

1 ***Plasmodium falciparum* adapts its investment into replication *versus* transmission**  
2 **according to the host environment**

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16

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18 **Abstract**

19 The malaria parasite life cycle includes asexual replication in human blood, with a proportion of  
20 parasites differentiating to gametocytes required for transmission to mosquitoes. Commitment to  
21 differentiate into gametocytes, which is marked by activation of the parasite transcription factor  
22 *ap2-g*, is known to be influenced by host factors but a comprehensive model remains uncertain.  
23 Here we analyze data from 828 children in Kilifi, Kenya with severe, uncomplicated, and  
24 asymptomatic malaria infection over 18 years of falling malaria transmission. We examine  
25 markers of host immunity and metabolism, and markers of parasite growth and transmission  
26 investment. We find that inflammatory responses and reduced plasma lysophosphatidylcholine  
27 levels are associated with markers of increased investment in parasite sexual reproduction (i.e.,  
28 transmission investment) and reduced growth (i.e., asexual replication). This association  
29 becomes stronger with falling transmission and suggests that parasites can rapidly respond to the  
30 within-host environment, which in turn is subject to changing transmission.

31

32

### 33      **Introduction**

34            Malaria remains one of the world's major public health problems. In 2020, an estimated  
35            627'000 deaths and 241 million cases were reported<sup>1</sup>. Around 70% of deaths are in African  
36            children under five years of age and are caused by a single parasite species, *Plasmodium*  
37            *falciparum*<sup>1</sup>.

38            *P. falciparum* has a complex life cycle, involving obligatory transmission through a  
39            mosquito vector and asexual replication within erythrocytes of the human host. Between-host  
40            transmission requires the formation of gametocytes from asexual blood stage forms, as  
41            gametocytes are the only parasite stage to progress the cycle in the mosquito. A series of recent  
42            studies has demonstrated that commitment to gametocyte formation (i.e., stage conversion) is  
43            epigenetically regulated and occurs via activation of the transcription factor, AP2-G that in turn  
44            induces transcription of the first set of gametocyte genes<sup>2,3</sup>.

45            The parasites that do not convert into gametocytes continue to replicate asexually,  
46            contributing to within-host parasite population growth (i.e., parasite burden) and determining *P.*  
47            *falciparum* infection outcome that ranges from asymptomatic infections to severe complications  
48            and death<sup>4-6</sup>. Cytoadhesion of infected erythrocytes (IE) to receptors on microvascular  
49            endothelium of deep tissues reduces the rate of parasite elimination in the spleen<sup>7,8</sup>, thus  
50            supporting the within-host expansion of the parasite population (i.e., parasite burden). As a side  
51            effect of this parasite survival strategy, cytoadhesion reduces the diameter of the vascular lumen,  
52            thus impairing perfusion and contributing to severe malaria pathology<sup>9-11</sup>. *P. falciparum*  
53            erythrocyte membrane protein 1 (PfEMP1), encoded by the *var* multi-gene family, plays a  
54            critical role in both pathogenesis (through cytoadhesion)<sup>12,13</sup> and establishment of chronic  
55            infection (through variant switching and immune evasion)<sup>14,15</sup>.

56 Both *var* gene transcription and stage conversion (and hence *ap2-g* transcription) are  
57 subject to within-host environmental pressures such as immunity<sup>16</sup>, febrile temperature<sup>17,18</sup>, and  
58 nutritional stress<sup>19</sup>, perhaps via a common epigenetic regulation mechanism<sup>20</sup>. For example, *in*  
59 *vitro* studies revealed that stage conversion can be induced by nutritional depletion such as spent  
60 culture media<sup>19,21</sup> and depletion of Lysophosphatidylcholine (LPC)<sup>22,23</sup>. Recent work from Kenya  
61 and Sudan provides some evidence that parasites in low relative to high transmission settings  
62 invest more in sexual commitment and less in replication and *vice versa*<sup>16</sup>. Altogether these  
63 studies suggest that the parasite can sense and rapidly adapt to its environment *in vitro* and *in*  
64 *vivo*. A family of protein deacetylases called sirtuins are known as signaling proteins linking  
65 environmental sensing to various cellular processes via metabolic regulation<sup>24-26</sup>. They do this  
66 through epigenetic control of gene expression<sup>26</sup> and post-translational modification of protein  
67 function<sup>25,27</sup>. The *P. falciparum* genome contains two sirtuins (Pfsir2a/b) which have been linked  
68 to the control of *var* gene transcription<sup>28,29</sup>, and their expression is influenced by febrile  
69 temperature<sup>17</sup> and low transmission intensity<sup>16</sup>.

70 Here we investigated the interplay between parasite and host environmental factors  
71 governing parasite investment in reproduction (to maximize between-host transmission) *versus*  
72 replication (to ensure within-host persistence) *in vivo*. We analyzed samples and clinical data  
73 collected from children in Kilifi county, Kenya, over changing malaria transmission intensity  
74 between 1994 and 2014. We quantified parasite transcripts for *ap2-g*, *PfSir2a*, and *var* genes, as  
75 well as *PfHRP2* protein levels (for parasite biomass) and levels of host inflammatory markers  
76 and lipid metabolites. We then integrated these host and parasite-derived parameters to  
77 interrogate their dynamics and interactions in the context of changing transmission intensity and  
78 immunity.

79

80 **Results**

81 **A clinical malaria patient cohort across changing transmission periods in Kilifi, Kenya**

82 The study included samples and clinical data collected from 828 children from Kilifi county,  
83 Kenya, over 18 years of changing malaria transmission<sup>30,31</sup>. The study period encompassed three  
84 defined transmission phases<sup>31</sup>: pre-decline (1990-2002), decline (2003-2008), and post-decline  
85 (2009-2014) (**Fig.1A**). During the study period, a total of 26'564 malaria admissions were  
86 recorded at Kilifi county hospital (**Fig.1A, 1B**). While the number of parasite-positive  
87 admissions decreased, the mean patient age at admission increased over time<sup>4,6,32</sup> (**Fig.1A**). For  
88 our study, 552 of the admissions were selected to ensure adequate sampling of the transmission  
89 periods and clinical phenotypes (**Fig.1C**). 150 patients presented with uncomplicated malaria and  
90 402 with one or a combination of the severe malaria syndromes: impaired consciousness (IC),  
91 respiratory distress (RD), and severe malaria anemia (SMA)<sup>33</sup> (**Fig.1D**). 223 samples from  
92 children presenting with mild malaria at outpatient clinics and 53 asymptomatic children from a  
93 longitudinal malaria cohort study were added to cover the full range of the possible outcomes of  
94 malaria infection (**Fig.1B-E**), bringing the total number included in this study to 828 children.  
95 The characteristics of participants and clinical parameters are summarized in **Table S1**. In the  
96 subsequent analysis, only clinical cases were considered. Asymptomatic patients were excluded  
97 except for analysis about clinical phenotype since asymptomatic sampling was limited to the  
98 decline and post-decline periods (**Fig.1C**).

99

100 **Dynamics of parasite parameters across transmission period and clinical phenotype**

101 First, we analyzed the dynamics of parasite parameters across transmission periods and clinical  
102 outcomes. For this purpose, we measured both total parasite biomass based on *Pf*HRP2 levels  
103 and peripheral parasitemia based on parasite counts from blood smears. Total parasite biomass  
104 decreased with declining transmission (**Fig.2A**). This decrease was significant in the patients  
105 presenting with mild malaria at outpatient clinics (**Fig.2A**) which is a more homogenous clinical  
106 subgroup as compared to admissions consisting of a range of clinical phenotypes (**Fig.1C-D**).

107 Parasite samples were subjected to qRT-PCR analysis to quantify transcription of *ap2-g*,  
108 *Pfsir2a*, and *var* gene subgroups relative to two housekeeping genes (fructose biphosphate  
109 aldolase and seryl tRNA synthetase)<sup>34,35</sup>. In line with recent findings<sup>16</sup>, *ap2-g* transcription  
110 increased significantly with declining malaria transmission (**Fig.2B**). Importantly, *ap2-g*  
111 transcription showed a highly significant correlation with transcription levels of the gametocyte  
112 marker *Pfs16* (**Fig. 2C**). This association validates *ap2-g* as a proxy for both, stage conversion  
113 and gametocyte levels. *Pfsir2a* transcription followed the same trend across transmission periods  
114 and was positively associated with *ap2-g* transcription (**Fig.2B,D**). *Pfsir2a* and *ap2-g*  
115 transcription also showed a positive association with fever (**Fig. 2E**), suggesting that both factors  
116 are sensitive to changes in the host inflammatory response. *Pfsir2a* but not *ap2-g* transcription  
117 also showed a significant negative association with *Pf*HRP2 (**Fig.2D**). Given this unexpected  
118 observation, we investigated the well-established associations between *Pfsir2a* transcription and  
119 *var* gene transcription patterns<sup>29,34</sup>. *Pfsir2a* transcription showed a positive association with  
120 global upregulation of *var* gene transcription, particularly with subgroup B (**Fig.2F**). Likewise,  
121 transcription of group B *var* subgroup, *Pfsir2a* and *ap2-g* transcription followed a similar pattern  
122 in relation to clinical phenotypes (**Fig.S1**). Altogether these data suggest co-regulation of *ap2-g*

123 and *Pfsir2a* and a negative association between *Pfsir2a* and *PfHRP2*, likely through host factors  
124 that are changing with the declining transmission.

125

126 ***ap2-g* and *Pfsir2a* transcription is associated with a distinct host inflammation profile**

127 We hypothesized that the observed variation in *ap2-g* and *Pfsir2a* levels across the transmission  
128 period and clinical phenotype is due to underlying differences in the host inflammatory response.  
129 To test this hypothesis, we quantified 34 inflammatory markers<sup>36</sup> with Luminex xMAP  
130 technology in the plasma of the 523 patients from the outpatient and admissions groups. These  
131 patients were selected from the original set of 828 to ensure adequate representation of the  
132 transmission periods and clinical phenotypes (including fever), as summarized in **Fig. 1C, E**. For  
133 this analysis, all associations were corrected for patient age and *PfHRP2* levels as possible  
134 confounders.

