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Repurposing of *Plasmodium falciparum* var genes beyond the blood stage

Eliana Real*, Flore Nardella*, Artur Scherf and Liliana Mancio-Silva

A commonly observed survival strategy in protozoan parasites is the sequential expression of clonally variant-surface antigens to avoid elimination by the host's immune response. In malaria-causing *P. falciparum*, the immunovariant erythrocyte-membrane protein-1 (PfEMP1) adhesin family, encoded by *var* genes, is responsible for both antigenic variation and cytoadherence of infected erythrocytes to the microvasculature. Until recently, the biological function of these variant genes was believed to be restricted to intraerythrocytic developmental stages. With the advent of new technologies, *var* gene expression has been confirmed in transmission and pre-erythrocytic stages. Here, we discuss how repurposing of *var* gene expression beyond chronic blood-stage infection may be critical for successful transmission.

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Introduction

Pathogens have evolved diverse immune-evasion strategies to establish prolonged or chronic infection in their hosts, thus ensuring enhanced transmission of the pathogen. A common strategy in protozoan parasites such as African trypanosomes, *Giardia lamblia*, or *P. falciparum* is the sequential expression of variant-surface antigens, so-called antigenic variation, to avoid clearance by the

host's adaptive immune response [1]. Mutually exclusive expression within large gene families is not a unique feature of pathogens, but a process conserved throughout the evolution of the eukaryotic lineage. In the case of mammalian olfactory genes, singular gene expression involves complex changes of nuclear architecture mediated by enhancer elements [2]. In *P. falciparum*, antigenic variation is mediated either by the expression of a single member of the *var* gene family (monoallelic expression) or a small subset of large gene families that encode small variant antigens, such as RIFIN and STEVOR (Table 1).

It has long been assumed that antigenic variation is a process uniquely evolved to evade the immune response of the vertebrate host. In African trypanosomes for instance, the expression of variant-surface glycoproteins is switched off upon parasite transmission from the mammalian host to the insect vector [3]. The recent discovery that a subset of *var* genes are expressed in life-cycle stages that develop in *Anopheles* mosquitoes (Figure 1) challenges this view [4,5••]. Here, we review the known roles of *Plasmodium* variant multigene families with a focus on *var* genes and discuss how the functional repurposing of *var* gene products beyond the infected erythrocyte might mediate host-parasite interactions essential for the establishment of infection in the human host.

Plasmodium clonally variant gene families

Plasmodium genomes encode highly polymorphic multiprotein families that underpin the extraordinary ability of the parasite to escape immune detection and continually adapt to changes in its environment. While the *var* gene family represents the best-studied example, other *Plasmodium* multigene families, many of which encode surface proteins that mediate adhesion to host-encoded receptors, also show variant expression and have been similarly linked to immune evasion and malaria pathogenesis (Table 1). Whole-genome sequencing has enabled us to explore their repertoire, genomic context, and evolutionary history. A key feature of many of these variant multigene families is that they are found on the highly recombinogenic subtelomeric regions of chromosomes, which is thought to contribute to the continual generation of new gene variants [6]. In *P. falciparum*, the *var*, *rif*, and *stevor* families are organized in

Table 1

Clonally variant multigene families in human-infective *Plasmodium* species.

