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5 **Understanding host-pathogen-vector interactions with chronic**  
6 **asymptomatic malaria infections**

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15 **Keywords:** Chronic asymptomatic malaria, *Plasmodium falciparum*, antigenic  
16 variation, var

18 **Abstract**

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

## 30 **Malaria pathogenesis**

31 Most of the half-a-million annual malaria deaths are due to *Plasmodium falciparum*  
32 [1]. This unicellular eukaryotic parasite is transmitted by female *Anopheles*  
33 mosquitoes. Malaria symptoms occur during the intra-erythrocytic part of the  
34 parasite's life cycle. Although the parasite is largely "hidden" within an infected  
35 red blood cell (iRBC) during the first ~20 hours (called ring-stage), it reveals itself  
36 to host immunity by exporting antigens to the surface of the RBC at the  
37 trophozoite-stage. Such antigens include ***P. falciparum* erythrocyte membrane**  
38 **protein 1 (PfEMP1, see Glossary)** [2], which enable the iRBC adhere to  
39 endothelial cells of the micro-vessels. This phenomenon, termed sequestration, is  
40 essential for late-stage iRBCs to avoid splenic clearance, but simultaneously leads  
41 to microvascular obstruction and release of pro-inflammatory cytokine, which are  
42 key features of malaria pathogenesis. A *P. falciparum* infection can result in  
43 multiple outcomes; from **asymptomatic** (afebrile in this context), to  
44 uncomplicated or severe malaria. What drives the disease one way or another is  
45 not fully understood, but does involve host, parasite and environmental factors.

46

## 47 ***P. falciparum* asymptomatic infections: looking for the elephant in the** 48 **room**

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

58

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

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

68

### 69 **Asymptomatic infections: out of sight, out of mind**

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

76

77 Today's technology is at a turning point to address these issues. Extremely low  
78 parasite densities can be detected with ultrasensitive PCR assays [9]. An entire  
79 *Plasmodium* genome can be sequenced from a dry blood spot [10], a single cell  
80 [11], or using a device as small as a USB-stick (Oxford Nanopore [12]). Thousands  
81 of transcriptomes can be individually tagged and pool-sequenced to drastically  
82 reduce sequencing cost [13]. Complex parasite populations may be resolved with  
83 single-cell approaches [14]. State-of-the-art techniques can now be applied to fully  
84 characterize *P. falciparum* parasites in low-density, asymptomatic infections.

85

### 86 **Fighting asymptomatic infections with asymptomatic infections**

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

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

99  
100 Practically, in collaboration with ethics committees and local health authorities  
101 (Box 2), a cohort is recruited by enrolling consenting *Plasmodium* asymptomatic  
102 carriers (Figure 1). Diagnosis is performed by qPCR as parasitaemia is often too  
103 low for microscopy detection. Each volunteer provides blood samples at regular  
104 intervals, unless he/she develops malaria symptoms. All volunteers must have  
105 immediate access to free professional medical care during the entire study. Blood  
106 samples can be used to address a variety of biological questions (Table 1). Should  
107 the volunteer develop symptoms or desire to withdraw from the cohort, anti-  
108 malarial treatment is given immediately. Regular genotyping will distinguish an  
109 ongoing infection from a new one. In the case of multi-clonal infections, a second  
110 sampling within 24-48 hours is necessary to detect circulating strains that would  
111 have been sequestered at the first timepoint. The period between each sampling  
112 timepoint can vary from days to months depending on the biological question(s)  
113 to be addressed. An alternative study design is to recruit non-infected volunteers,  
114 for example, just before the malaria transmission season starts, and sample them  
115 from the day of recruitment to catch the very early stages of the infection. This  
116 approach would be particularly suited to study the average duration of a *P.*  
117 *falciparum* infection.

