of fetal and infant immune cell populations, and has revealed a surprising degree of immune competence at, and even before, full-term gestation (2–5).

Placental malaria offers a valuable model to examine the response to pathogen-derived antigens *in utero*, and findings from this model bear relevance to other parasitic pathogens. Annually, more than 125 million pregnancies occur in areas where

malaria is endemic (6), and 25% of pregnancies in sub-Saharan Africa are complicated by malaria (7). While true congenital infection with malaria parasites is rare, malaria antigens gain access to the fetal circulation after crossing the placenta, and studies of cord blood from malaria-exposed neonates have demonstrated an impact on numerous immune cell populations (2, 8, 9). Thus, understanding the immunological implications of placental malaria exposure is of paramount importance to public health.

LEARNING TO ADAPT: T AND B CELLS

Not surprisingly, most T and B cells in umbilical cord blood are naïve in phenotype, reflecting the relative lack of antigen experience during intrauterine life. During early and mid-gestation, human fetal T cells are inclined toward tolerance, as has been reviewed elsewhere (10, 11). Upon encounter with non-self-antigens, naïve CD4 T cells in the mid-gestation fetus preferentially differentiate into FoxP3+ regulatory T-cells that, along with other regulatory populations, can actively suppress T cell activation and cytokine production (12–14). However, as the fetus approaches term, it must balance the demands of maternal tolerance with the need to mount an effector T cell response against potential pathogens encountered after birth. It was recently shown that even in the absence of intrauterine pathogen exposure, a sizeable subset of CD4 T cells in cord blood exhibit effector-memory differentiation and can produce both Th1 and Th2 cytokines (15). Indeed, TNFα and IFNγ production by fetal T cells that are alloreactive to maternal antigens may contribute to preterm labor (16). Whether fetal Th1 responses to pathogens such as malaria may contribute to preterm birth or poor growth *in utero* has not been adequately investigated.

Until recently, relatively few studies have applied single-cell analysis techniques to characterize the human T cell response to pathogen-derived antigens encountered *in utero*. In aggregate, these studies suggest that despite the numerous mechanisms enforcing fetal and neonatal tolerance, the fetus is capable of mounting robust T cell responses under particular conditions. Congenital viral infections such as CMV result in expansion and differentiation of virus-specific CD8 T cells that produce IFNγ, TNFα, and perform perforin-mediated cytolysis (17). Recently, we showed that in the setting of placental malaria, CD4 and CD8 T cells primed *in utero* demonstrate effector-memory differentiation, inflammatory cytokine production, and robust malaria antigen-specific T cell proliferation. These effector populations were associated with protection from both *P. falciparum* infection and symptomatic malaria during the first 2 years of life (2). The most profound effector T cell differentiation was observed in infants born to mothers with active placental malaria at the time of birth, possibly suggesting late gestation exposure (2). However, T cell responses were not observed in all exposed infants in this study, and other investigators have observed that *in utero P. falciparum* exposure tolerized fetal T cells in a subset of exposed infants (18). The timing, duration, and quantity of malaria antigen exposure (influenced by intermittent preventive therapy with antimalarials), as well as the degree of associated placental inflammation, may play a large role in influencing the balance between fetal T cell tolerance vs. effector differentiation.

Some evidence indicates that fetal B cells can also be primed *in utero*. Malaria-specific antibodies of the IgM class (which cannot cross the placenta) have been detected in the cord blood of malaria-exposed infants as early as 22 weeks gestation (8, 9, 19) and surprisingly, class-switching of malaria-specific B cells from IgM to IgG occurs in some infants prior to delivery (8). These findings raise the possibility that fetal B cells could be sensitized by maternal malaria vaccination, as has been reported with maternal tetanus and influenza vaccination (20, 21).

Together, these data indicate that the essential machinery for generation of robust T and B cell responses to pathogen-derived antigens is present during fetal life. They further suggest that the mechanisms that curb T cell inflammation *in utero* are not entirely cell-intrinsic, but also relate to extrinsic factors such as a lack of sufficient activating or co-stimulatory signals from antigen presenting cells (APCs) or from a tolerogenic cytokine environment. A better understanding of the conditions (e.g., timing, antigen load) that foster the priming and development of functionally competent pathogen-specific T cells (while avoiding induction of pathogen tolerance) could be of fundamental importance for efforts to develop vaccines that are optimally immunogenic in infancy.

APCs: PRESENTATION MATTERS

The maternal and fetal blood supply are separated by a single multinucleated cell layer termed the syncytiotrophoblast. Once malaria antigens or immune complexes cross the syncytiotrophoblast barrier, it is not clear where, how, and by "whom" (i.e., what cell type) they are taken up, processed, and presented to lymphocytes (**Figure 1**). This is a critical question, as APCs are key orchestrators of the immune response and play a paramount role in the initiation and regulation of adaptive immune responses through priming of antigen-specific T cells. Murine data indicate that neonatal T cells are extremely sensitive to the conditions of antigen presentation at priming, and small differences in the dose of antigen (22), type of APC (22, 23), and intensity of costimulation (22–24) strongly influence the efficacy of the ensuing T cell response. Given the many shortcomings of the neonatal mouse model (25), further studies are needed to confirm the relevance of these findings in human infants.

In adults, myeloid-lineage cells such as dendritic cells (DCs) and monocytes play a principal role in antigen presentation, although activated CD4 T and B cells also upregulate HLA-DR and can present antigen (26–28). In the fetus and neonate, dendritic cells and monocytes are both relatively inefficient in their ability to prime adaptive immune responses due to their reduced expression of MHC-II, co-stimulatory molecules, and Th1-polarizing cytokines (29–31). In particular, neonatal DC production of IL-12p70, the key cytokine required for Th1 polarization, is markedly reduced due to epigenetic regulation of the gene encoding its p35 subunit (29, 31–33). Th1 cytokine production by fetal DCs may be further inhibited by expression

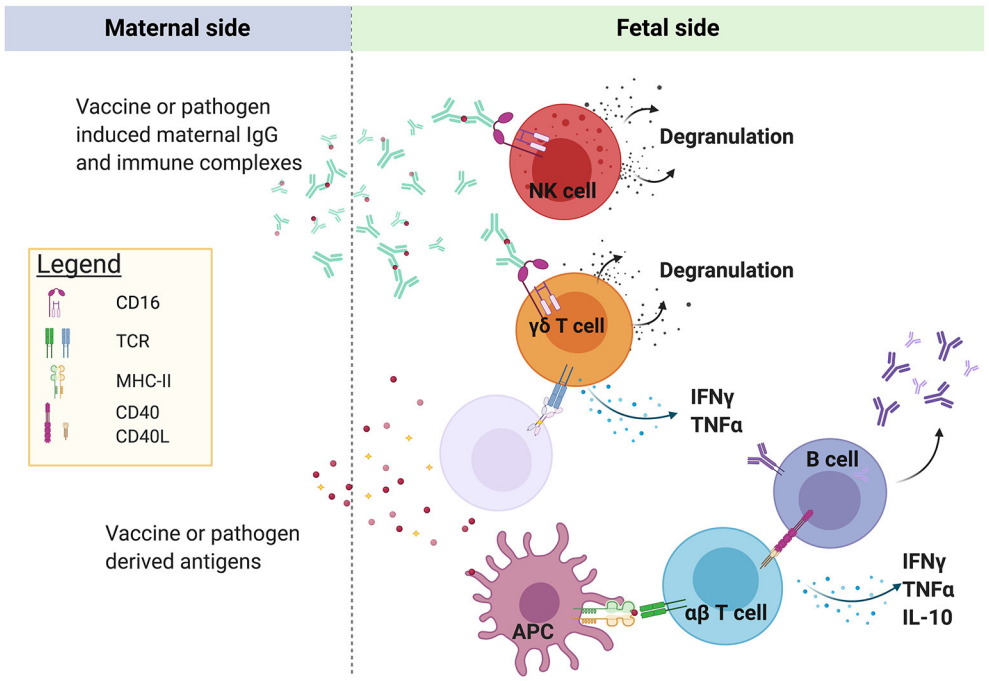

FIGURE 1 | Maternal-origin IgG is transported across the syncytiotrophoblast barrier of the placenta to the fetus via FcRn, possibly in the form of immune complexes. In areas of placental villous denudement or necrosis, unbound plasmodial antigen may also cross into the fetal circulation. *P. falciparum* antigens have been shown to prime fetal αβ T cells and B cells, the location and identity of the antigen-presenting cells remain unknown, but could include fetal Hofbauer cells, dendritic cells, or γδ T cells. Semi-innate Vγ9Vδ2 T cells can be directly activated by plasmodial-derived phosphoantigens via butyrophilin2a1 and butyrophilin3a1, even in the absence of prior antigen exposure. In addition, fetal lymphocytes expressing CD16/FcRγIIIa, including NK cells and possibly γδ T cells, may be activated by maternal IgG bound to antigen. Created with BioRender.com.

of arginase-2 (4). Fetal monocytes are also inefficient in their upregulation of costimulatory and antigen presentation machinery in response to IFNγ (34) despite enhanced sensitivity to inflammatory cytokines and increased expression of the IL6 receptor. Instead, inflammatory cytokines activate non-canonical signaling pathways in fetal monocytes, leading to upregulation of genes involved in the primitive antimicrobial response (34). This is likely a strategy to prevent activation of a potentially harmful anti-maternal adaptive response, which may trigger preterm labor and fetal expulsion (16).

It is possible that alternative cell populations may play a particularly important role in antigen presentation during fetal life. Macrophages termed Hofbauer cells reside within the placental villous stroma and express multiple Fc receptors, making them well-suited to a role in immune surveillance. Hofbauer cells are increased in placental malaria (35), but whether they play a role in antigen uptake is unclear. In an *ex vivo* human placental perfusion model, upon transplacental transfer immune-complexed MSP-1 was observed in the fetal villous stroma where it predominantly co-localized with fetal endothelial cells, not Hofbauer cells (36). Moreover, a recent and very detailed phenotypic analysis by Thomas et al. found that fetal-origin macrophages do not express HLA-DR (37). It is possible that non-myeloid cells, including non-classical lymphocytes, contribute to the induction of adaptive immune responses *in utero*. In adults, the Vγ9Vδ2 subset of γδ T cells (discussed in detail below) exhibit robust antigen presentation capabilities upon activation (38, 39) and can induce proliferation and differentiation of naïve αβ T cells comparable to that of mature DCs. Vγ9Vδ2 cells are highly prevalent during the second trimester (40), and their potential role in antigen presentation during fetal life merits further investigation.

INNATE ABILITY: NK CELLS AND NON-CLASSICAL LYMPHOCYTES

Innate immune populations may play a particularly important role in protecting the fetus and infant when little or no immunologic memory exists. In particular, semi-innate γδ T cells have several qualities that make them uniquely suited to protection of the fetus and infant. Unlike conventional αβ T cells, γδ T cells recognize conserved ligands and exhibit rapid, innate-like effector functions, including degranulation and cytokine production (41). This effector response is not dependent on prior antigen exposure nor on priming by dendritic cells, which are functionally immature in the fetus. γδ T cells are highly conserved across vertebrate species and are the first T cells to develop in the human fetus. It has been hypothesized that the primary selective advantage driving their remarkable conservation is their role in neonatal protection (39). Supporting

this hypothesis, γδ T cells are required for protection of young, but not mature, mice in models of parasitic infection (42).

During malaria infection, a specialized subset of γδ T cells, defined by use of the δ2 and γ9 TCR chains, can act as innate-like effectors that can indirectly recognize phosphoantigens that are produced by the *Plasmodium* apicoplast (43). These Vγ9Vδ2 T cells exhibit intrinsic reactivity to *Plasmodium*, with rapid degranulation and production of IFNγ and TNFα, even in malaria-naïve individuals (44). They are able to kill extracellular merozoites via release of granulysin and inhibit parasite growth *in vitro* (45). Moreover, they have been associated with protection from malaria in human clinical trials (46, 47). In the fetus, this subset has been shown to dominate the γδ T cell repertoire in the second trimester (40). Following *in utero* malaria exposure, cord blood Vγ9Vδ2 T cells are preferentially activated, produce more IFNγ (48), and exhibit greater memory differentiation (49). Thus, Vγ9Vδ2 T cells may be poised to respond to *Plasmodium* infection in the fetus and neonate.

The role of NK cells and other innate lymphoid cells (ILCs) in the fetal and infant immune response to malaria has received little research attention. Fetal NK cells that develop early in gestation can respond robustly to antibody mediated and cytokine-induced stimulation, but respond weakly to HLA-devoid cells and are more susceptible to TGF-β mediated suppression (50). At birth, NK cells are highly responsive to immune complexes and exhibit robust antibody-dependent functions, including IFNγ production and degranulation (51). Higher cord blood frequencies of $CD56^{dim}CD16^{+}$ NK cells have been observed following maternal *P. falciparum* infection (52). In light of recent studies demonstrating an association between NK cell antibody dependent cellular cytotoxicity (ADCC) and malaria outcomes in children and adults (53), these findings raise the possibility that NK cells could play an important antimalarial role *in utero* and in the newborn through engagement of transplacentally acquired malaria-specific antibodies.

MATERNOFETAL ANTIBODY TRANSFER: PASSIVE AND AGGRESSIVE?

During gestation, maternal antibodies are transferred across the placenta to the fetus via an active transport mechanism mediated by FcRn, the neonatal Fc receptor. FcRn is expressed on syncytiotrophoblast cells beginning at approximately 13 weeks gestation (54), and selectively transfers IgG. Transfer of IgG increases as pregnancy progresses, rising sharply in the final month of gestation, such that full-term infants have IgG levels that generally exceed those of the mother (54, 55). FcRn-mediated transport is influenced by maternal IgG concentration and saturates in the setting of high total IgG levels.

Maternally derived antibodies are essential for infant protection from some pathogens that are commonly encountered during infancy. In the case of malaria, the importance of this passive maternal antibody transfer is not clear. It has been widely promulgated that transplacental transfer of antimalarial antibodies is responsible for the relatively low incidence of symptomatic malaria during the first 6 months of life. Yet, a thorough review of this literature found scant evidence to support a protective role in infancy, concluding instead that malaria-specific IgG in cord blood merely serves as a biomarker of maternal exposure (56). It should be noted, however, that most studies to address this relationship evaluated antibodies to only a very limited number of malaria antigens, and measured only total IgG, and not individual IgG subclasses.

IgG subclass and glycosylation can both greatly influence the efficiency of transplacental antibody transfer by FcRn (55). The overall transfer efficiency is highest for the IgG1 subclass, followed by IgG4, IgG3, and IgG2. It is notable that IgG3 is transferred to the fetus with low efficiency, as it is the strongest activator of complement via recruitment of C1q, and its potent opsonizing ability has been linked to clinical protection from malaria in adults and children (57–59). IgG3 also binds more strongly to FcγRIIIa, which is important in ADCC and other antibody-dependent functions, than other IgG subclasses (60). Interestingly, a polymorphism in the FcRn-binding domain of IgG3 (H435) has been associated with increased transplacental transfer of malaria-specific IgG3, increased half-life of IgG3 in the infant, and protection from clinical malaria during infancy (61). Given this evidence, it will be important to understand the IgG subclass profile of transplacentally-transferred IgG in malaria-endemic regions (62), as well as the significance of opsonized antigen, complement activation, and FcR-expressing fetal cells in infant protection from malaria. Furthermore, secondary glycan structures, which can vary across pathogen-specific antibody repertoires within an individual (51, 63, 64), have also been shown to modulate the efficiency of transplacental IgG transfer. For example, digalactosylated Fc-glycans bind more strongly to FcRn leading to increased transplacental transfer (51), and they also preferentially bind FcγRIIIa leading to better activation of neonatal NK cells (51). As vaccine adjuvants can influence antibody subclass and glycosylation (65) which in turn influence placental transfer of antibody (55), vaccine design should prioritize adjuvants that enable better transplacental transfer and activation of neonatal effector cells.

In addition to conferring passive immunity, maternofetal antibody transfer may play a role in conveying pathogens and pathogen-derived antigens to the fetus in the form of antigen-antibody complexes. While some malaria antigens may breach the maternofetal barrier due to focal denudement of the syncytiotrophoblast cell layer, the more common route of transfer is likely via FcRn-mediated active transport of antigen-antibody complexes (8, 36), as supported by experimental evidence from an *ex vivo* placental perfusion model (36). There is also some evidence that FcRn can facilitate congenital infection by mediating transfer of CMV and ZIKV virions in the form of immune complexes (66–68). Hence, maternal immunoglobulins can at times act as a Trojan horse, ferrying pathogen-derived antigens (or even intact pathogens) to the fetus.

IMPLICATIONS FOR VACCINATION IN PREGNANCY AND EARLY INFANCY

The ultimate goal of better understanding the components of the fetal, neonatal and pregnant immune systems is to enable the translation of this knowledge to vaccine development. Late

stage trials are underway for vaccines to prevent placental malaria by inducing maternal antibodies against VAR2CSA, a pregnancy-specific adhesion ligand that is thought to be an important mediator of placental adherence (69). The goal of this vaccine approach is to stimulate maternal immunity and thus prevent placental malaria, sparing the infant from the clinical sequalae of placental insufficiency and inflammation, which include prematurity and poor growth. This distinguishes it from other vaccines targeting pregnant women, for which the rationale is to confer passive IgG-mediated immunity to the infant. Examples of this approach include vaccination against pertussis, influenza, and tetanus, all of which can be safely administered during pregnancy and induce high neutralizing titers of maternal antibodies that are transferred transplacentally and confer protection to the infant during the early months of life (70–73). Immunizing pregnant women against malaria (with RTS,S, whole sporozoites, or other vaccine candidates) could similarly induce transfer of malaria-specific IgG to the infant. However, whether this passive immunization approach would be of value hinges largely upon the still unanswered question of whether malaria-specific IgG antibodies, or a subclass thereof (e.g., IgG3), confer protection in infants. Moreover, it is important to note that maternal immunization can have adverse consequences by interfering with induction of antibodies in the infant. This has been most clearly demonstrated in the case of pertussis and viral pathogens such as measles, where maternal-origin IgG neutralizes vaccine antigen in the infant and thus preempts priming of infant B cells (74–77). However, even non-neutralizing antibodies can inhibit infant antibody production by mechanisms that may include masking of immunogenic epitopes, Fc-mediated engagement of inhibitory receptors, and/or clearance of maternal immune complexes; hence maternal antibody interference may be of concern for malaria. A recent meta-analysis estimated that maternal antibody inhibition results in a reduction of infant antibody responses to a broad range of antigens in common childhood vaccines (78). Interference by maternal-origin IgG has been hypothesized to contribute to the poor immunogenicity of RTS,S in infants (79). Ultimately such concerns may be mitigated by Fc engineering (currently being developed for monoclonal antibody therapies) and tailoring of adjuvants to induce antibodies of the desired subtype and glycosylation characteristics. As it is difficult to predict whether the benefits of maternal antibodies would outweigh the concern for antibody interference, the impact of maternal-origin IgG on infant vaccine responses should be an area of careful empiric investigation as malaria vaccine candidates enter field trials.

Mounting evidence that adaptive T and B cell responses can be primed *in utero* raises the additional question of whether *active immunization* of the fetus may indeed be possible. Such a strategy would be dependent upon delivery of antigen to the fetus in a context that favors priming of antigen-specific fetal T and/or B cells. The demonstration that fetal B and T cells are primed naturally in response to placental malaria in some infants (2, 8) should lend optimism to this prospect. Additionally, it has been shown that fetal T cells are primed in response to maternal influenza vaccination (20). While *in utero* vaccination may seem like a far horizon, these observations provide proof of concept that induction of protective immunity prior to birth may be possible. However, efforts toward fetal immunization would need to address the potential for induction of pathogen-specific tolerance *in utero*, which has been reported in some malaria-exposed infants (18, 80).

CONCLUSION

As pregnancy progresses, the fetal immune system gradually evolves from one that is skewed toward tolerance to one that is poised to fight foreign pathogens. The research agenda for translating recent advances in our understanding of fetal and neonatal immunology into vaccines that are safe and immunogenic when administered in early infancy is now coming into focus. Understanding how to best prime adaptive immune responses in the neonate is critical, due to both the increased susceptibility of newborns to infectious diseases and to the increased healthcare contact of this vulnerable population at the time of birth. This will be particularly important in the context of malaria vaccines, as the vast majority of malaria deaths occur in children under the age of five. Recent evidence from studies of infants exposed to perinatal pathogens indicates that fetal B and T cells can be primed and differentiate into effector cells *in utero*, providing grounds for optimism that they may respond to vaccination. In order to harness this intrinsic capability to engender durable antigen-specific memory in neonates, we will need to refine our understanding of which APCs are most able to prime fetal and neonatal immune responses and which adjuvants are best able to target and stimulate these APCs.

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PC, LF, and MF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Progress and Insights Toward an Effective Placental Malaria Vaccine

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In areas where *Plasmodium falciparum* transmission is endemic, clinical immunity against malaria is progressively acquired during childhood and adults are usually protected against the severe clinical consequences of the disease. Nevertheless, pregnant women, notably during their first pregnancies, are susceptible to placental malaria and the associated serious clinical outcomes. Placental malaria is characterized by the massive accumulation of *P. falciparum* infected erythrocytes and monocytes in the placental intervillous spaces leading to maternal anaemia, hypertension, stillbirth and low birth weight due to premature delivery, and foetal growth retardation. Remarkably, the prevalence of placental malaria sharply decreases with successive pregnancies. This protection is associated with the development of antibodies directed towards the surface of *P. falciparum*-infected erythrocytes from placental origin. Placental sequestration is mediated by the interaction between VAR2CSA, a member of the *P. falciparum* erythrocyte membrane protein 1 family expressed on the infected erythrocytes surface, and the placental receptor chondroitin sulfate A. VAR2CSA stands today as the leading candidate for a placental malaria vaccine. We recently reported the safety and immunogenicity of two VAR2CSA-derived placental malaria vaccines (PRIMVAC and PAMVAC), spanning the chondroitin sulfate A-binding region of VAR2CSA, in both malaria-naïve and *P. falciparum*-exposed non-pregnant women in two distinct Phase I clinical trials (ClinicalTrials.gov, NCT02658253 and NCT02647489). This review discusses recent advances in placental malaria vaccine development, with a focus on the recent clinical data, and discusses the next clinical steps to undertake in order to better comprehend vaccine-induced immunity and accelerate vaccine development.

Keywords: *Plasmodium falciparum*, placental malaria, VAR2CSA, PfEMP1, vaccine, pregnancy

INTRODUCTION

In 2019, approximately 11 million pregnant women were exposed to the risk of *P. falciparum* infection (1). Malaria contracted during pregnancy can lead to significant clinical complications for the mother, including anaemia (2, 3) and hypertension (4, 5), but also for the child. Indeed, malaria in pregnancy may account for over 200,000 stillbirths each year (6) and for the delivery of over 800,000 low birth weight babies (1). This clinical picture varies depending on the parity status and intensity of *P. falciparum* transmission in a given geographical area (7). Primigravid women are highly susceptible to develop severe clinical outcomes following *P. falciparum* infection. However, in high endemicity area, the incidence of illness sharply drops after successive pregnancies (7), demonstrating that protective immunity can be naturally acquired. These observations raised the hope of developing a vaccine that could protect pregnant women against the serious clinical manifestations of malaria in pregnancy.

P. falciparum infection in pregnancy can cause substantial morphological and immunological changes in the placenta, where a massive accumulation of infected erythrocytes (IEs) takes place (8–10), reshaping the cytokine profile of the local environment (11–13) and altering the maternofoetal exchanges (14). IEs from placental origin present a unique adhesive phenotype and do not bind to the host receptors (CD36, CD31, EPCR) commonly used by the parasite to cytoadhere to the microvasculature lining (15–17). Instead, placental IEs interact with a low-sulphated sugar only present at the surface of syncytiotrophoblasts, the chondroitin sulphate A (CSA) (18–21). This low-sulphated placental CSA is structurally distinct from CSA present in other organs or secreted into the extracellular matrix and body fluids (21). A single member of the *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) family, named VAR2CSA, has been identified as the main parasite-derived ligand mediating the interaction of IEs with placental CSA (22–27). Numerous studies have correlated the presence of antibodies reacting to VAR2CSA with protection against placental malaria (PM) (28–30). In a recent systematic review and meta-analysis, Cutts et al. showed that VAR2CSA derived antigens were positively associated with the presence of placental infections. However, they could not identify evidence that antibody response towards a specific VAR2CSA antigen is associated with protection from PM (31). The capacity of anti-VAR2CSA antibodies to block the adhesion of IEs to CSA is thought to play a major role in protection (32) but accumulating evidence suggests that other antibody-dependent effector mechanisms, such as opsonic phagocytosis (33–35), could also actively participate in parasite clearance.

Current malaria control strategies during pregnancy mainly consist in the use of long-lasting insecticidal bed nets and the administration of intermittent preventive treatments based on sulphadoxine-pyrimethamine (IPT-SP) as well as iron and folic acid supplementation [reviewed in (36, 37)]. Even if effective in reducing the malaria burden in pregnant women living in endemic areas (1), the global implementation of such approaches is threatened by the emergence of widespread resistance of both anopheles mosquitoes to insecticides (38) and of parasites to anti-malarial drugs (39). Furthermore, since IPT-SP is only initiated at the first antenatal care visit, usually in the second trimester, and that increasing evidence suggests that *P. falciparum* infection is particularly frequent and detrimental in the first trimester of pregnancy (40–43), complementary intervention regimens such as vaccines would therefore be extremely valuable. In the current global malaria vaccine landscape, predominated by anti-infection/transmission approaches, VAR2CSA-based PM vaccines stand as the main anti-disease strategy to reduce placental malaria morbidity and mortality.

IDENTIFYING THE BEST VAR2CSA-DERIVED VACCINE CANDIDATE

Prevention of placental infection in first pregnancies is not bestowed by the naturally acquired immune responses from previous *P. falciparum* infections, i.e., prior to pregnancy. Nevertheless, natural and protective immunity develops after one or two pregnancies (7, 44, 45). Vaccine candidates aiming to protect against PM should mimic, at least in part, the naturally acquired antibody response associated with protection (32). Ideally, the vaccine-induced immune responses should be boosted and even substantiated by natural infections.

Following the identification of VAR2CSA and the determination of its role in PM pathogenesis and immunity, researchers have been trying to identify the best VAR2CSA-derived antigen to include in a PM vaccine (27). Indeed, VAR2CSA is a complex cysteine-rich 350 kDa protein composed of an N-terminal segment (NTS) followed by six Duffy-binding like (DBL) domains interspaced by four inter-domains (ID) with less defined structures, a transmembrane region and a cytoplasmic ATS region (**Figure 1**). This complexity, associated to the difficulty in expressing sufficient material of the full-length VAR2CSA extracellular region to proceed to clinical trial, have spurred researchers to characterize and identify a subunit VAR2CSA vaccine. All *P. falciparum* genomes carry one or several *var2csa* genes (46, 47). *Var2csa* has also been identified in the genomes of *P. reichenowi*, which infects the chimpanzee, indicating that the CSA-binding phenotype has an ancient origin and that protein functionality might exert some degree of constraint on sequence variation (48). Among the various VAR2CSA variants, most of the functional studies have been performed on VAR2CSA derived from 3D7 and FCR3 laboratory strains. The secondary structure of the DBL fold is conserved, and variable regions are scattered in the primary sequence as mosaic blocks, which are generally the prime target of anti-VAR2CSA antibodies (49–51). The assumption that a structurally constrained adhesion-mediating region within VAR2CSA could be more conserved than others, led early research to focus on the determination of the single DBL domain(s) involved in CSA-binding (25, 51–57). The finding that single DBL domains, which appeared not to bind CSA, could induce inhibitory antibodies was a primary indication that the tertiary structure of the protein was not like DBL-pearls on a string (58, 59). When full-length recombinant VAR2CSA proteins became available, low-resolution structures obtained by small-angle X-ray scattering (SAXS) and CSA-binding kinetics of the full-length recombinant proteins indicated that VAR2CSA harboured a globular conformation rather than a linear-shaped structure

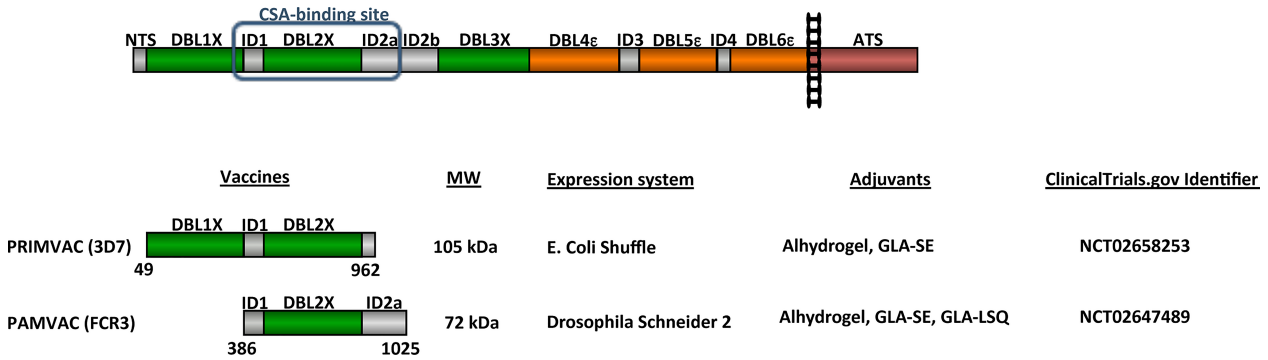

FIGURE 1 | Schematic representation of VAR2CSA and vaccines tested in clinical trials. VAR2CSA is a 350-kDa transmembrane protein composed of an N-terminal segment (NTS) followed by six Duffy-binding like (DBL) domains interspaced by 4 inter-domains (ID), a transmembrane region and a cytoplasmic Acidic C terminus sequence (ATS). PRIMVAC and PAMVAC schematic representation with sequences boundaries, predicted molecular weight, expression systems, and adjuvants used for the Phase I clinical trials. ClinicalTrials identifier for both Phase I are indicated.

(60, 61). The determination of the CSA-binding kinetics of the full-length recombinant VAR2CSA also revealed that the full-length protein was able to interact with CSA with high specificity and high affinity, yielding nano-molar affinity constants. This was markedly different to those obtained for single-domain constructs in the micromolar range (60, 61). Using SAXS and single particle reconstruction from negative stained electron micrographs, a recent study confirmed the globular conformation of VAR2CSA and suggested a model for CSA-binding (62). Dissection of the CSA-binding affinities of recombinant truncations of full-length VAR2CSA, as opposed to single DBL domains, identified the N-terminal part of VAR2CSA as the CSA-binding region (63–65). While for the 3D7 variant, a more specific binding to CSA was observed using the DBL1x-DBL3x multidomain protein (64), the DBL1x and DBL3x domains did not appear as essential for high affinity CSA binding for the FCR3 variant (65). The binding affinity of FCR3 DBL2x including small N and C terminal flanking ID1 and ID2 inter-domains was similar to the autologous full-length VAR2CSA. Thus, the minimal binding region of FCR3-VAR2CSA was designated as ID1-DBL2x-ID2a (65).

Although the identification of the high affinity CSA-binding site has orientated researchers to target this region to induce inhibitory antibodies, many studies have looked at the quality of the immune response generated by the different single DBL domains and multiple domains, including the full-length extracellular region of VAR2CSA. Indeed, due to the globular fold of the full-length molecule it appears possible that antibodies directed towards residues not directly involved in CSA-binding could i) be cross-reactive against different variants and/or ii) affect the molecular interaction of VAR2CSA with CSA by steric hindrance.

Interestingly, the generation of single domain nanobodies following immunization of camelids with the full-length VAR2CSA recombinant protein revealed a preferential targeting of the DBL1x domain and, to a lower extent, of DBL4ϵ, DBL5ϵ, and DBL6ϵ (66) suggesting the existence of immunodominant epitopes outside the CSA-binding region of the protein. This assumption is also strengthened by another study showing that monoclonal antibodies generated from naturally infected women are predominantly directed against DBL3x and DBL5x (67). Remarkably, none of these nanobodies and monoclonal antibodies presented CSA-binding inhibitory capability, which indicates that a single molecule targeting an epitope outside the binding region is unlikely to inhibit the adhesion of IEs to CSA.

Several small animal immunization studies, many of which have been carried out in collaboration between different laboratories, have established that the generation of antibodies targeting epitopes shared between different parasite lines could be achieved using various VAR2CSA domains (68–75). Although antibodies raised against the full-length VAR2CSA may not be cross-inhibitory (76), several studies performed in rodents have shown that shorter constructs including the N-terminal region of VAR2CSA are able to induce cross-inhibitory antibodies preventing IEs from binding to CSA (77–81).

Taken together, these data have evolved the rationale for developing a vaccine against PM based on the CSA binding N-terminal region, in the hope that it will prime the immune response towards semi-conserved antigenic determinants and then mimic the antibody repertoire acquired through natural exposure in first pregnancy (32).

PRIMVAC AND PAMVAC PHASE I CLINICAL TRIALS

VAR2CSA-based vaccines spanning the CSA-binding region (PRIMVAC and PAMVAC), designed to generate antibodies capable of inhibiting IEs adhesion to placental cells, have recently been assessed in two separate phase I clinical trials (ClinicalTrials.gov identifiers NCT02658253 and NCT02647489, respectively) (**Figure 1**). Together they represent an attractive intervention strategy to protect primigravid women from the serious clinical outcomes of PM. PAMVAC expressed in a Drosophila Schneider 2-derived cell line spans the interdomain region 1 through Duffy binding-like domain 2 to inter-domains 2 (ID1-DBL2x-ID2a) of the FCR3 variant of VAR2CSA (82), while PRIMVAC expressed in *E. coli* SHuffle® is based on the DBL1x-2x multidomain of

VAR2CSA from the *P. falciparum* 3D7 strain (**Figure 1**) (79, 80, 83). In order to allow comparative assessment of different placental malaria vaccine candidates, especially PRIMVAC and PAMVAC, two workshops hosted by the European Vaccine Initiative allowed the harmonization of clinical development plans and of assays for immunological assessment (84).

Based on these recommendations, both clinical trials were designed as randomized, double-blind, placebo-controlled, dose escalation trials and were meant to evaluate the safety and immunogenicity of three intramuscular vaccinations with progressively higher doses. PRIMVAC was adjuvanted with Alhydrogel or glucopyranosyl lipid A adjuvant in stable emulsion (GLA-SE) whereas PAMVAC was adjuvanted with Alhydrogel or GLA-SE or injected as a liposomal formulation in combination with QS21 (GLA-LSQ) (**Figure 1**). Volunteers were recruited into sequential cohorts receiving 20 μg, 50 μg, or 100 μg of vaccines at day 0, 28, and 56. PAMVAC volunteers were observed for 6 months following last immunization, while PRIMVAC volunteers were followed for one year after the last vaccination. While the safety and immunogenicity of PRIMVAC has been recently published in both malaria naïve (French volunteers) and *P. falciparum*-exposed non-pregnant women (Burkinabe women) (83), only the clinical evaluation of PAMVAC in malaria naïve volunteers (German volunteers) has been so far reported (82). All PAMVAC and PRIMVAC formulations were safe and well tolerated and none of the vaccines induced serious adverse events (82, 83).

PAMVAC and PRIMVAC were immunogenic in all participants and antibody levels were usually higher for both vaccines when adjuvanted with GLA-SE as compared to Alhydrogel and GLA-LSQ, at all dosages. In the case of PRIMVAC, all the volunteers seroconverted after two vaccine doses and a high proportion of them were still seroconverted one year after the third vaccination, which is indicative of a long-lasting immunity. Interestingly, IgG subclass analysis revealed that the induced antibodies were mostly IgG1 and IgG3 for PRIMVAC vaccinated volunteers. Therefore, the PRIMVAC-induced antibody response seems to mimic the naturally acquired immune response observed in multigravid women (85, 86).

Both vaccines generated antibodies reacting with the homologous VAR2CSA expressing parasites (NF54 for PRIMVAC and FCR3 for PAMVAC), however limited cross-reactivity was observed against heterologous VAR2CSA variants either recombinantly expressed (PAMVAC trial) or expressed on the surface of FCR3-CSA and 7G8-CSA IEs (PRIMVAC trial). The highest cross-reactivity was observed in sera collected from women that received the 100 μg PRIMVAC dose. Both vaccines induced functionally active antibodies inhibiting the interaction of their corresponding homologous VAR2CSA expressing IEs to CSA but low or no CSA-binding inhibition was observed for IEs expressing heterologous VAR2CSA variants (82, 83).

DISCREPANCIES BETWEEN PRECLINICAL AND CLINICAL STUDIES

Interestingly, the PAMVAC and PRIMVAC vaccines were able to induce antibodies in humans at significantly high titers after three doses received by the study participants. These antibodies were mainly vaccine-specific and mainly possessed functional activity against the homologous strains. Although these antibodies made it possible to describe a cross-reactivity with the constructs originating from other parasite variants, in particular those induced by vaccines adjuvanted with GLA-SE (82, 83), the lower cross-reactivity and the lack of cross-inhibition contrasts with the data generated in small animal models that guided the selection of these vaccine candidates (63, 77, 79, 80).

The observation that the responses in humans appear less cross-reactive than in rodents clearly raises the question of the transferability of the data generated in small animal models to humans. One confounding effect could be that the rodents received a higher dose per bodyweight than humans, so that the induced antibody titers are overall lower in humans compared to small animal models. A lower antibody titer would then possess less cross-reactive antibodies and then explain the lowest observed cross-reactivity. It is also possible that there is species-specific selection for cross-reactive epitopes in rodents versus humans. This would be a major obstacle in the placental malaria vaccine development process where the lack of an appropriate animal model remains. An animal model that would be as close as possible to humans, like non-human primates could be an asset in the preclinical validation phases. Efforts to develop such a model in the Aotus monkey is currently underway and could offer an interesting track in this perspective.

Interestingly, it was noted that some nulligravid women participating in the study in Burkina Faso to receive the PRIMVAC vaccine, possessed VAR2CSA antibodies before the administration of the vaccine doses (83). While these observations corroborate those of a few studies which have shown the presence of antibodies reacting with certain VAR2CSA antigens in some non-pregnant or male subjects living in endemic regions (87–89), their potential interference or benefit with vaccination deserves to be considered.

CURRENT VACCINE DEVELOPMENT GAPS AND POTENTIAL FUTURE DEVELOPMENTS

The preclinical and phase I clinical trial results for PAMVAC and PRIMVAC are highly encouraging and confirm the feasibility of developing a PM vaccine through further clinical testing. However, prior to embarking on costly, large-scale phase II clinical trials, it is essential to further evaluate the longevity of the immune response in vaccinated women and meanwhile assess its development upon natural infections. Novel strategies to enhance the immunogenicity of the PM vaccine candidates and to maximize the antibody cross-reactivity against different VAR2CSA variants could also be envisaged.

