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Prince B Nyarko, Antoine Claessens

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Understanding host-pathogen-vector interactions with chronic asymptomatic malaria infections

Prince B. Nyarko[1], Antoine Claessens[2*]

[1]Laboratory of Pathogen-Host Interaction (LPHI), CNRS, University of Montpellier, France.

[2]LPHI, MIVEGEC, CNRS, INSERM, University of Montpellier, France.

[*]Corresponding author: antoine.claessens@umontpellier.fr

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Abstract

The last malaria parasite standing will display effective adaptations to selective forces. While substantial progress has been made in reducing malaria mortality, eradication will require elimination of all *Plasmodium* parasites, including those in asymptomatic infections. These typically chronic, low-density infections, are difficult to detect, yet can persist for months. We argue that asymptomatic infection is the parasite's best asset for survival, but it can be exploited if studied as a new model for host-pathogen-vector interactions. Regular sampling from cohorts of asymptomatic individuals can provide a means to investigate continuous parasite development within its natural host. State-of-the-art techniques can now be applied to such infections. This approach may reveal key molecular drivers of chronic infections; a critical step for malaria eradication.

Malaria pathogenesis

Most of the half-a-million annual malaria deaths are due to *Plasmodium falciparum* [1]. This unicellular eukaryotic parasite is transmitted by female *Anopheles* mosquitoes. Malaria symptoms occur during the intra-erythrocytic part of the parasite's life cycle. Although the parasite is largely "hidden" within an infected red blood cell (iRBC) during the first ~20 hours (called ring-stage), it reveals itself to host immunity by exporting antigens to the surface of the RBC at the trophozoite-stage. Such antigens include ***P. falciparum* erythrocyte membrane protein 1 (PfEMP1**, see Glossary**)** [2], which enable the iRBC adhere to endothelial cells of the micro-vessels. This phenomenon, termed sequestration, is essential for late-stage iRBCs to avoid splenic clearance, but simultaneously leads to microvascular obstruction and release of pro-inflammatory cytokine, which are key features of malaria pathogenesis. A *P. falciparum* infection can result in multiple outcomes; from **asymptomatic** (afebrile in this context), to uncomplicated or severe malaria. What drives the disease one way or another is not fully understood, but does involve host, parasite and environmental factors.

P. falciparum asymptomatic infections: looking for the elephant in the room

The heavy malaria burden, in terms of clinical cases and deaths, is only the tip of the iceberg. Indeed, on any given day, the vast majority of all *P. falciparum* infections worldwide are asymptomatic [3]. Where transmission is seasonal, the dry season is characterised by limited transmission; hardly any clinical cases and fewer mosquitoes [4]. However, some parasites survive by establishing **chronic**, asymptomatic infections across the dry season. These infections, shown to produce and transmit gametocytes [5, 6], are the reservoir from which the seasonal peak will restart at the next transmission season, and arguably represent the biggest challenge for malaria eradication.

Clearing all infections would include treating carriers without clinical symptoms who are unlikely to seek treatment. A campaign that would only target clinical cases, is at risk of inadvertently selecting for a population with an "asymptomatic profile" (i.e. parasites epigenetically wired to maintain low–parasitaemic and

asymptomatic infections), thereby complicating the elimination effort. For example, mass screening with rapid diagnostic tests (RDTs) and systematic treatment, as has been trialled repeatedly [7, 8], is at high risk of selecting parasite populations that maintain a parasitaemia below the RDT detection level. These parasites will quickly re-emerge at the end of the campaign.

Asymptomatic infections: out of sight, out of mind

Despite the high prevalence of asymptomatic infections, our knowledge of the parasite biology is based on isolates derived from clinical cases and clonal culture-adapted parasites. How asymptomatic infections differ from clinical cases has hardly been investigated, with no genome, epigenome, transcriptome, proteome or phenotypic description of such parasites published to date; largely due to technical challenges associated with very low biological materials.

