AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology and Molecular Biology Reviews

Variable surface antigen expression, virulence, and persistent infection by *Plasmodium falciparum* malaria parasites

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SUMMARY The human malaria parasite *Plasmodium falciparum* is known for its ability to maintain lengthy infections that can extend for over a year. This property is derived from the parasite's capacity to continuously alter the antigens expressed on the surface of the infected red blood cell, thereby avoiding antibody recognition and immune destruction. The primary target of the immune system is an antigen called PfEMP1 that serves as a cell surface receptor and enables infected cells to adhere to the vascular endothelium and thus avoid filtration by the spleen. The parasite's genome encodes approximately 60 antigenically distinct forms of PfEMP1, each encoded by individual members of the multicopy *var* gene family. This provides the parasite with a repertoire of antigenic types that it systematically cycles through over the course of an infection, thereby maintaining an infection until the repertoire is exhausted. While this model of antigenic variation based on *var* gene switching explains the dynamics of acute infections in individuals with limited anti-malarial immunity, it fails to explain reports of chronic, asymptomatic infections that can last over a decade. Recent field studies have led to a re-evaluation of previous conclusions regarding the prevalence of chronic infections, and the application of new technologies has provided insights into the molecular mechanisms that enable chronic infections and how these processes evolved.

KEYWORDS PfEMP1, *var* genes, chronic infections, transcriptional regulation, immune evasion

INTRODUCTION

The burden of malaria on the health and economic productivity of large portions of the developing world remains substantial, especially in sub-Saharan Africa (1). Intervention strategies designed to reduce morbidity and mortality are largely focused on insecticide-treated bed nets and mosquito control to prevent infection combined

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The authors declare no conflict of interest.

See the funding table on p. 16.

Published 14 January 2025

with the use of effective antimalarial drugs to treat disease. Vaccines are now slowly being introduced; however, their availability and efficacy remain relatively low, reducing their overall impact on the burden of disease. The continued reliance on drug treatment to lessen the impact of malaria makes communities vulnerable to the ever-present threat of drug resistance, which is beginning to emerge in both Asia and sub-Saharan Africa to the current cohort of preferred antimalarial treatments (2, 3). These conditions indicate that malaria is likely to continue to be a major global health problem for the foreseeable future. Nonetheless, significant efforts are underway to eliminate malaria from specific regions of the world in hopes of eventually achieving eradication; therefore, a better understanding of exactly how malaria parasites are transmitted and maintain chronic infections, particularly as intervention strategies and climate change alter transmission frequencies in many geographical regions, is of paramount importance.

In regions of moderate to high levels of malaria transmission, the bulk of the morbidity and mortality is found within children under 10 years old (4). As transmission intensity increases, the age of vulnerability decreases, indicating that as individuals experience more infections as they age, they steadily gain some degree of clinically protective, but non-sterile immunity (5, 6). Thus, while the greatest emphasis for reducing malaria disease burden is focused on drug and vaccine development, it is important to note that the bulk of the population in an endemic area is not protected from disease by drugs, vaccines, or mosquito preventative measures, but rather by acquired immunity. Interestingly, the immunity acquired after repeated exposure to infection by malaria parasites is generally not sterile but rather protects against severe symptoms of the disease instead of infection. It is becoming evident that significant numbers of individuals in endemic regions harbor asymptomatic, chronic infections that are typically undiagnosed (7–9). In addition, recent work indicates that the bulk of transmission is likely from individuals with asymptomatic infections (10), who usually have very low parasitemias. Importantly, these individuals seldom seek treatment and, thus, can remain a source of disease transmission as long as their infections persist. Clinical data indicate that at least some such asymptomatic infections can last over a decade, only becoming evident upon splenectomy or the onset of immunosuppression (11, 12). Given the important role that asymptomatic individuals play in the persistent transmission of parasites, understanding the nature of these lengthy, untreated infections will be key for designing and assessing the effectiveness of intervention strategies as they are implemented.

Since the development of clinically protective, but non-sterile immunity is directly related to the number of infections a person has endured and develops sooner in regions with higher transmission rates (4–6), anything that leads to significant changes in malaria transmission frequency will have concomitant consequences for the acquisition of immunity. In recent decades, global efforts to lessen malaria incidence have greatly reduced the numbers of infections and deaths attributed to the disease (1), and it is hoped that maintaining and extending these efforts will continue to decrease the number of infections in highly endemic regions. While this is a decidedly desirable outcome, it could potentially also reduce the level of immunity and consequently increase the numbers of people who are vulnerable to symptomatic infections (13). Such changes in disease susceptibility were previously observed after unsuccessful malaria elimination campaigns (14), and similar effects could result from altered infection dynamics due to climate change and improved healthcare infrastructure and eradication efforts (15, 16). This provides additional impetus for better understanding of the nature of chronic infections, how they are maintained, and their contribution to disease transmission. These attributes will be particularly important as efforts to eliminate the disease move forward.

MAINTENANCE OF ACUTE INFECTIONS THROUGH ANTIGENIC VARIATION

Plasmodium falciparum is a species of malaria parasites responsible for the majority of morbidity and mortality globally and in sub-Saharan Africa (1). These parasites are transmitted by anopheline mosquitos and, after an asymptomatic liver stage, invade and replicate within the red blood cells (RBCs) of their host. Each replicative cycle takes approximately 48 hours and typically yields between 20 and 30 new parasites (called merozoites) that are released upon lysis of the host cell to invade new RBCs. During each replicative cycle, the parasite makes significant alterations to the host RBC that greatly change its shape and decrease its deformability. These altered properties make the infected cell vulnerable to filtration and destruction during passage through the spleen, a fate that must be avoided to perpetuate an infection. Parasites have therefore evolved the ability to modify the infected RBC through the placement of a parasite-produced receptor called Erythrocyte Membrane Protein 1 (PfEMP1) on the RBC surface where it binds to ligands on the vascular endothelium (17). The parasite-derived adhesive properties enable infected cells to avoid splenic clearance; however, these changes can cause vascular obstruction and inflammation through the adhesion of infected cells to the post-capillary endothelium (18, 19); thus, PfEMP1 is thought to be the primary virulence factor of malaria caused by infection with *P. falciparum*. The placement of PfEMP1 on the infected RBC surface makes it vulnerable to immune recognition and the generation of antibodies that can bind to the infected RBC, leading to its destruction and the death of the parasite. A high-titer antibody response typically takes approximately a week to develop, providing the parasites with an opportunity to proliferate and expand into populations that can number in the trillions of individual cells. Once antibody titers have risen to sufficient levels, the parasitemia is rapidly reduced and in some instances can fall below the limit of detection. However, small subpopulations of parasites can arise that express an alternative form of PfEMP1 that is not recognized by antibodies against the preceding form of the protein, thereby enabling the parasites to establish a new wave of the infection. This pattern of rising and falling populations of antigenically distinct parasites is the result of antigenic variation, the capacity of the parasite to systematically alter the antigen displayed to the immune system, and is typical of acute, symptomatic infections (20). The biology of PfEMP1, cytoadherence, and antigenic variation are summarized in Fig. 1.

