1 **Asymptomatic carriage of *Plasmodium falciparum* in children living in a hyperendemic area occurs**

2 **independently of IgG responses but is associated with a balanced inflammatory cytokine ratio.**

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18

19 **Footnote Page**

24 **Abstract**

25 **Background:** Asymptomatic carriage of infected red blood cells (iRBCs) can be prevalent in

26 communities regardless of transmission patterns and can occur with infection of different

27 *Plasmodium* species. Clinical immunity dampens the inflammatory responses leading to disease

28 symptoms in malaria. The aim of this study was to define the immunological correlates of

29 asymptomatic carriage of *P. falciparum* in a highly exposed population.

30 **Methods**: 142 asymptomatic *Plasmodium*-infected individuals greater than 2 years of age without

31 fever (body temperature $<37.5^{\circ}C$) were followed weekly for 10 weeks before being treated with

32 artemisinin-based combination treatment (ACT). Plasma levels of 38 cytokines were measured at

33 baseline by Luminex and the quantity and growth inhibitory activities of circulating parasite-reactive

34 antibodies measured. The *Plasmodium* antigen tested included *P. falciparum* merozoite extract (ME)

35 and schizont extract (SE), and the recombinant proteins Erythrocyte binding antigen 175 (EBA-175)

36 and merozoite surface protein 1 (MSP-1_{19}).

37 **Results:** Median levels of IgG against *P. falciparum* EBA-175 and MSP-1_{19} at baseline were

38 significantly higher in those older than 20 years of age compared with the younger age group and

39 appeared to correlate with better parasite control. Amongst all participants there were no discernible

40 changes in IgG levels over time. Parasite density was higher in the younger age group and associated

41 with IL-10, TNF-α and MCP-1 levels. A balanced IL-10:TNF-α ratio was associated with

42 asymptomatic malaria regardless of age, and balanced ratios of IL-10/TNF-α and IL-10/IFN-γ were

43 the only significant correlate of maintenance of asymptomatic malaria over the course of the study in

44 individuals 20 years of age and younger.

45 **Conclusion**: The above findings indicate that asymptomatic carriage of *P. falciparum* in children

46 living in a hyperendemic area occurs independently of IgG but is associated with a balanced

47 inflammatory cytokine ratio.

48 **Key words**: Asymptomatic malaria, *Plasmodium falciparum*, IgG and cytokine levels, TNF-α, IL-

49 10

50 **Introduction**

51 Malaria is still a significant global health problem with an estimated 249 million cases and 608 000

52 deaths in 2022 [1]. Although mortality rates from malaria have decreased considerably since the turn

53 of the century [2] this has been paralleled by high increases in proportions of asymptomatic infections,

54 known to contribute significantly to persistent malaria transmission in most endemic countries [3,4].

55 Asymptomatic infections can persist for weeks to years [5] and untreated *P. falciparum* infections

56 have been shown persist on average for 6 months, with fluctuations in parasite density over the course

57 of an infection [6,7]. The maintenance of chronic asymptomatic malaria will depend on the host

58 immune response to the blood stage of *Plasmodium* [3]. Anti-parasite immunity directly suppress

59 parasite growth resulting in low infection densities (below the pyrogenic threshold [3,8]) whilst anti-

60 disease immunity modulates inflammation-mediated clinical manifestations of the infection.

61 Older children and adults have more cumulative exposure to parasite variants circulating within the

62 community, developing adaptive responses which mediate both anti-parasite and anti-disease

63 immunity [9,10]. However, asymptomatic infections have been documented in younger children < 5

64 years who are too young to have developed robust adaptive immunity [11]. This suggests that

65 asymptomatic malaria may be differentially controlled by innate and adaptive immune responses

66 depending on age.

67 Anti-parasite immunity which is acquired with age or exposure [12,13], is mediated mainly by

68 antibodies which control parasite replication and mediate clearance of *Plasmodium*-infected red

69 blood cells (iRBCs) [14–16]. Suppression of inflammation associated with iRBCs depends on the

70 balance between anti- and pro-inflammatory cytokine responses [17,18] and it has been hypothesized

71 that asymptomatic infections are associated with a shift in host immune responses towards an anti-

72 inflammatory profile after multiple exposures to malaria [19]. This could occur via a decrease in

73 levels of pro-inflammatory immune mediators such as IFN-γ, TNF-α, or an increase in anti-

74 inflammatory mediators such as IL-10 or TGF-β [18,20–22].

75 The relative contributions of anti-parasite and anti-disease immunity to the maintenance of persistent

76 asymptomatic *Plasmodium* infections in younger individuals and older individuals remain unclear.

77 To determine the immunological associations of chronic asymptomatic malaria in younger (≤20

78 years) and older (>20 years) individuals we have undertaken a longitudinal study of highly exposed

79 individuals of all ages with asymptomatic malaria over a 3-month period where we monitored the

80 stability of IgG and cytokine responses. We also examined these responses upon clearance of

81 parasites to ascertain which responses depended on the presence of circulating parasites. Our study

82 demonstrates a lack of correlation of iRBC density and IgG responses in individuals ≤20 years old,

83 with an association of parasite carriage with IL-10 and an association of asymptomatic status with a

84 balanced ratio of IL-10:TNF-α and IL-10: IFN-γ.

85

86 **Material and methods**

87 **Ethics statement**

88 The study protocol was approved by the Cameroon National Ethics Committee for Human Health

89 Research (Ethical clearance N°: 2018/09/1104/CE/CNERSH/SP) and the Ministry of Public Health.

90 Written informed consent was obtained from each participant or parent/guardian of minor participants

91 (< 19 years old) prior to enrolment into the study. Assent was obtained from all children older than

92 12 years of age.

93 **Study area and population**

94 The study population consisted of individuals of both genders aged at least two years of age and

95 permanently living in one of the five selected villages of the Esse health district, Mefou-et-Afamba

96 Division, Centre Region of Cameroon where malaria transmission is perennial, stable and hyper-

97 endemic with *P. falciparum* as the predominant *Plasmodium* species. The study area has been

98   described elsewhere [23–25]. The inclusion criteria were: i) obtain the consent of each older

99   participant or parent or legal guardian for the children, ii) living permanently in the study area, iii)

100   not pregnant.

Study design and participants

102   This was a longitudinal household-based study that involved 353 individuals. Participants were

103   considered asymptomatic for malaria if positive for *P. falciparum* using multiplex nested PCR in the

104   absence of fever at least forty-eight hours prior to enrolment. Being febrile was assessed based on

105   axillary temperature $\geq 37.5\ °C$ or based on the history of fever as independently confirmed by another

106   household member. All symptomatic individuals (fever + rapid diagnostic test (RDT) +) were treated

107   with ACT-based drugs following recommended national guidelines. Participants with asymptomatic

108   carriage of *Plasmodium* parasites were monitored weekly until they either developed a fever, or until

109   the end of the follow up period at week 16 post-enrolment. Participant follow-up comprised of weekly

110   interviews regarding the occurrence of fever during the past week, measurement of axillary

111   temperatures on each visit, and laboratory testing for malaria at weeks 1, 4, 11 and 16 (PCR and

112   microscopy). All symptomatic cases occurring during the follow-up period were treated with ACT as

113   recommended and excluded from further follow-up. At week 11 post-enrolment all participants with

114   persisting asymptomatic parasitemia were treated as recommended, and monitored for five additional

115   weeks to assess the effect of ACT-based antimalarial treatment on selected host immune responses.

116   Participants with persistent asymptomatic parasitemia 10 weeks following enrolment were

117   considered as persistent asymptomatic malaria whereas those who developed malaria-associated

118   symptoms before week 11 post-enrolment were considered as developing uncomplicated malaria.

