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.

Introduction

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

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

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

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

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

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

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

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

Materials and Methods

Study design and sample collection

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

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

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

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

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

Antibody assays

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

Eight *P. falciparum* recombinant proteins were selected for our analysis: Duffy-binding like domains 3-4 (PfEMP1 DBL3-4 of var2csa PfEMP1, INSERM) [53], erythrocyte-binding antigen 140 (EBA140, Burnet Institute) [54], exported protein 1 (EXP1, Sanaria) [55], 42 kDA fragment of merozoite surface protein 1 ($MSP1_{42}$, WRAIR) [56], merozoite surface protein 1 block 2 (MSP1 bl2, University of Edinburgh) [57], merozoite surface protein 2 (MSP2, University of Edinburgh) [58], merozoite surface protein 5 (MSP5, Monash University) [59] and reticulocyte-binding-homologue-4.2 (Rh4.2, Burnet Institute) [60]. The proteins included in the panel are a selection of *P. falciparum* pregnancy-specific markers (DBL3-4) [53] and markers of malaria exposure (EXP1, $MSP1_{42}$ and MSP2) and immunity (EBA140, MSP1 bl2, MSP5 and Rh4.2) as defined in our previous study [38].

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

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

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

Statistical analysis

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

Univariable linear regression models were performed to determine the effect of covariables on the cord blood antibody levels or placental transfer of antibodies. The variables analysed in the univariable models were maternal antibody levels (log_{10} MFI), maternal HIV infection, PM, age, gravidity (defined as primigravidae and multigravidae), maternal anaemia (defined as haemoglobin levels <11g/dL), LBW (defined as <2500g at birth), prematurity (defined as delivery before 37 weeks of gestational age), gestational age (measured by Ballard score [65]), treatment arms (defined as MQ or placebo in the HIV+ women study and MQ full, MQ split or SP in HIV- women study), antiretroviral therapy (ART) received before pregnancy, started at recruitment or not received at all, $CD4^+$ T cell counts (<350 cells/μL or ≥350 cells/μL), HIV viral load (<400, 400-999, 1000-9999 and >9999 copies/mL), and seasonality (dry or rainy). Gravidity was defined as primigravidae and multigravidae following the approach used in previous studies and due to the lack of significant differences on antibody levels between secundigravidae and multigravidae in other studies [47, 48, 66–68]. Seasonality was defined as rainy if at least 4 of the pregnancy months fell under the category of rainy period (November-April), and defined as dry in any other case. Multivariable regression analyses were performed for each antigen and IgG or IgG subclass including always maternal antibody levels and maternal HIV infection (statistically significantly associated in all univariable analyses) and the additional predictors that resulted in the best fitted and simpler (less variables) models. Specifically, we tested exhaustively all possible combinations of the predictor variables and selected the models with lower Akaike information criterion (AIC) and Bayesian information criterion (BIC) and higher adjusted r-square. Then, variables that appeared significant in most of the best models for each antigen/subclass and that also had more significant associations in univariable analyses (significant adjusted p-values) were included in all the models, i.e. PM and LBW. The betas obtained in each case were transformed into a percentage for interpretation. For maternal antibody levels (log-log model) the beta transformed value (%) was calculated with the formula ((10^(beta*log10(1.1)))-1)*100. This represents the effect (in percentage) of a 10% increase in the corresponding predictor variable on IgG and IgG subclass cord blood levels. For maternal HIV infection, PM and LBW (log-linear models), the beta transformed value (%) was

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

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

Results

Description of participants

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

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

HIV+ women compared with HIV- women. IgG3 maternal levels were only lower among HIV+ women against DBL3-4, whereas IgG4 levels in HIV+ women were lower than HIV- women against EBA140, EXP1, $MSP1_{42}$ and MSP1 bl2. Statistically significant differences were found in the cord for the same antigens and IgG subclasses as in the mother, with the exception of DBL3-4 IgG3 and EXP1 IgG4 that were not significantly different in the cord. Regarding PM, there were no significant differences between women with and without PM in IgG subclass levels, although there was a general positive trend in women with PM (Additional file 1: Figure S2-S3).