Longevity

Longevity of the vaccine-induced immune response is an essential element in placental malaria vaccine development, since nulligravid women will be vaccinated many months or years prior to their first pregnancy. In 2012, Fowkes et al. suggested that half-lives of antibody responses in pregnant women induced by natural

P. falciparum infection were in general relatively short for merozoite antigens (0.8–7.6 years) (90). However, half-lives of antibodies to VAR2CSA were significantly longer (36–157 years), suggesting that antibodies acquired in one pregnancy may be maintained to protect subsequent pregnancies (90). This raises hope for PM vaccine development, a hope that is further bolstered by the results obtained in the pre-clinical and clinical PM vaccination studies. The PM vaccine candidates, especially PRIMVAC, produced a long-lasting immune response in PRIMVAC vaccinated women who developed an immune response that lasted for more than a year (83). To further characterize the longevity of the PRIMVAC-induced immune response in women in malaria-endemic areas and to define vaccination age and frequency of booster vaccinations, follow-up of the vaccinated women for several years will be crucial. Additionally, the capacity of the vaccine candidates to boost and broaden a naturally acquired immune response would provide valuable insight.

Immunogenicity

Recombinant soluble proteins are often thought to induce an immune response of insufficient strength and breadth to confer full protection. Therefore, alternative approaches for the presentation of the VAR2CSA antigens are considered. The most advanced approach is using a capsid-like particle (CLP) that has been added as a backbone to the PM antigen, thereby possibly inducing a stronger immune response than a vaccine based on soluble recombinant protein and thus potentially improving immunogenicity, cross-reactivity and longevity of the induced immune response (91, 92).

Cross-Reactivity

Each vaccine candidate, PAMVAC and PRIMVAC, consists of a single recombinant protein. However, VAR2CSA is a diverse antigen and in Benin alone, 57 haplotypes of the vaccine target were identified which phylogenetically cluster into five clades (93). The PM vaccine candidates PRIMVAC and PAMVAC are from two distinct clades; 3D7 (clade 1) and FCR3 (clade 2) respectively. Interestingly, the authors found an association between the 3D7-like clade and low birthweight (93). Because of the extensive VAR2CSA polymorphism, the development of a PM vaccine is challenging. While the correct part of VAR2CSA to target appears established, it may be needed to generate second-generation vaccines, which expands the possibility of immunizing against several variants to improve cross-reactivity.

Different approaches may be evaluated: 1) prime - boost vaccination using VAR2CSA variants from different clades, 2) combination vaccination approaches either using, e.g., soluble recombinant proteins or multi-VAR2CSA-CLPs, 3) chimeric VAR2CSA variants, either based on the PAMVAC or PRIMVAC protein boundaries or rational structure-based designs. Finally, the recently hyped mRNA vaccine technology deployed against Covid-19 may be a way to incorporate several variants due to the ease and low cost of manufacture (94).

CONCLUSION

Over the past years, a promising portfolio of PM vaccine candidates was developed and two VAR2CSA-derived vaccines were brought to clinical evaluation, confirming the feasibility of developing a PM vaccine. However, to evaluate and compare the results of the various PM vaccine approaches, the harmonization of clinical trial procedures and the standardization of immunoassays that was initiated before the start of the phase I clinical trials (84) has to be further strengthened. Stringent go/no-go criteria will be required to decide to enter in the next development stages based on production feasibility, safety, and induction of cross-reactive and functional immune responses. A major obstacle to transitioning a PM vaccine from preclinic to clinic is the lack of a suitable animal model (95). Evaluation of PM vaccine efficacy is particularly complex. Indeed, the lack of surrogates to predict PM vaccine-induced protection limits the potential of early clinical trials to provide indications on vaccine efficacy. The amplitude of the antibody response resulting from vaccination appears today as an indicator of putative efficacy, although the threshold levels of antibodies required for protection are undetermined. The lowest cross-reactivity observed during the clinical trials in comparison to the preclinical evaluation could be the consequence of a lower amplitude of the immune response upon antigen exposure in humans compared to rodents. Since sterile immunity is not required for a PM vaccine, the hope is that the vaccine-induced response will be boosted and even broadened by natural infection. Alternate schedules of immunization, antigen dosage, and combinations with other VAR2CSA-based vaccines are under development and will be assessed in future studies for their capacities to broaden the cross-reactivity of the induced immunity against heterologous VAR2CSA variants and then fully protect women from the negative outcomes of PM.

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The Development, Fine Specificity, and Importance of High-Avidity Antibodies to VAR2CSA in Pregnant Cameroonian Women Living in Yaoundé, an Urban City

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Pregnant women infected with *Plasmodium falciparum* often produce antibodies (**Abs**) to VAR2CSA, a ligand that binds to placental chondroitin sulfate A causing placental malaria (**PM**). Antibodies to VAR2CSA are associated with improved pregnancy outcomes. Antibody avidity is a surrogate marker for the extent of maturation of the humoral immune response. Little is known about high avidity Abs to VAR2CSA for women living in urban African cities. Therefore, this study sought to determine: i) if high avidity Abs to full-length VAR2CSA (**FV2**) increase with gravidity in women in Yaoundé, Cameroon exposed to ~ 0.3-1.1 infectious mosquito bites per month, ii) if high avidity Abs to FV2 are directed against a specific region of VAR2CSA, and iii) if having high avidity Abs to FV2 improve pregnancy outcomes. Plasma samples collected at delivery from 695 women who had Abs to FV2 were evaluated. Ab levels and the Avidity Index (**AI**), defined as the percent Abs remaining bound to FV2 after incubation with 3M NH_4SCN, were determined. Similar Ab levels to FV2 were present in women of all gravidities (G1 through 6+; p=0.80), except significantly lower levels were detected in PM–negative (PM–) primigravidae (p <0.001). Median Ab avidities increased between gravidity 1 and 2 (p<0.001) and remained stable thereafter (G3-G6+: p=0.51). These results suggest that B cell clonal expansion began during the first pregnancy, with clonal selection primarily occurring during the second. However, the majority of women (84%) had AI <35, a level of high avidity Abs previously reported to be associated with improved pregnancy outcomes. When plasma from 107 Cameroonian women was tested against 8 different regions of FV2, high avidity Abs were predominately restricted to DBL5 with median AI of 50 compared to AI <25 for the other domains. The only significance influence of high avidity Abs on pregnancy outcome was that babies born to mothers with AI above the median were 104 g heavier

than babies born to women with AI below the median (p=0.045). These results suggest that a vaccine that boosts maturation of the immune response to VAR2CSA may be beneficial for women residing in urban areas.

Keywords: *Plasmodium falciparum*, pregnant women, placental malaria, VAR2CSA, antibody avidity, urban cities

INTRODUCTION

Plasmodium falciparum infections during pregnancy may be harmful to both the mother and the developing fetus. Malaria-infected erythrocytes (**IE**) sequester in the intervillous space (**IVS**) of the placenta and stimulate an inflammatory response, thereby increasing the risk of maternal complications, stillbirths, premature deliveries, reduced infant birthweights, and low birthweight babies (1–4). Primigravidae are more susceptible to the effects of placental malaria (**PM**) than multigravida women. Sequestration is mediated primarily by an antigen expressed on the surface of IE called VAR2CSA that binds to chondroitin sulfate A (CSA) on syncytial trophoblasts lining the IVS (5, 6). Since CSA is only expressed in the placenta, IE expressing VAR2CSA are normally quickly eliminated in children and non-pregnant adults before an immune response is induced. However, when women are infected with *P. falciparum* for the first-time during pregnancy, they are exposed to VAR2CSA and a primary immune response is induced. The presence of antibodies (Abs) to VAR2CSA, especially in subsequent pregnancies, reduces the severity of PM and has been associated with increased infant birthweight, longer periods of pregnancy, reduced prevalence of PM, and reduced risk of low birthweight (LBW) babies (6–10) [reviewed in (11)]. Thus, immunity to PM and VAR2CSA is unique, in that it is pregnancy-associated and gravidity dependent.

Most studies assessing the benefits of Abs to VAR2CSA have been conducted in areas with high malaria transmission. The impact of Abs to VAR2CSA on pregnancy outcomes in urban settings with intermediate or low levels of transmission is less clear, in part, because a large sample size is needed to detect small changes. Currently, neither the amount, specificity nor immunological properties of Abs to VAR2CSA needed to improve pregnancy outcomes is known. In general, high Ab levels to VAR2CSA are associated with infection, not protection (11). Thus, many questions remain about the natural acquisition of Abs to VAR2CSA in pregnant women and their role in pregnancy outcomes in different transmission settings.

Antibody avidity, or functional affinity, is often listed as a functional assay, in that it measures the overall strength of binding of Abs circulating in plasma to an antigen (12). During affinity maturation, antigen is presented on follicular dendritic cells within germinal centers and B cells with B-cell receptors (sIg) that can outcompete circulating Abs and other maturing B cells for the antigen will be selected; thereby, increasing Ab avidity. Thus, Ab avidity is a marker for the extent of maturation of the humoral response against an antigen. Several studies have suggested that high avidity Abs to VAR2CSA might be beneficial to pregnant women residing in areas with intense malaria transmission. For example, pregnant women with high levels of high avidity Abs to VAR2CSA (i.e., >35% of Ab remained bound after treatment with 3M NH_4SCN) early in pregnancy, had a reduced risk of placental malaria at delivery; whereas, having high Ab levels to VAR2CSA *per* se was not associated with improved pregnancy outcome (13). A second study, designed to identify differences in the fine-specificity of Abs to VAR2CSA between PM -positive (**PM+**) and PM− multigravid women, found that the only significant difference was the presence of higher levels of high avidity Abs to full-length VAR2CSA (**FV2**) among the 21 assays studied using VAR2CSA-associated recombinant proteins (14). It remains unclear, however, if Ab avidity plays a direct role in enhancing inhibition or releasing IE bound to trophoblasts or if avidity is simply a marker of maturation of the overall immune response to VAR2CSA.

The role of Abs to VAR2CA in a low to intermediate transmission areas, such as the city of Yaoundé, Cameroon, where pregnant women were exposed to ~0.3 to 1.1 infectious mosquito bites per month, remains unclear (15, 16). Tutterrow et al., followed 50 women throughout pregnancy who were positive for *P. falciparum* (Pf) parasites either by microscopy or PCR early in the second trimester and reported that 60% of the women who were PM− at delivery, lacked Abs to FV2 (17). These data suggest that Abs to antigens other than VAR2CSA assist in clearing infected erythrocytes. Several studies have shown that Abs to other non-VAR2CSA *P. falciparum* antigens were associated with improved pregnancy outcomes in women residing in Yaoundé, Cameroon (8, 18). Thus, the prevalence, amount and importance of high avidity Abs to VAR2CSA in areas with relatively low malaria transmission remains to be determined.

The purpose of the current study was to determine if: i) high avidity Abs to full-length VAR2CSA (**FV2**) increased with gravidity in women in Yaoundé, Cameroon prior to implementation of intermittent preventive treatment (**IPTp**), ii) high avidity Abs to FV2 were directed against a specific region of FV2, and iii) having high avidity Abs to FV2 at delivery was associated with reduced prevalence of PM or improved infant birthweight.

MATERIALS AND METHODS

Ethical Approvals

The original study, conducted between 1995 and 2001, was approved by the National Ethics Committee, Cameroon and the IRB, Georgetown University (IRB#:1994-158) for collection of plasma, placental tissue and clinical information. Women participating in the study gave written informed consent. The use of the de-identified, archival plasma samples and clinical information in the current study were determined to be

exempt from human subjects' research by the Committee on Human Subjects, University of Hawaii-Manoa (CHS#21891).

The Study Design

Clinical and Laboratory Data

Plasma used in this study were consecutively collected at delivery between 1995 and 2001 in Yaoundé, Cameroon, at the Biyem Assi District Hospital where care was provided to women living in the adjacent area and at the Central Maternity Hospital, a referral hospital for a diverse group of women (19). At the time of recruitment, information on the woman's pregnancy and malaria histories were recorded on a standardized questionnaire, a heparinized peripheral blood sample was drawn, and a biopsy of the placenta was excised. In the laboratory, thick and thin slides were prepared of maternal peripheral and placental IVS blood and impression smears were made using the excised placental tissue for detection of *P. falciparum* infected erythrocytes (**IE**). Histological sections of the placenta were also prepared to confirm infection. A woman was considered to be PM+ if parasites were detected in either the placental blood, impression smears or by histology. Maternal anaemia was assessed by determining the packed cell volume (**PCV**) (19). All data were maintained in a password-protected archival database. The deidentified plasma samples were stored at −80° C until used, except during transportation on dry ice from the Biotechnology Center, Cameroon to Georgetown University and later from the Georgetown University to the University of Hawaii. A total of 1,649 plasma samples and corresponding clinical information were available. An advantage of using the clinical data and plasma from this group is that the women were recruited before the implementation in 2004 of IPT; therefore, the women developed natural immunity to PM that aided in clearing their placental infections. Post-IPTp, placental parasitemia were either prevented or eliminated by sulfadoxine–pyrimethamine treatment, making it impossible to assess the role of Abs to VAR2CSA in naturally-acquired immunity. HIV status of the women was not determined, but the prevalence in pregnant women attending urban antenatal clinics at the time of sample collection was 7.1% (20).

Sample Selection

The current study on Ab avidity is an extension of a study conducted in 2017 that used data from the archival samples in statistical predictive models to identify the best combination of antigens associated with absence of PM (18). The study design, sample selection, and results of that study have been reported previously (8, 18). In the 2017 study, plasma samples from 341 PM+ women who delivered live babies ≥28 weeks of gestation, along with three times the number (n=1,036) of randomly-selected PM−negative (PM−) women were screened at a 1:200 dilution in a bead-based multiplex assay for Abs to 17 VAR2CSA-associated antigens (FV2, DBL 1-6 of the FCR3, 3D7 and 7G8 lines, ID1-ID2a (FCR3 and 3D7) and 11 antigens reported to be associated with immunity to *P. falciparum* (AMA-1, CSP, EBA-175, LSA1, MSP1, MSP2, MSP3, MSP11, Pf41, Pf70 and RESA) (18). In addition, plasma from 30 males of equivalent age residing in Yaoundé served as negative controls for the var antigens. The resulting database, consisting of over 74,000 Ab data points, allowed us to evaluate each sample for potential Ab deterioration due to long-term storage and to identify the samples with Abs to FV2. The cut-off for Ab-positivity was the mean + 2SD of the male controls. A total of 695 women were identified who were Ab+ for FV2. Thus, plasma and clinical information from the 695 Ab+ women, and plasma from 20 age-matched sympatric males, were evaluated in the avidity assay.

The Multiplex Assay for Measuring Ab Levels and Avidity to FV2

The multiplex-immunoassay for measuring Abs to FV2 and the avidity assay were detailed previously (13, 14). In brief, FV2 of the FCR3 line was produced in baculovirus as previously described (21). The antigen was covalently coupled at saturating concentrations to SeroMap beads (Luminex) overnight according to the manufacturer's directions. Beads were then blocked with PBS+1% bovine serum albumin (BSA) and resuspended to 2,000 beads/50µl. Ab levels and avidity were determined by combining 50µl of plasma diluted 1:300 in PBS +1% BSA (pH 7.2) with 50µl of coupled beads (total dilution of plasma =1:600) in duplicate in filter microtitre wells. Following incubation at room temperature (**RT**) on a rotary shaker for 1 hr., beads were washed twice with PBS+0.05% Tween 20 and once with 1% BSA in PBS. Then, one of the paired wells was incubated with 100µl of PBS and one well with 100µl of 3M NH_4SCN for 30 min are RT. After washing, beads were incubated with 100µl of secondary antibody (R-phycoerythrin-conjugated, Affinity Pure F $(ab')_2$ fragment, Goat anti-human IgG Fc fragment specific, Jackson Immunoresearch, West Grove, PA) diluted to 2µg/ml in PBS-1% BSA was added to each well and incubated in the dark on a rotary shaker for 1 hr. Beads were washed, re-suspended in 100µl PBS-1% BSA and 85µl of the microsphere suspension was analyzed using a Liquichip M100 reader (Qiagen, Valencia, CA). The reader was programmed to read a minimum of 100 beads per spectral address, DD Gate 7,500–15,000. Results for Ab levels were expressed as median fluorescence intensity (**MFI**), where the linear part of the MFI binding curve extended from ~1,000 to >25,000 MFI (r coefficient of >0.90 for Ab dilution versus MFI) (22). Results from the avidity assay are expressed as an avidity index (**AI**) that equals the percentage of Abs that remains bound to FV2 after treatment with the chaotrope, using the formula (AI = [MFI beads with salt]/[MFI beads with no salt] x100). The entire assay was repeated by two investigators (KV, NB). When inadequate bead counts were obtained, the samples were re-run. The MFI for the two replicates were averaged and used for data analysis. Samples with MFI greater than the mean + 2 SD for the negative male controls were considered to be Ab+.

Measuring Ab Avidity to Different VAR2CSA Domains

The following Duffy-binding like (**DBL**) domains of VAR2CSA were coupled to SeroMap beads as described above: DBL1 (3D7), and the FCR3 lines of 1D1-2a, DBL 1 + 2, DBL2, DBL3, DBL4, DBL5, DBL6, and FV2. Characteristics of the recombinant proteins have been detailed previously (23, 24). The recombinant proteins were covalently coupled at saturating amounts to beads with different spectral addresses and then pooled to create a 10-plex.

In establishing the assay, results from the 10-plex were compared with each of the antigens as a mono-plex to confirm that competition among the antigens did not occur. For the assay, 50µl of plasma diluted 1:300 was combined with 50µl of the 10-plex (2,000 beads of each antigen) for 60 min at RT. The remainder of the methods was the same as that described above. A total of 107 plasma samples with known MFI to FV2 were screened, including 20 sample with MFI of 5,000–10,000; 30 samples with 10,000–15,000; 28 samples with 15,000–20,000; and 29 samples with 20,000–25,000 MFI.

Statistical Analysis

Demographic, clinical, and parasitological characteristics were first summarized by descriptive statistics: frequencies and percentages for categorical variables, i.e., maternal anaemia status; means with standard deviations (SD) or medians with interquartile ranges (IQR) and 25th and 75th percentiles for continuous variables based on data distribution. To compare the groups (e.g., by PM status or AI status), t-tests (or Wilcoxon-Rank-Sum tests) and analysis of variance [ANOVA or Kruskal-Wallis tests (**KW**)] were used for continuous variables depending on distribution and number of the groups. Chi Square tests were used for categorical variables. Post-hoc comparison test was used, when overall significance was identified, to examine the difference between two specific gravidity groups or AI groups. The relationships between birthweight and AI, and between PM and AI after adjusting for age and gravidity were examined by multivariable linear and logistic regression models, respectively. The relationship between MFI and AI was also assessed using linear regression. Statistical significance was determined by $p<0.05$.

RESULTS

Description of the Study Population

Among the 695 women studied, 460 women were PM− and 235 were PM+ (**Table 1**). PM+ women were younger (24.4 vs 26.3 years; p<0.001); had fewer pregnancies (2 vs. 3; p<0.001); had lower hematocrits (31.3% vs 33.9% PCV; p<0.001); and were more likely to be anemic (28.7% vs. 18.9%; $p = 0.011$) than PM− women. A minor difference in length of gestation was found between PM+ and PM− pregnancies (39.3 vs. 40 weeks, p=0.019), but no differences were evident in percentage of premature deliveries or proportion of LBW babies (**Table 1**). However, singleton babies born to PM+ mothers were an estimated 138 gm lower in birthweight and singletons born full-term were 141 gm lighter ($p = 0.007$, $p = 0.002$, respectively). Thus, among women who had Abs to FV2, mothers with PM+ appeared to have lower birthweight babies than those without PM.

TABLE 1 | Characteristics of women with antibodies to full-length VAR2CSA.

	PM− n = 460	PM+ n = 235	*P* value[a]
Age, Years [mean ± SD]	26.3 ± 5.6	24.4 ± 5.5	<0.001
Gravidity [median (IQR)]	3 (2,5)	2 (1,4)	<0.001
Placental parasitemia % [median (IQR)]	0	0.68 (0.16, 2.5)	NS
Maternal hematocrit, % PCV [mean ± SD][b]	33.9 ± 5.9	31.3 ± 5.6	<0.001
Maternal anemia status[c] [%]	18.9	28.7	0.011
Weeks of Gestation [median (IQR)][d]	40.0 (38.0, 41.0)	39.3 (38.0, 40.6)	0.019
Preterm deliveries [%][d,e]	19.9% (83/418)	21.6% (42/204)	0.915
Infant birth weight, g [mean ± SD][d]	3,131 ± 627	2,993 ± 600	0.007
Infant birthweight from term deliveries [mean ± SD][d]	3,297 ± 459	3,156 ± 482	0.002
Low-birthweight [%][d]	13.9	18.1	0.203

[a]*Statistical Tests: t-tests for age, maternal hematocrit, and infant birthweight; Wilcoxon-Rank-Sum tests for gravidity and weeks of gestation; Chi square tests of independence for anaemia, preterm delivery, and low birthweight.*

[b]*Analysis was performed on 569 subjects for whom data were available.*

[c]*Women with hematocrits <30% were defined as anemic.*

[d]*Twins were excluded from the analysis: n=622 women for weeks of gestation, preterm deliveries; n=648 for infant birthweight and low birthweight; n=493 for infant birthweight of full-term deliveries.*

[e]*Preterm delivery was defined as <37 weeks of delivery.*

Antibody Levels to FV2 Were Lower in PM− Primigravidae, but Similar Among Other Women

Plasma samples collected at delivery from the 695 pregnant women and 20 age-equivalent males were screened for Abs to FV2 at a 1:300 dilution in the avidity assay. Overall, 644/695 (93%) women had MFI to FV2 greater than the mean+2 SD of the male controls; whereas, 51 women, who originally tested positive at a 1:200 dilution, had MFI below the cut-off at a 1:300 dilution. Thus, results from only 644 women were included in the analysis.

Ab levels to FV2 for women with different gravidity (G1 through ≥G6) were compared (**Figure 1**). A difference in median Ab levels was found among the groups (Kruskal-Wallis test (**KW**): $p = 0.002$) with an apparent up-ward trend from primigravidae (G1) through grand-multigravid mothers (≥G6) (**Figure 1**). However, *post hoc* pairwise comparisons showed that the only significant difference in Ab levels among the groups was between G1 and G2 ($p = 0.005$). There was no difference in Ab levels between G2 and G6+ women (KW: $p = 0.57$: dotted rectangle in **Figure 1**).

When Ab levels were evaluated based on presence/absence of PM, only PM− primigravidae had significantly lower Ab levels than women in the other groups (e.g., primigravidae PM− vs PM+ p<0.001; PM− primi- vs PM− secondi-gravidae; p<0.001) (**Figure 2**). No difference in Ab levels was found among the other PM− gravidity groups (KW: $p = 0.78$) or among PM+ women G1 though G6+ (KW: $p = 0.54$). Thus, the distribution of Ab levels to VAR2CSA was similar among all the gravidity groups ($p = 0.80$), except for lower Ab levels in first-time mothers who were PM− at delivery.

Antibody Avidity to FV2 Increased Between Gravidity 1 and Gravidity 2

A significant increase in median AI occurred between G1 and G2 ($p < 0.001$) and then AI leveled off thereafter (KW test: $p = 0.50$) (**Figure 3**). Thus, the predominant increase in AI occurred between women in their first and second pregnancies.

When stratified by placental-malaria status at delivery, AI values were significantly lower in PM− primigravidae than

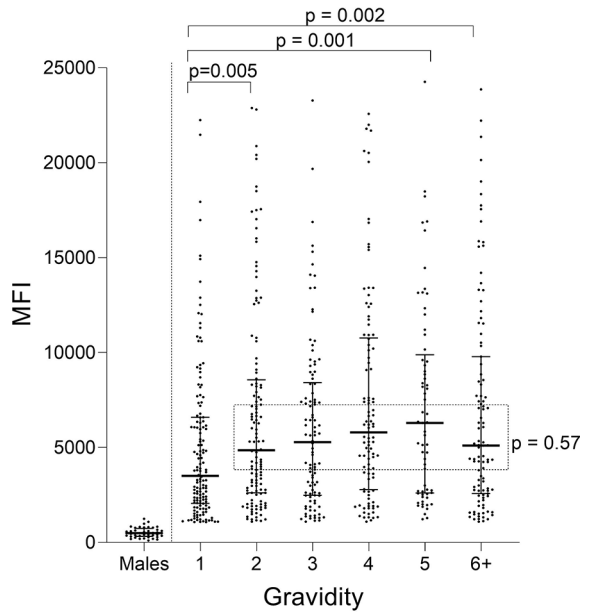

FIGURE 1 | Antibody levels to full-length FV2 at delivery in different gravidity groups. A significant difference in median Ab levels was detected among the six gravidity (G) groups (Kruskal-Wallis test: $p = 0.002$). Post hoc pairwise comparison showed that primigravidae (G1) had significantly lower Ab levels to FV2 than each of the other gravidity groups. Ab levels among groups shown in the rectangle did not differ significantly (Kruskal-Wallis test: $p = 0.57$). n = 644 women and 20 males (replicates shown, n=40 data points). Horizontal lines represent medians and IQR.

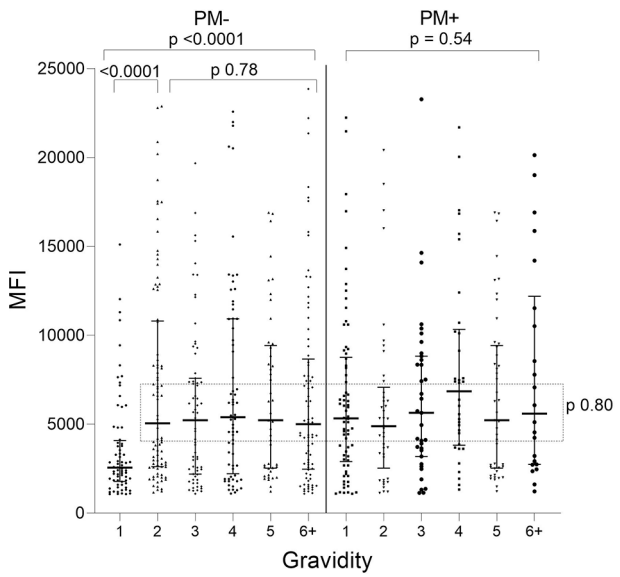

FIGURE 2 | Antibodies to FV2 at delivery in PM– and PM+ women. Antibody levels were significantly lower in PM– primigravidae (G1) than G2 (Mann-Whitney test: p <0.001), but no significant difference was found among the gravidity groups shown in the rectangle (Kruskal-Wallis test: $p = 0.80$). n = 644 women. Horizontal lines represent medians and IQR.

secundigravidae ($p < 0.001$) and lower in PM+ primigravidae than G3 women ($p = 0.050$) (**Figure 4**). Thereafter, the AI leveled off (KW test: PM– $p = 0.60$; PM+ $p = 0.28$). Thus, median AI values were lower in both PM– and PM+ primigravidae, but significantly increased during the second pregnancy in PM– mothers and between the first and third pregnancy in PM+ women.

Overall, AI values were low with 48% of the women having AI <20 and 75% having AI <30 (**Figure 5**). Only 16% of women had AI ≥35; 9.5% had AI ≥40; and only 2.8% had AI ≥50. Thus, the majority of women did not produce significant amounts of high avidity Abs, and only 2.8% had AI >50, which is considered to be a high avidity response. In a high transmission area, women with AI >35 early in pregnancy were reported to have a decreased risk of PM at delivery (13). Only 16% of women in Yaoundé had this level of high avidity Abs at delivery.

Relationship Between MFI and AI was Similar in Secundigravidae and Above

During affinity maturation, B cell clonal expansion and selection occur, resulting in an increase in both Ab levels (MFI) and avidity (AI). To help determine when affinity maturation began, the relationship between MFI and AI was assessed (**Figure 6**). No relationship existed between MFI and AI in PM– primigravidae, indicating that Abs were produced, but significant clonal selection had not occurred ($p = 0.31$, i.e., regression line was not significantly different from zero) (**Figure 6A**). A moderate association (r = 0.552), however, was detected in PM+

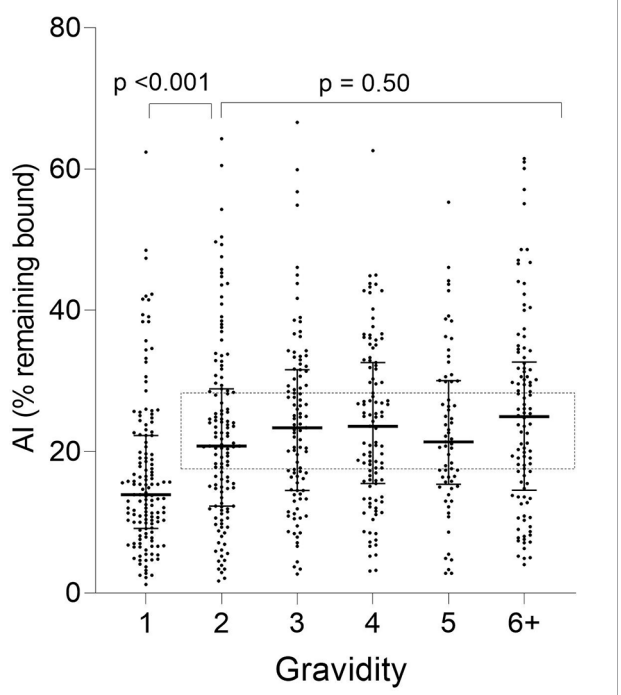

FIGURE 3 | Distribution of Ab avidity (AI) among pregnant women at delivery. AI were significantly lower in G1 than G2 (p <0.001), but AI did not change thereafter (rectangle: Kruskal-Wallis test: G2 through G6+ $p = 0.50$). Horizontal lines represent medians and IQR.

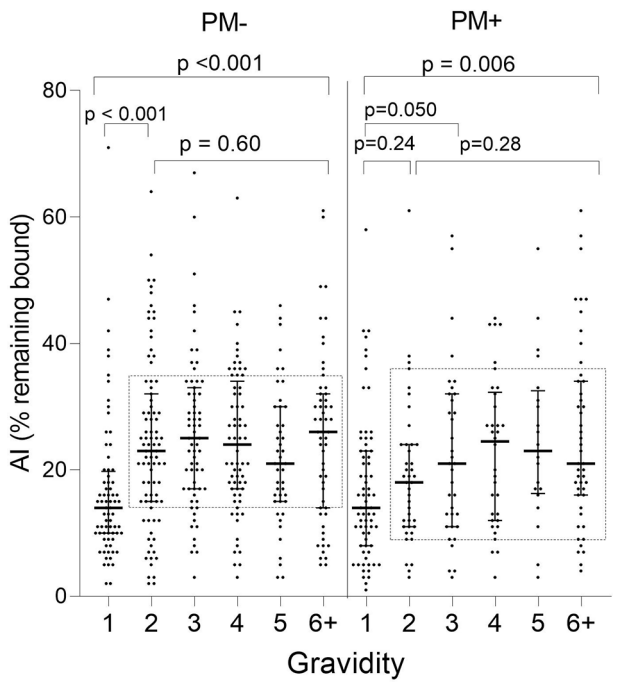

FIGURE 4 | Comparison of Ab avidity (AI) at delivery in PM– and PM+ women. A significant increase in AI occurred in PM– women between G1 and G2 women ($p < 0.001$) and between G1 and G3 in PM+ women ($p = 0.05$), but AI did not increase significantly thereafter (samples within rectangles for G2 through G6+: Kruskal-Wallis test: PM– $p = 0.60$; PM+ $p = 0.28$). Horizontal lines represent medians and IQR.

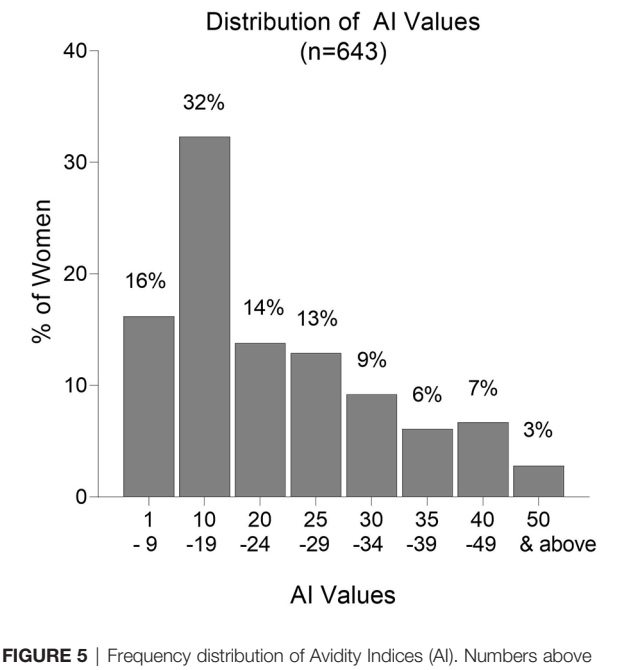

FIGURE 5 | Frequency distribution of Avidity Indices (AI). Numbers above each column are the percentage of samples in the AI category. n = 643 women.

primigravidae, suggesting that affinity maturation was occurring at term in some women (**Figure 6B**). A modest association was also seen in secundigravidae (G2) (**Figure 6C**), as well as in women gravidae G3 and above (n=373 women) (**Figure 6D**). Even among grand multi-gravid women (G6+), the association between MFI and AI still remained modest (r = 0.508). These data suggest that affinity maturation was occurring in some primigravidae who had PM at delivery, as well as in some women with prior pregnancies.

High Avidity Abs for Were Directed Mainly to DBL5

The extracellular part of VAR2CSA consists of six Duffy-binding-like domains (DBL) domains, a large intradomain (ID2) and a C-terminal region predicted to be intracellular (25). A total of 107 plasma samples with MFI to FV2 ranging from 5,000 to >20,000 were screened in the Ab avidity assay against the 6 DBL domains, ID1–ID2, and FV2 (**Figure 7**). The range of MFI for each domain, as well as the percentage of women who were Ab+, are shown in **Figure 7A**. Overall, 55% (e.g., DBL2) to 98% (e.g., DBL5) of the women had Abs to the DBL domains, with the highest Ab levels found for DBL5. Likewise, DBL5 had the highest AI compared to the other domains, in fact, the AI for DBL5 was significantly higher than the median AI to FV2 (median (25%,75% IQR): DBL5 52 (36,64) vs. FV2 24 (16,31); $p<0.001$). AI values for the other domains were much lower than to DBL5, with AI for the N-terminal domains DBL 1 through 3 having AI <20 and the more C-terminal domains, DBL 4 and DBL6 having slightly higher AI of 20 to 25. Thus, although most women had Abs to most of the domains, some regions of FV2 support affinity maturation better than others, with the majority of high avidity Abs induced by DBL5.

Mothers With High AI Appear to Have Higher Birthweight Babies

Since the frequency distribution of AI did not identify definable groups of women (**Figure 5**), the pregnant women were divided into two groups based on the median. Women in Group 1 had AI <20.5 and Group 2 had AI ≥20.5 (**Table 2**). Compared to Group 1, women in Group 2 were older (mean age: 26.4 vs. 24.9 years, p<0.001); had higher gravidities (median 3 vs 2, p<0.001); and higher hematocrits (mean PCV: 33.5% vs. 32.4%, p=0.038). Women with AI above the median appeared to have higher birthweight babies than those with AI below the median (average birthweight: 3,160 vs 3,020 g, p=0.006). After adjusting for age and gravidity, the difference remained statistically significant (p=0.045), with an average difference of 104 g. The percentage of women with PM at delivery was lower in Group than 2 than Group 1 (30% vs 39%; $p = 0.016$). However, after adjusting for age and gravidity, no significant association was found between AI and presence of PM. Likewise, no relationship between low and high AI and the distribution of placental parasitemia (p = 0.45) was found. Thus, these data suggest that having high avidity Abs to FV2 may be associated with increased infant birthweight, but additional factors could also influence the results.

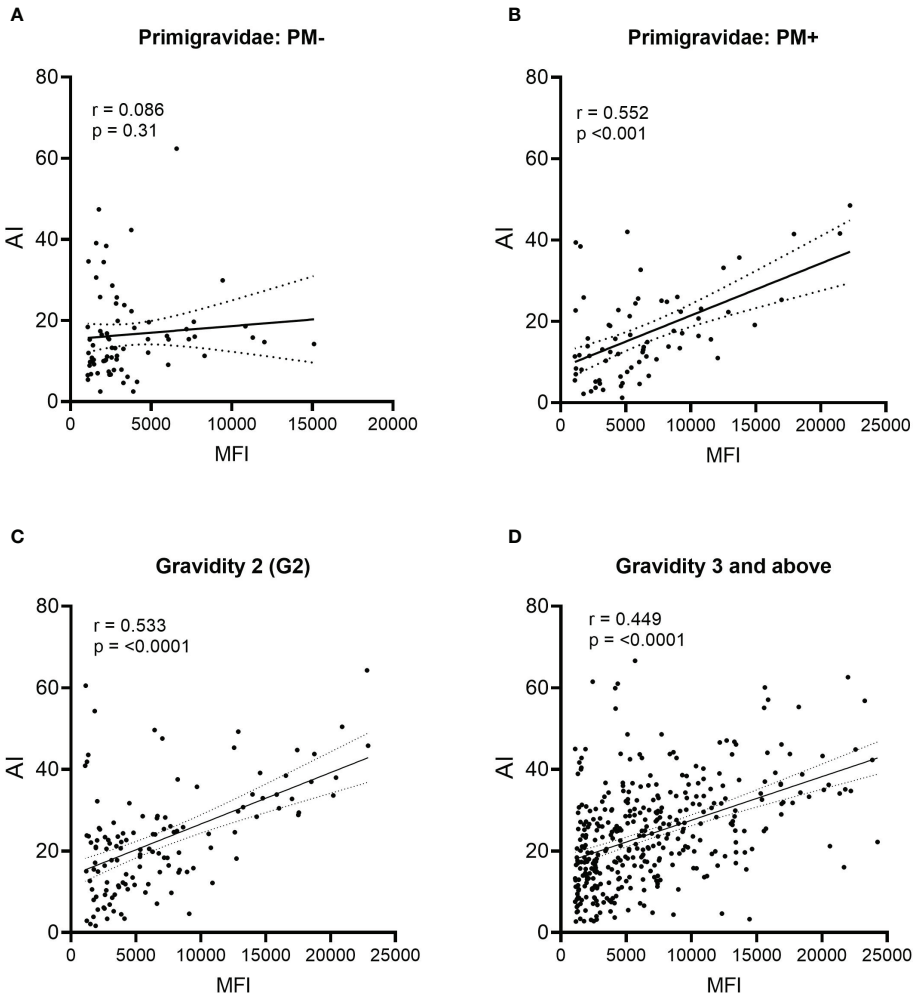

FIGURE 6 | Comparison of Ab levels (MFI) and avidity (AI) using linear regression. **(A)** Placental malaria-negative (PM–) primigravidae (n = 71), **(B)** Placental malaria-positive (PM+) primigravidae (n = 70); **(C)** all secundigravidae (G2) (n = 127); and **(D)** all women gravidity 3 (G3) and above (n = 373). Linear regression lines with 95% confidence bands are shown. P values indicate if the line is significantly different from zero.

