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# HIV infection and placental malaria reduce maternal transfer of multiple antimalarial antibodies in Mozambican women

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## **Summary**

### Objectives

Maternal *Plasmodium falciparum*-specific antibodies may contribute to protect infants against severe malaria. Our main objective was to evaluate the impact of maternal HIV infection and placental malaria on the cord blood levels and efficiency of placental transfer of IgG and IgG subclasses.

### Methods

In a cohort of 341 delivering HIV-negative and HIV-positive mothers from southern Mozambique, we measured total IgG and IgG subclasses in maternal and cord blood pairs by quantitative suspension array technology against eight *P. falciparum* antigens: Duffy-binding like domains 3-4 of VAR2CSA from the erythrocyte membrane protein 1, erythrocyte-binding antigen 140, exported protein 1 (EXP1), merozoite surface proteins 1, 2 and 5, and reticulocyte-binding-homologue-4.2 (Rh4.2). We performed univariable and multivariable regression models to assess the association of maternal HIV infection, placental malaria, maternal variables and pregnancy outcomes on cord antibody levels and antibody transplacental transfer.

### Results

Maternal antibody levels were the main determinants of cord antibody levels. HIV infection and placental malaria reduced the transfer and cord levels of IgG and IgG1, and this was antigen-dependent. Low birth weight was associated with an increase of IgG2 in cord against EXP1 and Rh4.2.

### Conclusions

We found lower maternally transferred antibodies in HIV-exposed infants and those born from mothers with placental malaria, which may underlie increased susceptibility to malaria in these children.

## **Keywords**

Maternal antibodies; cord blood antibodies; placental transfer; HIV; placental malaria; IgG; IgG subclasses.

## 1 **Introduction**

2 Each year, more than 200 million cases of malaria occur worldwide, the majority in Africa [1].  
3 Pregnant women and children older than 6 months of age are the most vulnerable groups  
4 affected by malaria. In fact, malaria in pregnancy is estimated to account for 100,000 neonatal  
5 deaths annually and it increases the risk of severe maternal anaemia, premature delivery, low  
6 birth weight (LBW) and perinatal mortality [2, 3]. The lower impact of malaria disease in  
7 infants younger than 6 months of age is thought to be due to a number of factors, such as  
8 passive transfer of maternal antibodies or higher presence of foetal haemoglobin associated with  
9 slower parasite growth [4–8]. However, recent reports show that the number of malaria cases  
10 may be underestimated [9, 10] and the risk of severe malaria increases when the transferred  
11 maternal antibodies start to wane [11].

12 Maternal antibodies contribute to protection of infants for the first 3-6 months of life by passive  
13 immunity, especially from severe malaria and its major complications [12, 13]. This immunity  
14 is acquired mainly through the transplacental transfer of antibodies that is facilitated by neonatal  
15 fragment crystallisable (Fc) region receptor (FcRn), expressed in the human syncytiotrophoblast  
16 [14]. Only IgG is transferred across the placenta, the majority during the third trimester [15].

17 The efficiency of transplacental transfer of antibodies is affected by many factors, such as  
18 maternal antibody levels, IgG subclass, avidity, antigen specificity, gestational age, parity,  
19 maternal infections, and differs between locations [16–20]. Maternal hypergammaglobulinemia,  
20 LBW and maternal infections have been inconsistently associated with reduced cord blood  
21 antibody levels and placental transfer [21–26]. Malaria in pregnancy, for example, has been  
22 reported to reduce transplacental IgG transfer against several common pathogen antigens in  
23 some studies [19, 21, 23, 27], although others have shown no impact [23, 25, 26, 28].

24 The effect of maternal HIV infection is also controversial. A study in Kenya showed that HIV-  
25 positive (HIV+) women had less transplacental transfer of IgG against the circumsporozoite  
26 protein (CSP) than HIV-negative (HIV-) women, but no differences were found for any other

27 malarial antigen [29]. Another study in Kenya assessed the effect of maternal HIV infection on  
28 the transplacental transfer of 14 *P. falciparum* antigen-specific IgG antibodies and reported that  
29 HIV+ women had a reduced transfer of IgG only against the merozoite surface protein 9  
30 (MSP9), CSP and erythrocyte binding antigen 181 (EBA181) [28]. In contrast, a study in  
31 Mozambique found that HIV+ women had a subclass-dependent reduction of cord blood IgG  
32 and placental transfer, with lower total IgG and IgG1 cord blood levels and placental transfer  
33 against erythrocyte binding antigen 175 (EBA 175), lower total IgG against apical membrane  
34 antigen 1 (AMA1) and lower IgG3 levels and placental transfer against merozoite surface  
35 protein 1 (MSP1) [30]. That study also assessed the effect of malaria in pregnancy, which  
36 reduced the transfer of antibodies against these antigens, and others have also reported reduced  
37 placental transfer of antibodies due to placental malaria [19]. Another study in Cameroon  
38 showed that there was a decreased transfer of CSP, MSP1 and AMA1 IgG antibodies in HIV+  
39 mothers [31]. Moreover, only a few studies assessed the effect of maternal HIV infection on  
40 IgG subclasses against malaria, and they had several limitations: a low number of HIV+  
41 women, a lack of viral load data, a small number of antigens tested and an absence of IgG2 and  
42 IgG4 analyses [30, 31]. Thus, further studies are needed to clarify the impact of maternal HIV  
43 infection on the transplacental transfer of antimalarial antibodies, especially IgG subclasses that  
44 have been reported to have differential associations with protection from malaria in childhood  
45 [32–36].

46 Maternal antibodies to *P. falciparum* antigens, could also interfere with the acquisition of a  
47 protective immune response after malaria vaccination, as suggested in previous studies [37–39],  
48 especially when the transferred antibodies are against a vaccine target antigen. This is known to  
49 be a significant issue for measles vaccines [40–44]. Therefore, it is important to decipher the  
50 factors that affect maternal antimalarial antibody transfer, not only because of their protective  
51 role in the infant, but also because of their implications on the antibody build-up against some  
52 vaccine target antigens and naturally acquired immunity (NAI) to malaria [45, 46].

53 Here, our main objective was to evaluate the effect of maternal HIV infection and placental  
54 malaria (PM), on the cord blood levels and placental transfer of total IgG and IgG subclasses to  
55 8 *P. falciparum* antigens associated with malaria exposure and protection in a large sample size  
56 cohort of Mozambican women. As exploratory objectives, we also aimed to assess the impact of  
57 maternal variables (age, gravidity, malaria treatment, antiretroviral therapy, CD4<sup>+</sup> T cell counts,  
58 HIV viral load), pregnancy outcomes (maternal anaemia, prematurity, gestational age and  
59 LBW) and seasonality.

60 A better understanding of factors affecting cord levels and placental transfer is essential towards  
61 the design and implementation of malaria vaccines, particularly in malaria endemic areas with  
62 high HIV prevalence.

## 63 **Materials and Methods**

### 64 Study design and sample collection

65 A total of 197 HIV- and 144 HIV+ pregnant women were recruited between May 2011 and  
66 September 2012 in the Manhiça District, Southern Mozambique, a semi-rural area in Maputo  
67 Province. These women were participants of two clinical trials of antimalarial intermittent  
68 preventive treatment in pregnancy (IPTp, ClinicalTrialGov NCT00811421) (Additional file 1:  
69 Figure S1) [47, 48] that evaluated i) mefloquine (MQ) as an alternative IPTp drug to  
70 sulfadoxine-pyrimethamine (SP) in HIV- pregnant women and ii) MQ as IPTp drug in HIV+  
71 pregnant women in whom SP is contraindicated and who received daily cotrimoxazole (CTX).  
72 Pregnant women of all gravidities and gestational age  $\leq 28$  weeks attending an antenatal care  
73 clinic for the first time and who had not received IPTp during their current pregnancy were  
74 invited to participate in the study after provision of informed consent. The study arms for the  
75 first trial were (1) SP, (2) single dose MQ (MQ full), and (3) split dose over two days MQ (MQ  
76 split), and for the second trial, women received either three monthly doses of MQ or placebo.  
77 Antiretroviral therapy (ART) with daily monotherapy with zidovudine (AZT) was  
78 recommended when CD4<sup>+</sup> T cell count was below  $<350$  cells/ $\mu$ L and/or when women were in

79 WHO HIV clinical stage III or IV [49]. At the time of the study, the intensity of malaria  
80 transmission was low/moderate and the HIV prevalence in pregnant women was 29% [50, 51].

