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Review

Variant antigen expression in malaria infections: posttranscriptional gene silencing, virulence and severe pathology

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1. Introduction

This review highlights features of two simian malarias, *Plasmodium knowlesi* and *Plasmodium coatneyi*, that make them attractive models for investigations relating to the molecular mechanisms governing antigenic variation and the development of human pathology in *Plasmodium falciparum* infections. This paper also reflects our current conclusions and working hypotheses as presented at the Severe Malaria meeting in November 2002 at the Karolinska Institute in Sweden. We propose that the 3′ regions of the variant antigen genes and their transcripts contain functional determinants that contribute to the regulation of protein expression. Our conclusions and hypotheses as presented are as follows:

- Numerous *SICAvar* genes are fully transcribed and detectable by RT-PCR during growth from the ring stage throughout the trophozoite stages of development.
- Full-length transcripts that are evident by northern blots represent the transcripts that become translated.
- If not destined for translation, *SICAvar* transcripts are rapidly subject to a post-transcriptional gene silencing (PTGS) mechanism.

Molecular studies relating to antigenic variation in *P. falciparum* have brought emphasis in recent years on transcriptional control mechanisms as a primary if not exclusive means to explain the observed allelic exclusion of *var* gene expression [1–11]. In our view, though, post-transcriptional regulatory mechanisms could also contribute to the observed allelic exclusion of variant antigen gene expression in *Plasmodium* infections. This review provides a historical perspective of the identification and characterization of the large surface-exposed variant antigens of *P. knowlesi*, *P. falciparum*, and *P. coatneyi*, and the genetic basis for advancing studies of gene expression relating to these antigens. Information pertaining to other gene families encoding small proteins predicted more recently to encode other putative variant antigens in multiple species of *Plasmodium*, although referred to here briefly, can be obtained in detail in recent papers and references therein (reviewed in [12,13]).

2. Antigenic variation in *Plasmodium knowlesi* and *P. falciparum*

That malaria infected erythrocytes could exhibit antigenic variation was first demonstrated by Brown and Brown in 1965 [14]. In this and subsequent research [15,16], it was shown by examining antibody mediated agglutination of schizont-infected erythrocytes [17] that the surface of *P. knowlesi* infected red blood cells (IRBCs) varied in antigenic phenotype during the course of blood-stage infections in *Macaca mulatta* (rhesus macaque) monkeys. In the early 1980s, the large surface-exposed parasite-encoded variant antigens of *P. knowlesi* infected erythrocytes were identified and their biochemical characteristics established [18–21]. These investigators named these proteins the schizont infected cell agglutination (SICA) antigens and designated infected cells expressing these antigens as parasites with a SICA[+] phenotype. The SICA antigens are >185 kDa, have distinct antigenic specificities, are extractable only in non-ionic detergents such as SDS, and can be radiolabeled metabolically or by I^{125} surface iodination. Further, the establishment of cloned *P. knowlesi* populations by micromanipulation of single schizont-infected cells and subsequent expansion in vivo enabled the first demonstration that the variation in antigenic phenotype in malaria parasites was clonal and that the phenotypic specificity of these clonal populations was stable when propagated in naïve rhesus monkeys [21,22]. A switch in the expressed phenotype was

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detected only in animals that had been previously infected with the homologous parasite clone; i.e., in monkeys that had generated a specific antibody response against the SICA antigens produced by the infecting parasites. Importantly, under appropriate conditions, the phenotypically 'switched' parasite populations, by and large, did not always appear to result from the selection and outgrowth of minor variant subpopulations, but seemed to result from an induced population-wide switch in phenotype [15,22]. These conclusions were based on precise monitoring of the growth kinetics of blood-stage infections where no differences in kinetics were noted between phenotypically switched populations and parasites propagated under conditions unfavorable for phenotypic variation. To date, although other *Plasmodium* gene families have been identified, which may encode other variant antigens [13,23–25], the *P. knowlesi* studies remain the only definitive in vivo experiments linking specific proteins with the property of antigenic variation.

