



HAL
open science

Understanding Host–Pathogen–Vector Interactions with Chronic Asymptomatic Malaria Infections

Prince B Nyarko, Antoine Claessens

► **To cite this version:**

Prince B Nyarko, Antoine Claessens. Understanding Host–Pathogen–Vector Interactions with Chronic Asymptomatic Malaria Infections. *Trends in Parasitology*, 2021, 37 (3), pp.195-204. 10.1016/j.pt.2020.09.017 . hal-02981651

HAL Id: hal-02981651

<https://hal.umontpellier.fr/hal-02981651>

Submitted on 24 Oct 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

8 Prince B. Nyarko¹, Antoine Claessens^{2*}

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

12 ²LPHI, MIVEGEC, CNRS, INSERM, University of Montpellier, France.

13 *Corresponding author: antoine.claessens@umontpellier.fr

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 α** 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-STEVAR [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

323 **Acknowledgements**

324 We would like to thank Dan Larremore, Kai Wengelnik, Ian Cheeseman and
325 anonymous reviewers for their critical review of this manuscript.

326

- 328 1. Bhatt, S. *et al.* (2015). The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature* 526, 207-211
- 329 2. Wahlgren, M. *et al.* (2017). Variant surface antigens of *Plasmodium falciparum* and their roles in severe malaria. *Nat. Rev. Microbiol.* 15, 479-491
- 330 3. Lindblade, K. A. *et al.* (2013). The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Rev. Anti-infect. Ther.* 11, 623-639
- 331 4. Ceessay, S. J. *et al.* (2008). Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. *The Lancet* 372, 1545-1554
- 332 5. Dunyo, S. *et al.* (2006). Gametocytaemia after drug treatment of asymptomatic *Plasmodium falciparum*. *PLOS Clin Trial* 1, e20
- 333 6. Stone, W. *et al.* (2015). Assessing the infectious reservoir of falciparum malaria: past and future. *Trends Parasitol.* 31, 287-296
- 334 7. Cook, J. *et al.* (2015). Mass screening and treatment on the basis of results of a *Plasmodium falciparum*-specific rapid diagnostic test did not reduce malaria incidence in Zanzibar. *J. Infect. Dis.* 211, 1476-1483
- 335 8. Larsen, D. A. *et al.* (2015). Population-wide malaria testing and treatment with rapid diagnostic tests and artemether-lumefantrine in southern Zambia: a community randomized step-wedge control trial design. *Am. J. Trop. Med. Hyg.* 92, 913-921
- 336 9. Imwong, M. *et al.* (2016). Numerical distributions of parasite densities during asymptomatic malaria. *J. Infect. Dis.* 213, 1322-1329
- 337 10. Oyola, S. O. *et al.* (2016). Whole genome sequencing of *Plasmodium falciparum* from dried blood spots using selective whole genome amplification. *Malar. J.* 15, 597
- 338 11. Nair, S. *et al.* (2014). Single-cell genomics for dissection of complex malaria infections. *Genome Res.* 24, 1028-1038
- 339 12. Quick, J. *et al.* (2016). Real-time, portable genome sequencing for Ebola surveillance. *Nature* 530, 228-232
- 340 13. Gierahn, T. M. *et al.* (2017). Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. *Nat. Methods* 14, 395-398
- 341 14. Nkhoma, S. C. *et al.* (2020). Co-transmission of Related Malaria Parasite Lineages Shapes Within-Host Parasite Diversity. *Cell Host Microbe* 27, 93-103. e104
- 342 15. Bull, P. C. and Abdi, A. I. (2016). The role of PfEMP1 as targets of naturally acquired immunity to childhood malaria: prospects for a vaccine. *Parasitology* 143, 171-186
- 343 16. Smith, J. D. (2014). The role of PfEMP1 adhesion domain classification in *Plasmodium falciparum* pathogenesis research. *Mol. Biochem. Parasitol.* 195, 82-87
- 344 17. Frank, M. *et al.* (2007). Variable switching rates of malaria virulence genes are associated with chromosomal position. *Mol. Microbiol.* 64, 1486-1498
- 345 18. Lavstsen, T. *et al.* (2012). *Plasmodium falciparum* erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1791-E1800
- 346 19. Claessens, A. *et al.* (2012). A subset of group A-like var genes encodes the malaria parasite ligands for binding to human brain endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1772-E1781
- 347 20. Turner, L. *et al.* (2013). Severe malaria is associated with parasite binding to endothelial protein C receptor. *Nature* 498, 502-505
- 348 21. Kaestli, M. *et al.* (2006). Virulence of malaria is associated with differential expression of *Plasmodium falciparum* var gene subgroups in a case-control study. *J. Infect. Dis.* 193, 1567-1574
- 349 22. Falk, N. *et al.* (2009). Analysis of *Plasmodium falciparum* var genes expressed in children from Papua New Guinea. *J. Infect. Dis.* 200, 347-356
- 350 23. Warimwe, G. M. *et al.* (2013). *Plasmodium falciparum* var gene expression homogeneity as a marker of the host-parasite relationship under different levels of naturally acquired immunity to malaria. *PLoS one* 8,
- 351 24. Abdi, A. I. *et al.* (2016). Global selection of *Plasmodium falciparum* virulence antigen expression by host antibodies. *Sci. Rep.* 6, 19882
- 352 25. Roberts, D. J. *et al.* (1992). Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature* 357, 689-692
- 353 26. Horrocks, P. *et al.* (2004). Variable var transition rates underlie antigenic variation in malaria. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11129-11134
- 354 27. Bachmann, A. *et al.* (2016). Mosquito passage dramatically changes var gene expression in controlled human *Plasmodium falciparum* infections. *PLoS Pathog.* 12, e1005538
- 355 28. Bachmann, A. *et al.* (2019). Controlled human malaria infection with *Plasmodium falciparum* demonstrates impact of naturally acquired immunity on virulence gene expression. *PLoS Pathog.* 15, e1007906
- 356 29. Kaestli, M. *et al.* (2004). Longitudinal assessment of *Plasmodium falciparum* var gene transcription in naturally infected asymptomatic children in Papua New Guinea. *J. Infect. Dis.* 189, 1942-1951
- 357 30. Taylor, H. M. *et al.* (2000). A study of var gene transcription in vitro using universal var gene primers. *Mol. Biochem. Parasitol.* 105, 13-23
- 358 31. Otto, T. D. *et al.* (2019). Evolutionary analysis of the most polymorphic gene family in falciparum malaria. *Wellcome Open Res.* 4, 193
- 359 32. Jespersen, J. S. *et al.* (2016). *Plasmodium falciparum* var genes expressed in children with severe malaria encode CIDR α 1 domains. *EMBO molecular medicine* 8, 839-850
- 360 33. Tonkin-Hill, G. Q. *et al.* (2018). The *Plasmodium falciparum* transcriptome in severe malaria reveals altered expression of genes involved in important processes including surface antigen-encoding var genes. *PLoS Biol.* 16, e2004328
- 361 34. Recker, M. *et al.* (2004). Transient cross-reactive immune responses can orchestrate antigenic variation in malaria. *Nature* 429, 555-558
- 362 35. Ofori, M. F. *et al.* (2002). Malaria-induced acquisition of antibodies to *Plasmodium falciparum* variant surface antigens. *Infect. Immun.* 70, 2982-2988