Different forms of PfEMP1 are encoded by individual members of the 45- to 90-member *var* gene family (21–23). These genes are distributed throughout the parasite's genome, with approximately two-thirds of the family found within subtelomeric regions and the remainder in tandem arrays in the central regions of the chromosomes (24). Based on their chromosomal location, promoter sequences, and domain composition, *var* genes are divided into different types including A, B, C, D, and E types (25, 26). Types A and B are typically found in the subtelomeric regions while type C genes are situated in internal chromosomal regions. D and E classes contain only one gene each, *var1csa* and *var2csa*, respectively. Each *var* gene is composed of two exons; exon 1 encodes the extracellular domain, which contains multiple Duffy binding-like and cysteine-rich interdomain region domains responsible for binding to host receptors, while exon 2 encodes a highly conserved transmembrane region and a cytoplasmic acidic terminal sequence that anchors the protein to the erythrocyte membrane (22, 24). The different forms of PfEMP1 encoded by each *var* gene exhibit unique binding phenotypes, allowing the parasite to bind to various host endothelial receptors, for example, CD36, ICAM-1, EPCR, and CSA, leading to the sequestration of infected RBCs within tissues that express these ligands. The different binding phenotypes enable the parasite to adhere within a variety of host tissues, influencing disease manifestations and complicating the host's immune response. Some examples of the best studied host receptors, the PfEMP1 types that bind to them, and the resulting disease manifestations are summarized in Table 1.

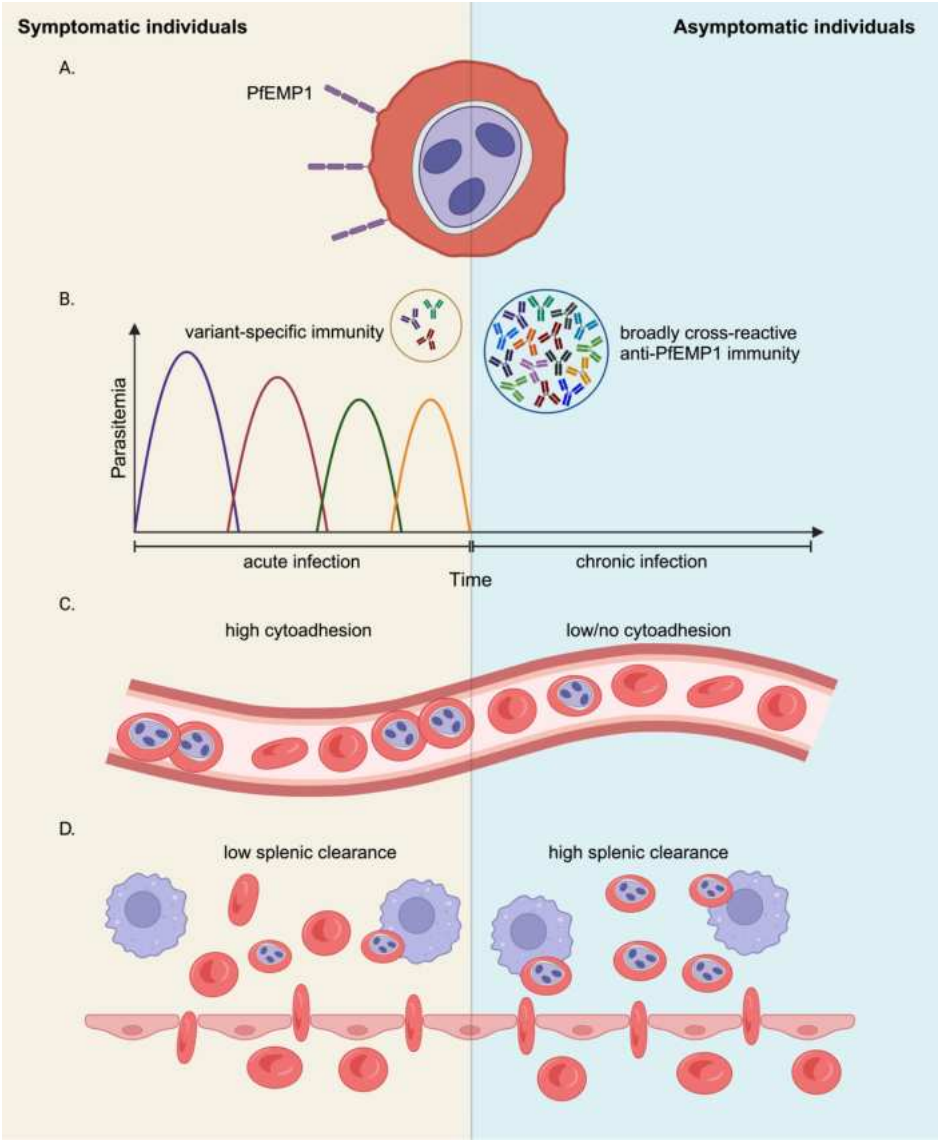

FIG 1 The role of PfEMP1, anti-PfEMP1-acquired immunity, cytoadherence, and mechanical splenic clearance in symptomatic and asymptomatic individuals. *P. falciparum* parasites can modify the surface of the infected RBC, enabling them to cytoadhere to several endothelial receptors and neighboring RBCs using a parasite-encoded protein called PfEMP1. (A) In symptomatic individuals (left, yellow background), PfEMP1 is anchored in knob-like structures on the surface of infected RBCs, enabling their cytoadherence to vascular endothelial receptors. This adhesion allows sequestration within the microvasculature of various organs, contributing to the development of malaria symptoms. In contrast, in asymptomatic individuals (right, blue background), mature parasites are under strong selection pressure from anti-PfEMP1 immunity, which forces them to silence their *var* gene repertoire, thereby halting PfEMP1 expression. This adaptation helps the parasite evade the host's immune response, allowing for a persistent, asymptomatic infection. (B) During acute infection, *P. falciparum* parasites undergo antigenic variation by switching the variant of PfEMP1 expressed on the surface of infected RBCs. This allows the parasite to evade the host's immune response, with each switching event corresponding to a new wave of parasitemia. In immunologically naïve individuals (left), the host's immune system generates variant-specific antibodies during each new wave, targeting infected RBCs and temporarily reducing parasitemia. However, a subset of parasites expressing a different PfEMP1 variant can escape immune detection and proliferate, causing the parasitemia to rise again. This cycle of immune evasion and parasite expansion leads to recurrent waves of parasitemia, enabling the infection to persist. Asymptomatic individuals (right) tend to be older and possess broadly cross-reactive anti-PfEMP1 immunity, acquired through repeated infections over their lifetime. In these individuals, parasites expressing PfEMP1 are efficiently targeted and eliminated by the immune system. Consequently, there is significant selection pressure favoring parasites that have silenced PfEMP1
(Continued on next page)