Blood sample collection, malaria diagnosis and parasite genotyping

120   Approximately 3 ml of venous blood was collected in EDTA-coated tubes and used for laboratory

121   testing for malaria and blood hemoglobin levels as well as to obtain plasma for immunological

122   analysis. Plasma was separated from the red blood cell (RBC) pellet by centrifugation (3500 RPM,

123 5 min) and stored at -80 °C until use. Blood hemoglobin levels were assessed using a portable Mission

124 Hb haemoglobinometer (ACON Laboratories, USA).

125 Parasite density (parasites/µl) was determined by microscopic examination of Giemsa-stained thick

126 blood smears. Parasite density was determined based on the number of parasites per at least 200 WBC

127 count on a thick film, assuming a total white blood cell count of 8000 cells/µL of whole blood. A

128 blood slide was considered positive when a concordant result was obtained by at least two

129 independent microscopists and when a discrepancy occurred, a third microscopist was used for

130 confirmation. A slide was considered negative if no parasites were detected after a count of 500 white

131 blood cells.

132 Genomic DNA was extracted from dried blood spots using the Chelex method [26], and used to

133 screen for *Plasmodium* infections by multiplex nested PCR targeting the 18S small subunit rRNA

134 (ssrRNA) genes as previously described [24]. Genotyping of *msp2* gene was performed by nested

135 PCR on *P. falciparum* PCR positive samples as previously described [24,27].

136 **Case definitions for the study**

137 Participants were defined as asymptomatic if positive for malaria parasites by PCR in the absence of

138 fever at least 48 hours prior to enrolment. Febrile malaria was determined based on RDT, light

139 microscopy or PCR positivity for malaria parasites and axillary temperature ≥ 37.5°C or a history of

140 fever in the past 48 hours. Those developing uncomplicated malaria were defined as asymptomatic

141 individuals who had developed fever associated with *Plasmodium* infection during the 10-week

142 infection follow-up period, whereas the persistent asymptomatic malaria classification represented

143 the asymptomatic individuals who remained asymptomatic until anti-malarial treatment at the end of

144 the infection follow-up period.

145 **Anti-*Plasmodium* antibody levels and avidity assessment**

146 Plasma IgG antibody levels and avidity were determined by indirect ELISA method using *P.*

147 *falciparum* Merozoite extract (ME), schizont extract (SE) or the recombinant proteins erythrocyte

148 binding antigen-175 (EBA-175) and Merozoite Surface Protein-1$_{19}$ (MSP1$_{19}$) as coating antigens.

149 ME and SE were isolated from laboratory maintained *P. falciparum* 3D7 parasites as described

150 previously [28], and used for protein extraction by freeze-thaw fractionation [23,29]. The

151 recombinant protein EBA-175 (F3 region) was obtained from BEI Resources (MRA-1162), whereas

152 *P. falciparum* MSP1$_{19}$ was produced using a baculovirus-insect cell expression system [30].

153

154 96-well microplates (F96 CERT-Maxisorp) were coated with each antigen (2 mg/ml of ME and SE,

155 0.20 mg/ml of EBA-175 and 0.5 mg/ml of MSP-1$_{19}$) in 0.1 M bicarbonate buffer (for ME, SE and

156 EBA-175) or PBS (for MSP-1$_{19}$) and incubated overnight at 4˚C. Plasma samples were diluted

157 (1/250) in PBS-T/1%BSA and used to measure anti-*Plasmodium* IgG as previously described [23,31].

158 Plasma from non-exposed European blood donors (10 samples) were used as naive controls and a

159 pool of IgG fractions from adults living in high malaria endemic areas was used as positive controls.

160 Results were expressed as optical-density ratios (OD sample/mean OD naive control). For avidity

161 testing, 100 µL of 8M urea in wash buffer (treated wells) or 100 µL of wash buffer (untreated wells)

162 were added for 15 minutes at room temperature to the respective wells following primary antibody

163 binding as previously described [32]. Avidity indices (AI) were calculated as proportion of bound

164 antibodies following urea treatment (AI = [mean OD of treated wells /mean OD of untreated wells]

165 × 100). Samples with OD ratios > 2, corresponding to mean OD naive control + 3SD were considered

166 as seropositive. In addition, only seropositive samples were used for avidity testing.

167

168 **Growth inhibition assay (GIA)**

169 GIA was performed using plasma from endemic and non-endemic individuals as described by

170 Duncan *et al.* [33]. Briefly, plasma samples were heat inactivated at 56 ° C for 20 min and a 1:20

171   ratio of 50% RBC was added to each sample and incubated at room temperature for 1 h to remove

172   the RBC-reactive antibody. After spinning for 2 min at 13,000 x g to pellet the RBCs, the

173   supernatant was transferred to a 96-well V-bottom plate. Synchronized late trophozoite/early

174   schizont (30-40 h) stages of 3D7 *P. falciparum* at 0.5% parasitemia were incubated with plasma

175   (1:10 ratio) for 48 h at 37 °C. The GIA harvest (parasite growth) was carried out by SYBR green-I

176   DNA quantification assay [34]. Parasite growth inhibition (GI) activities of plasma antibodies were

177   presented as: GI indices (%) = 100 x [1 - ((OD sample - OD RBC) / (OD parasitized RBC - OD

178   RBC))].

179   **Determination of plasma cytokine levels**

180   Plasma concentrations of 38 immune mediators including interleukin (IL)-1α, IL-1β, IL-1RA, IL-2

181   IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-p70, IL-13, IL-15, IL-17A, interferon

182   (IFN)-α2, IFN-γ, tumor necrosis factor (TNF)-α, lymphotoxin-α (LT-α), IFN-γ induced protein (IP-

183   10), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory protein (MIP)-

184   1α, MIP-1β, Eotaxin, Fractalkine, human growth-regulated oncogene (GRO), Macrophage-derived

185   chemokine (MDC), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte

186   colony-stimulating factor (G-CSF), sCD40L, FMS-like tyrosine kinase 3 ligand (Flt-3L), epidermal

187   growth factor (EGF), fibroblast growth factor 2 (FGF-2), transforming growth factor alpha (TGF-α)

188   and vascular endothelial growth factor (VEGF) were determined by Luminex-based method using

189   the Human Cytokine/Chemokine Magnetic Bead Panel MILLIPLEX MAP Kit (Cat. No.

190   HCYTMAG-60K-PX38, EMD Millipore Corporation) according to the manufacturer's instructions.

191   Briefly, 25 μL of premixed magnetic capture beads were incubated with equal volumes of plasma or

192   diluted standard in a 96-well microplate placed on a horizontal plate shaker for 2 hours. After washing

193   using a magnetic plate, plates were incubated with 25 μL/well of biotinylated detection antibodies

194   for 1 hour at room temperature followed by 25 μL of streptavidin-PE in each well for 30 minutes at

195   room temperature. Median fluorescence intensity was read on a Luminex MAGPIX Analyser (XMAP

196   Technology) equipped with xPONENT software version 4.2. The relative concentration of each

197   cytokine (pg/ml) in each sample was determined based on the automatically generated standard curve

198   for each analyte. Analyte amounts less than the limit of detection for each cytokine were attributed

199   the value of detection limit.