Today's technology is at a turning point to address these issues. Extremely low parasite densities can be detected with ultrasensitive PCR assays [9]. An entire *Plasmodium* genome can be sequenced from a dry blood spot [10], a single cell [11], or using a device as small as a USB-stick (Oxford Nanopore [12]). Thousands of transcriptomes can be individually tagged and pool-sequenced to drastically reduce sequencing cost [13]. Complex parasite populations may be resolved with single-cell approaches [14]. State-of-the-art techniques can now be applied to fully characterize *P. falciparum* parasites in low-density, asymptomatic infections.

Fighting asymptomatic infections with asymptomatic infections

Some long-standing biological questions such as the duration of a chronic infection, have been difficult to address outside the malariotherapy dataset (Box 1). The most relevant answers to host-pathogen-vector interaction questions will come from humans who are naturally infected with *Plasmodium* parasites. However, clinical studies typically collect a single sample from malaria patients on hospital arrivals, giving us only a snapshot of the host-pathogen interaction. Here, we argue that longitudinally sampled asymptomatic infections can provide blood samples at multiple timepoints to investigate the continuous development of the host-pathogen-vector interaction. Coupling this approach with state-of-the-art

technology opens a plethora of biological questions that are key to the elimination of malaria. This manuscript focuses on host-pathogen interactions, specifically, parasite **antigenic variation** and its associated immune response.

Practically, in collaboration with ethics committees and local health authorities (Box 2), a cohort is recruited by enrolling consenting *Plasmodium* asymptomatic carriers (Figure 1). Diagnosis is performed by qPCR as parasitaemia is often too low for microscopy detection. Each volunteer provides blood samples at regular intervals, unless he/she develops malaria symptoms. All volunteers must have immediate access to free professional medical care during the entire study. Blood samples can be used to address a variety of biological questions (Table 1). Should the volunteer develop symptoms or desire to withdraw from the cohort, anti-malarial treatment is given immediately. Regular genotyping will distinguish an ongoing infection from a new one. In the case of multi-clonal infections, a second sampling within 24-48 hours is necessary to detect circulating strains that would have been sequestered at the first timepoint. The period between each sampling timepoint can vary from days to months depending on the biological question(s) to be addressed. An alternative study design is to recruit non-infected volunteers, for example, just before the malaria transmission season starts, and sample them from the day of recruitment to catch the very early stages of the infection. This approach would be particularly suited to study the average duration of a *P. falciparum* infection.

Here, we describe gaps in our knowledge of host-pathogen-vector interactions which could be addressed with this study design.

Central dogma: antigenic variation and host response

General var gene background

Central to malaria pathogenesis is the infected erythrocytes' ability to **evade host immunity**; adhere to endothelial cells (sequestration) or uninfected erythrocytes (rosetting). These phenomena are mediated by highly polymorphic parasite-specific antigens, collectively termed **variant surface antigens** (VSAs); RIFINs, STEVORs, PfEMP1, etc., which are exported onto the surfaces of infected

erythrocytes [15]. The most prominent and well-studied VSA is PfEMP1, coded by the *var* gene family (Figure 2A). It has ~60 members per genome, classified into three main groups; A, B and C, based on their upstream 5' untranslated sequences (UpsA, UpsB, and UpsC, respectively), chromosomal locations and orientation [16]. Importantly, the grouping is associated with *in vitro* switching [17], **cytoadherence** phenotypes and pathogenesis. Notably, some Group A PfEMP1 bind EPCR1 and their expression is associated with cerebral malaria [18-20]. Group B and C PfEMP1 typically bind to CD36 and are associated with mild disease. Few cross-sectional studies which recorded *var* gene expression from asymptomatic infections identified low abundant, homogenous and mainly group C *var* gene expression [21-24]. To the best of our knowledge, the *ex vivo* cytoadherence phenotype of parasites derived from asymptomatic infections has not been addressed, most likely because current assays require much higher parasite density.

Does var gene expression switch in chronic infection?