Fig 1 (Continued)

expression, allowing them to evade immune detection and persist at lower parasitemias. (C) In symptomatic individuals, parasites adhere to the microvasculature, avoiding splenic clearance by using cytoadhesive properties mediated by PfEMP1. In contrast, in asymptomatic individuals, parasites are rarely observed due to low parasitemia, but in instances of splenectomy, they are found circulating freely in peripheral blood, indicating a lack of cytoadhesive properties. (D) Parasites that exploit their cytoadhesive properties can avoid passage through the spleen, effectively evading splenic clearance. As a result, in symptomatic individuals, the spleen's ability to filter and remove infected RBCs is significantly reduced during parasite expansion. However, parasites that lack cytoadhesive properties are unable to evade the spleen's filtering mechanism and are more readily eliminated by splenic macrophages, contributing to lower parasitemia levels.

var TRANSCRIPTIONAL SWITCHING AND THE DEVELOPMENT OF CLINICAL IMMUNITY

The standard paradigm of antigenic variation in *P. falciparum* posits that *var* genes are expressed in a mutually exclusive fashion, with the single expressed gene encoding the form of PfEMP1 found on the infected cell surface (35). Antigenic variation occurs when the transcriptionally active *var* gene is silenced and a new *var* gene is activated, resulting in expression of a new PfEMP1. *var* gene activation and silencing are an epigenetic process mediated by post-translational histone modifications and do not typically involve alterations to the DNA sequence of the parasite's genome (35–39). While recombination events between *var* genes are known to contribute to the generation of diversity within the family (40–43), these events are considered relatively rare and not to contribute substantially to the overall length of an infection. Therefore, once a parasite has cycled through its entire collection of *var* genes and exhausted its antigenic

TABLE 1 *P. falciparum* parasites can express distinct types of PfEMP1 proteins during RBC infection, enabling them to bind to various host receptors and tissues, which contributes to diverse disease phenotypes

Host receptor	PfEMP1 binders	Disease phenotypes
CD36 (Cluster of Differentiation 36)	Many isoforms of PfEMP1, particularly types B and C, bind to CD36 receptors that are found on various endothelial cells (Robinson et al., 27).	This interaction facilitates sequestration of infected RBCs to the microvasculature and is often associated with non-severe malaria.
ICAM-1 (Intercellular Adhesion Molecule 1)	PfEMP1s often encoded by type A *var* genes bind to ICAM-1 on endothelial cells (Bengtsson et al., 28; Carrington et al., 29).	Binding to ICAM-1 has been associated with cerebral malaria, where infected RBCs adhere to the brain microvasculature, often resulting in severe neurological complications.
EPCR (Endothelial Protein C Receptor)	Interactions of PfEMP1 variants with EPCR are often associated with group A *var* genes, particular those classified as DC8 and DC13 (Turner et al., 30; Avril et al., 31; Claessens et al., 32).	Binding to EPCR, a receptor that is involved in anti-inflammatory signaling, possibly contributes to the severity of the disease. It has been linked to severe forms of malaria, including cerebral malaria.
CSA (Chondroitin Sulfate A)	A unique form of PfEMP1, encoded by *var2csa,* binds to CSA in the placenta (Salanti et al., 33; Salanti et al., 34).	This binding phenotype is unique to pregnancy-associated malaria, a syndrome that leads to severe maternal anemia, fetal growth retardation, and premature delivery, as well as maternal and perinatal mortality.

repertoire, the infected individual is thought to clear their infection and recover from the disease (44). This model is generally well accepted and provides a likely explanation for the trajectory of infections in individuals lacking any pre-existing immunity.

While the genome of any individual parasite contains approximately 45–90 *var* genes, parasites isolated from different patients often possess *var* repertoires that diverge substantially in sequence (45, 46). Thus, even after clearing an initial infection, a person is vulnerable to subsequent infections by genetically distinct parasites. However, as mentioned above, individuals who live in malaria endemic regions of the world and experience repeated *P. falciparum* infections eventually develop a form of clinically protective, but non-sterile immunity (47), a phenomenon that is directly related to the number of infections an individual has had and develops sooner in regions with higher transmission rates (4–6). There is strong evidence that this clinically protective immunity significantly involves antibodies targeting PfEMP1 (47, 48); therefore, it appears that exposure to a broad range of PfEMP1 types through repeated infections provides the basis for protection against disease. Additional evidence for the importance of anti-PfEMP1 antibodies in clinical protection comes from observations of women in their first pregnancies. Specifically, it is well established that women from endemic regions who are immune to severe disease become highly vulnerable to severe malaria in their first pregnancies (49). This is due to the expression by parasites of a placental specific form of PfEMP1 called VAR2CSA (34) to which the women had not previously been exposed. However, once exposure to VAR2CSA stimulates antibodies against this antigen, they once again display clinical immunity during subsequent pregnancies. Thus, clinical immunity is strongly correlated with the acquisition of a broad, cross-reactive antibody response to the extensive repertoire of PfEMP1 variants expressed by locally transmitted parasites (50–52) (Fig. 1B). As demonstrated by placental malaria, disease can result whenever parasites express a form of PfEMP1 that escapes a person's collective antibody response.

CHRONIC, ASYMPTOMATIC INFECTIONS

Collectively, the studies mentioned in the previous section strongly indicate that anti-PfEMP1 immunity is responsible for protecting most people living in regions of moderate to high transmission from severe illness. The standard model of antigenic variation also proposes that once a parasite's repertoire of PfEMP1 forms has been exhausted, the infection should be cleared and that acquisition of extensive anti-PfEMP1 immunity should protect from infection. However, immunity is known to be non-sterile, indicating that parasites can infect individuals who possess antibodies against a broad range of PfEMP1s, albeit at very low parasitemias and with little or no symptoms. The simple model of *P. falciparum* immunity is further confounded by reports of asymptomatic infections that can last for years. For example, Ashley and White recently catalogued dozens of validated cases of asymptomatic infections lasting for up to 13 years (11). These infections remained untreated and unknown until revealed by splenectomy, by immunosuppression or through blood donation for transfusion.

Just how common are these asymptomatic infections in endemic regions and do they contribute to the ongoing transmission of the disease? Several methods have been used to identify asymptomatic infections under different conditions. In general, asymptomatic infection rates can be quite high for school-aged children, ranging from 18% to 75% in a study from Uganda, depending on the geographical region (53). A similar study in Ghana observed 40%–60% infection rates among children 3–15 years old (54), and a study of healthy individuals in a suburb of Lagos, Nigeria, detected parasites in ~35% of participants (55). Thus, rates of asymptomatic infections in places of high transmission can be substantial. Successful detection depends on the sensitivity of the diagnostic method used, typically either microscopy, rapid diagnostic tests, or PCR (55), and it is possible that some infections fall below the threshold of detection of even the most sensitive methods. Given the difficulties in estimating the rates of asymptomatic infections in any given area, their overall contribution to transmission is difficult to

determine. Nonetheless, a recent study has attributed the vast majority of transmission to asymptomatic individuals (10). While the frequency of asymptomatic infections will vary considerably in different places, it is clear that they are exceedingly common and contribute significantly to the stable transmission of malaria to susceptible individuals.