Gene family	Number of genes in each family and species (heterochromatin-associated) ^a			Life-cycle stage with proteomic evidence of expression ^b			Function	References
	<i>Pf</i>	<i>Pv</i>	<i>Pk</i>	<i>Po</i> ^c	<i>Pm</i>			
<i>var</i>	60 (60)	0	0	0	0	ABS; GAM; SPZ; LS	Cytoadherence; antigenic variation; immune evasion.	[7-9,35,38]
<i>pir</i>	0 ^d	1212 (633)	70 (60)	1949	255	ABS; SPZ	Putative role in immune evasion.	[7-10,38,39]
<i>rif</i>	160 (160)	0	0	0	0	ABS; GAM	Rosetting; antigenic variation; immune evasion; modulation of immune signalling.	[7-9,11,15,38]
<i>stevor</i>	30 (30)	0	0	0	0	ABS; GAM; SPZ	Rosetting; RBC invasion; modulation of host-cell rigidity; antigenic variation; immune evasion	[7-9,12,38,40]
<i>pfmc-2tm</i>	13 (13)	0	0	0	0	ABS	Unknown	[7-9,38,41]
<i>trag</i>	3 (0)	40 (1)	29 (0)	41	42	ABS	Binding to host-cell receptors; invasion of RBCs.	[7-10,38,42]
<i>SICAvar</i>	0	0	237 (56)	0	0	ABS	Antigenic variation; cytoadherence.	[7-9,38,43]
<i>fam-m</i>	0	0	0	0	283	-	Putative role in adhesion to RBCs based on structural similarity to Pf RH5.	[7-9,38]
<i>fam-l</i>	0	0	0	0	396	-	Putative role in adhesion to RBCs based on structural similarity to Pf RH5.	[7-9,38]
<i>surfin/STP1</i>	10 (2)	9 (7)	0	70	166	ABS; SPZ	Adhesion to and invasion of RBCs (SURFIN _{4,2}).	[7-10,13,38]
<i>rbp/rh</i>	5 (2)	5 (0)	2 (0)	9	3	ABS; GAM	RBC invasion; binding to host-cell receptors.	[7-10,38,44,45]
<i>6-cys</i>	12 (1)	13 (0)	13 (0)	13	11	ABS; GAM; SPZ	Stage-specific functions range from fertilization, hepatocyte invasion, PVM formation and maintenance, and mosquito immunity evasion.	[7-10,38,46]
<i>clag</i>	5 (2)	3 (1)	2 (0)	3	4	ABS	Nutrient uptake; solute transport (clag3.1, clag3.2).	[7-10,38,47]
<i>gbp</i>	3 (2)	0	0	0	0	ABS; GAM	Unknown; putative role in RBC binding.	[7-9,38,48]
<i>phist/rad</i>	81 (22)	82 (12)	44 (8)	54	30	ABS; GAM	Modulation of host-cell rigidity (phist b); protein traffic (phist c).	[7-10,38,49]
<i>hyp 1-17</i>	58 (12)	0	0	0	0	ABS; GAM	Unknown; Maurer's cleft localization (HYP4-5)	[7-9,38,50]
<i>fikk</i>	19 (0)	1 (0)	1 (0)	1	1	ABS; GAM; SPZ	RBC remodeling; modulation of host-cell cytoskeleton.	[7-10,38,51]
<i>acs</i>	12 (3)	4 (0)	4 (0)	5	4	ABS; GAM; SPZ	Binding to host-cell ankyrin (ACS1/3).	[7-10,38,52]
<i>etramp</i>	15 (0)	10 (1)	10 (1)	7	8	ABS; GAM; SPZ	Interactions with the host (usually at the host-PVM interface).	[7-10,38,53]
<i>lpl</i>	13 (1)	9 (1)	8 (1)	8	8	ABS; GAM; SPZ	Lipid homeostasis (PfLPL1); membrane biogenesis (PfLPL3).	[7-10,38,54,55]

Abbreviations: *clag*, cytoadherence-linked asexual gene; *etramp*, early transcribed membrane protein; *rif*, repetitive interspersed fragment; *stevor*, subtelomeric open-reading frame; *phist*, *Plasmodium* helical interspersed subtelomeric; *surfin*, surface-associated interspersed; *trag*, tryptophan-rich antigen; *6-cys*, 6-cysteine; *lpl*, lysophospholipase; *acs*, acetyl-CoA synthetase; *gbp*, glycoprotein-binding protein; *SICAvar*, Schizont infected cell-agglutination variant antigen; *RBC*, red blood cell; *ABS*, asexual blood stages; *GAM*, male and female gametocytes; *SPZ*, sporozoite; *LS*, liver stage; *Pf*, *P. falciparum*; *Pv*, *P. vivax*; *Pk*, *P. knowlesi*; *Po*, *P. ovale*; *Pm*, *P. malariae*.