200   **Statistical analysis**

201   Statistical analyses were performed using RStudio version 4.1.1 or version 4.2.2 [35] and Graphpad

202   Prism version 8.4.3 (Graphpad software, San Diego, California USA). Differences in antibody and

203   cytokine distribution between two or more groups were determined using Wilcoxon signed rank test

204   and Kruskal-Wallis test, respectively. Spearman rank correlation tests or linear regression were

205   used to determine the correlation between quantitative variables. Differences in categorical

206   variables were determined by chi-square tests. P-values < 0.05 were considered statistically

207   significant. Factor analysis of mixed data (FAMD) with FactoMineR and factoextra packages was

208   used to assess the association between cytokines levels and age and circulating parasite loads. The

209   complexHeatmap and ggpubr packages were used to draw the heatmaps and the line plots,

210   respectively. Given that the aim of this study was to determine the host immunological

211   determinants in the maintenance of the asymptomatic infections, among the 353 individuals

212   included, only participants with complete information on the study outcome during the longitudinal

213   follow-up period were included in the analysis (n= 211). Participants who missed at least one of the

214   follow-up points (the predominant reason being farming far away from the enrolment site) were not

215   included in our study. Three samples (2 symptomatic and 1 asymptomatic) were excluded because

216   no cytokines were detected in the plasma. For analysis of cytokines at the initial time point of our

217   study with respect to correlation with persistent asymptomatic malaria, we employed a mixed linear

218   model using the LME4 package [36]. Cytokine level was used as the response with conversion

219   status as the fixed-effect of the model. We used sex and age as random effects in the model and ran

220   the model separately for children ≤20 years old, adults >20 years old and all participants, regardless

221   of age. To obtain p-value estimates from the t-values we used the lmerTest package [37]. P values

222   were corrected for multiple comparisons using the Sattherthwaite correction. For analysis, parasite

223   densities were separated into three groups as follows, 1. submicroscopic parasitemia, 2. low levels

224   of microscopic parasitemia (parasitemia between 37.2 parasites/μl and 413.1 parasites/μl) and 3.

225   high levels of parasitemia (parasitemia> 413.1 parasites/μl, representing the median parasitemia in

226   the study population).

227

228   **Results**

229   **Asymptomatic malaria prevalence in the study area**

230   We carried out immunological analysis of a subset of 211 individuals from a previously reported

231   study of 353 individuals screened for malaria using PCR, where 328 (92.9%) were positive for

232   *Plasmodium falciparum* infection, of which 266 (81.1%) were asymptomatic and 62 (18.9%) were

233   symptomatic [24]. This subset had complete sampling from the follow-up study, and included 142

234   individuals with asymptomatic infection, 61 symptomatic individuals and 8 non-infected individuals.

235   At baseline when these individuals were first sampled, parasite densities were highly variable

236   between the symptomatic and asymptomatic groups, and no significant difference was observed in

237   the median parasite densities of the two groups at the initial time point of the study (**Table 1**). Age,

238   sex, and hemoglobin levels were also not different across the study groups at this initial time point

239   (**Table 1**).

240

Immunology of asymptomatic parasitemia

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Variables	Non detectable infection (n=8)		Asymptomatic (n=142)		Symptomatic (n=61)		P value	
Age groups	≤20	>20	≤20	>20	≤20	>20	≤20	>20
n	5	3	94	48	42	19		
Sex ratio (M:F)	0.67 (2:3)	50 (1:2)	0.82 (42:51)	41.2 (14:34)	1.1 (22:20)	58.3 (7:12)	0.7114	0.8280
Median age, years (range)	14 (4-20)	68 (50-68)	9 (2–19)	50 (22–86)	9 (2–20)	28 (21-64)	0.2193	0.0028
Median Hb, g/dl (range)	11.8 (10.7-12.7)	112.5 (11.9-14)	12 (4.9-14.3)	13.1 (10.9-16)	11.1 (6.8-14)	12.8 (9.4-16.8)	0.1866	0.9833
% of submicroscopic % (n)	-		22.3 (21)	60.4 (29)	9.5 (4)	57.9 (11)	0.0746	0.8495
Geometric mean parasitemia, parasites/µl (range)	-		575.7 (37.4-68190.5)	113.5 (38.3-660.2)	764.1 (38.1-240457.1)	183.3 (37.2-5058.8)	0.5139	0.6101

Table 1. Characteristics of the study population

The data presented in this table is from 211 individuals from our study at baseline. The subset of 142 asymptomatic individuals included in this table were those individuals with complete data from the follow-up study to 11 weeks and does not include baseline data from individuals who dropped out of the study. P-value was determined using Wilcoxon signed-rank test and Kruskal-Wallis test when comparing two or three groups, respectively. The median parasitemia presented in this table is only for the infected individuals with parasite densities at the detection limit of light microscopy. The sex ratio between groups were compared using chi-square test. Hb: hemoglobin. M: male. F: female.

245

Anti-parasite antibodies were inversely correlated with parasitemia but were not associated with asymptomatic parasite carriage

Consistent with the hypothesis that host immunity increases with repeated exposure to malaria parasites, baseline parasite densities above the detection limit of microscopy decreased significantly with age (r= -0.49, P< 0.0001) **(Figure 1A)** but only in individuals aged 20 years and younger (r= -0.39, P< 0.0001) (**Figure 1B**). Of the 142 participants who completed the follow-up study as designed, 78 (54.9%) remained asymptomatic during the 10-weeks follow-up period, whereas 63 (44.4%) developed malaria-associated fevers and 1 (0.7%) resolved the infection in the absence of any known antimalarial treatment. Based on the parasitemia distribution of those with microscopic *Plasmodium* infections at baseline the study population was divided into two groups aged ≤ 20 years and > 20 years old for further analyses. The proportion of submicroscopic infections was significantly higher (P<0.0001) in the older age group (age >20 years) at the baseline time point than in the younger age group (**Figure 1C**). At the baseline time point, median IgG levels against *P. falciparum* merozoite (ME) and schizont (SE) crude extracts were significantly higher in the >20-year age group than in the younger age group (P< 0.0001) (**Figure 2A**). This was mirrored by IgG reactive to *P. falciparum* recombinant proteins EBA-175 (P= 0.0250) and MSP-1$_{19}$ (P< 0.0001) (**Figure 2A**). Additionally, In the younger age group IgG levels against merozoite (r= 0.22, P= 0.0077) and schizont (r= 0.29, P= 0.0004) crude extracts as well as against MSP-1$_{19}$ (r= 0.15, P= 0.0764) but not EBA-175 (r= 0.01, P= 0.8525) were positively correlated with age at the baseline time point (**Figure 2B**). A negative correlation was observed between IgG levels to *P. falciparum* merozoite and schizont extracts at baseline and parasite density measured at the same time point (**Supplementary Figure 1**). Indeed, the multilevel linear regression model adjusted for village and household levels, using IgG levels at the baseline and age as predictors, showed that only age was the independent predictor of *Plasmodium* parasite densities in the study population (coef= -0.02, P= 0.0004 for age; coef= 0.01, P= 0.9170 for

270 ME; coef= -0.19, P = 0.1529 for SE; coef= -0.02, P= 0.4746 for EBA-175; coef= 0.02, P= 0.5362 for

271 MSP-1$_{19}$).

272 Median IgG levels were not significantly different between participants with sub-microscopic

273 parasitemia and those with microscopic parasites at baseline (**Supplementary Figure 2A**), and no

274 significant difference was observed in the *in vitro* growth inhibition potential of inactivated plasma

275 from submicroscopic parasite carriers and that of microscopic carriers (**Supplementary Figure 2B**).