How are these infections maintained for such lengthy time periods in the face of extensive anti-PfEMP1 immunity and why do parasitemias remain so low? Why are the infected individuals unable to fully clear their infections and why do they display little to no symptoms, despite persistent parasites that appear to be actively replicating? Given the important role of PfEMP1 in the acquisition of immunity, it is logical that changes in *var* gene expression are an important aspect of this phenomenon.

VARIABLE *var* GENE EXPRESION

While it is clear that anti-PfEMP1 immunity plays a key role in protecting people from severe and symptomatic infections, the state of PfEMP1 expression in parasites infecting asymptomatic individuals has not been well defined. The standard paradigm of *var* gene expression and antigenic variation presumes that all parasites express a single *var* gene at a time, thereby ensuring expression of PfEMP1 on the infected cell surface and avoidance of clearance by the spleen. Failure to express PfEMP1 is assumed to result in parasite destruction; however, recent studies indicate that this simple model might not be entirely correct, particularly in asymptomatic infections. For example, working in a region of Mali where transmission displays strong seasonality, Andrade and colleagues compared parasites obtained from symptomatic infections with those recovered from asymptomatic infections at the end of a dry season, thus ensuring that they were studying persistent infections (56). They found that parasites from the asymptomatic infections remained in the peripheral circulation longer, appeared to cytoadhere poorly, and were subject to increased splenic clearance, all characteristics implicating changes in PfEMP1 expression. Measurements of *var* gene expression were less conclusive, but suggestive of lower *var* transcripts in parasites from the asymptomatic infections. This study indicated that PfEMP1/*var* gene expression might be more flexible than suggested by standard models of antigenic variation.

A more drastic departure from the standard model of mutually exclusive *var* gene expression was described by Bachmann and colleagues (57). The authors obtained blood samples from an individual living in Germany who grew up in a malaria endemic region. Upon examination, this person was negative for malaria infection by blood smear and malaria antigen tests. However, serological testing revealed high titers of antibodies to *P. falciparum* antigens, indicating previous exposure and a high degree of immunity. The individual underwent a splenectomy, after which the patient displayed very high parasitemias, and blood smears showed large numbers of mature parasites (late trophozoites and schizonts) in the peripheral blood, indicating that the infected RBCs were not cytoadherent. Analysis of RNA extracted from parasites immediately after they were obtained from the patient detected no *var* gene expression, providing a likely explanation for the lack of cytoadherence of infected cells in the patient. Parasites were adapted to culture and maintained *in vitro* for at least 44 days. Interestingly, the cultures remained negative for *var* gene expression for 12 days, after which *var* gene expression could be detected. This study indicates that in the setting of a natural infection, parasites are capable of silencing their entire *var* repertoire and thus not displaying PfEMP1 on the infected cell surface. Presumably, this provided a selective advantage when encountering strong anti-PfEMP1 immunity as was likely present in this individual. Such parasites would be non-cytoadherent and therefore largely filtered by the spleen, explaining why the patient's parasitemia was initially not detected but expanded dramatically upon splenectomy. The fact that the parasites reverted to expressing *var* genes after replicating in culture for several generations further suggests that this non-PfEMP1-expressing state did not result from a genetic mutation but rather is a state that parasites can transition into and out of. Overall, this study provided a significant departure from the standard paradigm of mutually exclusive *var* gene expression and suggests a model

in which parasites face two significant selection pressures during a natural infection: splenic clearance and anti-PfEMP1 immunity. In individuals who have little immunity, parasites that express PfEMP1 avoid the spleen through cytoadherence and therefore have a strong growth advantage. They expand rapidly, causing symptomatic disease and displaying the classic waves of parasitemia seen in immunologically naïve individuals. However, in older people with extensive immunity, PfEMP1 expression is selected against by the broad range of anti-PfEMP1 antibodies acquired from previous infections. In this environment, parasites that do not express PfEMP1 are selected for, but due to splenic filtration, parasitemias remain low or undetectable and symptoms are rare. This hypothesis, shown schematically in Fig. 1, provides a possible explanation for chronic, asymptomatic infections (58).

The above studies provide examples in which *var* gene expression deviates from a simple model of transcriptional switching and mutually exclusive expression. However, it is not clear how common these situations are or how typical it is for parasites to reduce or eliminate PfEMP1/*var* gene expression. If parasites are able to adapt to the selective pressures of a natural infection by manipulating *var* gene expression levels, this should be evident in cultured parasites in which neither anti-PfEMP1 antibodies or splenic clearance are present. Past work examining this question discovered that in fact, populations of recently subcloned parasite populations can display drastically different *var* gene expression levels (59–61), consistent with much more flexibility than originally appreciated. However, these studies were completed on populations of parasites, so a true examination of mutually exclusive expression in individual parasites was not possible. To definitively examine this question, we recently employed single-cell RNA sequencing and discovered that individual parasites frequently reduce *var* gene expression to near undetectable levels, consistent with silencing of the entire family (62). RBCs infected with these parasites were not recognized by hyper-immune IgG obtained from people with extensive anti-malaria immunity (63), indicating they are indeed not expressing PfEMP1 on the infected cell surface. Such non-PfEMP1-expressing parasites were observed in all cultures examined in this study, suggesting that it is not an uncommon state for parasites reared *in vitro*. Taken together, these studies indicate that when confronted by extensive anti-PfEMP1 antibodies when infecting individuals who have experienced numerous prior malaria infections, parasites can significantly down-regulate or silence expression of PfEMP1, potentially enabling them to persist at very low levels and thus maintain asymptomatic infections.

var GENE MOLECULAR BIOLOGY

The ability of parasites to modify *var*/PfEMP1 expression levels to a greater extent than previously appreciated further complicates our understanding of the regulatory pathways that control this process. In addition to transcriptional switching between *var* genes, parasites appear to be able to switch in and out of a "*var*-null" state (62). A more complete understanding of how parasites adapt to extensive anti-PfEMP1 immunity will require a better understanding of the molecular basis of *var* gene transcriptional regulation. As previously mentioned, the transcriptional activation and silencing of *var* genes are epigenetically controlled, with the active *var* gene existing in a transcriptionally permissive chromatin region while the remaining silent *var* genes are found in a heterochromatin state (36, 39). The epigenetic marks associated with active and/or silent *var* genes include the following: H3K9ac, H3K4me2, and H3K4me3 are associated with the active *var* gene while H3K9me3 is associated with silent *var* genes (20). H3K36me3 is present on both active and silent *var* genes (64). These marks are deposited by specific histone methyltransferases including PfSET1, PfSET2, PfSET3, and PfSET10, and altering their activity has been shown to influence *var* gene expression (64–67). Non-coding RNAs are also associated with *var* gene expression (68–70), although with the exception of a specific anti-sense ncRNA associated with the active gene (71–73), their precise mechanism of action is unknown.

