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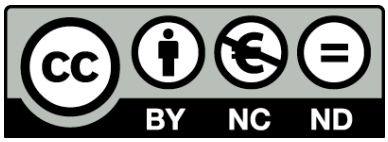
Selena Alonso Galindo



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Selena Alonso Galindo

Barcelona, 2021

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Doctoral Program in Medicine and Translational Research. Faculty of Medicine and Health Sciences. Universitat de Barcelona

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ISGlobal Barcelona Institute for Global Health

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Doctoral thesis by

Selena Alonso Galindo

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Dr. Carlota Dobaño, Research Professor of the Barcelona Institute for Global Health (ISGlobal) and Dr. Gemma Moncunill, Assistant Research Professor of the Barcelona Institute for Global Health (ISGlobal), certify that the doctoral thesis entitled "**Effect of maternal HIV and malaria on the transplacental transfer of antibodies against prevalent pathogens and vaccines in Mozambican women**" presented by Selena Alonso Galindo was conducted under their direction, and meets all the requirements dictated by current regulations for submission of dissertations as a compendium of articles from the Faculty of Medicine of the Universitat de Barcelona.

Barcelona, April 2021

Dr. Carlota Dobaño
Doctoral thesis codirector

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UNIVERSITAT DE BARCELONA

Ita fit, ut quod opus meum hoc inhibet, ad opus conductat; ad uiam conferat, quod uiae initae obstabat.

La reflexión en lo que hemos emprendido rodea y cambia cualquier impedimento a nuestra actuación, lo que impide nuestra acción se vuelve beneficioso y lo que se interponía en su camino favorable.

Marcus Aurelius, Ad se ipsum libri XII. 5.20(3).

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACT	Artemisinin-combination therapy
AIDS	Acquired immunodeficiency syndrome
AMA	Apical merozoite antigen
aP	Acellular pertussis
ART	Antiretroviral therapy
BCG	Bacille Calmette-Guerin
CMV	Cytomegalovirus
CSA	Chondroitin sulfate A
CSP	Circumsporozoite stage protein
CTL	Cytotoxic CD8+ T cells
DBL	Duffy-binding-like
DTP	Diphtheria, tetanus and pertussis
EBA	Erythrocyte binding antigen
EBL	Erythrocyte binding ligand
EPI	Expanded program of immunization
Fab	Fragment antigen-binding
Fc	Fragment crystallizable region
FcRn	Neonatal Fc receptor
GPI	Glycophosphatidylinositol
HBV	Hepatitis B virus
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
IPTp	Intermittent preventive treatment in pregnancy
IPV	Inactivated polio vaccine
LLINs	Long-lasting insecticide-treated nets
LPS	Lipopolysaccharide
LSA	Liver stage antigen
MHC	Major histocompatibility complex

MQ	Mefloquine
MSP	Merozoite surface protein
MV	Measles virus
NAI	Naturally acquired immunity
NK	Natural killer
OPV	Oral polio vaccine
PCR	Polymerase chain reaction
PCV	Pneumoccocal conjugate vaccine
pDC	Plasmacytoid dendritic cells
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfRh	*P. falciparum* reticulocyte-binding homologues
PM	Placental malaria
PspA	Pneumococcal surface protein A
PTEX	*Plasmodium* translocon of exported proteins
RBC	Red blood cells
RESA	Ring-infected erythrocyte surface antigen
RH	Rhoptry proteins
RSV	Respiratory syncytial virus
RV	Rotavirus
SP	Sulfadoxine-pyrimethamine
Tc	T cytotoxic
Tfh	T follicular helper
Th	T helper
TLR	toll-like receptor
TRAP	Thrombospondin-related adhesive protein
Treg	T regulatory
VSP	Variant-specific surface protein
WHO	World Health Organization
wP	Whole-cell pertussis

LIST OF PUBLICATIONS

Thesis in compendium of articles format. The thesis consists of two articles:

1. **Reduced placental transfer of antibodies against a wide range of microbial and vaccine antigens in HIV-infected women in Mozambique**

Selena Alonso, Marta Vidal[+], Gemma Ruiz-Olalla[+], Raquel González, M. Nelia Manaca, Chenjerai Jairoce, Miquel Vázquez-Santiago, Reyes Balcells, Anifa Vala, María Ruperez, Pau Cisteró, Laura Fuente-Soro, Marta Cova, Evelina Angov, Arsenio Nhacolo, Esperança Sevene, John J. Aponte, Eusébio Macete, Ruth Aguilar, Alfredo Mayor, Clara Menéndez, Carlota Dobaño[#], Gemma Moncunill[#]

+Contributed equally
#Contributed equally and share senior authorship.

Frontiers in Immunology. 2021 Mar 3;12:614246.

Impact factor (2019): 5.085. Quartile: 1.
Category: Immunology

2. **HIV infection and placental malaria reduce maternal transfer of multiple antimalarial antibodies in Mozambican women**

Selena Alonso, Marta Vidal[+], Gemma Ruiz-Olalla[+], Raquel González, Chenjerai Jairoce, M. Nelia Manaca, Miquel Vázquez-Santiago, Reyes Balcells, Anifa Vala, María Ruperez, Pau Cisteró, Laura Fuente-Soro, Evelina Angov, Ross L. Coppel, Benoit Gamain, David Cavanagh, James G. Beeson, Arsenio Nhacolo, Esperança Sevene, John J. Aponte, Eusébio Macete, Ruth Aguilar, Alfredo Mayor, Clara Menéndez, Carlota Dobaño[#], Gemma Moncunill[#]

+Contributed equally
#Contributed equally and share senior authorship.

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Thesis summary

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01

RESUMEN

INTRODUCCIÓN

1. Impacto de las enfermedades infecciosas en los niños

Cada año se producen más de 5 millones de muertes en niños menores de 5 años, de las cuales un 47% ocurren durante el primer mes de vida [1]. En regiones de baja renta, la mortalidad y morbididad es mayor. En África, el número de muertes en niños menores de un año es seis veces mayor que en Europa [2]. Entre las principales causas de muerte se encuentran las enfermedades infecciosas como la malaria, el virus de la inmunodeficiencia humana (VIH), la diarrea y la neumonía [3].

Los estudios realizados en esta tesis doctoral se llevaron a cabo en el distrito de Manhiça, una área semi-rural de Mozambique, país del sureste africano con elevadas incidencias y prevalencias de VIH y otras enfermedades infecciosas como la malaria, las enfermedades diarreicas y respiratorias, y la meningitis bacteriana [4, 5].

La neumonía es una infección de las vías respiratorias cuyos síntomas son la fiebre, los escalofríos y la fatiga, entre otros. Los patógenos asociados a la neumonía infantil son principalmente *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenza tipo b* (*Hib*) y el virus respiratorio sincitial (RSV).

S. pneumoniae es una bacteria que coloniza las mucosas del tracto respiratorio superior y fue responsable en 2015 de más de 290.000 muertes de niños de entre 1-59 meses de edad no infectados con VIH [6]. Las vacunas frente al neumococo generalmente utilizan proteínas o carbohidratos que se encuentran en la superficie bacteriana, como polisacáridos capsulares o la proteína de superficie A (PspA) [7]. *Hib* es una bacteria que afecta casi exclusivamente a niños menores de 5 años. Gracias a la vacunación, el número de muertes entre 2000 y 2015 se redujo en un 90% [6]. RSV es un virus que afecta principalmente al tracto respiratorio inferior y una de las mayores causas de morbididad y mortalidad en niños menores de 5 años [8]. Aunque todavía no existen vacunas disponibles contra el RSV, se están desarrollando varias cuya población diana son niños, mujeres embarazadas y ancianos [9].

La segunda causa principal de mortalidad en niños menores de 5 años son las enfermedades diarreicas, que provocaron más de 500.000 muertes en 2015 [10]. Entre los patógenos más frecuentes que causan enfermedad gastrointestinal se

encuentran bacterias como *Shigella* spp, virus como rotavirus (RV), o parásitos como *Cryptosporidium parvum* (*C. parvum*) o *Giardia intestinalis* (*G. intestinalis*) [11].

RV es un virus de RNA que infecta enterocitos y los destruye, causando diarrea y malabsorción. Este virus se asoció con más de 200.000 muertes en menores de 5 años en 2015. La vacunación frente a RV se ha ido implementado en diversos programas de inmunización desde 2006 y se ha demostrado su efectividad [12]. *C. parvum* es un protozoo parasitario que provoca diarreas profusas y prolongadas y que se transmite por la ruta oral-fecal o a través de material contaminado [13]. Las glicoproteínas de superficie Cp17 y Cp40 están implicadas en la invasión de células epiteliales y representan posibles dianas de vacunación, ya que son antígenos inmunodominantes [14, 15]. *G. intestinalis* es otro protozoo intestinal que afecta a 300 millones de niños menores de 5 años anualmente [16] y causó diarrea moderada/severa en un 18.6% de niños mozambiqueños entre 2001 y 2012 [17]. Su superficie está cubierta por proteínas altamente conservadas variante-específicas (VSP), que son reconocidas por los anticuerpos del huésped [18]. *Shigella dysenteriae* y *Vibrio cholerae* son dos bacterias que provocan diarreas severas debido a las toxinas que producen [19, 20]. Se han descrito más de 2,8 millones de casos y alrededor 90.000 muertes debido al cólera, principalmente en África subsahariana [21, 22]. Los anticuerpos contra el antígeno O de *V. cholerae* se han correlacionado con protección frente al cólera, siendo una potencial diana en el desarrollo de vacunas contra este patógeno [23, 24].

2. El sistema inmune

El sistema inmunitario está compuesto por células y moléculas cuyo papel es la protección frente a las infecciones. Existen dos tipos de respuestas en función de la velocidad y especificidad de la reacción: respuestas innatas y adaptativas.

La inmunidad innata incluye barreras físicas, químicas y microbiológicas contra las infecciones. También comprende diferentes tipos de células que proporcionan una defensa inmediata: células fagocíticas (neutrófilos, macrófagos y células dendríticas), células que liberan mediadores de inflamación (basófilos, eosinófilos y mastocitos), y células *natural killer* (NK). Asimismo, también incluye componentes moleculares como proteínas de complemento, proteínas de fase aguda y citocinas. La inmunidad innata proporciona una defensa inmediata y es capaz de eliminar agentes infecciosos en pocas horas. Aunque no tiene memoria inmunológica, estudios recientes sugieren que podría existir un tipo de "inmunidad entrenada" en la que previas

exposiciones a patógenos desencadenan una respuesta más potente en siguientes exposiciones [25].

La inmunidad adaptativa, por otra parte, produce una respuesta más específica, aunque tarda más en desarrollarse. Una de sus características principales es que tiene memoria, con lo que después de un encuentro con un patógeno puede "recordarlo" y en las siguientes exposiciones al mismo patógeno la respuesta será más rápida y vigorosa. Las células principales en la inmunidad adaptativa son los linfocitos B y T, que tienen receptores específicos de antígeno [26].

Los linfocitos B, cuando se activan, secretan inmunoglobulinas (anticuerpos específicos de antígeno), que pueden unirse a microorganismos y neutralizarlos. Esto se conoce como inmunidad humoral. Los linfocitos T activados se dividen en CD4+ o CD8+, y tienen diversas funciones, como la de regular las respuestas inmunitarias a patógenos intracelulares o extracelulares, mediar respuestas frente a hongos o modular la respuesta inflamatoria mediante la secreción de citocinas y quimiocinas.

Durante una infección, los linfocitos B generan anticuerpos de diferentes isotipos (IgD, IgM, IgA, IgG e IgE), y cada uno tiene una función efectora distinta. Los anticuerpos de tipo IgG son los más abundantes y de cuatro subclases diferentes: IgG1, IgG2, IgG3 e IgG4, numeradas en función de su abundancia (siendo la más prevalente IgG1 e IgG4 la menos abundante). Debido a pequeñas diferencias estructurales, cada subclase es funcionalmente distinta. Las subclases IgG1 e IgG3 están involucradas en la respuesta frente a antígenos proteicos, IgG2 en la respuesta frente a antígenos polisacáridos de bacterias encapsuladas e IgG4 suele ser inducida por exposiciones crónicas a alérgenos o helmintos [27–32].

2.1 El sistema inmune en neonatos

Los recién nacidos son más susceptibles a los patógenos y esto se traduce en una mayor morbilidad y mortalidad durante los primeros meses de vida. Esta mayor vulnerabilidad es debida a que el sistema inmunitario de los neonatos es distinto al de los adultos. Por ejemplo, en la fase fetal se ha visto que compuestos placentarios como la progesterona o la prostaglandina promueven una respuesta de tipo Th2, marcada por una ausencia de funciones inflamatorias que conduce a una inmunotolerancia y un menor control de las infecciones en los neonatos [33]. También se ha visto que las funciones de los neutrófilos, monocitos y macrófagos son más débiles durante los primeros meses de vida debido a una reducción de la adhesión a las células endoteliales, a una disminución de la quimiotaxis o a una reducción en la expresión de receptores

tipo “toll like receptor” (TLR) [34, 35]. Asimismo, también se ha descrito una menor proliferación de linfocitos B y un menor desarrollo de respuestas de anticuerpos [36].

Como resultado de esta disfunción del sistema inmunitario, la protección frente a patógenos en los neonatos depende mayoritariamente de la inmunidad conferida por los anticuerpos maternos.

3. Transferencia placentaria de anticuerpos

Los anticuerpos maternos se transfieren al feto a través de la placenta y son esenciales en la protección frente a las enfermedades infecciosas durante los primeros meses de vida [37]. La placenta es un órgano complejo que actúa como interfaz entre la madre y el feto y facilita el intercambio de sustancias entre ellos. La transferencia de anticuerpos se produce a través del receptor neonatal Fc (FcRn). Durante este proceso, los anticuerpos se unen al FcRn y son capaces de atravesar la placenta. Únicamente los anticuerpos de tipo IgG son transferidos y las distintas subclases lo hacen con diferente eficiencia. Comúnmente se ha descrito que la subclase IgG1 se transfiere con mayor eficiencia, seguida de IgG3, IgG4 y finalmente IgG2 [38], aunque otros estudios observaron un orden distinto [39].