276 Both IgG antibody avidity (**Supplementary Figure 3A&B**) and growth inhibition potentials

277 (**Supplementary Figure 2B**) were similar in the two groups (P> 0.05). Together, these findings

278 suggest that anti-parasite antibodies may control parasite density levels by mechanisms other than

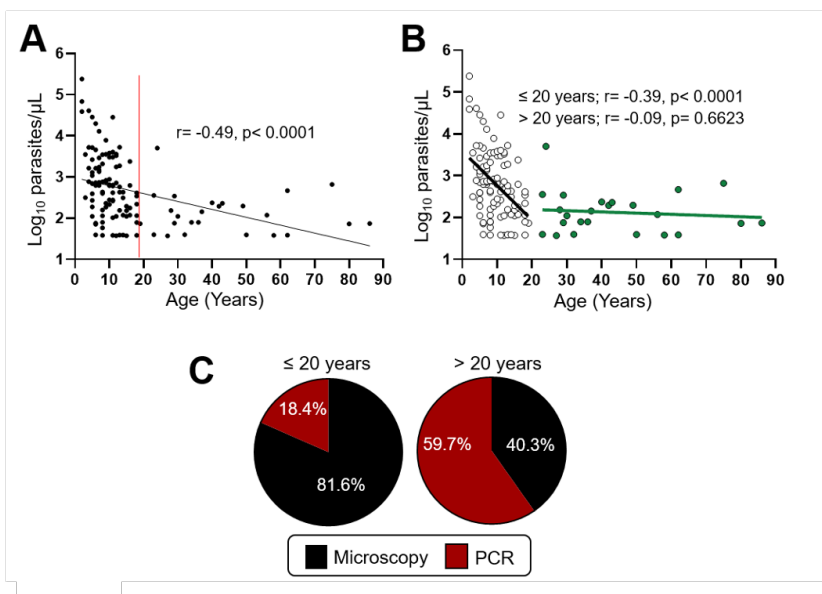279 direct inhibition of parasite growth *in vivo*.

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Figure 1. Older individuals (>20 years) had a better control of parasite densities. A) Scatterplot showing a correlation between baseline parasite densities and age in all the infected individuals with parasitemia at the detection limit of light microscopy (n= 138). Spearman rank correlation is presented as the best fit line and the coefficient (r), as well as the p-value (p), are shown. The red straight line was used to divide the study population into two groups aged ≤ 20 years and > 20 years old for further analyses. **B**) Scatterplot of correlation between baseline parasitemia detected by microscopy and age in different age groups (≤ 20 years and > 20 years) at the different time points of our study. Spearman rank correlation is presented as the best fit line and the coefficient (r), as well as the p-value (p), are shown for each age group. **C**) The pie chart shows the proportion of sub-microscopic infections (PCR+/microscopy-) versus microscopic (microscopy+) infections in each age group. PCR: Polymerase Chain Reaction.
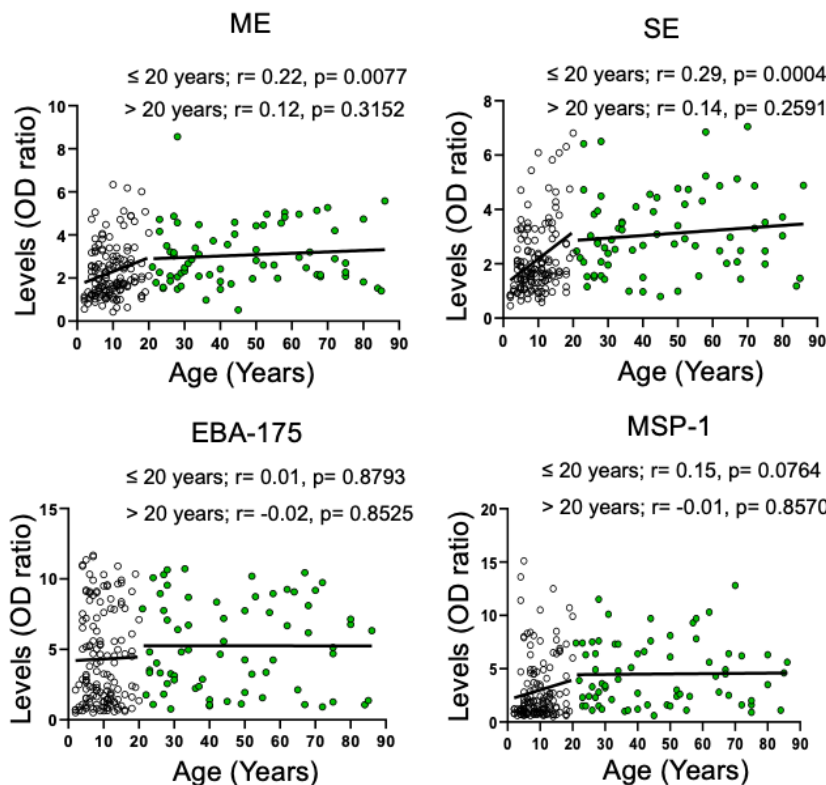
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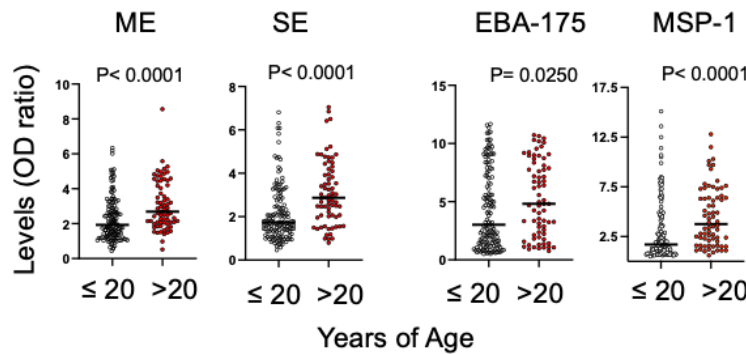
Figure 2. Individuals >20 years old had more anti-parasite IgG at baseline. A) Data presented in the scatterplot represent baseline IgG antibody levels against different *P. falciparum* antigens (ME, SE, EBA-175 and MSP-1$_{19}$) within age groups. Median levels between age groups were compared by Wilcoxon signed-rank test and the p-values are shown on the graph. **B**) The scatterplots on B show the correlation between IgG levels against different *P. falciparum* antigens (ME, SE, EBA-175 and MSP-1$_{19}$) and age in different age groups ($\leq$ 20 years and > 20 years). Spearman rank correlation is presented as the best fit line and the coefficient (r), as well as the p-values (p), are shown for each age group. SE: Schizont extract. ME: Merozoite extract. EBA-175: Erythrocyte binding antigen-175. MSP-1$_{19}$: Merozoite surface protein-1$_{19}$. OD: optical density.

308   Similarly, no significant difference was observed in malarial IgG antibody levels and avidity between

309   participants with symptomatic or asymptomatic parasitemia (**Supplementary Figure 4A**), and no

310   significant difference was seen in the blood parasitemia at the detection limit of microscopy

311   (Supplemental Figure 4B; **Table 1**). However, the *in vitro* growth inhibition potential of plasma from

312   asymptomatic malaria was greater than that of symptomatic individuals (P= 0.0245) despite no

313   significant differences in the parasitemia amongst those with parasite levels that were detectable by

314   microscopy (**Supplementary Figure 4B**). This may be a reflection of higher percentage of

315   asymptomatic children with submicroscopic parasitemias (77.7%) compared to symptomatic children

316   (90.5%) (**Supplementary Figure 4C**). Taken together, these data suggest that humoral responses

317   other than IgG responses to the tested parasite antigens contribute to the maintenance of

318   asymptomatic parasitemia in the study area.

