HHS Public Access

Author manuscript

Adv Parasitol. Author manuscript; available in PMC 2025 June 18.

Published in final edited form as:

Adv Parasitol. 2024 ; 125: 53–103. doi:10.1016/bs.apar.2024.02.001.

PfEMP1 and *var* genes – still of key importance in *Plasmodium falciparum* malaria pathogenesis and immunity

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Abstract

The most severe form of malaria, caused by infection with *Plasmodium falciparum* parasites, continues to be an important cause of human suffering and poverty. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of clonally variant antigens, which mediates the adhesion of infected erythrocytes to the vascular endothelium in various tissues and organs, is a central component of the pathogenesis of the disease and a key target of the acquired immune response to malaria. Much new knowledge has accumulated since we published a systematic overview of the PfEMP1 family almost ten years ago. In this chapter, we therefore aim to summarize research progress since 2015 on the structure, function, regulation etc. of this key protein family of arguably the most important human parasite. Recent insights regarding PfEMP1-specific immune responses and PfEMP1-specific vaccination against malaria, as well as an outlook for the coming years are also covered.

Keywords

immunity; malaria; pathogenesis; structure/function; PfEMP1; *Plasmodium falciparum* ; vaccine; *var* genes

2. Introduction

Almost ten years ago, we wrote a comprehensive overview of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) and the *var* gene family encoding them. The review also covered the role of PfEMP1 in the pathogenesis of malaria caused by that parasite and in the immune response to the disease it causes (Hviid and Jensen, 2015). The present work is intended as an update and a complement to the earlier review, and we therefore focus here on topics where substantial new insights have been gained in the meantime. Accordingly, we only cite few studies published before 2015, when needed to document key points or to correct inadvertent omissions in the earlier review.

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Erythrocytes infected by *P. falciparum* parasites older than approximately 18 h stick so efficiently to endothelium that only recently invaded erythrocytes (so-called “ring forms”) can be found in the peripheral circulation. The only exception is mature gametocytes, which are also present in the peripheral circulation, awaiting being picked up by a feeding *Anopheles* mosquito (Hviid and Jensen, 2015). The vascular adhesion (sequestration) of mature *P. falciparum*-infected erythrocytes (IEs) is mediated by PfEMP1. The other species of malaria parasites that cause disease in humans do not possess corresponding adhesins, and this fact goes a long way towards explaining the severity of *P. falciparum* malaria. Vascular sequestration undermines the ability of the spleen to remove IEs, and therefore leads to higher effective parasite multiplication rates and parasitaemias than seen in other types of human malaria. All members of *Laverania* subgenus, which includes *P. falciparum* and other related parasites of great apes, have large *var* gene families that encode orthologs of PfEMP1 (Brazier et al., 2017; Otto et al., 2018b; Otto et al., 2019; Gross et al., 2021), in contrast to the other species of malaria parasites infecting humans. The ancient origin of PfEMP1-mediated vascular sequestration of IEs is evidence of its value to *P. falciparum* as a survival strategy. To the host, the direct consequence of IE sequestration is tissue inflammation and circulatory impairment that can be clinically severe or even fatal.

3. The *var* genes

3.1. Structure

The multigene *var* family that encodes the PfEMP1 proteins has 45-90 members per haploid parasite genome (Gardner et al., 2002; Otto et al., 2019). Each *var* gene has two exons (Figure 1). The long 5’ Exon 1 encodes the extracellular part of PfEMP1, while exon 2 encodes the transmembrane and short intracellular parts. While exon 1 sequences are highly variable within and among genomes, exon 2 sequences are quite conserved. The *var* genes can be divided into groups according to their chromosomal location, their upstream promoter sequence, the amino acid sequence of the encoded protein, and the direction in which they are transcribed. The basic organization of the *var* gene family was originally described for the 3D7 reference strain (Kraemer and Smith, 2003; Lavstsen et al., 2003) and it includes approximately 10 Group A *var* genes located near the chromosome telomeres and transcribed towards them. These genes encode the largest PfEMP1 proteins with limited allelic diversity. A larger group of approximately 26 genes is referred to as Group B and Group B/A. These are also subtelomeric but are transcribed towards the centromere and are generally smaller and more diverse than the Group A genes. The Group C and Group B/C genes are highly diverse and located internally on a few chromosomes (Rask et al., 2010), while the atypical Group E genes (typically 1-2 per genome) encode the *var2csa*-type PfEMP1 involved in placental malaria.

Subsequent analysis of genome structure from field isolates, including more recent *de novo* assembly using long-read sequencing technology, indicates that the basic organization and structure of the *var* gene family is conserved globally (Otto et al., 2018a; Otto et al., 2019). However, the level of sequence diversity of the encoded PfEMP1 proteins displays geographical variability, with specific parasite populations displaying different degrees of structural diversity. For example, variability tends to be greatest in Africa and lowest in

South America, with intermediate diversity in Asia (Carlos et al., 2016; Day et al., 2017; Chaudhry and Singh, 2021; Dinzouna-Boutamba et al., 2023). Much of this work has focused on the genes encoding the most conserved region of PfEMP1, called DBLα, which can be easily amplified from small samples and compared. Extensive phylogenetic and similarity studies indicate geospatial clustering, both within specific regions of the world and among continents (Tessema et al., 2015; Rougeron et al., 2017; Sirisabhabhorn et al., 2021; Tonkin-Hill et al., 2021). Together, these studies indicate that *var* gene diversity reflects the underlying population structure within specific geographical regions, with different subsets of *var* genes circulating and recombining within different isolated populations. These findings provide tools for monitoring population movements and have implications for the acquisition of immunity.

3.2. Transcription

Over the course of an infection, *P. falciparum* is known to vary expression of PfEMP1 to enable the parasites to avoid the antibody response generated by the host to individual PfEMP1 variants. This process, called antigenic variation, can extend infections for years and enables transmission even when the mosquito vector is absent for long periods of time, for example in regions of unstable or seasonal transmission. Initial studies of *var* gene transcriptional regulation provided evidence underlying what has become the standard paradigm for antigenic variation in *P. falciparum* (reviewed in Petter and Duffy, 2015; Deitsch and Dzikowski, 2017; Zhang et al., 2022). In brief, each parasite transcribes and translates into protein only one *var* gene at a time (referred to as mutually exclusive transcription), thereby determining the antigenic and cytoadhesion phenotype of the IE. Which gene is transcribed is epigenetically determined, with the single active gene being marked by the histone modification H3K4me3 and acetylated versions of the alternative histones H2A.Z and H2B.Z (Azizan et al., 2023). The remaining and silent members of the family reside in condensed heterochromatin marked by H3K9me3 and bound by heterochromatin protein 1 (HP1) (Figure 1E). These epigenetic states are reinforced through the subnuclear three-dimensional organization. The silenced genes are clustered at the nuclear periphery, while the active gene is found within a specific subnuclear region of transcriptionally active euchromatin. This epigenetic state is maintained through cellular replication, thus providing epigenetic memory that ensures that the parasite can “remember” and maintain transcription of the active gene from one 48-h cycle to the next. However, the parasites occasionally switch transcription and translation from one *var* gene to another in a way that often results in a remarkable population-level coordination of which *var* gene is expressed. Together, these features form the basis of the clonal antigenic variation that sets *P. falciparum* apart from other human malaria parasites. Recent work has shed additional light on these processes (described below), providing a more detailed and mechanistic understanding of this aspect of *P. falciparum* biology.

3.2.1. Generation of diversity: The remarkable PfEMP1 sequence diversity encoded in the genomes of individual parasites enables persistence of infections for months to more than a decade (Ashley and White, 2014). However, equally important for the maintenance of malaria within a geographical region is the overall diversity of the *var* gene repertoire of the strains circulating in the area. This allows people to be infected repeatedly by

different strains and delays the acquisition of clinically protective but non-sterile immunity for years, often until young adulthood or later. The recent ability to assemble complete parasite genomes (Otto et al., 2019), including the repetitive subtelomeric regions in which much of the *var* gene family resides, has demonstrated that the *var* genes are a highly recombinogenic, rapidly evolving family that presents a continuous challenge to the immune systems of people living in endemic regions. These observations provide an explanation for the geospatial distribution and population structure previously described (Tessema et al., 2015; Tonkin-Hill et al., 2021).

The molecular mechanisms driving the continuous and rapid diversification of the *var* gene family was deduced to involve frequent ectopic recombination between family members (Freitas-Junior et al., 2000; Kraemer et al., 2007). Furthermore, the loss of the nonhomologous end-joining DNA repair pathway has been proposed as an evolutionary step selected specifically to promote more frequent recombination (Siao et al., 2020). Approximately two thirds of the *var* gene family are positioned within the subtelomeric chromosomal regions, which can easily recombine among chromosomes, thus accelerating the rate of gene diversification. However, recombination events between subtelomeric *var* genes require that they are oriented in the same direction (transcribed either toward or away from the telomere), a requirement that is thought to contribute to the divergence of *var* genes into A, B and C types (Gross et al., 2021). In addition, chromosome breaks within subtelomeric regions can initiate cascades of recombination, leading to the creation of multiple chimeric *var* genes initiated by a single DNA break (Arnot, 2019; Zhang et al., 2019). Similar rates of increased recombination are observed at internal *var* gene clusters, although the molecular basis for them is not as well understood.