- 399 36. Staalsoe, T. *et al.* (2002). In vivo switching between variant surface antigens in human *Plasmodium falciparum*
400 infection. *J. Infect. Dis* 186, 719-722
- 401 37. Nielsen, M. A. *et al.* (2002). *Plasmodium falciparum* variant surface antigen expression varies between isolates
402 causing severe and nonsevere malaria and is modified by acquired immunity. *J. Immunol.* 168, 3444-3450
- 403 38. Chattopadhyay, R. *et al.* (2003). *Plasmodium falciparum* infection elicits both variant-specific and cross-reactive
404 antibodies against variant surface antigens. *Infect. Immun.* 71, 597-604
- 405 39. Chan, J.-A. *et al.* (2012). Targets of antibodies against *Plasmodium falciparum*-infected erythrocytes in malaria
406 immunity. *J. Clin. Invest.* 122, 3227-3238
- 407 40. Goel, S. *et al.* (2015). RIFINs are adhesins implicated in severe *Plasmodium falciparum* malaria. *Nat. Med.* 21,
408 314
- 409 41. Niang, M. *et al.* (2014). STEVOR is a *Plasmodium falciparum* erythrocyte binding protein that mediates merozoite
410 invasion and rosetting. *Cell Host Microbe* 16, 81-93
- 411 42. Kinyanjui, S. M. *et al.* (2003). Kinetics of antibody responses to *Plasmodium falciparum*-infected erythrocyte
412 variant surface antigens. *J. Infect. Dis* 187, 667-674
- 413 43. Tan, J. and Bull, P. C., (2015). Agglutination Assays of the *Plasmodium falciparum*-Infected Erythrocyte. In
414 *Malaria Vaccines*, pp. 115-129, Springer
- 415 44. Travassos, M. A. *et al.* (2018). Children with cerebral malaria or severe malarial anaemia lack immunity to
416 distinct variant surface antigen subsets. *Sci. Rep.* 8, 1-14
- 417 45. Childs, L. M. and Buckee, C. O. (2015). Dissecting the determinants of malaria chronicity: why within-host models
418 struggle to reproduce infection dynamics. *J. R. Soc. Interface* 12, 20141379
- 419 46. Claessens, A. *et al.* (2014). Generation of antigenic diversity in *Plasmodium falciparum* by structured
420 rearrangement of Var genes during mitosis. *PLoS Genet.* 10, e1004812
- 421 47. Teuscher, F. *et al.* (2010). Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates,
422 and implications in treatment failure. *J. Infect. Dis* 202, 1362-1368
- 423 48. Talman, A. M. *et al.* (2019). Artemisinin Bioactivity and Resistance in Malaria Parasites. *Trends Parasitol.*
- 424 49. Chotivanich, K. *et al.* (2000). Parasite multiplication potential and the severity of falciparum malaria. *J. Infect. Dis*
425 181, 1206-1209
- 426 50. Deans, A.-M. *et al.* (2006). Low multiplication rates of African *Plasmodium falciparum* isolates and lack of
427 association of multiplication rate and red blood cell selectivity with malaria virulence. *Am. J. Trop. Med. Hyg.* 74,
428 554-563
- 429 51. Achan, J. *et al.* (2019). Serologic markers of previous malaria exposure and functional antibodies inhibiting
430 parasite growth are associated with parasite kinetics following a *Plasmodium falciparum* controlled human
431 infection. *Clin. Infect. Dis*
- 432 52. Murray, L. *et al.* (2017). Multiplication rate variation in the human malaria parasite *Plasmodium falciparum*. *Sci.*
433 *Rep.* 7, 1-8
- 434 53. Cornet, S. *et al.* (2014). Evolution of plastic transmission strategies in avian malaria. *PLoS Pathog.* 10,