Additionally, these investigations showed that the spleen of the host had a role to play in the expression of these antigens [22,26,27]. Passage of *P. knowlesi* IRBCs of different SICA[+] variant antigen phenotypes through splenectomized rhesus monkey hosts resulted in parasite populations with a SICA[−] phenotype, which do not express SICA antigens detectable at the surface of the infected cells. These parasites could no longer be agglutinated with specific antisera or antisera from monkeys with long-term chronic infections. Surface iodinated or metabolically labeled SICA antigens, also, could no longer be immunoprecipitated from detergent extracts prepared from these populations. Interestingly, cloned SICA[−] parasites were further shown to be less lethal than the SICA[+] clones in rhesus monkeys, suggesting an association between the expression of the SICA antigens and virulence [22,26]. Together these observations give support to the thesis that the natural in vivo environment may provide regulatory control signals that influence the normal expression and switching of variant antigen genes and their associated phenotypes.

Stable in vivo derived cloned populations of *P. knowlesi* can readily be produced by inoculating single schizont-infected cells into rhesus monkeys. Because of the high virulence of this parasite in this primate host, a single schizont-infected cell multiplies quite predictably at a nine to 10-fold multiplication rate each day, and after 10 days approaches a population of 10^{10} parasites, which verges on being a life-threatening infection ([22] and unpublished data). These infections are particularly virulent in rhesus monkeys of Indian origin, since *P. knowlesi* is not naturally transmitted in India (*P. knowlesi*, which is transmitted in South East Asia, is much less virulent in macaques of South East Asian origin). In contrast, efforts to expand single *P. falciparum* IRBCs directly in *Saimiri* or *Aotus* monkey hosts have not been successful, and human experimental studies do not deal with defined cloned populations that express known variant antigen types [8,28]. Human experimental studies also have other obvious limitations, including the inherent problem (expanded upon below) that variant antigen gene RT-PCR products obtained from blood samples do not reliably reflect 'expressed' variant antigens. Therefore, to date, *P. knowlesi* remains the only primate model that is well established for studying the molecular mechanisms that govern the clonal expression, switching and silencing of the variant antigens in vivo, with the potential to evaluate the influence and interplay of the host environment. Moreover, *P. knowlesi* can be easily manipulated for conducting in vivo and in vitro transfection studies to aid in these analyses ([29] and unpublished data). Our present studies have built upon investigations of two cloned parasite lines (clones Pk1(A+) and Pk1(B+)1+; from the H strain) among others, which were derived one from the other by an in vivo induced switch [21,26,27]. The Pk1(A+) parasites express a variant antigen doublet of 210/190 kDa and the Pk1(B+)1+ parasites express a variant antigen doublet of 205/200 kDa. A primary question being asked by us is what type of molecular events are consistently observed at the DNA, RNA and protein levels, when switches in expressed variant antigen phenotypes occur in a natural in vivo environment.

P. falciparum IRBCs can also be agglutinated by immune sera. Thus, following the identification and biochemical characterization of the *P. knowlesi* SICA antigens, a similar line of experimentation commenced to identify homologous antigens with similar characteristics in *P. falciparum*. This search lead to the description and characterization by Howard et al. [30,31] of the large surface-exposed protein that was named the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), which indeed shares all of the fundamental features of the SICA antigens. Subsequent field studies demonstrated the expression of PfEMP-1 in clinical isolates [32,33], while David, Hommel and coworkers [34,35] using the *Saimiri* monkey model demonstrated the potential importance of the spleen for the expression of *P. falciparum* variant antigens. A similar dependency of variant antigen expression on splenic factors was also shown for *P. fragile* in the toque monkey [36]. Furthermore, in vitro adhesion assays were employed to show that *P. falciparum* infected red blood cells adhere to a variety of specific receptors, including CD36, ICAM and CSA amongst others via the EMP1 variant antigens (reviewed in [37]), and some *P. falciparum* parasite populations grown in vitro were shown to rapidly and spontaneously switch to alternative adhesive and antigenic phenotypes [38]. These basic biological studies provided the background data from which later molecular-based investigations have developed, importantly relating PfEMP-1 with the properties of receptor adhesion and sequestration.