While it has become evident that malaria parasites can alter gene expression in response to environmental stimuli, it was long assumed that regulation of *var* gene expression was exempt from this phenomenon. Specifically, the standard model of *var* gene switching assumed that parasites had evolved a stochastic switching rate sufficient to counter the rate at which the human host mounted an antibody response to PfEMP1. Recently, our group challenged this paradigm and showed that *P. falciparum* parasites can sense environmental changes and alter their *var* gene expression in response (74). This study demonstrated that manipulating specific components of one-carbon metabolic pathways that alter the intracellular abundance of S-adenosylmethionine (SAM) can lead to changes in *var* gene expression. Increased levels of intracellular SAM induced either environmentally (i.e., altering nutrient availability in culture) or through genetic modifications (i.e., overexpression of SAM synthase), led to an increase in histone methylation, and significantly accelerated *var* gene switching. More specifically, excess choline/depleted serine, excess methionine, and overexpression of PfSAMS led to increased heterochromatin silencing and a switch in *var* gene expression to *var2csa*, a unique and highly conserved gene encoding a placental specific form of PfEMP1. Conditions that led to expression of *var2csa* also suppressed activation of a gene called *ap2-g* involved in sexual differentiation (75), demonstrating how environmental influences on epigenetic processes affect both these transcriptional regulatory pathways.

Interestingly, when levels of intracellular SAM were lowered through reducing the expression of PfSAMS, we observed activation of multiple *var* genes and an increase in the overall amount of *var* transcripts (74), suggesting the possibility that under these conditions, multiple *var* gene promoters are able to compete for activation. Additionally, low intracellular PfSAMS levels influenced the expression of other multi-copy gene families, including *rifin*, *stevor*, *clag*, and *phist*, whose expression has previously been associated with H3K9me3 (74). Notably, this modification did not affect the expression of other epigenetically silenced genes, such as those typically transcribed during different life cycle stages. Subsequent analysis at the single cell level demonstrated that in fact, under these conditions, mutually exclusive *var* expression is disrupted and each individual parasite expresses 6–8 *var* genes simultaneously, providing an additional example of the unappreciated flexibility of *var* gene expression (62). Interestingly, the PfSAMS-depleted parasites exhibited a positive growth phenotype, growing at a slightly faster rate than untransfected parasites, for reasons we don't yet understand. These findings illustrate that *var* gene expression in *P. falciparum* can be dynamically regulated by environmental cues, challenging the previous assumption that *var* gene regulation is "hardwired" or "programed" in some way.

EVIDENCE FOR A *var* SWITCHING "NETWORK"

As our understanding of the course of *P. falciparum* infections improves, a model arises in which the initial phase of the infection is characterized by rapidly rising waves of parasites expressing PfEMP1 that cytoadhere within the deep tissue vasculature. Upon exhaustion of the PfEMP1 repertoire, parasitemias become low or non-detectable and consist of parasites that do not express PfEMP1, enabling chronic, asymptomatic infections (58) (Fig. 1B). During the initial phase of a *P. falciparum* infection, it remains unknown how the enormous population of individual parasites in a person's circulation can coordinate and synchronize their PfEMP1 variant switching, thereby leading to relatively homogenous waves of parasitemia. Currently, no data indicate a specific order or pattern in *var* gene activation and silencing, and there is no evidence of communication between parasites that can influence *var* gene switching. On the other hand, in a wave of parasitemia early in an infection, the number of parasites within the circulation can number in the trillions. This makes it implausible that *var* switching is completely uncoordinated or random since this would result in the rapid exhaustion of the entire antigenic repertoire. In 2011, Recker et al. applied mathematical modeling to data sets derived from experimental human infections and proposed a model in which *var* genes

are organized into a hypothetic network, with each *var* gene representing a node within the network (76). For their analysis, they employed a genetic algorithm to determine an optimal switching strategy that balances immune evasion and repertoire protection, using parameters such as parasite growth rate, clearance efficacy, and variant switching rate. Importantly, expression switching was proposed to be coordinated through a "sink node," which was envisioned as a specific *var* gene that is transiently activated upon initiation of transcriptional switching. Expression then returns to either the originally active gene or switches to a previously silent *var* gene. Within the population, parasites that switch back to the originally active gene will be eliminated by rising antibody titers while those that switched to a new form of PfEMP1 will undergo clonal expansion, thus leading to a new wave of parasitemia (Fig. 2). If switching out of the "sink node" is not random, coordination of *var* gene expression can be achieved, leading to systematic cycling through the *var* gene repertoire. While the initial description of this model did not specify particular *var* genes that could serve the "sink node" function, recent work has implicated *var2csa* as a very attractive candidate.

var2csa is a unique member of the *var* gene family. Although *var* genes vary extensively between field isolates due to frequent DNA recombination events (40, 42, 43, 77), *var2csa* is highly conserved across all *P. falciparum* isolates and this conservation extends to the related species *Plasmodium reichenowi* and *Plasmodium praefalciparum* (45, 78). It is therefore estimated that *var2csa* has been preserved as a unique gene for approximately 9 million years (78). *P. falciparum*, along with seven other *Plasmodium* species, belong to the *Laverania* subgenous, and they all possess large *var* gene families. Otto and colleagues reported significant changes in the protein domains of the EMP1 proteins of the branch of the *Laverania* subgenus that includes *P. falciparum* when compared with the more distantly related species (79); however, VAR2CSA remains an exception, appearing as the sole remnant of the ancient type of EMP1. This suggests that strong selective pressure has preserved this particular *var* gene over millions of years of evolution and speciation, raising the possibility that *var2csa* may have a secondary function beyond its primary role in cytoadherence to the placenta—potentially in regulating *var* gene expression (78). However, it is unclear whether there is a link between these two functions. Additionally, unlike other *var* genes, *var2csa* has a unique promoter that appears to be highly competitive. For example, it was demonstrated that by altering the activity of histone modifiers (65, 80) or by simply culturing parasites long term (81), parasite populations converge to *var2csa* expression, suggesting a switching hierarchy that favors this locus. Interestingly, some parasite isolates have additional copies of *var2csa* duplicated into other regions of the genome. However, in experiments with one such line, HB3, we found that only the conserved locus on chromosome 12 is selectively activated in culture, suggesting that the competitive nature of this gene extends beyond the primary sequence of the promoter region and is also influenced by the chromosomal environment (80). Furthermore, in follow-up experiments, we observed that deletion or disruption of the *var2csa* promoter impaired *var* gene switching in cultured parasites (58). It is unclear how the *var2csa* locus contributes to regulating this transcriptional switching network or if the hypothetical "sink node" might apply to it, but given the importance of its promoter region, a model involving promoter competition is an intriguing possibility.