319   **Persistence of fluctuating asymptomatic parasitemia occurred in highly exposed individuals**

320   **without alteration in IgG levels**

321   Of the 142 participants who completed the follow-up study as designed, 72 (50.7%) remained

322   asymptomatic during the 10-weeks follow-up period, whereas 69 (48.6%) developed malaria-

323   associated fevers and 1 (0.7%) resolved the infection in the absence of any known antimalarial

324   treatment. Surprisingly, the propensity to convert was not associated with age (**Figure 3A**). Of the 72

325   participants who maintained chronic asymptomatic *P. falciparum* carriage, the proportion of

326   submicroscopic infections remained similar in both age groups with most participants becoming

327   microscopy positive on week 10 regardless of age (**Figure 3B**). Parasite densities fluctuated widely

328   over the follow-up period from ultra-low parasitemia (sub-microscopic) to high parasitemia (**Figure**

329   **3B**), possibly due to variation in the infecting clones from new infections as this is a high transmission

330   area (**Figure 3C**). However, no changes in IgG levels (**Figure 3D**) against all antigens tested were

331   observed during persistent asymptomatic infections indicating that persistent asymptomatic

332   parasitemia does not affect anti-*Plasmodium* IgG levels over this short 3-month time period. These

333   data suggests that asymptomatic carriage of *P. falciparum* occurs independently of circulating

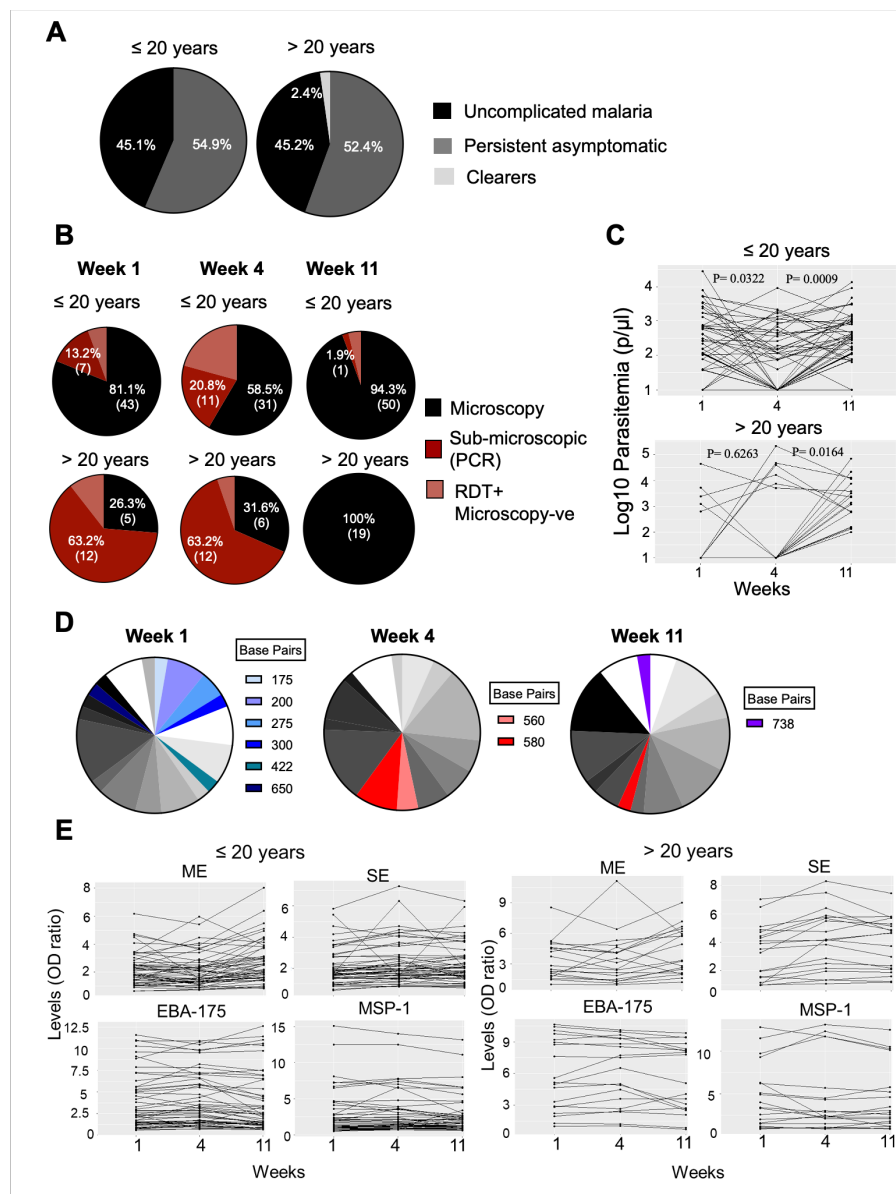334   parasite numbers, even within highly-exposed individuals.

336   **Figure 3. Asymptomatic malaria persisted in some individuals despite changes in the circulating**

337   **parasite densities and population over time, but did not affect the antibody levels**. **A**) The pie charts

338   show the proportion of individuals with initial asymptomatic infection (cross-sectional) who resolved the

339   infection (clearers), developed malaria-associated fever (uncomplicated malaria) or remained asymptomatic

340   (persistent asymptomatic) over the 10-week follow-up period, according to age groups. **B**) Panel B displays

341   the proportion of sub-microscopic/microscopic parasitemia in persistent asymptomatically infected

342   individuals at three different time points (weeks 1, 4 and 11) according to age groups. Individuals who were

343   RDT positive but without detectable micoroscopic parasitemia was detected are shown in pink. Of those who

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344   had microscopic parasitemia, the line plots parasitemia across the 3 time points (**C**). To generate the line

345   plots, the value 10 was assigned to submicroscopic parasitemia. The P values on the line plots refer to the

346   comparison of the median parasitemia between two time points using the Wilcoxon test. **D**) Pie charts show

347   the proportion of msp2 alleles (representing the clones) determined by nested PCR in individuals with

348   persistent asymptomatic infection per week. Numbers represent the band sizes obtained. Blue slices

349   represent the alleles only present at week 1. Red slices represent the alleles which appear at week4. Green

350   slices represent the allele only present at week 11. Grey slices represent the alleles that were present at all

351   three sampling time points (bands of 375, 400, 475, 500, 520, 534, 540, 600, 620, 633. 680, 700, 800 bp). **E**)

352   Data presented in the line plots represent the profile of IgG anti-*Plasmodium* antibody levels in each chronic

353   asymptomatically infected individuals at three different time points (week 1, 4 and 11) in the two age groups.

354   SE: Schizont extract. ME: Merozoite extract. EBA-175: Erythrocyte binding antigen-175. MSP-1$_{19}$:

355   Merozoite surface protein-1$_{19}$. OD: optical density.

356

357 **Parasite density was associated with inflammatory responses amongst younger individuals**

358 **with asymptomatic malaria**

359 Given that *Plasmodium* parasites are associated with induction of inflammatory responses we

360 measured the cytokine profiles of the 72 participants who had persistent asymptomatic carriage of

361 *P. falciparum* across the course of our study. There was no particular cytokine signature that

362 associated with asymptomatic carriage of *P. falciparum* in our study participants over the 11-week

363 follow-up period (**Figure 4A**). Using a factor analysis of mixed data (FAMD) on cytokine data

364 along with age and circulating parasite density, we found that levels of IL-10, TNF-α, the ratio of

365 IL-10:IFN-γ and MCP-1, along with age and parasite density explained most of the variation in the

366 peripheral cytokine response in asymptomatic individuals (**Figure 4B**). Multivariable analysis using

367 age and parasitemia as predictors showed that age independently predicted the levels of TNF-α

368 (coef= -0.31, P< 0.0001) peaking in younger study participants (**Figure 4C**). This is likely a

369 reflection of circulating parasite densities which were higher in the younger group (**Figure 1**) and

370 were also independently predictive of TNF-α levels (coef= 0.09, P< 0.0001) (**Figure 4D**).