81 Before delivery, 50 µl of maternal peripheral blood samples were collected on Whatman 903™  
82 filter paper at recruitment and in two visits (one during the second trimester and the other during  
83 the third trimester) for the detection of *P. falciparum* by real-time quantitative polymerase-  
84 chain-reaction (qPCR) targeting the 18S ribosomal RNA [52]. Data of qPCR were available for  
85 287 women (at recruitment), 240 women (visit 1) and 74 women (visit 2).

86 At delivery, a total of 332 plasma samples from peripheral blood (195 HIV- and 137 HIV+) and  
87 303 cord blood samples (178 HIV- and 125 HIV+) were available. Peripheral blood smears  
88 were performed according to standard procedures for the microscopic detection of *P. falciparum*  
89 species [47, 48] and data were available for 308 women (183 HIV- and 125 HIV+). 50 µl of  
90 maternal peripheral blood were also collected at delivery on Whatman 903™ filter paper for the  
91 detection of *P. falciparum* by qPCR, and data were available for 242 women (163 HIV- and 79  
92 HIV+).

93 To assess PM, placental blood was collected to perform blood smears and qPCR. Data of blood  
94 smears and qPCR were available for 340 (197 HIV- and 143 HIV+) and 236 (157 HIV- and 79  
95 HIV+) women, respectively. Tissue samples from the maternal side of the placenta were also  
96 collected and placental histology was performed on samples from 307 study participants. Acute  
97 PM was defined by the presence of parasites on sections without malaria pigment; chronic PM,  
98 by presence of parasites and pigment; or past PM, by the presence of pigment alone. PM was  
99 considered positive if any of the tests performed (blood smear, qPCR or histology) were  
100 positive, therefore the 341 women had PM data for at least one of the tests.

#### 101 Antibody assays

102 For the quantification of IgG, IgG1, IgG2, IgG3 and IgG4 responses, quantitative suspension  
103 array technology (qSAT) applying the xMAP™ technology (Luminex Corp., TX) was  
104 performed.

105 Eight *P. falciparum* recombinant proteins were selected for our analysis: Duffy-binding like  
106 domains 3-4 (PfEMP1 DBL3-4 of var2csa PfEMP1, INSERM) [53], erythrocyte-binding  
107 antigen 140 (EBA140, Burnet Institute) [54], exported protein 1 (EXP1, Sanaria) [55], 42 kDA  
108 fragment of merozoite surface protein 1 (MSP1<sub>42</sub>, WRAIR) [56], merozoite surface protein 1  
109 block 2 (MSP1 bl2, University of Edinburgh) [57], merozoite surface protein 2 (MSP2,  
110 University of Edinburgh) [58], merozoite surface protein 5 (MSP5, Monash University) [59]  
111 and reticulocyte-binding-homologue-4.2 (Rh4.2, Burnet Institute) [60]. The proteins included in  
112 the panel are a selection of *P. falciparum* pregnancy-specific markers (DBL3-4) [53] and  
113 markers of malaria exposure (EXP1, MSP1<sub>42</sub> and MSP2) and immunity (EBA140, MSP1 bl2,  
114 MSP5 and Rh4.2 ) as defined in our previous study [38].

115 Standardization and optimization of the qSAT assays were previously performed to control for  
116 sources of variability [61–63]. First, antigens covalently coupled to MagPlex beads and  
117 resuspended in 50µL of PBS, 1% BSA, 0.05% Azide pH 7.4 (PBS-BN) were added to a 96-well  
118 µClear® flat bottom plate (Greiner Bio-One) in multiplex. Fifty µL of test samples, negative or  
119 positive controls [64] were added to multiplex wells and incubated overnight at 4°C protected  
120 from light. After incubation, plates were washed three times with PBS-Tween 20 0.05%. Then,  
121 100µL of anti-human IgG (Sigma B1140, dilution 1/2500), anti-human IgG1 (Abcam ab99775,  
122 dilution 1/4000), anti-human IgG2 (Invitrogen MA1-34755, dilution 1/500), anti-human IgG3  
123 (Sigma B3523, dilution 1/1000) or anti-human IgG4 (Invitrogen MA5-16716, dilution 1/500)  
124 were added and incubated for 45 min. After another plate washing cycle, 100µL of streptavidin-  
125 R-phycoerythrin (Sigma 42250) at 1/1000 dilution was added and incubated 30 min for IgG,  
126 IgG1 and IgG3. For IgG2 and IgG4, 100 µL of anti-mouse IgG (Fc-specific)–biotin (Merck  
127 B7401, 1/40000 and 1/10000 dilution, respectively) was added and incubated for 45 min,  
128 followed by another washing cycle and then incubation with streptavidin-R-phycoerythrin for  
129 30 min. Finally, plates were washed and beads were resuspended in 100 µL/well of PBS-BN.  
130 The Luminex 100/200 analyser was used for reading the plates and at least 20 microspheres per



131 analyte were acquired per sample. Antibody levels were measured as median fluorescence  
132 intensity (MFI). Data were captured using xPonent software.

133 Test samples were assayed at 2 dilutions for IgG (1/250 and 1/10000), and IgG1 and IgG3  
134 (1/100 and 1/2500). Only 1 dilution was tested for IgG2 and IgG4 (1/50) because of their usual  
135 low levels. A positive control (WHO Reference Reagent for anti-malaria *P. falciparum* human  
136 serum, NIBSC code: 10/198) in twelve serial dilutions (1:3, starting at 1/25) was used for  
137 QA/QC and to select optimal sample dilution for data analysis. For quality control, two blanks  
138 were added to each plate. Test samples were distributed across plates ensuring balanced groups.

### 139 Statistical analysis

140 MFI data were  $\log_{10}$ -transformed. The Shapiro-Wilk test of normality and the quantile-quantile  
141 (Q-Q) plot were performed to evaluate the distribution of such  $\log_{10}$ -transformed MFI antibody  
142 data. Boxplots and radar charts were used to represent the differences on antibody levels ( $\log_{10}$   
143 MFI) and placental transfer (measured as the cord blood/mother ratio) between groups of  
144 categorical variables (HIV and PM). The non-parametric Mann-Whitney U test was used to  
145 compare antibody levels and placental transfer between groups as  $\log_{10}$  MFI data were not  
146 normally distributed. Due to the high dimensionality of the data regarding the number of  
147 variables (5 IgG and IgG subclasses and 8 antigen combinations), Principal Component  
148 Analysis (PCA) of the cord and maternal blood  $\log_{10}$  MFI data was performed to explore and  
149 visualize overall antibody patterns. Only individuals with complete data for all the antigens and  
150 antibodies were included in the PCA analysis. The aim of a PCA analysis is to find a new  
151 reduced set of variables (called principal components, or dimensions) that explain as much of  
152 the information in the dataset as possible. The first dimension contains the most information  
153 about the original dataset, and explains most of the variation, and the last contains the least. We  
154 selected the two principal components or dimensions that best explained the variance of the data  
155 and plotted the PCA scores. These plots allow visualizing clusters of samples based on their  
156 similarity.