3.2.2. Mutually exclusive transcription: Antigenic variation enables parasites to avoid clearance by the host's antibody response and substantially extend an infection. The process is most efficient if *var* gene expression is strictly mutually exclusive, with only a single *var* gene being expressed at any given time thereby preventing premature exhaustion of the PfEMP1 repertoire. Studies of *var* gene expression in the laboratory and in clinical samples indicate that this is largely the case, raising the question of how, at the molecular level, mutually exclusive transcription is achieved and maintained. Transcriptional activation and silencing of *var* genes depend on epigenetic modifications that control promoter access at each gene, but overall coordination to ensure that only a single gene is active at any time requires genome-level regulation. While the details of this mechanism are still missing, it appears to involve subnuclear organization and the separation of genes into regions of heterochromatin and euchromatin. The clustering of *var* genes into active and silent regions of the nuclear periphery was observed many years ago. More recent work implicates telomeric sequences (Duffy et al., 2017), *var*-specific antisense noncoding RNAs (Amit-Avraham et al., 2015; Jing et al., 2018), and a unique set of noncoding RNAs called RUF6 transcribed by RNA pol III from 15 genetic elements located within internal *var* gene clusters (Guizetti et al., 2016; Barcons-Simon et al., 2020) (Figure 1). Disruption of any of these elements leads to major changes in *var* gene expression, but how these individual components are integrated to mediate mutually exclusive expression is not known.

While there is ample evidence that the mutually exclusive *var* gene expression paradigm is largely true, the process might not be as strict as generally assumed. Active transcription of more than one *var* gene by individual parasites has been detected by *in situ* hybridization (Joergensen et al., 2010a), and more recent work examining the chromatin structure surrounding *var* genes implied that more than one gene can be active simultaneously (Michel-Todo et al., 2023). Similarly, detailed examination of subcloned parasite lines demonstrated that *var* gene expression can be highly variable, including both the level of *var* mRNA as well as its heterogeneity (Zhang et al., 2022), suggesting that *var* mutually exclusive expression might not be as strict as generally assumed. Additional studies employing single-cell RNA-seq verified that while most individual parasites express a single *var* gene at a time, parasites expressing multiple *var* genes or no *var* gene at all are also commonly observed in *P. falciparum in vitro* cultures (Florini *et al.* unpublished data). It also appears that parasites can violate the mutually exclusive expression paradigm *in vivo* (Bachmann et al., 2009), raising questions about the role of this in natural infections (Zhang and Deitsch, 2022).

3.2.3. Epigenetic memory: Once a specific *var* gene is chosen for activation, it must remain active for multiple replicative cycles, with transcriptional switching happening relatively rarely. This process, known as epigenetic memory, requires the reassembly of either active or silent chromatin at each locus as well as the subnuclear organization that contributes to *var* gene transcriptional regulation. The many individual elements mentioned previously, including histone modifications, noncoding RNAs, chromatin modifying enzymes and components that contribute to nuclear structure, all undoubtedly contribute to this process, although the precise choreography is not fully understood. Specifically, the histone marks H3K9me3 (for silent genes), H3K4me3 and H3K9ac (for active genes), along with the recruitment of HP1 to regions of silent chromatin, are thought to be reinstated through the process of genome replication, thereby maintaining the expression profile through multiple cycles of replication. An additional question concerns what happens to this memory as parasites differentiate into the sexual stages, which are not thought to express PfEMP1, and during the transition through the mosquito and subsequent liver stages before emerging as a blood stage infection in a new host.

Comprehensive analysis of *var* gene transcription in the South American *P. falciparum* clone 7G8 before and after controlled human malaria infection of unexposed volunteers (Wichers-Misterek et al., 2023) recently showed that *var* gene transcription is reset during the parasites' passage through mosquitoes. The study thereby confirmed earlier results with African NF54 parasites (Wang et al., 2009; Bachmann et al., 2016; Pickford et al., 2021). In these studies, *var* gene expression patterns are determined in a population of parasites prior to differentiation into gametocytes and subsequent transmission through mosquitos. This includes sexual recombination in the mosquito gut followed by transition through ookinete, oocyst, sporozoite and liver stages. Asexual parasites are then recovered from the circulation after the parasites have egressed from the liver, reestablished erythrocyte infection, and resumed *var* gene expression. By comparing *var* gene expression before and after transmission, the authors determined that expression patterns are reset. Specifically, they found that upon leaving the liver, expression of a broad range of *var* genes is detectable.

This begs the question of how the heterogeneous *var* transcription among the first-generation of parasites emerging from the liver is rapidly focussed on one or a few *var* genes.

3.2.4. Switching: Transcriptional switching between active *var* genes over the course of an infection is required for antigenic variation, however how parasites choose which gene to activate and when to switch are not fully understood. The mechanisms regulating gene choice are likely related to the selection of a dominant *var* gene at the initiation of an infection, although this has not been closely examined. To address this problem, Recker et al. (2011) suggested a "single-many-single" switching mechanism that does not require inter-parasite communication or a fixed switching hierarchy. It involves a two-step process of a switch from transcription of a single *var* gene to transient promiscuous transcription of many or all *var* genes followed by refocusing of transcription on another single *var* gene. The transient promiscuous transcriptional state proposed in this model has obvious parallels to the unrestricted *var* gene expression described at the onset of infection (Wang et al., 2009; Bachmann et al., 2016; Pickford et al., 2021; Wichers-Misterek et al., 2023). More recently, the proposed transient "many"-state was clearly documented in cultured parasites, including the ability of parasites to switch into and out of this state (Zhang et al., 2022), lending additional evidence in support of this model. While the mechanism determining which gene is ultimately activated was not defined in this model, other work potentially sheds light on this question. The propensity to switch from one *var* gene (the "off" rate) to another (the "on" rate) has been reported to vary substantially among different *var* genes, which could impose a switching hierarchy among them (Horrocks et al., 2004). This would lead to a transcriptional probability hierarchy among the *var* genes in the parasite's genome. A similar switching hierarchy was detected by Zhang et al. (2022), along with evidence that the propensity for activation shifts over time, thus providing a possible way for parasites to cycle through their *var* repertoire over the course of an infection.

Another aspect of the Recker switching model is the likely existence of "source" and "sink" nodes; genes that are frequently switched to or away from that lend structure to the proposed switching network. Over time, *var* gene transcription often converges to *var2csa*, the unusually structured *var* gene that encodes the VAR2CSA-type PfEMP1 that are key to the pathogenesis of placental malaria (see section 5.2) (Mok et al., 2008; Ukaegbu et al., 2015), indicating it has the properties of a hypothetical sink node. This gene is universally conserved among *P. falciparum* and related species, unlike the rest of the *var* gene family (Gross et al., 2021), suggesting a potential function in addition to encoding a placenta-specific adhesion ligand. This gene also is regulated at the level of mRNA translation (Amulic et al., 2009) enabling transcription without translation into protein. It may thus be transcriptionally activated many times in the course of an infection without being subject to immune selection. These properties suggest a possible role for *var2csa* in transcriptional switching, and indeed deletion of this gene profoundly alters the switching frequency *in vitro* (Zhang et al., 2022).

3.2.5. Population-level coordination of transcription: Symptomatic *P. falciparum* infections (i.e., malaria episodes) tend to be of low complexity, being completely dominated by one clone expressing one or a few dominant PfEMP1 variants (Kaestli et al., 2004;

Bachmann et al., 2011; Bachmann et al., 2019). In contrast, asymptomatic infections are often multiclonal with parasites expressing many PfEMP1 variants (Smith et al., 1999; Adomako-Ankomah et al., 2017; Eldh et al., 2019). The hypothesis described above that the switch from of one *var* gene to another involves a transient intermediate stage with transcription of many *var* genes provides a possible mechanistic explanation for these observations (Recker et al., 2011). In addition, *var2csa* transcripts are frequently detected in infections of nonpregnant individuals (Duffy et al., 2006; Rovira-Vallbona et al., 2011), consistent with this *var* gene acting as a transcriptional "switchboard". It is also worth noting that for practical reasons, it is common practice to assess *var* gene transcription of parasites freshly isolated from patients after short-term *in vitro* culture. However, analysis of paired samples obtained *ex vivo* and after short-term *in vitro* culture revealed unpredictable changes in *var* gene transcription within the first couple of multiplication cycles *in vitro* (Bachmann et al., 2011; Andradi-Brown et al., 2023). Thus, while *ex vivo* studies can be informative, it is important not to over-interpret the results of such studies.

The order of *var* gene switching is not random (Horrocks et al., 2004; Frank et al., 2007; Lemieux et al., 2013; Zhang et al., 2022). Initial models for *var* transcriptional switching assumed that switching occurs at a "hardwired" rate that evolved to meet the demands imposed by the antibody response of the host, similar to those suspected in early studies of non-human malaria parasites (Brown, 1973). However, recent studies suggest that parasites have the capacity to respond to changes in the local environment (Brancucci et al., 2017; Mancio-Silva et al., 2017; Harris et al., 2023), and environmental changes that alter the methylation capacity of parasites can induce *var* gene switching and activate *var2csa*, consistent with the putative role for this gene as a node in the *var* switching network (Heinberg et al., 2022; Schneider et al., 2023).

There appears to be no inherent growth advantage of expressing particular PfEMP1 proteins in a naïve host (Milne et al., 2021). It is thus tempting to hypothesize that *var* gene switching is a response to evade PfEMP1-specific immunity, similar to the mechanism proposed to drive changes in variant antigen expression in early work on *P. knowlesi* (Brown, 1973). Although a formal causal link between immune recognition and the metabolic conditions that induce *var* gene switching in culture is currently lacking, several studies concluded that the primary selection pressure on *var* gene transcription *in vivo* is PfEMP1-specific antibodies (Abdi et al., 2017; Bachmann et al., 2019).