From the time when the SICA and PfEMP-1 variant antigens were first identified, 10–15 years elapsed before the genes encoding these antigens were identified, characterized, and shown to comprise a large gene family [39–42]. The period between the identification of the proteins and the cloning of the genes comprising these large gene families was a challenging one, as a limited number of investigators

sought means to develop variant antigen-specific antibody reagents to immunoscreen gene expression libraries and verify the cloned gene products. This challenge was met by a few groups, ironically, at the time the genomics technological revolution arrived. In the past, identifying and confirming the identity of 'any' variant antigen gene(s) was an elusive goal. Now, PCR analyses of many *var* gene sequences from laboratory and field isolates (e.g., see [4,43,44]) and the completion of the *P. falciparum* genome project [45], have provided more variant antigen gene information than most, 10–20 years ago, probably ever thought possible. Today, numerous groups (∼30) worldwide are presently studying the sequence and expression patterns of the *P. falciparum* variant antigens, with the over-riding intent of furthering the understanding and prevention of severe malaria. A current challenge for this field is to rationally process and meaningfully utilize the large amount of incoming information.

A considerable volume of data has now been generated towards understanding the individual and genome-based make-up and organization of the *P. falciparum var* genes [6,44–48]. From such studies and the original reports [39–41], it is now known that the *P. falciparum var* genes range in size from 6 to 14 kb, have a two-exon structure, and encode proteins with molecular masses ranging from 200 to 400 kDa (Fig. 1). Exon I encodes the large external, cysteine-rich, highly variable portion of the protein followed by a transmembrane (TM) segment, and exon II encodes the cytoplasmic domain. *P. falciparum var* genes are present on all 14 chromosomes and are primarily but not exclusively located near the telomeres, where the presence of telomere-associated repeats are believed to enhance recombination. Fifty-nine *var* genes are present in the *P. falciparum* 3D7 genome [45], yet given the apparent high level of recombination among *var* gene sequences [43,49,50], there is the potential for a vast number of unique *var* gene repertoires in field isolates, as well as the presence of pseudogenes (see [45]). Additionally, related gene sequences and transcripts have been identified that have homology with exon II [40], yet which clearly represent other distinct proteins (reviewed in [9]).

A comparable body of data is now accumulating for the *P. knowlesi SICAvar* gene family ([42], and Corredor V. et al. submitted for publication). The first reported *SICAvar* gene was shown to encode the 205 kDa SICA antigen that is expressed by the Pk1(B+)1+ parasite clone [42]. This *SICAvar* gene is ∼14 kb, and like the *P. falciparum var* genes encodes a large external, cysteine-rich, and highly variable region followed by a TM domain and a cytoplasmic domain (Fig. 1). A unique difference compared to *P. falciparum var* genes is that this *SICAvar* gene has a 10-exon structure, with the first nine exons encoding the large external domain and the TM (encoded by exon IX), and the final exon X encoding the cytoplasmic region. Thus intron IX of this *SICAvar* gene can be viewed as the equivalent of the single intron present in the *P. falciparum var* genes. It is also noteworthy that each of these *SICAvar* introns are positioned precisely near the beginning of the coding regions of this protein's seven unique, yet related, cysteine-rich domain (CRD) modules. We have proposed that this structural arrangement could enhance the generation of diversity of the *SICAvar* genes in a population by facilitating the shuffling of *SICAvar* domains [42]. Importantly, it should also be noted that there are other examples of differences in the intron-exon structures within orthologous genes present in these two *Plasmodium* species [51]. Determining how (or when) they were generated and are maintained differently in each species is certainly intriguing and will perhaps be better understood with the analysis of such genes in a greater number of *Plasmodium* species.