- 435 54. Lin Ouédraogo, A. *et al.* (2016). Dynamics of the human infectious reservoir for malaria determined by mosquito
436 feeding assays and ultrasensitive malaria diagnosis in Burkina Faso. *J. Infect. Dis* 213, 90-99
- 437 55. Gadalla, A. A. *et al.* (2016). Associations between season and gametocyte dynamics in chronic *Plasmodium*
438 *falciparum* infections. *PLoS one* 11,
- 439 56. Reuling, I. J. *et al.* (2018). A randomized feasibility trial comparing four antimalarial drug regimens to induce
440 *Plasmodium falciparum* gametocytemia in the controlled human malaria infection model. *Elife* 7, e31549
- 441 57. Bousema, T. *et al.* (2012). Mosquito feeding assays to determine the infectiousness of naturally infected
442 *Plasmodium falciparum* gametocyte carriers. *PLoS one* 7,
- 443 58. Smit, M. R. *et al.* (2019). Human Direct Skin Feeding Versus Membrane Feeding to Assess the Mosquitocidal
444 Efficacy of High-Dose Ivermectin (IVERMAL Trial). *Clin. Infect. Dis* 69, 1112-1119
- 445 59. Talman, A. M. *et al.* (2020). Uptake of *Plasmodium falciparum* gametocytes during mosquito bloodmeal by direct
446 and membrane feeding. *Front Microbiol* 11, 246
- 447 60. Ashley, E. A. and White, N. J. (2014). The duration of *Plasmodium falciparum* infections. *Malar. J.* 13, 500
- 448 61. Hamad, A. *et al.* (2000). Chronic *Plasmodium falciparum* infections in an area of low intensity malaria
449 transmission in the Sudan. *Parasitology* 120, 447-456
- 450 62. Baliraine, F. N. *et al.* (2010). A cohort study of *Plasmodium falciparum* infection dynamics in Western Kenya
451 Highlands. *BMC Infect. Dis.* 10, 283
- 452 63. Bretscher, M. T. *et al.* (2011). The distribution of *Plasmodium falciparum* infection durations. *Epidemics* 3, 109-
453 118
- 454 64. Buchwald, A. G. *et al.* (2019). Clinical implications of asymptomatic *Plasmodium falciparum* infections in Malawi.
455 *Clin. Infect. Dis* 68, 106-112
- 456 65. Tuikue Ndam, N. *et al.* (2018). Persistent *Plasmodium falciparum* infection in women with an intent to become
457 pregnant as a risk factor for pregnancy-associated malaria. *Clin. Infect. Dis* 67, 1890-1896
- 458 66. Moormann, A. M. *et al.* (2019). Immune effector mechanisms in malaria: An update focusing on human immunity.
459 *Parasite Immunol.* 41, e12628
- 460 67. Moncunill, G. *et al.* (2020). Antigen-stimulated PBMC transcriptional protective signatures for malaria
461 immunization. *Sci. Transl. Med.* 12, eaay8924
- 462 68. Snounou, G. and Pérignon, J.-L., (2013). Malariotherapy-insanity at the service of malariology. In *Advances in*
463 *parasitology*, pp. 223-255, Elsevier
- 464 69. Sama, W. *et al.* (2006). Distribution of survival times of deliberate *Plasmodium falciparum* infections in tertiary
465 syphilis patients. *Trans. R. Soc. Trop. Med. Hyg.* 100, 811-816
- 466 70. Färnert, A. *et al.* (1999). Complexity of *Plasmodium falciparum* infections is consistent over time and protects
467 against clinical disease in Tanzanian children. *J. Infect. Dis* 179, 989-995
- 468 71. Portugal, S. *et al.* (2014). Exposure-dependent control of malaria-induced inflammation in children. *PLoS Pathog.*
469 10,
- 470 72. Njua-Yafi, C. *et al.* (2016). Malaria, helminths, co-infection and anaemia in a cohort of children from Mutengene,
471 south western Cameroon. *Malar. J.* 15, 69