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

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

Maternal HIV infection was negatively associated with cord blood antibody levels, reducing IgG to EXP1 and MSP5 by 3.84% and 1.47%, respectively; IgG1 to MSP2 and Rh4.2 by 9.09% and 3.12%, respectively; and IgG4 to $MSP1_{42}$ by 1.91%. No significant effect was found for IgG2 and IgG3 levels in cord blood (Fig. 3b). PM negatively impacted IgG cord blood levels against EBA140, MSP1 bl2 and Rh4.2 (2.19%, 2.53% and 3.52% reduction, respectively), and IgG2 to EBA140 (4.58% reduction) (Fig. 3c). When analysing HIV+ women only, PM was also associated with lower IgG2 to DBL3-4 (Additional file 1: Figure S4). LBW was positively associated with cord blood IgG2 levels against EBA140 and Rh4.2, with a 5.46% and 8.14%, increase, respectively (Fig. 3d). No significant associations were found for LBW and total IgG or the rest of the subclasses. Age, maternal anaemia, gravidity, IPTp treatment, prematurity,

seasonality, and $CD4^+$ T cell counts, ART and viral load for HIV+ women were not included in the models following the AIC, BIC and r-square criteria.

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

The radar charts (Fig. 4) showed that HIV+ women had a reduced placental transfer of antibodies compared to HIV- women. This was significant for IgG and IgG1 against DBL3-4, EBA140, EXP1, $MSP1_{42}$, MSP1 bl2, MSP2 and MSP, IgG1 against Rh4.2 (Fig. 4a-4b), and IgG4 against $MSP1_{42}$ (Additional file 1: Figure S4). However, HIV infection increased the transfer of IgG4 against DBL3-4 and also a trend was seen for IgG3 (Additional file 1: Figure S5). No significant differences in placental transfer between the two groups were found for IgG2 or IgG3.

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

Discussion

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

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

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

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

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

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

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

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

HIV infection reduced IgG1 cord levels against MSP2 and Rh4.2 due to an impairment of the IgG1 transplacental transfer. Although it has been previously reported that maternal HIV negatively affected MSP1 IgG1 [30, 31] and IgG3 [30] cord levels, we did not find any significant association between HIV infection and MSP1 IgG1-3 cord levels. However, we observed lower IgG4 cord levels against $MSP1_{42}$. Diminished levels of these antibodies could explain higher risk of infection, as cytophilic antibodies have been suggested to contribute to protection from clinical malaria in adults and children in endemic areas [34, 92] and IgG4 subclass has also been associated with malaria protection [34, 93]. LBW was previously associated with a reduction in cord blood levels and placental transfer of antibodies [94–96], but in this study we did not observe any association of LBW with lower cord levels or placental transfer. However, our results are consistent with other studies that did not show any impact of LBW on IgG and subclass cord levels against some antimalarial antigens [30, 31]. Surprisingly, LBW was associated with higher cord IgG2 levels against EXP1 and Rh4.2 and, to our knowledge, this is the first time that this observation has been reported. IgG2 antibodies are associated with increased risk of severe malaria [97] and, therefore, LBW infants may have higher risk to suffer from malaria complications than normal weight infants. No associations were found between maternal age, anaemia, gravidity and IPTp treatment and cord levels or placental transfer of antibodies against antimalarial antigens [28, 30]. We did not find either any significant differences between mothers who initiated ART before pregnancy, mothers who started during pregnancy, and mothers not taking ART. Previous studies on the effect of ART on placental transfer of antibodies are controversial and the effect varied depending upon the antigen, the initiation and type of treatment, and the dose. For example, Goetghebuer et al. observed the lowest maternal antibody transfer ratios against 5 vaccine and 2 pathogen antigens in HIV+ mothers who initiated ART during pregnancy, compared with those who initiated ART before pregnancy [98]. However, this study did not include *P. falciparum* antigens. Moro et al.