Existen diversas razones que pueden explicar las discrepancias en cuanto a la eficiencia de la transferencia placentaria entre las subclases de IgG, como por ejemplo las diferentes afinidades al FcRn, la glicosilación de los anticuerpos o la concentración de IgG materna [40]. Los niveles de IgG maternos e IgG del neonato suelen estar correlacionados, pero si los niveles maternos son demasiado altos, por ejemplo en mujeres con hipergammaglobulinemia, el FcRn puede saturarse y disminuir la eficiencia de la transferencia [41]. También la prematuridad se ha asociado con una reducción en la transferencia de anticuerpos, ya que la mayoría de IgG son transferidas durante el tercer trimestre y el mayor pico se alcanza a partir de la semana 36 del embarazo [41]. Por esta razón, los bebés prematuros suelen tener niveles menores de anticuerpos. Otros factores como la nutrición materna o la diabetes juegan un papel en la transferencia de anticuerpos, donde las mujeres hipoglucémicas o malnutridas podrían tener una menor transferencia [42–44]. Asimismo, la malaria y las infecciones por VIH también se han relacionado con una menor transferencia de anticuerpos [45, 46, 55, 47–54].

4. Malaria

La malaria es una enfermedad infecciosa causada por especies del parásito *Plasmodium*, que se transmite a los humanos a través de la picadura de mosquitos hembra del género *Anopheles*. Existen seis especies de *Plasmodium* que afectan al ser humano: *P. falciparum, P. vivax, P. ovale wallikeri, P. ovale curtisi, P. malariae* y *P. knowlesi* [56, 57], siendo *P. falciparum* la especie predominante en África y la que causa la enfermedad más severa.

En 2019 hubo casi 230 millones de casos de malaria en todo el mundo y más de 400,000 muertes, la mayoría en niños menores de 5 años. El 95% de los casos se producen en la región africana [58], siendo la prevalencia en Mozambique del 38.9% en 2018 [59].

El ciclo de vida del parásito de la malaria comienza con la picadura de un hembra *Anopheles* que inyecta esporozoitos en el torrente sanguíneo (1). Estos esporozoitos migran al hígado e invaden hepatocitos, donde se replican asexualmente bajo la forma de esquizontes hasta que se producen miles de merozoitos, que son liberados al torrente sanguíneo (2). Después de la liberación de merozoitos, éstos infectan eritrocitos y se reproducen asexualmente, liberando más merozoitos que infectarán nuevos eritrocitos (3). Algunos parásitos, en lugar de una replicación asexual, inician un ciclo de replicación sexual donde se generan gametocitos macho y hembra y se liberan al torrente sanguíneo (4). Estos gametocitos pueden ser ingeridos por el mosquito en una nueva picadura, y será en el mosquito donde el parásito complete su ciclo y migre a las glándulas salivares a la espera de ser inyectado en un ser humano (5).

Durante este ciclo el parásito expresa más de 5.000 proteínas que pueden ser reconocidas por el sistema inmunitario. Los anticuerpos pueden actuar sobre antígenos expresados en las diferentes fases:

- Antígenos de la fase pre-eritrocítica: Los esporozoitos expresan diferentes antígenos en su superficie, como la proteína de la etapa de circumsporozoito (CSP), que es el principal antígeno diana en vacunas frente a la malaria, como la RTS,S/AS01$_E$ [60].
- Antígenos de la fase sanguínea asexual: Los merozoitos liberados al torrente sanguíneo expresan diversas proteínas relevantes para la unión e invasión de eritrocitos. Estas proteínas se encuentran en la superficie del merozoito o contenidas dentro de orgánulos llamados roptrias, micronemas o gránulos densos. Algunos de los antígenos involucrados en el primer contacto y unión con la superficie del eritrocito son las proteínas de superficie del

merozoito (MSPs), antígenos de unión al eritrocito (EBA) o la familia de proteínas de unión al reticulocito (RHs) [61–63]. Otros antígenos relevantes son las proteínas exportadas (EXP), que son responsables de la absorción de nutrientes durante la etapa intraeritrocítica [64]. Dichas proteínas son las principales dianas de inmunidad natural frente a la malaria y anticuerpos frente a ellas son marcadores de exposición al parásito.

- Antígenos de la fase sexual: los anticuerpos también son capaces de reconocer proteínas del gametocito y bloquear su función, como por ejemplo la Pf230, esencial en la fertilidad del gameto y la formación del zigoto [65, 66].

4.1 Patogénesis

La enfermedad clínica de la malaria es principalmente el resultado de la replicación asexual del parásito, cuando los eritrocitos infectados se rompen y liberan merozoitos al torrente sanguíneo [67]. Como el ciclo asexual se repite cada 24-48h, la parasitemia va creciendo exponencialmente [68]. Los síntomas más comunes de la malaria incluyen fiebre intermitente, escalofríos y dolor de cabeza, pero las formas más graves de la enfermedad incluyen distrés respiratorio, convulsiones, shock y malaria severa, entre otros. Los niños pequeños que viven en áreas de alta transmisión suelen sufrir mayores complicaciones, como malaria cerebral, que puede derivar en muerte [69, 70]. Las mujeres embarazadas también sufren un mayor riesgo de complicaciones, ya que los eritrocitos infectados se pueden acumular en la placenta y causar malaria placentaria.

4.2 Diagnóstico y tratamiento

El diagnóstico de la malaria convencional es mediante microscopía óptica de un frotis sanguíneo [71]. También hay tests de diagnóstico rápido que son muy sencillos de utilizar y no requieren una formación previa, aunque la sensibilidad es más reducida [72, 73]. El diagnóstico por PCR, por otra parte, es muy sensible y específico ya que esta técnica consiste en amplificar y detectar el ADN del parásito, aunque es un método más caro y complejo [74].

El tratamiento consiste en la administración de fármacos como la cloroquina, quinina o mefloquina, tradicionalmente. Debido a la aparición de resistencias, se recomiendan las terapias combinadas con derivados de la artemisinina u otras combinaciones de fármacos como la sulfadoxina-pirimetamina [75–77]. En poblaciones vulnerables como mujeres embarazadas o niños se utilizan estos fármacos como quimioprofilaxis.

4.3 Inmunidad naturalmente adquirida

La inmunidad naturalmente adquirida frente a la malaria es un proceso en el que individuos que viven en zonas endémicas con una alta transmisión desarrollan gradualmente una protección en respuesta a repetidas infecciones [78]. Esta inmunidad, por tanto, es más alta en personas mayores que han sufrido mayor exposición, mientras que los niños menos expuestos tienen mayor riesgo de padecer malaria severa [79].

La respuesta inmunitaria puede generarse frente a cualquier fase del ciclo del parásito, aunque el desarrollo de anticuerpos contra antígenos del merozoito se ha asociado con protección contra la malaria clínica [80, 81]. Anticuerpos frente a los antígenos MSP o EBA pueden evitar la invasión a los eritrocitos. Específicamente, se han asociado las subclases IgG1 e IgG3 a la protección, probablemente debido a su capacidad de fijar el complemento o mediar la fagocitosis [82–84].

Durante el embarazo, la inmunidad naturalmente adquirida puede verse comprometida, haciendo que las mujeres embarazadas sean más susceptibles a las formas severas de la malaria, particularmente durante las primeras gestaciones. En mujeres embarazadas de áreas endémicas donde la exposición a la malaria es mayor, se generan anticuerpos capaces de bloquear la unión de eritrocitos infectados a los receptores de la placenta. Esto permite controlar mejor la infección y reducir la gravedad de la malaria en los siguientes embarazos [85, 86].

La transferencia placentaria de anticuerpos contra *P. falciparum* se ha correlacionado con protección frente a la malaria sintomática en neonatos [87–90], aunque la malaria materna puede tener un impacto negativo en esta transferencia [91].

4.4 Malaria placentaria

Los eritrocitos infectados pueden acumularse en la placenta junto con células del sistema inmunitario como monocitos y macrófagos, provocando inflamación [85, 86]. Además, en comparación con la sangre periférica, las densidades de parásitos en la placenta son mucho más altas [92]. Todo esto provoca un engrosamiento de la membrana de la placenta y se reduce el flujo de la arteria uterina, lo que altera el sistema de intercambio entre la madre y el feto [93]. La malaria placentaria además incrementa las respuestas Th1 de tipo proinflamatorio, dando lugar a complicaciones en el embarazo [94]. Los daños

causados a la placenta podrían disminuir la transferencia de anticuerpos maternos al feto [47, 48, 50, 95].

5. VIH

El VIH es el agente infeccioso causante del síndrome de la inmunodeficiencia adquirida (SIDA) y se divide en dos tipos: VIH-1 y VIH-2 [96]. En 2019 se estimó en 38 millones el número de personas con VIH, la mayoría en África subsahariana [4]. VIH-1 es el tipo de VIH más frecuente y provoca la mayoría de las infecciones [97]. El virus se transmite mediante fluidos sexuales, sangre, leche materna y también verticalmente (de madre a hijo).

Mozambique está entre los 10 países más afectados por VIH del mundo, con 2,2 millones de personas con VIH en 2019 [98]. La prevalencia entre mujeres adultas se estima en un 15,4%, mientras que para los hombres se estima en un 10,1% [99]. En el sur de Mozambique, entre 2010 y 2012, periodo en que se recogieron las muestras para la realización de los estudios de esta tesis doctoral, la prevalencia de VIH en mujeres era de un 43,1% [100].

5.1 VIH y el sistema inmunitario

El VIH infecta células del sistema inmunitario y establece una infección crónica que a la larga puede provocar SIDA. Existen cuatro fases durante el curso de la infección: eclipse, aguda, crónica y SIDA. Durante la fase de eclipse el virus se replica activamente sin ser detectado por el sistema inmunitario. La siguiente fase es la aguda, caracterizada por altos niveles de viremia y de linfocitos T CD4+ infectados. Durante esta fase se inician las respuestas inmunitarias humoral y celular. A continuación, se produce la fase crónica, donde los niveles de linfocitos T CD4+ van bajando y hay una inflamación y activación del sistema inmune crónicas. Si no se trata, la infección avanza hasta la fase de SIDA, que se caracteriza por un agotamiento del sistema inmunitario donde se pierden las funciones efectoras y la capacidad proliferativa de las células T de memoria. Esta deficiencia del sistema inmunitario provoca infecciones oportunistas, cáncer e incluso la muerte del individuo [101].

5.2 VIH en el embarazo

En 2015 había más de 1,4 millones de mujeres embarazadas infectadas con VIH [102]. En el distrito de Manhiça, el porcentaje de mujeres VIH-positivas era de aproximadamente el 30% [100]. Durante el embarazo, la infección con VIH aumenta el riesgo de complicaciones como anemia, infecciones concurrentes, abortos, prematuridad y bajo peso al nacer [103]. También se ha visto que las

mujeres embarazadas con VIH tienen un estado inflamatorio en la placenta, que puede incrementar en coinfecciones como malaria placentaria [104, 105].

Dado los efectos negativos del VIH en el sistema inmunitario y en la funcionalidad de la placenta, la transferencia placentaria de anticuerpos podría verse afectada. Existen diversos estudios que observaron una reducción en la eficiencia de transferencia de IgG en mujeres con VIH [49, 50, 54, 106], aunque todavía se desconoce el impacto del VIH materno en la transferencia de las subclases de IgG. Esta disminución de la transferencia de anticuerpos podría ser debida a la reducción de los niveles de anticuerpos maternos en mujeres VIH-positivas. Otra causa podría ser la hipergammaglobulinemia, condición que es común en infecciones crónicas y que se ha asociado con una menor transferencia de IgG, posiblemente debido a una saturación de los FcR de la placenta [41, 107].

5.3 Terapia antirretroviral

Para controlar las infecciones con VIH se introdujeron los fármacos antirretrovirales en 1987 [108]. El tratamiento con antirretrovirales es especialmente importante en África subsahariana, donde la transmisión es alta. Pese a que se ha ido aumentando la cobertura durante los últimos años, más de 9 millones de personas todavía siguen sin recibir tratamiento [109]. En Mozambique, en la fecha de los estudios de esta tesis doctoral, la cobertura con antirretrovirales era baja: únicamente un 50% de la población adulta recibió tratamiento [110, 111].

6. Vacunación

Los programas de inmunización representan un triunfo en la medicina preventiva y la salud pública, ya que han demostrado reducir la morbididad y mortalidad de las enfermedades infecciosas durante la infancia [112]. Esto tiene una especial importancia en regiones geográficas como África, donde los niños tienen 6 veces más probabilidades de morir durante el primer año de vida que los niños de países desarrollados [113]. En Mozambique, en 1979 se implementó el Programa Ampliado de Inmunización, que incluye la vacuna oral de la polio (OPV), la vacuna contra la hepatitis B, la vacuna contra el tétanos, difteria y tos ferina (DTP), la vacuna contra *Hib* (desde el 2009), la vacuna contra el neumococo (2013) y la vacuna contra RV (2015). También se administra la vacuna contra el tétanos a mujeres embarazadas [114]. En los estudios que comprenden esta tesis doctoral se analizaron las respuestas de anticuerpos de las vacunas DTP, sarampión, *Hib* y virus de la hepatitis B.

La vacuna DTP se introdujo en países de baja renta en la década de los 70, y requiere de tres dosis para lograr su máxima efectividad. Esto continúa siendo un reto en la región africana, con una cobertura de la tercera dosis del 69% [115]. Esta vacuna confiere inmunidad sobre todo gracias a la producción de anticuerpos neutralizantes de tipo IgG1 contra la toxina diftérica [116] e IgG1 e IgG4 contra la toxina inactivada del tétanos y contra *Bordetella pertussis* [117, 118]. En el caso del sarampión, la vacuna se introdujo en África en los años 60 [119] aunque todavía hay discrepancias entre estudios sobre la subclase de IgG que induce inmunidad [120, 121].

La vacuna conjugada contra *Hib* se introdujo en Mozambique en 2009 y se incluye en una formulación pentavalente junto con la DTP y la vacuna de la hepatitis B. Esta vacuna induce mayoritariamente anticuerpos de tipo IgG1 e IgG2 [116, 122, 123]. En cambio, la vacuna contra el virus de la hepatitis B contiene proteínas virales de superficie del virus e induce una respuesta de tipo IgG1 e IgG3 [124].