118

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

121

## 122 **Central dogma: antigenic variation and host response**

### 123 ***General var gene background***

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

129 erythrocytes [15]. The most prominent and well-studied VSA is PfEMP1, coded by  
130 the *var* gene family (Figure 2A). It has ~60 members per genome, classified into  
131 three main groups; A, B and C, based on their upstream 5' untranslated sequences  
132 (UpsA, UpsB, and UpsC, respectively), chromosomal locations and orientation  
133 [16]. Importantly, the grouping is associated with *in vitro* switching [17],  
134 **cytoadherence** phenotypes and pathogenesis. Notably, some Group A PfEMP1  
135 bind EPCR1 and their expression is associated with cerebral malaria [18-20].  
136 Group B and C PfEMP1 typically bind to CD36 and are associated with mild  
137 disease. Few cross-sectional studies which recorded *var* gene expression from  
138 asymptomatic infections identified low abundant, homogenous and mainly group  
139 C *var* gene expression [21-24]. To the best of our knowledge, the *ex vivo*  
140 cytoadherence phenotype of parasites derived from asymptomatic infections has  
141 not been addressed, most likely because current assays require much higher  
142 parasite density.

143

#### 144 ***Does var gene expression switch in chronic infection?***

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

158

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

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

171

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

173 Our current understanding of immune responses; both cellular and humoral, to *P.*  
174 *falciparum* infections is limited. Models suggest that the parasite-host relationship  
175 has evolved to favour some short-lived immune responses which allows the  
176 parasite to persist and the host to survive [34]. Several cross reactivity studies  
177 have demonstrated robust acquisition of antibodies to VSA of homologous  
178 parasites (from the same donor) during the course of the infection, with  
179 individuals having anti-VSA antibodies to both homologous and heterologous  
180 parasites (from a different donor) being more protected from severe or  
181 symptomatic disease [35-39]. Although anti-RIFIN [40] and anti-STEVEOR [41]  
182 antibodies have been shown to be functional; promoting immune effector  
183 mechanisms, PfEMP1 is thought to be the main target of both total and functional  
184 anti-VSA antibodies [39]. A body of knowledge highlight the emergence of both  
185 long-lived and short-lived anti-VSA antibody acquisition during, and after  
186 resolution of an infection [35, 42], with some individuals failing to switch antibody  
187 isotypes from IgM to IgG [42]. Nonetheless, these studies rely on samples taken  
188 either at the time of symptomatic disease or after resolution of the infection, and  
189 thus do not provide a holistic understanding of the kinetics of host immunity  
190 during sustained infection and how it contributes towards the establishment of  
191 chronic infection. It is equally important to know how the persistence of an  
192 infection shapes host immunity. Studies with cohorts of chronic asymptomatic  
193 infections followed over long periods could help fill this gap in knowledge (Figure  
194 2C).

195

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

204

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

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

223

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



228

229 ***Do parasites become dormant in the dry season?***

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

248

249 ***Gametocyte commitment and transmission with seasonality?***

250 Gametocytes are terminal blood stage parasites required for transmission. Thus,  
251 their formation needs to be timely to ensure successful transmission. Indeed, in  
252 birds, *Plasmodium* parasite density increases after repeated mosquito bites [53].  
253 This may also be the case in humans before the start of the transmission season  
254 [54, 55]. However, harbouring gametocytes does not necessarily equate to being  
255 mosquito infective. Successful transmission requires viable mature gametocytes  
256 in the right sex ratio which maximizes the chance of at least one female and one  
257 male being ingested. Data from CHMI studies predict gametocyte detection, on  
258 average, 10 days post blood-stage infection; suggesting gametocyte conversion  
259 within the first blood-stage generation [56]. These studies were however  
260 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-