- 472 73. Cornet, M. *et al.* (1998). Prevalence of and risk factors for anemia in young children in southern Cameroon. *Am.*
473 *J. Trop. Med. Hyg.* 58, 606-611
474 74. Males, S. *et al.* (2008). Long-term asymptomatic carriage of *Plasmodium falciparum* protects from malaria
475 attacks: a prospective study among Senegalese children. *Clin. Infect. Dis* 46, 516-522
476 75. Sondén, K. *et al.* (2015). Asymptomatic multiclonal *Plasmodium falciparum* infections carried through the dry
477 season predict protection against subsequent clinical malaria. *J. Infect. Dis* 212, 608-616
478 76. Portugal, S. *et al.* (2017). Treatment of chronic asymptomatic *Plasmodium falciparum* infection does not increase
479 the risk of clinical malaria upon reinfection. *Clin. Infect. Dis* 64, 645-653
480 77. Chen, I. *et al.* (2016). "Asymptomatic" malaria: a chronic and debilitating infection that should be treated. *PLoS*
481 *medicine* 13,
482 78. Quadt, K. A. *et al.* (2012). The density of knobs on *Plasmodium falciparum*-infected erythrocytes depends on
483 developmental age and varies among isolates. *PLoS one* 7, e45658

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 α :** The first Duffy Binding-Like sequence at the 5'-end of almost all *var* genes.
498 Although the total number of unique DBL α 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-20th 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.