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

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

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

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

Additional files

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

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

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Authors' contributions. SA, CD and GM wrote the first draft of the manuscript. SA, CD and GM conceived the immunological study and the experimental design and interpreted the data. CD, GM, RA and SA designed the analysis and selection of the antigens. SA and MV performed the antibody Luminex assay. EA, RLC, BG, DC and JGB contributed with the resources. GRO and MVS performed the statistical analysis. AM, CD and GM designed the immunology study ancillary to the clinical trials. MNM, RB and CJ processed the samples. PC and LFS performed the PCR. RG, MR, JJA, EM, AV, ES and CM designed and enrolled participants in the clinical trials. AN was the clinical trial data manager. JJA was the clinical trial statistician. RA, JB, PC, RG, CM, GRO and MVS contributed to the write up of the manuscript. All reviewed and approved the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

This study was carried out in accordance with ICH Good Clinical Practice guidelines and the Declaration of Helsinki. The study protocols and informed consent forms were reviewed and approved by the Comité Ètic d'Investigació Clínica (CEIC, Hospital Clínic, UB), Spain, and the Comité Nacional de Bioética (CNBS), Mozambique. Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table 1: Characteristics of study participants.

	All N=341	HIV- N=197	HIV+ N=144	p–value [a]
Age [a] (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
Multigravidae	259 (76.0)	128 (65.0)	131 (91.0)	
Primigravidae	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)	
$CD4^+$ 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 [b] (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 [c] (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.

[a] 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.

[b] Placental malaria was considered positive if there was any evidence of *P. falciparum* placental parasitaemia by any method.

[c] 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.