157 Univariable linear regression models were performed to determine the effect of covariables on  
158 the cord blood antibody levels or placental transfer of antibodies. The variables analysed in the  
159 univariable models were maternal antibody levels ( $\log_{10}$  MFI), maternal HIV infection, PM, age,  
160 gravidity (defined as primigravidae and multigravidae), maternal anaemia (defined as  
161 haemoglobin levels  $<11\text{g/dL}$ ), LBW (defined as  $<2500\text{g}$  at birth), prematurity (defined as  
162 delivery before 37 weeks of gestational age), gestational age (measured by Ballard score [65]),  
163 treatment arms (defined as MQ or placebo in the HIV+ women study and MQ full, MQ split or  
164 SP in HIV- women study), antiretroviral therapy (ART) received before pregnancy, started at  
165 recruitment or not received at all,  $\text{CD4}^+$  T cell counts ( $<350$  cells/ $\mu\text{L}$  or  $\geq 350$  cells/ $\mu\text{L}$ ), HIV  
166 viral load ( $<400$ , 400-999, 1000-9999 and  $>9999$  copies/mL), and seasonality (dry or rainy).  
167 Gravidity was defined as primigravidae and multigravidae following the approach used in  
168 previous studies and due to the lack of significant differences on antibody levels between  
169 secundigravidae and multigravidae in other studies [47, 48, 66–68]. Seasonality was defined as  
170 rainy if at least 4 of the pregnancy months fell under the category of rainy period (November-  
171 April), and defined as dry in any other case. Multivariable regression analyses were performed  
172 for each antigen and IgG or IgG subclass including always maternal antibody levels and  
173 maternal HIV infection (statistically significantly associated in all univariable analyses) and the  
174 additional predictors that resulted in the best fitted and simpler (less variables) models.  
175 Specifically, we tested exhaustively all possible combinations of the predictor variables and  
176 selected the models with lower Akaike information criterion (AIC) and Bayesian information  
177 criterion (BIC) and higher adjusted r-square. Then, variables that appeared significant in most of  
178 the best models for each antigen/subclass and that also had more significant associations in  
179 univariable analyses (significant adjusted p-values) were included in all the models, i.e. PM and  
180 LBW. The betas obtained in each case were transformed into a percentage for interpretation. For  
181 maternal antibody levels (log-log model) the beta transformed value (%) was calculated with the  
182 formula  $((10^{(\beta \cdot \log_{10}(1.1))} - 1) \cdot 100)$ . This represents the effect (in percentage) of a 10%  
183 increase in the corresponding predictor variable on IgG and IgG subclass cord blood levels. For  
184 maternal HIV infection, PM and LBW (log-linear models), the beta transformed value (%) was

185 calculated with the formula  $((10^{\beta})-1)*100$ . This gives the difference (in percentage) in IgG  
186 and IgG subclass cord blood levels or placental transfer between the reference group and the  
187 study group (e.g. the difference between cord antibody levels of HIV- women compared with  
188 HIV+ women cord antibody levels).

189 All p-values were considered statistically significant when  $<0.05$  after adjusting for multiple  
190 testing through Benjamini-Hochberg. Adjustments for multiple testing were done separately for  
191 each IgG subclass. Data were managed and analysed using the R software version 3.6.3 and its  
192 package devtools [69]. The ggplot2 package was used to perform boxplot graphs [70]. The  
193 FactoMineR [71] and factoextra [72] packages were used to perform PCA.

194

## 195 **Results**

### 196 Description of participants

197 Study participants consisted of 341 pregnant women (197 HIV- and 144 HIV+) (Table 1). Their  
198 median age was 25 years old (interquartile range [IQR] 19-29) and HIV+ women (median of 27  
199 years) were older than HIV- women (median of 21 years). Less than a fourth (24%) of the  
200 participants were primigravidae, and there were more primigravidae in the HIV- group (35%)  
201 compared to the HIV+ group (9%). Maternal anaemia was more prevalent among HIV+ women  
202 (68.8%) than HIV- women (56.2%). No significant differences were found in birth weight or  
203 prematurity between infants born to HIV+ and those born to HIV- women. Only 20 women had  
204 PM and the proportion of PM between HIV+ and HIV- women was similar: 13 HIV- (6.6%)  
205 and 7 HIV+ (4.9%). Among these 20 women, 3 had acute PM and 8 past PM (defined through  
206 histology), 5 had positive placental blood smears and 11 had positive placental qPCR, of which  
207 7 were only qPCR positive. A total of 51 women had peripheral malaria (positive in peripheral  
208 blood by microscopy and/or PCR at any of the visits during pregnancy) but there were no  
209 differences by HIV infection.

### 210 Profile of cord blood antibody levels and placental transfer to *P. falciparum* antigens

211 The PCA analysis of antibody levels in 303 cord and 332 maternal blood samples showed very  
212 similar patterns (maternal antibody PCA analyses not shown). Clusters showing similarity of  
213 responses were detected in cord antibody levels by IgG subclasses (Fig. 1a) and antigens (Fig.  
214 1b). While dimension 1 explained the majority of the variance and contributed to the separation  
215 of IgG4, IgG2 and IgG/IgG1/IgG3, dimension 2 contributed to the separation of the IgG1 and  
216 IgG3 responses (Fig. 1a) and MSP1 bl2, MSP2 from the rest of the antigens (Fig. 1b). DBL3-4  
217 greatly contributed to IgG1 whereas MSP1 bl2 and MSP2 contributed more to the IgG3  
218 responses (Fig. 1a). DBL3-4 was clearly separated from the rest indicating a different antibody  
219 profile (Fig. 1b). Consistently, DBL3-4 had lower IgG3 levels and MSP1 bl2 and MSP2 had  
220 lower IgG1 levels than the other antigens (Fig. 1c). Overall, IgG2 had lower median levels than  
221 IgG1 and IgG3 for most antigens, except for MSP1 bl2, MSP2 and DBL3-4. The lowest levels  
222 were shown for IgG4 in all antigens, with especially very low responses for DBL3-4, MSP5 and  
223 Rh4.2 (Fig. 1c).

224 For the placental transfer, DBL3-4 antibodies were the most efficiently transferred, especially  
225 IgG4 followed by IgG3 and finally IgG2 (Fig. 1d). For the rest of antigens, the four IgG  
226 subclasses showed similar placental transfer, of which IgG2 was the lowest.

#### 227 Altered maternal and cord blood anti-*P. falciparum* IgG levels by HIV and placental malaria

228 First, we compared total IgG levels in HIV+ and HIV- mothers for 332 maternal (137 HIV+ and  
229 195 HIV-), and 303 cord samples (125 HIV+ and 178 HIV-). In HIV+ women, both maternal  
230 and cord blood IgG levels were lower for EXP1 and MSP5 (Fig. 2a). Second, we assessed the  
231 differences between mothers with and without PM in maternal and cord total IgG levels (Fig.  
232 2b). IgG levels against MSP2 were higher among women with PM than those without PM.  
233 Also, cord blood IgG levels against EXP1 and MSP2 were higher among women with PM.

234 We also looked at the differences in maternal and cord IgG subclasses levels by HIV infection  
235 (Fig. 2c). In HIV+ women, maternal levels were lower for IgG1 DBL3-4, MSP2 and MSP5 than  
236 for HIV- women (Fig. 2c). Maternal levels of IgG2 against EXP1 and MSP2 were also lower in

237 HIV+ women compared with HIV- women. IgG3 maternal levels were only lower among HIV+  
238 women against DBL3-4, whereas IgG4 levels in HIV+ women were lower than HIV- women  
239 against EBA140, EXP1, MSP1<sub>42</sub> and MSP1 bl2. Statistically significant differences were found  
240 in the cord for the same antigens and IgG subclasses as in the mother, with the exception of  
241 DBL3-4 IgG3 and EXP1 IgG4 that were not significantly different in the cord. Regarding PM,  
242 there were no significant differences between women with and without PM in IgG subclass  
243 levels, although there was a general positive trend in women with PM (Additional file 1: Figure  
244 S2-S3).