Mutually exclusive expression and periodic switching of *var* genes (Figure 2A) at an approximately 2% rate per generation [17, 25, 26], likely ensure parasite survival in milieu of host immunity, and remains a major candidate to explain chronic infections. Nonetheless, very few studies have addressed *var* genes in asymptomatic infections or tried to address the *in vivo* switching mechanism. Though controlled human malaria infection (CHMI) studies have provided insight on *var* expression *in vivo*; reset after mosquito transmission, broad breadth *var* expression, etc. [27, 28], they are usually short-lived *in vivo* studies and also not necessarily representative of parasites in the wild. Known to us, only a single longitudinal study focused on *var* transcription over a 4-month period and showed that some *var* transcripts recur for up to 10 weeks [29]. Thus, more comprehensive studies are needed to define the role of *var* genes in the establishment of chronic infections (Figure 2B).

In practice, the well-established RT-qPCR method with **DBLα** universal primers may be used to record *var* gene expression over multiple timepoints [18, 30]. For full-length *var* sequence analysis, one approach is to whole-genome sequence[10],

followed by *de novo* assembly of *var* genes [31]. Alternatively, *var* gene gDNA may be amplified by long-range PCR and sequenced [32]. The entire transcriptome can also be sequenced [33]. Again, *var* gene-specific primers can be designed from the genomic sequence for RT-qPCR. Moreover, each timepoint isolate can be culture-adapted and its *var* gene expression recorded in a similar way (Figure 2B, lower panel). The *in vitro var* transcription can then be compared to the *in vivo* counterpart to investigate the *var* gene switching pattern with and without immune pressure. Furthermore, it remains to be tested whether a change in VSA leads to cytoadherence phenotype changes.

Do we observe specific antibody response against each wave of PfEMP1?

Our current understanding of immune responses; both cellular and humoral, to *P. falciparum* infections is limited. Models suggest that the parasite-host relationship has evolved to favour some short-lived immune responses which allows the parasite to persist and the host to survive [34]. Several cross reactivity studies have demonstrated robust acquisition of antibodies to VSA of homologous parasites (from the same donor) during the course of the infection, with individuals having anti-VSA antibodies to both homologous and heterologous parasites (from a different donor) being more protected from severe or symptomatic disease [35-39]. Although anti-RIFIN [40] and anti-STEVOR [41] antibodies have been shown to be functional; promoting immune effector mechanisms, PfEMP1 is thought to be the main target of both total and functional anti-VSA antibodies [39]. A body of knowledge highlight the emergence of both long-lived and short-lived anti-VSA antibody acquisition during, and after resolution of an infection [35, 42], with some individuals failing to switch antibody isotypes from IgM to IgG [42]. Nonetheless, these studies rely on samples taken either at the time of symptomatic disease or after resolution of the infection, and thus do not provide a holistic understanding of the kinetics of host immunity during sustained infection and how it contributes towards the establishment of chronic infection. It is equally important to know how the persistence of an infection shapes host immunity. Studies with cohorts of chronic asymptomatic infections followed over long periods could help fill this gap in knowledge (Figure 2C).

In this instance, total IgG from multi-timepoint samples collected from an individual could be used in a flow cytometry assay to identify surface expressed PfEMP1 of parasites from the same individual in a sequential manner [35, 37], to ascertain the possibility of PfEMP1 switching. Additionally, the specificity of these antibodies could be determine with agglutination assays [43]. More specifically, plasma-derived antibodies could be used to detect a protein microarray of recombinant PfEMP1 domains [44] to determine their specificity, the order and rate of PfEMP1 switching.

Does the parasite generate chimeric var gene during the course of an infection?