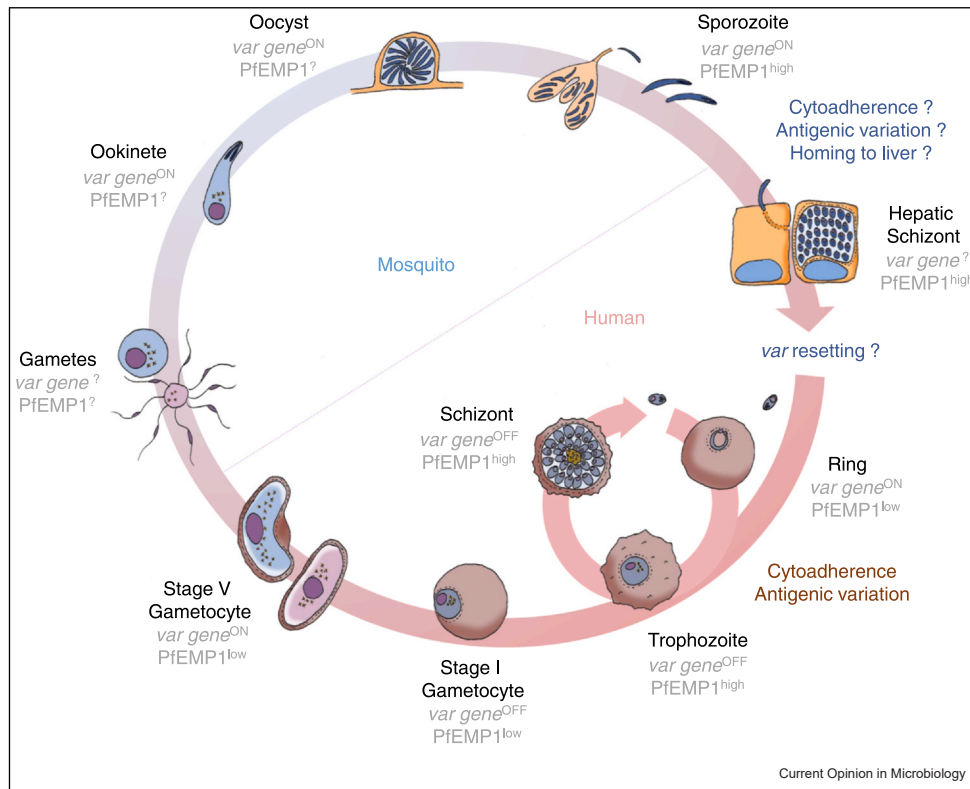
^a The number of family members with heterochromatin association is shown in parentheses. Data from [16••].

^b Life-cycle stages for which there is proteomic or functional evidence of expression. Data from [5••,26,30,41,56-59].

^c Data for *Plasmodium ovale curtisi*.

^d Based on protein-structure analysis, RIFIN and STEVOR are no longer considered members of the PIR protein family [60].

Figure 1



Model depicting known and putative roles of *var* genes across the *P. falciparum* life cycle. PfEMP1 proteins are expressed in knob-like structures on the surface of infected erythrocytes during the blood stages of infection, where they mediate cytoadherence and immune evasion. During sexual development or gametocytogenesis, *var* gene expression drops significantly. Mature gametocytes are taken up in the mosquito blood meal and undergo fertilization in the mosquito midgut. A series of developmental transformations then lead to the formation of the ookinete, which further develops into the oocyst upon crossing the midgut epithelium. Sporozoites initially develop within the oocyst, before being released to complete their maturation in the salivary glands. Expression of a single *var* gene has been reported in the oocyst stage and precedes surface PfEMP1 expression in salivary-gland sporozoites. Upon transmission to the human host, PfEMP1 expression might contribute to sporozoite homing to the liver and hepatocyte invasion. A second resetting of *var* gene expression during the liver stage likely produces a diverse repertoire at the onset of blood-stage infection, enabling the parasite to adapt to the immune status of the host.

clusters, with the *var* gene occupying the telomere-proximal position followed by multiple copies of *rif* and *stevor* family members [7]. Some *var* genes can also be found outside subtelomeres, in the internal regions of chromosomes. Both the chromosomal location and transcription orientation (whether a *var* gene is transcribed toward or away from the telomere) correspond to a specific promoter type upstream of the coding sequence (upsA, upsB, and upsC). The major variant gene families of *Plasmodium knowlesi*, *SICAvar* and *kir*, present an atypical chromosomal arrangement, as they are found exclusively outside subtelomeres [8].