135 The markers MCP-1, IL-10, IL-6, IL-1ra were significantly positively correlated with  
136 *ap2-g* and *Pfsir2a* transcription (**Fig. 3A and S2**). To cluster the inflammatory markers based on  
137 their correlation within the dataset, we used exploratory factor analysis and retained five factors  
138 with eigenvalues above 1 (**Fig. S3**). Factor loadings structured the inflammation markers into 5  
139 profiles with distinct inflammatory states (**Fig. 3B**). F1 consists of a mixture of inflammatory  
140 markers that support effector Th1/Th2/Th9/Th17 responses (i.e., hyperinflammatory state), F2  
141 represents a Th2 response, F3 represents markers that support follicular helper T cell  
142 development and Th17<sup>37</sup>, F4 represents markers of immune paralysis/tissue-injury linked to  
143 response to cellular/tissue injury<sup>38</sup> and F5 represents the inflammasome/Th1 response<sup>39</sup>. F4  
144 showed a significant positive association with *ap2-g* and *Pfsir2a* transcription and fever (**Fig.**  
145 **3C**). In contrast, F5 showed a negative association with *ap2-g* and fever while F1 was positively

146 associated with fever (**Fig.3C**). In parallel with the observed decrease in *Pf*HRP2 levels  
147 (**Fig.2A**), F1 and F5 significantly declined with falling transmission (**Fig.3D**).  
148 The data support our hypothesis and suggest that the host inflammatory response changes  
149 with the falling transmission. Of note, the observed negative association between *Pfsir2a*  
150 transcription and *Pf*HRP2 levels appears to be independent of the measured cytokine levels  
151 (**Fig.S4**) and is hence likely the result of parasite intrinsic regulation of replication.  
152

153 **Plasma phospholipids link variation in the host inflammatory profile to *ap2-g* and *Pfsir2a*  
154 transcription**

155 We have previously demonstrated *in vitro* that the serum phospholipid LPC serves as a substrate  
156 for parasite membrane biosynthesis during asexual replication, and as an environmental factor  
157 sensed by the parasite that triggers stage conversion<sup>22</sup>. Plasma LPC is mainly derived from the  
158 turnover of phosphatidylcholine (PC) via phospholipase A2, while in the presence of Acyl-CoA  
159 the enzyme LPC acyltransferase (LPCAT) can drive the reaction in the other direction<sup>40</sup>. LPC is  
160 an inflammatory mediator that boosts type 1 immune response to eliminate pathogens<sup>41,42</sup>. LPC  
161 turnover to PC can be triggered by inflammatory responses aimed to repair and restore tissue  
162 homeostasis rather than eliminate infection<sup>40</sup>. Here we performed an unbiased lipidomics  
163 analysis of plasma from a representative subset of the outpatient and admission patients (**Fig.1B-C, E, S5**)  
164 to explore whether the host inflammatory profile modifies the plasma lipid profile and  
165 consequently *ap2-g* and *Pfsir2a* transcription levels *in vivo*.

166 We examined associations between the host inflammatory factors (F1-F5) and the plasma  
167 lipidome data. Again, these associations were corrected for transmission period, patient age and  
168 *Pf*HRP2 levels. 24 lipid species dominated by phospholipids, showed significant association with

169 the inflammatory factors at a false discovery rate below 0.05 (**Fig.4A**). Similar to the observed  
170 associations with *ap2-g* and *Pfsir2a* transcription, cytokines in the F4 and F5 factors showed  
171 reciprocal associations with various LPC species and phosphatidylcholine/ethanolamine  
172 (PC/PE)(**Fig.4A**): F4 showed negative associations with LPC and positive associations with  
173 PC/PE, respectively, and *vice versa* for F5 (**Fig.4A**). The positive association of LPC with the F5  
174 inflammatory factor is consistent with previous findings that identified LPC as an  
175 immunomodulator that can enhance IFN- $\gamma$  production and the activation of the NLRP3  
176 inflammasome, which results in increased levels of cytokines such as IL-1 $\beta$ , IL-18, and IL-33<sup>40-</sup>  
177 <sup>45</sup> and is necessary for eliminating parasites. Depletion of LPC is also associated with elevated  
178 markers of tissue injury (F4), perhaps following uncontrolled parasite growth or maladaptive  
179 inflammation. In summary, the association of inflammatory factors with lipids identified LPC,  
180 PC and PE species as the most significant ones (**Fig.4A**), in line with their known  
181 immunomodulatory role. Importantly, we observed the same pattern in a controlled human  
182 infection model where parasite densities were allowed to rise to microscopic levels, both after  
183 sporozoite and blood-stage infection (**Fig.4B and S6**)<sup>46,47</sup>. Next, we examined the main lipid  
184 species associated with the 5 inflammatory factors with respect to *ap2-g* and *Pfsir2a*  
185 transcription. Indeed, LPC species showed a negative association with both *ap2-g* and *Pfsir2a*  
186 transcription levels (**Fig.4C-E**). The association was only significant in our data when  
187 inflammation is highest (and LPC level lowest), which is at low transmission (i.e., post decline).  
188 These data provide *in vivo* evidence for the previously observed link between LPC depletion and  
189 *ap2-g* activation and strongly suggest that LPC is both, a key immune modulator and a  
190 metabolite whose level is sensed by the parasite. Importantly, the key relationships described in  
191 figures 2-4 were independently significant in a structural equation model that examined how host

192 immunity modifies the host-parasite interaction, the within-host environment and parasite  
193 investment in transmission or replication (**Table S3**).

194

195 **Discussion**

196 Malaria parasites must adapt to changing environmental conditions across the life cycle in the  
197 mammalian and mosquito hosts. Similarly, changing conditions across seasons and transmission  
198 settings require both within- and between-host adaptation to optimize survival in the human host  
199 *versus* transmission to the next host. First, a recent transcriptomic study from Kenya and Sudan  
200 suggested that parasites in low transmission settings (where within-host competition is low)  
201 invest more in gametocyte production compared to high transmission settings (where within-host  
202 competition is high)<sup>16</sup>. Second, a longitudinal study from Senegal demonstrated that human-to-  
203 mosquito transmission efficiency (and gametocyte density) increases when parasite prevalence in  
204 the human population decreases, suggesting that parasites can adapt to changes in the  
205 environment<sup>48</sup>. However, the within-host mechanisms driving parasite adaptation to the  
206 prevailing environment remain unclear.

207 Here, we analysed parasite and host signatures in the plasma from a large malaria patient  
208 cohort over 18 years of declining malaria transmission in Kenya. This investigation allowed us to  
209 define some of the within-host environmental factors that change with transmission intensity and  
210 consequently influence the parasite decision to invest in reproduction *versus* replication. A major  
211 strength of our study is that observations are from a single site and are thus plausibly reflective  
212 of transmission-related changes in parasite investments, rather than differences between  
213 geographically distinct parasite populations. We show that high transmission is associated with a  
214 host immune response that promotes parasite killing without compromising the intrinsic

215 replicative ability of the individual parasite. In contrast, low transmission is associated with a  
216 host immune response that increases within-host stressors (fever, nutrient depletion), which  
217 trigger higher parasite investment into transmission (see also model in **Fig.5**). Importantly, the  
218 observed associations between the parasite parameters *ap2-g*, *Pfsir2a* and host inflammation  
219 remain significant if corrected for transmission, but they are strongest at low transmission (i.e.,  
220 post decline period) when inflammation and the risk of damaging the host are highest.

221 At a systemic level, inflammation can influence the within-host environment and  
222 modulate parasite investment in replication *versus* reproduction by altering the levels of  
223 environmental stressors (e.g., oxidative, thermal, or nutritional stress). Consistent with this  
224 hypothesis, we show that a pro-inflammatory response mediated by IFN- $\gamma$ /IL-18 (F5 in our  
225 analysis) promoting pathogen killing<sup>39,49,50</sup> is negatively associated with *ap2-g* and *Pfsir2a*  
226 transcription. In contrast, inflammatory markers that increase within-host environmental stress  
227 (e.g., fever) or reflect the extent of host tissue injury and are secreted to heal and restore  
228 homeostasis rather than kill pathogens (F4) are positively associated with *ap2-g* and *Pfsir2a*  
229 transcription. At a metabolic level, we previously demonstrated that LPC depletion induces *ap2-*  
230 *g* transcription and therefore gametocyte production *in vitro*<sup>22</sup>. A recent study has provided first  
231 indications of a possible association between LPC and *ap2-g* levels in a small malaria patient  
232 cohort<sup>51</sup>. Here, we reveal that LPC levels are negatively associated with *ap2-g* transcription in  
233 patient plasma, thus providing direct evidence for our *in vitro* findings<sup>22</sup> across a large malaria  
234 patient cohort. LPC is an immune effector molecule promoting macrophage polarization to M1  
235 phenotype that induces the secretion of various cytokines such as IFN- $\gamma$  and IL-1 family (i.e., IL-  
236 18) through activation of the NLRP3 inflammasome in endothelial cells and peripheral blood  
237 mononuclear cells (PBMCs)<sup>40-45</sup>. Furthermore, LPC is the main component of the oxidized form

238 of LDL (oxLDL) that induces inflammasome-mediated trained immunity in human  
239 monocytes<sup>44,45</sup>, resulting in increased responsiveness to LPS re-stimulation. Indeed, we  
240 demonstrate that LPC levels are positively associated with IFN- $\gamma$ /IL-18 levels (Factor 5). These  
241 observations are in line with recent data from experimentally infected macaques and malaria  
242 patients, where decreased LPC levels were associated with acute *versus* chronic malaria<sup>52</sup>. LPC  
243 is also a nutritional resource required by the parasite for replication<sup>22</sup> and hence scarcity is  
244 expected to promote reproduction, as gametocytes require less nutritional resource and therefore  
245 provide a better adaptation strategy.

246 Surprisingly, we also identified a link between *Pfsir2a* transcription, host inflammatory  
247 response and parasite biomass (*Pf*HRP2). *Pf*Sir2a belongs to the evolutionarily conserved family  
248 of sirtuins that act as environmental sensors to regulate various cellular processes<sup>24,25,53</sup>. In *P.*  
249 *falciparum*, *Pf*Sir2a and *Pf*Sir2b paralogues cooperate to regulate virulence gene transcription  
250 including *var* genes<sup>28,54</sup>. *In vitro* data have also demonstrated that increased *Pf*Sir2a levels are  
251 associated with reduced parasite replication (i.e., lower merozoite numbers)<sup>55</sup>. We hypothesise  
252 that the observed upregulation of *Pfsir2a* transcription in response to inflammation is part of an  
253 orchestrated stress response linking replication and antigenic variation (via *Pfsir2a*) to  
254 reproduction and transmission (via *ap2-g*), perhaps through a shared epigenetic control  
255 mechanism<sup>20</sup>. It is well known that host tolerance to malaria infection reduces with falling  
256 transmission<sup>56,57</sup>, as shown by the declining threshold of parasite biomass (*Pf*HRP2) required for  
257 clinical malaria. This suggests that parasites have more pronounced harmful consequences on the  
258 infected host (i.e., clinical symptoms) in low compared to high transmission settings, perhaps  
259 due to increasing host age<sup>58</sup>. Under this scenario, we propose that parasites experience increased  
260 within-host stress to which they respond through increased *ap2-g* transcription (to increase

261 reproduction, hence transmission) and increased *Pfsir2a* transcription (to affect antigenic  
262 variation and replication, hence the negative association with *PfHRP2*) – as part of a self-  
263 preservation strategy in the face of imminent risk of host death.