#### 245 Factors associated with anti-*P. falciparum* IgG cord blood levels

246 For the multivariable analyses, we selected log<sub>10</sub> MFI maternal antibodies, HIV infection, PM  
247 and LBW, as they were significant in univariable models (Supplementary material 1) and  
248 improved the model performances, having lower AIC and BIC, and higher adjusted r-squares.  
249 Maternal antibody levels had a high positive correlation with cord blood antibody levels for all  
250 the antigens and subclasses (Fig. 3a). A 10% increase in maternal total IgG levels and IgG  
251 subclasses was associated with 6.03% to 9.75% increases in total IgG and IgG subclass cord  
252 blood levels, depending on the antigen and IgG subclass.

253 Maternal HIV infection was negatively associated with cord blood antibody levels, reducing  
254 IgG to EXP1 and MSP5 by 3.84% and 1.47%, respectively; IgG1 to MSP2 and Rh4.2 by 9.09%  
255 and 3.12%, respectively; and IgG4 to MSP1<sub>42</sub> by 1.91%. No significant effect was found for  
256 IgG2 and IgG3 levels in cord blood (Fig. 3b). PM negatively impacted IgG cord blood levels  
257 against EBA140, MSP1 bl2 and Rh4.2 (2.19%, 2.53% and 3.52% reduction, respectively), and  
258 IgG2 to EBA140 (4.58% reduction) (Fig. 3c). When analysing HIV+ women only, PM was also  
259 associated with lower IgG2 to DBL3-4 (Additional file 1: Figure S4). LBW was positively  
260 associated with cord blood IgG2 levels against EBA140 and Rh4.2, with a 5.46% and 8.14%,  
261 increase, respectively (Fig. 3d). No significant associations were found for LBW and total IgG  
262 or the rest of the subclasses. Age, maternal anaemia, gravidity, IPTp treatment, prematurity,

263 seasonality, and CD4<sup>+</sup> T cell counts, ART and viral load for HIV<sup>+</sup> women were not included in  
264 the models following the AIC, BIC and r-square criteria.

#### 265 Decreased placental transfer of anti-*P. falciparum* IgGs by HIV and placental malaria

266 The radar charts (Fig. 4) showed that HIV<sup>+</sup> women had a reduced placental transfer of  
267 antibodies compared to HIV<sup>-</sup> women. This was significant for IgG and IgG1 against DBL3-4,  
268 EBA140, EXP1, MSP1<sub>42</sub>, MSP1 bl2, MSP2 and MSP, IgG1 against Rh4.2 (Fig. 4a-4b), and  
269 IgG4 against MSP1<sub>42</sub> (Additional file 1: Figure S4). However, HIV infection increased the  
270 transfer of IgG4 against DBL3-4 and also a trend was seen for IgG3 (Additional file 1: Figure  
271 S5). No significant differences in placental transfer between the two groups were found for  
272 IgG2 or IgG3.

273 In multivariable models including HIV, PM and LBW (variables showing an effect on placental  
274 antibody transfer in univariable models (Supplementary Material 1) and that when included in  
275 the models these had lower AIC and BIC and higher adjusted r-square), HIV infection was  
276 associated with a reduced placental transfer of IgG against EXP1 (3.10% reduction) and IgG1  
277 against MSP2 and Rh4.2 (8.01% and 2.84% reductions, respectively) (Fig. 5a). PM was  
278 associated with a diminished placental transfer of IgG to MSP1 bl2 and Rh4.2 (3.47% and  
279 4.46% reductions, respectively) (Fig. 5b). LBW did not have any significant impact on  
280 transplacental transfer of antibodies, although when considering raw p-values LBW was  
281 associated with higher placental transfer of IgG2 to EXP1, MSP5 and Rh4.2 (Fig. 5c). No  
282 additional variables were included in the multivariable analysis as they did not provide any  
283 added value to the models following the AIC, BIC and r-square criteria.

284

#### 285 **Discussion**

286 Our study provides a better understanding of the factors that affect placental transfer and cord  
287 blood levels of anti-malarial antibodies, especially IgG subclasses, which are relevant for  
288 malaria protection during the first months of life. We found that the main determinant of cord

289 antibody levels was the corresponding maternal levels, and that maternal HIV infection was  
290 generally associated with diminished cord IgG levels, although this effect was antigen-subclass  
291 dependent. Also, PM showed some association with lower cord blood IgG levels and placental  
292 transfer against malaria immunity-related antigens.

293 The highly associated mother and cord blood antibody levels are consistent with previous  
294 studies [19, 30, 73, 74]. The maternal antibodies transferred to the newborn are suggested to be  
295 protective against malaria infection during the first months of life. At the same time, these  
296 transferred antibodies may interfere with the acquisition of protective antibodies after malaria  
297 vaccination, as seen in RTS,S/AS01E immunisation against CSP and indirectly against non-CSP  
298 protection-related antigens [37–39].

299 Reaching protective cord antibody levels against malaria is essential for the newborn but HIV  
300 infection and PM could interfere with the efficiency of this passive immunity. Here, maternal  
301 HIV infection was associated with diminished antibody levels in the cord, but this was strongly  
302 antigen-subclass dependent, in line with previous studies in which maternal and cord IgG levels  
303 against some antigens related to malaria exposure and protection were lower in HIV+ women  
304 [28, 30, 31]. These previous studies show some discrepancies with the effect of maternal HIV  
305 infection on antimalarial cord antibody levels and placental transfer, and this could be due to  
306 different malaria prevalence, study sample sizes, sensitivities among the serological methods,  
307 and the variables used in the model adjustment [28, 30, 31].

308 Despite the low number of women with any evidence of PM in the study, PM also had an  
309 impact on the anti-malarial IgG transplacental transfer. Reduced transplacental transfer of  
310 antibodies associated with PM has been found in several studies [19, 26, 75] and may be due to  
311 damaged placental tissue. *P. falciparum*-infected erythrocytes and immune cells infiltrate within  
312 the intervillous spaces of the placenta causing inflammation, fibrinoid necrosis, basal membrane  
313 thickening and increase of the number of syncytial knots, and it may alter the exchange system  
314 between mother and foetus, including Fc receptors [76, 77].

315 Due to the importance of IgG subclasses on antimalarial effector immunity we wanted to assess  
316 their levels in the mother and their transfer to the foetus. Cord IgG1 and IgG3 levels were the  
317 highest and IgG4 the lowest for most antigens. In contrast, for most of the antigens, IgG4 was  
318 the most efficiently transferred, especially for the pregnancy-specific *P. falciparum* antigen  
319 DBL3-4 VAR2CSA, followed by IgG1 or IgG3 (depending on the antigen) and finally IgG2.  
320 This could be explained by lower maternal antibody concentrations having higher active  
321 placental transport [78]. Indeed, DBL3-4 had the highest placental transfer efficacy of IgG4  
322 despite cord IgG4 levels being the lowest. This ranking was unexpected because IgG1 followed  
323 by IgG4, IgG3 and finally IgG2 have been commonly stated as the best transferred subclasses  
324 [15, 79], although a recent manuscript reported a hierarchy of IgG1>IgG3>IgG4=IgG2 and  
325 identified a number of other studies that also observed different transfer efficiencies [80], such  
326 as our recent report [74]. This suggests that the IgG subclasses transfer efficiency may vary  
327 between study populations, as well as by maternal antigen exposure.

328 IgG1 and IgG3 are cytophilic antibodies, which can interact with complement and Fc $\gamma$ -receptors  
329 [81], and are considered to be protective [32, 33, 82]. Therefore, their high cord levels could be  
330 related with an effective induction of effector functions that are essential for *Plasmodium*  
331 clearance, as previously seen with members of the PfRh [83, 84], EBA invasion ligand families  
332 [35] and MSP5 [85]. IgG2 and IgG4 are non-cytophilic antibodies and have been classically  
333 correlated with disease [32, 86]. However, we recently proposed that the pattern of cytophilic  
334 and non-cytophilic IgG antibodies is antigen-dependent and both types could be involved in  
335 protection [34] since not all protective mechanisms require Fc-mediation [87]. A shift from anti-  
336 MSP2 IgG1 in primary malaria infections towards IgG3 in subsequent malaria infections  
337 indicates that IgG3 could be related with protection [88, 89], similarly to MSP1 bl2 IgG3 [90].  
338 Anti-IgG2 MSP2 increases with age and inversely associates with risk of infection, while IgG4  
339 levels have been positively associated with risk [91]. Thus, the high anti-MSP2 IgG2 and IgG3  
340 levels in the cord and lower IgG4 we observe could be associated with malaria protection in



341 infants. However, the relative importance of IgG subclasses in protective immunity is not clear  
342 and further research is needed in this regard.