3.3. Translation and export

During asexual development inside IEs, the parasites produce a large number of proteins that are specifically secreted into the host cell cytoplasm, and in the case of PfEMP1, exported to the IE surface. This requires transport across both the parasite plasma membrane and the parasitophorous vacuole membrane in a process that is relatively unique to apicomplexan parasites (reviewed in De Koning-Ward et al., 2016; Jonsdottir et al., 2021). For most of the proteins included in the "secretome/exportome", this involves a specific host cell targeting motif called a *Plasmodium* export element (PEXEL), which is cleaved by a protease called plasmepsin V prior to export through a novel translocon (Boddey et al., 2016; Batinovic et al., 2017; Beck and Ho, 2021; Gabriela et al., 2022). The discovery of

this trafficking pathway shed light on how parasites modify their environment. However, some exported proteins including PfEMP1 do not contain a canonical PEXEL motif. These PEXEL-negative exported proteins (PNEPs) share some aspects of trafficking with PEXEL containing proteins, but also have unique characteristics, including the involvement of a protein called PfJ23 (Kaur et al., 2018). Specifically for *var2csa*, which is critically involved in the pathogenesis of placental malaria (section 5.2), translation and trafficking is further complicated by the bicistronic nature of the mRNA, which includes an upstream open reading frame (uORF) (Amulic et al., 2009; Bancells and Deitsch, 2013) that alters translation of the *var2csa* mRNA to prevent IE surface expression of VAR2CSA protein in nonpregnant individuals. To overcome the translational block imposed by the uORF, parasites infecting pregnant women must express the translation-enhancing factor PTEF, which enables efficient translation and IE surface expression of VAR2CSA (Chan et al., 2017). Additional work is required to understand how parasites sense the presence of a placenta and alter translation appropriately. Pregnancy-associated hormones do not appear to be involved (Nunes et al., 2008).

3.4. Evolutionary origin

The *Laverania* subgenus encompasses several species of malaria parasites, which all infect great apes such as gorillas, chimpanzees, and humans (Sharp et al., 2020). *P. falciparum* is the only species in the subgenus that infects humans, but all maintain *var* gene families with 28-105 members within their genomes, with the genes found both in subtelomeric regions and in chromosomally internal tandem arrays (Otto et al., 2018b). The general structure of the genes is maintained throughout *Laverania*, but it can be divided into two clades (A and B) with different organization of the encoded proteins, suggesting that they might be cytoadhere to different endothelial receptors. The selective pressure that led to this split is unknown. The *var2csa* gene is found in only four members of the B clade, where it is the only *var* gene retaining the more ancient domain organization found in Clade A. This preservation may be related to its unique role in regulating transcriptional switching, as described above (Otto et al., 2018b; Gross et al., 2021).

4. The PfEMP1 proteins

4.1. Structure

The primary structure (the amino acid sequence) of the extracellular part of different PfEMP1 is highly variable, both among those encoded within a single genome (intraclonal diversity) and among the corresponding proteins in different genomes (allelic variation or polymorphism) (Hviid and Jensen, 2015 and references therein). The amino acid sequence determines the secondary structure of the PfEMP1, which arises from the 2-10 Duffy-binding-like (DBL) domains and cysteine-rich interdomain regions (CIDR) that can be recognized within the primary PfEMP1 structure. Based on so-called homology blocks, the DBL and CIDR domains can be subdivided into seven (α, β, γ, δ, ε, ζ, x) and three (α, β, γ) classes, respectively, most of which can be further sub-divided (Rask et al., 2010). Group A PfEMP1 contain a head structure (the short N-terminal segment (NTS) and the first DBL and CIDR domain that follow it) with a type A NTS (NTSA) followed by a DBLα1 and either a CIDRα1 or a β/γ/δ-type CIDR domain. Group B and Group C PfEMP1 have

head structures that contain a B-type NTS (NTSB) followed by either DBLα0 or DBLα2 and then a CIDR domain from other α-subclasses than α1. The head structure is followed by two to six additional DBL and CIDR domains of various types and sub-types. Group E (VAR2CSA-type PfEMP1) and the very short Type 3 PfEMP1 are exceptions to the above. Thus, VAR2CSA-type PfEMP1 are composed of six-eight divergent DBL domains (three DBLX domains followed by three-five DBLεPAM domains) (Doritchamou et al., 2019b). Type 3 PfEMP1 consist of just a DBLα1.3 and a DBLε8 domain.

Recurring tandem combinations of two or more DBL and CIDR domains are referred to as domain cassettes (DCs). The tertiary structure of individual domains and short tandem domains appear relatively conserved and consist of α-helix bundles connected by flexible loops (Lennartz et al., 2017; Lennartz et al., 2019). Finally, the high-resolution shape of the entire ectodomain (the quaternary structure) of VAR2CSA-type PfEMP1 proteins is now also known, extending the earlier low-resolution data regarding several PfEMP1 types summarized by Hviid and Jensen (2015). Specifically, two independent cryo-electron microscopy studies have shown that the DBL1X-DBL4ε domains of VAR2CSA-type PfEMP1 form a stable compact core that is stabilized by many interdomain interactions (Ma et al., 2021; Wang et al., 2021a). A flexible arm composed of DBL5ε and DBL6ε extends from this core to link the protein to the IE surface (Ma et al., 2021; Wang et al., 2021a). Corresponding high-resolution data regarding other full-length PfEMP1 proteins are not yet available.

4.2. Topology of PfEMP1 expression on the IE surface

After the merozoite-stage parasite has invaded an erythrocyte (reviewed in Cowman et al., 2017), it rapidly transforms into a trophozoite that matures and divides until the infected erythrocyte (IE) eventually bursts to release a new brood of merozoites. Over the 48-h period of intraerythrocytic parasite development, proteins exported by the parasites cause the IEs to become increasingly rigid and deformed (Cranston et al., 1984; Maier et al., 2008; Watermeyer et al., 2016), which makes them susceptible to detection and destruction in the spleen (Ghosh and Stumhofer, 2021). To avoid this, *P. falciparum* parasites have evolved to display PfEMP1 proteins on the IE surface, where they act as anchors that make the IEs stick to vascular receptors (sequestration) to avoid going through the spleen.

To reach the outer surface of the IE, the PfEMP1 proteins must first cross the surface membrane of the intra-erythrocytic parasite, then the parasitophorous vacuole membrane, and finally the IE surface membrane. As the erythrocyte has no protein synthesis and trafficking machinery of its own, the export of PfEMP1 molecules to the IE surface and their correct association with and display on the so-called knobs there involve several proteins and a parasite-derived exomembrane system called Maurer's clefts (Przyborski et al., 2016; Alampalli et al., 2018; Kumar et al., 2018; Looker et al., 2019; Diehl et al., 2021; Sanchez et al., 2022). After reaching the IE surface, a super-resolution microscopy study has now provided direct evidence that the PfEMP1 molecules are predominantly located near the tip of the knobs (Sanchez et al., 2019). Moreover, there may be just a handful PfEMP1 molecules there, which are fewer than estimated earlier and more indirectly (Joergensen et al., 2010b; Alampalli et al., 2018).

The number of knobs per IE varies considerably among studies, consistent with inter-strain and PfEMP1-dependent within-strain variation in knob density (Quadt et al., 2012; Subramani et al., 2015), with further modifications imposed by blood group, haemoglobinopathies, *et cetera* (Petersen et al., 2021). In addition, PfEMP1 expression tends to decline with *in vitro* culture time, consistent with PfEMP1 expression being a response to an *in vivo* selection pressure such as splenic clearance of IEs. In line with this, disruption of the Maurer's cleft-associated protein GEXP07, which causes aberrant knob formation and abrogated PfEMP1 IE surface expression, leads to faster parasite multiplication *in vitro* (McHugh et al., 2020). Furthermore, knob-less and PfEMP1-negative IEs show impaired infectivity (Stanisic et al., 2016), and splenectomy leads to emergence of knob-less, PfEMP1-negative IEs *in vivo* (Bachmann et al., 2009). However, it should be noted that early studies indicate that the latter may not always be true (David et al., 1983; Ho et al., 1992).

The main function of PfEMP1 is undoubtedly to prevent splenic destruction of IEs though sequestration. However, evidence is emerging that this family of proteins possibly also serves functions in the pre-erythrocytic stages (sporozoites and infected hepatocytes) as well as in sexually committed parasites (gametocytes and possibly even gametes) (reviewed in Real et al., 2022). The evidence remains limited, however (e.g., Zanghi et al., 2018), and will not be considered further in this text.

4.3. Interaction with tissue-bound receptors

PfEMP1 binding to a long list of endothelial receptors has been reported (Mahamar et al., 2017). As the distribution of many of these receptors vary considerably among different organs and tissues, the receptor specificity of the PfEMP1 expressed on the IE surface is an important determinant of the clinical presentation of malaria patients (see section 5). In most cases, the binding specificity of PfEMP1 molecules is determined by domain(s) in the IE membrane-distal, N-terminal head structure. It thus generally reflects the PfEMP1 structural categories (A-E) and characteristics (e.g., DCs) mentioned briefly above and discussed in more detail previously (Hviid and Jensen, 2015) and their association with malaria type and severity. In this and the following two sections, we briefly summarize insights obtained since our earlier review, regarding PfEMP1-specific interaction with host receptors.

Endothelial inflammation induced by adhering IEs can cause erosion of the endothelial glycocalyx (Yeo et al., 2019), which has been suggested to contribute to increased IE sequestration by making otherwise inaccessible receptors available for IE adhesion (Hempel et al., 2017). The pathogenic significance of this is unclear, however, as glycocalyx degradation and malaria severity are not obviously related (Yeo et al., 2019).