264 In summary, we propose a model where the falling host immunity with declining  
265 transmission modifies the predominant host immune response, and consequently, the within-host  
266 environment (e.g., LPC availability, fever), resulting in increased investment in transmission  
267 (i.e., higher *ap2-g* transcription) and limiting replication (i.e., higher *Pfsir2a* transcription). Our  
268 findings provide critical information to accurately model parasite population dynamics. They  
269 suggest that parasite populations in elimination scenarios may increase their transmission  
270 potential. Understanding how malaria parasites adapt to their environment, for example by  
271 increasing investment in transmission stages at low endemicity, is highly relevant for public  
272 health. Not only would this affect the timelines for successful elimination, but it would also form  
273 an important argument for the deployment of gametocytocidal drugs once transmission has been  
274 successfully reduced.

275

## 276 **Materials and Methods**

### 277 *Study design and participants*

278 Ethical approval was granted by the Scientific Ethics Review Unit of the Kenya Medical  
279 Research Institute under the protocol; KEMRI/SERU/3149, and informed consent was obtained  
280 from the parents/guardian of the children. The study was conducted at Kilifi county which is a  
281 malaria-endemic region along the Kenyan coast. Over the last three decades, Kilifi has  
282 experienced changes in the pattern of malaria transmission and clinical presentation spectrum<sup>30-</sup>  
283 <sup>32</sup>. The study included i) children admitted with malaria at Kilifi county hospital (KCH) between

284 1994-2012 and recruited as part of hospital admission surveillance system, ii) children presenting  
285 with mild malaria at out-patient clinic and iii) asymptomatic children which were part of a  
286 longitudinal malaria surveillance cohort which were sampled during annual cross-section bleed  
287 in 2007 and 2010. Clinical data, parasite isolates and plasma samples collected from the children  
288 were used to conduct the study. The selection of sub-samples for quantifying inflammatory  
289 markers and lipids was informed by availability of fever data and resource.

290

291 ***Clinical definitions***

292 Admission to malaria was defined as all hospitalized children with malaria parasitemia. The  
293 severe malaria syndromes: severe malarial anemia (SMA), impaired consciousness (IC) and  
294 respiratory distress (RD) were defined as haemoglobin <5 g/dl, Blantyre coma score (BCS) <5  
295 and deep breathing, respectively. Malaria admissions that did not present with either of the  
296 severe malaria syndromes were defined as moderate malaria. Mild malaria was defined as stable  
297 children presenting at outpatient clinic with peripheral parasitemia, and asymptomatic as those  
298 with positive malaria (Giemsa smear) but without fever or any other sign(s) of illness. The  
299 combination of mild and moderate were referred to as uncomplicated.

300

301 ***Controlled infection cohort***

302 Malaria naïve volunteers were infected by either bites from 5 *P. falciparum* 3D7–infected  
303 mosquitoes (n = 12) or by intravenous injection with approximately 2,800 *P. falciparum* 3D7–  
304 infected erythrocytes (n=12); treatment with piperaquine was provided at a parasite density of  
305 5000/mL or on day 8 following blood-stage exposure, respectively <sup>47</sup>.

306

307 ***Parasite parameters***

308 Thick and thin blood films were stained with Giemsa and examined for *Plasmodium falciparum*  
309 parasites according to standard methods. Data was presented as the number of infected RBCs per  
310 500, 200 or 100 RBC counted. This data was then used to calculate parasitemia per  $\mu\text{l}$  of blood  
311 using the formula described in “[2096-OMS-GMP-SOP-09-20160222\\_v2.indd \(who.int\)](#)”.  
312 Briefly, parasites/ $\mu\text{l}$ = number of parasitized RBCs x number of RBCs per  $\mu\text{l}$  /number of RBCs  
313 counted or number of parasites counted x number of WBCs per  $\mu\text{l}$ /number of WBCs counted.  
314 Where data on actual number of RBCs or number of WBCs per  $\mu\text{l}$  of blood is not available, 5  
315 million RBC and 8000 WBC per  $\mu\text{l}$  of blood was assumed.

316

317 ***Measurement of cytokine levels in the plasma samples***

318 The selection for this subset was primarily informed by availability of fever data but the  
319 transmission period and clinical phenotype were also considered. However, there were more  
320 children with fever data record in the post-decline period than pre-decline and decline periods  
321 which biased the sampling toward post-decline period. The plasma samples were analyzed using  
322 ProcartaPlex Human Cytokine & Chemokine Panel 1A(34plex) [Invitrogen/ThermoFisher  
323 Scientific; catalogue # EPX340-12167-901; Lot:188561049] following the manufacturer’s  
324 instructions. The following 34 cytokines were measured: GM-CSF, IFN- $\alpha$ , IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ ,  
325 IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, IL-  
326 18, IL-21, IL-22, IL-23, IL-27, IL-31, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 $\alpha$  (CCL3), MIP-  
327 1 $\beta$  (CCL4), TNF- $\alpha$ , TNF- $\beta$ , Eotaxin/CCL11, RANTES, GRO-a, and SDF-1a.

328 Briefly, 50 $\mu$ l of magnetic beads mix were added into each plate well and the 96-well plate  
329 securely placed on a hand-held magnetic plate washer for 2 minutes for the beads to settle. The  
330 liquid was then removed by carefully inverting the plate over a waste container while still on the  
331 magnet and lightly blotted on absorbent paper towels. The beads were then washed by adding  
332 150 $\mu$ l of 1 $\times$  wash buffer, left to settle for 2 minutes and the liquid removed as before followed by  
333 blotting. This was followed by adding 25 $\mu$ l of Universal Assay Buffer per well and then 25 $\mu$ l of  
334 plasma samples and standards into appropriate wells or 25 $\mu$ l of Universal Assay Buffer in blank  
335 wells. The plate was covered and shaken on a plate shaker at 500rpm for 30 minutes at room  
336 temperature followed by an overnight incubation at 4°C. After the overnight incubation, the plate  
337 was shaken on a plate shaker at 500rpm for 30 minutes at room temperature and the beads then  
338 washed twice while on a magnetic plate holder as outlined above. The beads were then incubated  
339 in the dark with 25 $\mu$ l of detection antibody mixture on a plate shaker at 500rpm for 30 minutes at  
340 room temperature followed by two washes as before. A 50 $\mu$ l of Streptavidin-Phycoerythrin  
341 (SAPE) solution was then added per well and similarly incubated for 30 minutes on a plate  
342 shaker at 500rpm and at room temperature followed by two washes. After the final wash, the  
343 beads were resuspended in 120 $\mu$ l of Reading Buffer per well, incubated for 5 minutes on a plate  
344 shaker at 500rpm before running on a MAGPIX reader running on MAGPIX xPOTENT 4.2  
345 software (Luminex Corporation). The instrument was set to count 100 beads for each analyte.  
346 The analyte concentrations were calculated (via Milliplex Analyst v5.1 [VigeneTech]) from the  
347 median fluorescence intensity (MdFI) expressed in pg/mL using the standard curves of each  
348 cytokine.  
349

350 ***Pf*HRP2 ELISA**

351 *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2) was quantified in the malaria acute  
352 plasma samples using ELISA as outlined. Nunc MaxiSorp™ flat-bottom 96-well plates  
353 (ThermoFisher Scientific) were coated with 100 $\mu$ l/well of the primary/capture antibody [Mouse  
354 anti-*Pf*HRP2 monoclonal antibody (MPFM-55A; MyBioscience)] in 1 $\times$ phosphate buffered  
355 saline (PBS) at a titrated final concentration of 0.9 $\mu$ g/ml (stock = 8.53mg/ml; dilution =  
356 1:10,000) and incubated overnight at 4°C. On the following day, the plates were washed thrice  
357 with 1 $\times$ PBS/0.05% Tween-20 (Sigma-Aldrich) using a BioTek ELx405 Select washer (BioTek  
358 Instruments, USA) and blotted on absorbent paper to remove residual buffer. These plates were  
359 then blocked with 200 $\mu$ l/well of 1 $\times$ PBS/3% Marvel skimmed milk (Premier Foods; Thame,  
360 Oxford) and incubated for 2 hours at room temperature (RT) on a shaker at 500rpm. The plates  
361 were then washed thrice as before. After the final wash, plasma samples and standards were then  
362 added at 100ul/well and in duplicates. The samples and standards (*Pf*HRP2 Recombinant  
363 protein; MBS232321, MyBioscience) had been appropriately diluted in 1 $\times$ PBS/2% bovine serum  
364 albumin (BSA). The samples and standards were incubated for 2 hours at RT on a shaker at  
365 500rpm followed by three washes with 1 $\times$ PBS/0.05% Tween-20 and blotted dry as before. This  
366 was followed by addition of a 100 $\mu$ l/well of the secondary/detection antibody [Mouse anti-  
367 *Pf*HRP2 HRP-conjugated antibody (MPFG-55P; MyBioscience) diluted in 1 $\times$ PBS/2% BSA and  
368 at a final titrated concentration of 0.2 $\mu$ g/ml (stock = 1mg/ml; dilution = 1:5,000). The plates  
369 were then incubated for 1 hour at RT on a shaker at 500rpm, washed thrice as before and dried  
370 on absorbent paper towels. o-Phenylenediamine dihydrochloride (OPD) (ThermoFisher  
371 Scientific) substrate was then added at 100 $\mu$ l/well and incubated for 15 minutes for colour

372 development. The reaction was stopped with 50µl/well of 2M sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) and optical  
373 densities (OD) read at 490nm with a BioTek Synergy4 reader (BioTek Instruments, USA).

374

375 ***Parasite transcript quantification using quantitative RT-PCR***

376 RNA was obtained from TRIzol™ reagent (Invitrogen, catalog number 15596026) preserved *P.*

377 *falciparum* positive venous blood samples obtained from the children recruited in the study.

378 RNA was extracted by Chloroform method<sup>59</sup> and cDNA synthesized using Superscript III kit

379 (Invitrogen, catalog number 18091050) following the manufacturer's protocol. Parasite gene

380 transcription analysis was carried out through quantitative real-time PCR as described below.