VAR2CSA protein is observed only in parasites obtained from the placenta of pregnant women; however, *var2csa* transcripts are also observed in non-pregnant individuals (82–84). This lack of correlation between transcription and translation is an indicator of post-transcriptional gene regulation, a distinctive feature of *var2csa* compared with other members of the *var* gene family. Post-transcriptional regulation is achieved by the presence of a 360-nucleotide-long upstream open-reading frame (uORF) in the 5′ untranslated region of the *var2csa* transcript (81, 85). The uORF is located between the transcription start site and the downstream ORF (dORF) that encodes VAR2CSA, and the two ORFs are separated by an intercistronic region ranging from 270 to 530 bp, resulting in a bicistronic mRNA. We demonstrated that the presence of the

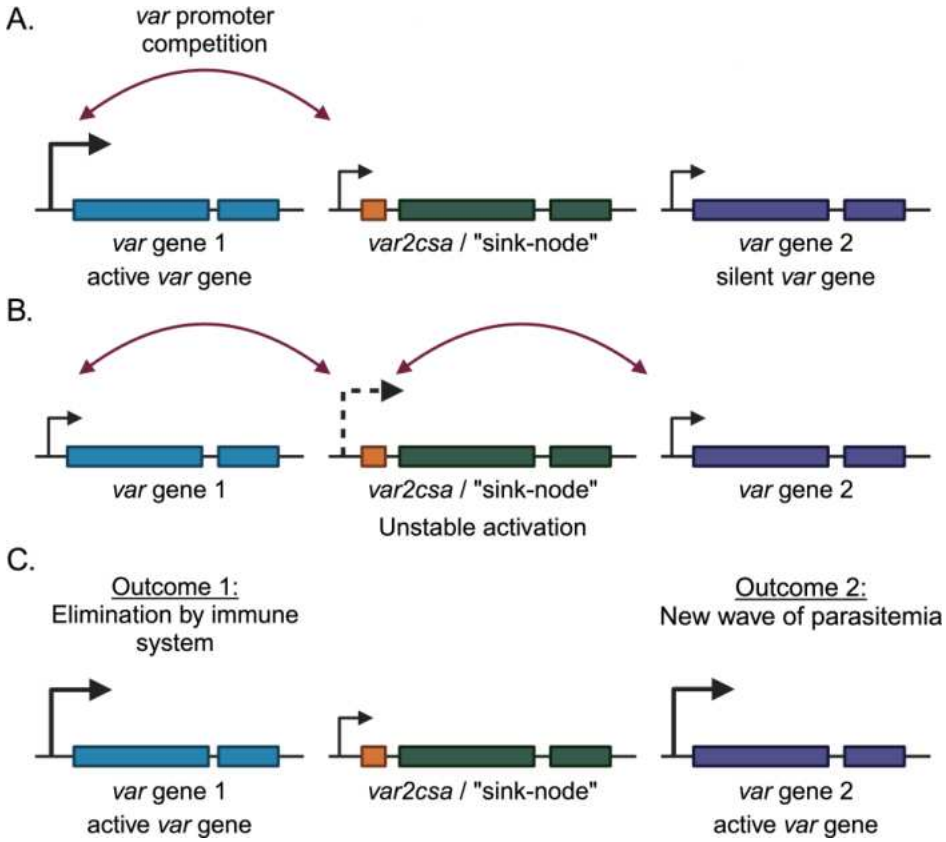

FIG 2 Model for the role of *var2csa* as a "sink-node" for *var* gene switching. (A) Upon initiation of transcriptional switching, *var2csa* competes for activation with the active *var* gene, *var* gene 1. (B) Given that *var2ca* has a unique promoter that appears to be highly competitive, switching to *var2csa* occurs and the previously active gene gets silenced. However, switching to *var2csa* is unstable due to its bicistronic mRNA that can lead to gene silencing. Thereby, other *var* genes will compete for activation. (C) Expression can then return to either the original active gene, *var* gene 1, or to a previously silent gene, *var* gene 2. Parasites that switch back to *var* gene 1 will get eliminated by the immune system, while parasites that switch to *var* gene 2 will expand and therefore lead to a new wave of parasitemia until the next switching event occurs.

uORF suppresses translation of the dORF encoding the VAR2CSA protein in cultured parasites, suggesting a mechanism by which the parasites achieve translational silencing of this mRNA when the protein is not needed, i.e., in the absence of a placenta (85). However, parasites can overcome this translational repression when infecting pregnant women, resulting in expression of this unique antigen and binding to chondroitin sulfate A (CSA) on placental syncytiotrophoblasts (Fig. 3). The precise translational switching mechanism that allows for VAR2CSA expression in pregnant individuals is unknown; however, we determined that translation of the dORF is achieved through translation re-initiation (81). How the parasites sense pregnancy or the presence of a placenta is unknown; however, as mentioned above, modifications to the media of cultured parasites can strongly influence the frequency of parasites to switch translational states (74), providing additional evidence for the ability to sense and respond to their environment. Interestingly, bicistronic mRNAs can be unstable and this instability can lead to gene silencing (86), providing a possible mechanism by which *var2csa* could be transiently activated, thus serving as a "sink node" and facilitating the activation of a new *var* gene. These data demonstrate a complex and tightly controlled regulatory mechanism that reinforces the unique importance of *var2csa* within the *var* gene family, emphasizing its potential as a key locus in regulating antigenic variation.

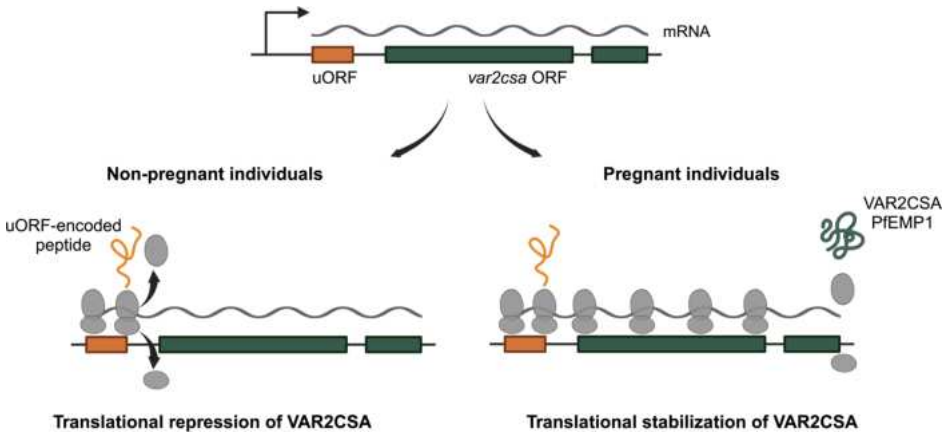

FIG 3 Translational regulation of *var2csa* mRNA in the absence (left panel) or presence (right panel) of a placenta. In non-pregnant individuals, the presence of a uORF prevents the VAR2CSA protein from being translated. In pregnant individuals, translational stabilization is achieved through translation re-initiation enabling expression of VAR2CSA that binds to CSA in the placenta.