371 Circulating parasite density also independently predicted the levels of IL-10 (coef= 0.21, P<

372 0.0001) and MCP-1 (coef= 0.06, P= 0.01) with a significant increase in those who had high levels

373 of parasitemia (parasitemia> 413.1 parasites/µl) relative to those who had low levels of microscopic

374 parasitemia (parasitemia between 37.2 parasites/µl and 413.1 parasites/µl) (**Figure 4D**). This

375 indicates that during asymptomatic carriage of *P. falciparum* circulating parasites simultaneously

376 induce anti-inflammatory cytokine production to counteract the inflammatory effects of TNF-α. To

377 determine which cytokines were dependent on parasite carriage we treated participants at week 11

378 of the study with ACT. This resulted in a significant decline in the IL-10 levels, but not TNF-α or

379 MCP-1 levels, in the younger age group (**Figure 5**). Thus, the levels of IL-10 measured is a

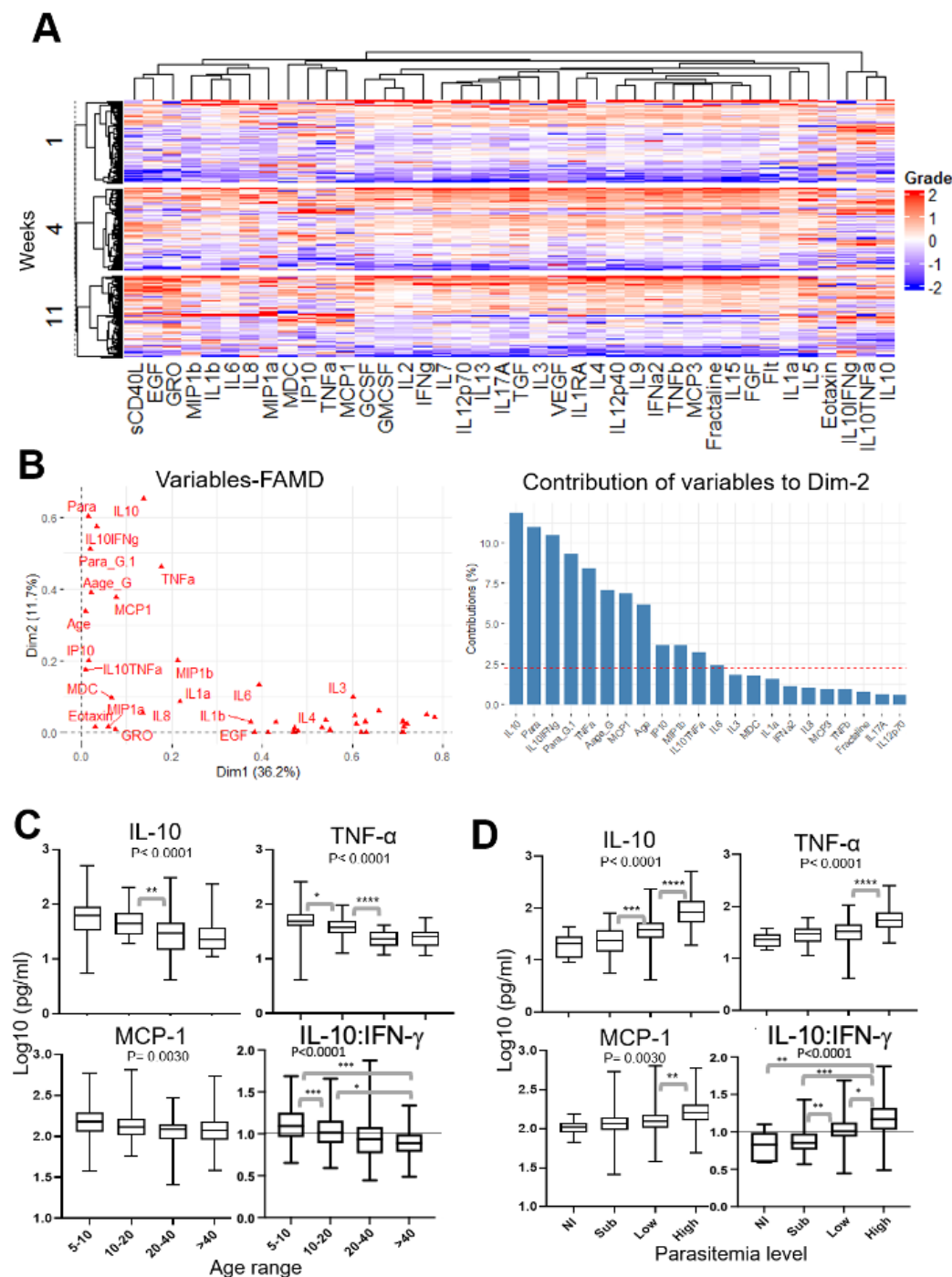380 reflection of parasite carriage in the younger age group.

Figure 4. Cytokine responses were blunted with increased age and correlated with parasite densities at baseline. A) Hierarchical clustering of the standardized least-square means of 38 differentially expressed cytokines/chemokines/growth factors across the three sampling time points in participants with persistent asymptomatic infection (n= 72). **B)** Principal component of cytokines, age and parasitemia from FAMD (left graph) analysis. The right graph of B shows the contribution of each variable on the principal component 2 (dimension 2). **C and D)** box plots representing interquartile ranges (25th, median and 75th percentile, whisker

388   percentile (1st and 99th)) of the levels (Log10 pg/ml) of some main regulatory cytokines measured by Luminex

389   according to age and parasite level groups at the baseline, respectively. Median levels of cytokines between

390   two or more groups were compared by Wilcoxon rank-sum test and Kruskal-Wallis test, respectively. In Panel

391   A, pairwise comparisons on neighboring groups were performed only when multiple comparisons were

392   significant at $p < 0.05$. Parasite densities were groups in three groups as follows: submicroscopic parasitemia,

393   low levels of microscopic parasitemia (parasitemia ≤ 413.1 parasites/µl, representing the median parasitemia

394   in the study population) and high levels of parasitemia (parasitemia > 413.1 parasites/µl). FAMD: Factor