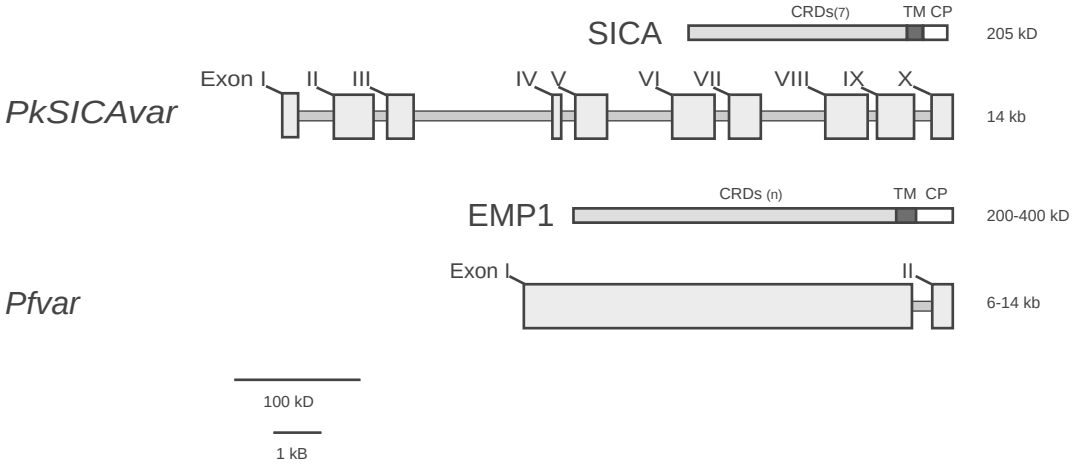

Fig. 1. Schematic depicting the *P. knowlesi SICAvar* gene encoding the 205 kDa SICA protein with its 10-exon structure compared to a representative *P. falciparum var* gene two-exon structure. As indicated, the resulting SICA and EMP1 antigens each have multiple CRDs, a transmembrane domain (TM) and a cytoplasmic region (CP). Exons are labeled with Roman numerals I to X. In *P. falciparum*, the CRDs have also been called Duffy Binding Like (DBL) and Cysteine InterDomain Regions (CIDR) (reviewed in [37]).

Five-fold sequencing coverage of the *P. knowlesi* genome is currently in progress (http://plasmodb.org/PlasmoDB.shtml). Recent BLAST analyses based on the well-conserved *SICAvar* exon X sequence identified at least 108 distinct contigs (Corredor et al., submitted for publication). These and related data developed in our laboratory show that there are defined regions of homology throughout the *SICAvar* gene family, with evidence of frequent recombination occurring among these sequences within this species over time. Next to and downstream of each exon X sequence, the 3′UTRs have a surprising level of conservation, with three regions showing clearly defined sequence patterns. Immediately downstream of the stop codon, a distinct region, which we have designated as Block I, consists of a variable number of imperfect heptad repeats, with similarity to the *Plasmodium* telomeric repeat sequence [GGGTT(T/C)A] (see [52–54]). This is followed by another polymorphic region, designated as Block II, containing a series of A and T homopolymers that vary in size. Many but not all Block II regions are then followed by a third region, Block III, which is characterized by interspersed GC tracts (Correder V. et al. submitted for publication). Different degrees of conservation and apparent sequence patterns within the 3′ non-coding sequences of the *P. falciparum var* genes was also noted recently [9]. The time lapse from 1995 until now for this information to be revealed for *P. falciparum* could be due to the high A/T content of *P. falciparum* (80%), which could camouflage the signatures of potentially important motifs. In contrast, the *P. knowlesi* genome has a more balanced nucleotide composition (∼65% A/T), which predictably would more readily enable the identification of the remarkable relationship of these sequences.