The repertoires of *Plasmodium* variant multigene families across the 5 human-infective species show striking examples of large species-specific gene expansions (Table 1). The *P. falciparum* Maurer's cleft two-transmembrane protein (*pfmc-2tm*) family, which

encodes several exported paralogous proteins of unknown function, is only found on the *Laverania* subgenus and has undergone expansion in *P. falciparum*. The surfin-related subtelomeric protein 1 (*STP1*) is highly expanded in *Plasmodium ovale* and *Plasmodium malariae*, whereas *fam-l* and *fam-m* are uniquely present in *P. malariae* (Table 1). The largest expansion, however, is observed in the *Plasmodium* interspersed repeat (*pir*) gene repertoire of *Plasmodium vivax* and *P. ovale* [9,10], which could be an indication of functional diversification within the *pir* superfamily of these hypnozoite-forming species.

Cytoadherence, invasion, and other functions

Several products of the variant multigene families have adhesive properties consistent with roles in erythrocyte invasion, rosette formation, and endothelial cytoadherence (Table 1). The RIFIN and STEVOR proteins of *P.*

falciparum have been implicated in rosetting through binding to group-A antigen and glycophorin C in erythrocytes, respectively [11,12]. SURFIN_{4,2}, which is closely related to the STP1 protein of *P. vivax*, has been shown to bind to erythrocytes through a cysteine-rich domain and may play a role in invasion through a putative interaction with rhoptry neck protein 4 at the parasite–host-cell-moving junction [13]. Other variant gene families with known roles in invasion include the reticulocyte-binding protein (RBP) and reticulocyte-binding homolog (RH). Interestingly, the predicted 3D structures of *P. malariae* *fam-l* and *fam-m* proteins were shown to overlap with that of RH5, the *P. falciparum* ligand for the basigin receptor expressed on the surface of erythrocytes [9,14]. It seems therefore plausible that *fam-l* and *fam-m* proteins may also have a role in invasion through their binding to host-cell receptors.

In addition to antigenic variation, variant multigene families encode other adaptive strategies that help the parasite to survive different types of environmental pressures. For instance, some RIFIN proteins bind to inhibitory immune receptors on myeloid and lymphoid cells to suppress immune signaling and evade the host immune system [15].

Regulation of clonally variant gene expression

Variant gene expression is orchestrated by multiple layers of transcriptional and post-transcriptional mechanisms that are regulated by diverse epigenetic and epitranscriptomic factors. Heterochromatin-dependent gene silencing is central to clonally variant gene expression during blood infection in humans [16••,17] and infected mosquitoes [4•,5••]. Chromatin-immunoprecipitation studies demonstrated differential and reversible enrichment of post-translational histone modifications in transcriptionally active and silent gene members. The silenced state is characterized by the presence of histone H3 lysine 9 trimethylation (H3K9me3) and heterochromatin protein 1 (HP1), whereas the active state is associated with acetylation of H3K9 (H3K9ac). Additional factors contributing to the monoallelic expression of *var* genes include differential nuclear positioning of the transcriptionally active gene [18] and long noncoding RNAs (lncRNA) [19,20] (Box 1; Figure 2). The pathways involved in the upstream regulation of each multigene family are not fully understood but are likely to be responsive to environmental factors. The cytoadherence-linked asexual protein (*clag*) paralogs *clag3.1* and *clag3.2*, which are components of an aqueous pore on the erythrocyte membrane involved in nutrient uptake [21], provide such an example. Expression of the two CLAG3 paralogs is mutually exclusive and dependent on H3K9m3 loss [22]. Switching between the two paralogs, enables the parasite to adapt to changes in nutrient availability.

Box 1 Calling *var* gene expression in transcriptomics studies.

var genes have a two-exon structure with the conserved intron directing the transcription of two lncRNA, one antisense and a second sense lncRNA (Figure 2) [20,35]. The existence of these lncRNAs can complicate the interpretation of transcriptomics data, especially as more data sets are being generated through scRNA-seq technologies that fail to simultaneously profile full-length transcripts and discriminate between sense and antisense transcripts. Without this information, assessing whether a *var* gene transcript might be coding or noncoding requires some caution. As pointed out by Reid et al. [25], only transcripts that span over the *var* intron and include the two exons constitute *bona fide* mRNA. Transcripts mapping to only one exon could represent lncRNAs transcribed from the *var* intron and as such should not be used to call *var* gene expression. scRNA-seq protocols enabling strand-specific full-length transcript profiling have been described [36] and might be considered as suitable alternatives to the commonly used SMARTseq2 and 10x protocols for surveying *var* transcript expression in single cells.