343 HIV infection reduced IgG1 cord levels against MSP2 and Rh4.2 due to an impairment of the  
344 IgG1 transplacental transfer. Although it has been previously reported that maternal HIV  
345 negatively affected MSP1 IgG1 [30, 31] and IgG3 [30] cord levels, we did not find any  
346 significant association between HIV infection and MSP1 IgG1-3 cord levels. However, we  
347 observed lower IgG4 cord levels against MSP1<sub>42</sub>. Diminished levels of these antibodies could  
348 explain higher risk of infection, as cytophilic antibodies have been suggested to contribute to  
349 protection from clinical malaria in adults and children in endemic areas [34, 92] and IgG4  
350 subclass has also been associated with malaria protection [34, 93]. LBW was previously  
351 associated with a reduction in cord blood levels and placental transfer of antibodies [94–96], but  
352 in this study we did not observe any association of LBW with lower cord levels or placental  
353 transfer. However, our results are consistent with other studies that did not show any impact of  
354 LBW on IgG and subclass cord levels against some antimalarial antigens [30, 31]. Surprisingly,  
355 LBW was associated with higher cord IgG2 levels against EXP1 and Rh4.2 and, to our  
356 knowledge, this is the first time that this observation has been reported. IgG2 antibodies are  
357 associated with increased risk of severe malaria [97] and, therefore, LBW infants may have  
358 higher risk to suffer from malaria complications than normal weight infants. No associations  
359 were found between maternal age, anaemia, gravidity and IPTp treatment and cord levels or  
360 placental transfer of antibodies against antimalarial antigens [28, 30]. We did not find either any  
361 significant differences between mothers who initiated ART before pregnancy, mothers who  
362 started during pregnancy, and mothers not taking ART. Previous studies on the effect of ART  
363 on placental transfer of antibodies are controversial and the effect varied depending upon the  
364 antigen, the initiation and type of treatment, and the dose. For example, Goetghebuer et al.  
365 observed the lowest maternal antibody transfer ratios against 5 vaccine and 2 pathogen antigens  
366 in HIV+ mothers who initiated ART during pregnancy, compared with those who initiated ART  
367 before pregnancy [98]. However, this study did not include *P. falciparum* antigens. Moro et al.

368 found reduced placental transfer of antibodies against MSP1, AMA1 and EBA175 in HIV+  
369 women receiving no ART, although in this cohort women with ART were not included [30].  
370 Ray et al. showed lower placental transfer of antibodies against the same antigens in women  
371 taking optimal ART treatment [28], suggesting that ART treatment did not make any difference  
372 in the transplacental transfer of these antimalarial antibodies. In the same line, Babakhanyan et  
373 al. reported lower placental transfer of antibodies against CSP, AMA1 and MSP1 in HIV+  
374 women taking only nevirapine at delivery than HIV- women [31]. On the contrary, Ayisi et al.  
375 found that HIV+ women not receiving ART had reduced transfer of antibodies against CSP but  
376 not against MSP1 or EBA175 [29].

377 Our study is subjected to some limitations. Specifically, hypergammaglobulinemia, which has  
378 been associated with a reduced transplacental transfer of antibodies [23, 25, 26], was not  
379 measured. Chronic infections such as HIV, but also malaria, induce hypergammaglobulinemia  
380 [99, 100], and it has been reported that 94% of women with hypergammaglobulinemia also had  
381 PM [25]. Consequently, the effect of maternal HIV and PM on cord blood levels and placental  
382 transfer might be in part due to hypergammaglobulinemia. Another limitation is that we had a  
383 low number of PM cases, which may result in low statistical power to detect significant  
384 associations. In addition, qPCR data were not available from all women and, consequently, we  
385 may have missed some cases of submicroscopic PM (only detected by qPCR). This is of  
386 specially importance as there are studies reporting that women with submicroscopic PM had  
387 higher inflammation markers than women without PM [101, 102], which could affect the  
388 placental transfer of antibodies. Finally, the impact of the observed differences in cord antibody  
389 levels on the malaria risk in the infants was not evaluated of this cohort and will be addressed  
390 on future studies.

391 In conclusion, our results demonstrate that maternal HIV infection was associated with reduced  
392 levels of antibodies, mostly IgG and IgG1, against some antimalarial antigens in cord blood.  
393 Part of this reduction in antibody levels was due to altered antibody levels in the mother, which  
394 is the main determinant of cord blood levels, but HIV-infection also diminished transplacental

395 transfer of antibodies. PM also reduced IgG cord levels to some malaria protection-related  
396 antigens, and LBW was associated with increased anti-malaria IgG2 cord levels, also related to  
397 a higher risk of severe malaria in the infant. Overall, the findings are important for better  
398 understanding the role of maternal HIV infection and malaria in the placental transfer of  
399 antimalarial antibodies, which is essential for protecting the infant against the severe  
400 consequences of malaria during the first months of life.

401

#### 402 **Additional files**

403 Additional file 1: Supplementary information including figures and methods. Figure S1. IPTp  
404 trial profile. Figure S2. Maternal blood antibody levels in women with PM and women without  
405 PM. Figure S3. Cord blood antibody levels in women with PM and women without PM. Figure  
406 S4. Effect of placental malaria on cord blood antibody levels in HIV+ women. Figure S5.  
407 Cord/mother ratios in HIV+ and HIV- women.

408 Supplementary material 1: Cord blood levels and placental transfer of antibodies univariable  
409 models.

410

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420 GM conceived the immunological study and the experimental design and interpreted the data.  
421 CD, GM, RA and SA designed the analysis and selection of the antigens. SA and MV  
422 performed the antibody Luminex assay. EA, RLC, BG, DC and JGB contributed with the  
423 resources. GRO and MVS performed the statistical analysis. AM, CD and GM designed the  
424 immunology study ancillary to the clinical trials. MNM, RB and CJ processed the samples. PC  
425 and LFS performed the PCR. RG, MR, JJA, EM, AV, ES and CM designed and enrolled  
426 participants in the clinical trials. AN was the clinical trial data manager. JJA was the clinical  
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445

446 **Availability of data and materials**

447 All data analysed during this study are included in this article and its supplementary information  
448 files or are available from the authors upon request.

449

450 **Ethics approval and consent to participate**

451 This study was carried out in accordance with ICH Good Clinical Practice guidelines and the  
452 Declaration of Helsinki. The study protocols and informed consent forms were reviewed and  
453 approved by the Comit   Ètic d'Investigaci   Cl  nica (CEIC, Hospital Cl  nic, UB), Spain, and the  
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456