The scavenger receptor (CD36) is the most common PfEMP1 receptor. It is widely expressed on endothelium, and is also found on many circulating cells, including erythrocytes and platelets. IE adhesion to CD36 has been associated with uncomplicated malaria and asymptomatic parasitaemia (reviewed in Hviid and Jensen, 2015) and is now known to be mediated via a conserved pocket in CIDR domain types α2 to α6, found in many otherwise diverse PfEMP1 proteins (Hsieh et al., 2016).

Since its discovery as an IE adhesion receptor (Turner et al., 2013), the endothelial protein C receptor (EPCR) has received much attention as an IE adhesion receptor because of the relationship of this phenotype with severe malaria in many, although not all, studies. IE adhesion to EPCR is mediated by the CIDRα1 domain in DC8- and DC13-containing PfEMP1, and the ligand-receptor interaction was recently resolved at high resolution (Raghavan et al., 2023). Binding to EPCR was shown to involve a large conformational change in PfEMP1, which opens its compact structure that is thought to protect the protein from immune recognition in the unbound state. Like many other studies of this kind, the study by Raghavan et al. (2023) relied on recombinant proteins, which is worth mentioning here, as the functional relationship between recombinant and native PfEMP1 proteins is not always straightforward (Azasi et al., 2018).

HABP1, also known as gC1qR or p32, is another widely distributed receptor with multiple functions (Ghebrehiwet and Peerschke, 1998), which can be exploited by *P. falciparum* for IE adhesion (Biswas et al., 2007). The interaction is mediated by DBLβ in DC8-containing PfEMP1 (Magallon-Tejada et al., 2016; Bakri et al., 2021). In a similar study, Chesnokov et al. (2018) showed that IE adhesion to the microvascular endothelial integrin αVβ3 (Siano et al., 1998) can be mediated by a DBLδ domain characterized two RGD motifs recognized by integrins like αVβ3. DBL domains containing a single or no RGD motifs did not bind to αVβ3.

While the consensus has been that PfEMP1-mediated IE adhesion only depends on one or a few tandem domains (mainly in the N-terminal head structure) (Smith et al., 2013), recent molecular high-resolution studies suggest that this may be an oversimplification. Thus, recent high-resolution studies of the binding of VAR2CSA to CSA have revealed that the interaction involves multiple domains (Ma et al., 2021; Wang et al., 2021a; Wang et al., 2021b) in this structurally compact PfEMP1 protein that is held together by several inter-domain sulphide bonds (Jagadeeshaprasad et al., 2022). These findings are consistent with the earlier lower-resolution model of the interaction (Bewley et al., 2020) and with the fact that the receptor affinity of full-length VAR2CSA is several orders of magnitude higher than that of any of its constituent domains (Clausen et al., 2012). Two of the recent cryo-electron microscopy studies (Ma et al., 2021; Wang et al., 2021a) reported no conformational difference in VAR2CSA after binding to CSA, whereas the third reported a significant change (Wang et al., 2021b).

4.4. Interaction with receptors on circulating cells

Rosetting is a special type of adhesive interaction that involves IEs adhering to receptors on circulating uninfected erythrocytes rather than on the vascular lining (reviewed in Lee et al., 2022). Rosetting is a complex phenotype that involves multiple receptors and both PfEMP1- and non-PfEMP1 ligands (McQuaid and Rowe, 2020). In *P. falciparum* malaria, it has been associated with disease severity and linked to parasite expression of particular PfEMP1 proteins and host carriage of specific blood group antigens. The functional significance of rosetting remains unclear, and it may simply be a convenient laboratory marker of IEs that can adhere to receptors shared between endothelial cells and erythrocytes (Wang and Hviid,

2015), as for example the ABO blood group antigens (Adams et al., 2014; Nain and Sharma, 2022).

4.5. Interaction with soluble molecules

In addition to interacting with membrane-bound host receptors in tissues and on circulating cells, PfEMP1 has also been found to bind various soluble molecules. The best described of these is circulating pentameric IgM (reviewed in Pleass et al., 2016). This binding of IgM is “non-immune”, meaning that it involves the Fc region of IgM (Fcμ) rather than the antigen-specific recognition element in the Fab region. The interaction is thus independent of the antigen-specificity of the IgM (hence “non-immune”), and it has been proposed to enhance the adhesive properties of IEs (Scholander et al., 1996; Stevenson et al., 2015a; Akhouri et al., 2016) or to inhibit recognition of IEs by PfEMP1-specific IgG (Barfod et al., 2011). The capacity for “non-immune” binding is widespread among PfEMP1 proteins and is mediated by C-terminal DBLε and DBLζ domains (in contrast to the N-terminal domains most often involved in binding to membrane-bound host receptors) (Jeppesen et al., 2015; Quintana et al., 2019). Recent high-resolution microscopy studies are consistent with earlier speculation that “non-immune” binding can serve to augment IE adhesiveness by aligning multiple individual PfEMP1 molecules, although some aspects of these studies are discordant (Akhouri et al., 2023; Ji et al., 2023). Similarly, the abundant and multifunctional serum protein α2-macroglobulin (Vandooren and Itoh, 2021) can also bind to the C-terminus of some PfEMP1 proteins. The function appears to be increasing IE adhesiveness through crosslinking of multiple PfEMP1 molecules, and each α2-macroglobulin molecule can bind four PfEMP1 molecules, whereas a pentameric IgM molecule can only accommodate two (Stevenson et al., 2015b). The clinical significance of “non-immune” IgM binding and binding of α2-macroglobulin to PfEMP1 remains unclear, although there is some evidence that these phenotypes are related to severity (Lopez-Perez et al., 2020).

5. PfEMP1-mediated pathogenesis

As outlined above, PfEMP1 proteins can bind to many different receptors that are differentially expressed in different tissues and organs. The resulting vascular sequestration of IEs can lead to circulatory disturbances and inflammation. As different PfEMP1 proteins have different receptor specificities, the type of PfEMP1 on the IE surface is almost certainly an important determinant of clinical outcome of *P. falciparum* infection, although the relation has mostly been studied at the level of *var* gene transcription (Rorick et al., 2013; Tarr et al., 2018; Tonkin-Hill et al., 2018; Duffy et al., 2019; Bhandari et al., 2021; Wichers et al., 2021). Many of the adhesion receptors exploited by *P. falciparum* are upregulated in response to inflammation and a vicious circle often ensues. This may be the explanation for the recurrent reports of malaria precipitated by traumatic or unrelated infectious insult (Church et al., 2016; reviewed in Hviid, 2020). The observation that exposure of IEs to febrile temperatures *in vitro* increases their adhesiveness to endothelium may also be related to this. However, it is not clear whether this involves temperature-dependent modulation of PfEMP1 expression or increased PfEMP1-independent adhesiveness of temperature-stressed IEs (Zhang et al., 2018; Dorpinghaus et al., 2020).

5.1. Severe and cerebral malaria

Group A and B/A PfEMP1 binding to EPCR and ICAM-1 have both been implicated in the sequestration of IEs in the cerebral microvasculature that causes the vessel occlusion, endothelial inflammation and impaired blood flow to the brain that are key elements in the pathogenesis of cerebral malaria (CM) (reviewed in Hviid and Jensen, 2015; Jensen et al., 2020). The ensuing shedding of EPCR (Moxon et al., 2013) fits the report by Moussiliou et al. (2015) of associations between plasma levels of soluble EPCR, CM, and fatal outcome. The role of genetic variants of EPCR in susceptibility to severe malaria is complex (Gill and Sharma, 2023), and specific EPCR alleles have been associated with protection in some studies, but not in others (Hansson et al., 2015; Shabani et al., 2016; Cespedes et al., 2020).

Adhesion to EPCR is mediated by the CIDRα domains of DC8- and DC13-type PfEMP1 proteins (Turner et al., 2013), and transcript levels of the *var* genes encoding them have repeatedly been shown to be increased in children and adults with severe malaria, including CM (Bernabeu et al., 2016; Jespersen et al., 2016; Mkumbaye et al., 2017; Sahu et al., 2021). Retinopathy is thought to be caused by brain-sequestering IEs (Beare et al., 2011), and the abundance of these transcripts has been reported to be highest among CM children with retinopathy in some (Kessler et al., 2017; Shabani et al., 2017) but not all studies (Abdi et al., 2015).

PfEMP1-mediated IE adhesion to ICAM-1 has long been implicated in the pathogenesis of CM, but as for EPCR, the association between this adhesion phenotype in isolation and CM is far from unequivocal (reviewed in Hviid and Jensen, 2015). By now, it seems clear that although PfEMP1-mediated binding to ICAM-1 can be mediated by many DBLβ domains, only those carrying a specific sequence motif appear relevant in the context of CM (Lennartz et al., 2017). PfEMP1 proteins with that type of DBLβ almost always also contain an EPCR-binding CIDRα domain. We have previously proposed that IEs first adhere to cerebral endothelium via EPCR-binding PfEMP1 variants, causing endothelial inflammation and shedding of the EPCR followed by increased ICAM-1 expression, which can then be exploited by IEs as an adhesion receptor (Jensen et al., 2020)[1]. Consistent with this idea, IEs obtained from CM patients were found to adhere stronger to brain microvascular endothelial cells than IEs obtained from uncomplicated malaria cases (Storm et al., 2019). Supportive *in vitro* evidence also exists (Bernabeu et al., 2019). Of particular interest, the authors reported that the increased adhesiveness was associated with higher levels of *var* gene transcripts predicted to encode EPCR- and ICAM-1-binding PfEMP1 proteins. In this scenario, it seems likely that variants that can mediate IE adhesion to both EPCR and ICAM-1 (“dual binders”) are particularly prone to cause CM (Avril et al., 2016; Lennartz et al., 2017; Tuikue Ndam et al., 2017), although conflicting evidence exists (Joste et al., 2020). Of note, dual-binding IEs were recently observed inside brain microvascular endothelial cells after *in vitro* co-culture (Adams et al., 2021). This process depended on ICAM-1 and caused endothelial cell swelling and disruption of the blood-brain-barrier in an organoid model. Analysis of postmortem brain tissue from CM patients also revealed IEs within brain

[1]A similar sequence of events has been proposed to be part of the pathogenesis of malaria-associated acute respiratory distress syndrome (Maknitikul *et al.*, 2017).

endothelial cells. Together, these findings suggest that endothelial internalization of IEs is part of the pathogenesis of CM (Adams et al., 2021).