381 Real-time PCR data was obtained as described<sup>34,60,61</sup>. Four primer pairs targeting DC8  
382 (named dc8-1, dc8-2, dc8-3, dc8-4), one primer pair targeting DC13 (dc13) and two primer pairs  
383 targeting the majority of group A *var* genes (gpA1 and gpA2) were used in real-time PCR  
384 analysis as described<sup>34</sup>. We also used two primer pairs, b1 and c2, targeting group B and C *var*  
385 genes respectively<sup>62</sup>. Primer pairs targeting *Pfsir2a* and *ap2-g* were also used<sup>34</sup>. Two  
386 housekeeping genes, Seryl tRNA synthetase and Fructose bisphosphate aldolase<sup>35,63,64</sup> were used  
387 for relative quantification of the expressed *var* genes, *Pfsir2a* and *ap2-g*. The PCR reaction and  
388 cycling conditions were carried out as described<sup>64</sup> using the Applied Biosystems 7500 Real-time  
389 PCR system. We set the cycle threshold (Ct) at 0.025. Controls with no template were included  
390 at the end of each batch of 22 samples per primer pairs and the melt-curves analysed for non-  
391 specific amplification. The *var* gene "transcript quantity" was determined relative to the mean  
392 transcript of the two housekeeping genes, Sery tRNA synthetase and Fructose biphosphate  
393 aldolase as decribed<sup>64</sup>. For each test primer, the  $\Delta$ Ct was calculated relative to the average Ct of  
394 the two housekeeping genes which was then transformed to arbitrary transcript unit (Tu<sub>s</sub>) using

395 the formula ( $T_{Us} = 2^{(5-\Delta Ct)}$ ) as described<sup>64</sup>. We assigned a zero  $T_{Us}$  value if a reaction did not  
396 result in detectable amplification after 40 cycles of amplification, i.e., if the Ct value was  
397 undetermined.

398

399 ***Lipidomics analysis***

400 Serum samples were preserved at -80°C until extraction with the chloroform/methanol method.  
401 25µL of serum were extracted with 1 mL of the extraction solvent chloroform/methanol/water  
402 (1:3:1 ratio), the tubes rocked for 10 min at 4°C and centrifuged for 3 min at 13'000g.  
403 Supernatant were collected and stored at -80°C in glass tubes until analysis.

404 Sample vials were placed in the autosampler tray in random order and kept at 5°C.  
405 Separation was performed using a Dionex UltiMate 3000 RSLC system (Thermo Scientific,  
406 Hemel Hempstead) by injection of 10 µl sample onto a silica gel column (150 mm × 3 mm × 3  
407 µm; HiChrom, Reading, UK) used in hydrophilic interaction chromatography (HILIC) mode  
408 held at 30°C<sup>65</sup>. Two solvents were used: solvent A [20% isopropyl alcohol (IPA) in acetonitrile]  
409 and solvent B [20% IPA in ammonium formate (20 mM)]. Elution was achieved using the  
410 following gradient at 0.3 ml/min: 0–1 min 8% B, 5 min 9% B, 10 min 20% B, 16 min 25% B, 23  
411 min 35% B, and 26–40 min 8% B. Detection of lipids was performed in a Thermo Orbitrap  
412 Fusion mass spectrometer (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) in polarity  
413 switching mode. The instrument was calibrated according to the manufacturer's specifications to  
414 give an rms mass error <1 ppm. The following electrospray ionization settings were used: source  
415 voltage, ±4.30 kV; capillary temp, 325°C; sheath gas flow, 40 arbitrary units (AU); auxiliary gas

416 flow, 5 AU; sweep gas flow, 1 AU. All LC-MS spectra were recorded in the range 100–1,200 at  
417 120,000 resolutions (FWHM at m/z 500).

418

419 ***Data preprocessing***

420 The raw data was converted to mzML files using proteowizard (v 3.0.9706 (2016-5-12)). These  
421 files were then analysed using R (v 4.2.1 ) libraries xcms (v 3.14.1) and mzmatch 2 (v 1.0 - 4) for  
422 peak picking, alignment, filtering and annotations<sup>66-68</sup>. Batch correction was applied as in  
423 (<https://www.mdpi.com/2218-1989/10/6/241/htm>), the data was then checked using PCA  
424 calculated using the R function prcomp (see supplementary figure 4B). Data was then range  
425 normalised and logged transformed using MetaboanalystR (v3.1.0). The CHMI lipidomics data  
426 was analysed the same way but did not require batch correction as the samples were run in one  
427 batch.

428

429 ***Statistical analysis***

430 All data were analyzed using R (v4.2.1). We normalized non-normally distributed variables by  
431 log transformation.

432 *qRT-PCR*: Zeros in qRT-PCR values were replaced by 0.001 (value before log transformation as  
433 the smallest measured value is about 0.0017). The median transcript units from qRT-PCR were  
434 calculated as follows: DC8 median from four primer pairs used (DC8-1, DC8-2, DC8-3 and  
435 DC8-4) and group A median from three primer pairs (gpA1, gpA2 and dc13). Samples for which  
436 *ap2-g* or *pfsir2a* arbitrary transcript unit was greater or equal to 32 (that is the transcript quantity  
437 of the reference genes based on the formula ( $Tu_s = 2^{(5-\Delta ct)}^{64}$  ) were deemed unreliable and  
438 excluded from the analysis that went into generating figures 2-4. Comparison between two

439 groups was done using two-sided wilcoxon test. All correlations were conducted using  
440 Spearman's rank correlation coefficient test. All forest plots were done using linear regressions  
441 adjusted for transmission period, *Pf*HRP2 and age of the patient (see figure legends) using R  
442 function lm. All multiple test corrections were done using Benjamini & Hochberg multiple test  
443 (using R function p.adjust).

444 *Principal factor analysis:* A measurement model (i.e., factor analytic model) was fitted to  
445 summarise the 34 analytes into fewer variables called factors. An exploratory factor analysis  
446 (EFA) was performed to explore the factor structure underlying the 34 analytes. Factors were  
447 retained based on the Kaiser's 'eigenvalue rule' of retaining eigenvalues larger than 1. In  
448 addition, we also considered the scree plot, parallel analysis, fit statistics and interpretability of  
449 the model/factors. This analysis resulted in the cytokine data being reduced to 5 factors. This  
450 analysis was done using the R "psych" library (v 2.1.9) available at  
451 <https://CRAN.R-project.org/package=psych>. The 34 analytes were individually linearly  
452 regressed to *ap2-g* or *Pfsir2* transcript levels with transmission, *Pf*HRP2 and age correction  
453 (model: analyte ~ transmission+*Pf*HRP2+age). Then each factor was analyzed the same way.  
454 *Lipidomics analysis:* The preprocessed lipidomics data was tested using transmission period,  
455 *Pf*HRP2 and age adjusted linear regression with any of the 5 factors. All m/z with a significant  
456 false discovery rate with any of the factors were then manually checked for peak quality and  
457 identified masses on mass and retention time<sup>69</sup>. The remaining identified lipids were then  
458 checked for relationship with *ap2-g* and *Pfsir2a* transcription levels (linear regression adjusted  
459 for transmission period, HRP and age, see methods above). The CHMI lipidomics data was  
460 analyzed the same way but the peaks retained were those significantly different pre and post  
461 treatment in either type of infection (student's t-tests corrected for multiple testing).

462 *Figures*: All heatmaps were done using the R library pheatmap (v 1.0.12) available at  
463 <https://CRAN.R-project.org/package=pheatmap>, and all other plots using the R libraries ggplot2  
464 (v 3.3.5) and ggpubr (v 0.4.0) available at <https://CRAN.R-project.org/package=ggpubr>.

465

## 466 **Data availability**

467 Raw data and script for all the analyses in this manuscript are available at  
468 <https://doi.org/10.7910/DVN/BXXVRY>. Raw mass spectrometry files for all lipidomics data sets  
469 are currently being submitted to MetaboLights.

470

## 471 **Acknowledgements**

472 This work was supported by Wolfson Merit Royal Society Award (to M.M.), Wellcome Trust  
473 Investigator Award 110166 (to F.A., L.S., J.L.S.F. and M.M.) and Wellcome Trust Center  
474 Award 104111 (to F.F.A., L.S., J.L.S.F. and M.M.), Glasgow-Radboud collaborative grant (to  
475 M.A., T.B. and M.M.), European Research Council (ERC) Consolidator Grant (to T.B., ERC-  
476 CoG 864180 QUANTUM), Wellcome Award 209289/Z/17/Z (to A.A.) and a core Wellcome  
477 award to KEMRI-Wellcome Trust (203077/Z/16/Z). This paper was published with permission  
478 of the director of Kenya Medical Research Institute.

479

## 480 **Competing interests**

481 The authors declare that they have no financial or non-financial competing interests.

482

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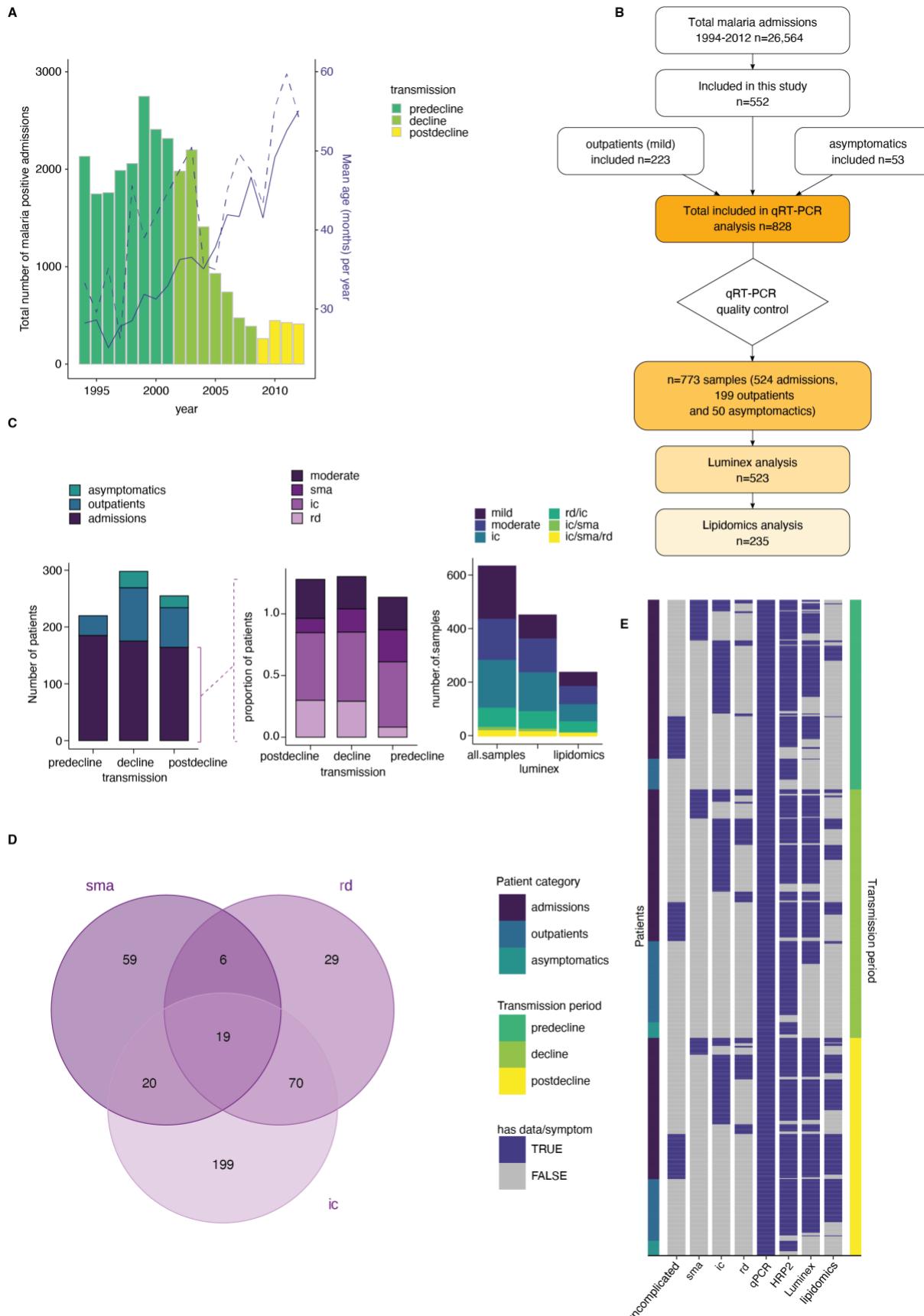
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655 **Figures and legends**



657 **Figure 1. A clinical malaria patient cohort during changing transmission in Kilifi, Kenya. A.**

658 Total malaria admissions and patient age of the parent cohort. Number of patients per year (grey

659 histogram, left axis). The solid blue line is the average patient age in the parent cohort, the

660 dashed line is the average patient age in this study (both right axis). **B.** Schematic of sample

661 selection for this study. **C.** Clinical presentation of patients selected for this study. Left: all

662 patients, middle: admissions only, right: subset selected for luminex and lipidomics analysis.