457 **Consent for publication**

458 Not applicable.

459

460 **Competing interests**

461 The authors declare that they have no competing interests.

462

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- 783

**Table 1:** Characteristics of study participants.

	All N=341	HIV- N=197	HIV+ N=144	p-value <sup>a</sup>
Age <sup>a</sup> (years median [IQR])	25.0 [19.0; 29.0]	21.0 [18.0; 28.0]	27.0 [22.0; 31.0]	< 0.001
Gravidity (n, %)				< 0.001
<i>Multigravidae</i>	259 (76.0)	128 (65.0)	131 (91.0)	
<i>Primigravidae</i>	82 (24.0)	69 (35.0)	13 (9.0)	
Maternal haemoglobin (n, %)				0.025
Anaemia (< 11 g/dL)	208 (61.5)	109 (56.2)	99 (68.8)	
Normal (≥ 11 g/dL)	130 (38.5)	85 (43.8)	45 (31.2)	
Birth weight (n, %)				1.000
Low (< 2500 g)	29 (8.5)	17 (8.6)	12 (8.33)	
Normal (≥ 2500 g)	312 (91.5)	180 (91.4)	132 (91.7)	
Prematurity (n, %)				0.502
No (≥ 37 weeks)	312 (94.3)	181 (95.3)	131 (92.9)	
Yes (< 37 weeks)	19 (5.7)	9 (4.7)	10 (7.1)	
Treatment				< 0.001
MQ	71 (20.9)	0 (0.0)	71 (49.7)	
MQ full	68 (20.8)	68 (34.5)	0 (0.0)	
MQ split	73 (21.5)	73 (37.1)	0 (0.0)	
Placebo	72 (21.2)	0 (0.0)	72 (50.3)	
SP	56 (16.5)	56 (28.4)	0 (0.0)	
ART (n, %)				NP
No	24 (7.1)	–	24 (17.1)	
Yes	116 (34.4)	–	116 (82.9)	
CD4 <sup>+</sup> T cell counts (n, %)				NP
Lower (< 350 c/μL)	40 (12.3)	–	40 (31.2)	
Higher (≥ 350 c/μL)	88 (27.1)	–	88 (68.8)	
HIV viral load (copies/mL)				NP
< 400	21 (6.4)	–	21 (16.0)	
(400–999)	41 (12.5)	–	41 (31.3)	
(1000–9999)	48 (14.6)	–	48 (36.6)	
> 9999	21 (6.4)	–	21 (16.0)	
Placental malaria <sup>b</sup> (n, %)				0.659
No	321 (94.1)	184 (93.4)	137 (95.1)	
Yes	20 (5.9)	13 (6.6)	7 (4.9)	
Peripheral malaria <sup>c</sup> (n, %)				0.531
No	290 (85.0)	165 (83.8)	125 (86.8)	
Yes	51 (15.0)	32 (16.2)	19 (13.2)	

For numerical variables, the median and first and third quantile, in brackets, are given. For the categorical variables the number of individuals for each group and percentages in parentheses, are given.

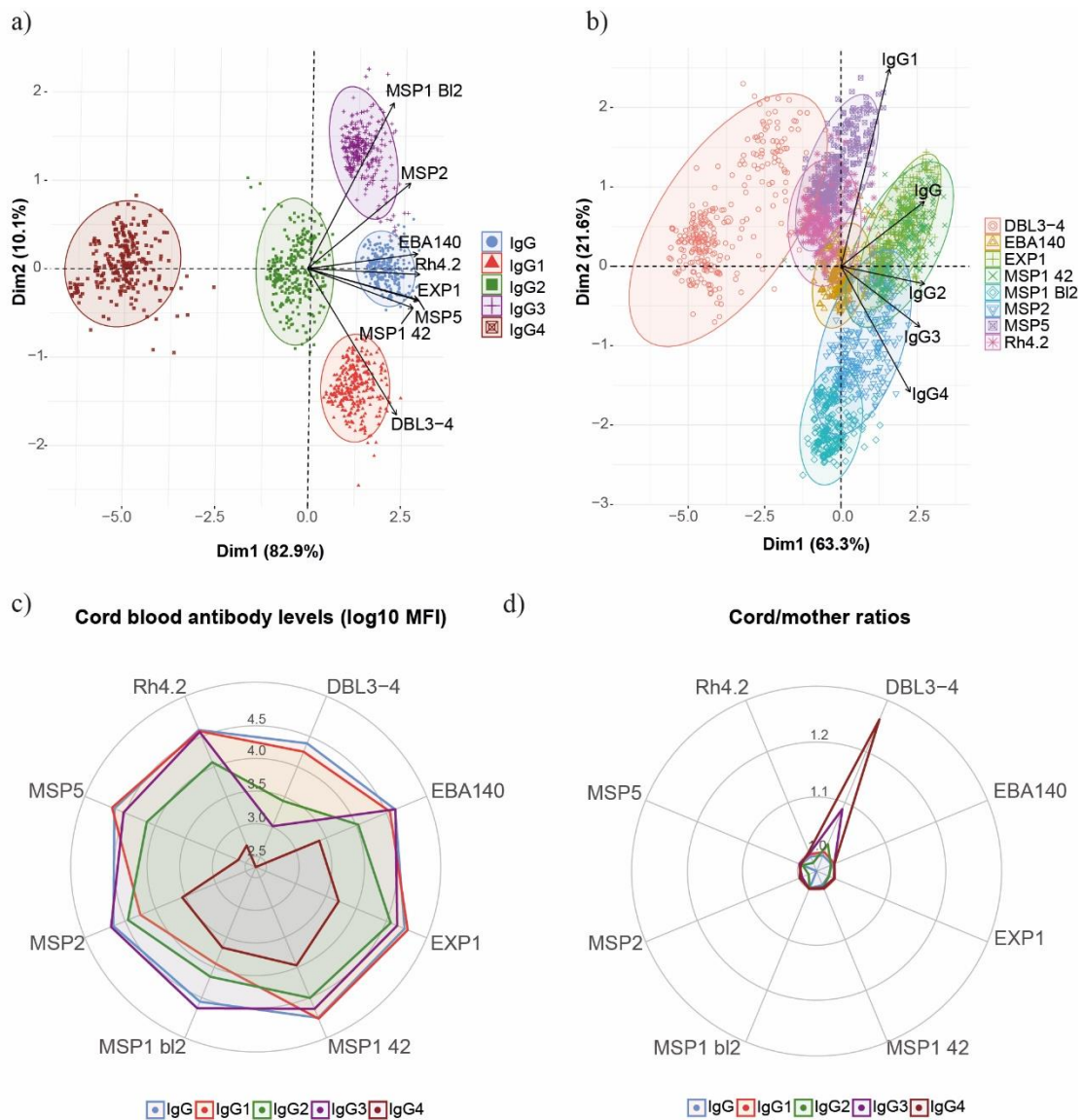
<sup>a</sup> For the age, the Mann-Whitney U test was used to compare differences between median values. For the categorical variables, the Chi-square test was used.

<sup>b</sup> Placental malaria was considered positive if there was any evidence of *P. falciparum* placental parasitaemia by any method.

<sup>c</sup> Peripheral malaria was considered positive if there was any evidence of *P. falciparum* peripheral parasitaemia by any method.

Statistical significance was considered when *p*-value ≤ 0.05; MQ, mefloquine; NP, not-performed tests; SP, sulfadoxine-pyrimethamine.