It remains to be determined to what extent recombination of *SICAvar* gene loci occurs during mitosis compared with meiosis. In any event, our ongoing analysis of genetic fingerprints of cloned parasites derived from repeated blood-stage passage in rhesus monkeys would suggest that spontaneous mitotic recombination during a blood-stage infection is an uncommon event. We know, nevertheless, that recombination events involving *SICAvar* gene sequences can in fact occur during the course of a blood-stage infection. As evidence of this point, a *SICAvar* 3′ genomic alteration has been associated with the switch in expression during a blood-stage infection from the 210/190 kDa SICA antigen doublet characteristic of the parental Pk1(A+) clonal line to the expression of the 205/200 kDa SICA antigen doublet characteristic of the Pk1(B+)1+ parasite [42]. This 3′ genomic alteration, while it could reflect a rare, random highly coincidental event, alternatively may reflect a specific event that could genetically ‘tag’ (i.e., mark) the 205 kDa SICA-encoding transcript so that it would be retained as a template for translation, and not be readily degraded [42]. Such a ‘tag’ could result from the simple addition or subtraction of important signature motifs that might, for example, alter the structure of the 3′UTR sequences or the polyadenylation characteristics of the resulting transcripts. Further investigation has shown that the observed 3′ genomic alteration resulted in the generation of a new *SICAvar* allele (i.e., the expressed *205B* allele) via a recombination event that incorporated an alternative 3′ intron IX, exon X and downstream sequences within the 205 kDa encoding gene locus in the Pk1(A+) parasites (i.e., within the *205A* allele) (Corredor et al., submitted for publication) (Fig. 2). Further studies are in progress to ascertain if such recombination events are generally associated with the exclusive expression of alternative SICA antigens in *P. knowlesi* and if particular signature motifs are present in the expressed genes. It is generally thought that recombination events are not associated with variant antigen switch events in *P. falciparum*, since the variant antigen genes are transcribed in situ and no large scale rearrangements have been detected [2,41]. However, we question if subtle alterations associated with the expressed variant antigen genes could perhaps be occurring in *P. falciparum*. Detection of a recombination event associated with the expression of the *P. knowlesi* 205 kDa SICA protein was in part fortuitous. Large-scale rearrangements or deletions were not observed; rather, a very small difference in the size of one restriction

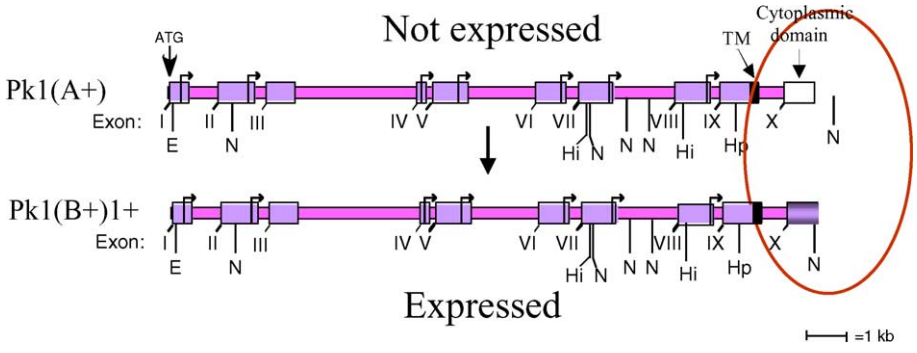

Fig. 2. Schematic depicting the *SICAvar* gene encoding the 205 kDa variant antigen in Pk1(A+) and Pk1(B+)1+ cloned parasite lines, with the altered 3′ region highlighted. The individual exons are noted by purple boxes and Roman numeral numbers I–X. Introns are depicted as pink bars, except for intron IX from the Pk1(B+)1+ allele. Bent arrows denote the beginning of cysteine-rich coding domains near the ends of most exons. The transmembrane (TM) encoding domains are labeled by solid black boxes and the ATG start sites and cytoplasmic encoding domains with an arrow head. The different intron IX, exon X and 3′UTRs that are the result of a DNA rearrangement associated with the switch in expression to the 205 kDa protein are depicted with different visual schemes within the oval. Restriction sites noted are: E: *Eco*R1; N: *Nci*I; Hi: *Hind*III; Hp: *Hpa*I.

enzyme fragment drew attention to this event. The identification of potentially cryptic rearrangement events in *P. falciparum* may require finer mapping than has necessarily been reported to date, along with the confirmed expression (transcription and translation) of the specific genes under investigation. Further investigation of both species could be important to verify whether specific recombination events are typically associated with switch events or not.