DISCUSSION

The timing of affinity maturation resulting in production of higher affinity Abs to VAR2CSA is an important component in acquiring immunity to PM. Previous studies have shown that the Ab response to VAR2CSA is different in urban cities from that in high transmission areas. For example, in Cameroon, >90% of women living in the rural village of Ngali II, where they receive ~200 infectious mosquito bites during pregnancy, had Abs to FV2 at delivery (13, 26); whereas, in Yaoundé, only 39% of women (n=1,337) were Ab+ (8). In Yaoundé, approximately half of the women who had PM at term do not have Abs to FV2 (8, 17). Thus, infection is not always accompanied by Ab production to FV2 in an urban setting. Clearly, when pregnant women receive only a few infectious mosquito bites during a single pregnancy, the resulting humoral response is limited. Results from the current study on Ab avidity help create a picture about the natural acquisition of Abs to VAR2CSA in pregnant women residing in Yaoundé. Although the data from this study are open to interpretation, the following picture is consistent with the current data set and data from past studies.

Antibody-Levels to FV2 Remain Low in PM–Negative Primigravidae, Suggesting That Abs to Other Malarial Antigens Aid in Parasite Clearance

Primigravidae lack Abs to FV2 prior to pregnancy. Thus, primigravidae with Abs to FV2 at delivery who were PM– must have become infected early enough in pregnancy to mount an Ab-response and clear their placental infections prior to delivery. However, since Ab levels to FV2 were low, Abs to other antigens may have helped limit parasite replication and aided in parasite clearance **(Figure 1)**. This possibility is supported by results from a study that measured Ab levels in 1,377 women living in Yaoundé, (i.e., the same initial set of plasma samples used herein) to 17 VAR2CSA-associated antigens, including FV2, DBL-1-6 of the

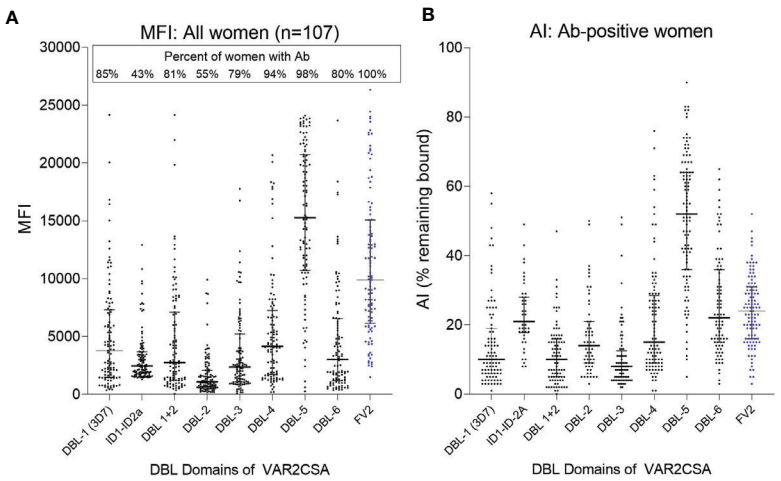

FIGURE 7 | Antibody MFI and AI to different regions of VAR2CSA. Plasma samples from 107 women were screened for Ab avidity against DBL1 (3D7 line), the FCR3 lines of ID1-ID2a, the DBL2 through DBL6, and FV2. **(A)** MFI for the 107 samples. Horizonal lines represent medians and IQR. **(B)** AI for women who had Ab against each of the domains (sample size: DBL1 n = 91; ID1-ID2a n = 46; DBL1+2 n = 87; DBL2 n= 59; DBL3 n = 84; DBL4 n = 101; DBL5 n = 105; DBL6 n = 86; FV2 n = 107). Horizontal lines represent medians and IQR.

TABLE 2 | Comparison of birth outcomes and AI to FV2.

	Group 1: AI <20.5 n = 319	Group 2AI: ≥20.5 n = 324	p-value[a]
Age, years [mean ± SD]	24.9 ± 6.0	26.4 ± 5.4	<0.001
Gravidity [median, (IQR)]	2 (1, 4)	3 (2, 5)	<0.001
Maternal hematocrit, % PCV [mean ± SD][b]	32.4 ± 6.0	33.5 ± 5.7	0.038
Maternal anaemia [%][b]	25.6 (69/270)	18.8 (48/256)	0.077
Weeks of gestation [median, (IQR)][c]	39.7 (37.8, 40.9)	39.9 (38.1, 40.9)	0.739
Infant birth weight, g [mean + SD][c]	3020 ± 633	3160 ± 615	0.006
Low birth weight babies [%][c]	16.9 (50/295)	13.2 (40/304)	0.236
Placental malaria-positive (%)	39.2 (125/319)	29.9 (97/324)	0.017

[a]*Statistical tests: t-tests for age, maternal hematocrit, and infant birthweight; Kruskal-Wallis tests for gravidity and weeks of gestation; Chi-square tests of independence for maternal anemia, placental malaria, and low birthweight.*
[b]*Analysis was performed on subjects for whom data were available, (n = 526).*
[c]*Only singleton deliveries were included: weeks of gestation (n = 576), infant birthweight and percent LBW (n = 599).*

FCR3, DBL, and 7G8 lines, IDI-ID2a (FCR3 and 3D7) and 11 non-VAR antigens associated with immunity to *P. falciparum* (AMA-1, CSP, EBA-175, LAS1, MSP1, MSP2, MSP3, MSP11, Pf41, Pf70, and RESA), and then used the data in seven statistical models to identify the combination of antigens that best correlated with absence of PM at term (18). A combination of Abs to AMA-1, MSP2, EBA-175, Pf41, and MSP11 was the best predictor of PM status. FV2 was among the top 10 individual predictors, but it did not improve the prediction when added to the combination. Likewise, in a parallel study, Abs to MSP3, EBA-175 and Pf41 were associated with reduced risk of high placental parasitemia and Abs to EBA-175 with reduced risk to premature deliveries in PM+ women (8). Thus, Abs to other malaria antigens, in conjunction with FV2, may play a significant role in improving pregnancy outcomes. Since Ab level were low (**Figure 2**) in PM− primigravidae and there was no evidence that affinity maturation had occurred (**Figures 3**, **4**), initial exposure to low levels of FV2 may be sufficient to activate short-lived plasma cells, but insufficient to induce strong clonal expansion of B cells, long-lived plasma cells, memory B cells, or affinity maturation.

Antibody-Levels to FV2 Reached Maximal Levels in PM+ Primigravidae

The anti-VAR2CSA response was significantly stronger in primigravidae who had placental parasitemia at term (**Figure 2**), possibly due to longer exposure to higher amounts of the antigen. Interestingly, distribution and median MFI of anti-FV2 Abs were similar at term in PM+ primigravidae and all other gravidity groups regardless of placental malaria status (**Figure 2**). After the first exposure to a sufficient concentration of FV2, Ab data are consistent with the speculation that B cell clonal expansion is similar in primigravidae to that of multigravida women. Thus, in primigravidae, exposure to an adequate amount of FV2 may be sufficient to induce substantial clonal expansion, but insufficient to induce affinity maturation (**Figure 3**).

Affinity Maturation Takes Place Primarily During the Second Pregnancy

The change in the Avidity Index between primigravidae and secundigravidae (**Figures 3**, **4**) is reminiscent of a primary and secondary Ab response. During second pregnancies (PM− women) and possibly into the third pregnancies (PM+ women), affinity maturation appeared to be occurring. At this time, women had both higher Ab levels and a higher proportion of higher avidity Abs (i.e., higher AI); however, the association between MFI and AI was only modest (r values ranging from 0.4 to 0.5) (**Figures 6B, C**). In general

r values >0.7 are considered to be strong associations. From the scattergrams, data show that a few secundigravidae and ≥G3 had high MFI but low AI (below the line) and a group of women with low AI and high MFI (values above the line) (**Figures 6C, D**). Thus, the two processes may not always occur simultaneously. Neither the quantity nor quality of Abs to FV2 appears to increase following additional exposure to VAR2CSA-expressing IE beyond the second pregnancy. These results suggest that in low transmission areas, two pregnancies are required for women to achieve the naturally acquired humoral response to VAR2CSA. However, the ultimate response is much less robust than that obtained by women living in rural areas with high exposure to VAR2CSA during pregnancy.

High Avidity Abs Appear to be Restricted Primarily to DBL5 in this Urban Setting

In Yaoundé, most women had detectable levels of Abs to the individual DBL domains, with the predominant Ab response to DBL5 (**Figure 7A**). That is, the median Ab level to DBL5 was 15,277 MFI; whereas, median Ab levels to the other domains were <4,000 MFI (**Figure 6**). In a high transmission village, the absence of PM at term was associated with women having high Ab levels to multiple domains and strains (lines) (17). Thus, the breadth of the Ab response to VAR2CSA is much higher in a rural, compared to the urban, site. Likewise, the median AI for DBL-5 was 52 compared to <25 for the other domains (**Figure 7B**). To our knowledge, similar data are not available on the avidity of Abs to DBL domains in women in high transmission areas. In summary, in Yaoundé the Ab response was similar to a primary Ab response when primigravidae are exposed to a new antigen and secundigravidae have a secondary exposure.

The above conclusions are based on the Ab response to the FCR3 line of *P. falciparum*. Like other malarial antigens, VAR2CSA is polymorphic with six DBL domains, each of which contains conserved and polymorphic sequences. Conserved epitopes on the molecular surface that bind Abs are most predominant in DBL5, thereby, inducing strain-transcending or cross-reactivity Abs (25). Among isolates, DBL5 domains average 86% amino acid identity, which may explain, in part, the high AIs to DBL5 (27). For a vaccine to VAR2CSA to be successful, the response must be against strain transcending epitopes. A limitation of the current study is the possibility that affinity maturation occurred to a few non-strain transcending epitopes that were not detected in this study. Likewise, this study used 3M NH_4SCN which is a very strong chaotrope for malarial antigens (22) and may efficiently remove Abs bound to more flexible epitopes. If a less stringent chaotrope had been used, additional evidence of affinity maturation may have been detected. Overall, these results suggest that in urban cities when women become infected only a few times during pregnancy, most high avidity Abs are directed against the DBL5 domain.

In high transmission areas, Abs to VAR2CSA have been reported to be associated with increase length of pregnancy, higher birthweight babies, lower placental parasitemia, and reduced prevalence of placental malaria at delivery (6, 7, 9, 10). The influence of Abs to VAR2CSA has been more difficult to assess in urban settings, in part, because a large sample size is need to detect small effects. Using data from 1,337 women, Abs to FV2 were found to be beneficial for PM+, but not PM−, women (8). That is, in PM+ women the presence of Abs to FV2 was associated with lower placental parasitemia and higher birth weight babies compared to PM+ women without Abs to FV2. In the current study, where all women had Abs to FV2, women whose AI to FV2 were above the median delivered higher birthweight babies compared to women whose AI was below the median (**Table 2**). The comparison remained significant after adjusting for age and gravidity. Thus, there is some evident that affinity maturation has a beneficial role in a low transmission setting; however, the increase of birthweight was only 104 g and may be of minimal clinical relevance. It remains unclear if Abs to FV2 with higher AI are better at blocking or reversing the attachment of infected erythrocytes to trophoblasts or if they are merely a surrogate marker for the overall maturation of the immune response to VAR2CSA.

The Ab response to VAR2CSA has been measured in other urban settings, e.g., Blantyre, (Malawi); Lambarene, Gabon; and Manjica, Mozambique (28–35). It is difficult to make direct comparisons between these cities and Yaoundé at the time the women were recruited, since factors that influence development of immunity differ among the cities, including time of the study, assays used, sample size, and range of intermediate malaria transmission. However, commonality among the results exist. In these urban cities, Ab levels in PM+ were higher than those in PM- primigravidae (28, 32, 36), Ab levels were higher in PM+ than PM − mothers at term, but there was no difference between PM+ and PM− multigravidae, regardless of the assay used to measure Abs to Var2CSA (18, 28, 33–35, 37). Similar to Yaoundé, Abs to FV2 were not associated with maternal peripheral parasitemia or percentage of LBW babies (29, 33); however, like higher avidity Abs, Abs that mediate phagocytosis of VAR2CSA-expressing IE were associated with increased infant birthweight (29). Abs, to a non-var antigen, AMA-1, were reported to contribute to improving infant birthweight (34). Thus, the results of this study may be applicable to other urban cities. With implementation of intervention strategies, such as IPT and insecticide treated bed nets, transmission of malaria is on the decline in many places (38). Today rural environments are becoming intermediate transmission areas similar to Yaoundé. Thus, the results reported herein are of both current and future interest.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The part of the study involving human subjects was reviewed and approved by the National Ethics Committee, Cameroon and IRB, Georgetown University (1994-158). The patients/participants provided their written informed consent to participate in the study. The use of archival materials used in the current study was approved by the Committee on Human Subject, University of Hawaii-Manoa (CH#21891).

AUTHORS CONTRIBUTIONS

The following were contributed by the authors: sample and data collection (RL), study design (NB and DT), conduction of the experiments (KV and NB), data analysis (MM and JC), provision of critical reagents and advice (AS), and manuscript preparation (RL, AS, and DT). All authors contributed to the article and approved the submitted version.

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Poor Birth Outcomes in Malaria in Pregnancy: Recent Insights Into Mechanisms and Prevention Approaches

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Pregnant women in malaria-endemic regions are susceptible to malaria in pregnancy, which has adverse consequences on birth outcomes, including having small for gestational age and preterm babies. These babies are likely to have low birthweights, which predisposes to infant mortality and lifelong morbidities. During malaria in pregnancy, *Plasmodium falciparum*-infected erythrocytes express a unique variant surface antigen, VAR2CSA, that mediates sequestration in the placenta. This process may initiate a range of host responses that contribute to placental inflammation and dysregulated placental development, which affects placental vasculogenesis, angiogenesis and nutrient transport. Collectively, these result in the impairment of placental functions, affecting fetal development. In this review, we provide an overview of malaria in pregnancy and the different pathological pathways leading to malaria in pregnancy-associated low birthweight. We also discuss current prevention and management strategies for malaria in pregnancy, and some potential therapeutic interventions that may improve birth outcomes. Lastly, we outline some priorities for future research that could bring us one step closer to reducing this health burden.

Keywords: low birthweight, preterm birth, small for gestational age, malaria, pregnancy, VAR2CSA

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INTRODUCTION

Global efforts to combat malaria have resulted in declining malaria transmission in many regions over the last decade. Unfortunately, young children and pregnant women remain vulnerable, especially in areas with sustained transmission. Malaria, a disease caused by the *Plasmodium* spp. parasite, can result in severe morbidity and mortality. Worldwide, infection with *Plasmodium falciparum* contributes to the highest burden, and it will be the main focus of this review (1). Despite childhood exposure that leads to acquisition of immunity against malaria, first time pregnant mothers or primigravidas are again susceptible to the disease due to a combination of host and parasitic factors (2, 3). Malaria in pregnancy (MiP) results in placental infection, termed placental malaria (PM), which predisposes to placental injury and insufficiency. Placental insufficiency refers to poor placental function and is commonly observed in PM. It is also hypothesized to be a leading cause of low birthweight (LBW). A baby with LBW is defined as a live born who is <2,500 g regardless of gestational age (4). LBW deliveries can be due to preterm birth (live birth < 37 gestational weeks) or small for gestational age (SGA) (birthweight < 10th percentile for its

gestational age). Of note, the precise mechanisms behind MiP-associated preterm birth and SGA remains unclear. *P. falciparum* infection can cause inflammation and potentially disrupt the fine immunological balance required to maintain pregnancy to term (5–7). On the other hand, SGA is often linked to placental insufficiency and there is also substantial evidence to suggest dysregulated placental development in mothers with MiP (8, 9). Interestingly, preterm birth does not commonly co-exist with SGA, further highlighting the complexity of MiP-associated birth outcomes (10).

LBW is an important indicator of infant mortality. MiP is estimated to cause approximately 900,000 LBW deliveries annually, with an estimated 100,000 MiP-related infant deaths (1, 11). Apart from high mortality risk, there is also increased morbidity in the surviving LBW infants, who are at elevated risk of poor cognitive and social development (12). LBW due to fetal growth restriction, the cause of SGA, is linked to increased incidence of adult diseases including type 2 diabetes and cardiovascular diseases (13). Hence, the prevention of MiP-associated LBW remains a priority in research. In this review, we discuss recent findings on the causes of LBW in *P. falciparum*-MiP and the different pathological pathways such as SGA and preterm birth in contributing to MiP-associated LBW. We also highlight existing gaps in our knowledge on the pathogenesis of LBW in MiP. Lastly, we review current interventions and provide suggestions for future work that allows us to better understand and manage MiP.

MALARIA IN PREGNANCY: AN OVERVIEW OF PATHOGENESIS AND IMMUNITY

Malaria in pregnancy threatens the well-being of the mother and her developing fetus, and an infected mother is likely to be an important reservoir of *Plasmodium* infection. One prominent feature of *P. falciparum*-infected erythrocytes (IEs) is their ability to adhere to endothelial and placental receptors. The latter allows them to sequester in the placenta, leading to placental infection and inflammation. Histological sections of *P. falciparum*-infected placentas reveal the presence of IEs, particularly at the surface of the syncytiotrophoblast, which is the main site of exchange for nutrient, waste and other metabolites between maternal and fetal circulations. Within the placental intervillous spaces, which are occupied by maternal blood, increased infiltrates of phagocytic cells with substantial amounts of ingested parasites can be observed; this is known as intervillositis (14, 15). The binding phenotype of IEs during pregnancy is mediated by their expression of variant surface antigens (VSA). Placental-binding parasites express VAR2CSA, a major VSA that is mainly expressed during pregnancy, and they bind specifically to the placental receptor known as chondroitin sulfate A (CSA) (16–19). Therefore, prior to pregnancy, antibodies against placental-binding IEs are uncommon, predisposing primigravidas to the adverse effects of PM (2, 20). In subsequent pregnancies, the protective anti-VAR2CSA antibodies can be naturally acquired in a gravidity-dependent manner and have been demonstrated to be effective against MiP and its consequences, thus preventing LBW (21). However, the specific antigenic targets and mechanisms of protection remain unclear. A recent systematic review showed that antibodies against different placental-binding antigens including VAR2CSA were associated with increased risk of PM and its consequences, suggesting that they may be markers of infection instead of correlates of protection (22). Nonetheless, it is likely that antibodies that are protective can inhibit binding of IEs to the placenta and/or facilitate clearance through opsonising phagocytosis, thus prevent placental inflammation and the subsequent adverse effects on fetal growth (**Figure 1**), and defining the characteristics of potentially protective antibodies remains a priority.

CAUSES OF MIP-ASSOCIATED LBW

The Link Between MiP, Dysregulated Placental Development and Small for Gestational Age Babies

Small for gestational age is commonly caused by fetal growth restriction. This condition significantly increases the risk of LBW, stillbirth, infant mortality and morbidity, and the risk of being diagnosed with chronic diseases in adulthood (13). Interestingly, SGA infants were two times more likely to experience *Plasmodium*-infection and clinical malaria by the age of 6 months, after adjusting for mosquito exposure (23). A recent study from an area of low malaria transmission reported several factors that were associated with increased odds of SGA during *P. falciparum*-related MiP, including symptomatic malaria and infection after 12–16 weeks' gestation (24), In addition, the risk of SGA was reported to increase by 1.13 times for every episode of infection during pregnancy (24). In contrast, in areas of high malaria transmission and particularly when pregnant women received anti-malarials in the form of intermittent preventive treatment in pregnancy (IPTp), there was no association between number of malaria episodes and SGA, suggesting that the timing of MiP may contribute to increased risk of SGA (25, 26). Indeed, placental pigment deposition (past-chronic infection) was associated with increased risk of SGA but not LBW or preterm birth, suggesting early chronic infection (usually outside of IPTp coverage) may drive SGA and not preterm labor (25, 27). Alterations in the downstream pathways of MiP such as growth factors and their regulators may further contribute to SGA. Recently, reduced plasma concentrations of placental growth factor in mid-pregnancy were associated with SGA and may indicate early placental insufficiency (9). MiP is known to be associated with reduced expression and bioavailability of placental growth factor, with the latter occurring through increased expression of their soluble inhibitor, soluble fms-like tyrosine kinase-1 (sFlt-1) (26, 28, 29). In addition, MiP leads to increased levels of asymmetric dimethylarginine, which is a competitive inhibitor of nitric oxide synthase (29). When nitric oxide synthase is inhibited, nitric oxide production is similarly affected, resulting in increased sFlt-1 levels and reduced levels of vascular endothelial growth factor (VEGF) family of proteins (30). This subsequently leads to

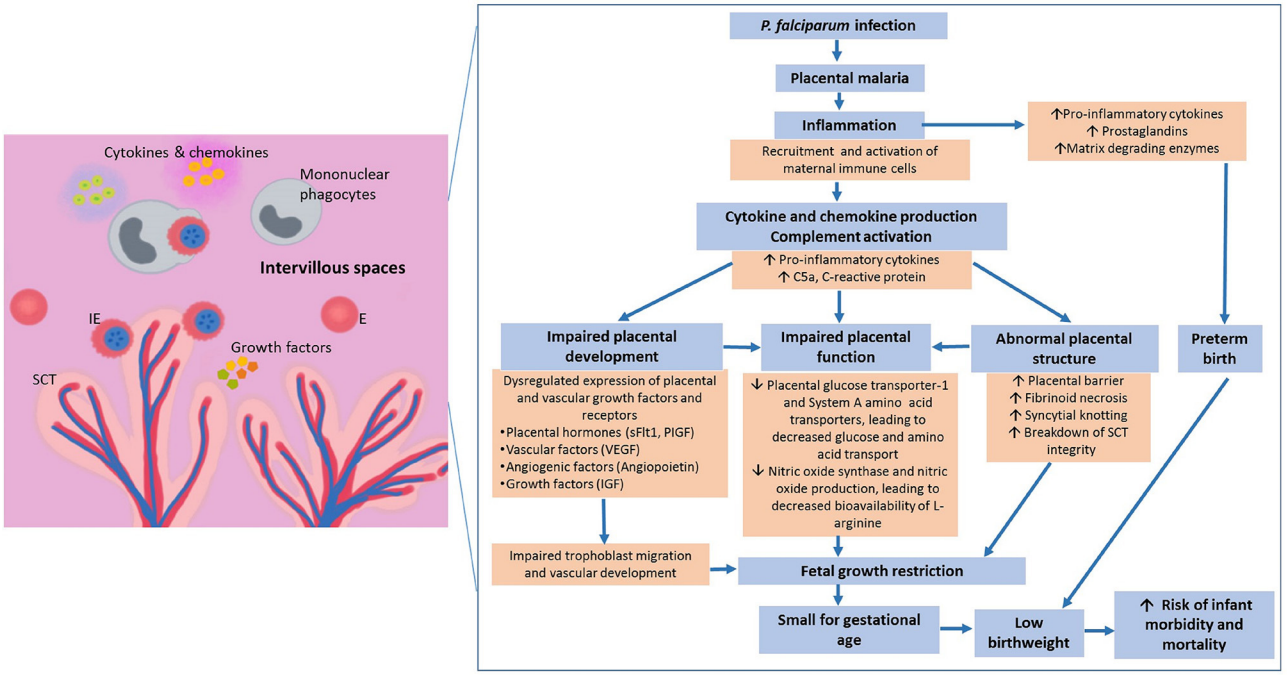

FIGURE 1 | Pathways leading to malaria in pregnancy-associated low birthweight. Infected erythrocytes express unique variant surface antigens that mediate binding to placental receptors. Sequestration leads to recruitment and activation of different immune cells that can result in a cascade of downstream inflammatory events. These inflammatory events include secretion of cytokines and chemokines that have been associated with poor pregnancy outcomes. The increased pro-inflammatory environment has been associated with – (1) dysregulated expression of growth hormones, vascular factors and angiopoietin-1 and -2; (2) abnormal placental development; and (3) reduced placental nutrient transport, overall contributing to placental insufficiency. Together, these factors contribute to malaria in pregnancy associated low birthweight which is the biggest predictor of infant morbidity and mortality globally and annually., ↑, increase; ↓, decrease; E, Erythrocytes; IE, Infected erythrocytes; SCT, Syncytiotrophoblast; IVS, Intervillous space; sFlt1, soluble fms-like tyrosine kinase-1; PlGF, Phosphatidylinositol-glycan biosynthesis class F protein; VEGF, Vascular endothelial growth factor; IGF, Insulin-like growth factor.

dysregulated placental vasculogenesis and angiogenesis that may affect placental functions, contributing to SGA.

The Link Between MiP, Inflammation and Preterm Babies

MiP is also associated with preterm birth, where these babies are usually born small and have increased risk of mortality due to complications such as brain hemorrhage, sepsis, acute respiratory illnesses and perinatal asphyxia (31). Surviving infants may develop life-long morbidities such as learning disabilities, which become obvious when the infant reaches school age (32). *Plasmodium* infection in both early and late pregnancies has been associated with preterm deliveries. A study from Malawi showed that earlier infection (before 24 weeks gestation) was associated with a higher risk of preterm delivery (26). In a Cameroon study, pregnant women who experienced MiP during the third trimester were four times more likely to have preterm deliveries (7). Similarly, in areas with low malaria transmission such as Southeast Asia, *P. falciparum* infection during late pregnancy was also associated with preterm delivery (24).

Studies on populations in malarious areas have identified several factors that significantly increase the risk of preterm birth, including PM, systemic inflammation (particularly CXCL9 and IL-1β), primigravidity, long-term iron supplementation and severe anemia (33–35). A recent systematic review found that maternal anemia in the first trimester, but not other trimesters, was associated with increased risk of preterm birth (36). Of note, the risk of MiP is highest during the first trimester, however, how severe maternal anemia contributes to preterm deliveries remains unclear. Interestingly, vertical parasite transmission, though uncommon, was also reported to contribute to preterm and LBW deliveries; this may partly be attributed to placental inflammation as higher complement activity was reported in cord blood infection (37, 38).

Several inflammatory mediators within the maternal peripheral circulation have also been associated with preterm delivery, but more investigations are required to understand whether they serve as biomarkers or play a pathological role. One of these markers is the soluble tumor necrosis factor receptor 2, which was found at increased levels in HIV positive women with MiP. Women who had elevated levels of this receptor throughout pregnancy also had preterm deliveries (6). Another study reported that in Papua New Guinean women who received one dose of IPTp with sulfadoxine-pyrimethamine (SP) and chloroquine, maternal serum levels of the acute phase protein α1-acid glycoprotein (AGP), an inflammatory marker, at delivery were positively correlated with several birth parameters including LBW, preterm and SGA deliveries (9). In

the same study, women with two or more doses of IPTp with SP and azithromycin (SPAZ) were observed to have reduced levels of AGP at delivery, suggesting that SPAZ may reduce inflammation thus preventing deleterious pregnancy outcomes (9). The levels of AGP were previously shown to be increased in *P. falciparum*-infected children and adults with uncomplicated malaria illness (39, 40), while the role of AGP in MiP, especially in higher transmission areas, requires further investigation. Our understanding of the pathways leading to preterm birth in MiP is still rather limited, although placental inflammation is likely to be a main contributing factor. During the initiation of labor, a pro-inflammatory environment is generated through the activation of maternal immune cells and placental stromal cells, which subsequently produce high levels of pro-inflammatory cytokines, prostaglandins and matrix-degrading enzymes. These are associated with the triggering of uterine contraction, cervical ripening and membrane rupture, signaling the beginning of the birthing process. In MiP, particularly PM with intervillositis, the massive leukocyte infiltrates may trigger an inflammatory environment similar to the one observed in labor, leading to the premature initiation of parturition. Various cytokines that are implicated in the induction of preterm birth such as IL-1β, IL-6, macrophage migration inhibitory factor and CXCL1 have also been found at elevated levels during MiP (33, 41). Recently, in a murine model of MiP, dysregulated expression of placental ATP-binding cassette transporters was associated with preterm labor (42). Further studies are required to determine if a similar mechanism operates in the human disease.

POTENTIAL PATHWAYS LEADING TO MIP-ASSOCIATED LBW

Effects of MiP on Placental Development

The placenta is an important organ that acts as an interface between the mother and her fetus. Successful placental development requires a fine immunological balance to be achieved between maternal, placental and fetal immunity. PM can alter nitric oxide bioavailability, and it may lead to host inflammatory responses. Either of these processes may result in poor placental development, leading to placental insufficiency when the placenta is unable to fully support the growth of the developing baby (26, 29).

The first trimester is associated with the highest risk of *Plasmodium*-infection especially in primigravid women (43, 44). Critically, the timing of infection appears to determine the risks and severity of adverse birth outcomes. For MiP, early exposure (<15 gestational weeks) was associated with reduced volume of placental transport villi and smaller surface areas for diffusion across the villi (45). Other abnormalities such as impaired development of placental vascular space, excessive syncytial knotting (aggregation of syncytiotrophoblast nuclei), increased fibrinoid necrosis areas and thickening of syncytiotrophoblast basal membrane have also been observed (46–48). The timing of infection that resulted in such placental pathologies, however, is unknown. Ultrastructural studies showed the breakdown of syncytiotrophoblast integrity in regions associated with IEs (49). These abnormal changes to the placenta were associated with decreased birth weight (45, 46, 49).

MiP and the subsequent immune activation are associated with placental pathologies that arise due to disruptions in placental tissue (trophoblast) and vascular development. An *in vitro* model demonstrated impaired trophoblast invasion and migration after incubation with sera from *P. falciparum*-infected women (50). In the same study, elevated levels of soluble factors that inhibit trophoblast invasion such as human chorionic gonadotropin and IL-10 were found in these sera. There was a corresponding decrease in the invasion stimulatory factors such as insulin-like growth factors and IL-8 (50). There are two main events in vascular development; vasculogenesis (formation of new blood vessels) and angiogenesis (development of blood vessels from existing vasculature). In a recent review, three main pathways that are associated with impaired vascular development and adverse birth outcomes in MiP were identified, including reduced bioavailability of L-arginine and nitric oxide, excessive complement activation and dysregulated hemoglobin-scavenging system due to increased intravascular haemolysis (51). In a Brazilian cohort, MiP altered the angiogenic profile of the placenta and was associated with increased placental barrier thickness, which is likely to affect nutrient and gas transfer (47). Often, *Plasmodium*-infected placentas become the foci of inflammation where maternal immune cells are recruited and retained. This concomitant inflammation generates various soluble inflammatory mediators including tumor necrosis factor-α, interferon-γ, complement C5a, and C-reactive protein (33, 37). In addition, higher levels of chemoattractant proteins such as macrophage inflammatory protein-1 (alpha and beta) and monocyte chemoattractant protein-1 were observed (41, 52). These inflammatory mediators and chemoattractants are known to regulate the levels of key placental growth factors and receptors, including insulin-like growth factors, angiopoietin (1 and 2), soluble endoglin, sFlt-1 and VEGF (8, 37), which in turn disturb the delicate balance required for normal placental development. All of these analytes have been associated with reduced birth weight and LBW deliveries (33, 53). Taken together, placental trophoblast development, vascular development pathways and inflammatory responses appear to be interdependent and may synergistically contribute to poor birth outcomes in MiP. However, it remains to be determined if inflammatory inhibitors and/or promoters of placental growth factors may alleviate the impact of MiP on placental development and birth outcome.

Intervillositis and Placental Insufficiency

Untreated *Plasmodium* infection during pregnancy increases the risk of intervillositis (inflammation in placental intervillous spaces). These spaces are occupied by maternal blood that surrounds the chorionic villi, serving as the materno-fetal exchange surface. Importantly, intervillositis, but not PM without intervillositis per se, was more commonly linked to adverse birth outcomes. This is particularly true in areas of high malaria transmission such as Tanzania and Malawi, where intervillositis was associated with abnormalities in the placenta and significantly higher rates of LBW (up to 50%)

(15, 54, 55). PM-associated intervillositis is characterized by the accumulation of mononuclear cells, predominantly monocytes/macrophages. The inflammatory foci serve as a physical barrier that may compromise placental transport function and cause hypoxia. These immune cells can also be activated to produce inflammatory molecules that affect various downstream signaling pathways in the placenta, leading to impairment of fetal growth.

Investigations on *Plasmodium*-infected placentas revealed that compromised nutrient transport is one of the molecular mechanisms contributing to growth restriction and reduced birth weight. In women with PM-associated intervillositis, reduced expression of glucose transporter isoform 1 (GLUT-1) on the syncytiotrophoblast basal membrane was reported (56). Transplacental glucose transport is primarily facilitated by GLUT-1 and its expression was positively correlated with birthweight. In addition, lower expression and activity of system A group of amino acid transporters were also found in PM-associated intervillositis. Maternal to cord blood ratios of several amino acids were lower in cases with intervillositis compared to uninfected controls and importantly, cord concentrations of amino acids were positively correlated with fetal/placental weight ratios (57). In a follow-up study, lower placental mammalian target of rapamycin (mTOR) activity was observed in infected placentas with intervillositis, which in turn is associated with reduced placental amino acid uptake and lower birthweight (58). Recently, increased placental autophagy was proposed as a possible mediator of placental insufficiency in PM and similarly, this has been reported in other non-infectious pregnancy complications such as preeclampsia and intrauterine growth restriction (59–61). The dysregulation of this process may negatively affect the uptake of amino acids by the placenta and future studies should further explore this association.

PREVENTION OF MIP-ASSOCIATED LBW: A PROSPECTIVE ON CURRENT AND FUTURE STRATEGIES

Insecticide-Treated Nets, Indoor Residual Spraying, and Intermittent Preventive Treatment

The World Health Organization recommends the prevention and management of MiP via the use insecticide-treated bed nets (ITN), IPTp and effective case management of malaria disease. Additionally, there are encouraging data to suggest that indoor residual spraying insecticide (IRS) may also be effective in reducing MiP and improving birth outcomes (62, 63). ITN and IRS, whether deployed individually or in combination, aim to reduce vector exposure and limit transmission, hence are likely to be beneficial (**Figure 2**). The use of ITN is a simple yet effective strategy to prevent *Plasmodium* infection and its consequences. There is substantial evidence from earlier studies suggesting that this strategy resulted in reduced prevalence of maternal parasitemia, improved birth weights and a 25% reduction in the risk of LBW deliveries (64–66). On the other hand, IRS, by decreasing vector contact, was associated with reduced risk of preterm birth, LBW delivery and fetal/neonatal deaths in both HIV-negative and HIV-infected women (62, 63).

Currently, all pregnant women residing in areas with high malaria transmission are recommended to receive three or more treatment courses of IPTp with SP, where each course should be administered at least one month apart, starting in the second trimester (67). This replaces the original recommendation of two doses of SP, following substantial data that demonstrated reduced risk of preterm birth and LBW as well as improved birth weight when three or more courses were administered (68–70). In mathematical modeling studies, it was estimated that three or more doses of IPTp-SP uptake would lead to a 30% relative reduction in LBW occurrence (70–73).

Despite these preventive strategies, the prevalence of PM and LBW remain a huge threat to pregnancies in malarious regions due to several reasons. Given that IPTp is only administered from the second trimester onwards, pregnant women are not protected during the first trimester and the later weeks of third trimester. This inadequate IPTp coverage throughout pregnancy, poor population coverage, development of drug and insecticide resistance, and the use of suboptimal drugs which results in the failure to clear submicroscopic infection, are likely to contribute to increased risk of preterm birth, SGA and LBW (26, 74, 75). Following reports on the increased prevalence of SP-resistant parasites, there are concerns regarding the future effectiveness of this prophylactic regimen in protecting against consequences of MiP (72, 76, 77). For this reason, other antimalarial drugs such as amodiaquine, mefloquine, chloroquine–azithromycin and dihydroartemisinin-piperaquine have been tested as potential alternatives to IPTp-SP (78, 79). Dihydroartemisinin-piperaquine appears to be a promising choice, as trial results showed that malaria burden including maternal parasitemia and PM was significantly lower in women who received this drug compared to those who received SP (80, 81). However, dihydroartemisinin-piperaquine did not lead to improved birth outcomes when compared with SP (81, 82). A recent systematic review showed that SP resistance decreased the effectiveness of IPTp especially in areas with high SP resistance, but this regimen is still associated with decreased cases of LBW (82).