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

299

### 300 **Concluding remarks**

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

309

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

322

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326

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484

## 485 Glossary

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

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

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

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

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

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

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

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

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

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

515

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

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

530

531 **Box 2. Ethical concerns**

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

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

544

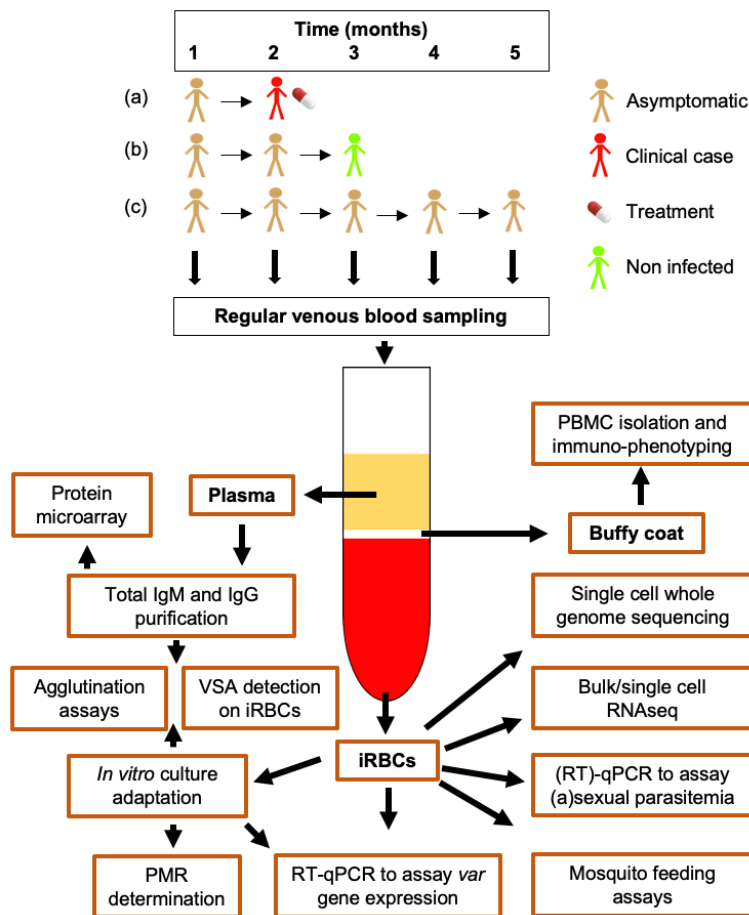
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546 **Table 1. Non-exhaustive list of biological questions to be addressed**  
 547 **specifically with a longitudinal approach.**

Theme	Specific question	Method	Comment
<b>Parasite sensing host state</b>	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).
<b>Antigenic variation</b>	What is the <i>in vivo</i> <i>var</i> gene switching rate? Can <i>var</i> genes alone explain chronic infection?	qRT-PCR	The switching rate of mutually exclusive expression of <i>var</i> genes can be assessed <i>in vivo</i> and <i>in vitro</i> (Figure 2B).
<b>Cytoadherence</b>	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.
<b>Duration of chronic infection</b>	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 <i>P. falciparum</i> .



<b>Cause of symptomatic onset</b>	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.
<b>Gametocyte transmission</b>	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.
<b>Host immunity to <i>P. falciparum</i> infections</b>	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 <i>ex vivo</i> or after stimulation with <i>Plasmodium</i> 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.
<b>Antibody response</b>	What is the specificity of the antibody response against <i>P.f.</i> VSA? How long does it last?	Flow cytometry, Luminex, protein microarray	Immunoglobulin recognition of iRBC can be studied at each time point, to test the hypothesis of a sequential antibody acquisition matching <i>var</i> 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.