Regardless, given the predicted high frequency of recombination at the *P. falciparum var* and *P. knowlesi SICAvar* loci ([4,50], and Corredor et al., submitted for publication), and the concomitant high rate of evolution of variant antigen genes, it is understandable why there are no distinct regions of identity between the variant antigen genes from these diverse *Plasmodium* species [42]. In fact, recombination within the *P. falciparum var* genes has resulted in the absence of hybridization among many gene family members, as exemplified by the lack of cross-hybridization between *var* gene tags of the HB3 line with the Dd2 or 3D7 parasites [4,50]. Generally, there is little, if any, high level of identity observed when comparing antigen encoding genes from *P. falciparum* and *P. knowlesi* or other simian malaria counterparts—or the human malaria *P. vivax*, which is phylogenetically close to the simian malaria species [55]. In fact a recent study showing the high level of recombination of the *P. vivax* merozoite surface protein-1 gene notes how few regions of this gene show homology with the counterpart genes from diverse species [56]. When identity is maintained, specific regions may be under common functional constraints. In the case of the coding regions of the variant antigens, maintaining a high level of antigenic diversity among gene family members may be of underlying importance to assure a large repertoire of variant antigens within a population. Given the extensive diversity in the variant domains of the *SICAvar* and *var* genes, or among the gene families encoding the small vir, yir, cir, or rifin family members [13,23–25], one might even question some of these relationships, despite the assorted genetic and biological similarities confirmed so far to date. Of special interest in this regard, *P. knowlesi* has both the *SICAvar* gene family and a '*kir*' gene family, which encodes the smaller-sized vir-related proteins mentioned above (unpublished data). The *P. knowlesi* in vivo and in vitro model systems therefore can be used to help differentiate the genetic and biological characteristics of these different *Plasmodium* families, and better establish the basic features that define the smaller proteins.

Importantly, current knowledge of transcription of the *P. knowlesi SICAvar* genes parallels in many respects the predominant observations generated from studies of *P. falciparum var* gene transcription [1,2,4–7]. Since the identification of the *SICAvar* multigene family in 1995, it was evident that numerous full-length transcripts can be produced by cloned parasite lines, despite the fact that only one SICA protein doublet is expressed at the surface of infected host cells ([21,42] and unpublished data). Initial awareness of the presence of numerous full-length *SICAvar* transcripts came from the fact that many different spliced *SICAvar* cDNAs containing the final few 3′ exons, and not just the Pk1(B+)1+ translated cDNAs, could be recovered by immunoscreening Pk1(B+)1+ cDNA expression libraries with a polyclonal antiserum [42]. Furthermore, numerous transcript segments representing many different members of the *SICAvar* gene family can also be readily recovered in RT-PCR amplifications using specific *SICAvar* primers, including primers that straddle the 3′ most intron IX sequence, i.e., spliced exon IX and exon X sequences are amplified at both the ring and trophozoite stages of development (unpublished data). From our perspective, these data are consistent with one out of several speculative possibilities put forth by Borst and others in 1995; in essence, that *Plasmodium* "transcribes all *var* genes all the time, but controls expression at the level of RNA processing by partial degradation of all mRNAs but one" [57]. Stage-specific northern blots depict a much higher apparent transcription level of *SICAvar* transcripts at the ring-stage compared to the level of trophozoite-stage transcripts [42], and, at either stage, full-length transcripts detected on northern blots seem to only represent the transcripts that are specifically translated (unpublished data). Nonetheless, multiple distinct smaller *SICAvar*-specific RNA bands can also be detected by northern blot analyses, as opposed to a continuous smear. Taking these and ongoing observations together, we propose that a PTGS mechanism, perhaps akin to RNAi mechanisms, (reviewed in [58]) is functioning to rapidly silence the many transcripts that are produced but not translated (and eventually the translated transcript as well). In further support of this hypothesis, antisense *SICAvar* RNA is present (unpublished data) that is clearly not simply a reflection of the occurrence of transcription on the antisense DNA strand, as has been noted for a number of other *Plasmodium* genes [59,60]. The *SICAvar* antisense clones recovered by RT-PCR reflect the sequences of spliced *SICAvar* messages as opposed to the genomic DNA counterpart with its introns present. We speculate that these antisense *SICAvar* RNAs may represent the products of RNA dependent RNA polymerase activity functioning to generate double stranded (ds) RNAs based on *SICAvar* RNA templates; as would occur in a functioning RNAi mechanism (reviewed in [58]).