The exact mechanisms by which SP leads to improved birth outcomes in the treated individuals remain elusive. In addition to malaria prevention, SP also serves as a broad-spectrum antibiotic that can resolve or prevent other infections of bacterial origin (sexually transmitted, reproductive tract and urinary tract infections) that have also been associated with adverse birth outcomes (69, 82). In addition, it was postulated that sulfadoxine can modulate bacterial microflora composition within the gut and vagina, which subsequently promotes weight gain (83) There is an ongoing study investigating the effect of SP on microbiota in the gut and/or vagina, in which the researchers proposed that higher birth weights following SP administration in pregnancy can be attributed to the modulation of these bacterial populations (84). To corroborate, antibiotic exposure during pregnancy led to increased gestational weight gain, which is an important predictor of birthweights (85). The overall reduction in malarial and other non-malarial infections during pregnancy is likely to

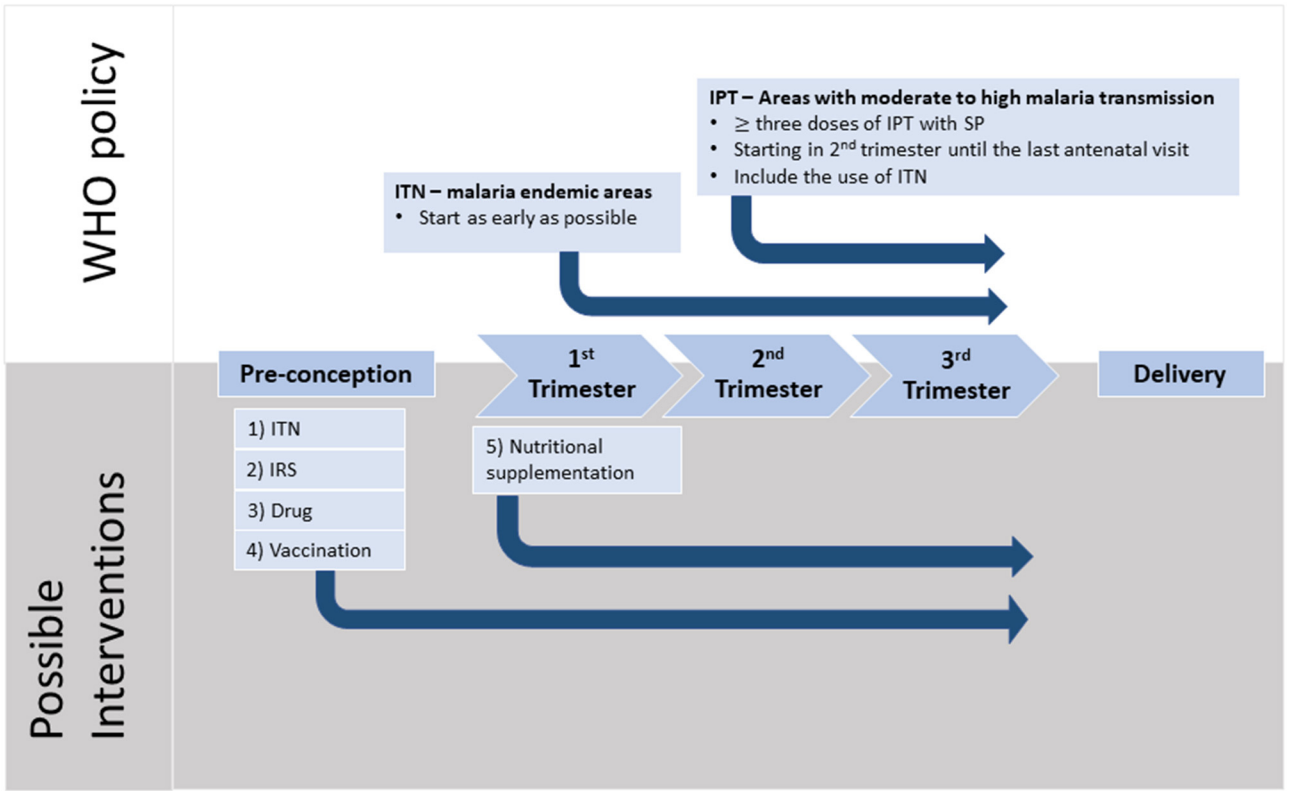

FIGURE 2 | Timeline of pregnancy events with WHO recommendations and possible interventions. Malaria in pregnancy (MiP) is preventable and the current WHO recommendations for Africa include three or more doses of intermittent preventive treatment during pregnancy (IPT) with sulfadoxine-pyrimethamine (SP) taken one month apart starting from fetus quickening. In addition, sleeping under insecticide treated nets (ITNs) has been shown to reduce parasite burden during pregnancy. Strong evidence indicates that MiP starts during the first trimester with irreversible damage and it is during this period of time that pregnant mothers are at the highest risk of parasitemia. Furthermore, recent evidence suggests that women often harbor parasites before conception, and these parasites may switch to a placental-binding phenotype, that is likely to contribute to placental inflammation, especially in women pregnant for the first time. We propose several potential interventions which may help to alleviate the harmful impact of MiP. (1) ITNs should be used from early pregnancy, or before conception, to reduce the risk of acquiring new infections. (2) Indoor residual spraying should be evaluated for its effectiveness against MiP. (3) Pre-pregnancy intermittent screening for malaria, with treatment by safe and effective drugs, should be considered. (4) Pregnant women need access to an effective vaccine that can induce protection against MiP. Currently, there are two vaccines that are in clinical trials. (5) The nutritional status of the mother should be optimized during pregnancy with appropriate micro- or macronutrient supplements to ensure optimal placental development and thus better birth outcomes.

minimize chronic maternal inflammation, thus improving birth outcomes (9, 82).

Given the limitations associated with current drugs, there is an urgent need to evaluate the safety and efficacy of new antimalarials or a combination of preventive strategies that are suitable for wider coverage and at earlier time points in pregnancy to protect against MiP in the first trimester.

Nutritional Interventions a Viable but Under-Interventions: Approach

Another major contributor to LBW is maternal undernutrition, a condition that is also highly prevalent in many areas with high malaria transmission. There is a growing number of studies suggesting that malaria and maternal undernutrition may worsen pregnancy outcomes (86, 87). Malnourishment during pregnancy, indicated by low body mass index and smaller mid-upper arm circumference, was associated with lower levels of antibodies to placental-binding IE and increased parasite load in the placenta (88). The impaired development of protective antibodies may reduce the host's ability to clear infection, thus prolonged infection is likely to result in chronic inflammation in the placenta. In addition, undernutrition during pregnancy may also affect placental size and its functional capacity, given that maternal weight and gestational weight gain are both mediators of placental weight. All three factors were positively correlated to birthweight (89). Furthermore, both MiP and maternal undernourishment have been reported to impair trophoblast invasion and migration, as well as increase uterine artery resistance (50, 90, 91). In recent studies, MiP and undernourishment during pregnancy were observed to affect the levels of L-arginine-nitric oxide biogenesis; this pathway is essential for placental vascular development (29, 92).

In a comprehensive study that analyzed 23 systematic reviews on nutritional interventions during pregnancy, a few factors including provision of vitamin A, low-dose calcium, zinc and multiple micronutrients were shown to be associated with

reduced risk of LBW (93). Similarly, several earlier macronutrient supplementation studies in malarious regions such as Sub-Saharan Africa provided evidence of improved birthweight, increased gestational length and reduced odds of LBW (94). Using a MiP animal model, dietary L-arginine supplementation resulted in improved fetal weights, reduced levels of pro-inflammatory mediators and increased levels of angiogenic factors, the last of which were associated with improved placental vascular development and remodeling (29). Nutrient intervention appears to be a promising strategy to prevent LBW, hence more studies should be carried out to evaluate the effectiveness of different types of nutrient supplementation in reducing MiP-associated LBW. Distinguishing between the potential beneficial impacts of malaria prevention with IPTp and of nutrient supplementation will require careful study designs.

Vaccines: the Pivotal Prevention Strategy

Efficacious malaria vaccines could potentially be one of the most effective strategies to prevent LBW in MiP. Previous studies have described the requirements of an effective vaccine for MiP, which include the ability to induce protective antibodies against placental-binding IEs to prevent their binding to CSA in the placenta *via* the parasites' VAR2CSA protein. These binding-inhibition antibodies have been associated with protection against preterm birth, increased gestational age, the delivery of heavier infants and importantly, reduced risk of LBW delivery (21). Furthermore, these binding-inhibition antibodies against whole parasites were demonstrated to be strain-independent, raising the possibility that targeting an immunogenically-conserved subunit or domain of VAR2CSA, which is a large (∼350 kDa) multidomain protein, could be effective (95). Given its large size, later studies then identified a minimal binding region on the VAR2CSA protein that binds with high affinity to CSA. Antibodies in the sera collected from malaria-exposed pregnant women were able to bind to recombinant VAR2CSA and purified antibodies against this protein exhibited binding-inhibitory activity (96–99). Importantly, a study from Benin then demonstrated strong antibody responses against the VAR2CSA N-terminal region of DBL3X to be associated with protection against SGA and reduced prevalence of both LBW babies and placental infection at delivery, possibly by reducing parasite burden and thus lowering the risk of placental insufficiency (97). However, recent studies have identified distinct pathogenicity among the parasite variants and diversity in functional antibodies against different placental binding variants, suggesting that a polyvalent VAR2CSA-based vaccine may provide better protection (100, 101). To corroborate, two isoforms of VAR2CSA subunit protein that recently completed phase 1 clinical trial, ID1-ID2a (PAMVAC) and DBL1x-DBL2x (PRIMVAC), only demonstrated binding-inhibition activities against homologous parasite variants and the duration of immune responses is unknown (102, 103). Critically, the duration of immune responses and whether vaccination translates to clinical protection remains to be determined.

FUTURE DIRECTIONS

Great advances have been made over the last two decades in understanding and reducing MiP, especially with effective anti-malarials and the use of ITNs during pregnancy. Despite this, our understanding of factors that contribute to LBW during MiP is still rather limited. In addition, with the increasing pressure of parasite resistance against existing drugs, alternatives such as a viable vaccine will be crucial to combat MiP. Hence, more research efforts should be channeled toward these areas of investigation. Herein, some areas of future research are proposed.

1. To better understand and manage the adverse birth consequences of MiP, there is a need to differentiate the relative contributions of SGA and preterm delivery to LBW. SGA and preterm delivery in MiP may be initiated by different molecular events, and hence may require different modes of interventions. In resource-limited malaria endemic areas where ultrasound is unavailable, pregnancy dating is based on recall of last menstrual period, abdominal palpation, or newborn assessments, all of which may be inaccurate. In addition, the reference curve for birth weights is based on those that have been established in the Western populations. Several studies have created population-specific charts detailing mean birth weights and percentiles at different gestational ages to have a better representation of their own populations (10, 104). In addition, a global healthcare project has developed standards and tools for assessing newborn weight, length and head circumference by gestational age and sex (105). These can lead to more accurate classifications of birth outcomes in MiP and studies on their effectiveness in differentiating SGA and preterm birth can be done.
2. Changes to the placenta in PM are only studied upon delivery, given the invasive nature of sampling placental blood or biopsies during pregnancy. However, this rarely captures the whole picture of placental events that occurred throughout pregnancy, limiting our interpretation of how placental changes during gestation can lead to LBW. Although the use of animal models may address this challenge, there are concerns regarding the relevance and transferability of the findings to human disease (106). One way to better understand the placental pathophysiology during MiP is to employ the use of Doppler ultrasound to investigate utero-and feto-placental blood flow and resistance. In earlier studies, MiP in the first half of gestation was associated with altered uteroplacental blood flow, suggesting the possibility of vascular deficiency (90, 107). This technique, together with measurement of various maternal analytes in the peripheral blood, may provide a clearer picture of the events in the placenta during MiP.
3. Our current understanding on inflammation in PM focuses on the roles of monocytes and macrophages as the main contributors of inflammatory mediators associated with LBW. However, there are recent studies suggesting that neutrophils, which are the most abundant leukocytes in the peripheral blood, may also play a role in orchestrating the inflammation (108, 109). Their role in MiP requires further clarification.

4. Our current understanding of protective antibodies in MiP is that these antibodies should be able to inhibit IEs binding to the placenta and promote opsonic phagocytosis (21). However, their importance and relative contribution to protection through other immune effector mechanisms such as antibody-dependent respiratory burst and antibody-dependent cellular cytotoxicity are less well-understood. This knowledge will be important in vaccine design and the evaluation of vaccine efficacy.
5. Early studies in The Gambia and Nigeria demonstrated that passive transfer of anti-malarial antibodies resolved malaria (110, 111). Currently, there are existing clinical trials that are investigating the protective efficacy of monoclonal antibodies as anti-infective agents (ClinicalTrials.gov Identifier: NCT04327310 and NCT04206332). Therefore, monoclonal antibodies, targeting either the domains of VAR2CSA or the circumsporozoite protein to prevent establishment of infection can be further developed as therapeutics for MiP.
6. In terms of improving vaccine design, there is a need to identify conserved subdomains of VAR2CSA that can induce protective antibodies, against the different parasite variants.
7. SP may be less efficacious in areas with high SP-resistant parasites. Moreover, SP is only given at the start of second trimester, but there is substantial evidence showing that infection during the first trimester can also greatly impact birth outcomes (24, 26). Hence new antimalarial drug combinations that can be administered in the first trimester need to be evaluated in future clinical trials.
8. SP, a broad-spectrum antibiotic, may be effective against sexually transmitted and reproductive tract infections (highly prevalent in malaria endemic regions), thus it may resolve infections other than malaria to improve adverse birth outcomes (69). Future studies to explore the use of effective antimalarials coupled with SP may be beneficial.
9. Malnutrition is an important contributor to LBW, and nutrient supplementations during pregnancy appear to be an attractive and feasible intervention to minimize the risk of LBW deliveries. However, the beneficial effect of nutrient supplementation may be masked if administered together with IPTp, thus undermining its impact on birth outcomes. Hence, future trials of nutrient supplementations require careful study designs such as having intermittent-screening and treatment with nutrient supplementations, or choosing a trial site in a lower transmission setting where IPTp is not a first line policy.

CONCLUSION

As of 2019, 38 countries and territories were granted the "malaria-free" status by WHO, while transmission continues in other regions. Hence, pregnant women remain at risk of MiP, which leads to increased mortality and morbidity in both the mother and her infant. MiP-associated LBW is preventable, but there needs to be a concerted effort in the adoption and implementation of current prevention strategies in malarious areas. Continuous efforts in research are needed, to better understand the molecular mechanisms associated with poor birth outcomes, and ultimately to enable design of specific targeted approaches for improving birth outcomes. In the face of increased parasite resistance, the effectiveness of IPT with SP in preventing this health burden may be at threat in the near future. Therefore, there is an urgent need to design and evaluate other therapeutic options that can be used to protect against MiP and its deleterious consequences.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Plasmodium falciparum VAR2CSA-Specific IgG Subclass Responses Reflect Protection Against Low Birth Weight and Pregnancy-Associated Malaria

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Sequestration of *Plasmodium falciparum*-infected erythrocytes expressing the VAR2CSA antigen in the placenta results in poor pregnancy outcomes, including low birth weight and maternal anemia. Antigen-specific antibody-mediated immunity is acquired during successive pregnancies. Thus, evaluating VAR2CSA-specific IgG profiles among pregnant women will increase knowledge on the immunological mechanisms associated with protection, and help in the development of VAR2CSA-based placental malaria vaccines. Using the PAMVAC candidate vaccine antigen, we assessed anti-VAR2CSA IgG subclass responses of a cohort of pregnant Beninese, and analyzed their relationships with pregnancy outcomes. Cytophilic IgG1 and IgG3 responses were the most frequent, with prevalences ranging from 28% (IgG3) up to 50% (IgG1). Elevated levels of VAR2CSA-specific total IgG and cytophilic IgG3 during pregnancy were consistently associated with higher birth weights, whilst high levels of IgG4 were associated with a reduced risk of placental infections. This suggests that protective anti-VAR2CSA IgG responses are coordinated between both cytophilic and non-cytophilic antibodies.

Keywords: pregnancy, malaria, VAR2CSA, IgG subclasses, IgG3, IgG4

INTRODUCTION

Plasmodium falciparum malaria continues to be a significant human disease burden throughout the tropics. In sub-Saharan Africa, placental malaria (PM) is known to cause low birth weight and maternal anemia (1, 2). The adverse effects associated with PM are due to the sequestration of infected erythrocytes (IEs) in the placental intervillous spaces. The often massive accumulation of IEs is mediated by VAR2CSA, a parasite ligand that promotes adhesion of IEs to placental

chondroitin sulfate A (CSA) (3–5). This pathophysiological feature, which led to the recognition of placental malaria as a specific syndrome, is particularly frequent in first pregnancies in areas where *P. falciparum* malaria is highly endemic (6). Women who have had PM develop anti-VAR2CSA IgG antibodies that protect them from the adverse effects of PM during subsequent pregnancies, making this protein an attractive target for vaccine development (4, 7). Anti-VAR2CSA IgGs have been shown to inhibit the binding of VAR2CSA-expressing infected erythrocytes to CSA (8–10). Further studies identified the Id1-DBL2X-Id2 region, in the N-terminal part of VAR2CSA, as being the minimal binding domain mediating binding between VAR2CSA and placental CSA (9), thus making it appropriate for the development of a VAR2CSA-based vaccine with an antigenic construct easier to produce in recombinant form. A Phase 1 clinical trial has recently been completed with such an antigenic construct (10).

Our team has already demonstrated in a longitudinal study that high levels of IgG with specificity for the N-terminal part of VAR2CSA present in women early in pregnancy were associated with better pregnancy outcomes (11). Additionally, a recent systematic review suggests that IgG levels against VAR2CSA antibodies are likely markers of infection rather than correlates of protection (12). However, information is lacking on which of the IgG subclasses targeting the vaccine construct are responsible for protective immunity and also on the dynamics of such protective antibody levels during pregnancy. Here, we used samples from a group of women whose pregnancy outcomes were well characterized in the Strategies to Prevent Pregnancy Associated Malaria (STOPPAM) study in Benin and from whom consecutive blood samples and clinical data were available from the time of inclusion until delivery. Using measurements of naturally acquired antibody targeting the PAMVAC vaccine Id1-Id2a recombinant antigen on consecutive plasma samples, we analyzed the link between IgG subclasses with different pathological outcomes of pregnancy.

METHODS

Study Site and Population

The current study made use of samples collected from a subgroup of women drawn from the STOPPAM project initially designed to characterize the pathology of placental malaria, to determine the factors important for optimal use of intermittent preventive treatment of malaria in pregnancy (IPTp) and for vaccine development against pregnancy-associated malaria. A detailed description of the STOPPAM study design has been reported (13). For the current work, 470 women were included based on their pregnancy outcome as previously reported (14). This sub-group included women who had placental infection at delivery, were anemic, whose infants had low birth weight, in whom intrauterine growth retardation was identified based on fetal growth curves derived from ultrasounds to define children born small-for-gestational-age (SGA), or whose infants were born prematurely. Another group of pregnant women from the same area with no pathological outcome at delivery was also included. Briefly, babies were classified as premature if their gestational age, assessed by ultrasound before 24 weeks of gestation, was <37 weeks at delivery. Fetal growth restriction was approximated by low birth weight for gestational age. A baby was defined as SGA if the birth weight was below the 10^{th} percentile of fetal weight for the gestational age (15). Maternal anemia at delivery was defined as a hemoglobin level below 10 g/dl. Placental malaria was defined as a positive placental blood smear (16). At inclusion and each antenatal or emergency visit, a thick blood smear was processed for malaria diagnosis. Venous blood was collected, and plasma samples were separated and frozen at −80°C for further use. All women diagnosed with *P. falciparum* infection by thick blood smear during the follow up were treated with quinine according to national guidelines applicable at the time of the study.

Ethical Statement

The STOPPAM study obtained ethical approval from two independent institutional ethics committees: the Comité Consultatif de Déontologie et d'Éthique of IRD in France and the Comité d'Éthique de la Faculté des Science de la Santé, Université d'Abomey Calavi, in Benin. All participants provided written consent.

Total IgG and Subclasses Quantification

To measure antibody response against VAR2CSA, we used the PAMVAC vaccine candidate, a recombinant protein representing the ID1-ID2a construct of VAR2CSA from the FCR3 variant of *P. falciparum* (10, 17). Antibody levels with specificity for the ID1-ID2a recombinant protein were determined by a previously described indirect ELISA protocol (7, 10). In brief, antigen-coated (0.5 μg/mL) and bovine serum albumin (BSA)-blocked 96-well ELISA plates (Maxisorp, NUNC, Denmark) were incubated overnight at 4°C with 100 μl/well of test plasma samples in duplicate at dilutions optimized for each subclass (1:200 for total IgG; 1:100 for IgG1 and IgG2 and 1:50 for IgG3 and IgG4) in phosphate-buffered saline (PBS) with 2% BSA. Pools of highly reactive plasma from exposed pregnant Beninese previously characterized on recombinant full-length ectodomain of VAR2CSA (FV2) (11), and non-immune plasma from unexposed French volunteers, were used on each plate as internal controls. Reactions were developed with goat anti-human IgG conjugated to horseradish peroxidase (SIGMA, France) followed by the substrate TMB (SIGMA, France). The color reaction was stopped by the addition of 0.2M H_2SO_4 and optical densities (ODs) were read at 450 nm. In between all incubation steps, plates were washed 3 times with PBS, pH 7.4, containing 0.1% Tween 20, using an automated plate washer.

ELISA for the subclasses followed the same procedure except using mouse anti-human IgG subclasses Fc secondary antibody conjugated to HRP (MH1715, MH1722, MH1732, or MH1742, all from Invitrogen, France). IgG4 antibody levels were also measure against ICAM1 binding DBL-β domain from IT4 VAR13 *Pf*EMP1 (18) donated by Professor Jensen. All reagents were used at predetermined concentrations and optical densities (OD) generated from the antibody measurements were converted to arbitrary units (AUs), as described (19). Briefly,

AUs were calculated as follows:

$$AU = 100 * \frac{\ln(0D\ test\ sample) - \ln(0D\ negative\ sample)}{\ln(0D\ positive\ sample) - \ln(0D\ negative\ sample)}$$

Statistical Analysis

For each measured IgG subtype, the mean ODs from non-immune plasma from each plate were calculated plus 2 standard deviations to define a common cut-off value for each IgG isotype above which samples were deemed antibody-positive. Seroprevalence was calculated as the proportion of samples with OD above this cut-off. The outcome variable was the level of IgG antibodies in peripheral maternal blood. Five principal outcome variables were created: the level of total IgG, the levels of IgG1, IgG2, IgG3, and IgG4. As multiple antibody measurements (inclusion, antenatal visits, and delivery) were available, appropriate hierarchical mixed models were used for univariate and multivariate analyses, performed with Stata® software, Version 12 (StatCorp LP, College Station, TX, USA). The level of antibodies was tested in relation to the following different covariates: maternal age, gravidity, malaria at inclusion, number of peripheral malaria infections identified until the date of plasma collection, parasite density, and at delivery according to pregnancy outcome including anemia and placental infection in mothers; low birth weight, prematurity, intrauterine growth retardation in newborns.

All factors with a p-value <0.20 during univariate analyses were included in the multivariate step. A backward stepwise procedure was used to select factors of the final model. Statistical significance was set at $p<0.05$.

TABLE 1 | Characteristics of the population.

Mothers	N=470
Maternal age (years)	26.48± 6.18
Primigravidae	78 (16.6%)
Multigravidae	392 (83.4%)
Malaria infection during the follow-up (BS)	
0	234 (49.8%)
1	132 (28.1%)
2	63 (13.4%)
≥3	41 (8.7%)
Placental malaria	
Yes	63 (13.4%)
No	405 (86.2%)
Unknown	2 (0.4%)
Maternal anemia at delivery	
Yes	82 (17.4%)
No	358 (76.2%)
Unknown	30 (6.4%)
Malaria at inclusion (BS and PCR)	
Negative	265 (56.4%)
Submicroscopic	121 (25.74%)
BS Positive	80 (17.0%)
Newborns	
Birth weight (grams)	2982.22± 434.23
Low birth weight	
Yes	49 (10.1%)
No	418 (88.9%)
Unknown	3 (0.6%)
SGA	
Yes	53 (11.3%)
No	417 (88.7%)
Prematurity	
Yes	24 (5.1%)
No	446 (94.9%)

BS, Blood smear; SGA, small for gestational age.

RESULTS

The STOPPAM cohort subgroup used in this work comprises Beninese pregnant women (mean age, 26 years) followed up during the course of pregnancy. Among the 470 women included in this study, 11 (2.3%) attended 3 antenatal visits (ANV) while 415 (88.3%) attended all the 5 scheduled visits (inclusion, ANV1, ANV2, ANV3, and delivery). Amongst the study cohort 78 (16.6%) were primigravidae, 63 (13.4%) had evidence of placental malaria at delivery, and 82 (17.4%) were anemic at delivery. Forty-nine (10.1%) women gave birth to infants with LBW (<2500g), 53 (11.3%) with SGA, 24 (5.1%) women gave birth prematurely (before 37 gestational weeks), and 272 women had no pathological outcomes. The characteristics of women who had their IgG profiles determined are presented in **Table 1**.

Antibody Profiling and Factors Associated

The distribution of IgG subclass responses shows that cytophilic IgG1 and IgG3 were measured in a higher number of women compared to IgG2 and IgG4 (**Figure 1A**). Analysis of the seroprevalence of the different IgG subclasses shows that IgG1 and IgG3 were the most prevalent with rates ranging from 49.6% to 53.8% for IgG1 and from 27.6% to 27.4% for IgG3 according to the time of pregnancy. IgG2 and IgG4 were less prevalent with rates ranging from 8.3% to 12.1% and from 3.4% to 8.9%, respectively (**Figure 1B**). The ranges were larger for subclass measurements compared to total IgG leading to larger dispersion of values (**Figure 1**). When taking into account repeated measurements made on consecutive samples of the same woman in the analyses, plasma levels of IgG (both total and subclasses) gradually decreased over the course of pregnancy, except for IgG4 which was consistently low throughout. (**Table 2**, **Figure 1A**).

When comparing levels by gravidity, it was clear that total IgG levels remained consistently higher in multigravid women compared to primigravidae, in both univariate and multivariate analysis. However, this observation was not so clear for individual IgG subclasses. When taking into account infections that were documented during the follow-up in relation to the time of antibody measurement, a high level of all subclasses were consistently observed in infected women, even though this reached significance only for IgG1 and IgG3. A similar observation was made for antibody subclass levels and parasite density of the infections documented during follow-up, but statistical significance in multivariate analysis was reached only for total IgG (**Table 2**) (P = 0.028) and IgG2 (P = 0.03).

Relationship With Pregnancy Outcomes

To compare the level of IgG during pregnancy in women with well-defined pregnancy outcomes, we performed a

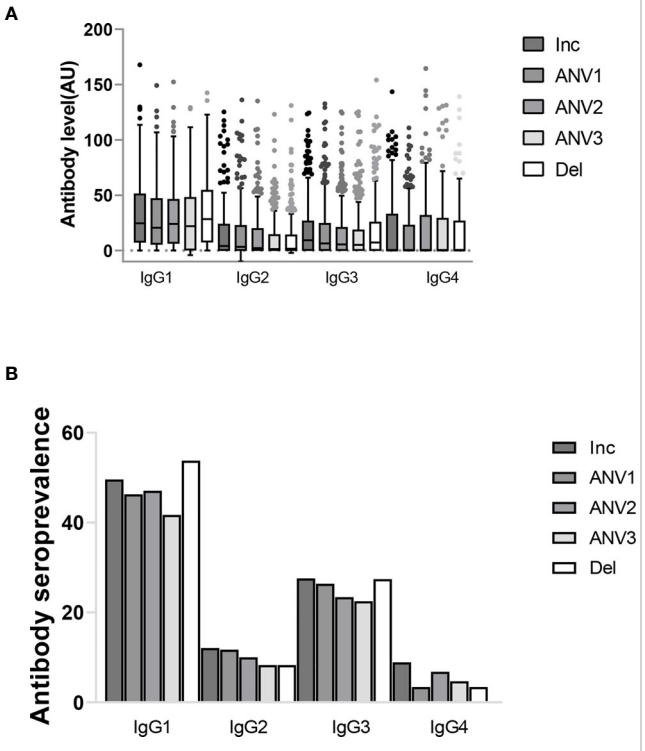

FIGURE 1 | IgG subclass profile to ID1-ID2a recombinant protein from pregnant women followed during the course of pregnancy (inclusion, antenatal visits (ANV) and delivery). **(A)** The distribution of IgG subclasses. Shown are IgG subclasses antibody levels (median and IQR) in arbitrary units. **(B)** IgG subclass seroprevalence. The profile of IgG subclasses clearly shows a cytophilic IgG's (IgG1 and IgG3) are the predominant IgGs while low prevalence of non-cytophilic IgG's (IgG2 and IgG4) were also observed.

hierarchical mixed model analysis and factors with p-value < 0.2 were further incorporated into the final multivariate model (**Table 3**). For total IgG, low levels of antibodies were consistently observed throughout the follow-up in women who delivered babies with low birth weight in univariate analysis (P = 0.014). This observation was further confirmed in the multivariate model (P = 0.004). However, for the other adverse pregnancy outcomes, no significant difference in the antibody levels of total IgG with specificity for the ID1-ID2a PAMVAC antigen was observed. We then tested in a similar way the antibody levels of the IgG subclasses. For IgG1 and IgG2, no significant differences were observed with the different pathological markers at delivery. Levels of IgG3 antibodies were consistently low in women who delivered low birth weight babies compared to those who delivered babies with normal birth weight (multivariate analysis, P = 0.01). Throughout pregnancy, IgG4 antibody levels were low in women presenting with placental infection at delivery compared to women without parasites in their placenta (multivariate analysis, P = 0.03). However, there was no association between IgG4 and placenta infection in data obtained in the same way using a comparable construct from a ICAM1 binding DBL-β domain from IT4 VAR13 *Pf*EMP1 which is not implicated in PM (β=-0.004; p-value = 0.33). Apart from LBW and placental malaria, we did not observe an association between the other pregnancy outcomes and antibody levels measured.

DISCUSSION

In this study of a cohort of pregnant Beninese, we determined the levels of total IgG and of IgG subclasses with specificity for

TABLE 2 | Relationship between antibody levels to ID1-ID2a construct of VAR2CSA and pregnancy outcomes (Total IgG and IgG1).

Covariates	Total IgG						IgG 1					
	Univariate			Multivariate(**)			Univariate			Multivariate(**)		
	β	95% CI	*p*	Adj β	95% CI	*p*	β	95% CI	*p*	Adj β	95% CI	*p*
Primiparity No	_			_			_					
Yes	-9.07	-14,94; -3,21	**0.002**	-9.9	-15,91; -3,90	**0.001**	-3.29	-12,68; 6,10	0.49			
Malaria Infections(*) 0	_			_			_			_		
1	5.18	3,62; 6,75		4.46	2,83; 6,09		6.16	3,24; 9,7	**$<10^{-3}$**	4.92		**$<10^{3}$**
2	7.73	3,75; 11,70	**$<10^{-3}$**	7.93	3,83; 12,02	**$<10^{-3}$**	12.92	4,50; 21,34		12.37		
≥3	13.56	3,13; 23,99		14.30	3,92; 24,68		7.66	-9,20; 24,52		5.44		
Parasite density	0.0002	0,00008; 0,0002	**$<10^{-3}$**	0.0001	0,000016; 0,00027	**0.028**	0.0004	0,0002; 0,0005	**$<10^{-3}$**	0,00002	-0,0003; 0,0003	0,89
Prematurity No	_			_			_					
Yes	-6.93	-16,93; 3,08	0.17	-4,04	-14,45; 6,38	0,45	0.05	-13,75; 13,84	0.99	-0,84	-18,01; 16;33	0,93
SGA No	_						_			_		
Yes	-4.45	-11,48; 2,58	0.22	-4,04	-12,04; 3,96	0,32	1.47	-9,39; 12,35	0.79	-1,58	-13,65; 10,48	0,80
Placental malaria No	_			_			_			_		
Yes	3.69	-2,77; 10,14	0.26	1,73	-4,70; 8,16	0,59	11.43	-0,88; 23,73	0.07	7,88	-4,65; 20,41	0,22
LBW No	_						_			_		
Yes	-9.04	-16,22; -1,85	**0.014**	-10.74	-18,04; -3,45	**0.004**	-0,59	-11,48; 10,30	0.92	-6,24	-17,33; 4,86	0,27
Maternal anemia No	_						_			_		
Yes	1,74	-3,99; 7,48	0.55	0,44	-5,18; 6,06	0,88	3.31	-5,87; 12,49	0.48	1,65	-7,52; 10,82	0,73

(*) number of documented blood smear positive during follow-up.
(**) Multivariate analysis were adjusted for timepoint.

TABLE 3 | Relationship between antibody levels to ID1-ID2a construct of VAR2CSA and pregnancy outcomes (IgG2, IgG3 and IgG4).

Covariates	IgG 2						IgG 3						IgG 4					
	Univariate			Multivariate(**)			Univariate			Multivariate(**)			Univariate			Multivariate(**)		
	β	95% CI	*p*	Adj β	95% CI	*p*	β	95% CI	*p*	Adj β	95% CI	*p*	β	95% CI	*p*	Adj β	95% CI	*p*
Primiparity	–						–						–					
No																		
Yes	-7.15	-15,52; 1,23	0.09	-7,32	-15,69; 1,05	0,09	2.17	-4,89; 9,23	0.55				-0.08	-6,32; 6,16	0.98			
Malaria Infections(*)	–						–			–			–					
0																		
1	2.21	0,09; 4,32	0.23				8.12	5,47; 10,77	**<10^{-3}**	7.94	5,21; 10,66	**<10^{-3}**	-1.81	-6,57; 2,96	0.7			
2	0.93	-4,78; 6,64					10.08	2,46; 17,69		10.12	2,47; 17,76		-1.54	-15,43; 12,36				
≥3	-0.35	-14,35; 13,64					2.97	-12,26; 18,22		2.93	-12,37; 18,22		-13.56	-41,33; 14,20				
Parasite density	0.0001	-0,0001; 0,0002	0.09	0.0001		**0.03**	0.00004	-0,0001; 0,0002	0,59				-0.0001	-0,0004; 0,0001	0.33			
Prematurity	–			–			–			–			–			–		
No																		
Yes	-2.9	-14,13; 8,33	0.61	-3,49	-15,17; 8,19	0,56	-0.46	-10,75; 9,82	0.92	9,29	-3,39; 21,98	0,15	-1.46	-10,54; 7,62	0.75	-0,26	-11,61; 11,09	0,96
SGA	–						–						–					
No																		
Yes	0.25	-9,10; 9,60	0.96	-1,48	-11,73; 8,77	0,78	1.87	-6,36; 10,09	0.66	6,62	-2,38; 15,61	0,15	-4.39	-11,54; 2,76	0,23	-3,33	-10,56; 3,89	0,36
Placental malaria	–			–			–			–			–					
No																		
Yes	8.99	-1,89; 19,88	0.10	10,09	-1,20; 21,39	0,08	-1.06	-10,17; 8,05	0.82	-2,18	-11,47; 7,11	0,65	-8.86	-16,97; -0,75	**0.03**	-8.86	-16,97; -0,75	**0.03**
LBW	–			–			–			–			–			–		
No																		
Yes	-1.01	-10,40; 8,38	0.83	-0,91	-14,75; 12,94	0,89	-5.58	-13,69; 2,52	0,18	-10.79	-19,15; -2,45	**0.01**	-4,15	-11,32; 3,01	0,26	-2,49	-10,23; 5,23	0,53
Maternal anemia	–			–			–			–			–			–		
No																		
Yes	-2.27	-10,03; 5,50	0.56	-2,04	-10,27; 6,18	0,63	1.04	-5,95; 8,03	0.77	-0,64	-7,64; 6,36	0,86	-0.92	-7,09; 5,25	0.77	-0,91	-7,27; 5,44	0,77

(*) number of documented blood smear positive during follow-up.

(**) Multivariate analysis were adjusted for timepoint.

the PAMVAC vaccine antigen. We focused on the PAMVAC antigen currently undergoing vaccine development to decipher the specific antibody response that women develop when exposed to placental-type parasites. The levels of total IgG with specificity for the PAMVAC antigen were consistently lower in women giving birth to low birth weight babies, unlike other women, regardless of the gestational age at which the plasma sample was taken. These results confirmed our initial observations that low levels of total IgG directed to recombinant DBL1X-Id1-DBL2X of VAR2CSA in early pregnancy were associated with an increased risk of low birth weight (11). The current study emphasizes the fact that this particular association with risk of low birth weight persists throughout pregnancy. Moreover, it should be noted that, as was expected, there were relationships between the antibody levels measured here and the level of exposure to malarial infections or the gravidity status of the women, further supporting the relevance of the observations. Importantly, a recent systematic review by Cutts and his colleagues (12) by analyzing antibody data, most of which was obtained at delivery suggests that IgG levels against VAR2CSA antigen are "likely markers of infection rather than correlates of protection". These observations are in line with those of this work which analyzed antibody levels during pregnancy on a longitudinal angle. This allowed underline the antigenic properties which can differ between different domains or epitopes of an antigen and thus provides additional justification for assessing whether VAR2CSA antibody subclass might better explain the discrepancies between studies.

One noteworthy observation is the association between low levels of cytophilic IgG3 antibody with low birth weight, although IgG1 was the predominant antibody subclass. This finding is similar to that of a study in Senegal (19) and suggests cytophilic IgG3 is the main effector in the prevention of LBW, reflecting the observation with total IgG. From a mechanistic point of view, IgG3 could be acting through either a direct inhibition of infected erythrocyte adhesion to CSA in the placenta, or an ability to mediate opsono-phagocytosis by effector cells, or through sensitization and activation of natural killer cells and the complement system (20–22). As LBW is among the most frequent detrimental outcomes of PM, this is an indication that any vaccine for PM should be demonstrably able to elicit IgG3.

Interestingly, we also observed that higher levels of IgG4 were associated with a reduced risk of placental infection. The IgG4 subclass, unlike IgG3, does not activate complement or act as an opsonin, suggesting that the role of such antibodies in any protective mechanism could only be achieved through the inhibition of infected erythrocyte adhesion to the placental receptor. In helminth infections, antibody isotype patterns are an indicator of the host's immune regulatory status, pointing to the relative level of IgG4 as a corollary of chronic parasite carriage favored by regulatory cytokines. Pathology of chronic helminth infections are characterized by elevated IL-10 and TGFβ that mediate IgG4 production by B cells (23–25). In the context of the STOPPAM study, sub-microscopic parasite carriage revealed by PCR-based diagnosis carried out with samples from this cohort (26) shows that the women who have elevated IgG4 levels are consistently those with low-density (sub-microscopic) parasite carriage at 4 different time points during pregnancy, strongly suggestive of persisting chronic infections. As we have always found, in all our studies, that IL-10 is elevated in the presence of *P. falciparum* infection, whether peripheral (27) or placental (14), we conclude that the conditions therefore likely favored IgG4 production. Further, data obtained using a ICAM-1 binding DBL-β domain protein showed no association with placental infection. This data suggest that our observation with PAMVAC antigen was pregnancy specific and not donor specific. It is also known that the IgG4 titers fall rapidly following curative drug treatment as compared to other subclasses (28). In the context of intermittent preventive treatment administered to pregnant women it is possible that this has contributed to disrupting the association with active infections detected by microscopy in this study. However, the plasma levels of IgG1 and IgG2 subclasses appeared here to represent markers of infection. The observations from this study thus further underline the complex mechanisms underlying antibody responses, the properties of which can both mark the presence of an infection and the success of its control. While this study clearly highlights the importance of the IgG3 subclass in the acquired protection against placental malaria, it also demonstrates for the first time the role that IgG4 could play in this protective mechanism.

One of the limitations of this study is based on the lack of functional data to demonstrate the particular properties of the various purified IgG subclasses. This study opens up on the need for some perspective to confirm the observations. The fact of having used other recombinant antigens reproducing the minimal binding domains of other PfEMP1 (ICAM-1 MBD) and for which we did not observe the same association suggests that the data of this work are of obvious interest. The other limitation is the absence of similar data on the full-length VAR2CSA or other epitopes of the VAR2CSA in this study which would have allowed verify if this association is specific to VAR2CSA in its entirety or just a portion.