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Figures

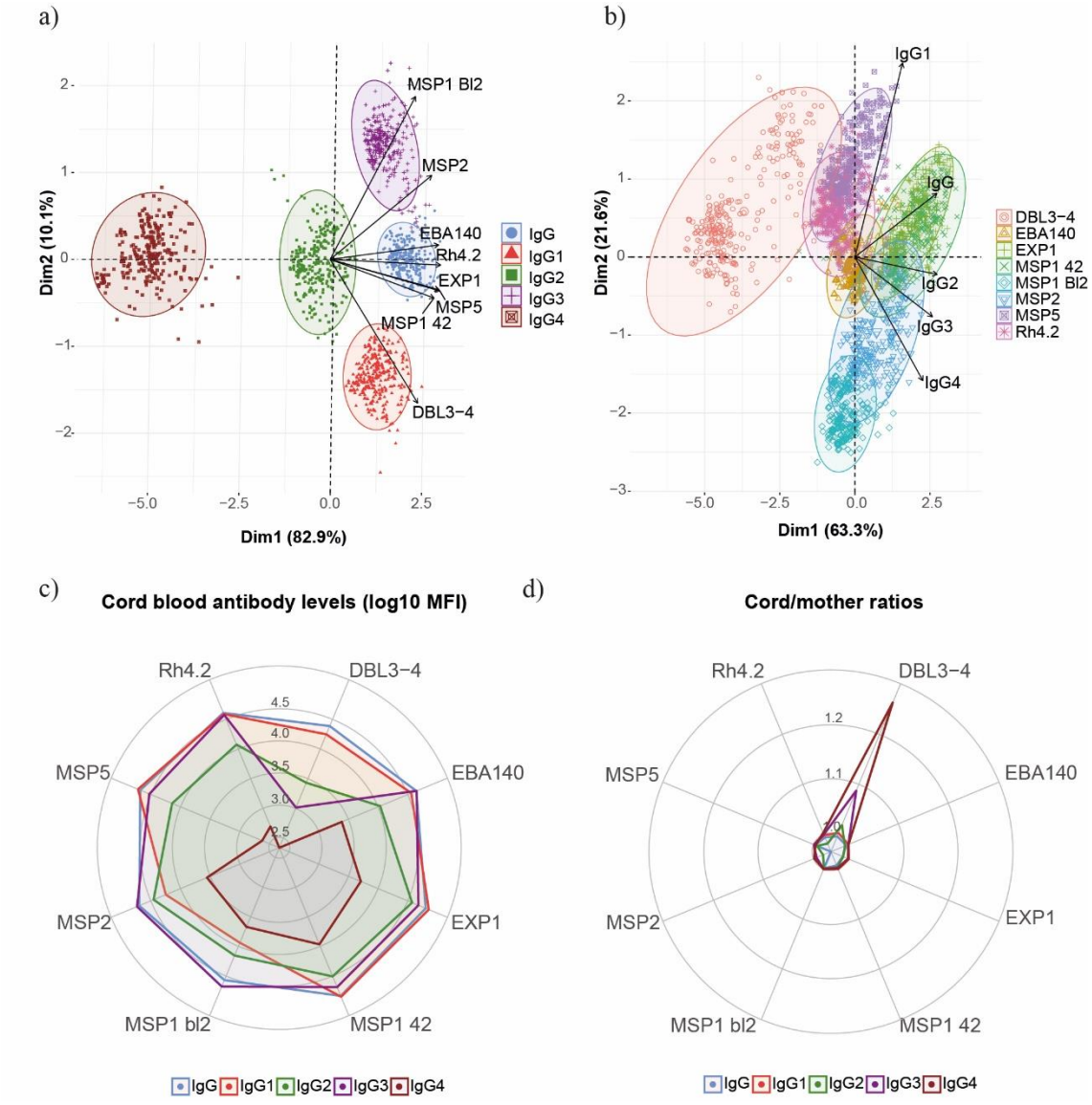
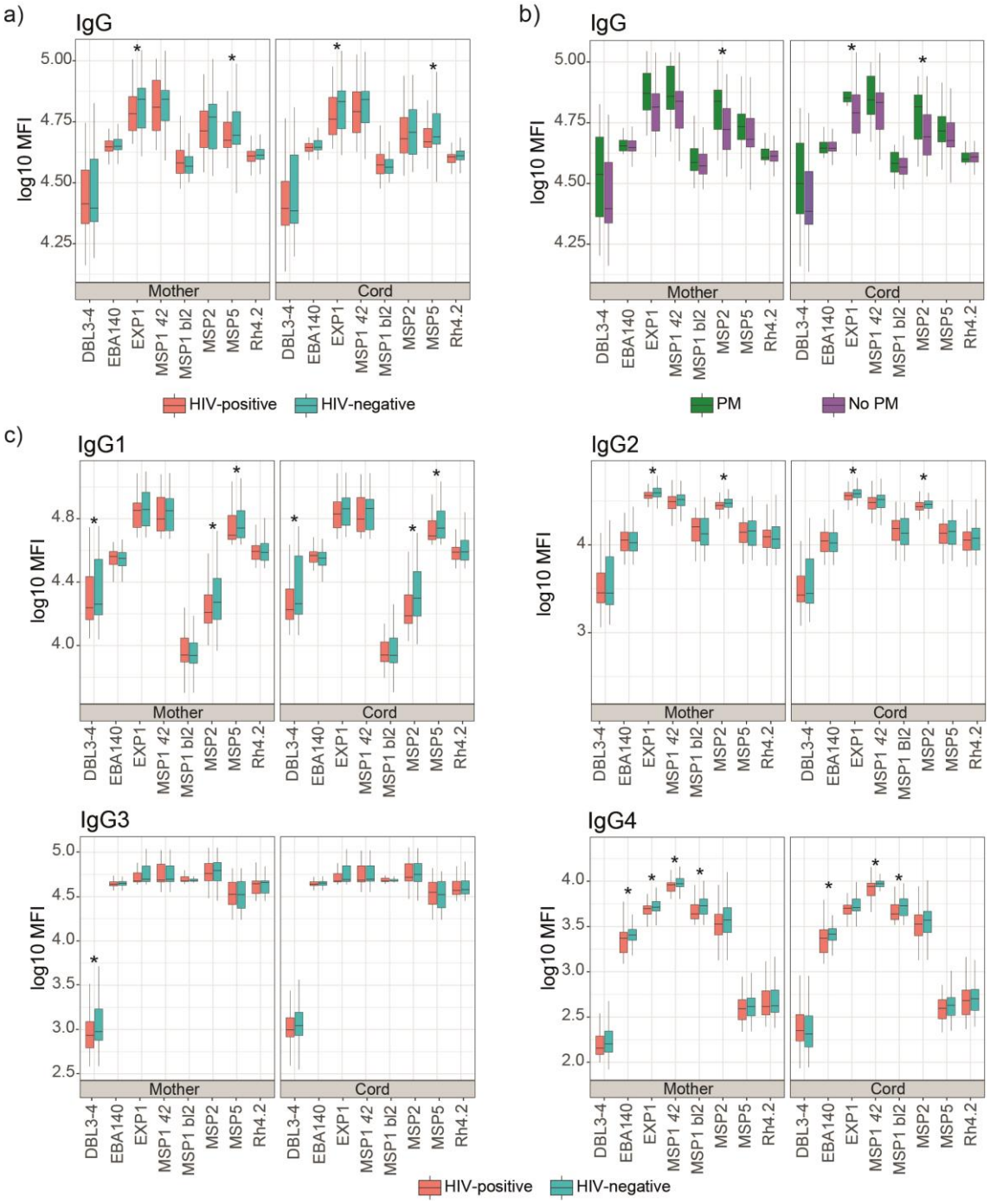