6.1 Estrategias de inmunización materna

La vacunación en mujeres embarazadas confiere protección tanto a la madre como a sus hijos gracias a la transferencia de IgG maternos. Actualmente se recomienda la vacunación contra la gripe, tétanos, difteria y tos ferina durante el embarazo, y otras vacunas como la del RSV y el neumococo están en desarrollo [125].

Se han descrito numerosos beneficios gracias a la inmunización materna, siendo aún más importante en mujeres con VIH en las que la transferencia placentaria de anticuerpos podría verse afectada [49, 50, 54, 106]. De forma similar, la malaria placentaria podría disminuir esta transferencia y limitar los efectos beneficiosos de la vacunación materna [126]. También las coinfecciones con helmintos podrían tener un efecto negativo, ya que se han observado niveles más bajos de anticuerpos en niños cuyas madres estaban infectadas [127].

HIPÓTESIS

La hipótesis principal de esta tesis doctoral es que las infecciones por VIH y *P. falciparum* durante el embarazo pueden reducir los niveles de anticuerpos en la sangre del cordón umbilical y disminuir la transferencia de anticuerpos maternos frente a patógenos clínicamente relevantes y a vacunas al neonato. Este efecto podría diferir entre las subclases de IgG y depender de la especificidad del antígeno.

Asimismo, otros factores maternos como la edad, la paridad, la anemia materna, el tratamiento antimalárico, la terapia antirretroviral, el recuento de linfocitos T CD4+ y la carga viral del VIH, pueden influir en los niveles de anticuerpos en el cordón umbilical y en la transferencia de anticuerpos.

Por último, los niveles de anticuerpos en el cordón umbilical y la transferencia placentaria pueden disminuir debido a otros factores como la prematuridad y el bajo peso al nacer.

OBJETIVOS

El OBJETIVO GENERAL de esta tesis doctoral es determinar el impacto de la infección materna por VIH y la malaria durante el embarazo en los niveles de anticuerpos en la sangre del cordón umbilical y la transferencia transplacentaria de anticuerpos frente a (i) patógenos prevalentes con alto impacto en la salud en el sur de Mozambique, en particular la malaria, y (ii) antígenos de vacunas utilizadas en la inmunización materna e incluidas en el Programa Ampliado de Inmunización de Mozambique.

Los objetivos específicos de la tesis son:

1. Evaluar el impacto de la infección materna por VIH en los niveles en el cordón umbilical y en la transferencia placentaria de IgG totales y subclases de IgG.
2. Evaluar el impacto de la malaria materna en los niveles en el cordón umbilical y en la transferencia placentaria de IgG totales y subclases de IgG.
3. Evaluar el impacto de factores maternos (edad, paridad, tratamiento antimalárico, terapia antirretroviral, recuento de linfocitos T CD4+ y carga viral de VIH), complicaciones del embarazo (anemia materna, prematuridad y bajo peso al nacer) y estacionalidad, en los niveles en el cordón umbilical y en la transferencia placentaria de IgG totales y subclases de IgG.

RESULTADOS

1. Reducción de la transferencia placentaria de anticuerpos contra una amplia gama de antígenos microbianos y de vacunas en mujeres infectadas por VIH en Mozambique.

INTRODUCCIÓN: La transferencia transplacentaria de anticuerpos es fundamental para conferir protección a los recién nacidos frente a enfermedades infecciosas. En este estudio se ha evaluado el impacto de diferentes factores, incluyendo la edad gestacional y las infecciones maternas por VIH y malaria, sobre la eficiencia de la transferencia placentaria de subclases de IgG, así como sobre los niveles de anticuerpos en cordón umbilical.

MÉTODOS: Mediante un ensayo cuantitativo multiplex con tecnología Luminex, medimos las IgG totales y las subclases de IgG frente a 14 antígenos de patógenos y vacunas en 341 parejas de madre e hijo de Mozambique infectadas y no infectadas por VIH. Estos antígenos representan patógenos prevalentes de Mozambique y vacunas incluidas en el Programa Ampliado de Inmunización, incluyendo dianas de inmunización materna. Analizamos la asociación de la infección materna por VIH, la exposición a *P. falciparum,* las variables maternas y las complicaciones del embarazo en los niveles de anticuerpos en el cordón umbilical y la transferencia transplacentaria.

RESULTADOS: Nuestros resultados muestran que los niveles de anticuerpos maternos fueron el principal determinante de los niveles de anticuerpos del cordón. Los análisis univariables mostraron que el VIH redujo los niveles en cordón de IgG1 contra *C. diphtheriae, C. tetani, B. pertussis, Hib, S. dysenteriae, V. cholerae* y sarampión, así como los niveles de IgG4 contra *C. diphtheriae* y *P. falciparum*. Los análisis multivariables incluyendo las variables de niveles de anticuerpos maternos, infección por VIH y exposición a *P. falciparum* también mostraron un efecto negativo de la infección por VIH. Las mujeres VIH positivas sufrieron una reducción del 1.83-7.17% de los niveles de IgG totales e IgG1 en cordón para la mayoría de los antígenos. También se redujeron en un 2.98%-7.02% los niveles de IgG2 en cordón contra *B. pertussis, S. dysenteriae,* HBV y *G. intestinalis*, mientras que hubo un incremento del 3.19% de IgG2 contra RSV. Asimismo, la infección por VIH se asoció con una reducción del 1.99-6.75% de las IgG totales e IgG1 en cordón contra la mayoría de los antígenos, y con una reducción de los niveles de IgG2 contra *B. pertussis,* HBV y *G. intestinalis*. En cambio, la transferencia de IgG2 contra RSV aumentó un 5.42%.

La exposición a *P. falciparum* y la prematuridad también se asociaron negativamente con los niveles de anticuerpos del cordón y con la transferencia placentaria, aunque de forma subclase-antígeno dependiente. Por ejemplo, en los análisis multivariados asociamos la exposición a *P. falciparum* con una reducción de los niveles de IgG totales de cordón contra *S. dysenteriae* y HBV, de IgG1 contra *S. pneumoniae* y RV, de IgG2 contra HBV y de IgG3 contra *C. diphtheriae* y RV. De forma similar, también se vio afectada la transferencia de IgG totales contra *S. dysenteriae* y HBV, IgG2 contra HBV e IgG3 contra *C. diphtheriae* debido a la exposición a *P. falciparum*.

CONCLUSIÓN: Nuestros hallazgos sugieren que una menor transferencia de anticuerpos maternos podría conllevar una mayor susceptibilidad a infecciones en bebés expuestos al VIH. Esto podría afectar a la eficacia de la vacunación materna, especialmente en África subsahariana, donde existe una alta prevalencia del VIH, la malaria y factores ambientales desfavorables.

2. **La infección por el VIH y la malaria placentaria reducen la transferencia materna de anticuerpos antimaláricos en mujeres mozambiqueñas.**

INTRODUCCIÓN: Los anticuerpos maternos contra *P. falciparum* podrían contribuir a la protección de los recién nacidos contra la malaria severa. Nuestro objetivo principal es evaluar el impacto de la infección materna por VIH y la malaria placentaria en los niveles de anticuerpos en cordón y en la eficiencia de la transferencia placentaria de IgG y sus subclases.

MÉTODOS: En una cohorte de 341 madres VIH negativas y VIH positivas del sur de Mozambique, medimos las subclases de IgG e IgG totales en sangre periférica materna y en cordón umbilical contra 8 antígenos de *P. falciparum* mediante un ensayo cuantitativo multiplex con tecnología Luminex. Los antígenos que se incluyeron en el panel fueron: DBL3-4, EBA140, EXP1, $MSP1_{42}$, MSP1 bl2, MSP2, MSP5 y RH4.2. Realizamos modelos de regresión univariables y multivariables para evaluar el impacto de la infección materna por VIH, la malaria placentaria, las variables maternas y las complicaciones del embarazo en los niveles de anticuerpos en cordón y en la transferencia placentaria de anticuerpos.

RESULTADOS: Los niveles de anticuerpos maternos fueron los principales determinantes de los niveles de anticuerpos en el cordón. Nuestros análisis univariables mostraron que la infección por VIH redujo los niveles maternos y en cordón de IgG totales para EXP1 y MSP5, de IgG1 contra DBL3-4, MSP2 y

MSP5, de IgG2 contra EXP1 y MSP2 y de IgG4 contra EBA140, $MSP1_{42}$ y MSP1 bl2. Asimismo, mostraron una reducción generalizada de la transferencia placentaria de IgG totales e IgG1 en mujeres VIH positivas. De forma similar, los modelos multivariables incluyendo las variables de niveles de anticuerpos maternos, infección por VIH, malaria placentaria y bajo peso al nacer mostraron una reducción de los niveles de anticuerpos de IgG totales contra EXP1 y MSP5, IgG1 contra MSP2 y Rh4.2 y de IgG4 contra $MSP1_{42}$. Estos modelos también mostraron una reducción de la transferencia placentaria causada por VIH en IgG totales contra EXP1 y en IgG1 contra MSP2 y Rh4.2.

En cuanto a malaria placentaria, en los análisis univariables observamos una asociación positiva de los niveles de IgG totales en cordón contra EXP1 y MSP2. En cambio, en modelos multivariables la malaria placentaria redujo los niveles en cordón de IgG totales contra EBA140, MSP1 bl2 y Rh4.2 y de IgG2 contra EBA140. La transferencia de IgG totales contra MSP1 bl2 y Rh4.2 también se vio disminuida.

Finalmente, el bajo peso al nacer se asoció con un incremento de los niveles de IgG2 en cordón contra EXP1 y Rh4.2

CONCLUSIÓN: Encontramos una menor transferencia de anticuerpos maternos protectores en los recién nacidos expuestos a VIH y en aquellos cuyas madres tienen malaria placentaria, lo que puede conllevar a un aumento de la susceptibilidad a la malaria en estos niños.

DISCUSIÓN

En esta tesis doctoral hemos medido los niveles de IgG total y de subclases de IgG en sangre materna y en el cordón umbilical frente a 21 antígenos de patógenos prevalentes de Mozambique y de diferentes vacunas. Hemos confirmado que los niveles en cordón de IgG totales y de subclases de IgG correlacionan positivamente con los niveles de anticuerpos maternos, sugiriendo que los niveles de anticuerpos maternos son los principales determinantes de los niveles en cordón. También hemos observado que la eficiencia de la transferencia placentaria era diferente dependiendo del antígeno y que la infección materna por VIH y la malaria tuvieron un efecto negativo en los niveles de anticuerpos en la madre y en cordón y en la transferencia de anticuerpos.

Las enfermedades infecciosas respiratorias, gastrointestinales y la malaria son las causas principales de mortalidad en recién nacidos en Mozambique [10, 58, 128]. En primer lugar, evaluamos el patrón general de los niveles de anticuerpos en la madre y en el cordón umbilical y la eficiencia de transferencia placentaria de los patógenos respiratorios *S. pneumoniae* y RSV. Los niveles de IgG1 maternos y en cordón contra *S. pneumoniae* fueron mayores que el resto de las subclases, mientras que la transferencia placentaria de esta subclase fue la menor. Esto podría ser debido a que las altas concentraciones de anticuerpos maternos saturan el receptor Fc de la placenta y disminuyen su transferencia. De forma similar, los niveles en la madre y en cordón de IgG1 contra RSV también fueron más altos que el resto de subclases. En infecciones naturales por RSV se inducen mayoritariamente anticuerpos de tipo IgG1, así como con la vacunación [129, 130], aunque los de tipo IgG4 fueron los que se transfirieron de forma más eficiente en nuestro estudio. Contra los patógenos gastrointestinales rotavirus, *C. parvum, G. intestinalis, S. dysenteriae* y *V. cholerae* también los niveles de IgG1 fueron mayores en la madre y en el cordón umbilical, que es la subclase que se induce predominantemente en estas infecciones [131–134].

En el caso de *P. falciparum*, observamos que los niveles en cordón de IgG1 e IgG3 eran los más altos para la todos los antígenos. Estas subclases se han relacionado con protección [135–137], por lo que altas concentraciones podrían proteger a los recién nacidos durante los primeros meses de vida.

La vacunación contra *C. diphtheriae, C. tetani, B. pertussis, Hib,* HBV y sarampión es esencial en la prevención de muertes infantiles y las mujeres embarazadas pueden proteger a sus bebés gracias a los programas de inmunización materna [37]. Los niveles de IgG1 en cordón contra la mayoría de estos patógenos fueron mayores que el resto de subclases, excepto para HBV y *Hib* donde los de tipo IgG2 predominaron. Esto podría deberse a que los anticuerpos inducidos en estas vacunas contra HBV y *Hib* son mayormente IgG2 [116, 122, 138], mientras que contra DTP y sarampión suelen inducirse respuestas de tipo IgG1 [117, 118, 121, 124].

En segundo lugar, estudiamos el efecto del VIH materno y observamos una reducción de los anticuerpos en la madre y en el cordón umbilical contra patógenos respiratorios como *S. pneumoniae* y RSV, así como una reducción de la transferencia placentaria. Esto sugiere que la infección por VIH podría disminuir la efectividad de las vacunas maternas contra RSV y *S. pneumoniae* que se encuentran actualmente en desarrollo, así como aumentar el riesgo de los recién nacidos a sufrir infecciones respiratorias. También observamos una reducción de los niveles IgG total materna y en cordón y una disminución de la

transferencia placentaria en antígenos contra *P. falciparum* en mujeres infectadas por VIH. Aunque el efecto entre subclases fue muy dependiente de antígeno, la reducción de la transferencia de IgG1 asociados con la protección de la malaria podría incrementar el riesgo de infección en recién nacidos. En cuanto a antígenos de vacunas, el VIH se asoció con una reducción de los niveles de IgG totales e IgG1 maternos, de IgG totales en cordón y de la transferencia placentaria IgG3 contra el tétanos. El VIH materno también redujo los niveles de anticuerpos contra otros antígenos de vacunas como la tos ferina, el sarampión, el virus de la hepatitis B y *Hib*. Asimismo, vimos mayores niveles de IgG total en mujeres VIH positivas contra el virus de la hepatitis B y contra *G. intestinalis*, coinfecciones muy comunes en personas con VIH [139–141], aunque la transferencia placentaria de estos anticuerpos fue menor que en mujeres VIH negativas.