663 sma=severe malarial anemia, ic=impaired consciousness, rd=respiratory distress. **D.** Number of

664 patients in this study with different clinical presentations (402 severe cases initially selected). **E.**

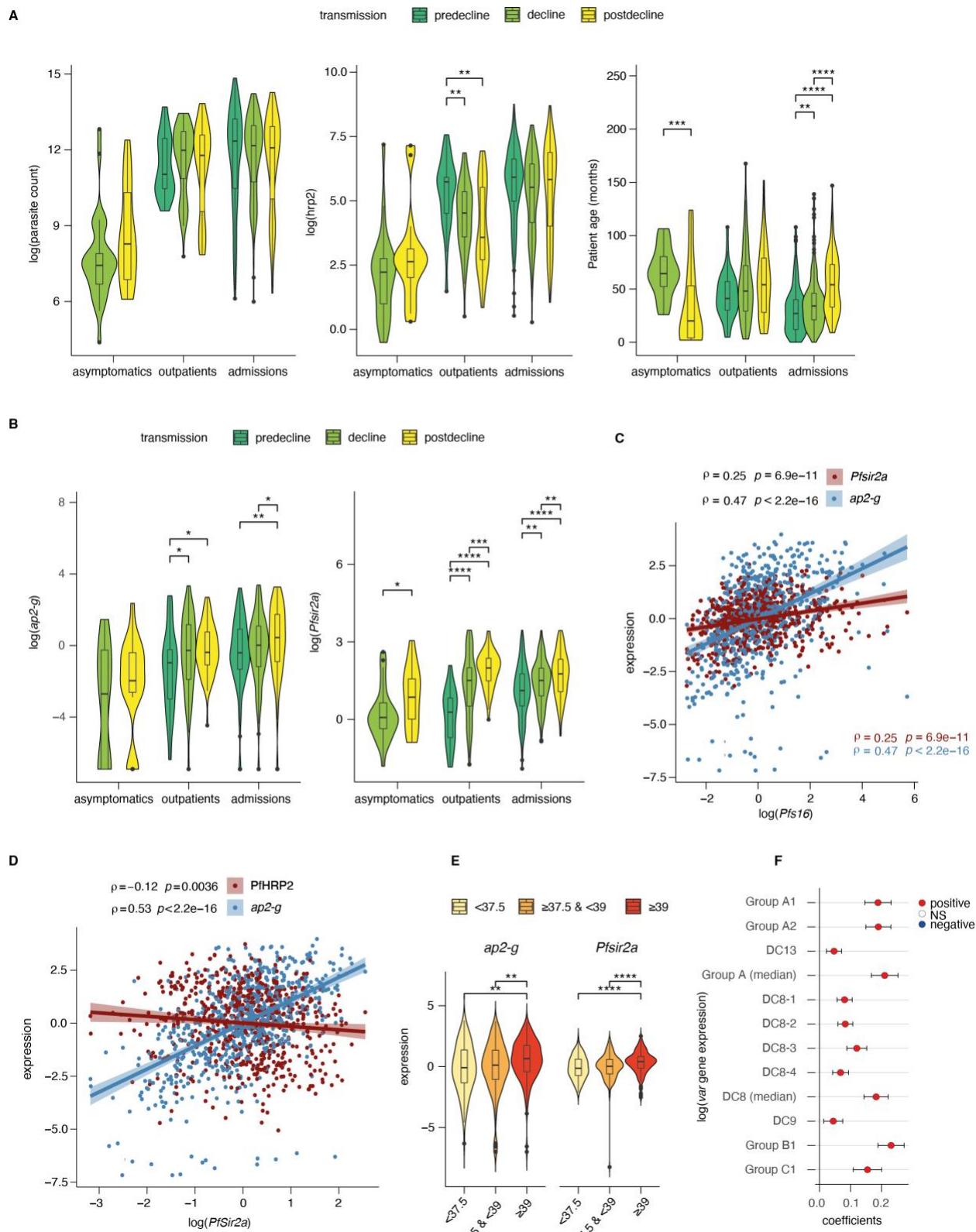
665 Overview of the data available for each patient of the study, after excluding samples with *Pfsir2a*

666 and *ap2-g* transcript transcription units greater or equal 32 as described in the methods. Each row

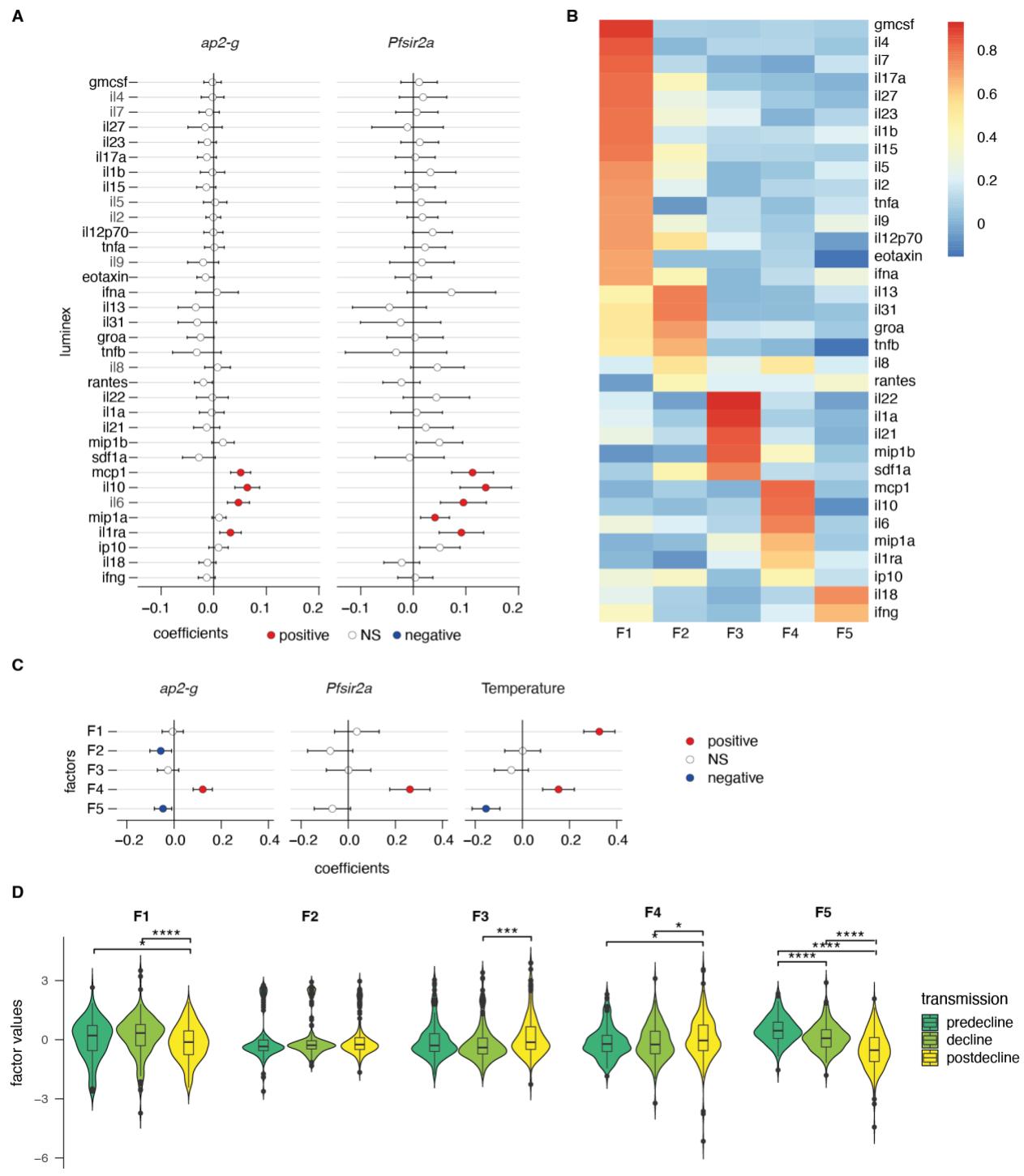
667 is one patient, organised by patient category (left axis) and transmission period (right axis).