In conclusion, the association of IgG3 subclass antibody levels with the risk of low birth weight and that of IgG4 to that of placental infections at delivery, strongly suggest mechanisms of protection mediated by antibody responses against VAR2CSA that rely on different IgG subclasses. This observation further suggests that PM vaccines should ideally target the elicitation of both IgG3 and IgG4 to achieve optimal protection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comité Consultatif de Déontologie et d'Éthique of IRD in France and the Comité d'Éthique de la Faculté des Science de la Santé, Université d'Abomey Calavi, in Benin. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NTN, PD and MA contributed to the design of the STOPPAM project. FV and NF collected the samples. BT and GE carried out all the lab experiments. MN provided us with the PAMVAC antigen. Td'A performed the statistical analysis. BT and NTN wrote the MS. AL reviewed the MS. All authors contributed to the article and approved the submitted version.

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Fetal Cytokine Balance, Erythropoietin and Thalassemia but Not Placental Malaria Contribute to Fetal Anemia Risk in Tanzania

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Fetal anemia is common in malaria-endemic areas and a risk factor for anemia as well as mortality during infancy. Placental malaria (PM) and red cell abnormalities have been proposed as possible etiologies, but the relationship between PM and fetal anemia has varied in earlier studies, and the role of red cell abnormalities has not been studied in malaria-endemic areas. In a Tanzanian birth cohort study designed to elucidate the pathogenesis of severe malaria in young infants, we performed a cross-sectional analysis of risk factors for fetal anemia. We determined PM status, newborn red cell abnormalities, and maternal and cord blood levels of iron regulatory proteins, erythropoietin (EPO), cytokines and cytokine receptors. We examined the relationship between these factors and fetal anemia. Fetal anemia was present in 46.2% of the neonates but was not related to PM. Maternal iron deficiency was common (81.6%), most frequent in multigravidae, and interacted with parity to modify risk of fetal anemia, but it was not directly related to risk. Among offspring of iron-deficient women, the odds of fetal anemia increased with fetal α^+-thalassemia, as well as these patterns of cord blood cytokines: increased cord IL-6, decreased TNF-RI, and decreased sTfR. The EPO response to fetal anemia was low or absent and EPO levels were significantly decreased in newborns with the most severe anemia. This study from an area of high malaria transmission provides evidence that 1) fetal α^+-thalassemia and cytokine balance, but not PM at delivery, are related to fetal anemia; 2) maternal iron deficiency increases the risk that other factors may cause fetal anemia; and 3) fetal anemia has a multifactorial etiology that may require a variety of interventions, although measures that reduce maternal iron deficiency may be generally beneficial.

Keywords: placental malaria, fetal anemia, thalassemia, cytokines, erythropoietin

INTRODUCTION

Fetal anemia can be the result of immune or non-immune insults. The most common immune etiology is maternal Rhesus (Rh) disease. If untreated, fetal anemia may lead to hydrops, multi-organ failure and fetal death (1, 2). In Malawi, fetal anemia at birth increased anemia risk during the first half of infancy, as well as shorter time to first illness, higher cumulative morbidity, and infant mortality (3, 4). In Kenya, anemia during infancy is a risk factor for infant mortality (5).

Fetal anemia, defined as cord blood hemoglobin less than 12.5g/dl, is common in areas where malaria is transmitted (6). Placental malaria (PM) and thalassemia are also common in areas of stable transmission, and have been proposed but not proven to explain the high incidence of fetal anemia in such areas (6). The association of fetal anemia and PM has varied in earlier studies with some showing association (7–9) but others reporting no association (10–12). In an area of high parasite resistance to sulfadoxine-pyrimethamine (SP) in Tanzania, the use of SP for intermittent preventive therapy (IPTp) to prevent PM and the parasite *dhps* c581 SP resistance allele are independently related to decreased levels of cord blood hemoglobin in the newborn (13).

α^+-thalassemia has not been examined as a cause of fetal anemia in areas of malaria transmission (6). Because malaria is rare in African newborns (14), hemolysis of infected red cells cannot explain fetal anemia, but other mechanisms related to malarial anemia could be involved. In older children and adults, malarial anemia has been associated with inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) that can inhibit erythropoiesis (15), cause dyserythropoiesis and promote erythrophagocytosis (16). Levels of the anti-inflammatory cytokine interleukin (IL)-10 (17) and the ratio of IL-10 to TNF-α (18) are significantly lower in African children during severe malarial anemia, and placental levels of TNF-α are significantly higher in African mothers with severe malarial anemia (19).

Iron deficiency is another major cause of anemia, is common among pregnant women living in resource-poor settings of both malarious and non-malarious countries, and is more common in multigravid than primigravid women (20, 21). Neonatal iron stores are influenced by maternal iron storage levels (22) and maternal iron deficiency anemia was found to be a risk factor for fetal anemia in Malawi (7). Transplacental transfer of iron from the mother is the only source of iron to the developing fetus. This is achieved through the formation of an iron-transferrin complex (Fe-Tf), with iron from maternal circulation binding to transferrin receptor 1 (TFR1) on the apical surface of the syncytiotrophoblast (23). This complex is endocytosed, then iron is released into the endosome through ferroportin on the basal side of the syncytiotrophoblast, and finally into fetal circulation (24).

Erythropoietin (EPO) is a major regulator of RBC production in the bone marrow. EPO binds to its receptor on the red blood cell (RBC) progenitor surface, inducing proliferation and terminal differentiation of erythroid precursor cells while also protecting against RBC precursor apoptosis (25). Several studies have reported increases in cord blood EPO concentration in response to fetal hypoxia caused by a variety of conditions including intrauterine growth restriction (26) and maternal smoking (27). Increased levels of EPO in blood or amniotic fluid have also been reported in neonates with severe anemia caused by hemolytic anemia (28) and Rh immunization (29).

In the present study, we searched for potential causes and mediators of fetal anemia in an area of Tanzania with intense malaria transmission. We determined PM status, iron status in mothers and newborns, and inherited red cell abnormalities in newborns. We measured plasma levels of inflammatory and anti-inflammatory cytokines that have been previously implicated in the pathogenesis of anemia (TNF-α and its receptors (soluble TNF-RI and TNF-RII), IFN-γ, IL-6, and IL-10) in cord blood. Levels of sTfR and erythropoietin (EPO) were also measured in these samples. We examined the relationship of all these factors to the risk of fetal anemia.

MATERIALS AND METHODS

Study Cohort and Laboratory Procedures

Mothers and newborns included in this analysis were participating in a birth cohort study known locally as the Mother-Offspring Malaria Studies (MOMS) Project at Muheza Designated District Hospital, Muheza, Tanga-Tanzania. The study protocols were approved by the Division of Microbiology and Infectious Diseases at the U.S. National Institutes of Health and by the institutional review boards of Seattle Biomedical Research Institute (contracted to the Western Institutional Review Board, Puyallup, WA, USA, now WCGH IRB) and the Medical Research Coordinating Committee in Tanzania. Written informed consent was obtained from each child's mother before participation for herself and her newborn. Clinical procedures for the MOMS Project have been previously described (30–38). Hemoglobin was measured using a Cell Dyne 1200 hematology analyzer (Abbot Diagnostics Division, Abbot Park, IL-60064, USA).

Maternal peripheral blood was obtained by venipuncture from women immediately after delivery and anticoagulated with citrate phosphate dextrose. Plasma was obtained by centrifugation at 3,000 *g* for 3 min and stored at -70°C until thawed on the day that assays were performed. Thick and thin smears were prepared; thin smears were fixed with methanol. Blood slides were stained for 10 minutes in 10% Giemsa, washed in tap water, air-dried, then examined using light microscopy at 100 × magnification. Ten thousand red cells were examined in the thin smear before concluding that a placental blood slide was negative.

Levels of ferritin, sTfR, EPO, cytokines and cytokine receptors were analyzed using a multiplexed, bead-based platform (BioPlex®, BioRad, Irvine, CA) and custom-made assay kits as previously described (39, 40). Detection limits for these assays were as follows: ferritin - 0.07 ng/ml, soluble transferrin receptor (sTfR) - 0.03 ng/ml, EPO - 0.1 mIU/ml, TNF-α - 0.1 pg/ml, TNF receptor (R) I - 1.58 pg/ml, TNF-RII - 0.21 pg/ml, IFN-γ - 0.04

pg/ml, IL-6 - 1.45 pg/ml, IL-10 - 0.02 pg/ml. Levels of soluble factors were adjusted to account for dilution in anticoagulant at the time of sample collection. For each plasma sample, all analytes were assayed in a single day, thus eliminating freeze/thaw cycles.

Sickle cell variants (HbAA, HbAS and HbSS) were determined by cellulose acetate paper electrophoresis according to the manufacturer's instructions (Helena Laboratories, Beaumont, Texas, USA). Genotyping for α^+-thalassemia and glucose-6-phosphate dehydrogenase (G6PD) variants was performed by polymerase chain reaction (PCR) techniques. DNA was isolated from blood spots on filter paper according to the manufacturer's instructions (Generation® Capture Card Kit, Gentra Inc.). α^+-thalassemia typing was done according to the protocol described by Chong et al. (41).

To type G6PD variants (G6PD B, G6PD A, and G6PD A-), a region spanning the third to fifth exon was amplified by an outer PCR (Forward 5'- GGT GGA TGA TGT ATG TAG-3' and Reverse 5'- GCA ACG CTG CCA CCT TGT G-3'), followed by nested multiplex PCR. Nested primers to detect the residue 202 polymorphism were G6PD-202 F (5'-CCT TCT GCC CGA AAA CAC CTT CACC-3') and G6PD-202 R (5'- GTC CCC GAA GCT GGC CAT GCT GG -3'); and primers to detect the residue 376 polymorphism were G6PD-376 F (5'- TAC CAG CGC CTC AAC AGC CCC ATG –3') and G6PD-376 R (5'- GGA CTC GTG AAT GTT CTT GGT GAC G-3'). The PCR conditions for the first reaction were as follows: samples were subjected to initial denaturation for 60 seconds at 94°C followed by 30 cycles of 94°C for 60 seconds, 58°C for 45 seconds and 72°C for 60 seconds, followed by 7 minutes at 72°C. The nested multiplex PCR conditions were similar to the above except the annealing temperature was set at 56°C. Mismatches in the primers (at the underlined residues) introduced NcoI digestion sites into the amplified products of the variant alleles, leading to digestion of the G6PD-202 product in G6PD A individuals and digestion of the G6PD-376 product in G6PD A- individuals.

Clinical and Parasitological Definitions

Maternal anemia was defined as maternal venous blood hemoglobin concentration less than 11.0 g/dl. Fetal anemia was defined as cord blood hemoglobin concentration less than 12.5 g/dl (6). Placental malaria status was determined from thick and thin smear of mechanically extracted placental blood, and the presence of placental parasites, inflammation and pigment in PM+ women was characterized by histology as previously described (42). Iron deficiency was defined as ferritin concentration <30 ng/ml when C-reactive protein (CRP) was ≤8.2 μg/ml (iron deficiency in the absence of inflammation), or ferritin concentration <70 ng/ml when CRP was >8.2 μg/ml (iron deficiency in the presence of inflammation) (42).

Statistical Analysis

Analyses were performed using Statview 5.0.1 (SAS Institute, Cary, NC, USA). Differences between proportions were compared by χ^2 test. Normally distributed continuous data were compared by the Student's *t* test and analysis of variance. Data that did not conform to a normal distribution were compared by Mann-Whitney or Kruskal-Wallis tests. Simple and multiple logistic regression models were used to test for associations of maternal and fetal factors to fetal anemia. Two-sided $p \leq 0.05$ was considered to be statistically significant.

In a stepwise approach (see **Supplementary Figure S1**), we searched for maternal and fetal factors related to fetal anemia. We first used a Chi-square test for categorical variables and a Student's t-test for continuous variables to examine their direct relationship to fetal anemia. Because parity was strongly related to fetal anemia risk in that analysis, and is known to impact numerous pregnancy outcomes in malarious areas, we used ANOVA to examine the interactions of parity with other baseline maternal and fetal factors for their relationships to cord hemoglobin level. Secondly, we employed a simple logistic regression analysis to identify fetal soluble factors (iron regulatory proteins, erythropoietin, cytokines and cytokine receptors) related to fetal anemia. We then examined maternal and fetal factors that were seen to be significant in univariate analysis, for their independent association with fetal anemia in multiple logistic regression analysis. Finally, because the interaction term parity*maternal iron deficiency was related to cord hemoglobin level, we stratified the multivariate logistic regression analysis by maternal iron deficiency, and then by parity groups among mothers with iron deficiency.

RESULTS

Fetal Anemia Is Related to Parity but Not to Maternal Anemia

We determined the hemoglobin level in 610 cord blood and 658 maternal blood samples (90 with PM) obtained at delivery. The overall prevalence of fetal anemia defined as cord hemoglobin below 12.5g/dL (6) was 46.2% and increased with successive pregnancies. 95.7% of deliveries were spontaneous vaginal deliveries without any recorded complications of labor. 26/610 (4.3%) were delivered *via* C-section, and 8/26 (30.8%) C-sections had fetal anemia. Mean (SD) cord hemoglobin (g/dL) was 12.5 (2.7), 12.6 (2.3) and 12.1 (3.0) in offspring of primigravidae, secundigravidae and multigravidae, respectively. Fetal anemia was present in 38.9%, 41.3% and 63.3% of first, second and later offspring, respectively (χ^2=9.4, p = 0.008). Cord hemoglobin tended to be inversely correlated with increasing parity although this relationship was not statistically significant (r = - 0.071; p = 0.07).

Most women (356/658 (57.1%)) were anemic at delivery (hemoglobin < 11 g/dL), and this did not differ by parity (54.6%, 56.5% and 58.9% (χ^2 = 0.93, p = 0.62) in primigravidae, secundigravidae and multigravidae respectively). The proportion of infants with fetal anemia did not differ significantly among offspring of mothers with anemia versus offspring of mothers without anemia (50.6% versus 43.6%; χ^2 = 2.7, p = 0.1). This pattern was similar in the different parity groups (primigravid, 46.2% versus 36.6%, χ^2 = 1.4, p = 0.2; secundigravid, 44.7% versus 41.3%, χ^2 = 0.14, p = 0.7; multigravid, 55.6% versus 49.1, χ^2 = 1.1, p = 0.3). When

treated as a continuous variable, maternal and fetal hemoglobin were related, but the correlation was weak (Linear correlation coefficient = 0.15; p = 0.04)

Fetal Anemia Is Not Related to Placental Malaria (PM) at Delivery

Fetal anemia was present in 240/520 (46.1%) offspring of PM- versus 49/90 (46.4%) offspring of PM+ mothers (p = 0.9). Although fetal anemia was more frequent in the PM+ than the PM- group in each parity category, none of these differences were statistically significant, using cord hemoglobin of either 12.5 g/dL (**Supplementary Table S1**) or of 10.0 g/dL (**Supplementary Table S2**) to stratify the population. Cord hemoglobin levels did not differ significantly between offspring of PM+ (mean (SD) 12.0 (2.9) g/dL) versus PM- women (mean (SD) 12.3 (2.7) g/dL). Mean (SD) cord hemoglobin levels were 12.3 (2.8) g/dL and 12.2 (2.8) g/dL in offspring of PM+ mothers with inflammation and PM+ mothers without inflammation respectively. The proportions of newborns with fetal anemia did not differ significantly between PM- (46.1%), PM+ without placental inflammation (46.4%), and PM+ with placental inflammation (57.6%), defined histologically as presence of intervillous inflammatory cells (χ^2 = 1.3, p = 0.51). The proportion with fetal anemia did not differ between offspring of mothers with active, past or no placental malaria by histology (p = 0.8).

TABLE 1 | Maternal and newborn characteristics in relation to fetal anemia*.

Characteristic	Fetal Anemia	No Fetal Anemia	P value
	MATERNAL		
Age (Years), Mean (SD)	26.46 (6.3)	25.25 (6.0)	**0.02**
Iron deficiency (n = 461)	211 (45.8%)	250 (54.2%)	0.8
Anemia (n = 314)	159 (50.6%)	155 (49.4%)	0.1
Parity			
Primigravidae (n = 167)	65 (38.9%)	102 (61.1%)	**0.009**
Secundigravidae (n = 146)	61 (41.8%)	85 (58.2%)	
Multigravidae (n = 297)	156 (52.5%)	141 (47.5%)	
PM (Histology) (n=610)			
No infection (n = 498)	227 (45.6%)	271 (54.4%)	0.8
Active infection (n = 78)	38 (48.7%)	40 (51.3%)	
Past infection (n = 34)	17 (50.0%)	17 (50.0%)	
PM (Microscopy)			
PM+ (n = 90)	42/90 (46.7%)	48/90 (53.3%)	0.9
PM– (n = 520)	240/520 (46.2%)	280/520 (53.8%)	
	NEWBORN		
Birth Weight (g), Mean (SD)	3162 (0.41)	3203 (0.43)	0.3
Female/Male (n)	157/171	142/139	0.6
Iron deficiency (n =73)	33 (45.2%)	40 (54.8%)	1.0
Sickle hemoglobin			
AA (n = 488)	232 (84.0%)	256 (80%)	0.4
AS (n = 103)	42 (15.3%)	61 (19.1%)	
SS (n = 5)	2 (0.7%)	3 (0.9%)	
G6PD status			
A (n = 72)	35 (12.65%)	37 (11.7%)	0.2
A- (n = 72)	27 (9.7%)	45 (14.2%)	
A Heterozygous (n =52)	24 (8.6%)	15 (4.7%)	
A- Heterozygous (n = 39)	22 (8.0%)	30 (9.4%)	
B (n = 361)	170 (61.1%)	191 (60.0%)	
Thalassemia			
αα/ αα (n = 247)	104 (44.9%)	143 (54.2%)	**0.0005**
−α/ αα (n = 199)	92 (39.6%)	107 (40.5%)	
−α/ −α (n = 50)	36 (15.5%)	14 (5.3%)	

All variables were analyzed by Chi-square t-test except for maternal age and birth weight (unpaired t-test).
Bold values represent significant differences.

Maternal Iron Status and Parity Interact to Modify Risk of Fetal Anemia

Parity was significantly related to fetal anemia (**Table 1**) and is well-known to impact numerous maternal and fetal outcomes in malarious areas, including its strong effect on placental malaria risk as women acquire protective immunity over successive pregnancies (43). We therefore examined whether the interaction of parity with other maternal and fetal baseline factors was related to cord hemoglobin levels, as an indication whether these interactions might be confounding analyses of fetal anemia risk. Among all interactions examined (**Supplementary Table S3**), only the interaction of parity with maternal iron status was significantly related to cord blood hemoglobin levels (factorial ANOVA, p = 0.03), suggesting that fetal anemia risk factors should be examined after stratification by maternal iron status and parity.

Among mothers with available measurements, 81.6% (618/757) were iron-deficient, and iron deficiency was less common in primigravid (76.6%) than in secundigravid (85.7%) or multigravid women (82.7%; χ^2 = 5.9, p = 0.05). By comparison, only 12.2% (93/726) of newborns had iron deficiency and this did not differ significantly by parity (12.2% in first, 10.2% in second, and 14.3% in third or later offspring; χ^2 = 1.6, P = 0.4). The frequency of maternal or fetal iron deficiency did not differ between newborns with or without fetal anemia, whether defined as hemoglobin below 12.5 g/dL (**Table 1**) or below 10 g/dL (**Supplementary Tables S4** and **S5**).

When mothers were iron-deficient, cord hemoglobin levels decreased (**Figure 1A**, p = 0.01) and the prevalence of fetal anemia increased over successive pregnancies (35.0%, 44.6% and 52.0% of first, second and later offspring, respectively; χ^2 = 8.9, p = 0.001). The opposite pattern was observed when mothers had adequate iron stores: cord hemoglobin levels increased (**Figure 1B**, p = 0.15) and the prevalence of fetal anemia decreased with increasing parity, although this was not statistically significant (51.1%, 31.6% and 42.8% of first, second and later offspring, respectively; χ^2 = 2.1, p = 0.34).

Thalassemia but No Other Red Cell Disorder Is Associated With Fetal Anemia

Samples from 496 newborns were genotyped for α^+-thalassemia, 596 for G6PD deficiency and 596 for sickle cell hemoglobin phenotype. The frequency of neonatal genotypes/phenotypes did not vary by PM or by parity (**Supplementary Table S6**).

α^+-thalassemia but not G6PD deficiency nor sickle hemoglobin increased fetal anemia risk. Overall, thalassemia homozygous (−α/ −α) offspring had the lowest hemoglobin levels (mean (SD) g/dL of 12.5 (2.9), 12.4 (2.7) and 10.9 (2.1) in normal, heterozygous or homozygous offspring; Kruskal Wallis test, p < 0.0001) and the highest frequency of fetal anemia (**Table 1**) compared to heterozygous (−α/ αα) or normal newborns.

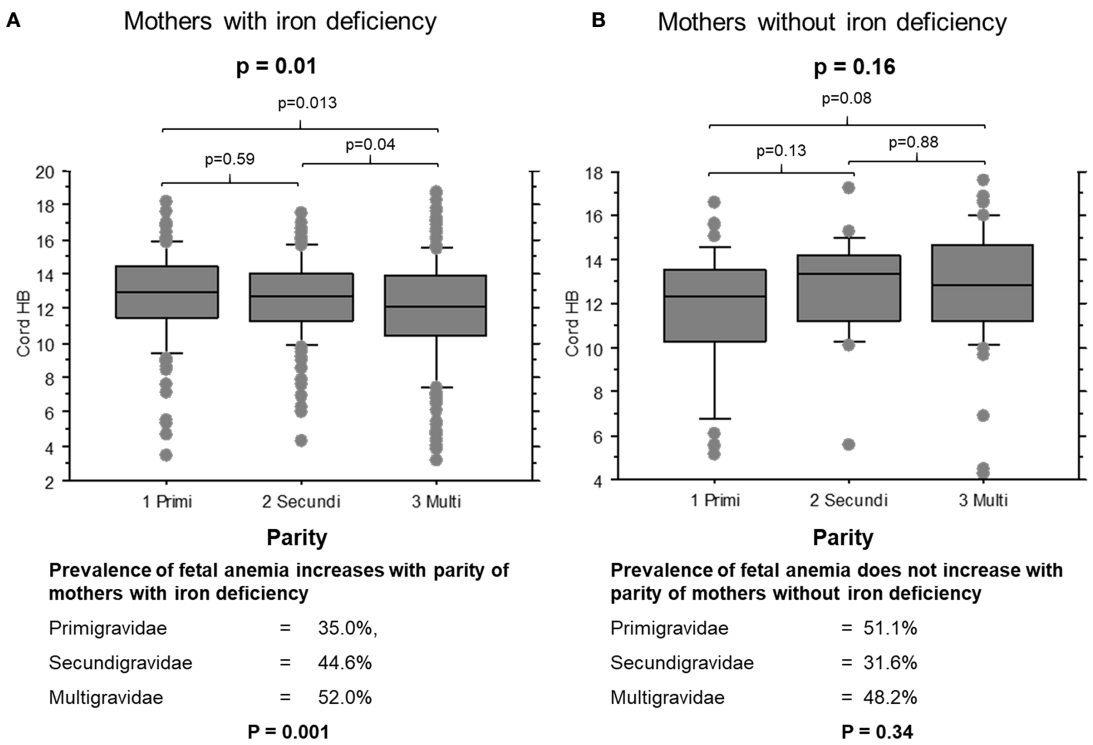

FIGURE 1 | Cord blood hemoglobin levels in offspring of mothers with and without iron deficiency, stratified by parity. **(A)** Cord samples from mothers with iron deficiency: 1, primigravidae (n = 53); 2, secundigravidae (n = 72); 3, multigravidae (n = 132). **(B)** Cord samples from mothers with normal iron status: 1, primigravidae (n = 29); 2, secundigravidae (n = 14); 3, multigravidae (n = 31). The box plots indicate the median (horizontal line) and interquartile range (box), and the whiskers represent 10^{th}/90^{th} percentiles. The differences between groups were analyzed by the Kruskal-Wallis test. Associated P values are shown.

Thalassemia significantly decreased fetal hemoglobin levels in offspring of multigravidae but not in offspring of primigravidae or secundigravidae. Mean (SD) cord hemoglobin (g/dL) was 12.5 (3.0), 12.4 (2.8) or 10.4 (1.8) in normal, heterozygous or homozygous offspring, respectively, of multigravidae (Kruskal-Wallis test, p = 0.0002), versus 12.4 (2.7), 12.4 (3.0) or 12.4 (2.7) in those of primigravidae (Kruskal-Wallis test, p = 0.86) and 12.7 (2.7), 12.8 (2.0) or 11.4 (2.0) in those of secundigravidae (Kruskal-Wallis test, p = 0.06). Thalassemia increased the frequency of fetal anemia in offspring of multigravidae (χ^2 = 5.64, p = 0.02) but not primigravidae (χ^2 = 0.001, p = 0.9) or secundigravidae (χ^2 = 0.56, p = 0.45).

Cytokine Balance, sTfR and Fetal Thalassemia Are Associated With Fetal Anemia Risk in Univariate and Multivariate Analyses

We performed a simple logistic regression analysis of cord soluble factors and risk of fetal anemia. Higher levels of TNFRII and IL-6 increased the risk of fetal anemia, whereas higher levels of TNF-RI and sTfR decreased the risk (**Table 2**). In an un-stratified multiple logistic regression analysis of these significant factors, −a/−a thalassemia and higher levels of IL-6 and TNF-RII independently increased, whereas higher levels of TNF-RI and sTfR decreased, the risk of fetal anemia (**Table 3**).

TABLE 2 | Simple logistic regression analysis of soluble fetal factors in relation to fetal anemia.

FETAL FACTOR	OR	95% CI	P value
TNF-α (n = 576)	0.80	(0.49 - 1.21)	0.3
TNF-RI (n = 583)	0.46	(0.23 - 0.90)	**0.02**
TNF-RII (n = 583)	2.19	(1.24 - 3.90)	**0.007**
IFN-γ (n = 121)	1.71	(0.82 - 3.56)	0.2
IL-10 (n = 523)	1.08	(0.63 - 1.85)	0.8
IL-6 (n = 476)	1.62	(1.21 - 2.17)	**0.001**
sTfR (n = 583)	0.30	(0.14 - 0.62)	**0.001**
EPO (n = 402)	1.40	(0.78 - 2.52)	0.3

Bold values represent significant differences.

TABLE 3 | Multiple logistic regression analysis of factors in relation to fetal anemia risk (n = 358).

FACTOR	aOR*	95% CI	P value
Secundigravidae	0.41	0.12-1.47	0.2
Multigravidae	0.27	0.02-2.72	0.3
Maternal iron deficiency	0.29	0.07-1.26	0.1
Maternal iron deficiency * Parity	1.63	0.89-3.08	0.1
−α/ αα thalassemia	1.34	0.84-2.15	0.2
−α/ −α thalassemia	4.30	1.78-10.34	**0.001**
TNF-RI	0.26	0.09-0.70	**0.008**
TNF-RII	2.31	1.08-4.94	**0.03**
sTfR	0.24	0.07-0.63	**0.005**
IL-6	2.05	1.38-3.02	**0.0003**

**Adjusted Odds Ratio.*
Bold values represent significant differences.

TABLE 4 | Multivariate logistic regression analysis of risk factors for fetal anemia in offspring of mothers with OR without iron deficiency.

FACTOR	IRON DEFICIENCY (n = 287)			NO IRON DEFICIENCY (n = 71)		
	aOR*	95% CI	P value	aOR	95% CI	P value
Secundigravidae	1.54	0.72-3.28	0.3	0.13	0.03-0.69	**0.02**
Multigravidae	2.12	1.13-4.00	**0.02**	0.57	0.18-1.82	0.3
−α/ αα	1.68	0.97-2.91	0.1	0.58	0.20-1.69	0.3
−α/ −α	7.02	2.62-18.84	**0.0001**	0.22	0.01-4.24	0.3
TNF-RI	0.19	0.06-0.59	**0.005**	1.10	0.10-10.32	1.0
TNF-RII	2.07	0.90-4.79	0.1	5.64	0.55-57.70	0.1
sTfR	0.12	0.03-0.43	**0.001**	1.68	0.15-18.30	0.7
IL-6	2.60	1.60-4.21	**0.0001**	1.47	0.70-3.12	0.3

**Adjusted Odds Ratio.*
Bold values represent significant differences.

TABLE 5 | Multivariate logistic regression analysis of risk factors for fetal anemia in offspring of mothers with iron deficiency in different parity groups.

FACTOR	PRIMIGRAVIDAE (n = 75)			SECUNDIGRAVIDAE (n = 66)			MULTIGRAVIDAE (n = 146)		
	aOR*	95% CI	P value	aOR	95% CI	P value	aOR	95% CI	P value
−α/ αα	1.68	0.50-5.69	0.4	2.21	0.57-8.59	0.3	1.79	0.85-3.75	0.1
−α/ −α	29.92	1.88-475.28	**0.02**	7.92	1.14-55.17	**0.04**	7.87	1.87-33.10	**0.005**
TNF-RI	0.02	0.001-0.33	**0.006**	0.18	0.01-2.49	0.2	0.23	0.05-1.20	0.1
TNF-RII	3.19	0.37-27.84	0.3	0.96	0.15-6.25	1.0	1.83	0.62-5.40	0.3
sTfR	0.006	0.0001-0.24	**0.007**	0.003	0.0004-0.19	**0.006**	0.40	0.08-1.96	0.3
IL-6	1.92	0.68-5.45	0.2	2.50	0.84-7.41	0.1	2.67	1.33-5.47	**0.006**

**Adjusted Odds Ratio.*
Bold values represent significant differences.

Since maternal iron deficiency interacted with parity to modify the levels of cord blood hemoglobin, we stratified the multiple regression analysis by maternal iron status (**Table 4**). All relationships between newborn risk factors and fetal anemia occurred in the maternal iron deficiency group and were absent in the group with normal maternal iron status.

We therefore conducted analyses after stratifying only the maternal iron deficiency group by parity (**Table 5**). Among all parity groups, fetal anemia risk was higher in newborns with thalassemia, higher cord levels of IL-6, and lower cord levels of TNF-RI and sTfR (**Table 5**). Other than −α/ −α thalassemia, these relationships achieved statistical significance in only some parity groups: increased cord TNF-RI and sTfR significantly reduced fetal anemia risk in first pregnancies, increased cord sTfR significantly reduced fetal anemia risk in second pregnancies, and increased cord IL-6 significantly increased fetal anemia risk in third pregnancies. Of note, reduced sample sizes may limit the power of these subgroup analyses to identify significant relationships.

Erythropoietin Levels Are Decreased During Severe Fetal Anemia

Cord EPO levels did not differ significantly between anemic (median (interquartile range), 70.8 mIU/mL (35.2 - 85.3)) and non-anemic newborns (62.3 mIU/mL (37.5 – 95.9); p = 0.1, Mann-Whitney test) indicating that EPO is not responding

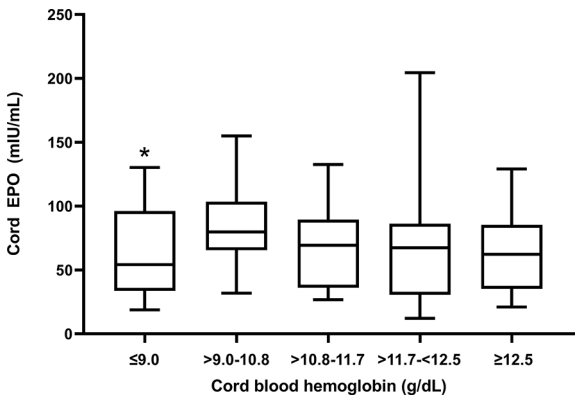

FIGURE 2 | Cord blood EPO levels in relation to degree of fetal anemia. Q1 (Hb ≤ 9 mg/dl); Q2 (Hb > 9 -10.8 mg/dl); Q3 (Hb >10.8-11.7 mg/dl); Q4 (Hb > 11.7-<12.5 mg/dl; and Q5, no fetal anemia (≥12.5). The box plots indicate the median (horizontal line) and interquartile range (box), and the whiskers represent 10^{th}/90^{th} percentiles. The differences between groups were analyzed by the Kruskal-Wallis test; *Q1 was significantly lower than the reference group Q5 (p = 0.03).

appropriately to declining hemoglobin levels. We stratified anemic newborns in quartiles by fetal hemoglobin levels and compared them to normal newborns for their EPO levels (**Figure 2**). Newborns with fetal anemia showed a non-significant trend to increase EPO as cord hemoglobin decreased, except for newborns in the lowest Hb quartile (Hb ≤ 9.0 g/dl) in whom EPO was significantly lower versus normal children.

DISCUSSION

Fetal anemia predisposes to anemia in infancy (4) and is a risk factor for infant mortality (5). Fetal anemia is more frequent in malarious areas (6) but the cause is unclear, so we sought to identify maternal and fetal factors that increase the risk of fetal anemia in Tanzania. We confirmed that fetal anemia is common in an area of intense malaria transmission, but that it is not related to PM at the time of delivery. Instead, inappropriately low EPO levels were associated with the most severe fetal anemia in this population. Further, α^+-thalassemia, sTfR and the cytokine balance (involving TNF-RI, TNF-RII, and IL-6) of the newborn were related to fetal anemia, but these relationships were limited to offspring of iron-deficient mothers in whom all but thalassemia were modified by parity.

We found a high proportion (46.2%) of infants with fetal anemia in our population. The mean cord hemoglobin level of 12.3 g/dl in the present study is lower than levels reported in developing countries without malaria but is comparable to levels from other malarious countries (6). However, placental malaria at delivery was not related to fetal anemia in this study, suggesting that active malaria is not a direct cause. Contradictory findings on the association between malaria in pregnancy and fetal anemia have been reported with some studies showing no association (7–9) but others reporting an association (10–12). Our study suggests that malaria may increase fetal anemia risk through indirect means, such as by causing maternal iron deficiency (44) or by its evolutionary selection of thalassemia (10–12).

Iron is an essential micronutrient for erythropoiesis. The fetus obtains iron from the maternal circulation, and iron deficiency in the mother reduces iron stores in the newborns (45). Recently, animal and human studies have indicated that in the setting of maternal iron deficiency, regulation of placental iron uptake and export to the fetus favors preservation of placental iron sufficiency rather than fetal iron content in order to maintain global function of the healthy placenta (46). We expected that iron deficiency would increase the risk of fetal anemia. However, cord hemoglobin levels did not differ between iron-deficient mothers and mothers with normal iron stores.

Instead, the interaction of maternal iron status and parity was related to cord hemoglobin levels (**Supplementary Table S3**). Fetal anemia prevalence increased significantly with parity when mothers were iron-deficient but tended to decrease when mothers had normal iron stores (**Figure 1**). Maternal iron status modified the relationships of several other factors to fetal anemia. For example, increased levels of cord IL-6 increased the odds and cord TNF-RI decreased the odds of fetal anemia, but only when the mother was iron-deficient. This suggests roles for fetal cytokine balance in the pathogenesis of fetal anemia only during maternal iron deficiency, which suggests that distinct mechanisms converge in the fetus to cause anemia.

Fetal immune activation has been proposed as a mechanism by which placental malaria itself might increase the risk of fetal anemia (6), albeit this and earlier studies (10–12) do not find a direct relationship of placental malaria to fetal anemia. Notably, inflammatory cytokines do not increase in fetal blood during placental malaria (47) and this may explain in part why active placental malaria does not influence the immediate risk of fetal anemia. We cannot rule out that infections prior to delivery may contribute to fetal anemia, as mothers were enrolled in this study at the time of delivery. Future studies should explore whether malaria episodes occurring earlier during pregnancy contribute to the risk of fetal anemia.

The mechanism through which the fetal cytokine milieu may be mediating anemia requires further study. IL-6 is known to induce hypoferremia of infection and inflammation by stimulating the synthesis of hepcidin (48). Hepcidin limits the iron that is available for erythropoiesis by inhibiting its efflux through ferroportin, an iron exporter that is expressed in the small intestine, hepatocytes, macrophages, and placental trophoblast cells (23). Studies to examine IL-6 and hepcidin for their interrelationships to fetal anemia are warranted. Elevated fetal IL-6 was reported in fetal anemia due to maternal Rh alloimmunisation, and presumed to be a marker of fetal systemic inflammatory response syndrome (SIRS) secondary to red blood cell destruction in the reticuloendothelial system (49, 50). Conversely, TNF-RI protects against fetal anemia and might do so by binding and neutralizing TNF-α, an inflammatory cytokine that has been associated with anemia. For this reason, free TNF-α levels in cord blood should be examined in future studies to see whether they have a stronger relationship with fetal anemia than total TNF-α levels.

Maternal iron deficiency and parity also modified the effect of α^+-thalassemia to cause fetal anemia (**Table 4**). α^+-thalassemia is the most common hemoglobinopathy of humans and leads to mild hypochromic microcytic anemia. The prevalence of fetal thalassemia in this population (10.1% $-\alpha/-\alpha$ and 40.1% $-\alpha/\alpha\alpha$) is comparable to that found in other malarious areas of Africa (49). Thalassaemia has been related to hematological status of the fetus in a non-malarious area. In New Zealand, cord levels of hemoglobin, mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) were significantly lower in heterozygous and homozygous thalassemic newborns compared to normal newborns (51, 52).

In the present study, decreased sTfR levels were associated with increased odds of fetal anemia. Erythropoietic activity has been found to be the most important determinant of sTfR levels (51, 53). In conditions such as α-thalassemia which are characterized by stimulated erythropoiesis, sTfR levels are increased; elevated levels of sTfR in older children with thalassemia were previously reported from Vanuatu where malaria is endemic (54). Elevated sTfR may reflect stimulated erythropoiesis in the fetus with thalassemia and anemia.

EPO levels were not significantly increased in newborns with fetal anemia, and this inappropriately blunted response may have contributed to anemia in our study population. EPO is the primary growth factor regulating red blood cell production, and increased cord blood EPO levels are a marker of chronic fetal hypoxia and intrauterine fetal growth retardation (55), as well as Rh isoimmunization (28). EPO does not cross the placenta (56) and therefore must emanate from fetal sources, such as kidneys, liver, and placenta which are known to express the EPO gene (57). We speculate that the degree of fetal anemia observed in Tanzanian newborns may not cause sufficient hypoxia to induce EPO production. Rodent studies suggest that the EPO response to hypoxia is markedly reduced in newborn compared to adult rats (58), possibly because the high oxygen-affinity fetal hemoglobin mitigates tissue hypoxia due to anemia. Alternatively, TNF-α and IL-1 have been shown to inhibit EPO production (59) and therefore the inflammatory environment could suppress EPO production by the fetus during anemia. Indeed, EPO levels were significantly decreased in newborns with the most severe anemia (**Figure 2**), suggesting that a failed EPO response might actively contribute to fetal anemia.