Fig. 1: Overview of cord blood levels of IgG and IgG subclasses to *P. falciparum* antigens for all women. a) Principal component analysis (PCA) plots of cord IgG and IgG subclass levels against all antigens clustered by subclass type. b) PCA plots of cord IgG and IgG subclass levels clustered by antigen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



797

798 **Fig. 2**: Mother and cord blood antibody levels (log_{10} 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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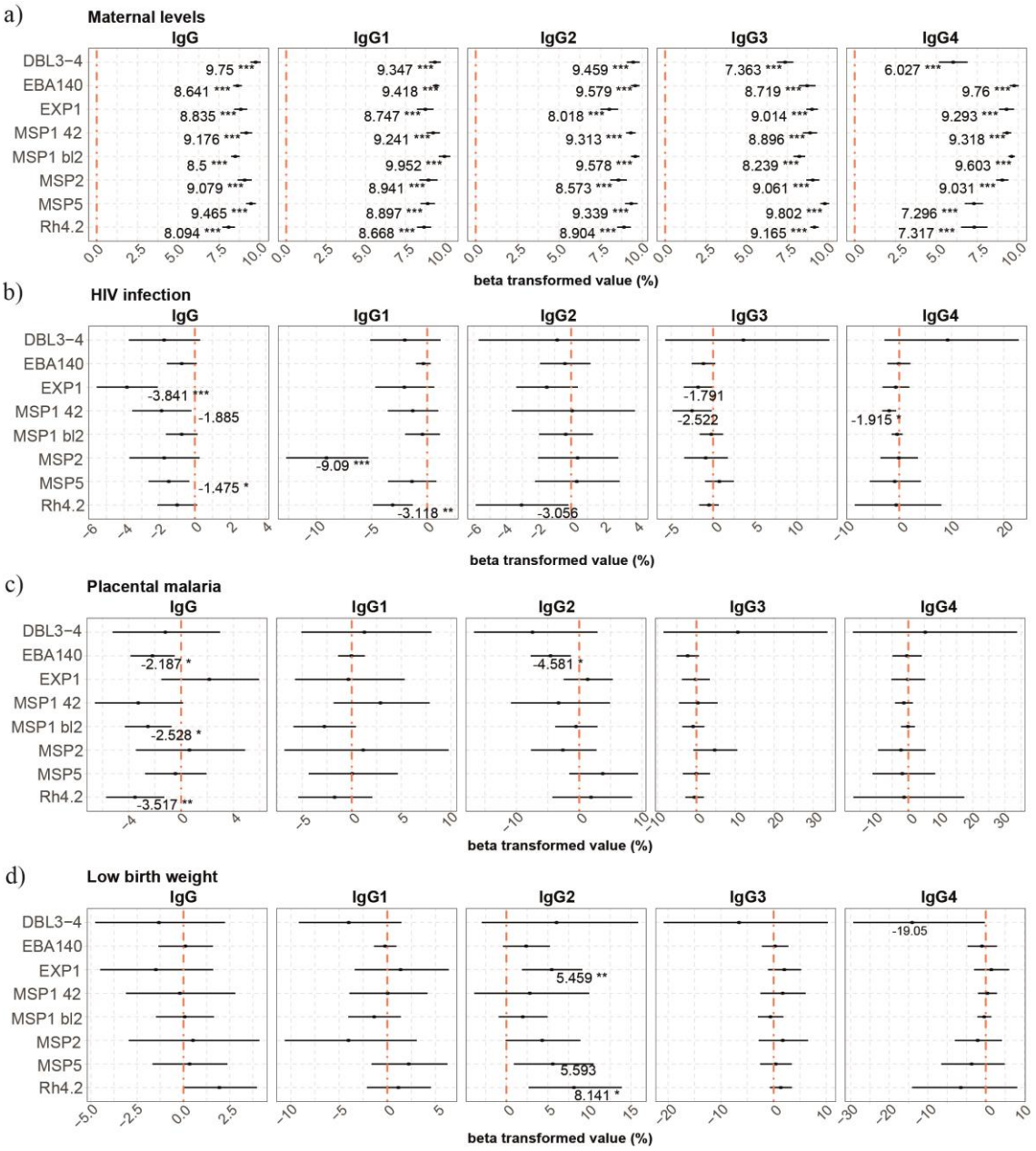
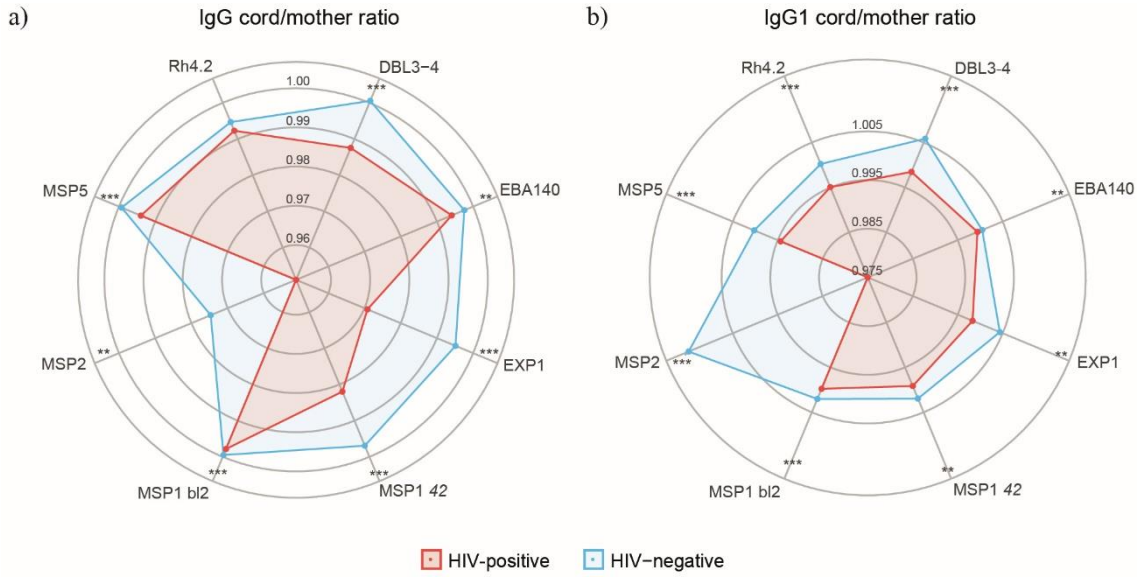


Fig. 3: Difference of IgG and IgG subclass levels in cord blood by study factors. Forest plots show the effect (in percentage) of a) maternal antibody levels, b) HIV infection, c) placental malaria and d) low birth weight, on cord blood levels of IgG and IgG subclasses for all the antigens tested. The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