El tratamiento con antirretrovirales es muy beneficioso en cuanto a la reducción de la morbididad y mortalidad por HIV, especialmente en África donde existe una alta incidencia de VIH [142]. Por ello, evaluamos el impacto del tratamiento con antirretrovirales en la transferencia placentaria de anticuerpos, ya que podría prevenir los efectos negativos de la infección por VIH, pero no encontramos ninguna asociación con la transferencia placentaria de anticuerpos.

En tercer lugar, evaluamos el efecto de la malaria durante el embarazo en la transferencia de anticuerpos. Vimos una correlación negativa entre la exposición a *P. falciparum* y los niveles de anticuerpos en cordón y en la transferencia de anticuerpos contra algunos antígenos de patógenos y vacunas. Estos resultados sugieren que la malaria podría reducir la protección conferida por inmunidad pasiva a los neonatos. También observamos un impacto negativo de la malaria placentaria en la transferencia de IgG contra antígenos de *P. falciparum* relacionados con inmunidad. Este efecto, junto con la reducción de la transferencia en mujeres VIH positivas, podría aumentar el riesgo de los recién nacidos a sufrir malaria severa. En contraste con la exposición a *P. falciparum*, la malaria placentaria no tuvo ningún impacto en la transferencia de anticuerpos contra el resto de patógenos ni vacunas testadas.

Finalmente, evaluamos el efecto de la prematuridad y el bajo peso al nacer en la eficiencia de la transferencia de anticuerpos maternos y en los niveles en cordón umbilical. Únicamente encontramos una asociación de la prematuridad con menores niveles de IgG totales contra *Hib, V. cholerae*, sarampión y *C. parvum*. Tampoco observamos ningún efecto negativo del bajo peso al nacer, aunque si vimos un aumento de IgG2 contra los antígenos de *P. falciparum* EXP1

y Rh4.2 en el cordón umbilical en los bebés con bajo peso. Estos niveles más altos de IgG2 podrían estar asociados a un incremento del riesgo a sufrir complicaciones de malaria en estos bebés.

CONCLUSIONES

- Los niveles de anticuerpos maternos fueron los principales determinantes de los niveles de anticuerpos en el cordón, y la eficacia de la transferencia de subclases de IgG fue diferente según el antígeno y puede variar con la exposición materna a los antígenos.

- La infección materna por VIH se asoció con una reducción de los niveles de anticuerpos en el cordón umbilical y con una disminución de la transferencia placentaria, principalmente IgG1, contra una gama amplia de patógenos, incluyendo *P. falciparum*, y antígenos de vacunas. Esto puede explicar en parte la mayor morbididad de los recién nacidos VIH expuestos no infectados.

- La exposición a la malaria se asoció negativamente con los niveles de anticuerpos en cordón umbilical y con la transferencia de anticuerpos contra algunos patógenos, excluyendo *P. falciparum,* y contra antígenos de vacunas de una manera antígeno-anticuerpo dependiente.

- La malaria placentaria también se asoció con una reducción de los niveles totales de IgG en cordón umbilical contra algunos antígenos relacionados con la protección de la malaria, pero no tuvo efecto sobre las subclases de IgG. Esta reducción puede aumentar el riesgo de que los recién nacidos contraigan la malaria.

- La prematuridad se asoció con niveles más bajos de IgG total en cordón umbilical y con una reducción de la transferencia placentaria contra algunos antígenos de patógenos, excluyendo *P. falciparum*, aunque el efecto no fue consistente entre las subclases.

- El bajo peso al nacer se asoció con un aumento de IgG2 contra antígenos de *P. falciparum* en el cordón umbilical, que están asociados con un mayor riesgo de complicaciones de la malaria.

Introduction

Effect of maternal HIV and malaria on the transplacental transfer of antibodies against prevalent pathogens and vaccines in Mozambican women

02

INTRODUCTION

1. BURDEN OF INFECTIOUS DISEASES IN INFANTS

Each year, 5.2 million children under five years of age die [1]. Among them, 47% occur in the first month of life and 28% during the first year [1]. The vast majority of deaths take place in low and middle-income countries: the risk of a child dying before completing the first year of age was highest in the World Health Organization (WHO) African Region (51 per 1000 live births), over six times higher than that in the WHO European Region (8 per 1000 live births) [2]. Infectious diseases, such as malaria, the human immunodeficiency virus (HIV), diarrhea and pneumonia, are the leading cause of mortality [3]. Among neonates, the main causes of death are preterm birth complications, intrapartum-related events, sepsis or meningitis, congenital abnormalities, pneumonia, tetanus and diarrhea (**Figure 1**) [10].

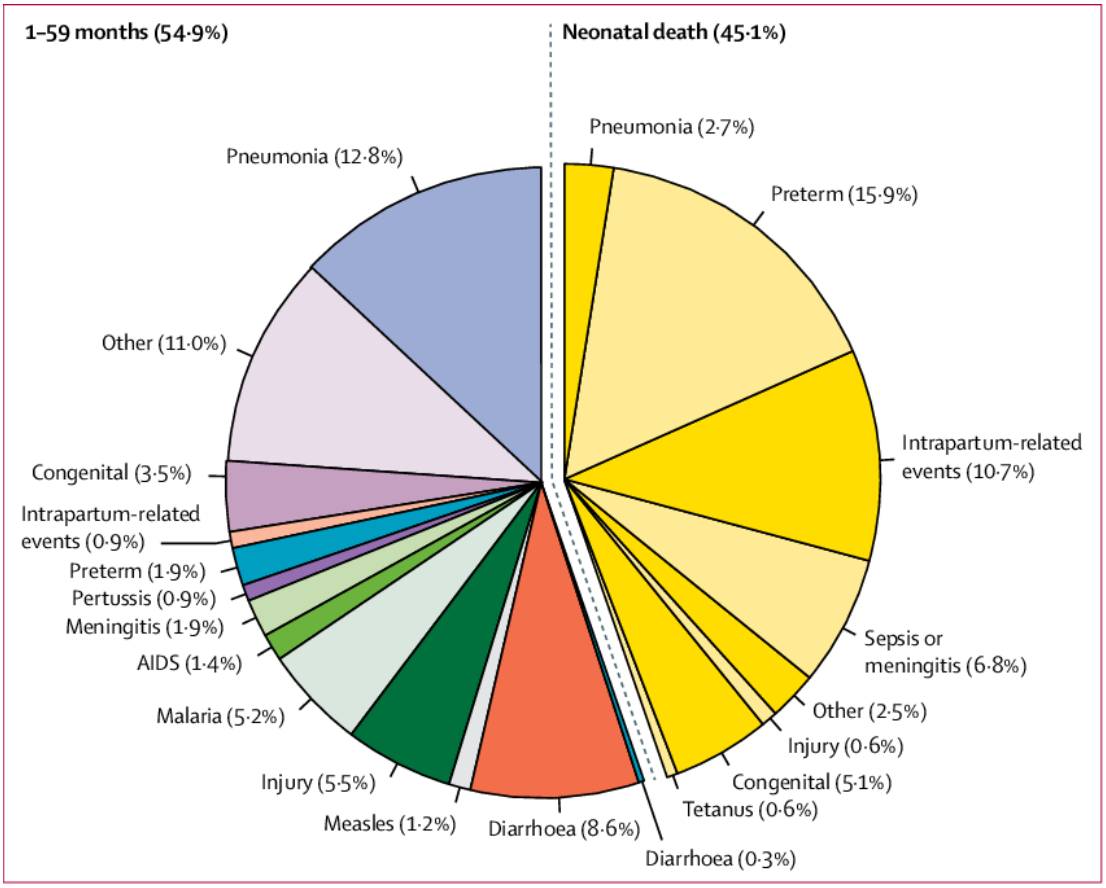

Figure 1: Global causes of under-5 deaths in 2015. Liu L, et al. (2016). *The Lancet,* 388(10063), 3027-3035.

The social determinants that contribute to the high burden of infectious diseases in low-income countries include revenue, social status and education. Risk factors

include undernutrition and injury. In particular, the determinants that affect infants under 5 years of age include poverty, malnutrition, inequity, complications of labor and low birth weight [143].

Public health initiatives such as breastfeeding promotion and maternal and infant immunization are low cost and highly effective at reducing the effect of infectious diseases on both fetal and neonatal death and long-term morbidity [144]. Therapeutic interventions demonstrated to be effective for viral, bacterial and parasitic infections are *Haemophilus influenzae* type b (*Hib*) and pneumococcal vaccines for pneumonia, rotavirus (RV) vaccine, improved water and sanitation, zinc supplementation, oral rehydration, and community case management for diarrhea, insecticide treated bed nets and malaria intermittent preventive treatment in pregnancy (IPTp) [10]. For example, regions with high rates of infant morbidity and mortality like sub-Saharan Africa benefit from the implementation of the Expanded Program of Immunization (EPI) [113]. Thanks to these interventions, the under-5 mortality rate declined from 77.8 to 42.5 per 1000 livebirths over the 2000-2015 period and mortality rates for pneumonia, diarrhea, neonatal intrapartum related events, malaria and measles were all reduced by more than 30% [10].

Despite these efforts, sub-Saharan Africa remains the region with the highest under-five mortality rate in the world. Although the global number of neonatal deaths declined from 5 million in 1990 to 2.4 million in 2019, neonates are in increased risk of death compared to older children [1].

The country where the field studies of this doctoral thesis were performed is Mozambique. Mozambique is located in Southeast Africa and divided by 11 provinces and 128 districts. Our studies were conducted in the Manhiça District, a semi-rural area in Maputo Province, in the South of the country. Mozambique is one of the countries with the highest burden of HIV worldwide [4] and the most prevalent infectious diseases are malaria, which will be further explained in chapter 4, diarrheal diseases, tuberculosis, lower respiratory infections and bacterial meningitis [5].

1.1 Respiratory pathogens

Pneumonia is an infectious disease of the lung parenchyma that caused 12.8% of the deaths in children between 1-59 months and was responsible of 2.7% neonatal deaths in 2015 [10]. The symptoms of pneumonia include coughing, fever, chills,

wheezing and fatigue, among others. *Streptococcus pneumoniae* (*S. pneumoniae*), *Hib* and respiratory syncytial virus (RSV) are the main pathogens associated with childhood pneumonia [128].

S. pneumoniae is a Gram-positive, extracellular bacteria that colonizes the mucosal surfaces of the human upper respiratory tract. It is the leading cause of pneumonia, sepsis, meningitis, bacteremia and otitis media [145], causing more than 290,000 deaths in HIV-uninfected children aged 1-59 months in 2015 [6]. Antibiotics can treat pneumococcal disease, although the emergence of resistance strains has raised concerns [146]. Prevention is, therefore, the best strategy against this disease. Pneumococcal vaccines use proteins and carbohydrates that are on the pneumococcal surface, such as capsular polysaccharides [147] or pneumococcal surface protein A (PspA) [7]. Currently, there are two types of vaccines recommended by the WHO, one unconjugated polysaccharide vaccine and a conjugated polysaccharide vaccine, which have shown to be effective in African countries [148].

Hib is a Gram-negative encapsulated bacterium that causes several infections including pneumonia, meningitis, sepsis and pericarditis. It affects almost exclusively children aged less than 5 years old and caused more than 29,000 deaths in HIV-uninfected children in 2015 [6]. Vaccination against *Hib* declined the number of deaths by 90% from 2000 to 2015 [6], demonstrating the effectiveness of this vaccine.

RSV is a virus that causes lower respiratory tract illness in infants, one of the leading causes of morbidity and mortality in children younger than 5 years [8], but no vaccine is currently available. Vaccines under development target different populations, including infants younger than six months of age, children older than six months of age, pregnant women and the elderly [9]. Vaccine candidates are live-attenuated, protein-based or nucleic acid vaccines. One promising vaccine currently in a global phase 3 trial in pregnant women targets the fusion (F) protein of RSV, that plays a fundamental role in infectivity, and has been shown to be safe and immunogenic [149].

1.2 Gastrointestinal pathogens

After respiratory infections, the second leading infectious etiology of mortality globally among children under 5 years is diarrheal disease, causing more than 500,000 deaths in 2015 [10]. Some of the risk factors for diarrhea include no

handwashing with soap, low vaccine coverage, unsafe sanitation, unsafe water, zinc deficiency, childhood underweight, low use of oral rehydration and vitamin A deficiency [150].

Pathogens causing gastrointestinal diseases worldwide belong to different groups, for example: bacterial organisms such as *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *Listeria monocytogenes* and different groups of enteropathogenic *Escherichia coli;* fungi such as *Candida* spp. which is the most common cause of esophagitis in immunocompromised hosts; viruses such as cytomegalovirus (CMV), RV, norovirus or adenovirus; protist or helminth parasites such as *Cryptosporidium* spp., *Giardia intestinalis* (*G. intestinalis*, also known as *G. lamblia*) or *Strongyloides* [11].

Low-income countries that suffer limited access to sanitation and have poor hygiene behaviors, bear the highest burden of diarrheal disease morbidity and mortality in young children [151]. In sub-Saharan Africa and South Asia, most attributable cases of moderate-to-severe diarrhea were due to four pathogens: RV, *Cryptosporidium,* enterotoxigenic *E. coli* and *Shigella*. Other pathogens also important were *Aeromonas, Vibrio cholerae* (*V. cholerae*), *Campylobacter jejuni, G. intestinalis* and *Entamoeba histolytica* [152, 153].

RV is a non-enveloped double-stranded RNA virus that primarily infects enterocytes and induces diarrhea through the destruction of absorptive enterocytes (leading to malabsorption). In addition, RV also can induce vomiting, malaise and fever. RV was associated with about 200,000 under-5 years deaths globally in 2015 [154]. RV vaccination has been implemented in several vaccination programs worldwide since 2006 and demonstrated effectiveness reducing the disease burden [12].