668

669



671 **Figure 2. Dynamics of parasite parameters across transmission periods. A.** Peripheral  
672 parasitemia (smear, left), total parasite biomass (*Pf*HRP2, middle) and patient age (right) across  
673 patients. **B.** *ap2-g* transcript levels (left) and *Pfsir2a* levels (right) across patients. **C.** Spearman's  
674 correlation between *Pfs16* and *ap2-g* (blue) or *PfSir2a* transcription (red) across patients  
675 (corrected for transmission). The lines fitted are linear regressions for visualisation only. **D.**  
676 Spearman's correlation between *Pfsir2a* and *ap2-g* transcription (blue) or *Pf*HRP2 levels (red)  
677 across patients (corrected for transmission). The lines fitted are linear regressions for  
678 visualisation only. **E.** *ap2-g* and *Pfsir2a* transcription (corrected for transmission) stratified by  
679 patient temperature. **F.** Linear regression of *var* gene transcription levels with *Pfsir2a* levels  
680 (adjusted for transmission). 95% confidence intervals are shown. The color indicates whether the  
681 relationship is statistically significant (with Benjamini & Hochberg multiple tests correction).  
682 Positive correlations in red, negative in blue. In above figures, asymptomatics were excluded in  
683 analyses involving transmission period since they are not represented in the pre-decline period.  
684 All pairwise statistical tests indicated in the graphs are wilcoxon tests corrected for multiple  
685 testing (Benjamini & Hochberg, \* $=$ FDR $<0.05$ , \*\* $=$  $<0.01$ , \*\*\* $=$  $<0.001$  and \*\*\*\* $=$  $<0.0001$ ).  
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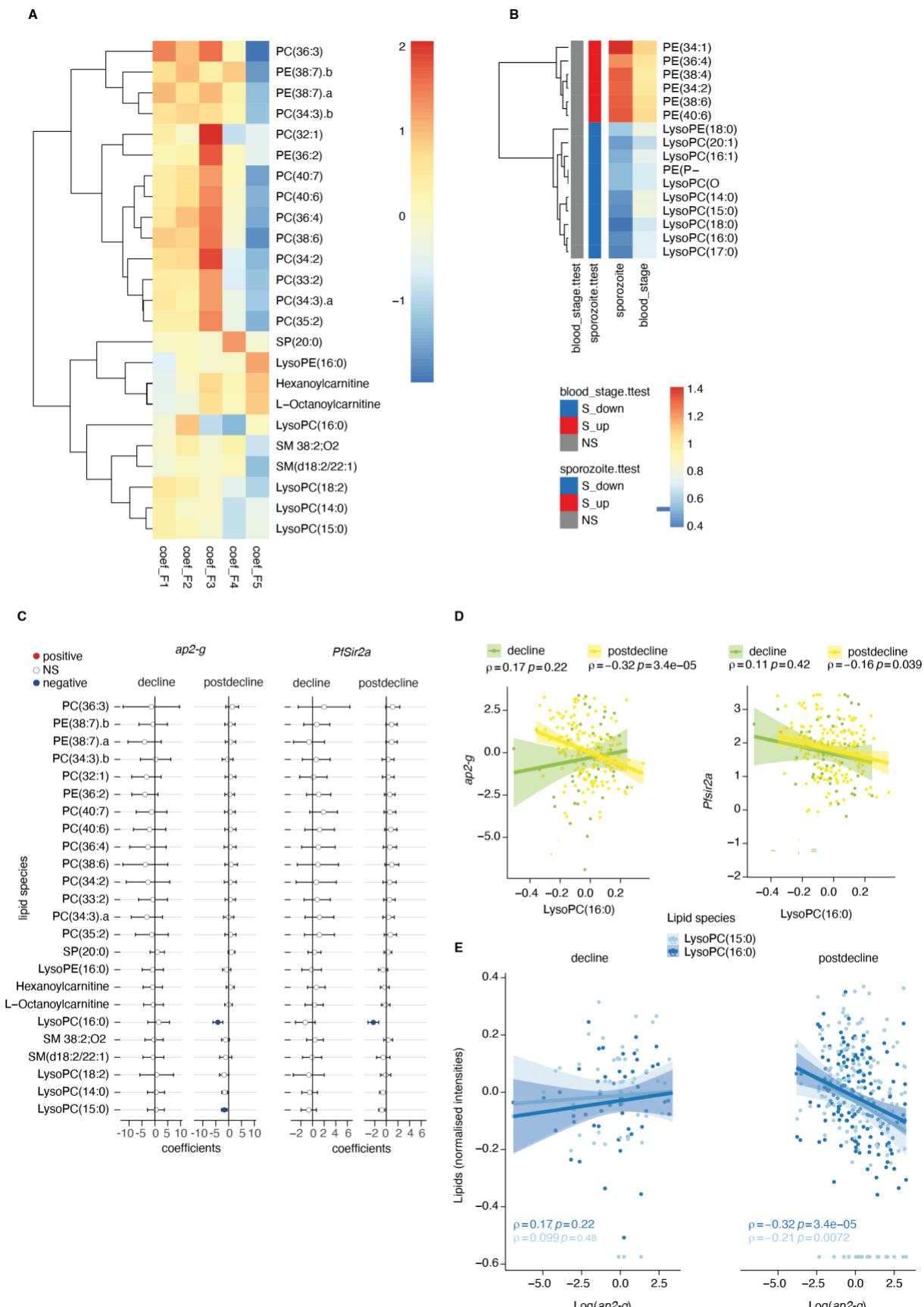


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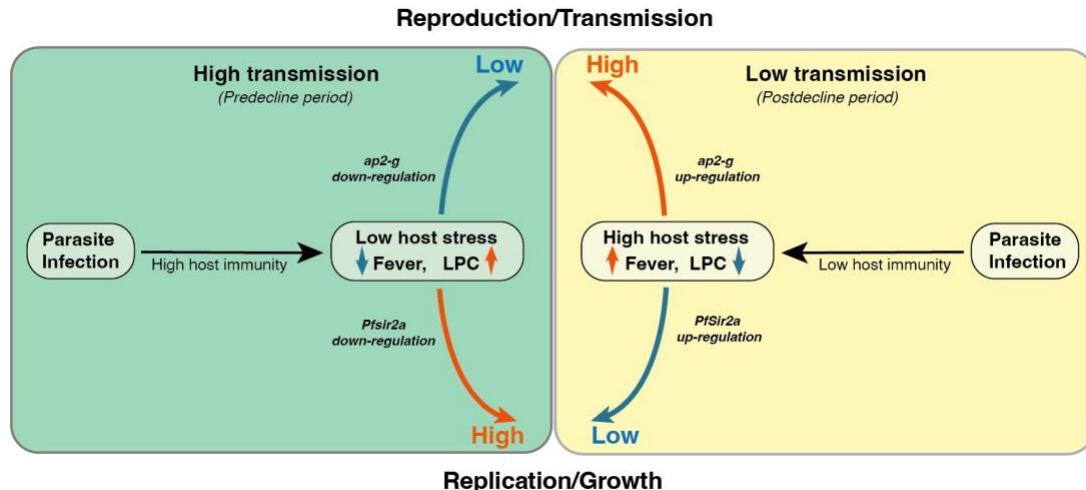
689 **Figure 3. *ap2-g* and *Pfsir2a* transcription levels are associated with the host inflammation**

690 **profile. A.** Association of inflammatory markers with *ap2-g* and *Pfsir2a* transcripts, tested using  
 691 transmission period, age and *Pf*HRP2 adjusted linear regression (*p*-values adjusted for multiple  
 692 testing using Benjamini & Hochberg multiple tests correction). Plotted is the regression

693 coefficient (estimate) and 95%CI. Above and below zero indicate statistically significant positive  
694 (red) and negative association (blue), respectively. **B.** Principal exploratory factor analysis. The  
695 figure shows the inflammatory marker loadings on the five factors (F1-F5) identified to have  
696 eigenvalue above 1. **C.** Linear regression between inflammatory factors (F1-F5) and *ap2-g* and  
697 *Pfsir2a* transcription and patient temperature (adjusted for transmission, *PfHRP2* and age).  
698 Plotted is the coefficient between the factor and the parameter (estimate) and 95%CI. The  
699 association is significant if the correlation FDR < 0.05, in which case the positive associations  
700 are marked in red and the negative ones in blue. **D.** Inflammatory factors stratified by  
701 transmission period. Pairwise tests are wilcoxon tests (Benjamini & Hochberg, \*=FDR<0.05,  
702 \*\*=<0.01, \*\*=<0.001 and \*\*\*\*=<0.0001).  
703  
704



706 **Figure 4. Plasma LPC links host inflammation to *ap2-g* and *Pfsir2a* transcription. A.**  
707 Heatmap of the linear regression coefficients between lipids and inflammatory factors (F1-F5,  
708 adjusted for transmission period and corrected for multiple testing). Shown are all lipids that are  
709 significantly associated (positive or negative) with factors F1-F5, clustered using R hclust  
710 (distance=Euclidean, method=centroid) and that have been manually identified and filtered for  
711 peak quality (isotopes and fragments were also filtered out). **B.** Shown are the lipids with  
712 significant differences (student's t-test corrected for multiple testing) between pre- and post-  
713 treatment in the controlled human malaria infections (CHMI) for either infection type (blood or  
714 sporozoite infection). Plotted is the fold-change post-treatment *vs* pre-treatment. On the left is  
715 indicated whether the lipid is significantly increased (red) or decreased (blue) in either type of  
716 infection. **C.** Linear regression between the lipids from A and *ap2-g* or *Pfsir2a* transcription  
717 levels. Plotted is the coefficient and 95%CI. Blue dots are the statistically significant negative  
718 correlations, red are the statically significant positive correlations (FDR<0.05). **D.** Correlation  
719 between LPC (16:0) (top) and *ap2-g* (top) or *Pfsir2a* (bottom) transcription (Spearman's  
720 correlations corrected for multiple testing). **E.** Correlations between identified LPCs and *ap2-g*  
721 transcription by transmission period (Spearman's correlations corrected for multiple testing).  
722 Note that predecline period is not plotted separately in panels C-E due to insufficient sample  
723 numbers for the statistical analysis.  
724  
725