Despite the overwhelming evidence in support of PfEMP1 variant surface display as a major contributor to **immune evasion** and the subsequent establishment of chronic infections in semi-immune individuals, the limited number of *var* genes per genome, coupled with the seemingly high switch rate does not support the maintenance of infections over several months [45]. One hypothesis to reconcile these facts is that the parasite is able to generate novel antigenic sequences, termed "**chimeric *var*** genes", in the course of an infection. A chimeric *var* is formed by mitotic ectopic recombination during asexual growth when two *var* genes which share short (~50bp) homologous sequences undergo single or multiple crossovers to exchange sequences, resulting in the generation of a novel *var* which share parts of its sequence with the two "parental" *var* genes [46] (Figure 2D). *In vitro*, the new *var* gene (chimera) maintains its sequence architecture and presumably, function, but differs from the "parental" *var* genes in sequence identity [46]. The *in vivo* generation of such sequences remains to be tested, and if so, whether the recombination occurs solely to sustain a chronic infection, increase *var* polymorphism at the population level, or both.

The hypothesis may be tested with single-cell whole genome sequencing [11] and de novo assembly, with a chimeric *var* gene being defined as a recombined sequence unique to time-point X and X+n, but not detected in time-point X-n. Expression can be determined with single-cell RNA sequencing.

Do parasites become dormant in the dry season?

P. falciparum could establish long-term infections by delaying ring-stage development, possibly even entering **dormancy/quiescence** (G0 in the cell cycle), and only completing the entire cycle weeks/months later [47, 48]. The parasite multiplication rate (PMR), a proxy for growth, was 3-fold higher in severe malaria cases compared to uncomplicated cases in Thailand [49], but this was not the case in Malian or Kenyan children [50]. The PMR in long-term chronic infections has not been measured yet. In general, the mechanisms allowing parasites to survive during the dry season before restarting transmission as vector population increases in the ensuing wet season remain to be investigated. Importantly, transcriptomic studies so far have only been performed in bulk, measuring the average gene expression of potentially heterogeneous parasite populations. A single-cell approach could reveal subpopulations of circulating parasites. Additionally, single-cell methods can identify clones within multi-clonal infections and track their progression and potential competition from one timepoint to another. With a cohort of chronic asymptomatic infections, PMR can be measured *in vivo* [51] and/or *in vitro* [52]. Also, the possibility of dormancy formation in chronic infections could be probed with single-cell RNA sequencing and epigenetic approaches.

Gametocyte commitment and transmission with seasonality?

Gametocytes are terminal blood stage parasites required for transmission. Thus, their formation needs to be timely to ensure successful transmission. Indeed, in birds, *Plasmodium* parasite density increases after repeated mosquito bites [53]. This may also be the case in humans before the start of the transmission season [54, 55]. However, harbouring gametocytes does not necessarily equate to being mosquito infective. Successful transmission requires viable mature gametocytes in the right sex ratio which maximizes the chance of at least one female and one male being ingested. Data from CHMI studies predict gametocyte detection, on average, 10 days post blood-stage infection; suggesting gametocyte conversion within the first blood-stage generation [56]. These studies were however performed in malaria-naïve individuals and thus the dynamics could be different

261 in malaria-exposed persons, given that host immunity may impact
262 gametocytogenesis, maturation or viability for transmission. Malaria control
263 efforts will benefit enormously from a better understanding of the rate of
264 gametocyte conversion (kinetics and density) and transmission feasibility in
265 natural chronic asymptomatic infections, particularly in regions where
266 transmission is seasonal.
267
268 With a multi-timepoint sampling strategy from chronic infections across different
269 transmission seasons, gametocyte carriage and turnover can be determined with
270 RT-qPCR [56] and their infectiousness ascertained either by direct membrane
271 feeding assays with fresh blood samples or direct mosquito bites of infected
272 individuals [57-59].
273
274 ***Other host-parasite interaction questions to be investigated***
275 The duration of asymptomatic infection prior to onset of symptoms could vary
276 from few days to several years [60, 61]. Conditions accounting for the disparities
277 are not fully understood, although multiplicity of infections, exposure and host
278 immunity have been implicated [62, 63]. The duration of an infection is essential
279 for transmission dynamics, especially in areas of seasonal transmission. Thus,
280 unraveling the underlying mechanisms influencing how long an infection can
281 persist will be central to future malaria control strategies.
282
283 Tightly linked to the duration of chronic asymptomatic infections is the onset of
284 symptomatic disease. A recent longitudinal study in Malawi showed that
285 asymptomatic infections rarely progress to clinical disease, as 92% of malaria
286 illnesses in chronically infected individuals were due to a novel infection [64]. On
287 the other hand, in the case of pregnancy associated malaria, most women who
288 suffered from such diseases had been infected prior to getting pregnant [65].
289 These two examples nicely illustrate the power of using longitudinal approaches.
290
291 A major hindrance to malaria vaccine development is our scanty understanding of
292 host immunological responses to the parasite. Despite partial antibody transfer
293 studies and other serological studies making a claim for the pivotal role anti-