POSSIBLE HIDDEN RESERVOIRS OF PARASITES

As described above, in asymptomatic individuals, parasites are typically rare or difficult to detect, often falling below the level of detection (57, 87). In principle, only a small number of parasites that can avoid immune recognition or destruction in the spleen would be sufficient to maintain an infection. This has led to speculation that there might be a hidden reservoir of parasites largely removed from the peripheral circulation where infected cells reside in people with asymptomatic infections. This is particularly true if parasites in lengthy, chronic asymptomatic infections are not expressing PfEMP1 and are not cytoadhesive (57). How could such parasites avoid filtration and destruction from passage through the spleen? One possibility is that the parasites sequester within anatomical niches that are somewhat removed from the peripheral circulation and thus where non-cytoadhesive infected cells could reside. It is also possible that such niches could provide microenvironments that the parasites can sense and respond to, thereby reinforcing specific *var* expression (or non-expression) patterns. Three tissues that have been implicated as possible niches that influence *var* gene expression are the bone marrow, spleen, and placenta. All three have unique characteristics (summarized in Table 2) that could influence the metabolic environment in which parasites develop, which could in turn influence *var* gene expression.

The bone marrow is a highly specialized and dynamic environment that plays a critical role in hematopoiesis. It is characterized by a unique microenvironment that supports both the maintenance of hematopoietic stem cells and the maturation of various blood cell lineages. It is highly vascularized, containing a network of sinusoids, large-diameter blood vessels that facilitate the exchange of cells and nutrients between the bone marrow and the peripheral blood (99). Blood flow within the bone marrow is typically slower compared with other tissues, facilitating prolonged interactions between hematopoietic cells and the supportive stromal cells (88). This reduced flow rate promotes the retention and migration of various cells within the bone marrow, creating an optimal environment for the sequestration of *P. falciparum*-infected erythrocytes. This sequestration is likely advantageous for the parasite, as the bone marrow provides a sanctuary where it can develop and mature, protected from the host's immune system and mechanical clearance mechanisms. Oxygen levels in the bone marrow are generally lower compared with those in peripheral blood, resulting in a hypoxic environment that is vital for the regulation and maintenance of hematopoietic cells (91). The parasite's replication and survival within the bone marrow may be directly affected by this hypoxia,

TABLE 2 Key environmental characteristics of the bone marrow, spleen, and placenta—tissues implicated as potential niches for *P. falciparum* parasites[a]

	Bone marrow	Spleen	Placenta
Blood flow	Low: slower blood flow facilitates prolonged interactions between hematopoietic cells and supportive stromal cells (Bixel et al., 88).	Low: blood flow slows as it moves through the narrow vessels of the spleen (Moreau et al., 89).	High: flow of blood increases significantly in the second and third trimesters (Jauniaux et al., 90).
Oxygen levels	Low: hypoxic environment is vital for the maintenance of hematopoietic cells (Parmar et al., 91).	Low: slow blood flow leads to low oxygen tension in the spleen.	High: oxygen-rich maternal blood flows into the intervillous space of the placenta from the second trimester onwards (Jauniaux et al., 90; Filippi et al., 92).
Nutrient availability	High: ample glucose and amino acids such as glutamate and aspartate (Pham et al., 93; Suchacki et al., 94; Devignes et al., 95).	Low: nutrient-deprived environment due to the high metabolic demands of immune cells (Vijay et al., 96).	High: nutrient-rich, high levels of glucose, lipids, and amino acids such as glutamine to support fetal growth (Gaccioli et al., 97; Day et al., 98).

[a]These tissues exhibit variations in blood flow, oxygen levels, and nutrient availability, which may influence parasite sequestration and *var* gene expression.

as the low oxygen levels could alter its metabolic pathways and potentially influence its ability to evade the host's immune responses by altering *var* gene expression.

It is well established that the bone marrow represents a major site of *P. falciparum* and *Plasmodium vivax* sequestration, especially of immature gametocytes (100–103). Interestingly, gametocytes are not thought to express PfEMP1 on the infected cell surface (104, 105) and thus are not cytoadhesive and likely not susceptible to the anti-PfEMP1 antibodies proposed to play a significant role in the immunity responsible for asymptomatic infections. The nutrient-rich environment of the bone marrow provides essential factors required for cell growth and differentiation, which are not as abundantly available in peripheral blood (106), including ample glucose (93, 94) and amino acids, such as glutamate and aspartate (95). The rich supply of nutrients, coupled with the relatively protected environment, makes the bone marrow a favorable niche for *P. falciparum* during certain stages of its life cycle. Differentiation from asexual into male and female gametocytes requires expression of the stage-specific transcription factor AP2-G (107, 108). The process of gametocytogenesis is generally repressed due to epigenetic silencing of the *ap2-g* locus through H3K9me3 and recruitment of the heterochromatic protein 1 (109) and histone deacetylase 2 (110), epigenetic marks that are similarly known to maintain *var* gene silencing (36, 39, 109, 110). Several metabolites have been shown to alter the rate of sexual commitment, including serine, homocysteine (111, 112), and phosphocholine or lysophosphatidylcholine (75, 113), and these metabolites can also influence *var* gene expression (74). These findings highlight the intricate connection between environmental factors, metabolite availability, and epigenetic regulation within the environment of the bone marrow that likely enable parasites to specifically sense and take advantage of this unique anatomical niche. Given that the same epigenetic marks that control *ap2-g* silencing and activation also influence *var* gene expression (36, 39, 109, 110), it is possible that the environment of the bone marrow could similarly influence *var* gene transcription or switching.

Another tissue that is considered a niche for malaria parasites, especially in asymptomatic individuals, is the spleen. The spleen is a highly vascular organ, primarily involved in filtering blood and mounting immune responses. The splenic artery delivers blood to the spleen, where it slows down as it passes through a network of narrow arterioles and sinusoids (89). This results in the spleen having a relatively lower oxygen tension compared with peripheral blood. Its structure can be divided into two main regions: the red pulp and the white pulp. The red pulp is responsible for blood filtration and removal of abnormal/unhealthy RBCs from the circulation (114). During this filtration, RBCs must leave the blood vessels and enter the red pulp. To reenter the circulation, RBCs need to squeeze into very narrow slits of vinous sinuses, something that only healthy cells are sufficiently deformable to do. Unhealthy RBCs, such as those infected by *P. falciparum*,

are unable to squeeze through the venous sinuses and will be engulfed by macrophages. Thus, the spleen is typically thought of as a place where parasites are destroyed rather than a possible niche that could host a reservoir of infected RBCs. Notably however, 2%–3% of the total human RBC mass is removed from the circulation and resides in the spleen (115), perhaps providing an opportunity for parasite-infected RBCs to sequester from the circulation without the need for cytoadherence. These splenic erythrocytes are continuously exchanging with those in circulation, forming a slowly cycling pool of RBCs.