In summary, this study has shown that fetal α-thalassemia, cytokine balance, and sTfR levels, but not PM, are related to fetal anemia risk in an area of high malaria transmission. The relationship of these factors to fetal anemia was only seen when mothers were iron-deficient, suggesting that measures to prevent maternal iron deficiency may reduce fetal anemia risk, albeit maternal iron status was not directly related to fetal anemia. The opposing effects of inflammatory (IL-6) and anti-inflammatory (soluble TNF-RI) factors suggest that conditions that alter fetal cytokine balance may be involved in the pathogenesis of fetal anemia. Future studies are needed to elucidate the causes of altered fetal cytokine balance and their roles in the pathogenesis of fetal anemia. Inadequate EPO may also be a contributor to the most severe cases of anemia in Tanzanian newborns.

DATA AVAILABILITY STATEMENT

All relevant data are included in the manuscript and are available from the authors upon reasonable request and execution of inter-institutional agreements for sharing of human data.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Division of Microbiology and Infectious Diseases at the U.S. National Institutes of Health and by the institutional review boards of Seattle BioMed and the Medical Research Coordinating Committee in Tanzania. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PD and MF designed the MOMS Project. JK and EK completed the cytokine assays. GM determined variants of red cell abnormalities. AM performed histological studies. EK and PD analyzed the data and wrote the manuscript with the contribution of all other authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.624136/full#supplementary-material

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High Antibodies to VAR2CSA in Response to Malaria Infection Are Associated With Improved Birthweight in a Longitudinal Study of Pregnant Women

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Introduction: Pregnant women have an increased risk of *P. falciparum* infection, which is associated with low birth weight and preterm delivery. VAR2CSA, a variant surface antigen expressed on the parasitized erythrocyte surface, enables sequestration in the placenta. Few studies have prospectively examined relationships between antibody responses during pregnancy and subsequent adverse birth outcomes, and there are limited data outside Africa.

Methods: Levels of IgG against VAR2CSA domains (DBL3; DBL5) and a VAR2CSA-expressing placental-binding *P. falciparum* isolate (PfCS2-IE) were measured in 301 women enrolled at their first visit to antenatal care which occurred mid-pregnancy (median = 26 weeks, lower and upper quartiles = 22, 28). Associations between antibody levels at enrolment and placental infection, birthweight and estimated gestational age at delivery were assessed by linear and logistic regression with adjustment for confounders. For all outcomes, effect modification by gravidity and peripheral blood *P. falciparum* infection at enrolment was assessed.

Results: Among women who had acquired *P. falciparum* infection at enrolment, those with higher levels of VAR2CSA antibodies (75th percentile) had infants with higher mean birthweight (estimates varied from +35g to +149g depending on antibody response) and reduced adjusted odds of placental infection (aOR estimates varied from 0.17 to 0.80), relative to women with lower levels (25th percentile) of VAR2CSA antibodies. However,

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among women who had not acquired an infection at enrolment, higher VAR2CSA antibodies were associated with increased odds of placental infection (aOR estimates varied from 1.10 to 2.24).

Conclusions: When infected by mid-pregnancy, a better immune response to VAR2CSA-expressing parasites may contribute to protecting against adverse pregnancy outcomes.

Keywords: VAR2CSA antibodies, birthweight, placental infection, Papua New Guinea, malaria in pregnancy (MiP), *Plasmodium falciparum*

INTRODUCTION

Plasmodium falciparum infections during pregnancy are associated with low birth weight and preterm delivery (1). Pregnant women are at an increased risk of detected *P. falciparum* infection relative to non-pregnant women and are at greatest risk during their first pregnancy (2). The variant surface antigen VAR2CSA, expressed on the infected erythrocyte (IE) surface (3–5), mediates adhesion to chondroitin sulfate A (CSA) (6) and thus enables sequestration of IEs in the placenta (7). VAR2CSA is a specific variant of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). Primigravid women and men in malaria endemic settings generally have low levels of antibodies specific for VAR2CSA but levels increase among women with increasing gravidity (8–12). It has been suggested that the reduced risk of malaria infection in multigravid women relative to primigravid women can be partly explained by the acquisition of naturally acquired antibodies to VAR2CSA with each pregnancy, providing protection against placental *P. falciparum* infection and its adverse consequences (11). On this basis, efforts are underway to design a vaccine to induce immunity against VAR2CSA to protect against placental infection (13, 14). However, there is very limited evidence from population studies to indicate that VAR2CSA antibodies are protective against adverse maternal and birth outcomes (15), especially in Asia-Pacific where a large population is at risk of malaria.

Most cross-sectional studies investigating antibodies in women at delivery have not found significant protective associations between levels of VAR2CSA antibodies and birthweight (15–18) or gestational age at delivery (15, 16) and a limited number of studies have noted protective associations in subsets of women according to clinical outcome or gravidity (3, 19–22). As VAR2CSA antibodies arise in response to placental infection but are also putatively protective against placental infection and its adverse outcomes, cross-sectional studies are limited in their ability to determine the protective effect of VAR2CSA immune responses.

The majority of longitudinal studies have been undertaken in Africa and have not observed significant associations between VAR2CSA antibodies measured mid-pregnancy and birthweight (23–26) or gestational age at delivery (26, 27). However, the presence of malaria infection can confound these associations as they are associated with both VAR2CSA antibodies and birth outcomes. Indeed, one African study demonstrated a positive association between VAR2CSA antibodies and birthweight, but only in women who had peripheral blood *P. falciparum* infection at enrolment; no association was observed in *P. falciparum* negative women (27). The probability of malaria infection is heterogeneous across individuals and in populations of varying malaria transmission. Investigating the potential modification of *P. falciparum* infection on associations between VAR2CSA and birth outcomes is needed. In addition, investigations in other regions that experience a high burden of malaria in pregnancy but may differ from Africa with respect to parasite and host genetics and human behaviour, such as the Asia-Pacific (28), are required.

In this study, we investigated whether antibodies measured at the first visit to ANC were associated with protection against adverse pregnancy outcomes in a longitudinal study in a malaria-endemic region of Papua New Guinea. We evaluated antibodies to VAR2CSA expressed on the surface of IEs and to specific domains expressed as recombinant proteins. We hypothesized that women who have higher magnitude antibody responses when infected during pregnancy will have better pregnancy outcomes than those with lower responses. Because of the heterogeneity of exposure to malaria in populations, we investigated how infection status at enrolment in mid-pregnancy influenced protective associations.

MATERIALS AND METHODS

Study Population and Sample Collection

The study was carried out in the malaria endemic province of Madang in Papua New Guinea (PNG) as described in detail previously (29). Between September 2005 and October 2007, 470 pregnant women >16 years of age attending their first antenatal care visit at Alexishafen Health Centre were recruited following written informed voluntary consent. Women were followed up at 30-34 weeks gestation, at delivery and 6-8 weeks postpartum. Gestational age was estimated from fundal height measurements. At enrolment, women received chloroquine (9 or 12 tablets, 150 mg base) and (when available) sulphadoxine pyrimethamine (500/25 mg, three tablets), followed by weekly chloroquine prophylaxis (two 150 mg tablets weekly), and ferrous sulphate 270 mg and folic acid 0.3 mg daily, according to local policy. Prophylaxis was not monitored. Inclusion criteria included no

history of multiple births (e.g. past delivery of twins) and delivery complications, intention to deliver at the Alexishafen Health Centre, haemoglobin (Hb) >5g/dl and evidence of foetal movement. This study included 301 women who had delivery data and peripheral blood samples available at the enrolment visit for antibody analysis. Peripheral blood samples were collected at each visit and plasma and serum samples were separated and frozen. Placental blood and placental biopsy samples were collected at delivery if the delivery occurred at the clinic (n = 233). At each visit peripheral parasitemia was determined by microscopy on thick and thin blood films and *Plasmodium spp* confirmed by PCR. The presence of placental infection was determined by placental histology of fixed Giemsa-stained placental biopsies by light microscopy and samples were classified as no infection (no malaria parasites present) or active infection (presence of malaria parasites) as previously described (30, 31). Data on human genetic polymorphisms of South-East Asian Ovalocytosis (SAO), Complement Receptor 1 (CR1) and α+thalassaemia were available as described (29). Samples from malaria-naive residents of Melbourne, Australia were used as malaria naive controls in all assays. Male Madang samples were collected between 2001 and 2002 from Modilon Hospital, the Madang town clinic, and the Yagaum immunization service (32).

This study received ethical approval from the PNG Medical Research Advisory Council, the Melbourne Health Human Research Ethics Committee and Alfred Health Human Research Ethics Committee.

Parasite Culture and Selection

The CS2 *P. falciparum* parasite line that binds to CSA and predominantly express the *var2csa* transcript (33–35) was used in this study. *P. falciparum* CS2 parasites were cultured in O+ human red blood cells (RBCs) in RPMI-HEPES culture medium supplemented with 5% pooled non-exposed human serum and 0.1% Albumax (32). Parasites were routinely selected for knob expression by gelatin floatation (36).

Measurement of Antibody Levels to Recombinant Proteins

Antibody levels were determined for 3 VAR2CSA recombinant proteins representing 2 allelic variants; DBL5 (3D7), DBL5 (7G8) and DBL3 (7G8) by standard ELISA assays (37). All recombinant proteins were cloned and produced in *Pichia pastoris* (38). DBL5 and DBL3 recombinant proteins were selected because compared to other VAR2CSA domains they are highly immunogenic in natural infections and elicit some degree of cross-reactive and adhesion-blocking antibodies (25, 39–43), and also promote opsonic phagocytosis by monocytes (44). Antibodies to apical membrane antigen 1 were assessed as a broad measure of blood-stage immunity (45). Recombinant proteins were coated onto plates overnight at concentrations of 0.5 μg/ml followed by 2 hour incubation with sera samples (1/500) and 1 hour incubation with goat anti-human IgG conjugated to horseradish peroxidase (HRP) (Millipore) (1/2500). Reactivity was determined by measuring the optical density (OD) at 405 nm following the addition of ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (Thermo Fisher Scientific) for fifteen minutes and the reaction stopped with the addition of 1% sodium dodecyl sulfate in PBS. Results are presented as ODs standardized to five positive control samples (individuals from the study identified as having high IgG reactivity to VAR2CSA during assay optimisation) run on each assay plate to account for inter-plate variability.

Measurement of Antibody Levels to VAR2CSA on the Surface of IEs

We used the CS2 parasite line to measure antibody levels to VAR2CSA expressed on the surface of IEs. The CS2 parasite line predominantly expresses the *var2csa* transcript (32, 33), binds to CSA and is recognized by serum from malaria exposed pregnant women (46, 47). Total IgG reactivity to the surface of CS2 IEs was assessed by flow cytometry as previously described (36, 48). Total IgG binding for each sample was determined by subtracting the geometric Mean Fluorescence Intensity (MFI) of uninfected erythrocytes from that of IEs. Geometric MFI were then expressed as a percentage of the mean geometric MFI (Arbitrary Units) of pooled serum from five positive high responders.

Statistical Analysis

Statistical analyses were performed using Stata Version 16.1 (StataCorp, College Station, TX, USA).

Descriptive statistics were used to describe the included women, categorical variables were summarised with proportions and frequencies; continuous variables were summarised with quartiles. Linear regression was used to assess the associations between the exposures (antibody levels) and outcomes [birth weight (grams) and estimated gestational age at delivery (weeks)]. Logistic regression was used to assess associations between antibody levels and odds of placental infection (defined as presence of *P. falciparum* parasites in placental histology). Potential confounders for all analyses were selected *a priori* using causal diagrams (49). All analyses were adjusted for the following confounders: *P. falciparum* infection at enrolment (from peripheral blood sample, diagnosis by light microscopy of blood smears with confirmation by PCR), gravidity (primigravid/multigravid), haemoglobin at enrolment (g/dl; determined using Hemocue), middle upper arm circumference (cm), SAO (yes/no; by PCR), alpha thalassaemia (yes/no; by PCR). In the birth weight regression model, sex of baby (male/female) was included as a covariate known to be independently predictive of the outcome.

For all models, the assumption of a linear association between antibody levels and outcome was assessed both visually and by testing regression models with categorical (groups cut at quartiles) and pseudo-continuous antibody variables by likelihood ratio tests. As there was no evidence of non-linearity of associations between antibodies and outcomes in all models, antibody measures were fitted as continuous exposures. To aid interpretation, coefficients were presented representing the difference in outcome of a high responder (75^{th} percentile) to a low responder (25^{th} percentile). To assess whether the relationship between antibody levels and the outcome of

interest was modified by gravidity (primigravid/multigravid) or *P. falciparum* infection at enrolment detected by light microscopy (yes/no), likelihood ratio tests were performed comparing the model with and without interaction terms. Where evidence supporting an interaction was present ($p < 0.1$ for at least two pregnancy-specific antibody exposures) this model was presented in the main text; results from all models are reported in **Supplementary Material**.

RESULTS

The median age of women was 24 years (lower, upper quartiles = 21, 28); 115 (38%) women were primigravid (**Table 1**). Median estimated gestational age at enrolment was 26 (22, 28) weeks. Primigravid women were more likely to present with peripheral blood *P. falciparum* infection [49/115 (43%); by light microscopy) than multigravid women [54/186 (29%)]. There were 103 (34%) women who had *P. falciparum* infection. Women infected at enrolment were more likely to experience adverse birth outcomes than women uninfected at enrolment with a higher percentage of low birth weight births (20% *vs* 15%) and preterm deliveries (30% *vs* 18%) and were more likely to experience placental *P. falciparum* infection (65% *vs* 53%).

Antibodies to VAR2CSA Were Associated With Gravidity and Exposure

Antibodies to VAR2CSA were assessed by quantifying IgG to recombinant DBL3 and DBL5 domains, and antibodies to the surface of CS2-IEs expressing VAR2CSA. VAR2CSA antibody levels were higher in multigravid women than primigravid women ($p = 0.03$), and higher in pregnant women compared to malaria-exposed men from the same population ($p < 0.001$) or malaria non-exposed adults ($p < 0.001$, **Figure 1A**). Women who were infected at enrolment had higher levels of VAR2CSA antibodies than women who were uninfected at enrolment ($p < 0.001$, **Figure 1B**).

VAR2CSA IgG at Enrolment Was Differentially Associated With Placental Infection Among Women Infected Versus Non-Infected at Enrolment

There was evidence of effect modification of the association between VAR2CSA antibodies and odds of placental infection

TABLE 1 | Study population characteristics in women with and without microscopic peripheral *P. falciparum* infection at enrolment.

	Total (N=301)	Uninfected at enrolment (N=198)	Infected at enrolment (N=103)
Enrolment demographics			
Age (years)	24 (21, 28)	25 (21, 30)	23 (20, 27)
Gravidity			
Primigravid	115/301 (38%)	66/198 (33%)	49/103 (48%)
Multigravid	186/301 (62%)	132/198 (67%)	54/103 (52%)
Gestational age (weeks)	26 (22, 28)	26 (22, 28)	26 (22, 29)
Middle Upper Arm Circumference (cm)	22 (21, 24)	23 (21, 24)	22 (22, 23)
Smokes	60/300 (20%)	40/198 (20%)	20/102 (20%)
Maternal genetics			
South East Asian Ovalocytosis	41/301 (14%)	23/198 (12%)	18/103 (17%)
α+thalassaemia			
Normal	59/301 (20%)	40/198 (20%)	19/103 (18%)
Heterozygous	112/301 (37%)	72/198 (36%)	40/103 (39%)
Homozygous	130/301 (43%)	86/198 (43%)	44/103 (43%)
Complement Receptor 1 (exon 22)			
AA	30/301 (10%)	21/198 (11%)	9/103 (8.7%)
AG	120/301 (40%)	84/198 (42%)	36/103 (35%)
GG	151/301 (50%)	93/198 (47%)	58/103 (56%)
Haemoglobin at enrolment			
Haemoglobin (g/dl)	8.4 (7.6, 9.4)	8.7 (7.9, 9.7)	8.1 (7.2, 9.0)
Severe anaemia (<8g/dl)	111/301 (37%)	60/198 (30%)	51/103 (50%)
Infections at enrolment			
Peripheral *P. falciparum*[a]	103/301 (34%)	0/198 (0%)	103/103 (100%)
Infections at delivery			
Peripheral *P. falciparum*[a]	33/288 (11%)	22/190 (12%)	11/98 (11%)
Placental *P. falciparum*[b]	132/233 (57%)	81/154 (53%)	51/79 (65%)
Haemoglobin at delivery			
Haemoglobin (g/dl)	9.4 (8.2, 10.3)	9.4 (8.1, 10.4)	9.2 (8.2, 10.2)
Severe anaemia (<8g/dl)	68/285 (24%)	45/188 (24%)	23/97 (24%)
Birth Outcomes			
Birth weight (kg)	2.9 (2.6, 3.2)	3.0 (2.6, 3.2)	2.8 (2.5, 3.0)
Low birth weight (<2500g)	50/299 (17%)	30/197 (15%)	20/102 (20%)
Gestational age	38 (37, 40)	38 (37, 40)	38 (36, 40)
Preterm delivery	63/285 (22%)	35/191 (18%)	28/94 (30%)

Data are presented as median (25th percentile, 75th percentile) and n/total (%) for categorical variables. [a]Parasite positive by light microscopy, confirmed by PCR. [b]Parasite positive by placental histology. Smoker missing for 1 participant, Middle Upper Arm Circumference at enrolment missing for 12 participants, Age missing for 10 participants.

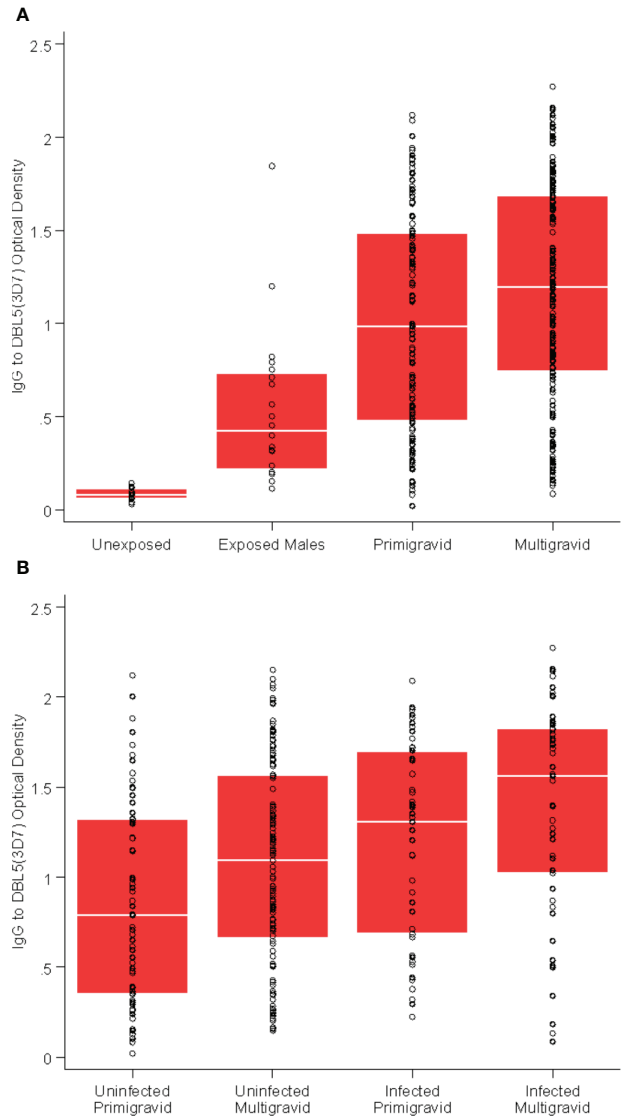

FIGURE 1 | VAR2CSA-specific antibodies are associated with gravidity and exposure. **(A)** Total IgG to DBL5 (3D7) in unexposed Melbourne controls (n = 30); exposed males from Papua New Guinea (n = 20); primigravid women (n = 115); and multigravid (n = 186) women. p = 0.03 for multigravid versus primigravid; p < 0.01 for all other comparisons. **(B)** Total IgG to DBL5 (3D7) in primigravid uninfected at enrolment (n = 66); primigravid infected at enrolment (n = 49); multigravid uninfected at enrolment (n = 132); and multigravid infected at enrolment (n = 54). p = 0.02 and p = 0.08 for multigravid versus primigravid (uninfected and infected respectively); p < 0.01 for infected versus uninfected (primigravid and multigravid respectively).

by *P. falciparum* infection at enrolment (**Supplementary Table S1**, likelihood ratio tests, p < 0.02 for DBL3 and DBL5; p = 0.11 for CS2-IE). Among women with infection detected at enrolment, women with higher levels of antibodies to VAR2CSA had lower adjusted odds of placental infection at delivery [**Figure 2**, Odds Ratio (OR) [95% confidence interval (CI)] = 0.6 (0.2,1.4); 0.2 (0.1,0.5); 0.6 (0.3,1.2); 0.8 (0.6,1.2) for DBL3 (7G8), DBL5 (3D7) and DBL5 (7G8) and CS2-IE respectively]. However, 95% CIs were wide and associations were generally not statistically significant [with the exception of DBL5 (3D7)]. In contrast, among women who were not infected at enrolment, higher levels of VAR2CSA antibodies were associated with an increased adjusted odds of placental infection (**Figure 2**, OR = 2.2 (1.2,4.3); 2.0 (1.0,3.8); 1.7 (1.0,3.1); 1.1 (0.8,1.5) for DBL3 (7G8), DBL5 (3D7) and DBL5 (7G8) and CS2-IE respectively). Levels of AMA1 antibodies (not specific to malaria in pregnancy), were not associated with odds of placental infection among infected women or uninfected women [OR = 0.9 (0.5,1.9) and 1.0 (0.6,1.7) respectively].

VAR2CSA IgG Levels Associated With Increased Birth Weight in Women Infected During Pregnancy

Moderate evidence for effect modification by infection at enrolment was observed for some VAR2CSA antibodies.

Among women infected at enrolment, higher levels of IgG to CS2-IEs, which represents IgG to full-length VAR2CSA (50), were associated with higher birth weight [+90g (20g-150g)] relative to women with lower antibody levels (**Figure 3** and **Supplementary Table S4**). Higher antibodies to VAR2CSA domains DBL5 (3D7 and 7G8) were also associated with higher birth weight [+150g (95% CI: 0g, +300g); +120g (-10g, +240g), respectively]. There was no clear association for DBL3 antibodies (+40g (-110g, +180g)). The estimated association between levels of antibodies to AMA1 was not significant (-60g (95% CI: -180g, +60g). Among women without infection detected at enrolment, the estimated associations between higher levels of VAR2CSA antibodies and birth weight were close to zero (estimates -10g to +0g). There was very little evidence to support effect modification by gravidity (**Supplementary Table S5**, likelihood ratio tests, p >0.3). Estimates of the associations between VAR2CSA antibodies and gestational age at delivery were of small magnitude and were not significant (**Supplementary Tables S7-9**).

DISCUSSION

In this study, we investigated the association between anti-VAR2CSA antibody responses and pregnancy outcomes, examining the influence of infection with *P. falciparum* at enrolment as well as gravidity on these associations. Women infected at enrolment and women who had been pregnant before had higher levels of anti-VAR2CSA antibodies at enrolment compared to women who were uninfected at enrolment, and women in their first pregnancy, respectively. Among women who were infected with *P. falciparum* at enrolment, those with higher VAR2CSA antibody levels had higher birthweight babies and lower odds of odds of LBW relative to those with low levels. These findings suggest that when women experience *P. falciparum* infection during pregnancy, the ability to generate higher levels of VAR2CSA antibodies may lead to better pregnancy outcomes.

Findings from previous studies investigating the associations between anti-VAR2CSA antibody responses and improved birth outcomes have been inconsistent and contradictory (3, 16–27, 51). Most of the literature reporting positive associations

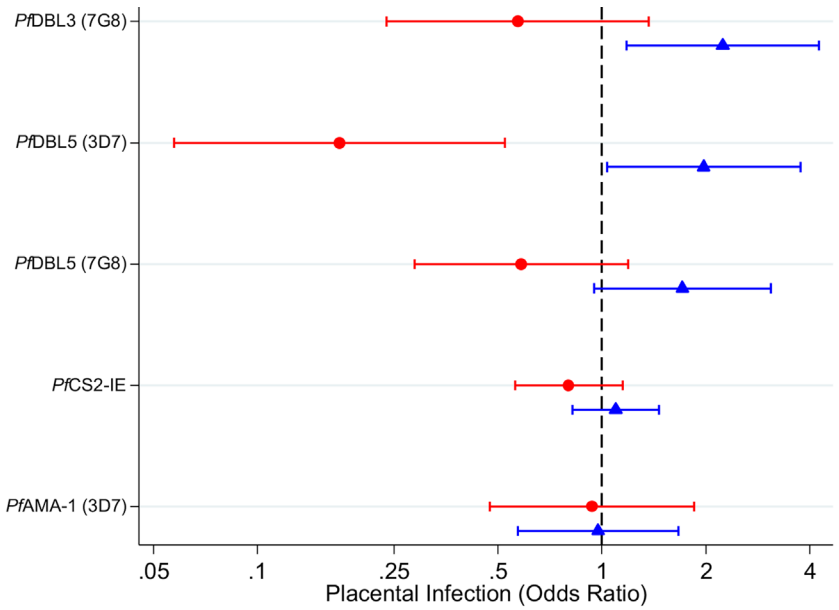

FIGURE 2 | Adjusted odds ratios for placental *P. falciparum* infection in individuals with high antibody levels (75th percentile) relative to individuals with low antibody levels (25th percentile) in women infected at enrolment (red circles) and uninfected at enrolment (blue triangles). Capped bars indicate 95% confidence intervals. See **Supplementary Table 1** for a table with p values. See **Supplementary Tables 2** and **3** for estimates from models fitted with an interaction between antibodies and gravidity; and for estimates from models fitted without an interaction term.

between anti-VAR2CSA antibodies and better birth outcomes have concentrated on particular subsets of infected women (3, 20–22, 27) or in particular strata of gravidity (19, 21), and often analyses were conducted as a cross-sectional study at delivery (15). Importantly, our analysis examined a cohort of pregnant women prospectively and formally tested for effect modification by *P. falciparum* infection and gravidity. This study supports the hypothesis that antibody responses to VAR2CSA are protective against lower birth weight, and possibly placental *P. falciparum* infection, but this protective association is only observed in women with evidence of *P. falciparum* infection at enrolment. Therefore, our findings suggest that women who generate higher antibody responses when infected in pregnancy have a reduced risk of low birthweight. That antibody levels in women with no peripheral blood *P. falciparum* infection at enrolment were instead associated with poor outcomes suggests that in this subset of women, antibodies are likely correlated with unmeasured recent infection, confounding any underlying protective association. Alternatively, it may be important to quantify antibodies during an active infection to better assess the nature and potential beneficial effects of antibody responses.

The strength of associations between antibody response to VAR2CSA and birth outcomes were broadly comparable across each measure of antibody response to VAR2CSA. It is beyond the scope of our immune-epidemiological study to be able to distinguish antibodies that have a truly protective role from antibodies that do not play a protective role but are associated with protection. However, a plausible role for antibodies to VAR2CSA in protective immunity is supported by the absence of any association between antibodies to AMA-1 and birth outcomes as well as observations that VAR2CSA antibodies can function to inhibit placental adhesion of PfIEs and promote phagocytosis (23, 52, 53). There may be other mechanisms that are not yet defined. A stronger association of antibodies specific for one domain relative to another domain may not indicate that these antibodies play a stronger direct role in protection. Strong associations with protection may indicate that these antibodies may be useful biomarkers for protective immunity. Further studies in multiple populations are needed to estimate the causal effect of single antibody responses and determine the importance of a repertoire of antibodies for maximal immunity. This knowledge will be valuable for informing vaccine approaches. Further assessment of functional mechanisms of protection (54, 55) for different epitopes or targets is warranted to clarify their role in the immune system in mediating protection. In this study, we only assessed antibodies to two VAR2CSA domains, DBL3 and DBL5. While these both are targets of acquired immunity and functionally-relevant antibodies (25, 39–44) and served the purpose of providing an assessment of responses to VAR2CSA in our study population, other domains have also been identified as targets of acquired immunity (25, 27). Detailed studies including an evaluation of all VAR2CSA domains and sub-domain could be valuable in future studies.

The longitudinal nature of this study allowed us to investigate a temporal relationship between the presence of antibodies mid-pregnancy at enrolment and subsequent adverse pregnancy outcomes, measured at delivery. Given that antibodies arise in

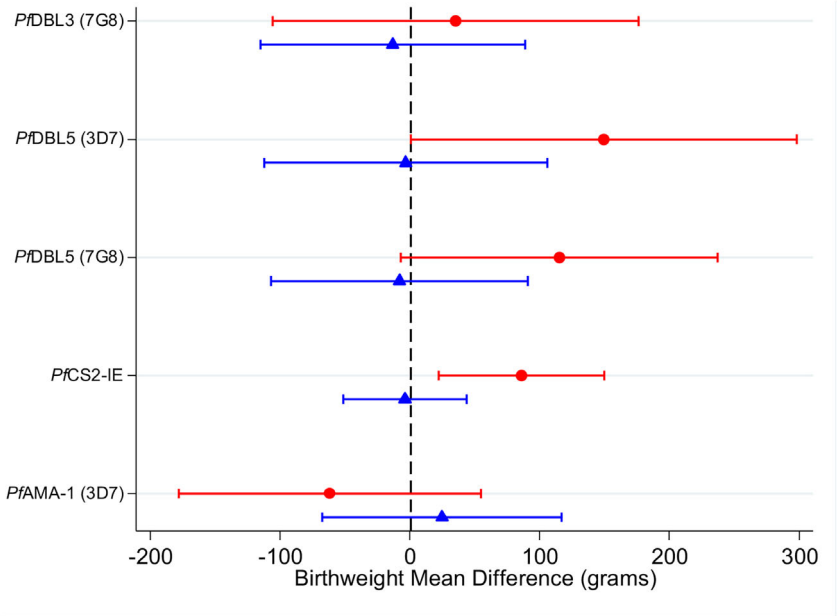

FIGURE 3 | Adjusted mean difference in birthweight (grams) in individuals with high antibody levels (75th percentile) relative to individuals with low antibody levels (25th percentile) in women infected at enrolment (red circles) and uninfected at enrolment (blue triangles). Capped bars indicate 95% confidence intervals. See **Supplementary Table 4** for a table with p values. See **Supplementary Tables 5** and **6** for estimates from models fitted with an interaction between antibodies and gravidity; and for estimates from models fitted without an interaction term.

response to exposure but may also serve a protective role against clinical malaria and adverse outcomes, it is difficult for cross-sectional studies (3, 16–22) to distinguish between women who have high antibody levels that reflect a recent infection and women who have high antibody levels that have successfully kept them infection free. In the absence of detailed exposure history, these studies will likely be subject to residual confounding by unmeasured exposure. A limitation of our study was measurement of *P. falciparum* exposure during, and prior to, the study; *P. falciparum* infection was only measured once using light microscopy and more frequent measurement of infection may have provided more accurate exposure data. Although these *P. falciparum* infections were observed during pregnancy, the acquisition of the infection will predate the moment it was detected, and some infections may have been present prior to conception. It is also possible that some women experienced undetected infections between recruitment and delivery and/or experienced and cleared infection prior to recruitment. This study was conducted in a setting where women received prophylaxis as part of their antenatal care which likely reduced *P. falciparum* infection and its negative effects during pregnancy. As such the magnitudes of protective effects of VAR2CSA immunity we observed may not be generalisable to other populations where malaria prophylaxis is not routinely given to pregnant women or in areas of different malaria endemicity.

In summary, we found that associations between VAR2CSA specific antibodies and birth outcomes varied across groups experiencing different levels of exposure. Among women who had already acquired infection by mid-pregnancy at enrolment, higher levels of antibodies to VAR2CSA were associated with a reduced risk of adverse outcomes. In contrast, among women uninfected at enrolment, anti-VAR2CSA antibodies may indicate women at increased risk of *P. falciparum* exposure and the adverse outcomes that follow. These differences in antibody associations due to exposure history might explain some of the conflicting reports in the published literature. Given the close relationship between exposure, antibodies and protection, ignoring heterogeneous exposure may obscure relevant protective associations. Future population studies should ensure the design and analysis accounts for the possibility of heterogeneous exposure.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request and dependent on approval by the National Department of Health, Papua New Guinea.

ETHICS STATEMENT

This study received ethical approval from the PNG Medical Research Advisory Council, the Melbourne Health Human Research Ethics Committee and Alfred Health Human Research Ethics Committee.Written informed consent to participate in this study was provided by the participants.

AUTHOR CONTRIBUTIONS

JB, FF, SR, and IM designed the research. AM, HO, DS, GF, and AU performed the research, AM and HO analysed the data, and AM, HO, JC, JB, and FF wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.644563/full#supplementary-material

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Myeloperoxidase and Other Markers of Neutrophil Activation Associate With Malaria and Malaria/HIV Coinfection in the Human Placenta

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[†]This manuscript is dedicated to the memory of our dear friend and colleague

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Introduction: Placental malaria (PM) is characterized by accumulation of inflammatory leukocytes in the placenta, leading to poor pregnancy outcomes. Understanding of the underlying mechanisms remains incomplete. Neutrophils respond to malaria parasites by phagocytosis, generation of oxidants, and externalization of Neutrophil Extracellular Traps (NETs). NETs drive inflammation in malaria but evidence of NETosis in PM has not been reported. Neutrophil activity in the placenta has not been directly investigated in the context of PM and PM/HIV-co-infection.

Methods: Using peripheral and placental plasma samples and placental tissue collected from Kenyan women at risk for malaria and HIV infections, we assessed granulocyte levels across all gravidities and markers of neutrophil activation, including NET formation, in primi- and secundigravid women, by ELISA, western blot, immunohistochemistry and immunofluorescence.

Results: Reduced peripheral blood granulocyte numbers are observed with PM and PM/HIV co-infection in association with increasing parasite density and placental leukocyte hemozoin accumulation. In contrast, placental granulocyte levels are unchanged across infection groups, resulting in enhanced placental: peripheral count ratios with PM. Within individuals, PM- women have reduced granulocyte counts in placental relative to peripheral blood; in contrast, PM stabilizes these relative counts, with HIV coinfection tending to elevate placental counts relative to the periphery. In placental blood, indicators of neutrophil activation, myeloperoxidase (MPO) and proteinase 3 (PRTN3), are significantly elevated with PM and, more profoundly, with PM/HIV co-infection, in association with placental parasite density and hemozoin-bearing leukocyte accumulation. Another neutrophil marker, matrix

metalloproteinase (MMP9), together with MPO and PRTN3, is elevated with self-reported fever. None of these factors, including the neutrophil chemoattractant, CXCL8, differs in relation to infant birth weight or gestational age. CXCL8 and MPO levels in the peripheral blood do not differ with infection status nor associate with birth outcomes. Indicators of NETosis in the placental plasma do not vary with infection, and while structures consistent with NETs are observed in placental tissue, the results do not support an association with PM.

Conclusions: Granulocyte levels are differentially regulated in the peripheral and placental blood in the presence and absence of PM. PM, both with and without pre-existing HIV infection, enhances neutrophil activation in the placenta. The impact of local neutrophil activation on placental function and maternal and fetal health remains unclear. Additional investigations exploring how neutrophil activation and NETosis participate in the pathogenesis of malaria in pregnant women are needed.

Keywords: neutrophils, pregnancy, NETs (neutrophil extracellular traps), placental malaria, myeloperoxidase, *Plasmodium falciparum*

INTRODUCTION

Malaria infection during pregnancy is a significant public health problem with substantial effects on the mother, her fetus, and the newborn child [reviewed by (1)]. Accumulation of parasites in the placenta is a common feature of *Plasmodium falciparum* infection in pregnant women, mediated by VAR2CSA, a parasite protein exported to the surface of infected red blood cells, which binds to chondroitin sulfate A on proteoglycans, including syndecan-1 (2), in the placenta. Paucigravid women (in the first or second pregnancy) are especially vulnerable and are more likely than multigravidae (three or more pregnancies) and nonpregnant women to develop severe malaria (1). The interplay between parasites and the placenta is associated with inflammation characterized by the recruitment, retention, and activation of innate immune cells including polymorphonuclear leukocytes (neutrophils) (3) and is known as placental malaria (PM) (4). PM is associated with adverse pregnancy outcomes such as maternal anemia, stillbirth, and low birth weight (LBW) due to intrauterine growth restriction, and is most severe in the first pregnancy (5). How neutrophils in particular may affect placental pathology and fetal growth in the context of malaria remains a mystery.

Previous studies addressing potential interactions between PM and HIV infection suggest that the latter impairs immunity against malaria (6). HIV-infected pregnant women have more frequent and higher density parasitemia than HIV-negative pregnant women (7–11). Importantly, malaria accelerates HIV disease progression and higher viral load among pregnant women. Fetal complications in PM and association between maternal HIV status and fetal outcome have also been addressed (11–17). A definitive role for neutrophils in pathogenesis of PM alone and co-infections with HIV has been rarely studied. In one study, the number of circulating pigmented (Hz-bearing) neutrophils negatively correlated with birth weight, suggesting that these cells may have a pathogenic role in PM and thus may serve as prognostic markers for malaria-associated low birth weight (18). Another study reported that circulating neutrophils were reduced in pregnant women with *P. falciparum* malaria compared to negative controls (3). Others have found elevated neutrophil levels in placental relative to peripheral blood in malaria-infected women (19). Limited studies that performed direct measures have noted increased neutrophil levels by placental histopathology (20, 21). Consistent with this, cytokines and chemokines that can attract neutrophils, namely MIF, CXCL8/IL-8, and CCL3, are elevated in human PM (22–27).

Neutrophils are essential effector cells of the innate immune system. In humans, neutrophils are the most abundant type of white blood cell, accounting for 70% of all leukocytes in the blood of healthy adults (28). During pregnancy, the neutrophil count begins to increase in the second month and plateaus in the second or third trimester, a time at which the total number of white blood cells ranges from 9,000 to 15,000 $X10^6$/L (29). These cells are classically considered to be short-lived and act as the first line of defense in innate immunity, ensuring tissue restitution following resolution of infection (30–32). Neutrophils can rapidly be recruited to sites of infection and tissue injury (33), where they generate reactive oxygen species (ROS) through the activity of NADPH oxidase, thereby initiating antibacterial/antiparasitic defense (34). Neutrophils clear infections by phagocytosis, generation of ROS, release of potent bactericidal enzymes by degranulation, and formation of neutrophil extracellular traps (NETs) (35).