Plasmodium antibodies play in mitigating disease severity, malaria immunity transcends the antibody repertoire [66, 67]. Although a full review of malaria immunity is outside the scope of this manuscript, it is clear that comprehensive studies of host immunity in individuals over an extended period is required to put in perspective, host effector immunity to *Plasmodium* infections.

Concluding remarks

Risks associated with cohorts of untreated asymptomatic carriers should be the first concern (Box 2), in agreement with Ethics Committees and National Malaria Control Programs. Dozens of such cohorts have been investigated in the past, but few 'bench research projects' were associated with them, presumably because the appropriate technology was not available. We are hoping this review will promote greater interactions between bench-based and field-based malariologists so that when such cohort studies are designed, blood samples are tapped to their full potential (see Outstanding Questions).

Of all the biological discoveries to be made from samples derived from human chronic infections, the ultimate one is the comprehension of the chronic infection itself, as it is arguably the biggest challenge faced by malaria elimination efforts. Not only do they represent an 'invisible' reservoir from which a malaria epidemic could originate, any campaign that focuses on treating clinical cases would likely select for a population with an "asymptomatic profile" (low-parasitaemia, chronic infections). Deciphering the biology of *P. falciparum* chronic infection is required to outcompete the selective pressure we exert on the disease. In the long term, a better understanding of the human host and the *Plasmodium* pathogen interaction will help reduce the huge disease burden and socio-economic impact of malaria in endemic countries, thus having a direct impact on the people who had volunteered to donate their blood for research.

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Glossary

Antigenic variation: The recurrent variation of surface exposed antigens by successive parasite generations to evade the host immune system.

Asymptomatic: The presence of circulating *Plasmodium* parasites in the blood of an individual with a body temperature < 38 degrees for more than 48 hours; also referred to as "afebrile".

Chimeric *var*: A *var* allele generated from the mitotic ectopic recombination of two *var* genes.

Chronic: Persistence of multiplying malaria parasites over a long period of time without resolution.

Cytoadherence: The binding of *P. falciparum* infected erythrocytes to other cells such as endothelial cells.

DBLα: The first Duffy Binding-Like sequence at the 5'-end of almost all *var* genes. Although the total number of unique DBLα sequences in the *P. falciparum* population is virtually infinite, two ~30bp regions on either end of the sequence are highly conserved. "Universal primers" targeting these regions are used to amplify the polymorphic region in between, for sequence identification and transcript quantitation.

Dormancy/quiescence: A temporary halt of development of the parasite's intra-erythrocytic cycle.

Immune evasion: In this context, strategies by parasites to avoid being detected and/or removed by the host's immune system, particularly via antigenic variation.

Variant surface antigens (VSA): A group of highly polymorphic parasite antigens displayed on the surface of infected erythrocytes. They include the *var* (~60 copies), *rif* (~180 copies) and *stevor* gene families (~40 copies).

***Var*/PfEMP1**: A family of highly polymorphic genes, with mutually exclusive expression, coding for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is displayed on the surface of infected erythrocytes to mediate cytoadherence to endothelial cells,or uninfected erythrocytes. The protein is typically composed of 4 to 7 DBL and CIDR domains.