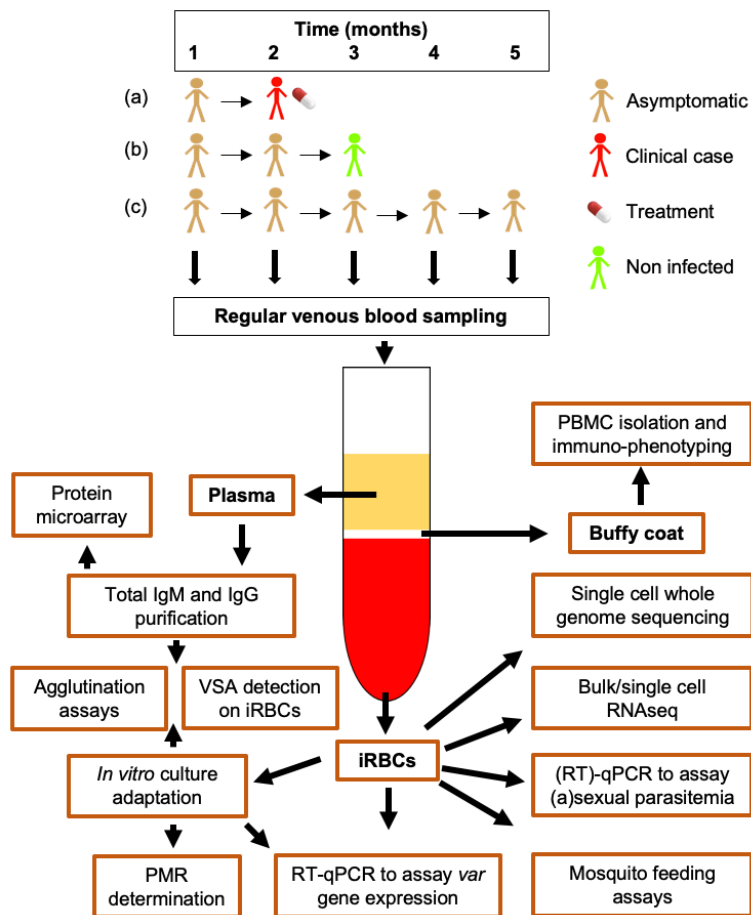
544

545

546 **Table 1. Non-exhaustive list of biological questions to be addressed**
 547 **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 <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).
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 <i>P. falciparum</i> .

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 <i>P. falciparum</i> 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 <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.
Antibody response	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/

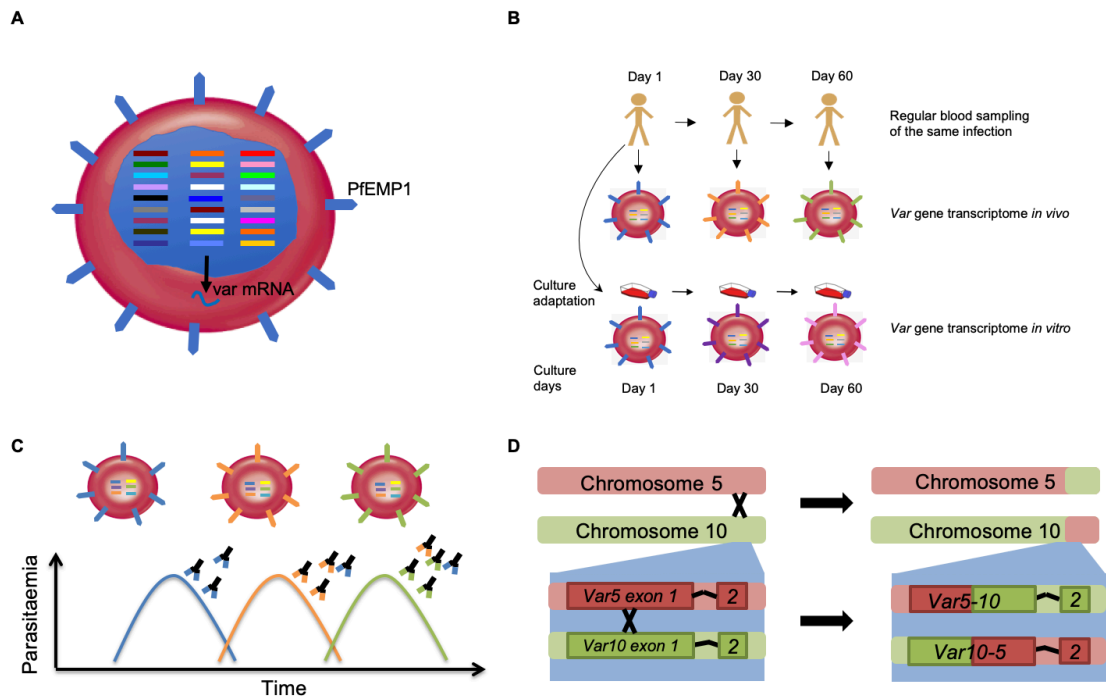
555

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