As evidenced in malaria, however, neutrophils represent a double-edged sword. These cells are activated and are capable of clearing malaria parasites by a variety of mechanisms (reviewed by (36), yet they are implicated in pathogenic mechanisms as well (37). Mice developing malaria-associated acute lung injury/acute respiratory distress syndrome (ALI/ARDS) had greater neutrophil accumulation in the lungs compared to mice that did not develop pulmonary complications (38). In these mice, targeting of neutrophils decreased the development of malaria-associated ALI/ARDS and significantly increased mouse survival (38), suggesting that neutrophils play a significant role in the pathogenesis of ALI/ARDS during experimental severe malaria and could be targeted to improve disease outcome.

Oxidative damage to tissues is also a key attribute of malaria pathogenesis that may be in part attributable to neutrophils.

Retinopathy-positive cerebral malaria is associated with accumulation of neutrophils (39). Likewise, previous work with *P. chabaudi* and *P. berghei* ANKA infection in mice indicated that neutrophils were responsible for liver damage, cerebral complications, and ALI/ARDS (38, 40, 41). Uptake of hemozoin (Hz)-containing digestive vacuoles by neutrophils drives a rapid oxidative burst but suppresses subsequent neutrophil activity (42). Oxidative damage in PM has been reported in humans and in mouse models, but key drivers remain unclear (43–46).

NETs are generated by the extrusion of DNA strands into the extracellular milieu, where they can entrap invasive pathogens (47, 48). The most common method for NET detection *in vitro* is microscopic observation (49), with immunodetection of neutrophil-derived proteins such as myeloperoxidase (MPO) and proteinase 3 (PRTN3) (50, 51) colocalized with DNA (49). NETs in tissue samples have similarly been shown as extracellular DNA colocalized with neutrophil-derived proteins (49). NET remnants such as DNA and neutrophil-derived protein complexes (MPO-DNA; neutrophil elastase (NE)-DNA) and citrullinated histones can be determined by ELISA in fluid samples (50, 51) or detected by flow cytometry (52, 53) as indicators of NETosis.

A role for NETosis in both protection and pathogenesis in malaria is emerging. *P. falciparum*-infected red blood cells reportedly stimulate human neutrophils to release NETs *in vitro* (38). The latest mechanistic investigations of NETs in malaria show that they are released by neutrophils exposed to malaria parasites and impede parasite spread thereby controlling infection (54). Furthermore, these studies provide evidence that NET release in malaria is independent of cell-cell contact and is mediated by macrophage migration inhibitory factor and peptidylarginine deiminase 4 (PAD4)-dependent histone citrullination (54). Interestingly, malaria parasite species have been shown to produce DNase that degrades NETs and the deficiency of this enzyme resulted in lower parasitemia in mice (55). Importantly, NETosis has been linked to severe malaria in human infection and in mouse models (38, 56–58). Using human samples and a mouse model for malaria, Knackstedt et al. demonstrated that heme-induced NETs are essential for malaria pathogenesis, with granulopoiesis and endothelial cell activation as two mechanisms of NET-mediated inflammation of the vasculature (58).

The present study investigates granulocyte levels and neutrophil activity in the peripheral and placental blood and tissue of parturient Kenyan women exposed to malaria and HIV. Neutrophils and associated markers appear to be influenced by these infections and preliminary evidence of NETosis in the placenta blood is offered. These data show a potential implication of neutrophils in the pathogenesis of PM but further studies are required to characterize the mechanisms by which this occurs.

MATERIAL AND METHODS

Ethics Statement

The study supporting collection of samples used in this report was approved by the Kenya Medical Research Institute, the Centers for Disease Control and Prevention, and the University of Georgia Institutional Review Boards. All study participants provided written informed consent before enrollment and procedures and instruments involving human subjects, sample collection and data analysis, processing, and testing were approved throughout the conduct of patient recruitment. All samples and data are anonymized.

Study Participants and Sample Collection

Participant recruitment and sample collection have been previously described (11, 59) (Matthias et al., manuscript in preparation). Briefly, the recruitment of 222 participants was performed at New Nyanza Provincial General Hospital, a public referral hospital, in Kisumu from November, 2002 to May, 2004. Subsequently, 825 participants were recruited at Siaya District Hospital, a public secondary health facility in Siaya until September, 2008. Women of all gravidities and uncomplicated vaginal deliveries were randomly recruited from patients admitted to the Delivery Ward of these hospitals. Only women with no health issues aside from malaria or HIV were eligible for full participation in the study. Maternal demographic and clinical information was collected and summarized, including whether or not participants self-reported fever within the two weeks prior to delivery. Infant gestational age was estimated using the modified Dubowitz score, and birth weight in grams was measured within eight hours after delivery. Maternal placental (intervillous) blood (IVB) was collected by the prick method within five minutes of placental expulsion (60). Peripheral blood was collected by venipuncture of the cubital vein within 12 hours post-partum. Platelet-free plasma was prepared as described (59) and stored continuously at -80°C, avoiding multiple cycles of freeze-thaw. Complete blood count (CBC) of both peripheral blood and IVB to estimate total white blood cell (WBC) and granulocyte counts was performed simultaneously with a Beckman Coulter AcT diff2 (Beckman Coulter Corporation, Miami, FL) within eight hours of blood collection. Although the majority of granulocytes detected by CBC are expected to be neutrophils, differential analysis for granulocytes was not available; thus, granulocyte levels are reported for this study. Full thickness placental tissue sections were collected from three unique regions of the placental disk, and fixed in Streck Tissue Fixative (Streck Inc., Omaha, NE). Five-micron sections were stained with hematoxylin and eosin for histopathological examination or left unstained for immunohistochemical analysis. From the same placental regions, 125 mm^3 sections of villus tissue underlying the placental basal plate were collected and flash frozen in liquid nitrogen for future molecular analysis and with Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA) for immunofluorescence assessments and stored continuously at ≤-80°C until use.

Parasitemia was assessed on thick and thin smears of peripheral and placental blood and estimation of thick smear parasite density assumed 8,000 WBCs per μL of blood for both peripheral and placental blood. Percentage of leukocytes in the maternal placental vascular space bearing Hz was calculated from these thick smears. HIV serostatus was determined by rapid tests as previously described (11, 59).

Study Designs

Among the 1047 women recruited into the study, samples and data from a subset of 379 women are included in the work described here. Sample selection and use are summarized in **Figure 1** and **Supplemental Figure 1**, and patient characteristics are summarized in **Table 1**. All malaria infections are attributable to *P. falciparum;* a single participant was diagnosed with a *P. falciparum/P. malariae* mixed infection. Paired peripheral and placental data from Complete Blood Count were available from 224 women (79 primigravidae, 54 secundigravidae and 91 multigravidae, representing four infection groups (uninfected, malaria only (PM+HIV-), HIV only (PM-HIV +), and co-infected (PM+HIV+)). Gravidity differs significantly across the infection groups providing CBC data (P=0.0093 by Kruskal-Wallis test with post-hoc group-wise comparisons by Dunn's multiple comparisons test; median, interquartile range (IQR): PM-HIV-, 2, 1 – 3; PM+HIV-, 1, 1 - 2; PM-HIV+, 3, 2 – 4; PM+HIV+, 2, 1 – 3). Subsequent analyses focused on primigravid and secundigravid (paucigravid) women who experience the most significant outcomes with malaria and HIV infections in this setting (10, 61) (Matthias et al., manuscript in preparation).

Pilot data to detect markers of neutrophil activation and NETosis (cell-free DNA, DNA-human neutrophil elastase (NE) complexes, histones) in placental plasma were generated using HIV seronegative primigravid placental plasma (**Figure 1**), selected on the basis of placental histopathological status (uninfected, and infected: acute, chronic, chronic inflammatory). Acute infection is defined as the presence of infected red blood cells (iRBC), white blood cell (WBC) count by CBC <13,000/uL, and hemozoin scores [as described in (Avery et al., 2012)] ≤1 in WBCs and in fibrin. Chronic infection is defined as presence of iRBC, WBC count <13,000/uL, and hemozoin scores ≥2 in WBCs and in fibrin. Chronic inflammatory infection is defined as presence of iRBC, WBC count >13,000/uL, and hemozoin scores ≥2 in WBCs and in fibrin. Uninfected samples were confirmed parasite PCR negative and lacked iRBCs in histological sections. Subsequent ELISA data were generated from paucigravid women (**Figure 1**), representing malaria and HIV positive and negative women, with balanced selection of primigravid and secundigravid women within each infection group (PM-HIV-, PM+HIV-, PM-HIV+, PM+HIV+), and with matched selection across groups of infant birth weights, in both cases to the extent that sample availability allowed. Frozen placental tissues for immunofluorescence were selected to represent each infection group from among samples with MPO data, based on sample availability (**Figure 1**). Immunohistochemistry and western blot experiments utilized paucigravid samples (**Figure 1**), representing the four infection groups, with matched selection for granulocyte count (by CBC) ranges across each group.

ELISA

Levels of myeloperoxidase (MPO), proteinase 3 (PRTN3), matrix metalloproteinase (MMP9), and CXCL8 were quantified in peripheral and placental blood using a commercial ELISA kit

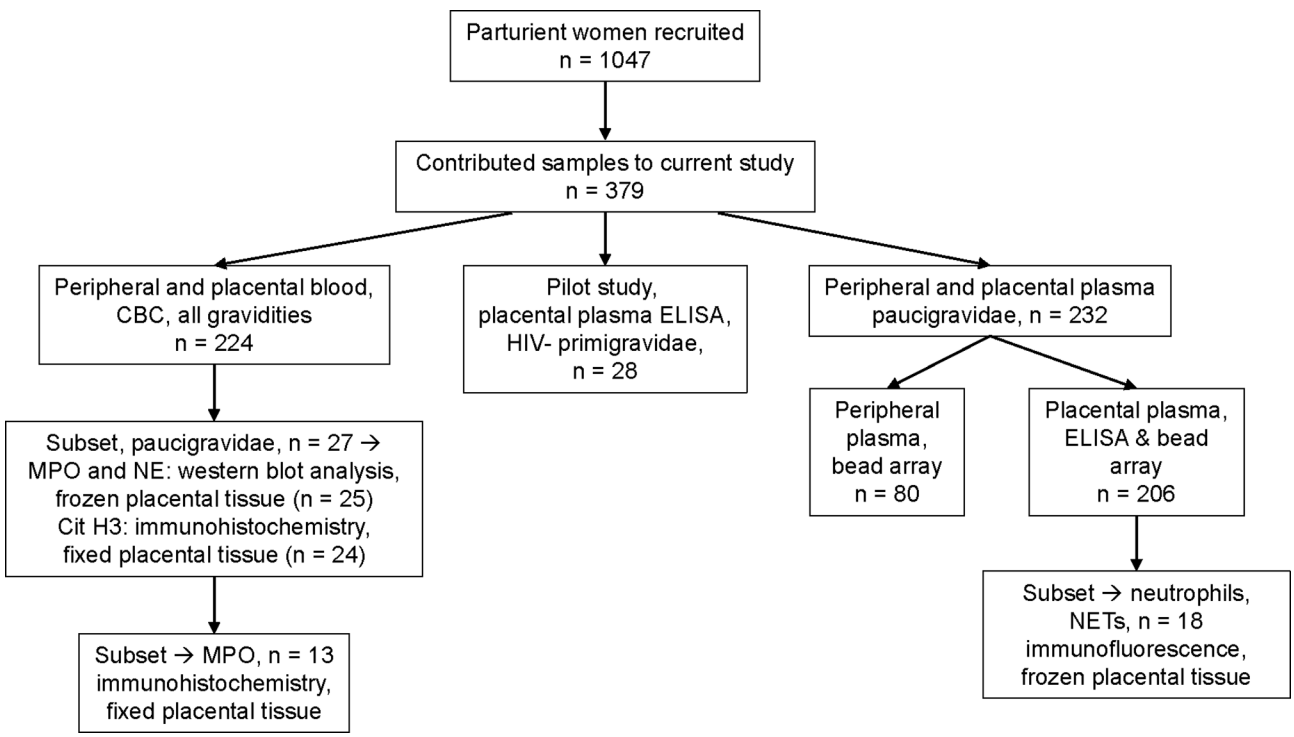

FIGURE 1 | Diagram of sample collection and testing. Shown are the number of patients recruited in a large cohort of parturient women in western Kenya, and samples contributed and tested in this study. Details of sample selection criteria are described in Materials and methods. Overlap of sample assessment on a per patient basis is depicted in **Supplemental Figure 1**.

TABLE 1 | Descriptive characteristics of study population stratified by gravidity.

Characteristics	All Women (n = 379)	Paucigravid (n = 288)	Multigravid[a] (n = 91)	*P*-value[b]
Maternal Sociodemographic				
Gravidity	1 (1-11)	1 (1-2)	4 (3-11)	-
Primigravid (%)	192 (50.6)	192 (66.7)	-	-
Age (years)	21 (13-39)	19 (13-32)	26 (20-39)	<0.0001
Married (%)	258 (68.1)	171 (59.4)	87 (95.6)	<0.0001
Luo ethnicity (%)	348 (91.8) (n=377)	259 (90.5) (n=286)	89 (97.8)	0.0230
Siaya[c] residence (%)	342 (90.2)	251 (87.1)	91 (100)	<0.0001
Laboratory				
Fever[d] at admission (%)	4 (3.3) (n=123)	3 (3.1) (n=97)	1 (3.8) (n=26)	1
HIV[e] seropositive* (%)	103 (27.2)	75 (26)	28 (30.8)	0.4177
Malaria smear positive* (%)	131 (34.6)	113 (39.2)	18 (19.8)	0.0006
Parasite density/μL[f] (range; interquartile range)	4,976 (40-226,208; 618-25,345) (n=131)	5,208 (40-226,208; 958-31,188) (n=113)	2,433 (83-119,107; 479-7,521) (n=18)	0.0752
Peripheral hemoglobin[g] (g/dL)	11.3 (5.30-20.4) (n=319)	11.3 (5.30-20.2) (n=228)	11.4 (5.80-20.4) (n=91)	0.5433
Placental hemozoin load[h]	3.5 (0.3-82) (n=127)	3.7 (0.3-82) (n=108)	1.6 (0.6-54) (n=19)	0.1820
Newborn				
Birth weight (g)	3,200 (2,000-4,500)	3,000 (2,000-4,400)	3,400 (2,600-4,500)	<0.0001
Low birth weight (≤ 2500 g) (%)	61 (16.1)	61 (21.2)	0 (0)	<0.0001
Gestational age (weeks)	38 (34-42) (n=376)	38 (34-40) (n=285)	38 (35-42)	0.0116
Preterm birth (<37 weeks) (%)	56 (14.9) (n=376)	49 (17.2) (n=285)	7 (7.7)	0.0277
Male infant (%)	193 (50.9)	150 (52.1)	43 (47.2)	0.4709
Self-reported history				
Fever, past two weeks[i] (%)	77 (20.3)	63 (21.9)	14 (15.4)	0.2315

Data are shown as number (percent) or median (range) unless otherwise noted. Sample sizes are shown where missing data reduce group numbers for specific parameters or only a subset of the group have values >0.

[a]*Multigravidae contribute complete blood count data only;*

[b]*Comparison of paucigravidae with multigravidae by two-tailed Fisher's exact test (proportions), Mann Whitney test (continuous data) or Welch's t test (log transformed continuous data).*

[c]*remainder were recruited in Kisumu;*

[d]*defined as >37.6°C.*

[e]*HIV, human immunodeficiency virus;*

[f]*parasitemia measured in placental blood, analysis done on log-transformed data;*

[g]*by complete blood count.*

[h]*percent white blood cells observed on placental blood thick smear with engulfed hemozoin;*

[i]*self-reported fever or malaria in the last two weeks;*

**factors used to guide sample selection.*

(R&D Systems, DuoSet, Minneapolis, MN) or bead arrays (R&D Systems) following manufacturer instructions and as previously described (62, 63). The experimental lower limits of detection for MPO were 125 pg/ml (ELISA) and 130 pg/mL (bead array), 125 pg/mL for MMP9, 10 pg/mL for PRTN3 and 3.9 pg/mL for CXCL8. Citrullinated histone H3 was measured using a kit from Cayman Chemical (Ann Arbor, MI), with a lower limit of detection at 0.1 ng/mL. Levels of NE-DNA complexes were assessed using an in-house protocol established to detect NET formation (64). In this assay, rabbit anti-NE (1:2,000, Calbiochem, San Diego, CA) was used as capture antibody and horseradish peroxidase-conjugated anti-DNA antibody (1:500, Roche, Indianapolis, IN) as detection antibody. Cell-free double stranded DNA was detected using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Grand Island, NY, USA) according to the manufacturer's instructions. DNA concentrations were quantitated using a known DNA standard with a lower limit of detection of 5 ng/mL as previously described (65). Total histone H3 levels were detected using a commercial kit (Active Motif, Carlsbad, CA) according to manufacturer instructions; the lower limit of detection was 150 ng/mL.

Immunohistochemistry and Immunofluorescence

Unstained 5 μm placental tissue sections were dewaxed for 15 minutes at 65°C followed by two incubations in xylene (2x5 minutes). Sections were then rehydrated in alcohol and antigen-retrieved with Sodium Citrate Buffer as previously described (66). Sections were then brought to room temperature and exposed to endogenous peroxidase activity block (DAKO, Cat#S2023) for 15 minutes. After washing with 1X TBST, sections were incubated with 10% Goat Serum for 10 minutes and incubated with the primary antibody (1/500 for MPO or 1/500 for citH3 in 1% Goat Serum) overnight at 4°C. The next day, samples were washed with 1X TBST (3x5 minutes) and incubated with polymer HRP anti-rabbit IgG for 30 minutes at RT. After three washes in 1X TBST, sections were exposed to DAB for 5 minutes, washed with distilled water, counterstained

with hematoxylin (Cat#H3401-500, Vector Laboratories), dehydrated, and mounted with acrytol mounting medium (Cat#13518, Electron Microscopy Sciences, Hatfield, USA).

Immunofluorescent analysis was performed on 5 μm cryo-sections of OCT-preserved placental tissue. The sections were fixed in ice-cold acetone for 10 minutes and air dried for 1 hour. Following a rehydration step with 0.05 mM Tris-buffered saline (TBS; pH 7.5), sections were incubated with Protein Block, Serum-Free solution from Agilent DAKO (X0909, Santa Clara, CA, USA) for 1 hour. Rabbit anti-human MPO was purchased from Agilent DAKO (A039829-2, Santa Clara, CA, USA) and rabbit -anti-NE was purchased from Millipore-Sigma (481001, Burlington, MA, USA). Antibodies were pre-labeled using the Zenon Alexa Fluor 488 rabbit IgG1 labeling kit for anti-human MPO and Zenon Alexa Fluor 594 rabbit IgG1 labeling kit for anti-NE from Thermo Fisher Scientific (Waltham, MA USA). The labeled antibodies were diluted in Antibody Diluent reagent from Agilent DAKO (X0909, Santa Clara, CA, USA) at 1:100 and the sections incubated overnight a 4° C. Negative controls were labelled with an irrelevant prelabeled isotype control at the same concentration and same labeling as the primary antibody. The sections were then washed three times in TBS and labeled with Hoescht 33342 reagent (2.3 μg/ml) for 15 minutes. The slides were washed again, and cover slips were mounted using Prolong Antifade mounting medium (Thermo Fisher Scientific, Waltham, MA USA).

Slides were examined using a Leica DM2500 LED microscope with filters 02 (DAPI filter), 03 (FITC filter) and 15 (rhodamine filter) at 20 X magnification. Digital images were acquired using Leica LASX software and a high-resolution Leica DMC6200 digital camera. Five random fields within each villi section were captured for morphometric analysis within a 197.7 mm^2 field of view (FOV). A total cell number was generated and analyzed for each sample based on the sum of the average number of cells per FOV in each sample.

For image analysis of immunohistochemistry samples, several random FOV were acquired in RBG format and exported as tag image format (*tif) with the respective metadata. Using QuPath v0.2.3-m4 software (67), the digital images were preprocessed using the built-in visual stain editor to estimate and adjust stain vectors to improve staining quality. The round cells in the intervillous spaces were manually annotated based on hematoxylin filter to warrant the selection of all nucleated cells in the analyzed FOV. The intensity of MPO staining was measured as optical density (OD) and classified by the module "positive cell detection" using adjusted pixel size (0.1465 μM) to match the image resolution and automatic thresholds. The cell detection measurements were compiled. The number of positive cells and the intensity of DAB staining (per mm^2) were used in the statistical analysis. All analyzed images were blindly evaluated by an observer for quality control purposes prior to data export. Due to the variability of preservation of intervillous blood among the samples, the minimum for evaluation was three FOV or at least 100 intervillous round cells.

Analysis of immunofluorescence samples was also performed using QuPath v0.2.3-m4 software (67). Images of three fluorescence channels (green, red and blue) were overlaid. The positive cells for either MPO or NE or dually positive cells were manually annotated. The annotated cells were always associated with blue stained nuclei (DNA material). The fluorescence intensity of MPO staining was measured in the green channel and intensity of NE staining was measured in the red channel using the "Analysis" and "Calculate features" with automatic thresholds. Additional overlays of negative controls were exported, and random areas were analyzed to set a threshold of nonspecific fluorescence by average of intensity of fluorescence in these areas in the green and red channels. An annotated cell was considered positive when the fluorescence intensity was higher than the average fluorescence intensity in either or both of the green and red channels in the negative controls. NETs were identified as dual positive for MPO and NE, associated with DNA staining, and with a size higher than 102 μM^2 as reported (68). All analyzed images were blindly evaluated by an observer for quality control purposes prior to data export.

Western Blotting

Placental villous tissue (30 mg frozen weight) was homogenized in RIPA buffer supplemented with proteinase inhibitor cocktail. Samples were homogenized with Tissue Lyser (Qiagen, Valencia, USA) and centrifuged at 10,000g for 15 minutes. Protein concentrations were determined by bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, USA) with bovine serum albumin (BSA) as a standard. Equal individual protein samples were prepared and stored at -80°C until use. Proteins (30 μg/sample) were separated by SDS-PAGE, blotted onto nitrocellulose membranes (Biorad, Hercules, USA) and probed with monoclonal or polyclonal rabbit antibodies specific for MPO (Agilent Technology, Santa Clara, USA), NE (Abcam, Cambridge, USA), citrullinated histone H3 (Abcam), and Hsp 90 (Cell Signaling Technologies) (as a loading control). Overnight incubation with primary antibody at 4°C was followed by one-hour incubation with anti-rabbit horseradish peroxidase secondary conjugates (Vector Laboratories). Proteins were detected using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). ChemiDoc Touch Imaging System with Image Lab Touch Software (BioRad) was used for image acquisition and densitometry analysis. Densitometry data are presented as ratio of target protein to Hsp90.

Statistical Analysis

Graph Pad Prism 9.1 software was used for all graphical data presentation and statistical analysis. Data are presented as scatter plot (correlation analysis and categorical analysis, with median line). Details for statistical analysis are indicated in the text or in figure legends as appropriate. Parasite density and percent Hz-bearing WBCs were log transformed prior to analyses. Binary analysis of non-normally distributed data of matched samples utilized the Wilcoxon matched-pairs signed rank test; unpaired analyses utilized the Mann-Whitney test. Correlation analysis was performed using the Spearman's correlation test. Multiple group comparisons were performed with Kruskal-Wallis test with post-hoc group-wise comparisons by Dunn's multiple

comparisons test. Two-tailed Fisher's exact test was used to compare proportions. Statistical significance was set at $P < 0.05$.

RESULTS

Granulocyte Counts Are Differentially Impacted by Malaria and HIV Infection in the Peripheral and Placental Blood

To determine the extent to which granulocyte levels are influenced by PM, and how pre-existing HIV infection may modify this response, granulocyte counts derived from CBC analysis of peripheral and placental blood from women of all gravidities were assessed. Counts do not vary with gravidity in peripheral (median, IQR: paucigravid, 10.0 (6.90 – 13.7) x10^3/μL; multigravid, 9.10 (7.30 – 11.5) x10^3/μL; $P = 0.6543$, Mann Whitney test) or placental blood (paucigravid, 7.80 (5.70 – 11.9) x10^3/μL; multigravid, 7.80 (5.70 – 10.4); $P = 0.4889$). Compared with control PM-HIV- women, peripheral granulocyte counts are reduced in PM+HIV- and PM+HIV+ women (**Figure 2A**). The lower levels in the latter are strongly attributable to HIV infection, as levels between the two HIV+ groups are not significantly different ($P = 0.5044$). In contrast to peripheral blood, no differences in neutrophil counts in the placenta are evident (**Figure 2B**). Interestingly, the ratio of placental to peripheral blood granulocyte counts is enhanced by PM in both HIV- and HIV+ women (**Figure 2C**). Pairwise comparison of peripheral to placental granulocyte counts among individuals within the infection groups shows that while placental granulocyte levels are reduced relative to the periphery in uninfected and HIV seronegative women, this difference is lost with PM, with a tendency toward a reversed pattern in co-infected women (**Figure 2D** and **Supplemental Table 1**). Additionally, with the exception of PM+HIV+ women, in whom total WBC counts are strongly elevated in placental relative to peripheral blood, relative patterns of granulocyte levels between the peripheral and placental blood vary independently of total WBC counts (**Supplemental Table 1** and **Supplemental Figure 2A**). In terms of proportion of total WBCs, granulocytes are universally substantially reduced in the placenta relative to the peripheral blood (**Supplemental Figure 2B** and **Supplemental Table 1**). However, whereas granulocyte percentages in the peripheral blood (**Supplemental Figure 2C**) follow the same trends observed with granulocyte counts (**Figure 2A**), placental granulocyte percentages reveal a distinct pattern of reduction in HIV+PM+ women (**Supplemental Figure 2D**) that is not observed in placental granulocyte counts (**Figure 2B**).

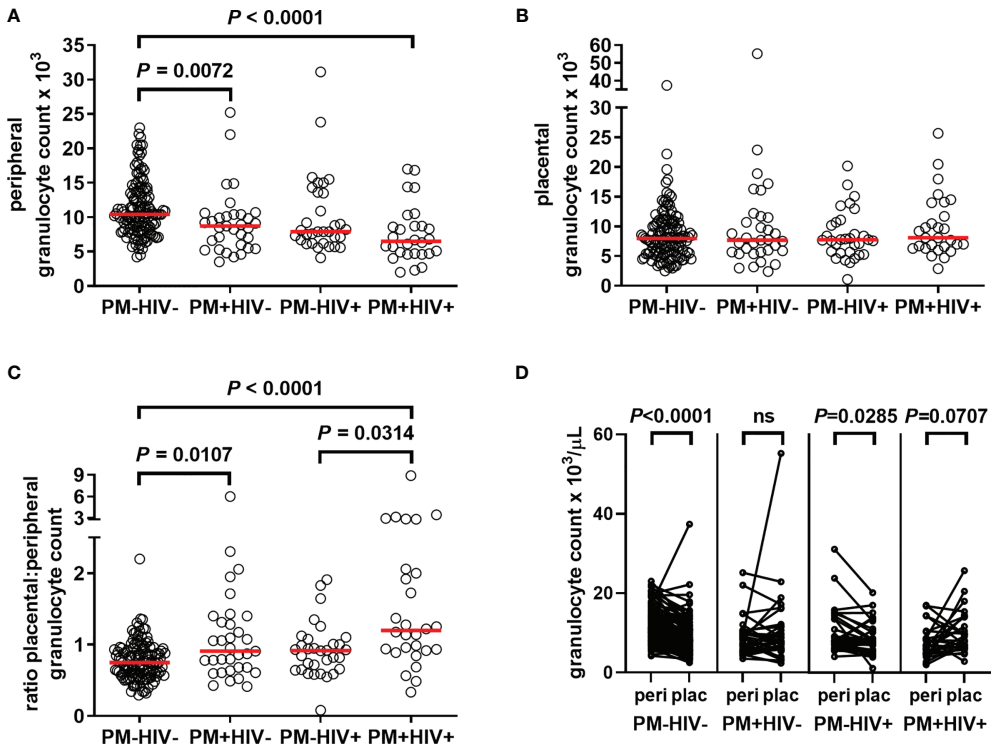

FIGURE 2 | Placental malaria and HIV infections differentially alter granulocyte levels in peripheral and placental blood. Peripheral and placental blood were subjected to complete blood count. Granulocyte numbers in **(A)** peripheral and **(B)** placental blood are shown. **(C)** Depicts the ratio of placental to peripheral blood granulocyte numbers, HIV-/PM-, n = 131; HIV-/PM+, n = 33; HIV+/PM-, n = 32; HIV+/PM+, n = 28. **(A–C)** Statistics by Kruskal-Wallis test with post-hoc group-wise comparisons by Dunn's multiple comparisons test. **(D)**, pairwise comparisons by Wilcoxon matched-pairs signed rank test. PM-=placental malaria negative; PM+=placental malaria positive; HIV-=human immunodeficiency virus seronegative; HIV+=human immunodeficiency virus seropositive.

Granulocyte Counts Correlate With Indicators of PM Severity but Not Birth Outcomes

PM often manifests as a chronic maternal inflammatory response dominated by accumulation of monocytes (5, 20, 26, 69). To assess the extent to which granulocyte counts might be influenced by severity of PM, correlation analysis of counts among PM+ women (combined HIV- and HIV+) were performed with parasite density and the percentage of WBCs bearing Hz in the placental blood space, which is taken as an indicator of chronicity of placental infection. The placental granulocyte count is unrelated to placental parasite density (**Figure 3A**) but positively correlates with Hz-bearing WBCs (**Figure 3C**). In contrast, the peripheral granulocyte count is inversely related to peripheral parasite density (**Figure 3B**) and Hz-bearing WBCs in the placenta (**Figure 3D**).

While granulocyte levels appear to be influenced by the intensity of the PM infection, no relationship with infant birth weight or gestational age is evident (**Supplemental Table 2**) nor do counts vary as a function of maternal self-reported fever (data not shown).

Placental Blood MPO Increases With Placental Inflammation

Based upon parameters including presence of parasites, inflammatory cell infiltration, and presence of Hz, PM has been variously categorized in histopathological examination in attempts to summarize severity and longevity of PM and associated birth outcomes (70–73). As a first step toward probing functional attributes of neutrophils in PM, a pilot study was conducted to measure levels of MPO in placental plasma from HIV-seronegative primigravidae whose placentae were histologically categorized into four groups (uninfected, acute, chronic, and chronic, inflammatory infection). MPO is released upon activation of neutrophils in the blood and tissues into both the phagolysosomal compartment and the extracellular environment, as well as in NETs (74, 75). Relative to uninfected samples, placental plasma from tissues with evidence of chronic, inflammatory infection shows significantly elevated MPO levels (**Figure 4A**). Correspondingly, this group tends toward lower infant birth weights relative to uninfected women (**Figure 4B**). Placental MPO levels correlate positively with percent of Hz-bearing WBCs in the placenta (**Figure 4C**). MPO levels are

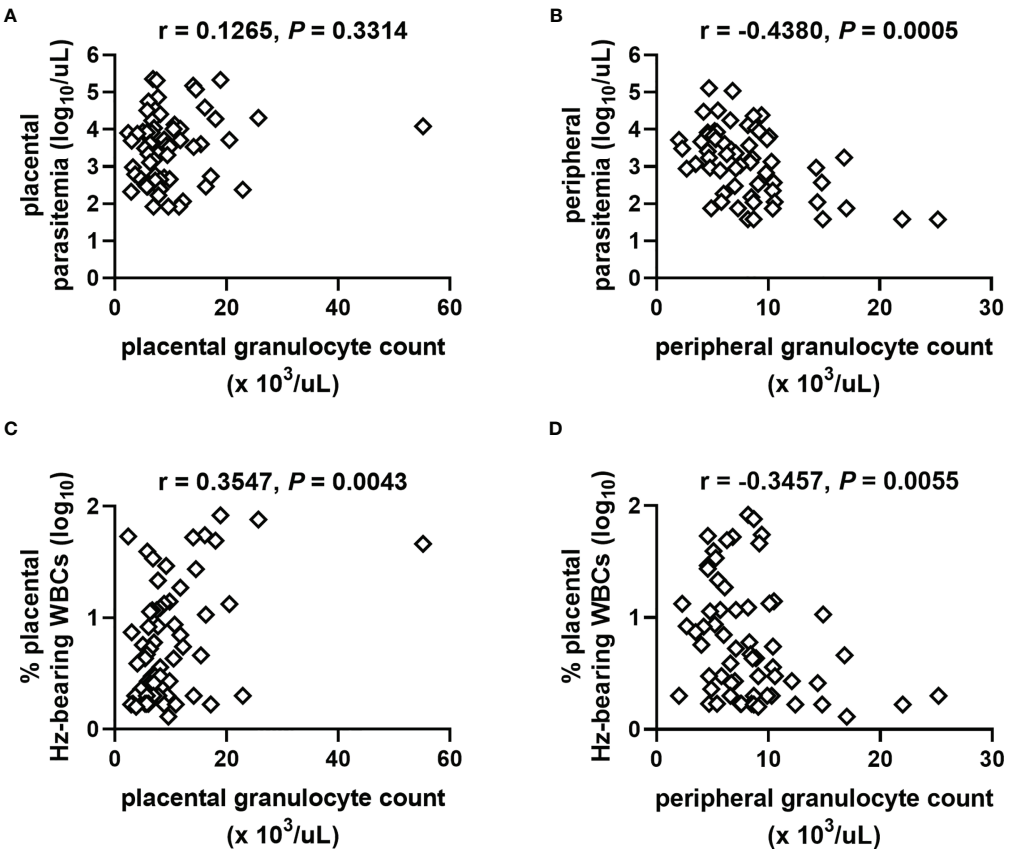

FIGURE 3 | Relationships of granulocyte numbers with measures of placental malaria vary as a function of blood source. Placental granulocyte counts measured by complete blood count in women with PM are unrelated to **(A)** placental parasite density but positively correlate with **(C)** the level of Hz-bearing WBCs in the placental intervillous space. Peripheral granulocyte counts negatively correlate with **(B)** peripheral parasite density and **(D)** level of Hz-bearing WBCs in the placental intervillous space. Both infection measures are log transformed. Results of Spearman's correlation analysis are shown. **(A, B)** n = 61; **(C, D)** n = 63.

elevated with self-reported fever in these women and tend toward higher levels in those delivering low birth weight infants (**Figure 4D**). Overall, these data indicate that high MPO concentrations are associated with PM, especially chronic, inflammatory infection, and relevant clinical outcomes.

Malaria and HIV Infection Impact Placental but Not Peripheral Levels of Soluble Markers of Neutrophil Activation

Building upon these initial observations, a larger analysis was undertaken to identify the extent to which malaria and PM/HIV co-infections in pregnant women impact indicators of neutrophil function. In addition to MPO, key markers of activated neutrophils, MMP9, and PRTN3, and a key neutrophil chemoattractant, CXCL8, were measured in peripheral and placental plasma of paucigravid women. In peripheral blood, MPO and CXCL8 levels are not impacted by PM, regardless of HIV infection status (**Figures 5A, B**). Additionally, peripheral MPO and CXCL8 levels are unrelated to infant birth weight and gestational age at birth (**Supplemental Figures 3E, F**). In contrast, placental blood levels of MPO are significantly elevated with PM and PM/HIV co-infection relative to uninfected and HIV+ women, respectively (**Figure 6A**), and in mothers who reported recent fever (**Figure 6B**). CXCL8 levels in PM+HIV+ women are elevated relative to levels in uninfected women (**Figure 6C**), and tend toward a weak enhancement with reported fever (**Figure 6D**). While MMP9 levels remain unchanged with infection (**Figure 6E**), levels are enhanced with reported fever (**Figure 6F**). Finally, like MPO, PRTN3 levels are enhanced by PM in both HIV- and HIV+ women (**Figure 6G**), and are increased with recent fever (**Figure 6H**). Despite associations with PM, none of the measured markers differ as a function of birth weight or gestational age (**Supplemental Figure 3**). Placental levels of all of these factors significantly positively correlate with each other (**Table 2**), and with the exception of CXCL8, all positively correlate with placental parasite density (**Figures 7A–D**), and percent Hz-bearing WBCs in the placenta (**Figures 7E–H**). However, contrary to expectation, none of these factors correlate with granulocyte counts or percentages (data not shown).

Placental MPO Detection by Immunohistochemistry and Western Blot

To further characterize MPO expression in the placenta, tissue sections from a subgroup of patients were probed using

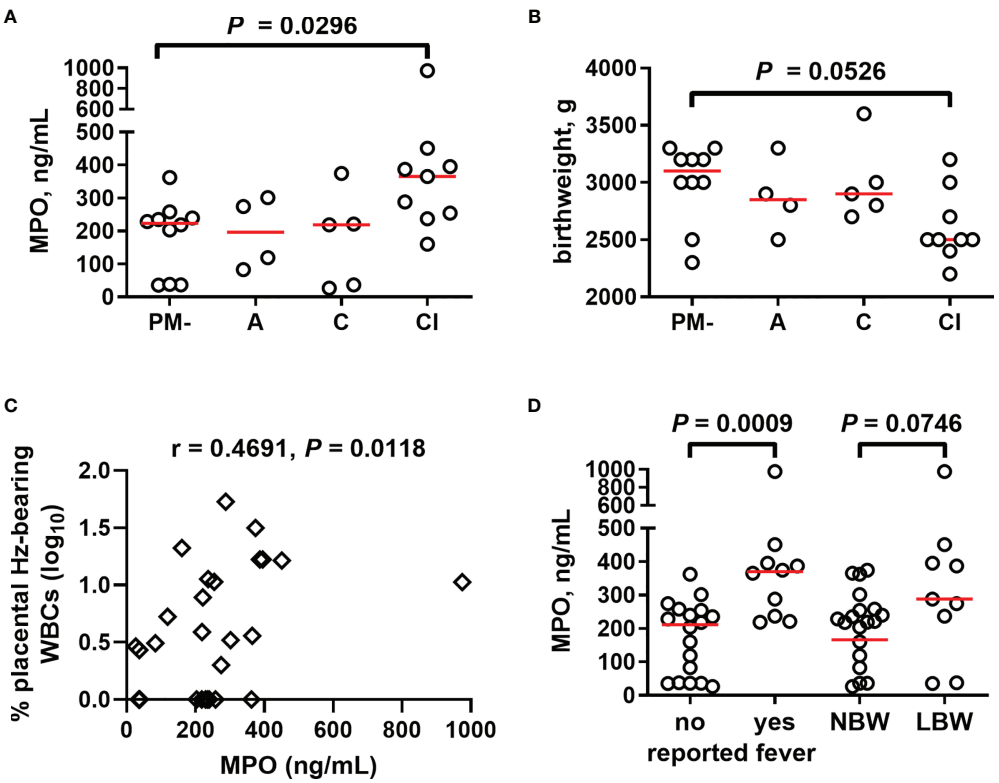

FIGURE 4 | Placental blood MPO levels increase in chronic, inflammatory placental malaria, together with reduced birth weight. **(A)** MPO measured in placental plasma by ELISA from primigravid women with and without PM stratified by placental histopathological status (see Methods). **(B)** Infant birth weight from the same women is similarly stratified. **(C)** MPO levels collectively analyzed by Spearman's correlation test with percent placental leukocytes (WBCs) bearing engulfed hemozoin. **(D)** Placental MPO levels measured by ELISA assessed for relationships with self-reported fever and infant birth weight. Statistics by Kruskal-Wallis test with post-hoc group-wise comparisons by Dunn's multiple comparisons test. PM- = placental malaria negative (n = 10); A = acute infection (n = 4); C = chronic infection (n = 5); CI = chronic inflammatory infection (n = 9); NBW= normal birth weight (n = 19); LBW = low birth weight (n = 9); no self-reported fever (n = 18), reported fever (n = 10).