All the above notwithstanding, it is important to accept that the pathogenesis of CM is complex and multifactorial (reviewed in Hadjilaou et al., 2023), and that PfEMP1-mediated IE adhesion is one component among many (Guillochon et al., 2022). Even within the brain, expression of known and suspected receptors for PfEMP1-mediated IE adhesion differs substantially among its different regions, adding another potential layer of complexity (Mbagwu and Filgueira, 2020).

5.2. Placental malaria

Placental malaria (PM) is caused by parasites expressing VAR2CSA-type PfEMP1 that are functionally distinct from all other types of PfEMP1 in their unique affinity for the particular type of chondroitin sulphate (CSA)[2] expressed on the syncytiotrophoblast and in the intervillous space of the placenta (reviewed in Hviid and Jensen, 2015). Susceptibility begins early in pregnancy (<120 days of gestation) and PM can have a profound negative impact on pregnancy outcome (Schmiegelow et al., 2017; Accrombessi et al., 2019). In fact, several independent lines of evidence show that PM is often caused by low-grade (even sub-microscopic) infections already present at conception (Berry et al., 2018; Ofori et al., 2018; Tuikue Ndam et al., 2018). This “reversed causality” (infected women becoming pregnant rather than pregnant women becoming infected) can explain the remarkable inefficiency of insecticide-treated bed nets against PM as opposed to their efficiency against malaria in general (Eisele et al., 2012).

Although pregnant women in areas with stable transmission of *P. falciparum* are often infected with parasites expressing several different types of PfEMP1, VAR2CSA generally dominates (Rovira-Vallbona et al., 2011; Anabire et al., 2023). This is because VAR2CSA is immunologically distinct from other PfEMP1 types, and the IEs can therefore escape PfEMP1-specific immunity capable of controlling parasitemia outside pregnancy (see section 6). As the placenta is expulsed after birth, VAR2CSA-expressing IEs lose their sequestration focus, which is the likely explanation for the precipitous and spontaneous clearance of parasitemia among infected pregnant women within a couple of days of delivery (Nguyen-Dinh et al., 1988; Bottero et al., 2011; Anabire et al., 2023).

The ability of VAR2CSA to bind strongly to CSA depends on the so-called “minimal binding region” (MBR), composed of the DBL2X domain, its upstream interdomain (ID) region ID1 and part of its downstream ID2 region (Clausen et al., 2012). VAR2CSA in general, and the MBR in particular, shows very substantial interclonal diversity that may be clinically significant (Doritchamou et al., 2015; Patel et al., 2017; Renn et al., 2021; Talundzic et al., 2022). The detailed interaction of VAR2CSA with its cognate ligand was recently resolved in molecular detail in two elegant studies (Ma et al., 2021; Wang et al.,

[2]Chondroitin sulphate in the placenta (referred to here as CSA) and on many tumour cells are structurally similar to each other and different from chondroitin sulphate at other locations (Clausen et al. 2016; Spliid et al. 2021). This similarity is emphasized in the term onchofoetal chondroitin sulphate (ofCS), which is used increasingly. However, we have maintained the traditional acronym CSA in the present text.

2021a). The structural basis for the apparent importance of phosphorylation of residues in MBR (Dorin-Semblat et al., 2019) remains unclear, however.

5.3. Burkitt lymphoma

Burkitt lymphoma (BL) was first described as an aggressive facial tumour of African children (Burkitt, 1958). The geographical distribution of BL shows a striking overlap with areas of stable transmission of *P. falciparum* parasites (Burkitt, 1962). In such areas, this aggressive non-Hodgkin lymphoma is ten-fold more prevalent than elsewhere and is the most common childhood cancer (endemic BL; eBL) (Hämmerl et al., 2019). The disease is also linked to early and heavy exposure to Epstein-Barr virus (de-Thé et al., 1978).

The role of *P. Falciparum* infection in the pathogenesis of eBL is not well understood, but it has been proposed to involve a polyclonal B-cell activator located in PfEMP1 CIDRα domains (Donati et al., 2004), triggering malignant transformation of B cell clones already destabilized by EBV infection (Torgbor et al., 2014). However, no direct serological evidence linking responses to specific PfEMP1 proteins, such as the above-mentioned CIDRα domains, to eBL has been found (Derkach et al., 2019). It should be noted, however, that the prevalence of eBL peaks several years after that of severe malaria in areas where eBL and *P. falciparum* malaria are endemic. The catastrophic mutation leading to eBL may thus be the result of a relentless pressure on the immune system imposed by chronic low-grade and largely asymptomatic *P. falciparum* infections that persist for years after clinical immunity to severe disease has been established. Considering that scenario (which remains speculative, but is consistent with the available evidence), we have proposed that the putative PfEMP1-specific link to eBL should be found among the very diverse antigens in Group B and Group C (Section 4.1 and Hviid and Jensen (2015)) rather than among those linked to severe malaria (section 5.1) and thus most studied (Quintana et al., 2020).

Finally, it should be mentioned that because chondroitin sulphate on malignant cells resembles placental CSA (see footnote 2), VAR2CSA binds to many tumours (Salanti et al., 2015), including eBL. It is thus conceivable that eBL patients (and indeed patients with many forms of cancers) are at risk of malaria caused by VAR2CSA-expressing parasites. However, as far as we are aware, this hypothesis has only been tested once – in eBL – and the study did not find any supportive evidence (Agerbaek et al., 2017).

6. PfEMP1-specific immune responses

PfEMP1 is an important target of the protective immunity acquired by children in areas with stable parasite transmission, first against severe malaria (including CM, see section 5.1), later also against uncomplicated malaria. The evidence, however, is largely associative, making it hard to establish causal links between IgG responses to specific PfEMP1 antigens and clinical protection (Duffy et al., 2016; Tessema et al., 2018; Travassos et al., 2018; Chan et al., 2019; Tessema et al., 2019; Araj et al., 2021). PfEMP1-specific IgG also appears of importance in the passive immunization of the unborn baby *in utero* (Kivisi et al., 2019; Moussiliou et al., 2020). In contrast, the evidence in favour of a causal role for VAR2CSA-specific IgG in protection against PM (section 5.2) by now appears solid.

Inhibition of the vascular adhesion of IEs by neutralizing PfEMP1-specific IgG is the effector function that has received most attention so far (Attaher et al., 2019). However, recent evidence emphasizes that opsonization of IEs by PfEMP1-specific IgG (independent of adhesion-blocking) for subsequent cell-mediated or complement-mediated destruction are additional mechanisms of potentially major significance.

6.1. Effector functions of PfEMP1-specific antibodies

The antibody-mediated, including the PfEMP1-specific, antibody response to *P. falciparum* is completely dominated by the cytophilic and complement-fixing IgG classes IgG1 and IgG3. As alluded to above, antibody effector functions can be broadly divided into neutralization and opsonization. The function of neutralizing antibodies is direct (independent of effector cells or additional soluble effectors) and is principally independent of antibody class/sub-class. For PfEMP1-specific antibodies, neutralization means inhibition (prevention and/or reversal) of IE sequestration and of rosette formation. In contrast, opsonizing antibodies function through their ability to attract non-antibody effector molecules (primarily complement) or cellular effectors that can recognize the antibody Fc region via their Fc receptors, most of which are IgG-specific. It should be noted that the two antibody effector functions are not mutually exclusive.

6.1.1. Complement-dependent effector functions: The role of complement in the immune response to PfEMP1 is not fully resolved, but it does not appear to affect parasite multiplication *in vitro*. In a study of VAR2CSA-specific IgG, Opi et al. (2021) reported binding of C1q and C3, but no formation of membrane attack complex or cell lysis, in assays using VAR2CSA^{+} IEs opsonized by immune sera from PM-exposed women. There was no binding to erythrocytes infected by parasites engineered to disrupt VAR2CSA expression. In a similar study, we reported very limited binding of C1q to VAR2CSA^{+} IEs opsonized by immune IgG and even less with a VAR2CSA-specific human monoclonal IgG1 antibody, even after modification to enhance its on-target hexamerization (Larsen et al., 2019). A separate potential effector function of complement is direct enzymatic cleavage of IE PfEMP1 by complement component C1s, which appears not to require binding of C1q to IE-opsonizing IgG (Azasi et al., 2021). This C1s-mediated “shaving” inhibited subsequent PfEMP1-dependent adhesion of IEs to their cognate receptors *in vitro*, but the *in vivo* relevance of this process remains unexplored.

6.1.2. Antibody-dependent phagocytosis: Early *ex vivo* studies demonstrated monocytic phagocytosis of IEs that were presumably opsonized by IgG (Vernes, 1980; Ordi et al., 1998). All circulating CD14^{+} monocytes express the high-affinity IgG-specific Fc receptor FcγRI (CD64), whereas “non-classical” (CD14^{lo}CD16^{hi}) and “intermediate” (CD14^{hi}CD16^{lo}) monocytes that constitute about a fifth of them also express the low-affinity FcγRIIIa (CD16) (Ziegler-Heitbrock et al., 2010). Although purified “classical” (CD16^{neg}) and “intermediate” monocytes can both engulf IgG-opsonized IEs *in vitro*, the latter were the most efficient on a per cell basis when complement was present (as it obviously is *in vivo*) (Passlick et al., 1989). The above studies were not PfEMP1-specific, but PfEMP1 is clearly involved in monocytic phagocytosis of IEs opsonized by immune IgG (Chan et al.,

2019; Suurbaar et al., 2022), as are “intermediate” monocytes and FcγRIIIa (Zhou et al., 2015).