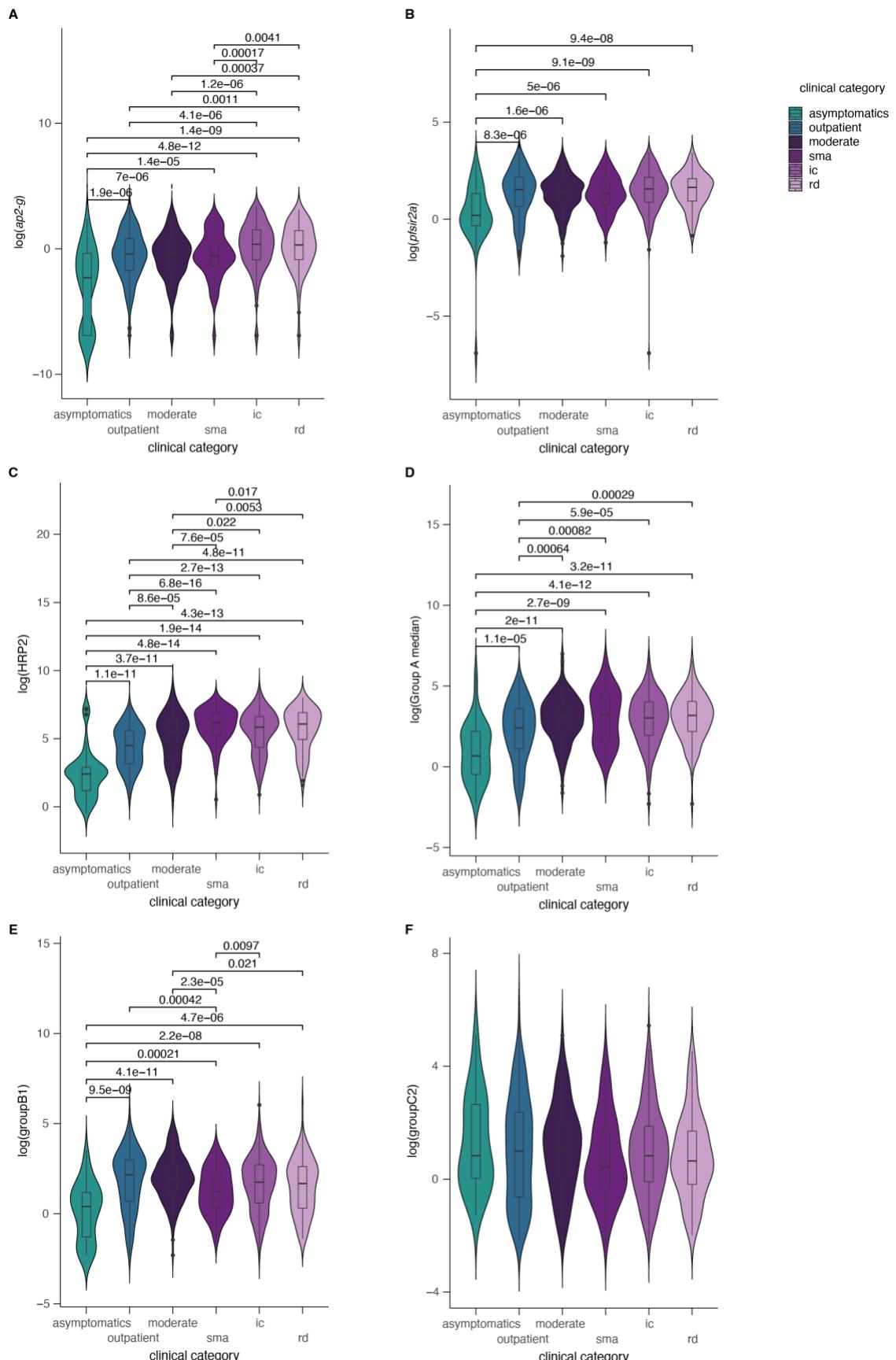
726

727 **Figure 5. Proposed model on within-host adaptation of the parasite to changing**  
728 **environments.** The model is based on the interaction between the different host and parasite  
729 parameters described in this study. It proposes that declining transmission reduces host  
730 immunity, resulting in increased inflammation (including reduced LPC availability, fever) and  
731 susceptibility to clinical symptoms/damage. The altered host response modifies the parasite  
732 response during infection, resulting in increased investment in transmission (as indicated by the  
733 elevated *ap2-g* levels) and reduced replication (as indicated by elevated *Pfsir2a* levels and  
734 reduced parasite burden/*Pf*HRP2 levels).

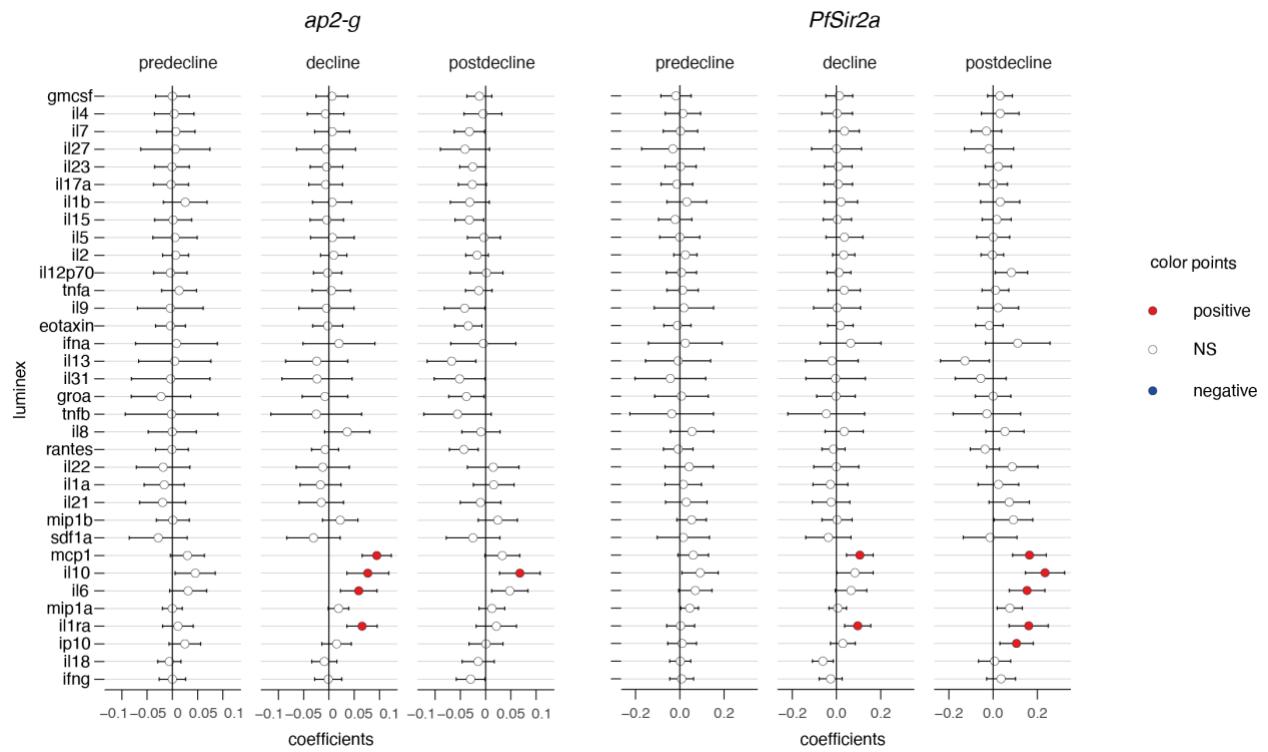
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737 **Supplementary figures and tables**



739 **Figure S1. Parasite parameters stratified by clinical categories.** *ap2-g*, *Pfsir2a*, *var* gene  
740 transcription and *PfHRP2* levels stratified by clinical categories. Significant wilcoxon test *p*-  
741 values (corrected for multiple testing) marked with \* $<0.05$ , \*\* $<0.01$ , \*\*\* $<0.001$ , \*\*\*\* $<0.0001$ .  
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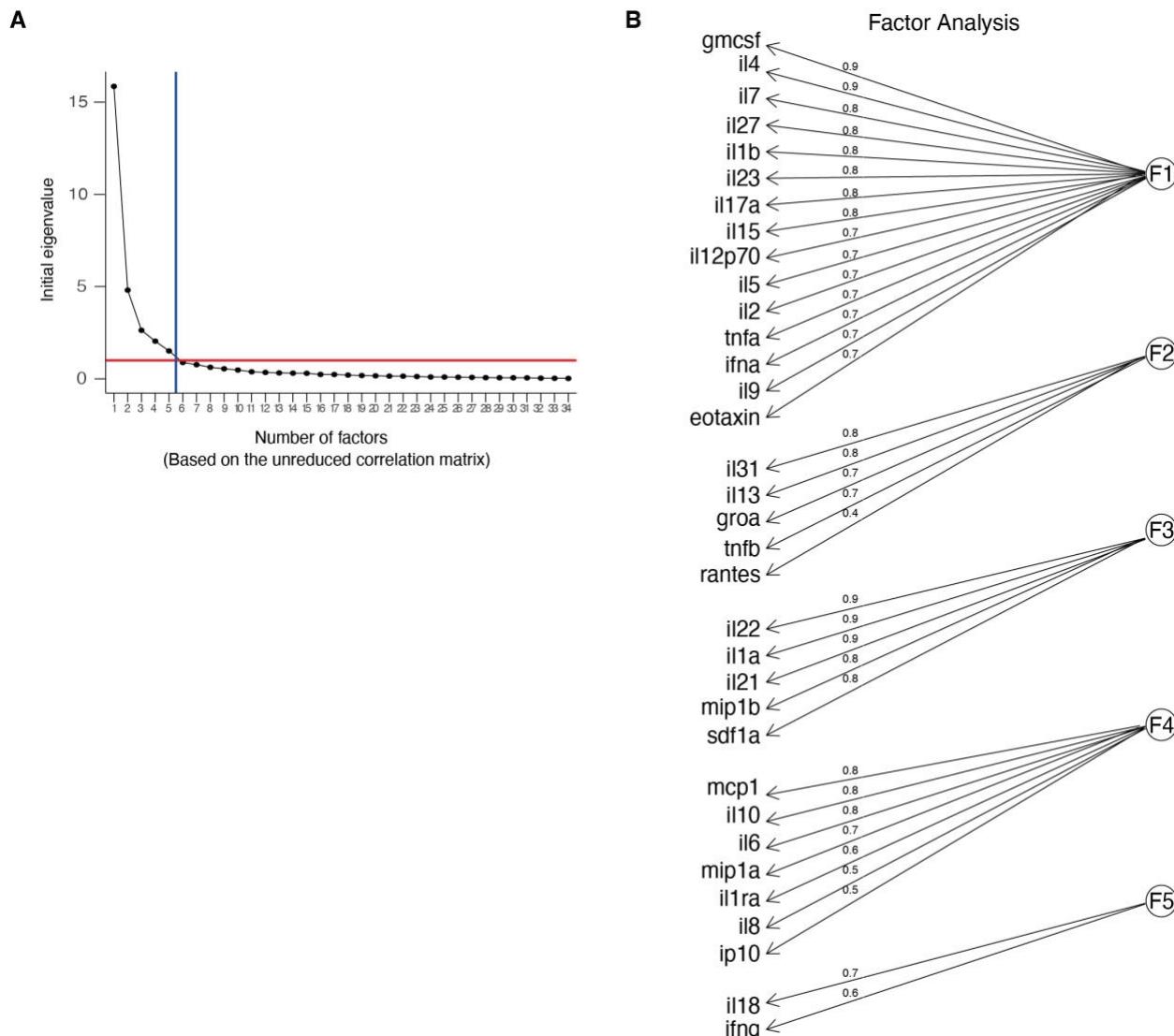
745 **Figure S2. Inflammatory markers stratified by transmission period.** Linear regressions

746 between *ap2-g* (A) or *PfSir2a* (B) transcription and luminex markers as per figure 3A, stratified

747 by transmission period.