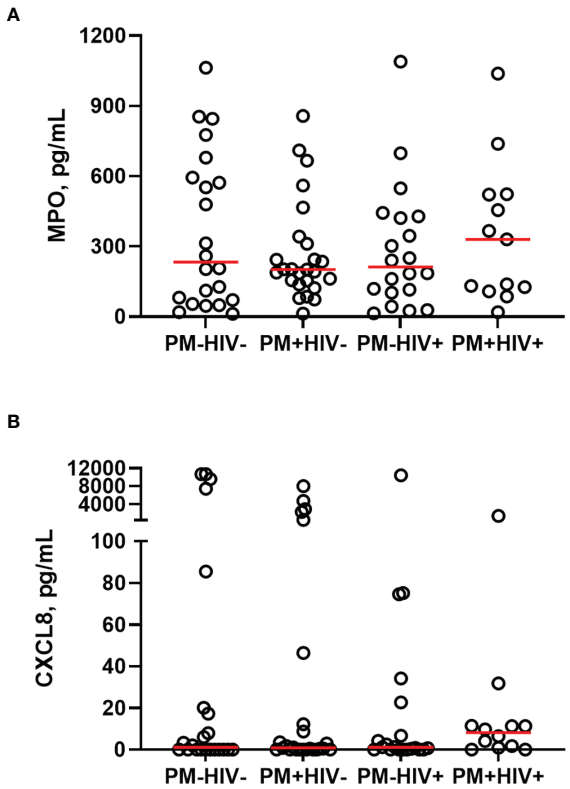

FIGURE 5 | Peripheral blood MPO and CXCL8 levels do not change as a function of placental malaria and HIV seropositivity. Peripheral plasma samples from paucigravid women were analyzed for **(A)** MPO and **(B)** CXCL8 levels by bead array. No statistically significant differences among the groups are evident (Kruskal-Wallis test with post-hoc group-wise comparisons by Dunn's multiple comparisons test). HIV-/PM-, n = 22; HIV-/PM+, n = 25; HIV+/PM-, n = 20; HIV +/PM+, n = 14 (MPO), n = 13 (CXCL8.) Groups as defined in **Figure 2**.

immunohistochemistry. MPO+ cells are frequently observed in these placentae, even in the absence of PM (**Figures 8A–D**). Frequency (**Figure 8E**) and intensity of MPO expression (**Figure 8F**), however, tend to be enhanced in PM+HIV- but not PM+HIV+ tissues. Probing for MPO by western blot of proteins derived from whole placental tissue may serve as a proxy for intensity of MPO expression, but preliminary analysis thereto does not reveal compelling evidence for influence of protein levels by infection status (**Supplemental Figures 4A, C**). Similarly, NE protein levels in whole placental tissue extracts are independent of infection status (**Supplemental Figure 4B, D**).

Plasma Markers of NETosis Are Not Affected by PM

Although NETosis has been linked to severe malaria in nonpregnant patients (38, 56, 57), this mechanism has not to our knowledge been explored in PM. Using detection of nonspecific (cell-free DNA; **Supplemental Figure 5A**) and known markers of NET formation (NE-DNA, **Supplemental Figure 5B;** cell-free histones, **Supplemental Figure 5C**), no evidence of significant PM-induced NETosis in placental plasma is observed in HIV-primigravidae (as in **Figure 4**), regardless of placental histopathological status. Similarly, citrullinated histone H3 (citH3), an additional marker of NET formation, does not differ in paucigravid placental plasma as a function of PM regardless of HIV infection (**Supplemental Figure 6A**), although elevated levels are evident in two PM+ women. Nonetheless, citrullinated histone H3 levels tend to positively correlate with placental granulocyte count (**Supplemental Figure 6B**).

In Situ Evidence for Netosis in Placental Tissue

As an alternate indicator of NETosis, fixed placental tissue was probed by immunohistochemistry for citH3. Some weakly positive cells are evident in the intervillous space, and, surprisingly, occasional stronger staining is seen in syncytiotrophoblast (**Supplemental Figure 7A, B**). Extracellular evidence of citH3, structures that would be consistent with NETosis, however, is rarely observed (**Supplemental Figure 4B**).

Although attempts to identify soluble components of NETosis in placental plasma and *in situ* in fixed, paraffin-embedded placental tissue does not provide compelling evidence of PM with or without HIV co-infection as a driver of placental NETosis, it is not possible to conclude that this process does not occur in PM, since sample collection, processing and storage, for example, could impede such detection. As an alternate approach, placental tissues flash-frozen in OCT were assessed by immunofluorescence staining for MPO, NE and DNA. In most samples, regardless of infection status, structures that are suggestive of NETs are evident (**Figure 9; Supplemental Figure 8**). The presumptive NETs are highly pleomorphic. Some structures are composed of single cells in the intervillous space that have streaky, comet-like nuclei and cytoplasm containing blue fluorescence (DNA) that colocalizes with green fluorescence (MPO) and red fluorescence (NE) (**Figures 9M–P, R–T**). Most frequently, the NETs are characterized by small to large aggregates of two or more pleomorphic cells with indistinct cell limits and pleomorphic nuclei encroached in a large MPO+/NE+ cytoplasm (**Figures 9M–O, Q–S**). Frequently, the cytoplasmic projections colocalize with MPO, NE and DNA material (**Figures 9M–O, Q–S**). These presumptive NETs are observed in maternal intervillous blood spaces, within the villus stroma, and occasionally inside the fetal vasculature (**Figures 9S, T**).

The small sample size of this experiment impacts statistical power; however, counts of singly stained MPO+, NE+ and MPO +/NE+ double stained cells do not vary with PM, including with stratification by HIV serostatus (**Supplemental Figures 8A–C**). Small, non-NETosing neutrophils (double MPO+/NE+, <102 μm^2) tend to be more numerous with PM (median, IQR, PM-: 12, 4.5 – 60; PM+: 22, 18 – 41; $P = 0.0928$) whereas larger putatively NETosing MPO+/NE+ cells are unchanged (median, IQR, PM-: 9.0, 2.0 – 35; PM+: 19, 15 – 34; $P = 0.3735$). Further stratification by HIV infection status yields no significant differences between the groups for small and large cells, including assessment of large NETosing cells as a percent of all MPO+/NE+ cells (**Supplemental Figures 8D–F**). Counts of MPO+/NE+ cells, including those classified as large, positively correlate with placental levels of CXCL8 (**Figures 10A, B**), with a similar tendency for small MPO+/NE+ neutrophils

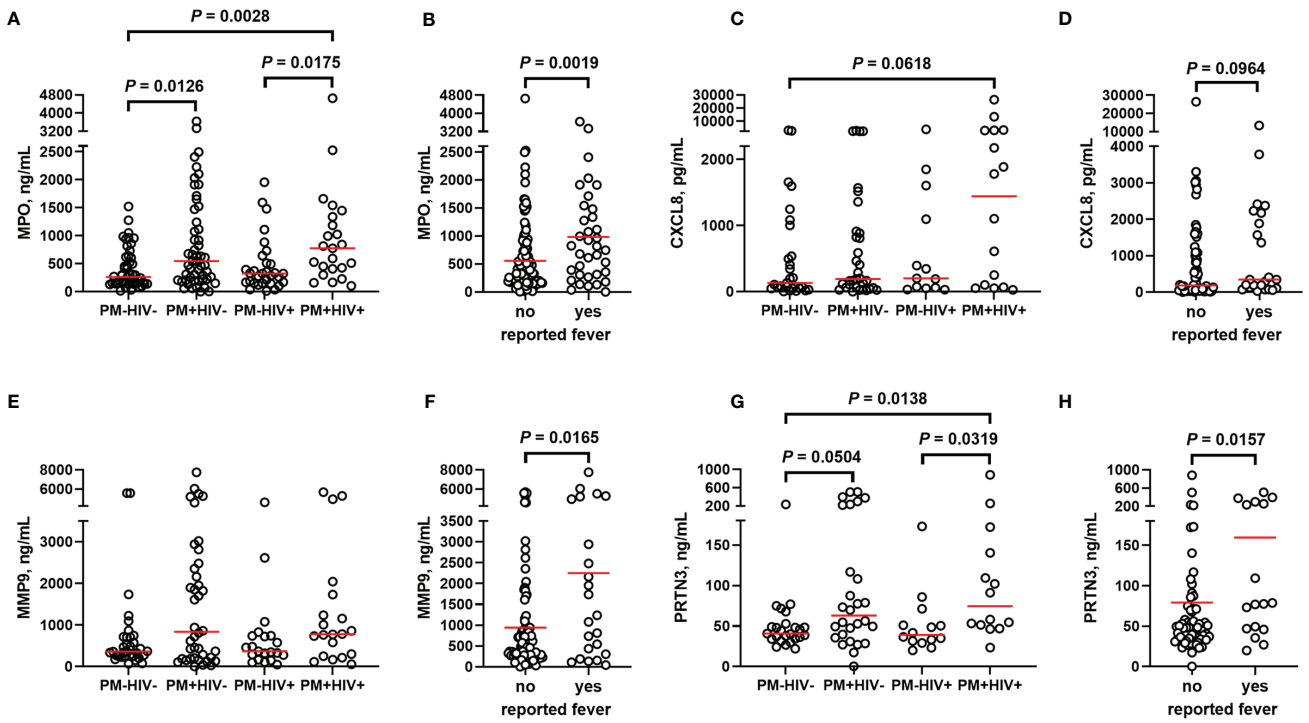

FIGURE 6 | Markers of neutrophil activation are variably altered by placental malaria and HIV seropositivity. Placental plasma from paucigravid women was analyzed for **(A, B)** myeloperoxidase (MPO), **(C, D)** CXCL8, **(E, F)** matrix metalloproteinase 9 (MMP9), and **(G, H)** proteinase 3 (PRTN3) levels by bead array and ELISA and stratified by **(A, C, E, G)** infection status and **(B, D, F, H)** self-reported fever (independent of infection status). **(A, C, E, G)** Statistics by Kruskal-Wallis test with post-hoc group-wise comparisons by Dunn's multiple comparisons test and **(B, D, F, H)** by Mann-Whitney U test. MPO: HIV-/PM-, n = 50; HIV-/PM+, n = 53; HIV+/PM-, n = 31; HIV+/PM+, n = 24. CXCL8, HIV-/PM-, n = 27; HIV-/PM+, n = 33; HIV+/PM-, n = 13; HIV+/PM+, n = 16. MMP9, HIV-/PM-, n = 35; HIV-/PM+, n = 40; HIV+/PM-, n = 21; HIV+/PM+, n = 21. PRTN3, HIV-/PM-, n = 25; HIV-/PM+, n = 28; HIV+/PM-, n = 14; HIV+/PM+, n = 15. Groups as defined in **Figure 2**.

(**Figure 10C**). Likewise, weak positive correlations are observed between MMP9 and MPO+/NE+ cells as well as small neutrophils (**Figures 10D, F**) but not between MMP9 and large cell count (**Figure 10E**). Placental levels of MPO are unrelated to these cell counts (**Supplemental Figure 9**).

DISCUSSION

In this study, we analyzed maternal peripheral and placental granulocyte levels as well as markers of neutrophil activation, including NET-specific markers and structures, in women with and without PM and HIV infection at the time of delivery. The results show that maternal peripheral granulocyte counts decrease with PM in the presence and absence of pre-existing HIV infection. Although a differential to discern neutrophil counts among the granulocytes was not performed, these findings are consistent with previous work that reported reduced neutrophil counts in malaria-infected pregnant compared to uninfected women (3, 76). A study in rhesus monkeys (*Macaca mulatta*) infected with *P. coatneyi* also showed a significant decrease in neutrophil levels starting at gestation week 9 (77). This contrasts with the observation that neutrophil counts are enhanced with HIV infection in pregnant women (78). Our results suggest that PM overcomes this apparent HIV-driven neutrophilia, since HIV seropositive women with PM

TABLE 2 | Correlation matrix of placental markers of neutrophil activation.

	MPO		MMP9		PRTN3		CXCL8	
	r, IQR*	*P*	r, IQR	*P*	r, IQR	*P*	r, IQR	*P*
MPO	1	-	0.7287, 0.6273 - 0.8058	<0.0001	0.9179, 0.8737 - 0.9471	<0.0001	0.4485, 0.2002 - 0.6426	0.0006
MMP9	0.7287, 0.6273 - 0.8058	<0.0001	1	-	0.7626, 0.6500 - 0.8424	<0.0001	0.3797, 0.0804 - 0.6161	0.0120
PRTN3	0.9179, 0.8737 - 0.9471	<0.0001	0.7626, 0.6500 - 0.8424	<0.0001	1	-	0.4106, 0.0615 - 0.6701	0.0196
CXCL8	0.4485, 0.2002 - 0.6426	0.0006	0.3796, 0.0804 - 0.6161	0.0120	0.4106, 0.0615 - 0.6701	0.0196	1	-

**Data represent results of Spearman's test with IQR, interquartile range.*

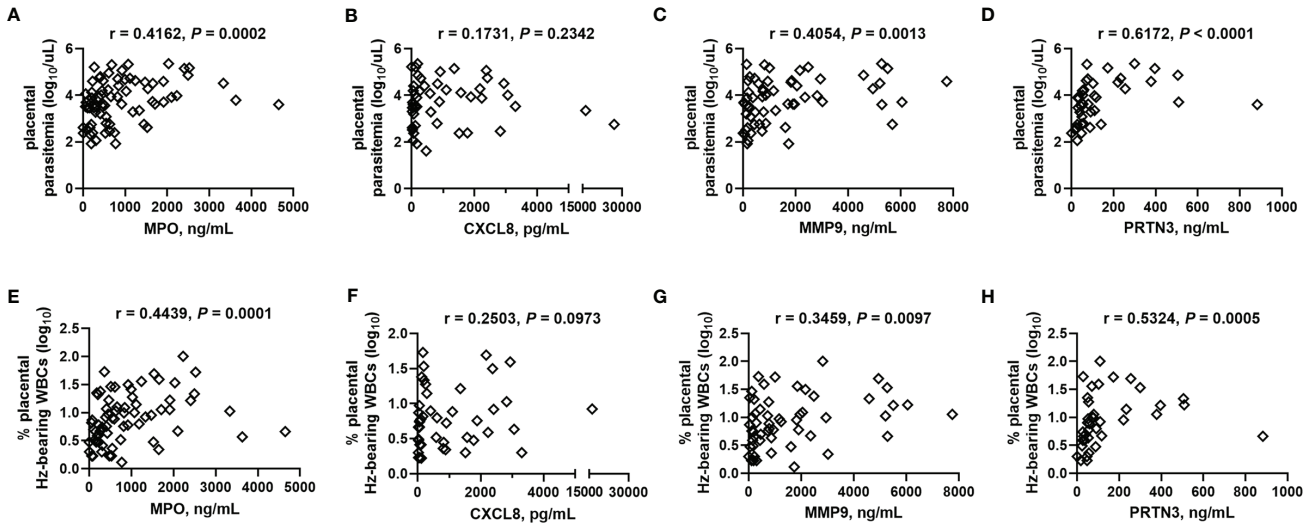

FIGURE 7 | Placental blood MPO, MMP9, and PRTN3 levels positively correlate with placental parasite density and hemozoin-bearing white blood cells. **(A, E)** MPO, **(B, F)** CXCL8, **(C, G)** MMP9, and **(D, H)** PRTN3 levels measured by bead array in plasma are collectively analyzed by Spearman's correlation test with **(A–D)** thick smear placental parasite density (log transformed) and **(E–H)** percent placental WBCs bearing engulfed hemozoin (log transformed). **(A)**, n = 77; **(B)**, n = 49; **(C)**, n = 61; **(D)**, n = 43; **(E)**, n = 73; **(F)**, n = 47; **(G)**, n = 57; **(H)**, n = 40.

have significantly suppressed granulocyte counts in the peripheral blood.

Contrary to expectation, no differences in granulocyte counts in the placenta are evident in this cohort of women. However, the enhanced ratios of placental to peripheral blood granulocyte numbers at the population level support the conclusion that during PM and PM/HIV co-infection, granulocytes accumulate in the placenta at the expense of the periphery, or, at minimum, are relatively more stable in the placenta. The physiological control of this phenomenon is worthy of further investigation, particularly given our observation that overall, granulocytes make up a lower proportion of total leukocytes in the placenta than in the peripheral blood and thus may be differentially regulated in these two blood spaces. Neutrophils are a key component of the normal process of labor, with accumulation of these cells in the uterine wall; the reductions observed here may be indicative of an exodus of neutrophils from the intervillous space to the myometrium [reviewed in (79)]. Despite the tendency toward lower granulocyte percentages and counts in placental relative to peripheral blood in the absence of infection, pairwise comparison at the individual patient level shows an overall tendency for granulocyte counts in placenta to increase uniquely with PM/HIV co-infection. However, granulocytes as a percent of total WBCs in the placenta are significantly reduced with co-infection. This may indicate that the massive overall increase in placental WBCs seen in this group (**Supplemental Table 1**) is heavily attributable to other cell subsets, likely monocytes (20).

Other researchers have reported significant accumulation of neutrophils in the placenta with PM (19, 20). What may be driving accumulation or preservation of these cells in the maternal placental blood, and why the current study shows placental granulocyte count stability but not accumulation with PM is unclear. Aside from the obvious differences in methodology and cell identification, placental granulocyte levels as measured here appear to be influenced by infection intensity, as counts positively correlate with the percent of placental WBCs bearing phagocytosed Hz. Thus, differences across studies could also be due to differences in infection intensity or chronicity. In general, local production of chemokines, including CXCL8, a factor that promotes neutrophil chemotaxis (80), may be an important determining factor in granulocyte/neutrophil presence in the intervillous blood, as is the case for recruitment to the uterus at parturition [reviewed in (79)]. Importantly, CXCL8 is elevated in PM+HIV+ placentae. The source of CXCL8 in the placenta that might participate in this response is not clear but could be maternal monocytes (22, 26), fetal syncytiotrophoblast (3, 22, 81, 82), or uterine/decidual stromal cells (83).

Previous studies have indicated that neutrophils may play a pathogenic role in PM and could serve as prognostic markers for malaria-associated low birth weight (18). We report that in this population of parturient women, granulocyte counts do not associate with infant birth outcomes. This may be due to inadequate sample size, lack of differential analysis to directly count neutrophils, or may be related to the overall patient recruitment strategy, which excluded complicated pregnancies and deliveries and health issues other than malaria and HIV infections. Because an interesting relationship between neutrophils and malaria parasites is emerging [reviewed in (36)] and neutrophils are key cells at multiple stages of normal and abnormal pregnancy [reviewed in (79)], it is imperative for future studies to definitively identify neutrophils in the granulocyte population, and to further consider neutrophil

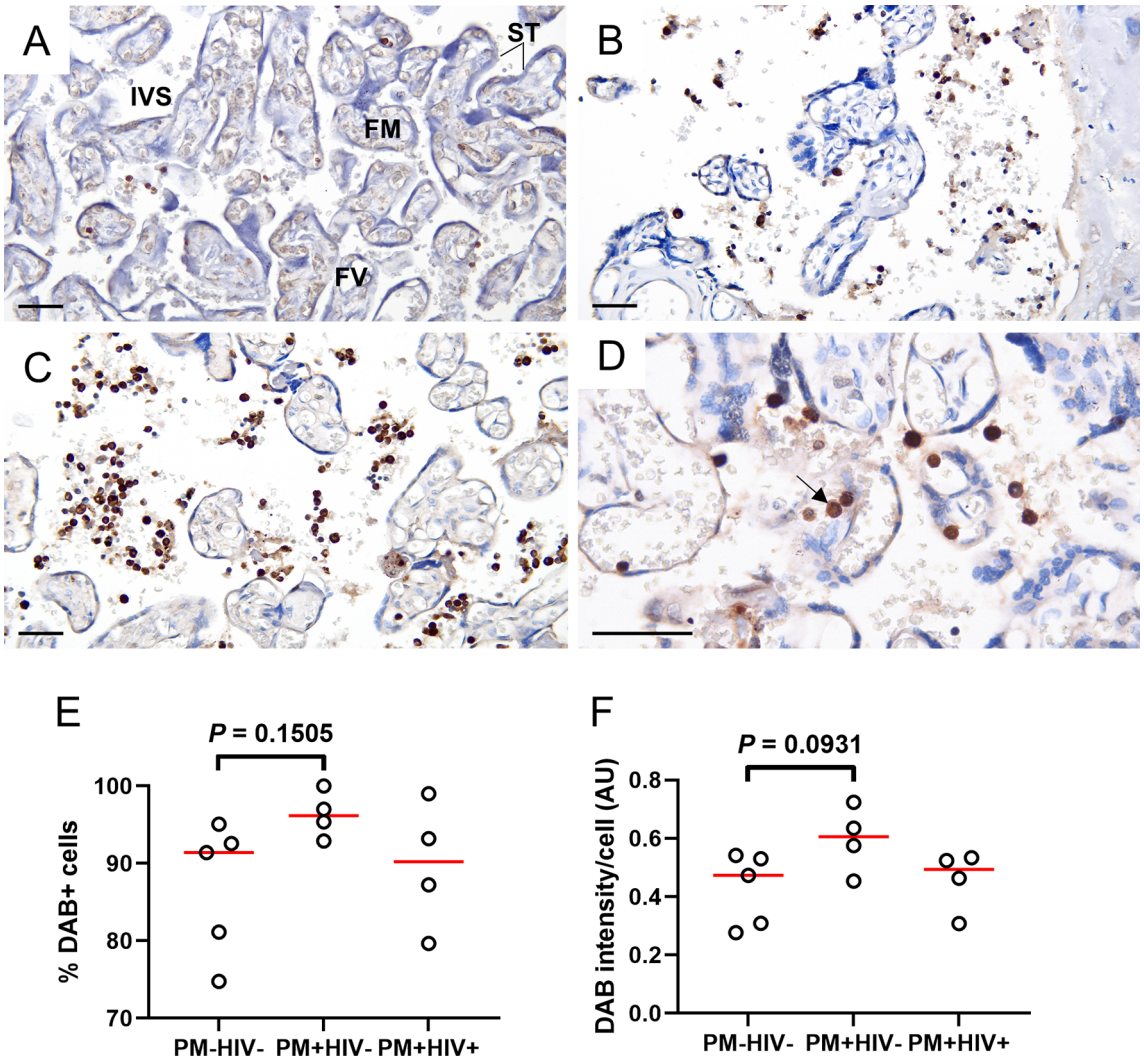

FIGURE 8 | Placental WBCs from PM+HIV- women show more robust MPO expression by immunohistochemistry relative to uninfected and co-infected women. Frequency and intensity of MPO staining is lower in **(A)** uninfected placenta and **(B)** PM+HIV+ placenta relative to **(C)** PM+HIV- placenta. **(D)** Segmented nucleus clearly delineates MPO staining in a neutrophil (arrow). ST, syncytiotrophoblast; FM, fetal mesenchyme; FV, fetal vessel; IVS, intervillous space. Scale bars represent 50 µm. **(E)** Percent cells expressing MPO weakly tends to be higher in PM+HIV- relative to uninfected women. **(F)** MPO staining intensity tends to be higher in PM+HIV- relative to uninfected women. Statistics by Kruskal-Wallis test with post-hoc group-wise comparisons by Dunn's multiple comparisons test. PM-/HIV-, n = 5; PM+/HIV-, n = 4; PM+/HIV+, n = 4. Groups as defined in **Figure 2**.

subsets and functional parameters to discern potential relationships with pathogenic outcomes of PM.

One of the many consequences of neutrophil activation is the secretion of granules containing MPO, MMP9, NE, and PRTN3. Neutrophils produce massive amounts of these proteins, in the case of MPO, representing 5% of the total cellular protein (84). Our data show for the first time that placental blood MPO and PRTN3 levels are elevated with PM in both HIV- and HIV+ paucigravid women relative to PM-HIV- women. It was unexpected to find that none of these markers correlates with placental granulocyte counts, emphasizing the need for further work to definitively identify the source of these factors in placental plasma and fully characterize neutrophil function in PM. Importantly, observations of MPO in fixed placental tissue sections suggest that this factor may be produced by both intervillous neutrophils and monocytes, yet most MPO-expressing cells observed by immunofluorescence are co-stained for NE, identifying them as neutrophils.

Placental levels of MPO, MMP9, and PRTN3 all positively correlate with placental parasitemia as well as placental hemozoin bearing WBCs, suggesting that production is enhanced by chronic infection. Since MPO attenuates pathogen clearance during *P. yoelii* nonlethal infection (85), it is tempting to speculate that this enzyme may inhibit parasite clearance in PM as well. In an initial pilot study, we found placental blood MPO levels to be increased significantly with

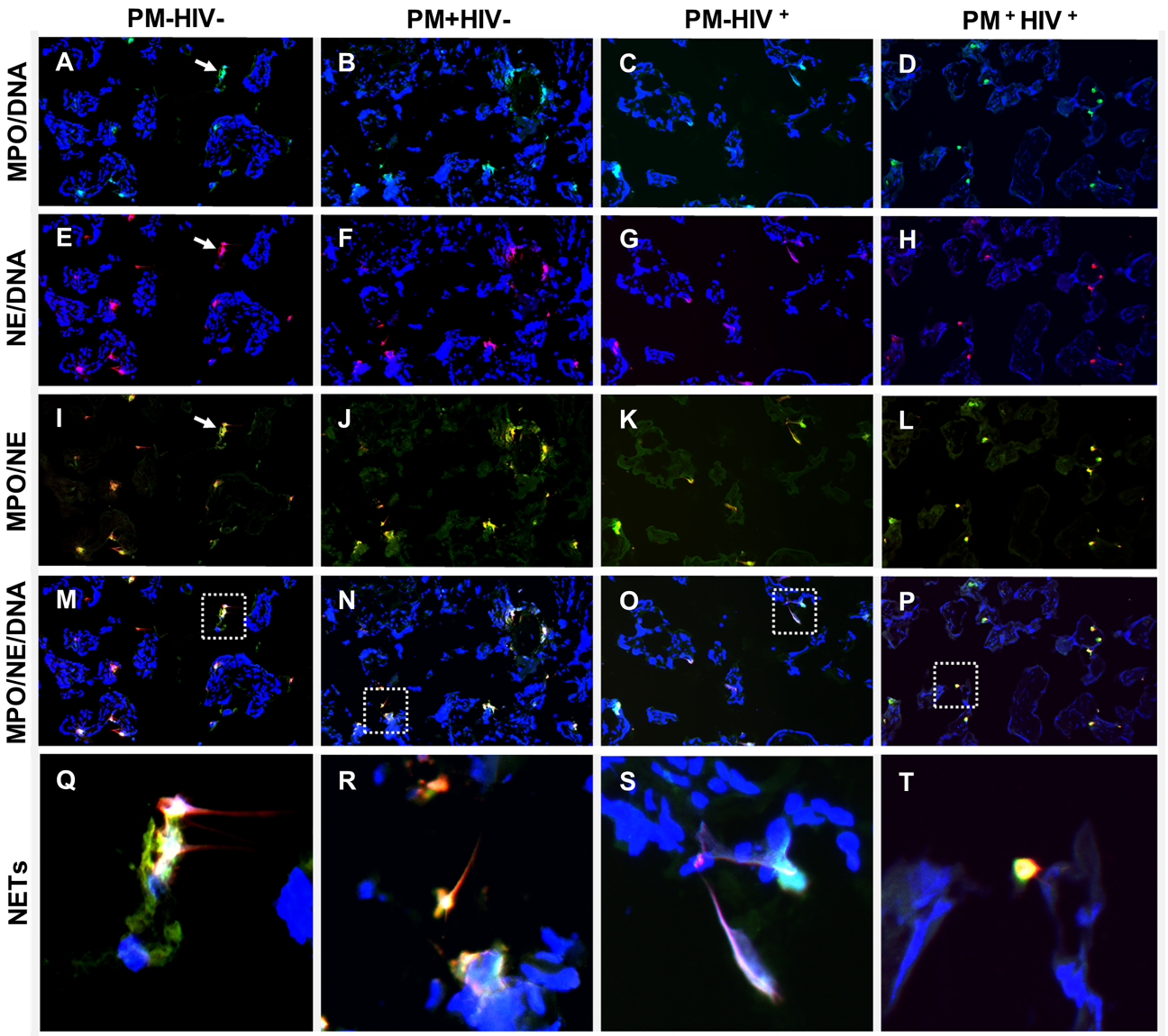

FIGURE 9 | Detection of NETs by immunfluorescence in placental tissue. Immunolocalization of MPO (green), NE (red) in term placenta. The DNA was labeled with Hoescht 33342 (blue). **(A, E, I, M, Q)** uninfected placenta; **(B, F, J, N, R)** PM+HIV- placenta; **(C, G, K, O, S)** PM-HIV+ placenta; and **(D, H, L, P, T)** PM+HIV+ placenta. The first row **(A–D)** shows the colocalization of MPO with DNA, the second row **(E–H)**, colocalization of NE and DNA, the third row **(I–L)**, colocalization of MPO and NE and the fourth row **(M–P)**, colocalization of MPO, NE and DNA. The last row shows in higher magnification the dotted areas in panels **(M–P)** that contain putative NET-like structures **(Q–T)**. Arrows in panels **(A, E, I)** indicate NET-like structures. Groups are as defined in **Figure 2**.

chronic, inflammatory PM in primigravidae. MPO is associated with vascular dysfunction (86) and underlies the pathophysiology of numerous vascular inflammatory diseases including arteriosclerosis and coronary artery disease (87, 88). Inflammation and endothelial dysfunction are characteristics of preeclampsia, and increased MPO levels in placental and peripheral circulation in preeclamptic women have been described (89, 90). The extent to which PM and PM/HIV coinfection may contribute to preeclampsia *via* MPO production or modification of other neutrophil functions remains to be determined. This may be an exciting avenue to pursue given increasing evidence that malaria predisposes women to this hypertensive disorder (91, 92).

Elevated neutrophil activation, as evidenced by higher plasma concentrations not only of MPO but also of PRTN3 and NE, is associated with severe pediatric malaria (39). Likewise, MMP9, an endopeptidase released by neutrophils and monocytes, is implicated in the pathogenesis of severe malaria (93–95). An MMP9 polymorphism protects against PM, further implicating an important role for this enzyme in *P. falciparum* infection (96). Importantly, MPO, MMP9 and PRTN3 levels in the placenta associate with self-reported fever in this cohort of parturient

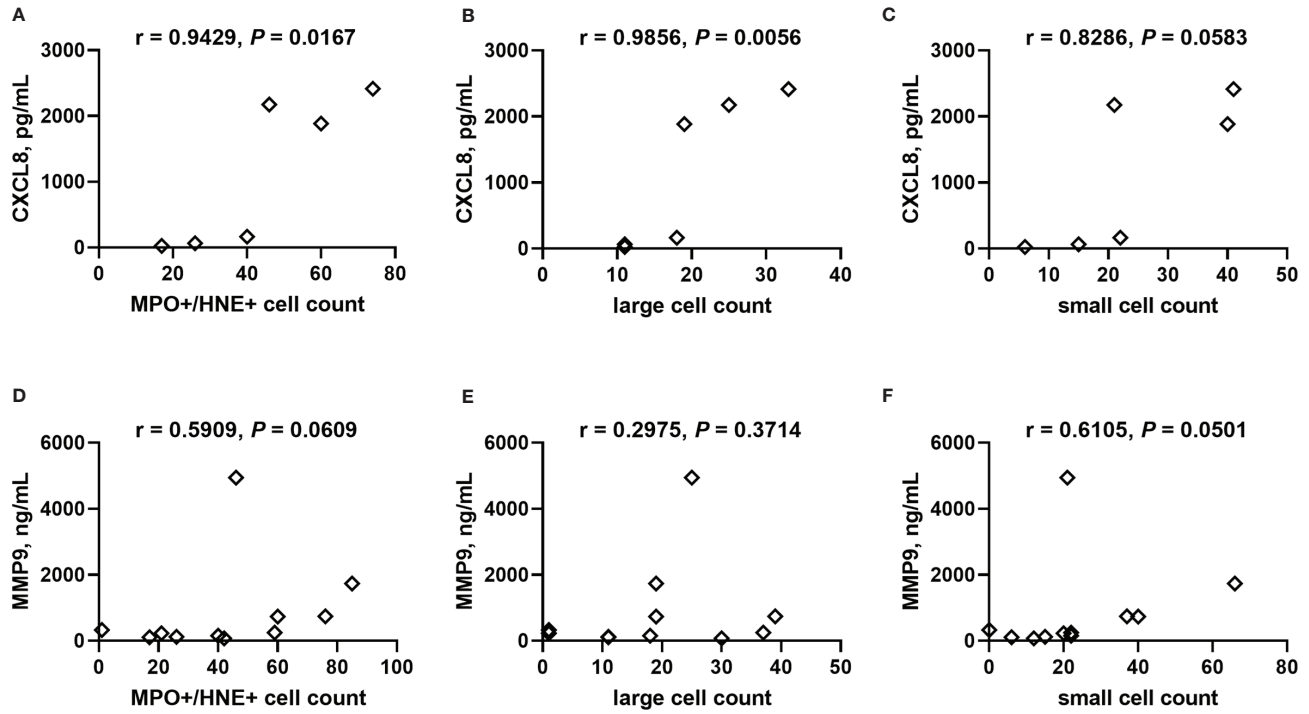

FIGURE 10 | Correlation analysis of CXCL8 and MMP9 with neutrophils in placental tissue. Counts of neutrophils detected in frozen cryosections as **(A, D)** MPO+/NE+, and MPO+/NE+ cells stratified by size as **(B, E)** large: >102 μm^2, and **(C, F)** small: <102 μm^2 collectively correlated with placental plasma levels of **(A–C)** CXCL8 and **(D–F)** MMP9. Cell counts determined by analysis of immunofluorescence of tissues. Statistics by Spearman's correlation test. PM-/HIV-, n = 5; PM+/HIV-, n = 5; PM-/HIV+, n = 4; PM+/HIV+, n = 4. Groups as defined in **Figure 2**.

women. However, while pilot data in HIV seronegative primigravidae suggest a tendency for placental MPO to be higher in women with low birth weight infants, in a larger cohort of paucigravid malaria-exposed women that also considered HIV infection, no relationship with birth weight or gestational age emerges for any of these markers. This is unexpected given that the factors do correlate with parameters (placental parasite density and Hz-bearing WBCs) that are typically associated with poor outcomes in PM. Indeed, Hz-bearing neutrophils in the peripheral blood of pregnant women predict low birth weight (18). While additional work will be required to resolve this discrepancy, these results suggest that the mechanism by which neutrophils mediate poor birth outcomes may not be directly related to the release of neutrophil granules in the placental blood space. One potential alternate mechanism may be downstream effects of these factors, which were not measured here. High concentrations of neutrophil-derived antimicrobial compounds can make these cells detrimental to the host (97–99). For example, activated neutrophils exacerbate preeclampsia by releasing ROS (100–105). Of note, MPO catalyzes the formation of aggressive reactive oxygen intermediates, including hypochlorous, hypobromous, and hypothiocyanous acids, respectively (106, 107), which contribute to oxidative killing (108). Oxidative stress is a feature of PM (43, 44), but the extent to which neutrophils contribute to it remains to be established.

Contrary to expectation, this study finds no evidence of a relationship between PM and levels of soluble markers of NETosis in the placenta. Similarly, while structures consistent with NETs (MPO, NE, and DNA co-localization) are observed in the intervillous space, a relationship with PM is not evident. Alternatively, non-activated, resting, "small" neutrophils tend to be elevated with PM in this study. Because NETs have been observed in the placental intervillous space in pregnancies complicated by preeclampsia (103, 105), and given parallels between PM and preeclampsia, it was our expectation that NETosis would be enhanced in placentae of infected women. While further research designed specifically to address this question is warranted, it is tempting to speculate that NETosis may be subject to unique and as yet poorly understood control mechanisms in the placenta that are not specifically activated or perturbed by PM. The overall reduction of granulocyte counts in the placenta relative to the peripheral blood hints at this possibility.

In conclusion, the present study demonstrates that PM and PM/HIV co-infection perturb granulocyte levels, and soluble signatures of neutrophil activation associate with indicators of PM infection and associated symptoms. The findings do not authoritatively distinguish between a protective or pathogenic role for neutrophils or products of their activation, nor is an association of PM with NETosis established. Further exploration of neutrophil function in the context of malaria and HIV in pregnant women, particularly direct assessment of activity, is required to fill remaining gaps in knowledge.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study supporting collection of samples used in this report was approved by the Kenya Medical Research Institute Ethical Review Committee, and the Centers for Disease Control and Prevention and the University of Georgia Institutional Review Boards. All study participants provided written informed consent before enrollment and procedures and instruments involving human subjects, sample collection and data analysis, processing, and testing were approved throughout the conduct of patient recruitment. All samples and data are anonymized.

AUTHOR CONTRIBUTIONS

JMM, LJO, BR, and DS conceived and designed the experiments. LA, JDM, JMM, SM, and SO coordinated sample and clinical data collection. FA, JMM, LJO, SO, BR, BNR, and DS performed the experiments. LJO performed the Qupath analysis and prepared micrographs. JMM, LJO, BNR, and DS prepared the figures and tables. JMM performed descriptive statistical analyses. JMM, LJO, and DS wrote the manuscript. JV provided logistical and infrastructural support for sample collection, processing, storage and shipment, and facilitated local ethical reviews in Kenya. All authors (with the exception of JV, deceased) contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.682668/full#supplementary-material

Supplementary Table 1 | Pairwise comparison of peripheral and placental white blood cell (WBC) and granulocyte counts. PM = placental malaria; HIV = human immunodeficiency virus. Data are shown in graphical form in **Supplemental Figure 2A**, and **Supplemental Figure 2B**.

Supplementary Table 2 | Granulocyte numbers as a function of infant birth weight and gestational age at birth. NBW, normal birth weight, LBW, low birth weight; TB, term birth; PTB, preterm birth.

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