Variant gene families across the malaria life cycle

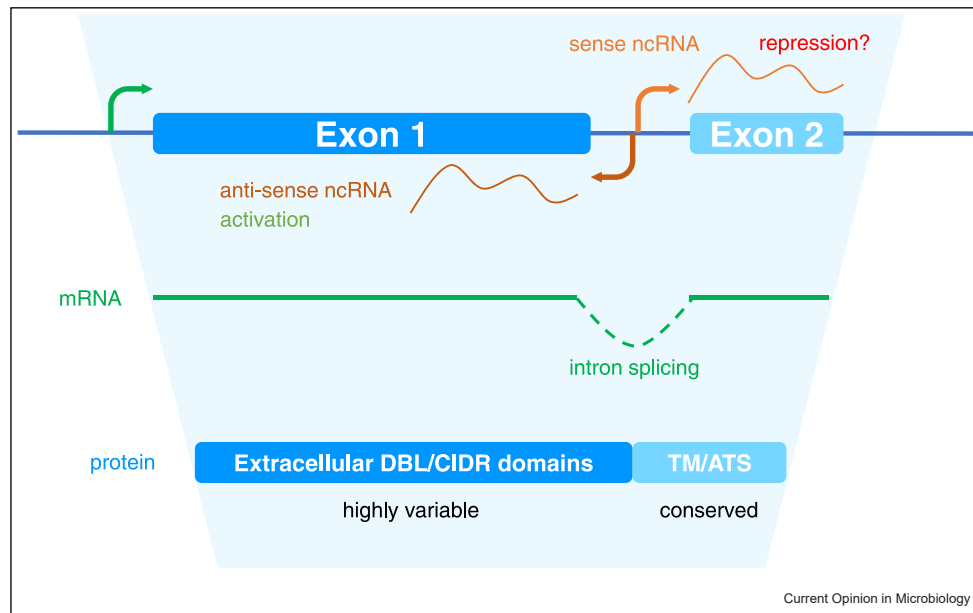
Beyond their roles in the blood stage, there is mounting evidence that *Plasmodium* variant multigene families could play additional roles across the life cycle (Table 1). In the rodent parasite *Plasmodium berghei*, multiple proteins encoded by the *pir*, *fam-a*, and *fam-b* families are exported to the parasitophorous vacuole membrane (PVM) of hepatic schizonts, where at least Fam-a proteins have been implicated in the transfer of host lipids across the vacuole [23]. Expression of *pfmc-2tm* transcripts, which was thought to be restricted to infected erythrocytes, is highly abundant in *P. falciparum* ookinetes [24•], raising intriguing questions about a possible role for this poorly understood multigene family in mediating immune evasion in the mosquito midgut. It is equally likely that it could be a response to other environmental pressures. The picture emerging from these and the example of the *var* gene family that will be discussed below is that variant families constitute versatile multistage actors of host–parasite interactions.

Repurposing and resetting of *var* genes

Although it is commonly admitted that expression of *var* genes and of the PfEMP1 proteins they encode is restricted to asexual blood stages of infection, there is evidence that single members of the *var* gene family can be transcribed in gametocytes, ookinetes, oocysts, and sporozoites [4•,5••,24•,25] (Figure 1). The implications of this functional repurposing of a variant-surface adhesin with respect to transmission and the establishment of infection in the human host are starting to emerge.

In gametocytes, single-cell RNA sequencing (scRNA-seq) profiling of gene usage revealed that *var* gene expression is mutually exclusive in both male and female

Figure 2



Basic architecture of *var* genes and their encoded proteins. All *var* genes are composed of two exons. In addition to the 5' upstream promoter, a second bidirectional promoter is located in the *var* intron, leading to transcription of sense and antisense lncRNAs. Although there is experimental evidence linking antisense lncRNA with *var* gene expression [20], deletion of the *var* intron does not affect *var* gene activation or silencing [37]. The intron is spliced out during mRNA maturation and leads to a high-molecular-weight protein composed of highly variable extracellular (DBL/CIDR) domains, a transmembrane (TM) domain, and an acidic terminal sequence (ATS) that is conserved among PfEMP1 proteins. Different types of CIDR domains mediate PfEMP1 binding to major host receptors, namely the scavenger receptor CD36 and the endothelial protein C receptor (EPCR).

sexual stages. Interestingly, the detected *var* transcripts are of the UpsB and UpsC types [24,25]. While proteomics data further confirm the presence of several PfEMP1 proteins in gametocytes [26], their postulated role in cytoadherence and spleen and bone marrow sequestration during gametocytogenesis remains contentious. The lack of knobs in sexual stages suggests that the PfEMP1 variants on the surface of stage-I gametocytes might be insufficient to mediate cytoadherence on endothelial cells [27–29].