Intestinal parasites causing diarrhea are mostly reported in low-income countries, such as Mozambique. ***Cryptosporidium parvum*** is a protozoan parasite causing abdominal pain and watery diarrhea that can be profuse and prolonged, as well as nausea, vomiting and fever. Transmission can occur directly by the fecal-oral route from infected hosts, and indirectly involving contact with contaminated material [13]. Two immunodominant antigens, Cp17 and Cp40, are major surface glycoproteins involved in invasion of the host epithelial cells and represent potential vaccine targets [14, 15]. In Mozambique, *Cryptosporidium* spp. caused 17.7% of the moderate-to-severe diarrhea cases during the period 2001-2012 [17].

G. intestinalis is another intestinal protozoan parasite that affected 18.6% of children with moderate-to-severe diarrhea between 2001 and 2012 in Mozambique [17] and was detected in 9.7% of children hospitalized with diarrhea from 2014 to

2018 [153]. Worldwide, *G. intestinalis* is the third most common agent of diarrheal disease in children ‹5 years of age and more than 300 million cases are reported annually [16]. Like *Cryptosporidium* spp, this protozoan is spread through the fecal-oral route and alter gastrointestinal motility. The *G. intestinalis* trophozoite surface is covered by highly conserved variant-specific surface proteins (VSPs) that are recognized by host antibodies [18].

Shigella dysenteriae is a Gram-negative intracellular bacterium that initiates infection by invading cells and causing intense inflammation in the colonic and rectal epithelium. *Shigella* toxin is responsible for the most severe manifestations of shigellosis, such as hemolytic anemia, thrombocytopenia and renal failure [19]. It can cause severe illness and persistent diarrhea, which makes young infants a vulnerable group.

V. cholerae is the bacteria responsible for cholera, a severe disease that affects 2.8 million people annually and causes more than 90,000 deaths [21]. Sub-Saharan Africa accounts for the majority of this burden [22]. To date, more than 200 serogroups of *V. cholerae* have been identified, based on variations in O-antigen structure of the lipopolysaccharide (LPS). The serogroups O1 and O139 can produce the cholera toxin and case epidemic cholera [20]. The serogroup O1 has two main serotypes, Ogawa and Inaba [155]. In Mozambique, for example, there have been regular outbreaks with the majority of cases belonging to *V. cholerae* O1 Ogawa [156]. Current strategies to treat and prevent cholera are oral rehydration therapy, antibiotics and vaccination. Both live-attenuated and inactivated oral whole cell vaccines are safe and well tolerated, although the most significant limitation is that they offer relatively limited protection in young children [157]. Antibodies against the O antigen have been correlated with long-term protection, pointing the O antigen as a potential target for new cholera vaccines [23, 24].

2. THE IMMUNE SYSTEM

The immune system is essential for the protecting the host against pathogenic agents. Immune responses vary depending on the pathogen. The increased susceptibility of newborns and young children to infectious diseases could be explained by the differences of the infant immune system compared with adults. Understanding the immune system and the factors that may modulate the immune function is essential towards designing effective maternal and infant immunization strategies that can confer protection against the major global infectious diseases.

2.1 Overview of the immune system

The immune system is a highly adaptable organization of cells and molecules with specialized roles in protection against infections. There are two different types of responses depending on the speed and specificity of the reaction: the innate and the adaptive responses.

Innate immunity includes physical, chemical and microbiological barriers to infection, as well as phagocytic cells (neutrophils, monocytes, macrophages and dendritic cells), cells that release inflammatory mediators (basophils, mast cells and eosinophils) and natural killer (NK) cells, which provide immediate host defense. The molecular components include complement (series of preexisting serum proteins that bind common pathogen-associated structures and initiate a cascade of labeling and destruction events), acute-phase proteins (serum proteins whose levels change in response to inflammation) and cytokines (molecules mediating communication among cells of the immune system and are key modulators of inflammation). The innate immunity provides immediate host defense and eliminates infectious agents within hours of encounter, but it lacks immunological memory [26, 158–162]. However, recent studies suggested that innate immune cells can also have a type of memory called "trained immunity", in which previous encounters with pathogens result in enhanced responses upon subsequent exposure [25].

The innate immune system is key for the activation of the **adaptive immunity**, as phagocytic cells, particularly dendritic cells, are also professional antigen-presenting cells. They make contact with a pathogen at the site of infection, phagocyte it and present the antigen to T lymphocytes in major histocompatibility complex (MHC) class I and class II receptors on their membrane surface, leading to the activation of an adaptive immune response [163]. This response is more precise, but takes longer to develop. It also has memory, therefore after an encounter with a new pathogen, the adaptive immune system can "remember" it and subsequent exposure leads to a more vigorous and rapid response. The characteristic of adaptive immunity is the use of antigen-specific receptors on B lymphocytes (B cells) and T lymphocytes (T cells) [26].

B cells originate from bone marrow, a primary lymphoid organ that supports self-renewal and differentiation of hematopoietic stem cells into mature blood cells. Activated B cells proliferate into plasma cells, that secrete immunoglobulins (Ig), the antigen-specific antibodies, into the extracellular space where they can bind and neutralize microorganisms, and into memory cells, which have a long life and

mount a faster antibody production in future infections (**Figure 2**). This antibody-mediated immunity is called humoral immunity, but B cells also contribute to cellular immunity serving as antigen-presenting cells that enhance T cells mediated immunity [159, 164–166]. Furthermore, a subgroup of B cells, known as Bregs, can also modulate immune responses, supporting immunological tolerance [167].

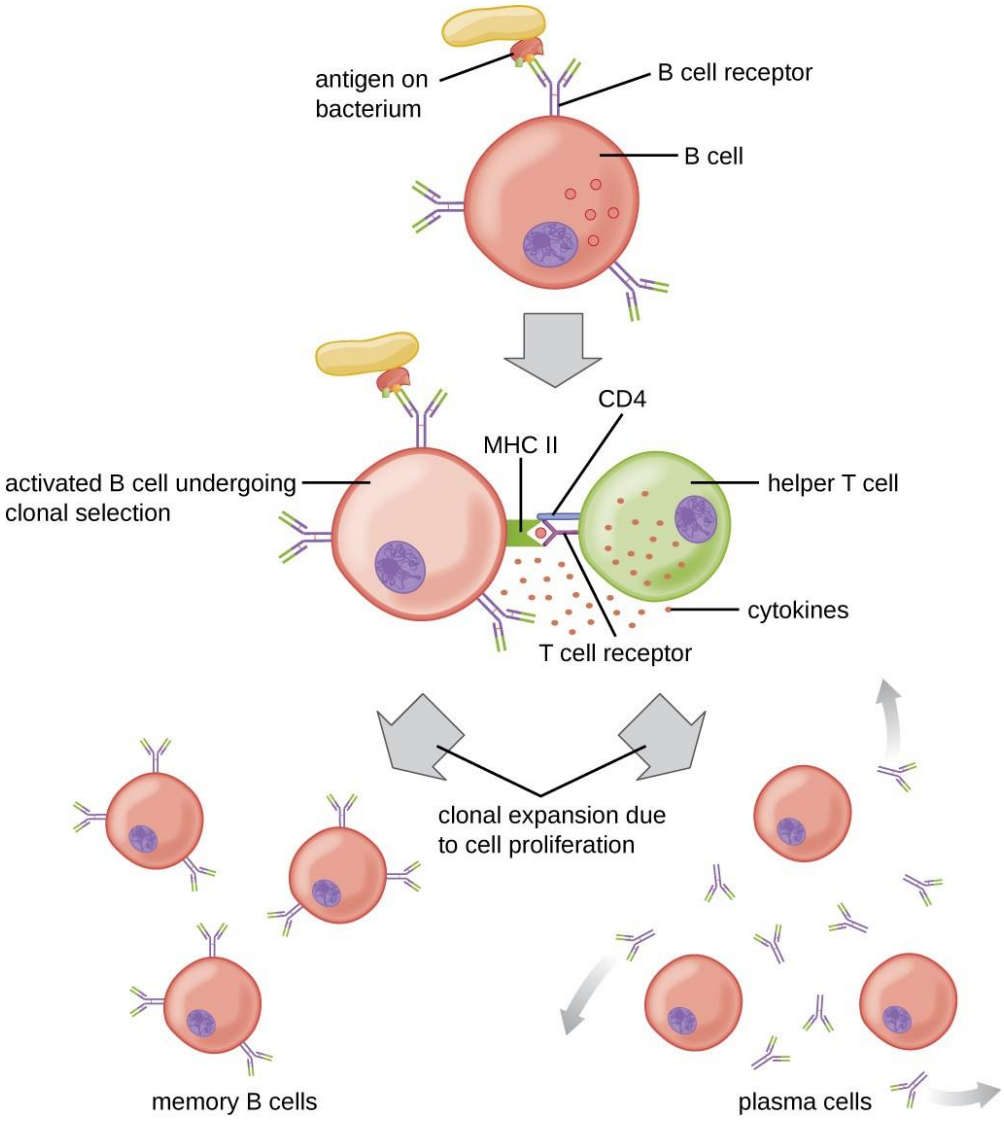

Figure 2: B cell activation and proliferation into memory B cells and plasma cells. Lumen Microbiology, B lymphocytes and humoral immunity. https://courses.lumenlearning.com/microbiology/chapter/b-lymphocytes-and-humoral-immunity/ (accessed 12 October 2020)

T cells originate from bone marrow progenitors that migrate to the thymus for maturation, selection and subsequent export to the periphery [168, 169]. They are divided into two major cell types, T helper (T_H) cells and T cytotoxic (T_C) cells, that can be distinguished from one another by the presence of either CD4 or CD8 membrane glycoproteins on their surfaces, respectively [159, 168].

Activated T CD4⁺ cells, depending on the cytokines present in the surrounding environment, can differentiate into i) T helper type 1 (T_{H1}) cells, which regulate the immune response to intracellular pathogens; ii) T helper type 2 (T_{H2}) cells, which regulate the response to many extracellular pathogens; iii) T helper type 17 cells (T_{H17}), which have an important role in cell-mediated immunity and help the defense against fungi; iv) T follicular helper cells (T_{FH}), which play a role in humoral immunity and regulate B-cell development in germinal centers; and v) regulatory T cells (T_{REG}), which have the unique capacity to inhibit immune responses (**Figure 3**). Each of these CD4+ T-cell subtypes produce a different set of cytokines that enable the activation of B cells, T_C cells, macrophages and other cells that participate in the immune responses [26, 158, 159, 168, 170].

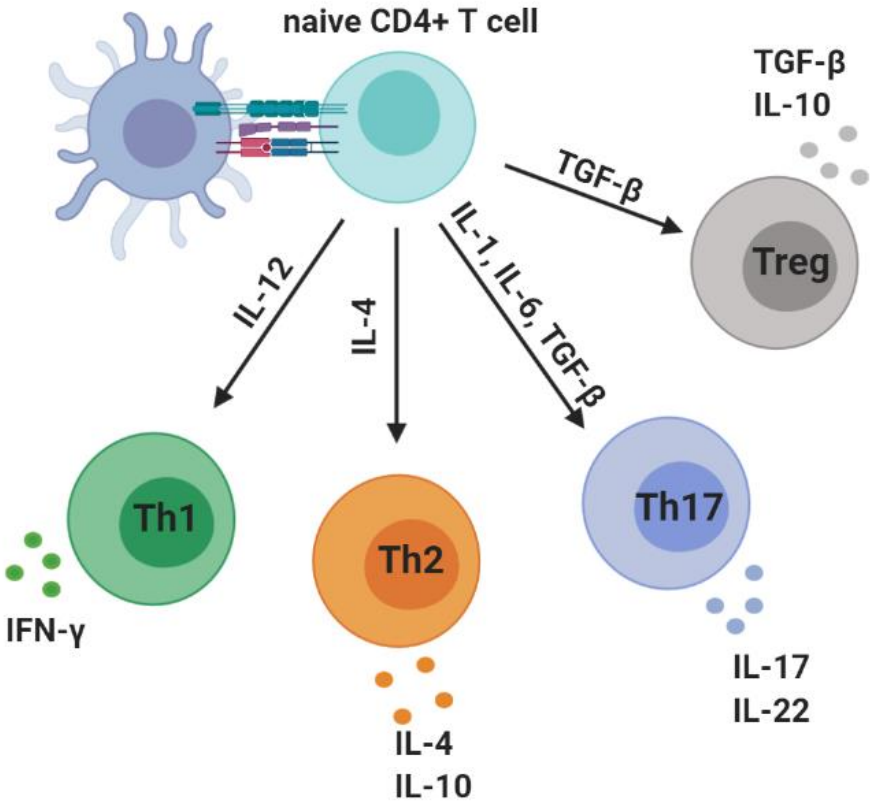

Figure 3: T CD4+ cell differentiation. Julie Bettke, ImmunoBites: Bite-sized Immunology. https://immunobites.com/2020/04/13/the-many-flavors-of-t-cells/ (2020, accessed 12 October 2020).

Activated T CD8⁺ cells differentiate into T cytotoxic cells (T_C), that are able to cytolyze target cells that are infected with intracellular pathogens and secrete cytokines that play important roles in antimicrobial defense. They can also express chemokines that attract inflammatory cells to sites of infection [159, 168, 171].

2.1.1 Antibodies

Antibodies, key components of protective immunity, are glycoproteins composed of two identical heavy chains and two identical light chains that are covalently bonded together by disulfide bonds forming an "Y" shape. The N-terminal ends of the light and heavy chains are extremely variable and they are referred to as the variable regions. The less variable part of the sequence is termed constant region (**Figure 4**). The two regions are linked with each other by a flexible domain called hinge region containing disulfide bonds [172, 173]. The fragment antigen-binding (Fab) is composed by one constant and one variable domain of each of the heavy chain and light chain, and this region binds specifically to pathogen antigens. The fragment crystallizable region (Fc) is the tail region of the antibody composed by the constant domains that interact with the Fc receptors of effector cells and molecules, although it is suggested that it could also influence affinity and specificity [174–178]. Affinity is defined as the strength of the interaction between an antibody receptor and its respective ligand and it increases with time, in a process known as affinity maturation. This maturation primarily occurs in germinal centers and relies on somatic hypermutation of immunoglobulin genes in B cells, resulting in the production of antibodies of higher affinities [179]. The affinity maturation and somatic hypermutation also increase antibody avidity for antigens. Avidity is the measure of the overall strength of the antigen-antibody binding complex. As antibodies and antigens possess more than one binding site, avidity is defined as the total strength of all interactions between an antibody binding to its ligands. It has been used to measure the functional maturation of the humoral immune response [180]. High antibody affinity and avidity are important for acquiring protective immunity against infections.