818

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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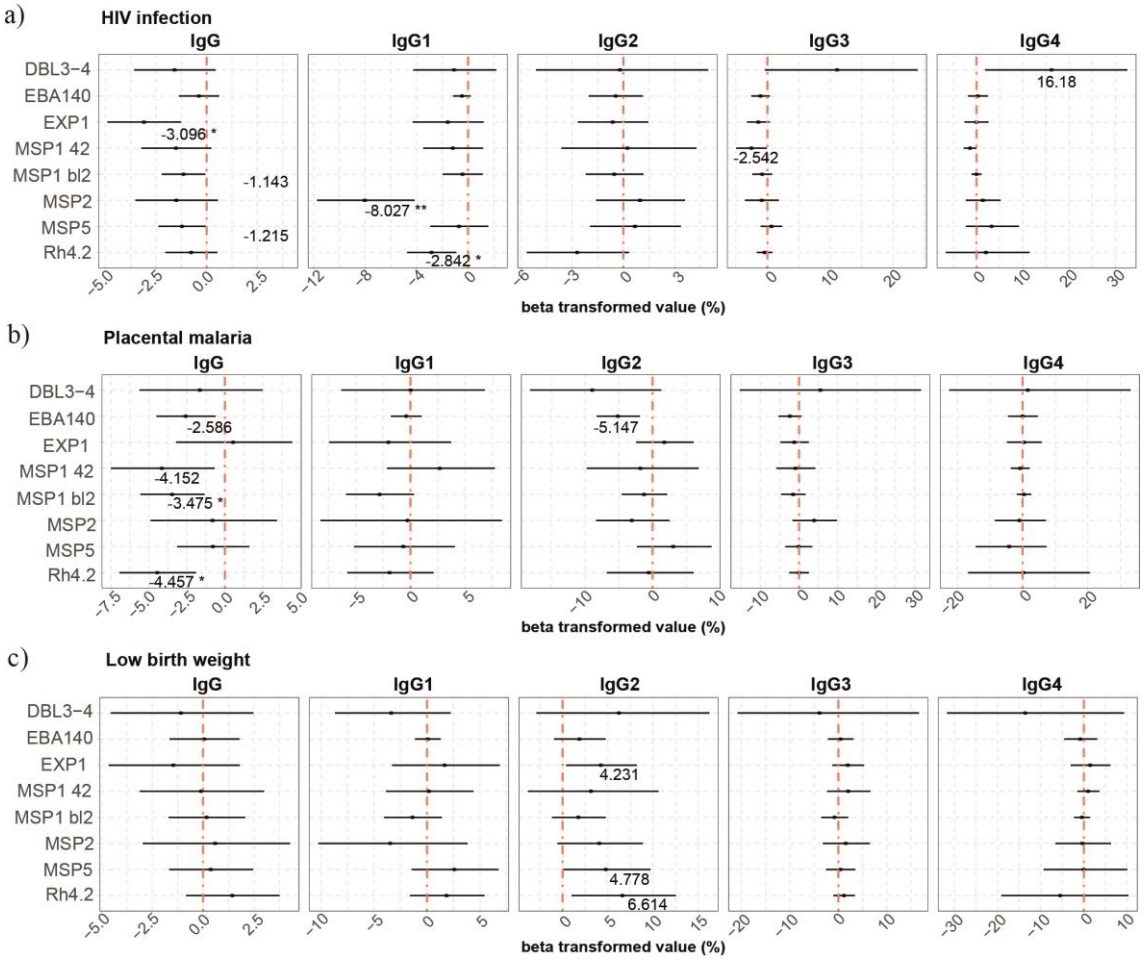

Fig. 5: Difference of IgG and IgG subclass placental transfer by study factors. Forest plots show the effect (in percentage) of a) HIV infection, b) placental malaria and c) low birth weight, on placental transfer of IgG and IgG subclasses for all the antigens tested. The differences in percentage correspond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