Box 1. Malariotherapy, the lesser of two evils, has been immensely informative

In the early to mid-20th century, tens of thousands of neurosyphilis patients were treated by inoculation of *P. vivax* or *P. falciparum* strains. The occurrence of malaria-induced fever helped the patient's immune system kill off the bacteria. The discovery of antibiotics and a drastic change in medical ethics definitively stopped malariotherapy in 1963. Although neither parasite culture nor molecular biology tools were available, this unique dataset of controlled *Plasmodium* infections is still the basis of our understanding of chronic infections, as demonstrated by continued re-analysis of the data [45, 63, 68]. Of particular interest, the average untreated *P. falciparum* infection in non-immune American syphilis patients lasted just over 7 months (range 14 to 417 days) [69]. Novel approaches to research the long-term effect of *P. falciparum* in the human host are needed.

Box 2. Ethical concerns

Cohorts of asymptomatic carriers, without giving immediate treatment after detection of *P. falciparum*, have been used in the past without any reported major incident [29, 61, 70-73]. Moreover, in places where malaria is seasonal, asymptomatic infections during the dry season reduce the risk of developing clinical malaria during the following wet season [70, 74-76]. However, the lack of malaria-like symptoms, such as fever, does not exclude long-term effects of asymptomatic infections. Asymptomatically infected individuals are at an increased risk of systemic bacterial infections and are more likely to be anaemic, which could impair cognitive function (reviewed in [77]). An exhaustive assessment of the long-term impact of *Plasmodium* infections is greatly needed.

Research projects as described in Table 1 could be piggybacked on such longitudinal studies.

Table 1. Non-exhaustive list of biological questions to be addressed specifically with a longitudinal approach.

Theme	Specific question	Method	Comment
Parasite sensing host state	Do parasites regulate their multiplication rate during the course of an infection?	qPCR	The Parasite Multiplication Rate is measured by qPCR to determine parasitaemia at regular time interval
	What genes are differentially expressed to establish a long-term infection? And do parasites enter dormancy during the dry season?	Single-Cell RNAseq	The multiplex single-cell approaches (such as DropSeq or Seq-Well) have the potential to identify subpopulations within a single infection. For example, parasites that would have entered a quiescent state (G0 of the life cycle).
Antigenic variation	What is the in vivo *var* gene switching rate? Can *var* genes alone explain chronic infection?	qRT-PCR	The switching rate of mutually exclusive expression of *var* genes can be assessed *in vivo* and *in vitro* (Figure 2B).
Cytoadherence	What type, and quantity of PfEMP1 is expressed on the surface of infected erythrocytes during a chronic infection? Does it correlate with cytoadherence phenotype?	Atomic force microscopy	Knobs can be quantified by microscopy [78]. Current cytoadherence assays, under static or flow conditions, will need to be greatly optimised before addressing such questions.
Duration of chronic infection	How long does a chronic asymptomatic infection last? What proportion becomes febrile? What host, parasite and environmental factor are associated with duration of infection?	qPCR	PCR and microscopy based probing of finger-prick blood samples for the presence or absence of *P. falciparum*.