A study in 2008 showed for the first time that a subpopulation of ring-stage parasites is retained in the spleen red pulp at each spleen passage (116). Ten percent of ring-infected erythrocytes is retained in the spleen due to the spleen's unique microcirculatory environment. The slow blood flow through the spleen's narrow arterioles and sinusoids allows these infected cells to stay in the spleen longer than in other parts of the circulation. More recently, a study of asymptomatic adults in a malaria-endemic region of Papua, Indonesia, discovered a large, previously unrecognized biomass of parasite-infected RBCs in the spleens of individuals in which this organ was removed due to trauma (87). The parasite densities were hundreds or thousands of times higher than that observed in the peripheral circulation, consistent with this anatomical site serving as a reservoir for infected cells. These large accumulations of infected cells in the spleen were observed even in two patients whose infections were undetectable by PCR performed from peripheral blood samples. Although distinguishing between parasites filtered by the spleen and destined to be destroyed from those productively infecting splenic RBC's is challenging, the authors noted that these parasites appeared to be viable and replicating. This finding suggests that the spleen might serve as a privileged site for parasite-infected RBCs in individuals with significant anti-malarial immunity. This is despite lower nutrient availability, as the metabolic demands of immune cells in the spleen can deprive the parasites of crucial amino acids like glutamine (96), potentially slowing parasite growth or multiplication and further facilitating their prolonged survival in this environment. The discovery of hidden populations of parasites replicating within the spleens of asymptomatic patients (87) and the detection of non-PfEMP1 expressing parasites in a chronic, asymptomatic infection (57) suggest the intriguing possibility that the micro-environment of the spleen might suppress *var* gene expression, thereby providing a niche where non-circulating, non-PfEMP1-expressing parasites can reside.

While the spleen provides a potential reservoir for parasites in a chronic infection, during pregnancy, *P. falciparum* parasites take advantage of the placenta as a particularly favorable niche that supports their survival and proliferation due to the placenta's rich supply of essential nutrients such as glucose, amino acids, and lipids (97). The placenta's enhanced blood flow ensures the consistent delivery of nutrients and oxygen, creating an optimal setting for the parasite's replication. Throughout the second and third trimesters of pregnancy, the oxygenation of the placenta undergoes substantial changes. Initially, blood flow through the placenta is restricted, which limits oxygen exposure to the developing tissue and fetus (117). However, as pregnancy progresses, the flow of oxygen-rich maternal blood into the intervillous space of the placenta increases (90). This adjustment is crucial as it enhances oxygen delivery to the syncytiotrophoblasts and, consequently, to the developing fetus. By the third trimester, placental oxygenation reaches its peak as both the placenta and fetus approach full development (92). This results in the intervillous space being filled with maternal blood, which maximizes the efficiency of oxygen transfer to the fetus.

Glucose availability in the placenta is crucial for fetal growth and development. The placenta facilitates the transfer of glucose from the maternal to the fetal circulation via specific glucose transporters, primarily GLUT1, which is highly expressed on the syncytiotrophoblast where *P. falciparum* parasites cytoadhere (118). Glucose diffuses or is transported from regions with high (i.e., maternal blood) to low (i.e., fetal blood) concentration and creates a concentration gradient through the placenta, between maternal and fetal blood. As pregnancy progresses, the demand for glucose increases significantly due to the rapid growth and development of the fetus. Therefore,

the placenta adapts to these increasing demands by enhancing its glucose transport capacity, ensuring that the fetus receives an adequate supply of glucose. MacRae et al. studied the central carbon metabolism of *P. falciparum* parasites and found that both asexual and sexual blood stages utilize a conventional TCA cycle to catabolize glucose and glutamine (119). Interestingly, they observed that flux of glucose carbon skeletons into the TCA cycle is low in the asexual blood stages, with glutamine providing most of the carbon skeletons. The amino acid composition in placental tissue differs significantly from that in peripheral blood, reflecting the specialized functions of the placenta in supporting fetal development. In 2013, Day et al. demonstrated that glutamate taken up from the maternal and fetal circulations was primarily converted into glutamine by the placenta, and unexpectedly, the majority of this glutamine is released into the maternal rather than the fetal circulation, leading to a glutamine-rich environment in the placenta (98). In the context of placental malaria, the altered amino acid environment could be advantageous for the parasite. The unique amino acid profile of the placenta, compared with peripheral blood, could help fuel parasite growth and persistence during placental malaria. Given that asexual parasites primarily utilize glutamine for energy and carbon metabolism, the placenta provides a glutamine-rich environment that the parasite may exploit for its own metabolic needs. In addition to the presence of a placenta, pregnancy involves numerous systemic changes that could potentially be sensed by the parasite. However, no data currently distinguish between the effects of the placenta itself and these broader systemic changes. Nevertheless, the placenta's unique characteristics as an organ make it a particularly compelling factor in modulating *var2csa* activation. Considering the strong correlation between VAR2CSA translation in pregnancy, the unique amino acid and oxygen environment in the placenta may play a crucial role in favoring the translational switch to VAR2CSA expression. The placenta's distinctive metabolic environment may not only support parasite survival but also act as a trigger for this expression of this specific PfEMP1.

FLEXIBLE ANTIGEN EXPRESSION, CHRONIC INFECTIONS, AND EFFICIENT TRANSMISSION

As work in both the laboratory setting and in the field continue to refine our understanding of antigenic variation in *P. falciparum*, it is becoming clear that these parasites have evolved a highly flexible network of surface antigen encoding *var* genes that enables them to successfully maintain chronic infections and be efficiently transmitted between human hosts. The ability to maintain lengthy infections is particularly important in regions of the world that experience significant dry seasons when the mosquito vector is absent. The parasite must be able to perpetuate an infection of sufficient length to ensure transmission upon return of the vector. Other species of malaria parasites, for example, *P. vivax* or *Plasmodium ovale*, appear to have solved this problem through the formation of dormant hypnozoites; however, *P. falciparum* must maintain continuous blood stage replication and thus relies on antigenic variation. The surprisingly flexible nature of *var* gene expression, including the discovery that parasites can enter a state in which they don't express *var* genes, has altered our view of this important process and the mechanisms that regulate it. Similarly, the possibility that the metabolic environment within various anatomical niches might influence *var* gene expression adds a new layer of complexity to an already-complicated system. The unique and highly conserved gene *var2csa* appears to occupy a key position within the *var* gene network, serving roles in both binding within the placenta and coordinating *var* gene transcriptional switching. Altogether, the *var* gene regulatory pathway represents the evolution of an efficient, elegant, and important example of how parasites have adapted to life in the hostile environment of their human hosts. As our understanding of the molecular details of this pathway deepens, we hope to be able to use the resulting knowledge to lessen the disease burden in regions of the world that continue to be strongly affected by malaria.

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FUNDING

Funder	Grant(s)	Author(s)
HHS | National Institutes of Health (NIH)	AI161299	Kirk W. Deitsch
HHS | National Institutes of Health (NIH)	AI138499	Kirk W. Deitsch

AUTHOR CONTRIBUTIONS

Evi Hadjimichael, Conceptualization, Writing – original draft | Kirk W. Deitsch, Conceptualization, Funding acquisition, Supervision, Writing – original draft

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