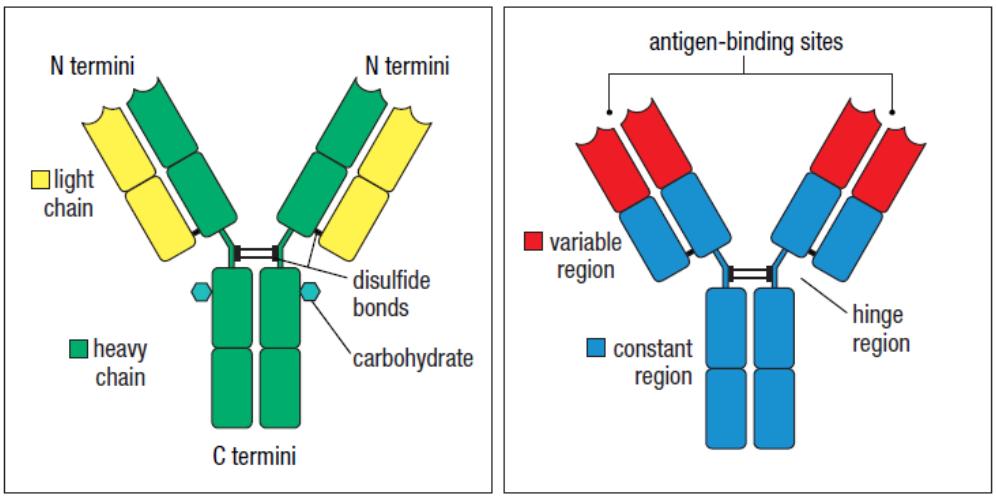

Figure 4. The immunoglobulin G (IgG) molecule. The Immune System, 4th edition (© Garland Science 2015)

During an infection, B cells generate different antibody isotypes (IgD, IgM, IgA, IgG, and IgE). The class and the effector function of an antibody is defined by the structure of its heavy chain. IgDs have heavy chains known as delta(δ)-chains, IgMs have mu(μ)-chains, IgGs have gamma(γ)-chains, IgAs have alpha(α)-chain and IgEs have epsilon(ε)-chains (**Figure 5**). While IgGs, IgDs and IgEs are monomeric, IgAs have two mayor forms, monomeric in serum and dimeric in mucosa, and IgMs form a predominantly pentameric complex that also contains a polypeptide joining chain (J), although hexamers have also been found in plasma [181–183].

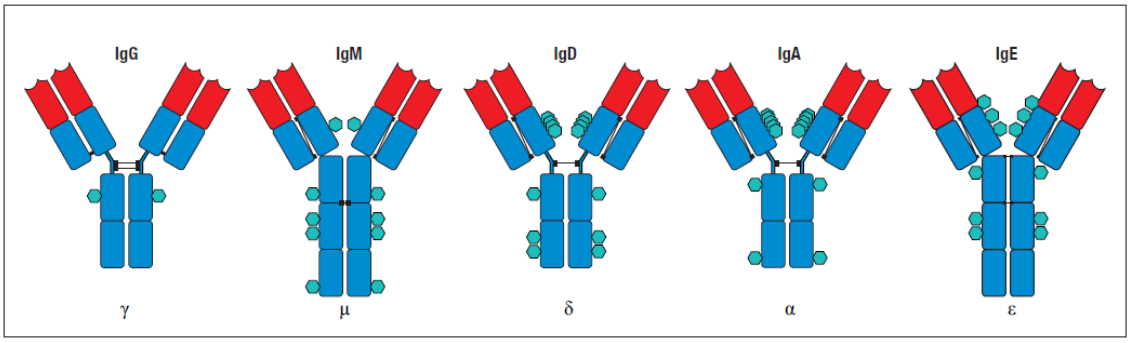

Figure 5: The structures of the human immunoglobulin classes. The Immune System, 4th edition (© Garland Science 2015)

All naïve B cells express cell-surface IgM and IgD, but after an infection, activated naïve B cells can switch from expressing them to express IgG, IgE or IgA on their surface. This process is called class switch recombination and involves a replacement of the μ and δ heavy chain constant regions of the expressed immunoglobulin with γ, ε or α heavy chain constant regions by a DNA recombination event (somatic recombination) [184]. IgM is the first antibody secreted by activated B cells but is less than 10% of the immunoglobulin found in plasma. Little IgD is produced at any time, while IgE contributes a small but biologically important part of the immune response. IgG and IgA are the predominant antibody classes [185]. In the body, IgA can be found predominantly in mucus secretions, IgM in the blood, IgG in body tissues, IgE in blood or extracellular fluid and IgD in the circulation, nasopharyngeal, oral and lachrymal secretions [186–190]. Antibody classes have different affinities for Fc-receptors and, consequently, their functional activities and distribution vary **(Figure 6).** The principal role of IgM is the activation of the complement cascade by directly binding the C1q component and initiating the cascade that results in opsonization and lysis of the pathogen [191]. IgG principal functions are neutralization, interfering with the pathogen attachment to host tissues, and non-neutralizing functions such as opsonization, in which the antibody binds to the pathogen and enhance its phagocytosis, and activation of the complement system [192]. IgA acts mainly as a neutralizing antibody [193]. IgE is predominantly associated with allergic reactions

and immunity to helminth parasites by the activation of mast cells and basophils [194, 195]. Finally, the function of IgD is not well understood yet but it is thought to be involved in mucosal immunity [196, 197].

Functional activity	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Neutralization	+	–	++	++	++	++	++	–
Opsonization	+	–	++	*	++	+	+	–
Sensitization for killing by NK cells	–	–	++	–	++	–	–	–
Sensitization of mast cells	–	–	+	–	+	–	–	+++
Activates complement system	+++	–	++	+	+++	–	+	–

Distribution	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Transport across epithelium	+	–	–	–	–	–	+++ (dimer)	–
Transport across placenta	–	–	+++	+	++	+/–	–	–
Diffusion into extravascular sites	+/–	–	+++	+++	+++	+++	++ (monomer)	+
Mean serum level ($mg \bullet ml^{-1}$)	1.5	0.04	9	3	1	0.5	2.1	3×10^{-5}

Figure 6: Each human immunoglobulin class has specialized functions and a unique distribution. The major effector functions of each class (+++) are shaded in dark red, whereas lesser functions (++) are shown in dark pink, and very minor functions (+) in pale pink. The distributions are marked similarly, with actual average levels in serum being shown in the bottom row. IgA has two subclasses, IgA1 and IgA2. The IgA column refers to both. *IgG2 can act as an opsonin in the presence of an Fc receptor of the appropriate allotype, found in about 50% of people of Caucasian descent. Janeway's Immunobiology, 9th edition (© Garland Science 2017)

IgG is the most abundant immunoglobulin and it has four subclasses, IgG1, IgG2, IgG3 and IgG4. The most prevalent subclass is IgG1 (60-70% of the total IgG), followed by IgG2 (20-30%), IgG3 (5-8%) and IgG4 (1-4%) [198]. The structures of the four IgG subclasses are very similar, but they have differences in the hinge region and N-terminal domain and heavy chain genes. These structural differences provide IgG subclasses a unique profile as they have different affinities for Fc-receptors, making them functionally distinct in antigen binding, immune complex formation, complement activation, triggering of effector cells, half-life, and

placental transport [29, 199]. IgG1 and IgG3 are the predominant subclasses involved in the response to protein antigens, while IgG2 is involved in the response to bacterial capsular polysaccharide antigens and IgG4 is often induced by chronic exposure to allergens or helminths [27–32].

2.2 Immune system in neonates

The reasons for the higher susceptibility of newborns and young infants to pathogens and the infection-induced increase in morbidity and mortality in early life remain poorly defined. In part this vulnerability is due to the lack of previous exposure to pathogens and external antigens, and therefore their immune systems rely on innate responses. In addition, the newborn's host immune system has been considered to be "immature", meaning that the newborn is less capable of protecting from infectious agents than adults due to "deficient" responses compared to adults [200]. However, newborns can mount pro-inflammatory and adaptive immune responses. Nevertheless, the magnitude and quality of responses differ from that in adults [201].

The development of the immune system starts at the fetal stage. Placental mediators such as progesterone and prostaglandins promote Th2-type responses, that may be responsible for the absence of inflammatory functions, leading to immunotolerance and low control over infections [33]. Neutrophil, monocyte and macrophage frequencies steeply increase in number shortly before birth [202] and their functions are weak compared with later life as a result of reduced adhesion to endothelial cells, diminished chemotaxis and lower expression of TLRs [34, 35]. Also, the complement system components are decreased [33]. Overall, neonatal innate immunity cells have impaired antigen-presenting functions and T cell stimulatory abilities [203]. NK cells numbers also increase during gestation and reach the highest number at birth, but they exhibit lower cytotoxicity due to reduced degranulation abilities [204].

Innate lymphoid cells are immune cells that, similar to T cells, mediate both pro-inflammatory and anti-inflammatory responses. However, they target conserved components of the pathogens without the requirement of recombination or expansion from memory cells [205], which makes them key for neonatal immunity.

Neonatal CD4+ T-cells are polarized towards a Th2 response and an important population develops into Th17 cells involved in tolerance [206]. Compared to adults, neonates also show reduced T_{FH} cells in their frequency and secretion of IL-

21, essential at B-cell proliferation and development of antibody responses [36]. Neonatal CD8+ T-cell response is also impaired, mainly as a result of reduced expression of APC receptors [207] and less production of IL-12 from neonatal APCs compared to adults [208]. Also, antibody responses in neonates are delayed in onset, reach lower peak levels, are of shorter duration, differ in the distribution of IgG isotypes, and are of lower average affinity and reduced heterogeneity compared to adult responses [209].

As a result, neonates exhibit an increased susceptibility to infections and protection from pathogens mainly depends on the immunity conferred by maternal antibodies.

3. PLACENTAL TRANSFER OF ANTIBODIES

Maternal antibodies, transferred through the placenta to the fetus, are essential in the protection of infants against infectious diseases during their first months of life [37]. The human placenta is a complex organ that acts as the interface between the mother and fetus and facilitates the transport of substances between them, allowing the exchange of gas, nutrients and the elimination of waste products. It has a protective function against some infectious agents and prevents the rejection of the mother's immune system towards the fetus. The placenta is composed of a fetal part, including the chorionic plate and chorionic villi, and a maternal part consisting of endometrium (decidua basalis). The villi are vascular projections of fetal tissue surrounded by chorion, which consist of a layer of syncytiotrophoblasts in direct contact with maternal blood within the intervillous space, and the inner cytotrophoblast [210]. Two umbilical arteries and a umbilical vein form the umbilical cord, transporting blood from the placenta to the fetus and *vice versa* [211] (**Figure 7**).

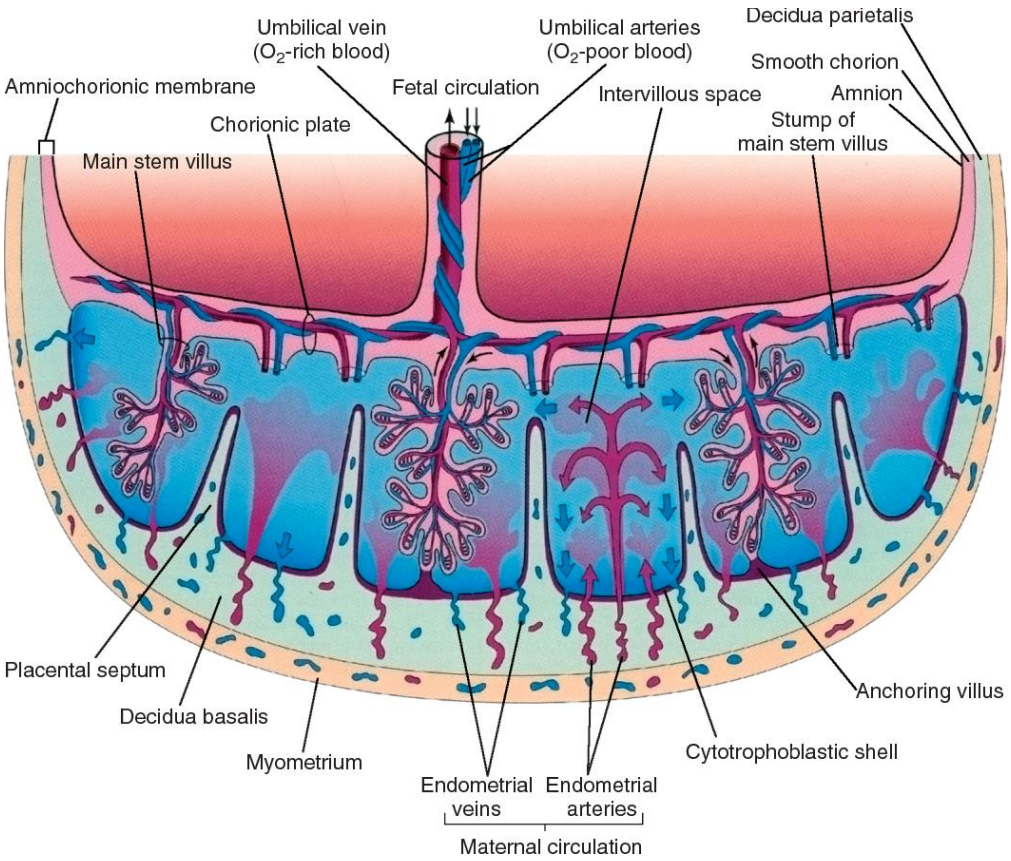

Figure 7: Schematic drawing of a transverse section through a full-term placenta. The Developing Human: Clinically Oriented Embryology, 8th Edition by K.L. Moore and T.V.N. Persaud (Elsevier Inc.)