Cause of symptomatic onset	Should symptomatic disease occur, will it be due to the parasites in the chronic infection or a newly infecting parasite?	qPCR	Parasites can be genotyped during asymptomatic stage and upon onset of symptoms.
Gametocyte transmission	After how many days of infection are humans most infectious? Does transmission efficacy vary with seasonality?	Mosquito feeding assays	Mosquitoes are fed with blood from asymptomatic infection to determine the rate of gametocyte infectivity over time.
Host immunity to *P. falciparum* infections	What is the dynamics and contribution of the various arms of the immune system during a sustained infection?	Flow cytometry, Luminex assays, ELISpot assays, RNA sequencing (bulk or single cell)	Peripheral Blood Mononuclear Cells (PBMC) can be immuno-phenotyped to identify and track the expansion and/or activation state of immune cell subpopulations. Plasma cytokine levels and cell-based antigen recognition and reactivity can be measured with Luminex and ELISpot assays, respectively. Gene expression levels in each cell type can be assayed, directly *ex vivo* or after stimulation with *Plasmodium* antigens.
	How is B and T cell receptor affinity shaped by chronic infections?	Single cell genomic sequencing	The immunoglobulin genes of isolated B and T cells can be sequence to ascertain their affinity maturation over time.
Antibody response	What is the specificity of the antibody response against *P.f.* VSA? How long does it last?	Flow cytometry, Luminex, protein microarray	Immunoglubin recognition of iRBC can be studied at each time point, to test the hypothesis of a sequential antibody acquisition matching *var* gene switching
	How effective is the antibody response to asymptomatic/low parasitaemia infections? How long does it last?	Opsonisation assays, Flow cytometry	Antibodies from asymptomatic infections could be used in opsonisation assay to test their effectiveness in inducing host responses.

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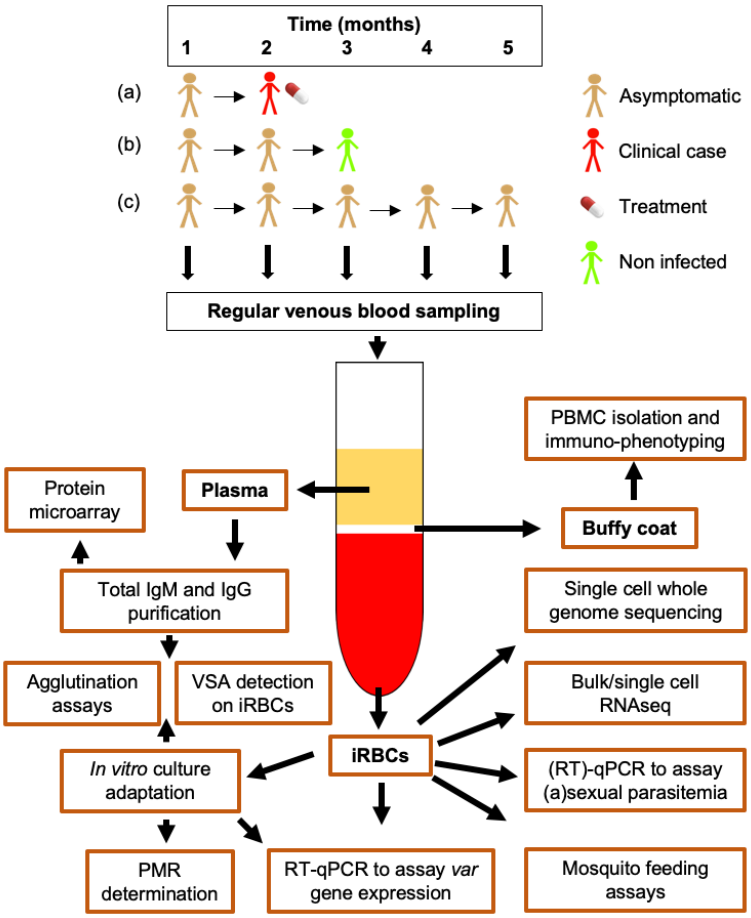

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550 **Figure 1. Cohort of asymptomatic *Plasmodium* positive volunteers.** A
551 *Plasmodium* infection results in three possible outcomes (A) febrile malaria, in
552 which case the volunteer is treated by anti-malarials immediately, (B) the
553 infection is cleared by the host, (C) the infection is still on going at the end of the
554 study period. Note that the blood sampling frequency could be in days/ weeks/
555 months based on study requirements. The lower panel indicates the usage of
556 each component of a blood sample.