395   analysis of mixed data. *$p < 0.05$; **$p < 0.001$, ***$p < 0.0001$, ****$p < 0.00001$.
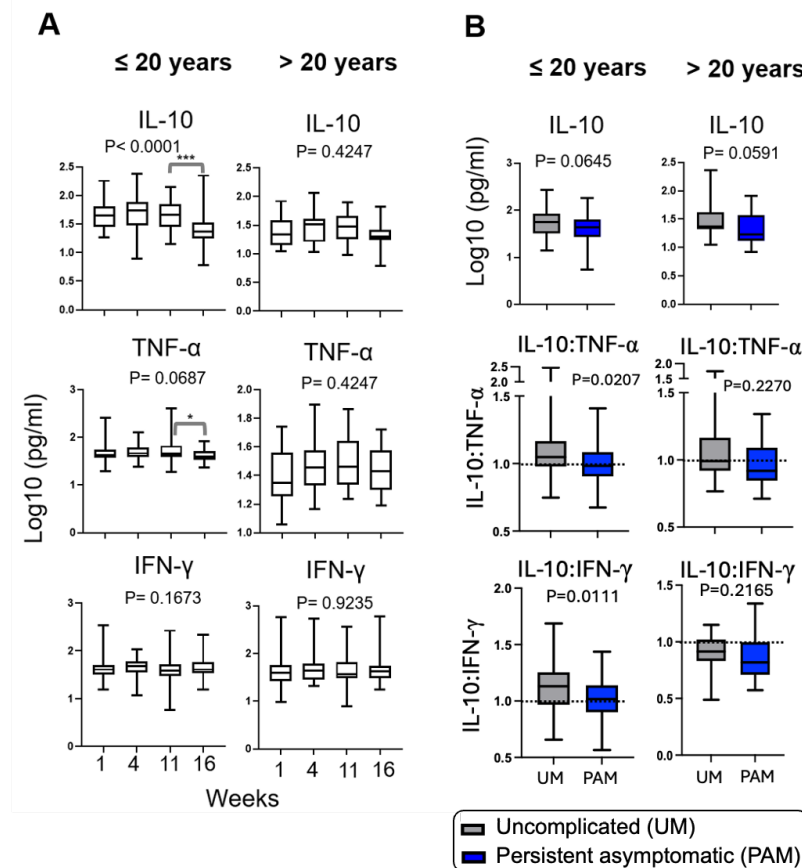
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Figure 5. IL-10 and TNF-α levels were a reflection of parasite carriage, and the balance of inflammatory cytokines at baseline was associated with the maintenance of asymptomatic infections in the younger age, ≤ 20 years. A) Box plots representing interquartile ranges (25th, median and 75th percentile, whisker percentile (1st and 99th)) of cytokine levels (Log$_{10}$ pg/ml) during persistent asymptomatic infections (week 1, 4 and 11) and after anti-malarial treatment (week 16). **B**) Box plots representing interquartile ranges (25th, median and 75th percentile, whisker percentile (1st and 99th)) of cytokine levels (Log$_{10}$ pg/ml) at the baseline between in those who developed uncomplicated malaria (UM; grey) and those who went on to display persistent asymptomatic malaria throughout the course of the study (PAM; blue). Analyses were performed including values from individuals with submicroscopic and microscopic parasitemia. Median cytokines levels between two or more groups were compared by Wilcoxon rank-sum test and Kruskal-Wallis test, respectively and significant between group differences indicated by the P value on the plot. In Panel A, pairwise comparisons on neighboring groups were performed only when multiple comparisons between the 4 groups were significant and are shown by the grey brackets. *p< 0.05 and ***p< 0.0001.

413 **Maintenance of asymptomatic carriage of *P. falciparum* is associated with a more balanced**

414 **inflammatory cytokine ratio**

415 There was no particular cytokine signature of asymptomatic individuals relative to symptomatic

416 individuals in our study (**Figure 6A**). However, given that anti-inflammatory cytokines may be

417 important in down-regulating the pathogenic inflammatory response in malaria, we tested the

418 hypothesis that asymptomatic infection would be associated with higher levels of IL-10. To our

419 surprise, at the initial time point of our study there was a non-significant trend towards higher levels

420 of IL-10 in the symptomatic compared to asymptomatic participants (**Figure 6B**). This may be a

421 reflection of higher inflammation levels in symptomatic individuals as other pro-inflammatory

422 markers (IL-6 and MCP-1) were significantly higher in individuals with symptomatic malaria

423 compared to those with asymptomatic *P. falciparum* infections in the younger age group (**Figure 6B**).

424 There was an IL-10-skewed ratio of IL-10:TNF-α and IL-10:IFN-γ in the symptomatic group

425 compared to the asymptomatic group in both age groups (**Figure 6C**). This is in agreement with

426 previously published data in individuals with uncomplicated malaria and the IL-10 skewed ratio of

427 IL-10 to TNF-α that can be measured in those with severe symptoms of malaria [38,39].

428 Multivariable logistic regression analysis showed that the IL-10:TNF-α ratio and IL-1RA levels were

429 independently associated with developing symptoms during *P. falciparum* infection in this study
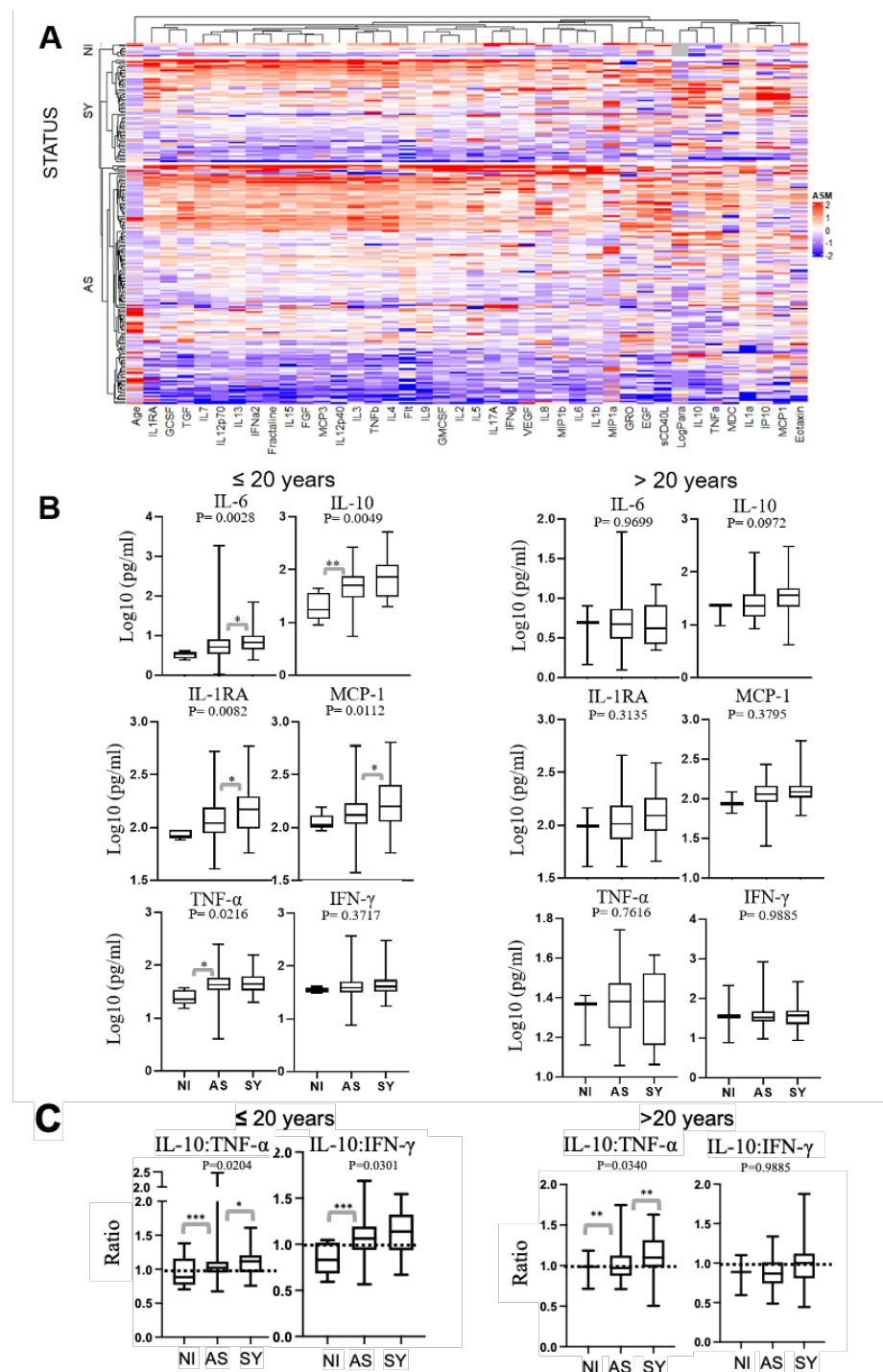
430 (**Supplementary Table S1**).