In support of the possibility that RNAi is an inherent mechanism that functions in *Plasmodium*, Malhotra et al. [61] recently reported the intrinsic capability of *Plasmodium* to process dsRNAs into 25nt small RNAs, which are characteristic downstream components of RNAi mechanisms. Moreover, it is worth noting that Slavin, Gottig and Lujan (2002) have recently proposed RNAi as a mechanism functioning in the post-transcriptional silencing of variant antigen gene transcripts in *Giardia lamblia* (Slavin H., Gottig N., Lujan H.D., 2002. Molecular Parasitology Meeting XIII, Marine Biological Laboratory, Woods Hole, MA USA.[Abstract]). The possibility that a PTGS mechanism functions to silence the majority of *P. knowlesi SICAvar* and *P. falciparum var* gene transcripts likewise follows from

our reflections upon the possible functional importance of conserved 3′UTR variant antigen gene sequences ([9], Corredor et al., submitted for publication). We speculate that the conserved 3′ UTR sequences may be maintained to support the initiation of a post-transcriptional silencing mechanism.

3. Molecular mechanisms impact investigations relating to severe malaria

If a PTGS mechanism is functioning to silence variant antigen gene transcripts, in a fashion that leaves single or dsRNA remnants that can be amplified using RT-PCR procedures, then data generated by such means cannot be interpreted to reflect those genes that are turned on and ultimately expressed as proteins at the surface of the infected host cells. Usage of the term ‘expression’ in our analyses is thus restricted to those variant antigen gene transcripts that are in fact translated and result in protein expression. An increasing number of published reports utilize RT-PCR to describe the ‘expression’ of *P. falciparum var* genes, with the explicit inference that the transcripts reported reflect protein expression. Moreover, in a number of cases the detection of such transcripts has been related to the clinical status of patients or the propensity for certain *var* gene sequences to be associated with severe disease, including placental malaria [62–66]. While the detection of dominant transcripts may represent expressed genes, this is not necessarily the case, and such associations could be misleading if in fact what is being detected are products of regulated degradatory processes. A similar note of caution was put forth recently by Michael Duffy and colleagues who definitively showed that multiple full-length transcripts are produced in individual trophozoites [7]. Accordingly, an understanding of how expression of variant antigens in *Plasmodium* is genetically controlled is important for basic molecular biologists, field scientists, and health workers alike, as this knowledge will influence how data is interpreted. Not only are current RT-PCR studies of variant antigen gene expression potentially misleading, but associations being made between disease, epidemiology, and population structure based on such data without verification of the expressed proteins could inadvertently prove to be irrelevant.

4. Cytoadherence and the pathogenesis of severe disease, including placental malaria

While immune evasion is a primary function in common between the *P. knowlesi* and *P. falciparum* variant antigens, the cytoadherence properties of the classic SICA and PfEMP1 variant antigens could be viewed as a secondary adaptation, which has evolved as a much stronger characteristic in *P. falciparum* than *P. knowlesi* (all *P. knowlesi* blood-stage forms circulate; yet *P. knowlesi* nonetheless exhibits some sequestration characteristics ([67] and unpublished data). We have observed that different clonal populations of *P. knowlesi* exhibit striking differences in the degree to which the older parasites retreat from the circulation and sequester (unpublished data). Future analysis of the cytoadherent properties of individual clones of *P. knowlesi*, expressing different known *SICAvar* genes, will help to define potential cytoadherent properties of a subgroup of the CRDs of the *P. knowlesi* variant antigens. Furthermore, phylogenetic analyses show that the simian malaria *P. coatneyi* [68] is a sister taxa of *P. knowlesi* [69]. Given the close phylogenetic relationship of these two species, we have been able to clone members of the orthologous *var* gene family from *P. coatneyi* (unpublished data). *P. coatneyi* sequesters to a similar high degree and exhibits cytoadhesion to vascular endothelium in similar tissue sites as *P. falciparum* [67,70]. It also adheres to uninfected red blood cells forming rosettes [71], and has been associated with severe disease and possibly cerebral malaria in macaques [72,73]. *P. coatneyi* infected trophozoites and schizonts also have knob protrusions and many morphological and biological characteristics indistinguishable from *P. falciparum* [67,74,75]. Continued investigations of the *P. coatneyi var* gene family, recombination properties and functional characteristics in parallel with the *P. knowlesi* variant antigens holds much promise towards understanding the basic biology, immunophysiopathology and molecular mechanisms associated with the variant antigen gene family in *Plasmodium*.