549

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/

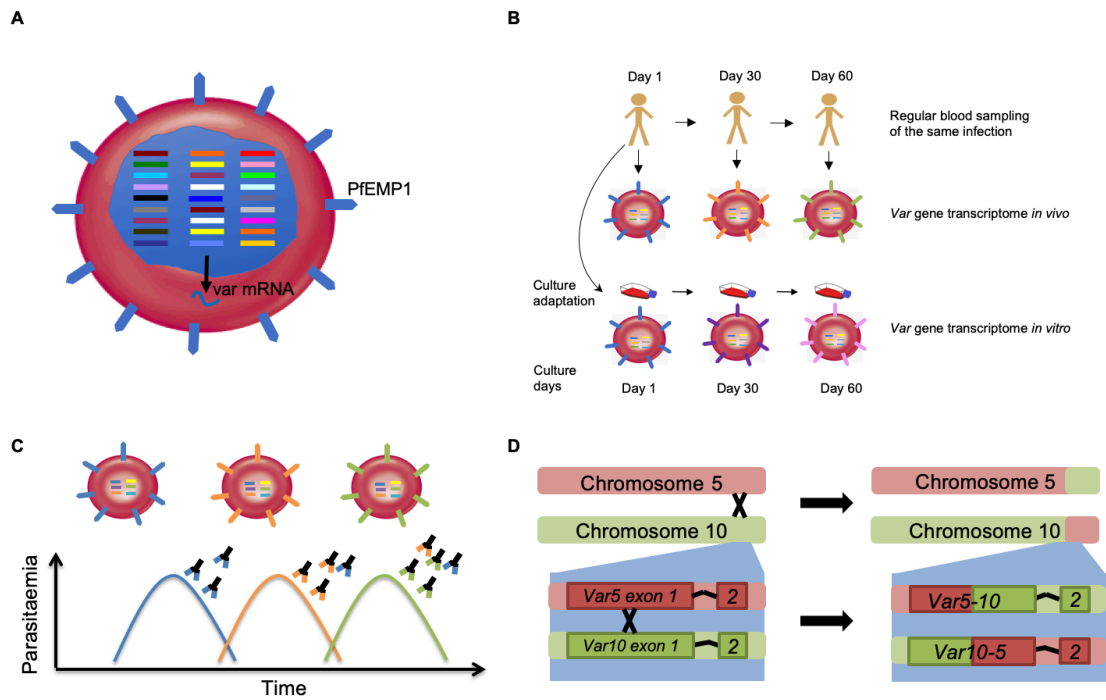
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months based on study requirements. The lower panel indicates the usage of

556

each component of a blood sample.

557



558

559 **Figure 2. Var genes and antigenic variation.** Panel (A) depicts the mutually exclusive

560 expression of the ~60 var genes in the *P. falciparum* genome. Only one member of the

561 family is expressed at the ring stage, with a single type of PfEMP1 molecule at the surface

562 of the red blood cell at late-pigmented trophozoite stage. Each isolate of *P. falciparum*

563 typically contains a distinct set of var, making the total repertoire of sequences virtually

564 infinite. This may explain why sterile immunity against malaria is rarely acquired. Panel

565 (B) illustrates how var gene transcription could be recorded in the host and *in vitro*.

566 Regular blood sampling of a *P. falciparum*-infected asymptomatic volunteer to determine

567 the most commonly expressed var genes at each timepoint. In parallel, an isolate from the

568 first timepoint is cultured in a flask, to record var gene switching in the absence of

569 immune selection. This hypothetical and simplified example depicts a different

570 transcription pattern in the host and *in vitro*. Panel (C) exemplifies the antigenic variation

571 hypothesis. Regular switching of surface-exposed PfEMP1 would lead to burst of

572 parasitaemia immediately followed by sequential acquisition of specific antibodies. For

573 example, plasma samples from timepoint 2 would recognize infected red blood cell from

574 timepoint 1 but not from timepoint 3. Panel (D) portrays ectopic recombination

575 generating a chimeric var gene. In this hypothetical example, a recombination between

576 two subtelomeric var genes leads to the replacement of the var10 gene by a chimeric

577 sequence containing the 5' end of var10 and 3' end of var5. Multiple crossing-over events

578 can lead to more complex, and potentially antigenically distinct, sequences.

579