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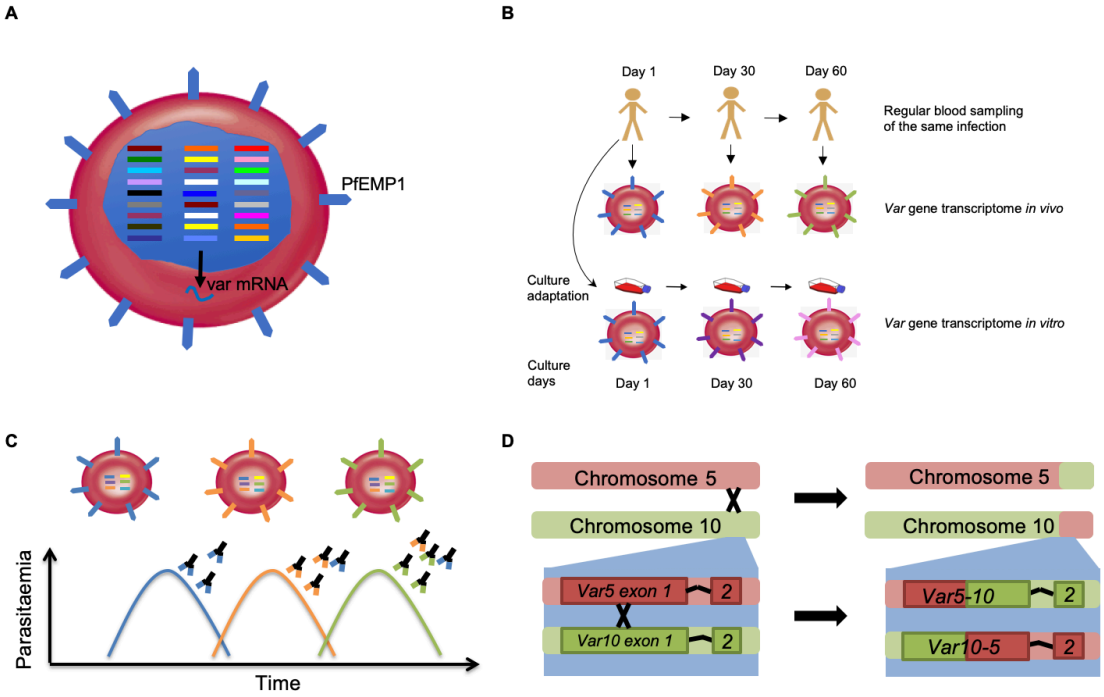

Figure 2. *Var* genes and antigenic variation. Panel (A) depicts the mutually exclusive expression of the ~60 *var* genes in the *P. falciparum* genome. Only one member of the family is expressed at the ring stage, with a single type of PfEMP1 molecule at the surface of the red blood cell at late-pigmented trophozoite stage. Each isolate of *P. falciparum* typically contains a distinct set of *var*, making the total repertoire of sequences virtually infinite. This may explain why sterile immunity against malaria is rarely acquired. Panel (B) illustrates how *var* gene transcription could be recorded in the host and *in vitro*. Regular blood sampling of a *P. falciparum*-infected asymptomatic volunteer to determine the most commonly expressed *var* genes at each timepoint. In parallel, an isolate from the first timepoint is cultured in a flask, to record *var* gene switching in the absence of immune selection. This hypothetical and simplified example depicts a different transcription pattern in the host and *in vitro*. Panel (C) exemplifies the antigenic variation hypothesis. Regular switching of surface-exposed PfEMP1 would lead to burst of parasitaemia immediately followed by sequential acquisition of specific antibodies. For example, plasma samples from timepoint 2 would recognize infected red blood cell from timepoint 1 but not from timepoint 3. Panel (D) portrays ectopic recombination generating a chimeric *var* gene. In this hypothetical example, a recombination between two subtelomeric *var* genes leads to the replacement of the *var10* gene by a chimeric sequence containing the 5' end of *var10* and 3' end of *var5*. Multiple crossing-over events can lead to more complex, and potentially antigenically distinct, sequences.