Experimental malaria infections during pregnancy have been carried out in rhesus macaque monkeys in a few studies using *P. knowlesi*, *P. cynomolgi* or *P. coatneyi*, with the first report dating back as early as 1939. In 1939, Das Gupta reported the initial observation of the apparent compartmentalization of simian malaria parasites in the placenta [76]. A pregnant rhesus monkey experimentally infected with *P. knowlesi* during the latter stage of gestation developed severe signs of infection with severe anemia. Upon histological examination the fetal blood was free of parasites, and the maternal blood films had a “a fair number” of parasites. The maternal side of the placenta, though, showed a “stupendous number” (>95%) of IRBCs, with parasites in late stages of development filling the placental intervillous space. Only a few IRBCs of this multitude appeared to be actually apposed to the surface of the syncytiotrophoblast (SCT). Four decades later, 22 pregnant rhesus monkeys were infected with *P. cynomolgi* [77], the simian malaria parasite that is closely related to *P. vivax* [55]. Like *P. vivax*, *P. cynomolgi* has neither knob protrusions like *P. falciparum* nor is it noted for any characteristic cytoadhesion or sequestration. These infections lead to four maternal deaths, five fetal deaths, and four post-natal deaths. In a later study, an additional 15 pregnant rhesus monkeys were infected with *P. cynomolgi* in the second trimester and exhibited severe complications with one maternal death and five abortions [78]. Electron microscopic analysis of the placentas from this study showed attachment of macrophages, fibrin

deposition on the surface of SCT, and necrosis, but no adhesion of IRBCs to the SCT could be determined with any degree of certainty. *P. cynomolgi* in rhesus monkeys, like *P. vivax* in humans or New World primates, normally causes a non-lethal infection without critically severe disease manifestations. These outcomes therefore raise important questions: (1) how much placental malaria damage is actually typical with the so-called 'benign' human malaria, *P. vivax*, especially in non-immune individuals in endemic communities; and (2) what factors other than the expression of the variant antigens with their adhesion receptors are associated with the development of placental pathology and the variety of associated poor fetal outcomes (reviewed in [79–81]).

We have begun to address these questions by performing a number of pilot studies, infecting pregnant rhesus monkeys with *P. knowlesi*, *P. cynomolgi* or *P. coatneyi*. It is known that *P. coatneyi* malaria in pregnant *M. mulatta* monkeys leads to poor fetal outcomes and associated placental pathology [82,83]. Our preliminary data confirms that the development of placental pathology in rhesus macaques occurs regardless of whether the infecting parasite species is one that is known for its cytoadherence and sequestration, as is the case with *P. coatneyi*, or not. Preliminary in vitro binding studies also indicate that *P. coatneyi* parasites obtained from the placental tissues and from the peripheral blood at the time of C-section have different adhesion characteristics (Moreno et al., unpublished data), as has been shown to be the case for human *P. falciparum* infections in pregnant women (reviewed in [80,81]).

5. Concluding remarks

The well-established *P. knowlesi*–rhesus monkey model system and other primate malarias can reveal valuable information relating to the phenomena of antigenic variation and severe malaria. With the associated morbidity and mortality for millions of individuals at extraordinary levels [84,85], any potentially promising avenue for breakthroughs in our understanding and the development of medical solutions should be explored. The answers being sought today can potentially be uncovered by comparing the survival mechanisms developed by a number of species of *Plasmodium*, as well as related parasitic blood-borne pathogens, such as *Babesia bovis* [86,87], which have also chosen the erythrocyte as their host cell.

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