431

432

Figure 6. IL-10 at baseline was associated with clinical malaria in the younger age group (≤ 20 years).
A) Hierarchical clustering (heatmap) of the standardized least-square means of 38 differentially expressed cytokines/chemokines/growth factors by infection status at the baseline of this study. **B** and **C** box plots represent interquartile ranges (25th, median and 75th percentile, whisker percentile (1st and 99th)) of the baseline cytokine levels (Log_{10} pg/ml) measured by Luminex according to infection status. Analyses were performed including both individuals submicroscopic and microscopic parasitemia. Median levels of cytokines between two or three groups were compared by Wilcoxon rank-sum test and Kruskal-Wallis test, respectively.

25

441 In Panel B and C, pairwise comparisons were performed only when multiple comparisons were significant. P

442 values refer to the Kruskal-Wallis test comparison between the three groups. NI: Non-infected. AS:

443 Asymptomatic. SY: Symptomatic. *p< 0.05; **p< 0.001; ***p< 0.0001.

444

445 To further test the hypothesis that asymptomatic carriage of *P. falciparum* may be related to IL-10

446 production, we compared the cytokine ratios in the younger group who converted to symptomatic

447 malaria throughout our study with those who remained asymptomatic. At the initial time point of the

448 study there was a trend towards increased IL-10 levels in those who subsequently went on to develop

449 uncomplicated malaria compared to those who experienced persistent asymptomatic malaria in both

450 age groups. In the younger age group IL-10/pro-inflammatory cytokine ratios were significantly

451 higher and IL-10-skewed (P= 0.0207 and P= 0.0111 for IL-10:TNF-α and IL-10:IFN-γ ratios,

452 respectively) in the those developing uncomplicated malaria compared to those with persistent

453 asymptomatic malaria in younger age group (**Figure 5B**). In this group those with persistent

454 asymptomatic malaria had a ratios of 1.001±0.1355 for IL-10:TNF-α and 1.016±0.1794 for IL-

455 10:IFN-γ) displaying an even balance of IL-10: proinflammatory cytokine. On the other hand adults

456 did not have IL-10 skewed ratios with TNF-α or IFN-γ suggesting that a different or additional

457 mechanism may facilitate the persistence of asymptomatic malaria in the older age group.

458 Collectively this data suggests that IL-10 is not sufficient to dampen inflammatory responses in

459 *Plasmodium* infection that lead to the fevers that define uncomplicated malaria.

460

461 **Discussion**

462 Significant gaps exist in the understanding of the contribution of anti-parasite and anti-disease

463 immunity in the persistent asymptomatic parasite carriage in areas with high transmission of *P.*

464 *falciparum*. Mathematical modelling of existing data indicates that asymptomatic *P.*

465 *falciparum* infections could last for as long as 13-years [40] and field data documents the longevity

466 of asymptomatic infections ranges from at least 2-9 months [7,41,42]. As we have previously

467 published, our study area has high rate of persistent asymptomatic parasite carriage over the 3 month

468 time period of study [24]. However, a proportion of individuals in our study converted to symptomatic

469 malaria as defined by the development of fever. Thus, we have assessed immune correlates of

470 asymptomatic carriage of *P. falciparum*, as well as the predictors of conversion to symptomatic

471 malaria.

472 Consistent with the acquisition of immune response with age and exposure [13,43] in our study, older

473 asymptomatic individuals had better control of circulating iRBCs with a greater proportion of sub-

474 microscopic infections than younger individuals. This was associated with an increase in the quantity

475 of circulating anti-parasite IgG which appeared to saturate at around 20 years old. Our data is in

476 agreement with an age-structured mathematical model of malaria transmission which showed that

477 anti-parasite immunity which results in more rapid clearance of parasitemia has an approximate half-

478 life of 20 years [44]. With a focus on the younger age group, there did not appear to be any correlations

479 with respect to parasitemia levels and IgG levels or the avidity of the IgG. The *in vitro* invasion-

480 blocking capability of the plasma in young individuals had some functionality with respect to invasion

481 blocking, but only at higher parasitemia levels. In this assay plasma factors other than IgG, such as

482 IgM [45,46], may contribute to this observation and control of *P. falciparum* parasitemia in children.

483 Collectively these data support a role for IgG in exerting control of iRBCs in *P. falciparum* infections,

484 but it may not be the principal factor that dictates asymptomatic carriage of parasites in younger

485 individuals.

486 Surprisingly the proportion of those developing uncomplicated malaria over the course of our study

487 was comparable between the younger and the older age groups, suggesting that the conversion from

488 an asymptomatic to symptomatic infection is independent of age in highly exposed individuals. The

489 reasons for this finding are not clear but may be related to heterogeneity of the circulating *P.*

490 *falciparum* parasite population and emergence of highly inflammatory parasite clonotypes [47,48]

491 that overcome the pyrogenic threshold of individuals. Consistent with the oscillation of parasite

492 densities over time reported in a Vietnam cohort [7], parasitemia in persistent asymptomatic

493   infections in our study fluctuated widely and independently of IgG levels, and different clonotypes

494   of *P. falciparum* were observed at different time points in this study.

495   Cytokine profiles are considerably more dynamic than IgG responses. In line with the hypothesis that

496   clinical immunity to malaria is mediated by a tempering of inflammation, previous studies have

497   shown that the clinical outcome of malaria infection is largely dictated by the balance between pro-

498   inflammatory and anti-inflammatory markers [17,18]. Although our study highlighted that carriage

499   of *P. falciparum* across the duration of this study was associated with a consistent inflammatory

500   response characterized by TNF-α, IL-10 and MCP-1, we found no obvious cytokine signature

501   associated with the asymptomatic carriage of *P. falciparum* when compared to symptomatic

502   individuals. Similar to findings in a study of visceral leishmaniasis [49], where increased IL-10 levels

503   were the primary predictor of clinical conversion from asymptomatic to symptomatic

504   visceral leishmaniasis, the conversion from asymptomatic to symptomatic malaria in our study was

505   linked to increased baseline levels of IL-10 skewed ratios with TNF-α and IFN-γ in children at the

506   initial time point of our study.

507   Although IL-10 is known to exert anti-inflammatory effects that include inhibition of monocytes and

508   macrophages in the production of inflammatory cytokines such as IL-6, TNF-α, and IL-l [50], in

509   agreement with a recent study from the Gambia [51] in our study IL-10 was associated with the

510   presence of *Plasmodium* parasites. Given that higher levels of IL-6, IL-1RA and MCP-1 were also

511   associated with symptomatic infections in the younger age group of our study, IL-10 levels are likely

512   a consequence of increased inflammation. Notably, IL-10 induces IL1RA, a potent anti-inflammatory

513   cytokine that regulates the pro-inflammatory actions of IL-1 [52] that has previously been shown to

514   be associated with severity of malarial disease [53]. At the baseline time point in our study, we found

515   that balanced inflammatory cytokine ratios were correlated with developing symptoms from *P.*

516   *falciparum* infection in our younger age group. Specifically, those who developed uncomplicated

517   malaria had a significant IL-10 skewed ratio at the initial time point compared to those who had

518   persistent asymptomatic malaria. An IL-10 skewed ratio may indicate that an individual will shortly

519   develop symptoms of *P. falciparum* infection.

520   Most of the association between biomarkers and clinical malaria in this study were only found in the

521   younger age group, but not in adults, indicating that the mechanisms of the protection against clinical

522   malaria may be different according to age. As such, more studies into how asymptomatic carriage of

523   *Plasmodium* occurs are warranted and should include non-antibody mediated mechanisms of parasite

524   control and clinical immunity which may be relevant in children.

525

540

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