Neutrophils are also potential effector cells against IEs (reviewed in Aitken et al., 2018). In a PfEMP1-specific context, this is mainly via phagocytosis of IgG-opsonized IEs (Celada et al., 1983), consistent with the fact that neutrophils express several types of FcγRs. In addition, it was recently reported that neutrophils can recognize IEs expressing ICAM-1-binding PfEMP1 variants (see section 4.3) directly, i.e., independent of IgG, and engulf them (Zelter et al., 2022). This is potentially significant, as IEs expressing PfEMP1 adhering to ICAM-1 are implicated in the pathogenesis of CM (see section 5.1). However, the clinical significance of this finding remains unexplored.

6.1.3. Antibody-dependent cellular cytotoxicity: Recognition of IgG by FcγRIIIa (see the previous section) is strongly influenced by the glycosylation of the Fc region of the antibody (Ferrara et al., 2011). Only the small proportion (usually 10% or less) of total IgG1 that lacks proximal fucose at the single Fc glycosylation site (“Fc-afucosylated” IgG1) binds efficiently to FcγRIIIa (hence the overall low affinity of this receptor). This is of interest in the present context, as we could recently show that half or more of naturally acquired PfEMP1-specific IgG1 can be Fc-afucosylated (Larsen et al., 2021b). The finding is reminiscent of a corresponding marked Fc-afucosylation of antigen-specific IgG in the allo-response of RhD-negative women to paternally encoded RhD on foetal erythrocytes (Kapur et al., 2014). IgG specific for enveloped virus antigens (Larsen et al., 2021a) are also selectively Fc-afucosylated, although mostly to a much smaller degree. Together, these findings indicate that the human IgG1 response to foreign antigen is responsive to the location of foreign antigen, in a way that results in selective Fc-afucosylation of IgG specific for foreign antigen expressed of self-membranes (Larsen et al., 2021a). Natural killer cells (NK cells) rely on FcγRIIIa for their antibody-dependent cellular cytotoxicity (ADCC) effector response. Because of the low affinity of FcγRIIIa for IgG, NK cells have not been much studied as effector cells against antibody-opsonized IEs. However, Arora et al. (2018) reported that IEs opsonized by immune plasma are efficiently lysed by ADCC in *in vitro* co-cultures with NK cells. This fits well with the selective Fc-afucosylation of PfEMP1-specific IgG and with the observation that IEs opsonized by Fc-afucosylated polyclonal and monoclonal VAR2CSA-specific IgG were efficiently killed by ADCC, whereas corresponding Fc-fucosylated IgG was ineffective (Larsen et al., 2021b). In a follow-up of the study by Arora et al. (2018), the same authors found that so-called “adaptive” NK cells within the major $CD56^+$ NK-cell subset, but characterized by low expression of CD56 (Sun et al., 2009), were the most efficient ADCC effectors, and that the frequency of those cells increased with age in an endemic setting (Hart et al., 2019). Most recently, it was reported that NK cells entirely devoid of CD56, and thus not considered in the above studies, share many (but not all) characteristics with “adaptive” NK cells and are in fact the most efficient mediators of ADCC against IgG-opsonized IEs (Ty et al., 2023). Furthermore, the frequency of CD56-negative NK cells increased with age in the study area having the most intense transmission. Importantly, NK cells are not the only effector cells expressing FcγRIIIa. Indeed, frequent exposure to *P. falciparum* infection drives expansion of γδ T cells expressing this receptor (Farrington et al., 2016), and those

cells can also kill IgG-opsonized IEs by ADCC *in vitro* (Farrington et al., 2020). Together, these studies point to an important role of NK cells and other Fc FcγRIIIa-bearing cell subsets in protection against *P. falciparum* malaria. However, there is currently no direct evidence linking Fc-afucosylation of PfEMP1-specific IgG to clinical immunity.

6.2. Severe and cerebral malaria

Available evidence supports a role for IgG interfering with PfEMP1-mediated adhesion of IEs to EPCR in naturally acquired clinical immunity to *P. falciparum* malaria, including severe disease (Rambhatla et al., 2019; Obeng-Adjei et al., 2020; Tewey et al., 2023), although the evidence specifically with respect to CM is less convincing (Nunes-Silva et al., 2015). Regarding CM, Olsen et al. (2018) found that acquisition of IgG specific for DBLβ domains containing the motif characteristic of "dual-binding" PfEMP1 proteins (DBLβ_motif) coincided broadly with the age of peak susceptibility to CM in a cohort of Ghanaian children. These antibodies were mostly broadly cross-reactive (recognizing multiple DBLβ_motif domains). Badaut et al. (2021) studied levels of IgG specific for recombinant proteins representing sequences in CIDRα1.4 and DBLβ domains of the "dual binding" PfEMP1 protein Pf3D7_1150400 (see section 5.1) in Beninese children with asymptomatic and symptomatic (including CM). The authors reported increased levels of specific IgG when comparing convalescence samples to samples obtained during the acute disease episode. Children with asymptomatic infections had the highest IgG levels. However, like some other studies (Joste et al., 2020), they did not find difference in these IgG responses between children with CM and those with uncomplicated malaria.

Together, the above findings are consistent with a role for IgG specific for EPCR-binding, ICAM-1-binding and "dual binding" PfEMP1 proteins in acquired protection against severe childhood malaria and CM. However, they also underscore the difficulty of establishing causal relationships with protection of antibodies that can potentially reflect exposure as well as protection.

6.3. Placental malaria

PM is caused by the selective accumulation of VAR2CSA$^+$ IEs that adhere to CSA on the syncytiotrophoblast and the intervillous space. This can cause placental inflammation and dysfunction, threatening the wellbeing of both mother and foetus (reviewed in Rogerson et al., 2007). As explained in Section 5.2, the type of CSA that acts as a receptor for VAR2CSA is normally restricted to the placenta, and VAR2CSA is completely specific for that receptor. VAR2CSA-expressing parasites consequently do not thrive in non-pregnant hosts. As far as we know, no other PfEMP1 antigens bind to placental CSA. Because VAR2CSA is both functionally and immunologically distinct from all other PfEMP1 variants, women who have never been pregnant before, as well as children and men, do not possess VAR2CSA-specific immunity. First-time pregnant women are therefore fully susceptible to PM, even if they possess substantial protective immunity to other types of PfEMP1 (reviewed in Hviid and Salanti, 2007).

6.3.1. The importance of conformational epitopes: As is the case for all PfEMP1 variants, there is substantial allelic variation of VAR2CSA (Verity et al., 2018).

Nevertheless, the protective immunity to PM that is acquired following exposure to VAR2CSA$^+$ IEs during pregnancy often causes a sharp decline in susceptibility to PM with increasing parity – particularly where endemicity is high (McGregor, 1984). Furthermore, even the first pregnancy can result in IgG levels to CSA-adhering IEs that are as high as those seen among women of high parity (Ricke et al., 2000). This suggests the presence of functionally important epitopes in VAR2CSA that are shared by many allelic variants. There is evidence to support that inference (Barfod et al., 2010), although there is also evidence of potentially clinically significant diversity (Doritchamou et al., 2019a; Renn et al., 2021). In any case, it was unexpected, when Doritchamou et al. (2016) reported that it was not possible to deplete broadly cross-reactive IgG inhibiting IE adhesion to CSA by sequential removal of IgG binding to recombinant proteins representing the MBR (see Section 5.2) and other domains from several allelic variants of VAR2CSA. We recently confirmed this high degree of allele-specificity of single-domain reactive (including MBR-reactive) antibodies at the monoclonal IgG level (Hoffmann-Veltung et al., 2022). In sharp contrast to these findings, broadly cross-reactive IgG inhibiting IE adhesion to CSA can be removed by panning on just a single allelic variant of full-length recombinant VAR2CSA (Doritchamou et al., 2022). These findings indicate that the epitopes recognized by broadly cross-reactive VAR2CSA-specific IgG following exposure to placenta-sequestering IEs during pregnancy often are complex – conformational, multi-domain, and discontinuous. This was theorized by us early on (Barfod et al., 2007), and recently shown directly for a single VAR2CSA-specific monoclonal IgG (Raghavan et al., 2022). The findings furthermore suggest that functionally critical epitopes recognized by the immune system after exposure to native VAR2CSA are not retained in recombinant proteins representing individual domains or short tandem repeats (Fried et al., 2018; Doritchamou et al., 2023).

6.3.2. *Cross-reactivity with epitopes in* Plasmodium vivax: As mentioned above, acquisition of VAR2CSA-specific IgG reactivity is generally though to require exposure to VAR2CSA$^+$ IEs during pregnancy. However, Gnidehou et al. (2014) reported high serum levels of VAR2CSA-specific IgG reactivity in Colombians (including men and children) independent of pregnancy history and equally among individuals exposed to *P. falciparum* and *P. vivax*. The conventional VAR2CSA-specific IgG reactivity pattern (confined to women and related to parity) was observed among African control samples. The authors suggested that their surprising finding was due to widespread exposure to *P. vivax* in Colombia, a parasite that is rare in Africa. They further reported cross-reactivity between an epitope in the *P. vivax* antigen, PvDBP, which is unrelated to PfEMP1 (*P. vivax* parasites do not possess PfEMP1-like antigens) and a conserved epitope in VAR2CSA (Gnidehou et al., 2017; Gnidehou et al., 2019; Mitran et al., 2019; Iyamu et al., 2023). The data remain controversial and may at least in part be due to technical difficulties (Lopez-Perez et al., 2018).