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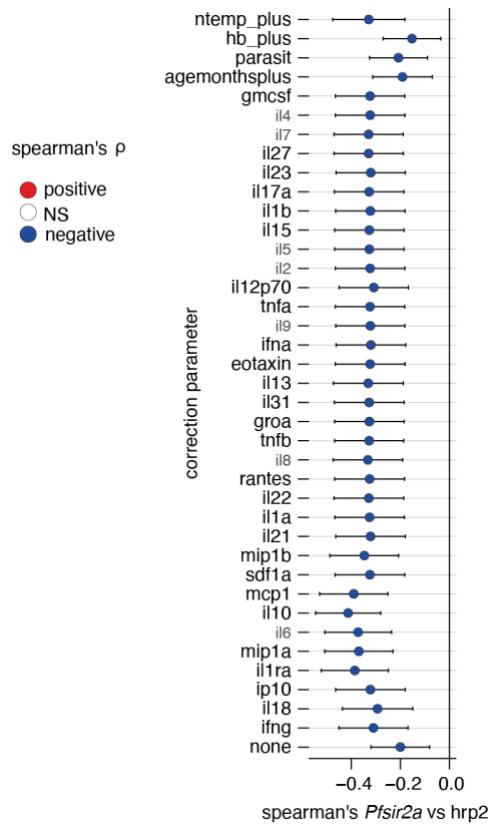


750

751 **Figure S3. Factor loadings. A.** Scree plot showing the eigen values *vs* the number of factors  
752 (factor analysis of the luminex data). **B.** Major loadings of each factor calculated by factor  
753 analysis of the luminex data (values $\geq 0.3$ ). Loading values are noted on the edges.

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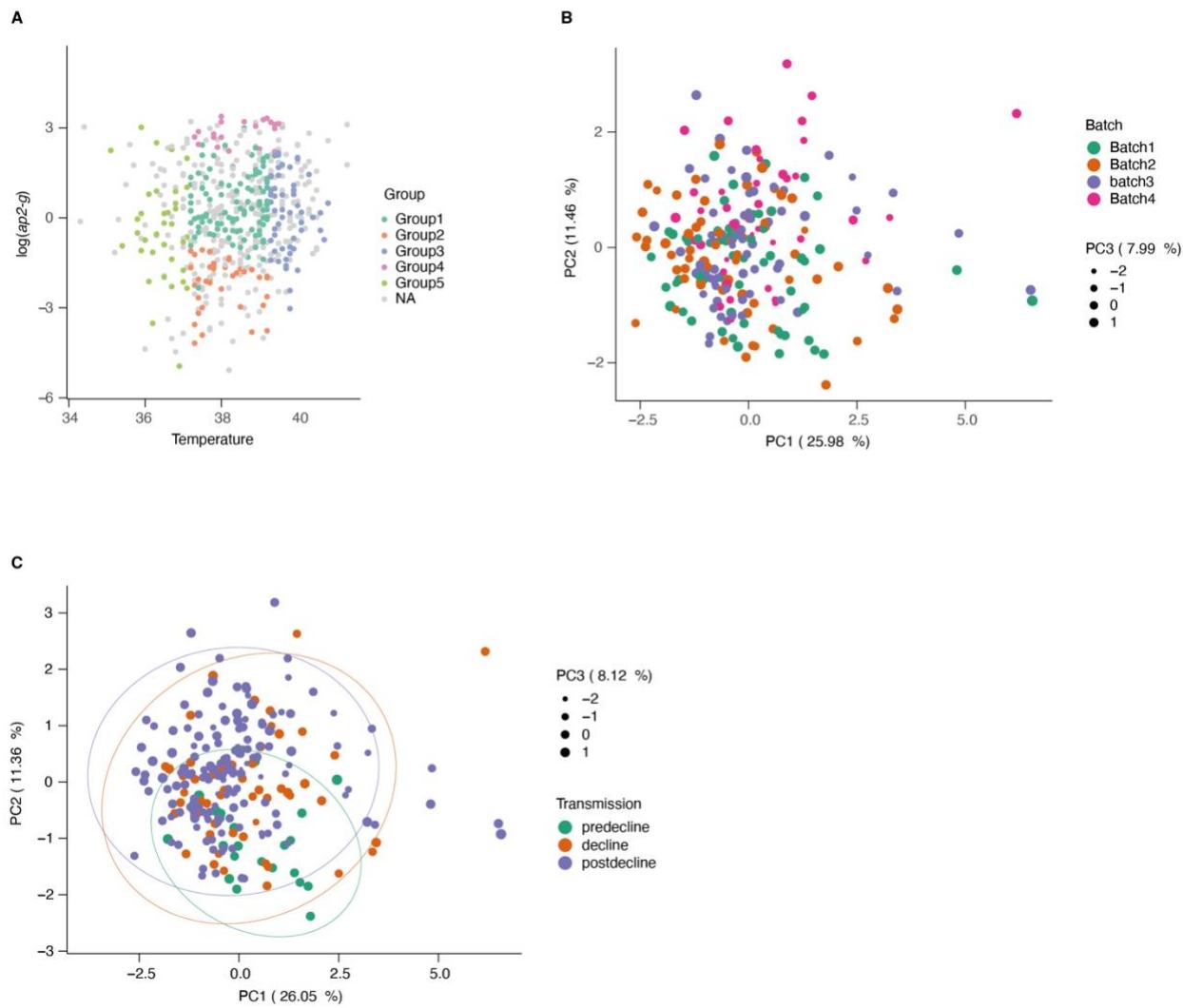


756

757 **Figure S4. Correcting *Pf*HRP2 vs *Pfsir2a* associations for external factors.** Plotted is the  
758 linear regression coefficient (estimate) and 95%CI. Blue indicates a significant negative  
759 correlation between *Pf*HRP2 levels and *Pfsir2a* transcription with the additional correction  
760 indicated on the left.

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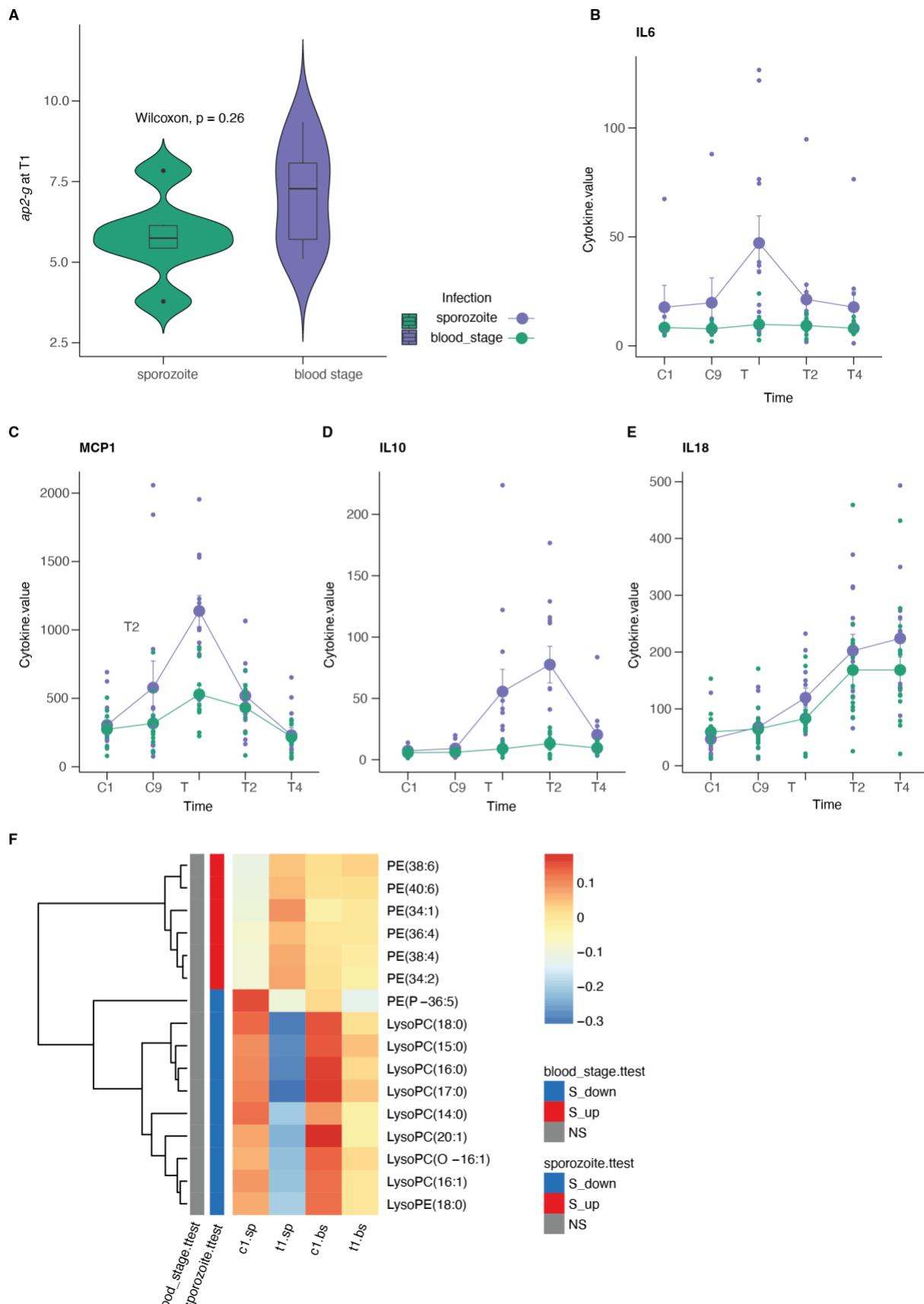


763

764 **Figure S5. Sample subsetting and batch correction for lipidomics data. A.** Patient  
765 temperature,  $ap2-g$  transcription level and disease type were used to subset samples for  
766 metabolomics. This resulted in 5 groups from severe disease categories and matching mild cases  
767 (outpatients, in grey). **B.** PCA of the lipidomics data colored by batch number (post batch  
768 correction). **C.** PCA of the lipidomics data colored by transmission period.

769

770



772 **Figure S6. CHMI data.** **A.** *ap2-g* transcription measured on day 1 of treatment (T1) and  
773 stratified by type of infection. **B-E:** Average (and standard deviation) cytokine levels during the  
774 experiment per infection type. Shown are those markers shared with the luminex analysis of the  
775 Kilifi cohort. **F.** Average normalized lipid levels are significantly different pre and post treatment  
776 (sp=sporozoite infection, bs=blood stage infection). (C=days post infection, T=days post  
777 treatment). On the left is indicated whether the lipid is significantly increased (red) or decreased  
778 (blue) in either type of infection.

779

780 **Table S1.** Associations between parasite parameters *ap2-g*, *Pfsir2-a* and *PfHRP2* and clinical  
781 parameters.

782

783 **Table S2.** Associations between parasite parameters *ap2-g*, *Pfsir2-a* and *PfHRP2*, host luminex  
784 markers and lipidomics data.

785

786 **Table S3.** Structural equation model (SEM). The model assumes that pre-existing host immunity  
787 affects the interaction between host (i.e., altered within host environment including inflammatory  
788 response, fever, nutritional resource availability) and parasite (i.e., altered investment in  
789 reproduction vs replication). Significant *p*-values are highlighted in bold and negative and  
790 positive estimates of associations are highlighted in blue and red, respectively.