Transplacental antibody transfer is facilitated by the neonatal Fc receptor (FcRn) in the placental syncytiotrophoblast. In this process, antibodies bind to FcRn and cross the syncytiotrophoblast layer, the villous stroma and the fetal vessel endothelium by a FcRn-mediated transcytosis in which antibodies are protected from degradation [212, 213]. Only IgG is transferred across the placenta with the highest rate occurring during the third trimester of pregnancy [214], although some studies suggest that maternal IgE may also be transferred to the fetus as IgG/IgE complexes [215]. The different IgG subclasses are transferred with different efficiency: it has been commonly stated that the order is IgG1›IgG4›IgG3›IgG2 [38], although a recent study on transplacental transfer of antibodies showed a hierarchy of IgG1›IgG3›IgG4=IgG2 and reported many other studies with different transfer efficiencies [39]. There are many reasons that could explain these differences between studies, such as different study populations or the type of antigen tested.

The disparities in transplacental antibody transfer efficiency between IgG subclasses could be associated with their affinities to the FcRn, but it may be more complex. For example, it has been reported that the IgG hinge region is different in length and flexibility between subclasses [40], which could affect the possible

conformations of the Fab arms relative to the Fc domain, changing their orientation and movement. This could alter the binding to the FcRn, specially for IgG2 that has a shorter and less flexible hinge region [29, 216]. Another hypothesis is that, apart from FcRn, several other Fcγ receptors expressed in the placenta, such as FcγRIIb, could play a role in modulating transplacental antibody transport [217], although the mechanism is not yet elucidated. As IgG2 has a very low binding affinity to FcγRIIb [218], this hypothesis could explain the low IgG2 transfer. Other factors affecting the FcRn-mediated transplacental transfer of antibodies are glycosylation of antibodies, maternal IgG concentration, maternal infections, maternal nutrition and birthweight [40].

Maternal IgG levels and neonatal IgG levels are usually correlated, but if maternal IgG levels are too high, the FcRn could be saturated and IgGs must then compete for a finite number of FcRn receptors [41]. For this reason, hypergammaglobulinemia has been associated with a reduction on the transplacental transfer of antibodies in many studies [47, 219–222]. Prematurity, on the other hand, has also been shown to have a detrimental effect on placental transfer of antibodies [223–228]. The largest amount of IgG is transferred in the third trimester, reaching 50% of maternal concentrations at weeks 28-32, but a sharp increase in cord blood levels occurs after the 36th week of gestation [41]. Therefore, preterm newborns usually have lower antibody levels than term newborns. Low birthweight may also negatively affect the transplacental transfer of antibodies, as reported in many studies [227–229].

Maternal nutrition and non-communicable diseases also play a role in the transplacental transfer of antibodies. It has been described that placental transfer of *Hib* antibodies is lower among malnourished pregnant women [44] and it could be related with an impaired growth in utero associated with the lower placental nutrient supply [230]. Diabetes mellitus, the most common medical complication of pregnancy [231], has been demonstrated to have an effect on the placental transfer of antibodies. Some studies showed an increase rate of IgG transfer in hyperglycemic mothers [232, 233] and others demonstrated that the FcRn expression in cells from maternal blood, cord blood and placenta is compromised among women with diabetes, as well as the total IgG and IgG subclass levels [234]. Hyperglycemia is also associated with structural alterations of the placenta, that could affect the transfer of antibodies [42, 43].

Maternal malaria and HIV infections have also been related with lower transplacental transfer of antibodies [45, 46, 55, 47–54]. Further details on the influence of these infections on the transplacental transfer of antibodies are explained in the following chapters.

4. MALARIA

Malaria is an infectious disease caused by protozoan *Plasmodium* spp. parasites of the phylum Apicomplexa, which are transmitted to humans by female mosquitoes of the genus *Anopheles* [235].

The term malaria originates from *mal'aria* in Medieval Italian ("bad air"), inspired by the condition known as Roman fever [236]. Historical references of malaria are dated from about 2700BC in Chinese medical reports and 1200 years later in Egyptian papyri [237]. The parasite was first discovered by Charles Louis Alphonse Laveran in Algeria in 1880. He performed necropsies on malaria victims and found moveable filamented or flagellated pigmented bodies in their blood, concluding that they were the cause of malaria and calling them *Oscillaria malariae* [238]. However, his discovery produced skepticism as Corrado Tommasi-Crudeli claimed that the pathogen of malaria was the bacteria *Bacillus malariae*. Few years later, the Italian scientists Giovanni Battista Grassi, Amico Bignami, Giusseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava confirmed Laveran's observations and incriminated mosquitoes as the vectors for human malaria [239]. Laveran was awarded the Nobel Prize in Physiology or Medicine in 1907 in recognition of his work on the role played by protozoa in causing diseases.

4.1 Epidemiology

Malaria affected 229 million people worldwide in 2019 and caused more than 400,000 deaths, the majority were children aged under 5 years. The WHO African Region is the largest burden of malaria morbidity, with the 95% of the cases(**Figure 8**) [58]. In Mozambique, the prevalence of malaria was 38.9% in 2018 [59]. Between 2010-2012, the time of the study of this doctoral thesis, malaria transmission was low/moderate in Manhiça District, with a prevalence among pregnant women of 2-4% [240].

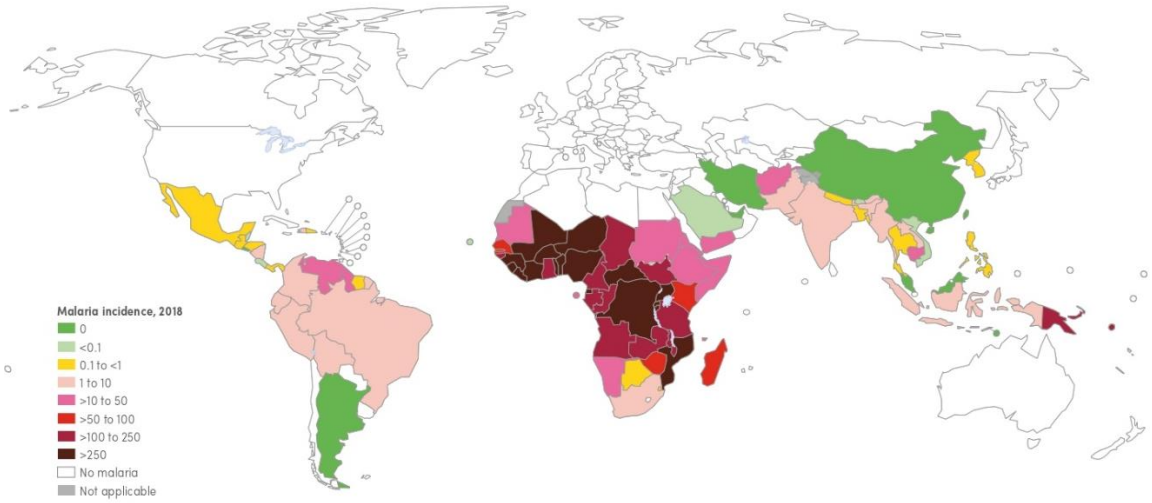

Figure 8: Malaria incidence rate in 2018. *WHO Malaria Report 2018.*

There are six *Plasmodium* species known to affect humans: *P. falciparum, P. vivax, P. ovale wallikeri, P. ovale curtisi, P. malariae* and *P. knowlesi* [56, 57]. *P. falciparum* is the most widespread in Africa and causes the most severe form of the disease. *P. vivax* is predominant in the WHO Region of the Americas and is responsible for 50% of the cases in the WHO South-East Asia Region and 29% in the WHO Eastern Mediterranean Region [241]. *P. ovale* species and *P. malariae* can be found in Asia and in West Africa [242, 243], while *P. knowlesi* is located in Southeast Asia [244]. Co-infections are frequent in areas where different species are prevalent, and mixed infections of *P. falciparum* and *P. vivax* or *P. ovale* species and *P. malariae* have been reported [245–249].

Transmission occurs primarily in tropical and subtropical areas where the environment is optimal for the mosquito. In endemic areas though, the intensity and risk of infections is highly variable and influenced by many factors, including climate variables such as temperature, rainfall and humidity, that affect the development of the mosquito larva and the activity of mosquitoes [250–252]. Seasonality, therefore, has a direct effect on malaria transmission: the mosquito reservoir is lower during the dry season, and increases exponentially during the rainy or wet season [253, 254].

Drug resistance to antimalarials is a concern as it results in increased malaria morbidity and mortality and also favors the spread of the parasite. It occurs when the parasite acquires the ability to survive to what it was an effective treatment, normally due to mutations that affect the susceptibility to the drug [255]. Similar to antimalarial resistance, insecticide tolerance is widespread and has negative implications in the control of the disease vectors [256, 257]. The application of insecticide-based vector control interventions for public health such as long-

lasting insecticide-treated nets (LLINs) or indoor residual spraying (IRS) have achieved substantial reductions in malaria prevalence [258]. However, the resistance to insecticides in African malaria vector population is increasing [259, 260].

4.2 Life cycle

The life cycle of the malaria parasite is complex and involves a human and mosquito host (**Figure 9)**. It begins when an infected female *Anopheles* mosquito bites a human and inject *Plasmodium* sporozoites into the bloodstream (1), although a small part invade lymphatic vessels and arrive to lymph nodes [261]. Then, sporozoites migrate to the liver and invade hepatocytes (2), in which they replicate asexually as hepatic schizonts until thousands of merozoites are produced and released in the bloodstream. In *P. vivax* and *P. ovale* infections, some parasites remain in the liver as hypnozoites, a dormant stage that can persist quiescent during weeks and then cause relapses. After this liver replication stage, known as exo-erythrocytic schizogony, merozoites infect erythrocytes and the ring stage starts (3), in which merozoites undergo asexual replication and develop into trophozoites, that mature into schizonts whose rupture releases merozoites that invade new erythrocytes. Some parasites, instead of asexual replication, differentiate into sexual erythrocytic stage and release male and female gametocytes into the bloodstream. When a female *Anopheles* bites a person, those gametocytes can be ingested during the blood meal and develop into mature sexual cells called gametes (4). In the mosquito midgut male gametocytes penetrate female gametocytes and generate zygotes. This is known as the sporogonic cycle. Zygotes develop into ookinetes, a motile stage that can traverse the midgut wall of the mosquito and develop into oocyst (5), where thousands of sporozoites are produced. After the oocyst growing and rupture, sporozoites migrate to the mosquito salivary glands and are injected to a human by the insect bite.

More than 5,000 putative proteins are expressed during the malaria parasite life cycle and they are structurally different in each stage [262]. The immune system is capable of recognizing some of them, most of them during the blood stage.

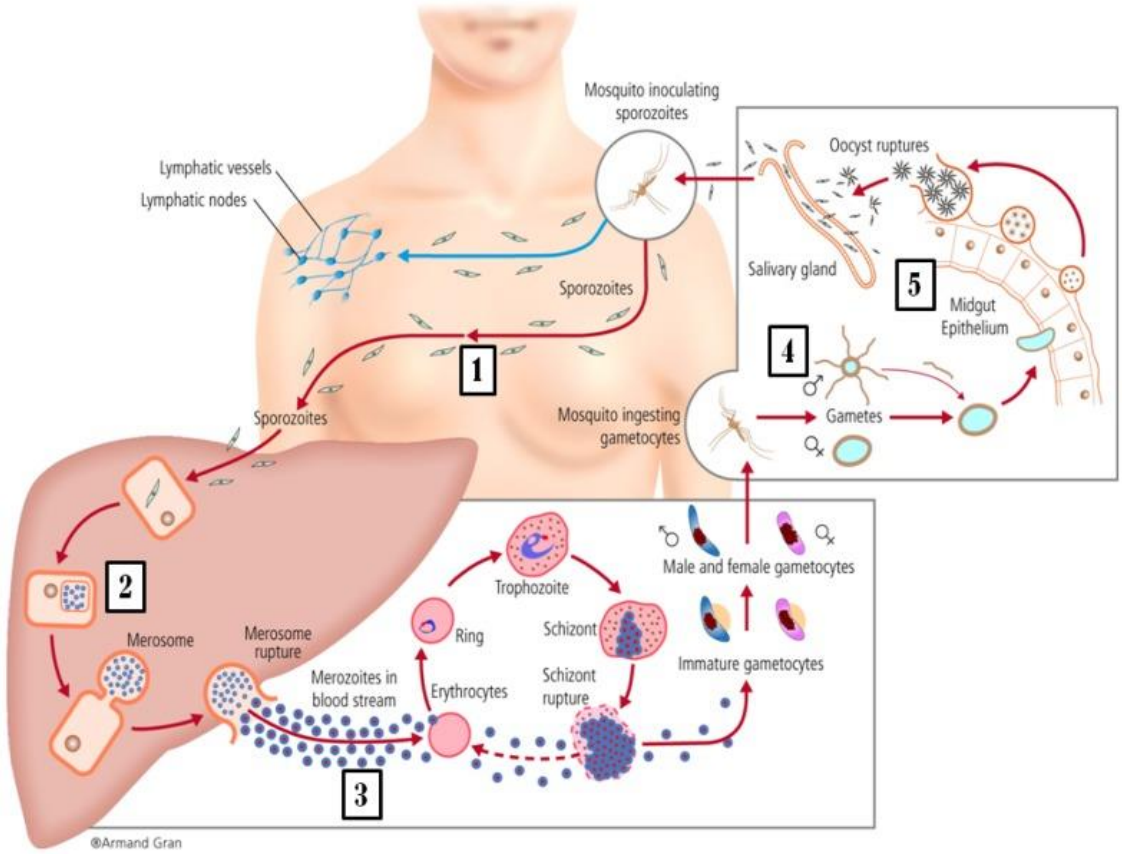

Figure 9*:* The *Plasmodium falciparum* life cycle (ISGlobal).