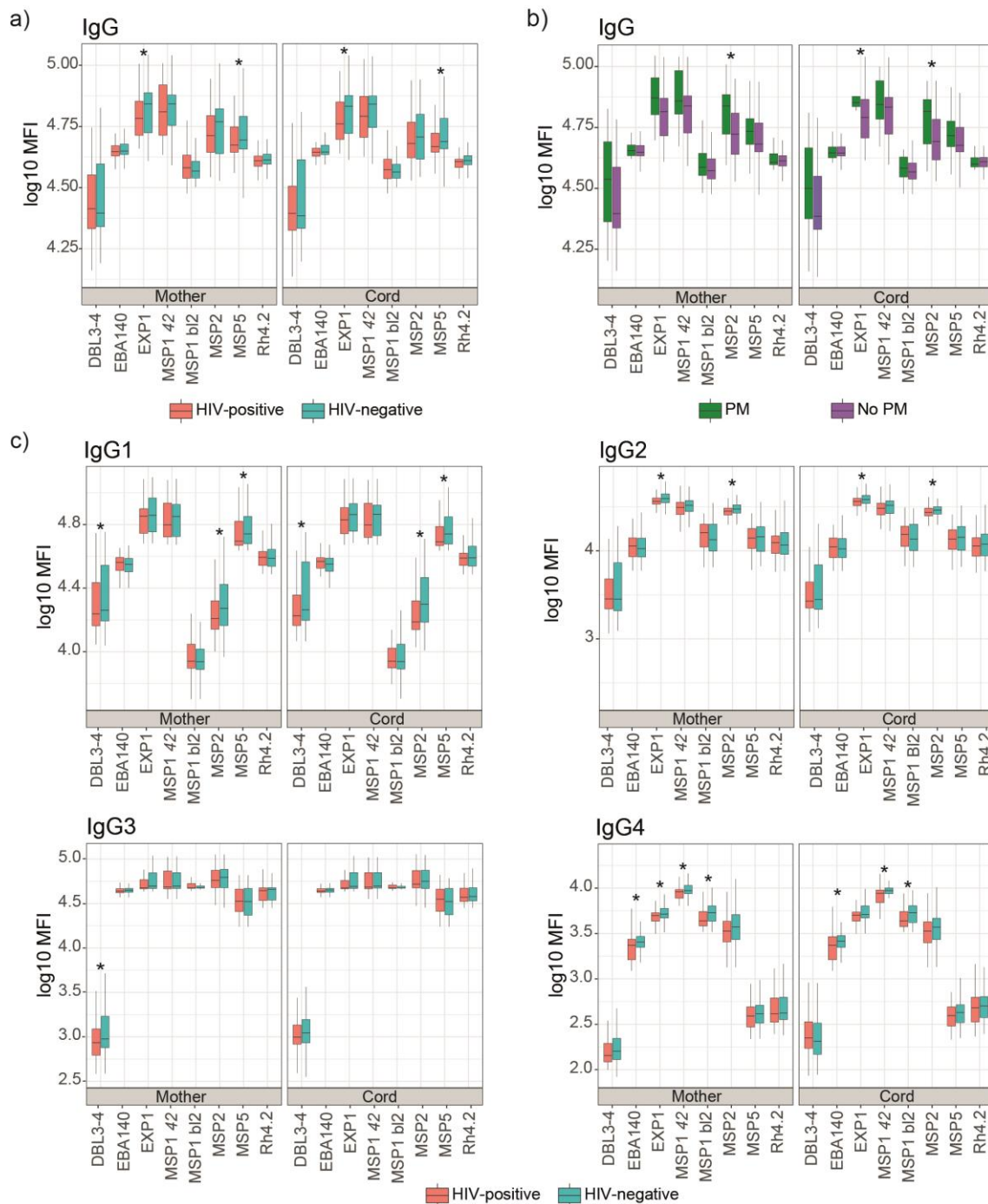
785 **Figures**



786

787 **Fig. 1:** Overview of cord blood levels of IgG and IgG subclasses to *P. falciparum* antigens for all women.  
 788 a) Principal component analysis (PCA) plots of cord IgG and IgG subclass levels  
 789 against all antigens clustered by subclass type. b) PCA plots of cord IgG and IgG subclass levels  
 790 clustered by antigen type. The two principal components (Dim 1, Dim 2) that explained the  
 791 highest percentage of the variance of the data (percentage in parenthesis) were chosen for  
 792 representation. The arrows in a) and b) represent how the variables contribute to each of the two  
 793 principal components. c) Medians of IgG and IgG subclass levels (log<sub>10</sub> MFI) in cord blood for  
 794 each antigen. d) Medians of IgG and IgG subclass placental transfer for each antigen,  
 795 represented as the cord/mother ratios.

796

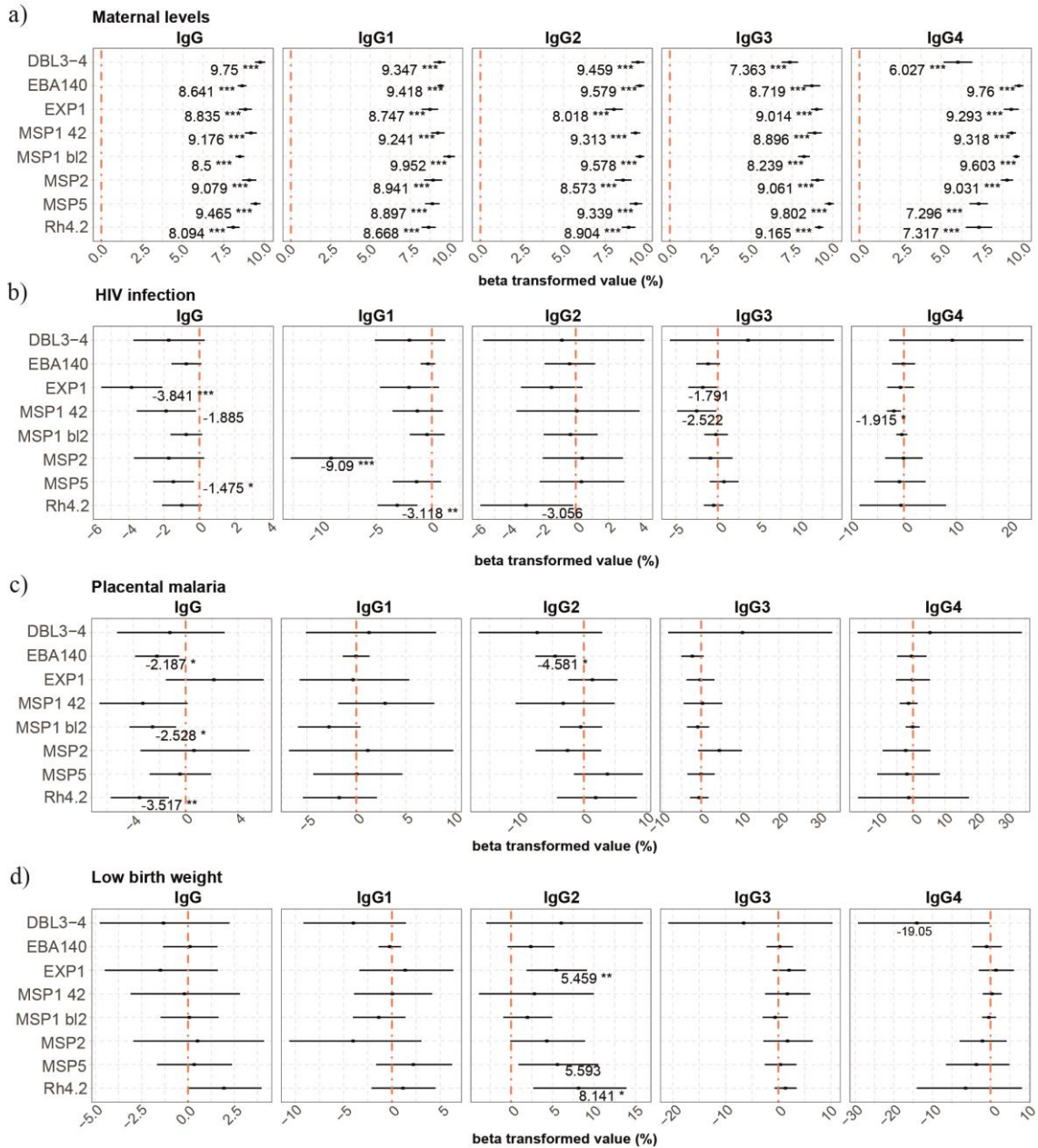


797

798 **Fig. 2:** Mother and cord blood antibody levels (log<sub>10</sub> MFI) in HIV-positive and HIV-negative  
 799 women and women with PM and without PM. Boxplots illustrate the medians and the  
 800 interquartile range for IgG in HIV-positive and HIV-negative women (a), IgG in women with  
 801 PM and women without PM (b), and IgG1, IgG2, IgG3 and IgG4 subclasses in HIV-positive  
 802 and HIV-negative women (c). Levels between groups were compared by the non-parametric  
 803 Mann–Whitney U test and p-values were adjusted for multiple testing by the Benjamini-  
 804 Hochberg approach. Statistically significant differences are highlighted with an asterisk. HIV-  
 805 positive women are represented in red, HIV-negative women in blue, women with PM in green  
 806 and women without PM in purple.