Regarding the mosquito stages, several transcriptomics and epigenetic studies reported the presence of *var* transcripts in the ookinete and oocysts [4,5,24,30], although at low levels and mostly expressed as sense lncRNAs (Figure 2). The exception is the *var* gene encoding the PfEMP1 expressed in the sporozoite stage, named *var*^{spor} [5]. Independent validation that sporozoites express a specific PfEMP1 on their surface was obtained using antibodies recognizing the extracellular domain of the protein [5]. Exposure of sporozoites to anti-*var*^{spor}-specific antibodies prevented infection of primary human hepatocytes. The effect, however, was shown to be strain-dependent, suggesting that other PfEMP1 proteins not recognized by these specific

antibodies may be expressed in sporozoites. Likewise, it remains unclear whether PfEMP1 expression in sporozoites is associated with a specific subset of *var* genes and what their distinguishing features might be. It also remains to be determined whether PfEMP1 proteins expressed at this stage could play additional roles along the sporozoite journey to the liver. Based on the known adhesive functions of this protein family, the possibility that PfEMP1 could mediate the homing of sporozoites to the liver is an appealing idea. Indeed, previously identified PfEMP1 host receptors are also expressed in the liver, namely on the surface of hepatocytes. Expression of this highly polymorphic protein on the sporozoite surface could also play a role in immune avoidance, thereby protecting the PfEMP1 for an essential role in receptor engagement during hepatocyte invasion. This may be particularly relevant in endemic areas where the host may harbor anti-PfEMP1 antibodies from previous malaria episodes. In this context, it is tempting to speculate that parasites with *var* repertoires not previously seen by the host have better chance to invade hepatocytes and establish new infections.

The lack of genome-wide transcriptomic data for *P. falciparum* intrahepatic stages precludes a thorough

analysis of the *var* gene repertoire in the liver. However, the analysis of the first generations of blood-stage parasites from malaria-naïve human volunteers infected with *P. falciparum* sporozoites have demonstrated transcription of a consistent broad repertoire of *var* genes and other variant multigene families [31,32••]. Similar results were observed in the rodent *Plasmodium chabaudi* for the *cir* multigenic family after mosquito passage [33], pointing to a conserved epigenetic resetting occurring during transmission. Despite the absence of transcriptional data, PfEMP1 expression has been confirmed in *P. falciparum* liver schizonts using an antibody recognizing the intracellular conserved domain (G Zanghi, PhD thesis, Sorbonne University, Paris, 2016). Taken together, these observations suggest that the liver stage may promote a general resetting of *var* gene expression, so that the parasites released into circulation are equipped with a diverse repertoire (Figure 1) and may enhance blood-stage infection, regardless of pre-existing host immunity against PfEMP1 proteins. The resetting model, which could be viewed as an adaptive strategy, raises fundamental questions about the regulation of variant gene expression in these underexplored steps of the *Plasmodium* life cycle.

Conclusion

It is becoming increasingly apparent that *Plasmodium* variant multigene families are not exclusively associated with blood-stage infection and may play additional roles across the life cycle. Cell atlasing initiatives have unveiled previously unsuspected gene-expression patterns for members of these gene families (reviewed in Ref. [34]), but due to the prevalence of post-transcriptional repression mechanisms in *Plasmodium*, it remains unclear whether this transcriptional activity translates into protein expression and a gain in function. Future work should address whether the recently characterized *var*^{sporo} [5••] confers additional adhesive properties to the sporozoite transmissive form, potentially altering the way sporozoites interact with their environment during the journey to the liver. Identification of *var*^{sporo} host-interacting factors as well as a potential role in antigenic variation and hepatocyte invasion should be further investigated. It will also be important to validate the resetting model by profiling the *var* gene repertoire at single-cell resolution during liver stages.

Conflict of interest statement

We declare that all authors of this paper have no conflict of interest.

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