6.3.3. Intermittent preventive treatment and acquisition of VAR2CSA-specific IgG: An early study reported that intermittent presumptive treatment of malaria in pregnant women (IPTp) with sulphadoxin-pyrimethamine (S/P) interfered with acquisition of immunity to PM (Staalsoe et al., 2004) and completely prevented it among primigravidae receiving three doses. Later studies have reported much lower negative impact of S/P-IPTp

on acquisition of VAR2CSA-specific immunity (Djontu et al., 2020), probably reflecting increasing levels of S/P resistance. Nevertheless, S/P remains surprisingly efficient in prevention of PM (Gutman et al., 2021). A possible explanation is that S/P-related suppression of placental parasitaemia is sufficient to alleviate pathology, while allowing acquisition of protective VAR2CSA-specific immunity. Further studies to address this question appear warranted.

6.4. PfEMP1-specific immunity and the ABO blood type

ABO blood group has been reported to affect susceptibility to *P. falciparum* malaria. Although recent meta-analyses do not provide strong support for such a relationship (Degarege et al., 2019a; Degarege et al., 2019b), it is of interest here because blood group A and B have been implicated as receptors for PfEMP1, e.g., in rosetting that is considered a marker of severe malaria (reviewed in McQuaid and Rowe, 2020). Specifically, it has been proposed that blood group A allows formation of bigger and tighter rosettes that can exclude recognition of PfEMP1 on the central IE by specific IgG and cause more severe pathology (Hedberg et al., 2021; Opi et al., 2023). In a study from Papua New Guinea, (Barua et al., 2023) reported higher IgG reactivity to IEs among children with blood group O than among other children. In another study, *in vitro* selection of IEs for adhesion to purified ABO antigens did lead to increased adhesiveness, but it did not cause marked changes in transcription of *var* genes (van der Puije et al., 2020).

6.5. The impact of haemoglobinopathies on PfEMP1-specific immune responses

Heterozygous carriers (sickle-cell trait; HbAS) of sickle haemoglobin (HbS) are markedly resistant to severe malaria, although they are as susceptible to infection with *P. falciparum* parasites as individuals with unmutated haemoglobin (HbAA). This likely involves reduced sequestration and increased clearance of HbAS IEs (Lansche et al., 2018), although the evidence is not unanimous (Mahamar et al., 2017). However, the impaired *P. falciparum* growth rate in HbAS erythrocytes at the low oxygen tensions prevailing in the postcapillary venules where IEs often accumulate is also likely to be protective (Raventos-Suarez et al., 1985; Archer et al., 2018). This is supported by the finding that HbAS women are not protected from placental malaria, where VAR2CSA-mediated IE sequestration occurs in a high-oxygen tension tissue (Patel et al., 2016; Tetard et al., 2017; Chauvet et al., 2019). It furthermore suggests that preferential expression of PfEMP1 variants mediating selective IE sequestration in high oxygen tension tissues is advantageous to *P. falciparum* infecting HbAS individuals. While this conceivably would be reflected as qualitative differences in the PfEMP1-specific antibody response of individuals with and without HbAS, current evidence is inconclusive, both for HbS and for HbC, another malaria-protective haemoglobinopathy (Lopez-Perez et al., 2021; Petersen et al., 2021; Lopez-Perez et al., 2022).

6.6. PfEMP1-dependent parasite evasion of host immunity

P. falciparum parasites somehow “know” which antigens to express on the IE surface to allow sequestration while crucially avoiding the IEs being recognized by specific antibodies (Marsh and Howard, 1986; Bull et al., 1998). Clearly, they can accomplish this feat, but how they do it remains obscure. As already mentioned, early studies of non-human infections

suggested that the intra-erythrocytic parasites can somehow "sense" being recognized and respond by switching from the currently expressed adhesion ligand to one not recognized (Brown, 1973). However, not much hard evidence supporting this intuitive idea exists.

6.6.1. IgM cloaking: As mentioned earlier (section 4.5), pentameric IgM can bind to some PfEMP1 proteins via the constant (Fcμ) regions of the antibody ("non-immune" binding). This was first demonstrated for VAR2CSA-type PfEMP1 (Rasti et al., 2006), but it also applies to other PfEMP1 types (Stevenson et al., 2015a; Quintana et al., 2019). At least in the case of VAR2CSA, one function of the Fcμ-mediated binding of IgM to IEs appears to be immune-evasion. It thus impedes the ability of VAR2CSA-specific ("immune") IgG to recognize VAR2CSA$^+$ IEs while the ability of IEs to adhere to CSA remains unimpeded (Barfod et al., 2011). The affinity of "non-immune" IgM for VAR2CSA is in the nanomolar range and was recently shown by high-resolution cryo-electron microscopy to involve the Fcμ4 domain of IgM and the DBL3X and DBL5ε domains of VAR2CSA (Akhouri et al., 2023; Ji et al., 2023). This finding is consistent with the ability of "non-immune" IgM to inhibit the binding of DBL3X- and DBL5ε-specific monoclonal IgG to VAR2CSA, but not the binding of another VAR2CSA-specific monoclonal antibody (PAM1.4) that is recognizing an epitope not involving DBL3X and only marginally involving DBL5ε (Barfod et al., 2011; Raghavan et al., 2022). It is also consistent with the unimpaired adhesion of VAR2CSA$^+$ IEs after "non-immune" IgM binding, as the CSA-binding pocket of VAR2CSA does not involve DBL3X and DBL5ε (Ma et al., 2021; Wang et al., 2021a).

6.6.2. PfEMP1-specific responses to seasonal variation in transmission: In areas of seasonal parasite transmission, the malaria parasites must be able to persist in the human host during the dry season, where transmission is essentially absent, until the following rainy season, when it resumes. A *P. falciparum* infection can certainly last that long, as cases of asymptomatic infections lasting years and even decades are regularly reported in the literature (reviewed in Ashley and White, 2014; White, 2017; Marino et al., 2023). Chronic asymptomatic infections are common in endemic areas, but the parasitaemias are often extremely low (Mooney et al., 2022). It is plausible that they are kept that way by acquired PfEMP1-specific immunity that can control parasite densities at very low levels, unless the equilibrium is upset by superinfection with parasites expressing PfEMP1 antigens, to which protective immunity is absent (Lines and Armstrong, 1992; Bull et al., 1998)[3]. Parasitemia and clinical symptoms are thus largely controlled by the immune system, consistent with the observation that clinical disease is largely confined to the wet season in areas where transmission varies seasonally, whereas parasite prevalence is much less affected (Greenwood et al., 1987; Dodoo et al., 1999). However, Andrade et al. (2020) recently proposed that *P. falciparum* parasites can actively regulate parasitaemia by transcriptional regulation of PfEMP1 expression to adapt to seasonal changes, largely independent of the pressure imposed by adaptive immunity. Specifically, that by switching to poorly adhesive PfEMP1 variants during the dry season, the parasites reduce inflammation and activation of immune responses, concomitantly reducing parasitaemia through enhanced

[3]Alternatively, the control of existing parasitaemia can break down if the availability of receptors for PfEMP1-specific IE adhesion changes (reviewed in Hviid, 2020).

splenic clearance of non-adherent IEs (Thomson-Luque et al., 2021). This suggests the existence of some sort of parasite sensing mechanism affecting *var* gene switching, and several such have been reported, although their relevance in the present context has not been explored (Wu et al., 2016; Chou et al., 2018; Abdi et al., 2023; Schneider et al., 2023).

6.6.3. Sequence diversity versus epitope diversity: The diversity of *var* genes is immense. Thousands of variants can be present among the parasites within small geographical areas (Barry et al., 2007; Chen et al., 2011), although a small set of variants can dominate in areas with low parasite transmission intensity (Albrecht et al., 2010; Carlos et al., 2016). Furthermore, new variants are continuously being generated (section 3.2.1). These features are often used to explain the slow acquisition of protective immunity. They are also used as evidence of the futility of PfEMP1-based vaccines. Nevertheless, protective immunity to severe forms of malaria can be acquired quite rapidly (Fried et al., 1998; Gupta et al., 1999), even after making allowance for the relative conservation of the placental malaria-associated *var2csa* and the genes encoding the Group A PfEMP1 proteins involved in severe malaria in children. Furthermore, plasma IgG from exposed individuals can efficiently recognize IEs collected at distant locations and decades apart (Nielsen et al., 2004). Finally, even a single *P. falciparum*-exposed pregnancy can result in as high IgG levels as those achievable after 10 pregnancies or more (Ricke et al., 2000), and a single allelic variant of VAR2CSA efficiently depletes broadly neutralizing IgG reactivity from immune plasma (Doritchamou et al., 2022). Taken together, these observations suggest that the diversity of clinically relevant PfEMP1 antibody epitopes is much smaller than intuitively anticipated from the diversity of *var* gene sequences and the amino acid sequences they encode. A similar conclusion was drawn from a study of the hypervariable variant surface glycoproteins of *Trypanosoma brucei* many years ago (Blum et al., 1993), but the inference has yet to be explored in depth in malaria.