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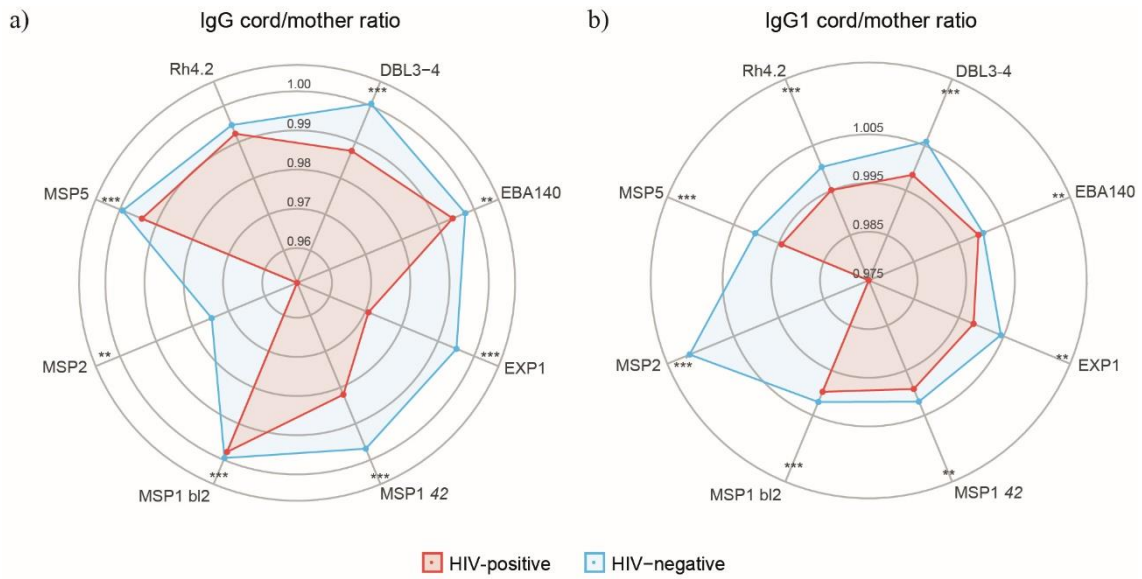




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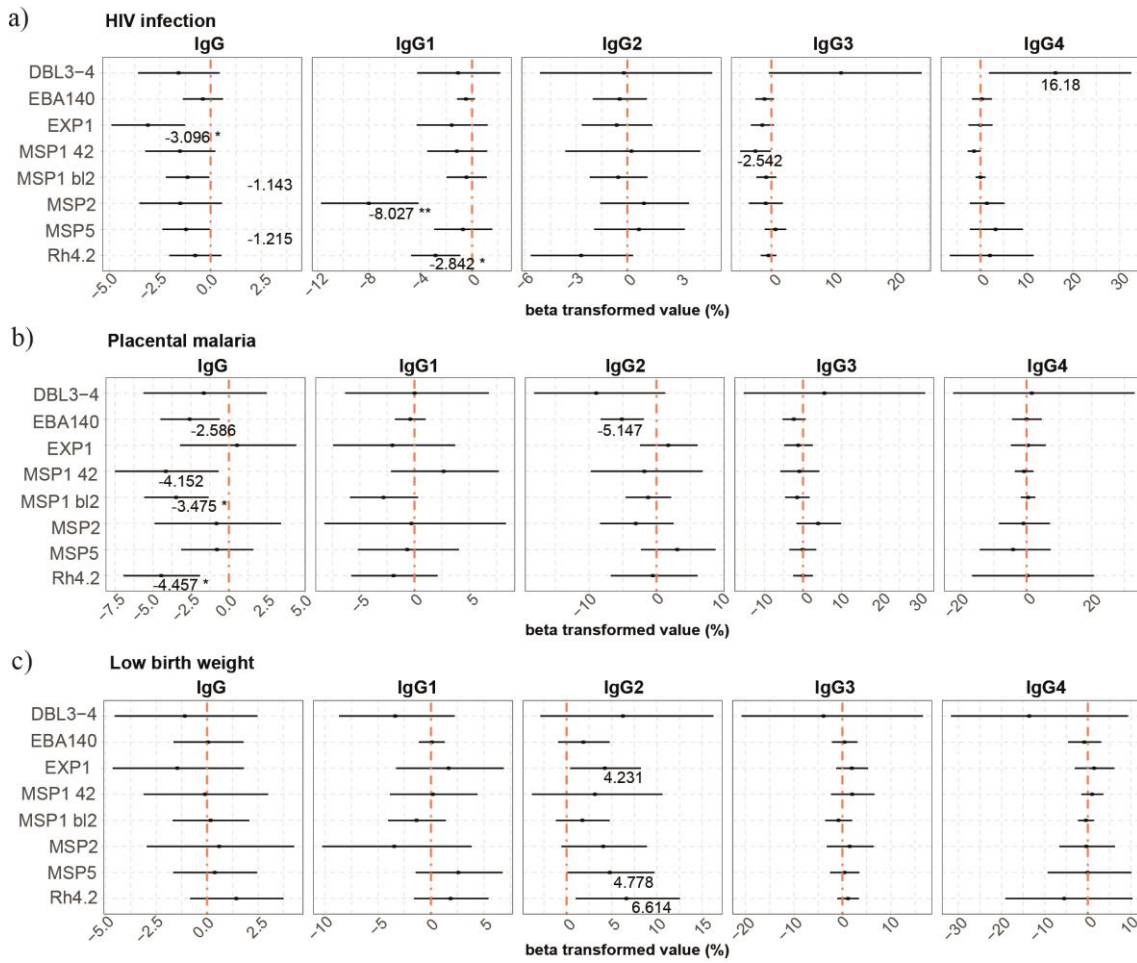
809 **Fig. 3:** Difference of IgG and IgG subclass levels in cord blood by study factors. Forest plots  
 810 show the effect (in percentage) of a) maternal antibody levels, b) HIV infection, c) placental  
 811 malaria and d) low birth weight, on cord blood levels of IgG and IgG subclasses for all the  
 812 antigens tested. The differences in percentage correspond to beta transformed values (%) that  
 813 were calculated from the beta values obtained in the multivariable models. Beta transformed  
 814 values (%) are displayed when raw p-values are significant. Asterisks are shown when adjusted  
 815 p-values by Benjamini-Hochberg are significant. \*\*\* = p-value  $\leq$  0.001, \*\* = p-value  $\leq$  0.01, \*  
 816 = p-value  $\leq$  0.05.

817



819 **Fig. 4:** Antibody placental transfer in HIV-positive and HIV-negative women. Radar charts  
 820 representing the medians of each analyte antibody cord/mother ratio in HIV-positive and HIV-  
 821 negative women for IgG (a) and IgG1 subclass (b). Ratios between HIV-positive and negative  
 822 women were compared by the non-parametric Mann-Whitney U test and p-values were adjusted  
 823 for multiple testing by the Benjamini-Hochberg approach. Statistically significant differences  
 824 between HIV-positive and negative women ratios are highlighted with asterisks. \*\*\* = p-value  
 825  $\leq 0.001$ , \*\* = p-value  $\leq 0.01$ , \* = p-value  $\leq 0.05$ . HIV-positive women are represented in red  
 826 and HIV-negative women in blue.

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831

832 **Fig. 5:** Difference of IgG and IgG subclass placental transfer by study factors. Forest plots show  
 833 the effect (in percentage) of a) HIV infection, b) placental malaria and c) low birth weight, on  
 834 placental transfer of IgG and IgG subclasses for all the antigens tested. The differences in  
 835 percentage correspond to beta transformed values (%) that were calculated from the beta values  
 836 obtained in the multivariable models. Beta transformed values (%) are displayed when raw p-  
 837 values are significant. Asterisks are shown when adjusted p-values by Benjamini-Hochberg are  
 838 significant. \*\*\* = p-value  $\leq 0.001$ , \*\* = p-value  $\leq 0.01$ , \* = p-value  $\leq 0.05$ .

839