7. PfEMP1 and vaccination against malaria

The key importance of particular PfEMP1 antigens in severe malaria pathogenesis and immunity provides the rationale for the development of PfEMP1-based vaccines designed to prevent specific and severe clinical presentations of *P. falciparum* malaria, such as CM (reviewed in Bull and Abdi, 2016; Hviid et al., 2018; Jensen et al., 2020) and PM (reviewed in Hviid, 2011; Doritchamou et al., 2021). Durable, practical, and sterile protection against *P. falciparum* infection through vaccination would clearly be optimal, but it has yet to be achieved. PfEMP1-based vaccination offers the alternative strategy of focussing on preventing disease; aiming to emulate and preferably accelerate naturally acquired immunity rather than to achieve something that nature has not conceived. It seems plausible that successful PfEMP1-based vaccines would markedly reduce the burden of malaria and that the immunity induced would be more durable than that of non-PfEMP1 vaccines. However, obstacles clearly remain.

The development of a PfEMP1-based vaccine to protect against PM is most advanced, as two VAR2CSA-based vaccines have already been tested in clinical trials (Mordmuller et al., 2019; Sirima et al., 2020). Both vaccines elicited IgG that was largely specific for the allelic variant used in the vaccine. Furthermore, the vaccine-induced IgG showed only

very limited ability to inhibit IE adhesion to CSA, despite being based on the minimal binding region of VAR2CSA. These findings were disappointing, considering the prevalence of broadly cross-reactive and neutralizing (IE adhesion-inhibiting) IgG after exposure to $VAR2CSA^+$ IEs during pregnancy (see section 6.3). As discussed in section 6.3.1, the difference could be due to the absence in the vaccines of the conformation-dependent epitopes that induce the broadly cross-reactive and neutralizing IgG acquired after exposure to native VAR2CSA, but this remains to be assessed directly (Figure 2). In the meantime, the results of experimental vaccination with EPCR-binding CIDRα1 domains indicate that this issue may apply to PfEMP1 antigens in general (Turner et al., 2018). If the importance of conformation-dependent, including discontinuous and multi-domain epitopes is confirmed, novel strategies to overcome this difficulty may be needed, for example through the design of synthetic antigens displaying relevant antibody mimotopes (Barber et al., 2021).

In addition to the above shortcomings of the current PfEMP1-based subunit vaccines, it is also of concern that they elicit fully Fc-fucosylated VAR2CSA-specific IgG, rather than the markedly Fc-afucosylated IgG induced by natural exposure to PM (Larsen et al., 2021b) (Figure 2). However, the clinical significance of FcγRIIIa-ADCC, which is only elicited by Fc-afucosylated IgG (see section 6.1.3) in protective acquired immunity to *P. falciparum* malaria remains to be established (Damelang et al., 2021). If confirmed, revised strategies for vaccine delivery of PfEMP1 antigens may be required, for example via recombinant enveloped viruses.

8. Conclusions and future directions

The understanding of how PfEMP1 function as adhesive proteins, and their role in malaria pathogenesis and as targets of protective immunity has continued to deepen with great speed in the last decade, not least thanks to dramatic technological developments in super-resolution microscopy. The same applies to the molecular details of the *var* gene switching that underpins it all. Concurrently, the development of PfEMP1-based vaccines has been met with unforeseen obstacles. However, new insights regarding the characteristics of broadly cross-reactive and neutralizing PfEMP1-specific IgG as well as identification of immune effector functions in addition to neutralization give hope that these difficulties can be overcome. Exciting times lie ahead for students of the *var* genes and the PfEMP1 proteins they encode.

Acknowledgements

Research in the authors' laboratories is supported by National Institutes of Health, Commission of the European Communities, The Consultative Committee for Development Research, the Lundbeck Foundation, and the Danish Council for Independent Research.

Abbreviations used:

CIDR	Cysteine-rich interdomain region
CSA	chondroitin sulphate 1
DBL	Duffy-binding-like

IE	infected erythrocyte
MS	mass spectrometry
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1

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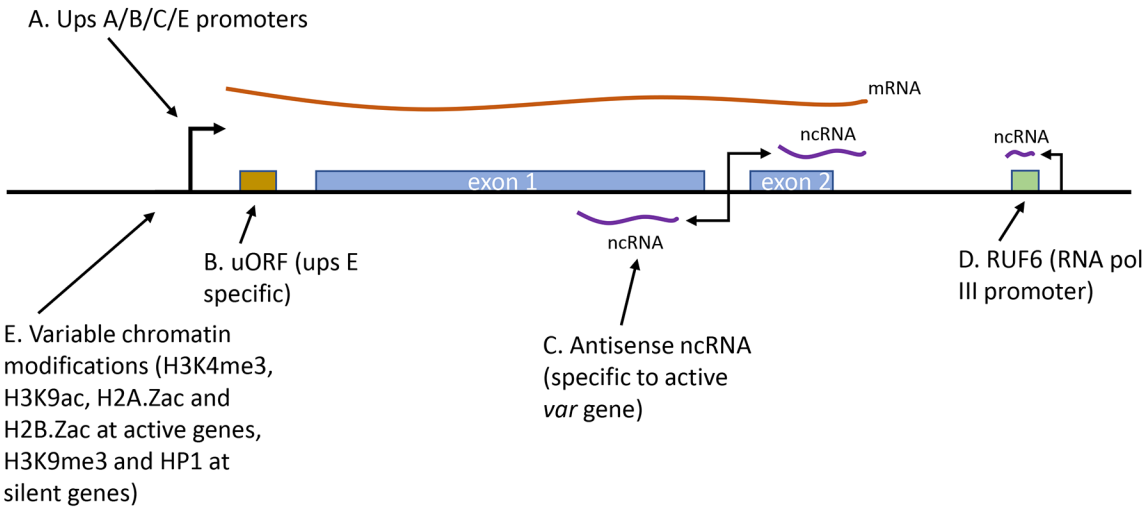

Figure 1. General organization of *var* genes and associated regulatory elements.

Transcription of the PfEMP1 encoding mRNA initiates at upstream promoter regions of four basic types, referred to as Ups A/B/C or E [A]. Ups E promoter types are associated with an upstream open reading frame (uORF) positioned between the transcription start site and the PfEMP1 coding region. This serves as a translational repressor and a protein export motif [B]. All *var* genes contain a bidirectional promoter situated within a conserved intron that gives rise to both sense and antisense noncoding RNAs (ncRNAs). The antisense ncRNA has been associated specifically with the active *var* gene [C]. A unique class of ncRNAs called RUF6 transcribed by RNA polymerase III are found at 15 loci within the parasite's genome at positions within or adjacent to clusters of *var* genes. Transcription of these ncRNAs has been associated with *var* transcriptional activation [D]. Variable chromatin structure is associated with activation and silencing of *var* gene transcription. These histone modifications extend across the entire promoter region, including the transcription start site and most of exon 1 [E].

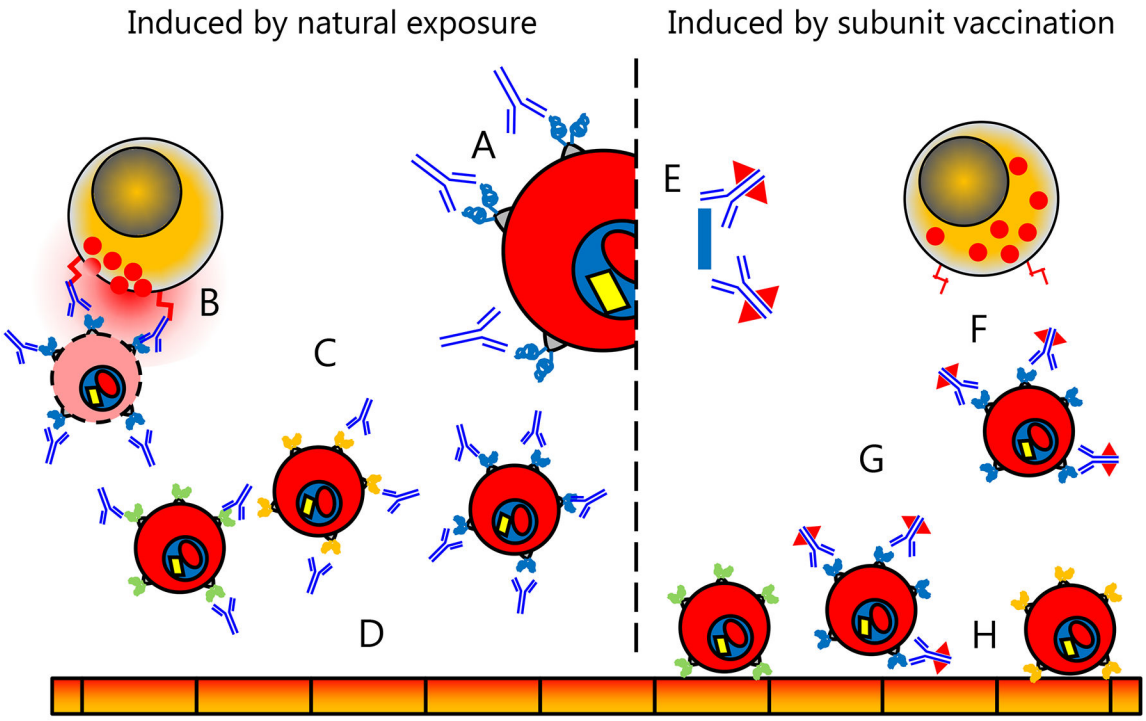

Figure 2. PfEMP1-specific IgG induced by natural infection and by subunit vaccination.

Exposure to native, conformationally intact PfEMP1 [A] induces afucosylated IgG that induces ADCC [B], that can recognize complex and inter-clonally conserved epitopes [C], and that inhibits IE adhesion [D]. Subunit vaccination with soluble, recombinant oligo-domain, conformationally compromised PfEMP1 [E] induces fucosylated IgG that cannot induce ADCC [F], recognizes epitopes that are often simple and allelic variant-specific [G], and with limited capacity to neutralize IE adhesion [H].