4.2.1 Pre-erythrocytic stage antigens

The pre-erythrocytic stage starts with the inoculation of sporozoites in the bloodstream. Sporozoites express different antigens that can be recognized by antibodies. A key protein in this stage is the circumsporozoite stage protein (CSP), which is expressed in the surface of sporozoites forming a dense coat that seems to be involved in the migration to the midgut in mosquitoes and in hepatocyte binding in the human host [263]. This is the antigenic target of the RTS,S/AS01$_E$, the most advanced malaria vaccine [60]. Other important proteins are the thrombospondin-related adhesive protein (TRAP), expressed on the surface of sporozoites during hepatocyte invasion [264] and included in the vaccine candidate ME-TRAP [265], and the liver stage antigen 1 (LSA1), expressed on schizonts shortly after hepatocyte invasion and also a target for a malaria vaccine [266].

4.2.2 Asexual blood stage antigens

The asexual blood stage starts with the release of merozoites from the liver hepatocytes to the bloodstream, where they invade erythrocytes and undergo asexual replication, causing symptoms and complications in human infections. Bloodstream merozoites rapidly attach to red blood cell (RBC) surface and the process of invasion and internalization within the RBC occurs [267]. *In vitro* studies demonstrated that outside the erythrocyte, merozoites cannot replicate and only remain infective for a few minutes, probably for less time *in vivo* where they are subjected to host immune responses, so this process must be rapid; in fact, it is completed in less than 1 minute by most merozoites [268].

During this stage, different antigens are expressed and recognized by the immune system. Proteins relevant to the RBC attachment and invasion are present in the surface of the merozoite or contained within organelles called rhoptries, micronemes and dense granules (**Figure 10**). These organelles, located inside the merozoite, are secretory specialized organelles involved in the invasion and formation of the vacuole in which the parasite grows [269].

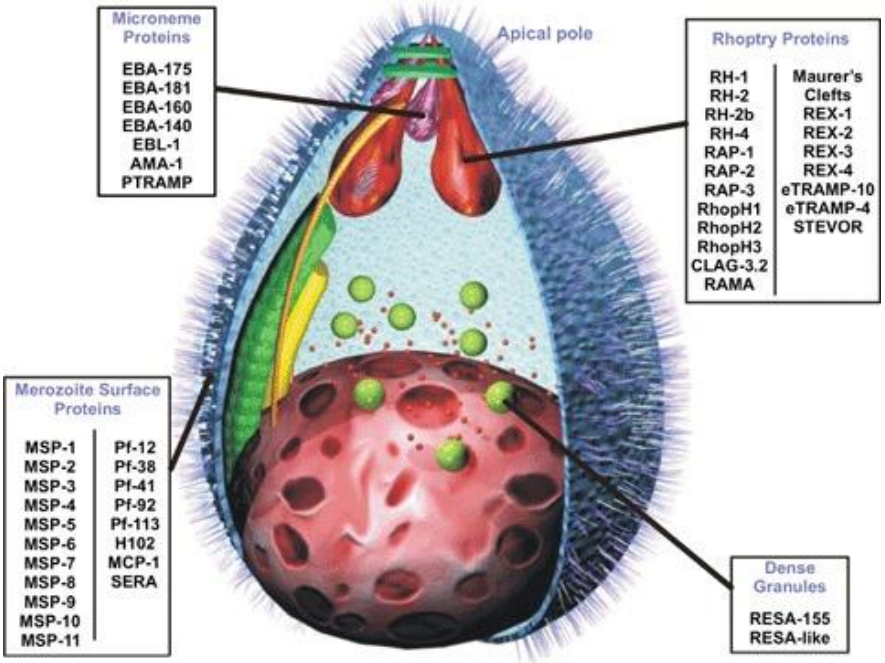

Figure 10: The merozoite structure and molecules localized in organelles involved in merozoite invasion of RBCs. Patarroyo ME, et al. (2018). *Journal of Cellular and Molecular Medicine*, 12(5B):1915-1935.

The erythrocyte invasion is a complex process that involves multiple stages (**Figure 11**). Merozoite surface proteins (MSPs) are 180 kDA proteins involved in the first contact and attachment to the RBC surface. MSPs can be either

glycophosphatidylinositol (GPI)-anchored proteins, integral membrane proteins or peripherally-associated proteins. The initial binding to the RBC seems to be mediated by the GPI-anchored MSPs, principally merozoite surface protein 1 (MSP1). MSP1 is the most abundant surface antigen in the blood stage of *P. falciparum*. The gene has been divided into diverse blocks of which block 2 (MSP1 bl2) shows extensive allelic polymorphism worldwide [270]. During schizogony, MSP1 is processed into four proteolytic products named accordingly to their mass weight ($MSP1_{83}$, $MSP1_{30}$, $MSP1_{38}$ and $MSP1_{42}$) forming the MSP1 complex. This MSP1 complex bind to the human erythrocyte membrane and then the GPI-anchored C-terminal 42 kDa fragment ($MSP1_{42}$) is processed by a second protease to give the 19 kDa fragment ($MSP1_{19}$), which is carried into the RBCs and seems to be involved in intraerythrocytic development. The function of the rest of MSPs is still unknown, although they appear to be essential in the erythrocyte binding step [61, 62]. MSP2, which is the second most abundant GPI anchored MSP, is a highly polymorphic 25kDa protein with two main allelic forms (3D7-like and FC27-like). Although its precise role is still not deciphered, it has been reported that MSP2 can polymerize to form amyloid-like fibrils, which has been associated with attachment and invasion roles in other microorganisms as well as evasion of host immune responses [271, 272]. Another important protein is MSP5, a 272-residue protein with a low polymorphism. Anti-MSP5 antibody response has been associated with reduced incidence of clinical malaria, suggesting that it could be a potential vaccine target [273]. MSP5 is expressed not only in merozoites, but also in sporozoites and infected hepatocytes [274].

The next step of the erythrocyte invasion is a process called reorientation, in which the apical end of the merozoite directly contact the RBC membrane. Then rhoptries, micronemes and dense granules discharge their contents, that interact with erythrocyte surface receptors forming a “moving junction”, a belt-like structure anchored to the merozoite’s inner membrane complex, which contributes to the formation and maintenance of the merozoite’s characteristic ovoid shape. The invasion ends when the moving junction closes behind the merozoite, leaving the merozoite enclosed within a parasitophorous vacuole. In the process of moving junction formation and erythrocyte invasion, the reticulocyte-binding-like family rhoptry proteins (RHs), erythrocyte-binding antigens (EBAs) and apical merozoite antigen-1 (AMA1) are involved [63].

The process of invasion can be sialic acid-dependent or independent. The sialic acid-dependent pathway involves high affinity recognition of sialic acid on cell surface receptors. In contrast, with the sialic acid-independent pathway the recognition of sialic acid to invade erythrocytes is not needed [275].

P. falciparum reticulocyte-binding homologues (PfRh) proteins are located in the rhoptries and include PfRh1, 2a, 2b, 4 and 5. Whereas PfRh1 is involved in sialic acid dependent invasion, PfRh2 and PfRh4 are important in sialic acid independent invasion, although it has been suggested that PfRh2 may also play a role in the sialic acid dependent pathway. On the contrary, EBA proteins are involved in sialic dependent invasion pathway, as the majority binds to glycophorins that are the major sialylated proteins on the erythrocyte. EBA proteins are located in the micronemes and include EBA175, EBA140, EBA181 and erythrocyte binding ligand 1 (EBL1). EBA175 was the first characterized protein of the EBA family and bind to the erythrocyte surface molecule glycophorin A. EBL1 has been shown to bind to glycophorin B, EBA140 bind to glycophorin C and EBA181 bind to sialic acid on the erythrocyte surface and to band 4.1 protein [276].

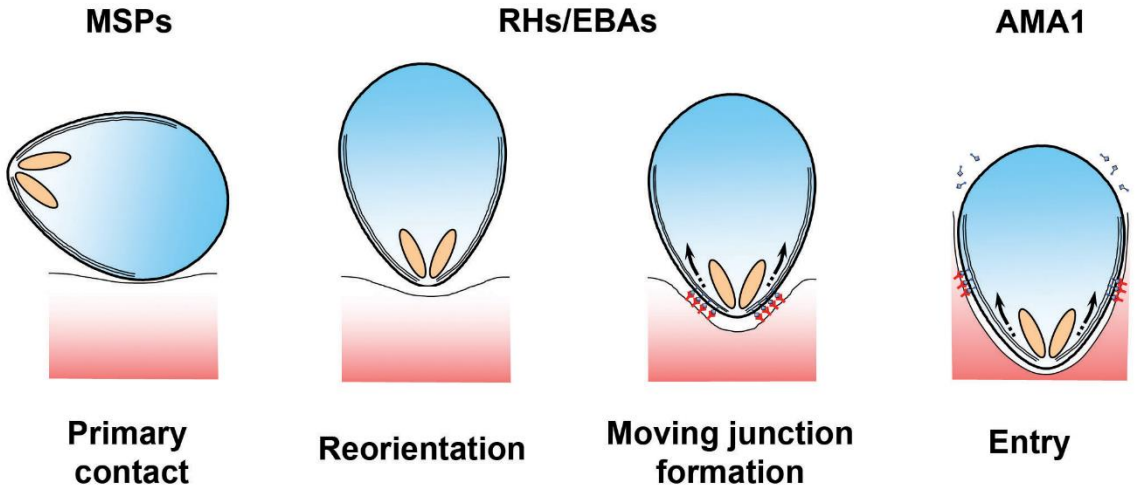

Figure 11: Summary of the proteins involved in erythrocyte invasion and their function. Wright GJ, Rayner JC (2014). *PLoS Pathog* 10(3).

Once RBCs are infected, the intra-erythrocytic parasites are surrounded by the parasitophorous vacuole membrane, which serves as a protective barrier for the protozoa and functions as a communication interface between parasite and host cell. Exported proteins EXP1 and EXP2 have been described as components of *Plasmodium* translocon of exported proteins (PTEX) complex, which is responsible for the export of parasite proteins and nutrient uptake during the intraerythrocytic stage and liver stage [64]. EXP1 is a highly conserved 17kDa membrane protein essential for parasite proliferation in this stage and it has been hypothesized that is localized in the merozoite's dense granules, as well as EXP2 [277]. EXP1 is critical for nutrient uptake across the parasitophorous vacuole membrane, as it is required for EXP2's proper distribution and function as a nutrient-permeable channel [278]. Some parasite proteins, such as ring-infected erythrocyte surface antigen family (RESA) and *P. falciparum* erythrocyte membrane protein 1 family (PfEMP1), are exported to the erythrocyte surface across the parasitophorous vacuole

membrane via the PTEX. The RESA proteins are stored within dense granules and their function is to stabilize the infected RBC cytoskeleton [279]. PfEMP1 is encoded by the *var* multigene family and it is involved in cytoadhesion, a process in which the infected erythrocytes bind to the vasculature as an immune evasion mechanism [280]. Each parasite genome contains about 60 different *var* genes, but only one *var* gene is expressed within a single infected erythrocyte [281]. This is especially important in pregnant women, as placental *P. falciparum* parasites express only *var2csa* [282]. VAR2CSA is a transmembrane protein composed of six Duffy-Binding-Like domains (DBL 1-6) that binds to chondroitin sulfate A (CSA). CSA is found mainly in the placental intervillous space and on syncytiotrophoblasts [283], resulting in an accumulation of infected erythrocytes at the maternal-fetal interface, causing placental malaria (PM).

4.2.3 Sexual stage antigens

Antibodies are also able to recognize gametocyte antigens and have the capability to block their function. Because of that, there have been efforts on vaccine development against these proteins in order to acquire potential transmission reducing immunity. Some of the sexual stage antigens include gametocyte/gamete proteins such as Pfs230 and Pfs45/48 and zygote/ookinete proteins Pfs25 and Pfs28. The names of these antigens are due to their observed molecular weight after separation by SDS-PAGE. Pfs230 and Pfs48/45 proteins are thought to be essential for gamete fertility and zygote formation, while Pfs25 seems to play a role in ookinete to oocyst transition [65, 66].

4.3 Malaria pathogenesis

Malaria clinical disease is mainly the result of the parasite asexual replication, when the rupture of infected RBCs occur and merozoites are released into the bloodstream [67]. The asexual cycle repeats every 24 to 48 hours and parasitemia rises exponentially, leading to an increase of the immune response that is usually associated with inflammatory responses to eliminate parasites [68]. After the incubation period of 10-14 days (longer for *P. vivax, P. ovale* and *P. malariae*) the clinical effects of the infection appear [284]. The disease can be separated into two presentations: uncomplicated and severe malaria, although some individuals

from endemic areas have low parasitemia with no symptoms, which is known as asymptomatic malaria.

Uncomplicated malaria most common symptoms include intermittent fever that coincide with the rupture of parasite schizonts in infected RBCs during the asexual blood stage cycle. Other symptoms such as chills, body-aches, headache, cough and diarrhea can also be present [284].

Severe malaria signs and symptoms include impaired consciousness, respiratory distress, multiple convulsions, prostration, shock, pulmonary edema, abnormal bleeding and jaundice, as well as severe anemia, hypoglycemia, acidosis, hyperlactatemia and renal impairment [285]. Among the complications of severe malaria, severe anemia is more frequent among infants and young children in high transmission areas [69], while in areas with less intense or seasonal transmission, cerebral malaria is more common in older children [286]. Severe anemia is defined as hemoglobin concentration ‹5g/dl in children ‹12 years of age (‹7g/dl in adults). Hyperparasitemia is associated with the severity of illness and it is defined as ›250.000 asexual parasites/μl blood or ›4% parasitized erythrocytes. A threshold of ›10% infected RBCs is an indicator of high risk [285].

Cerebral malaria is the most severe complication and includes neurological symptoms. Although the mechanisms that leads to neurological complications and death are still not clear, it has been postulated that infected RBCs accumulate into brain capillaries causing occlusion and a reduction of microvascular flow, leading to a vessel wall damage and hemorrhages [70].

Malaria infection is particularly dangerous during pregnancy, as pregnant women are at greater risk of malaria complication, such as severe anemia. *P. falciparum* parasite densities are higher in pregnant women than non-pregnant adults and placenta is frequently damaged by the parasite, that results in lower birth weight babies, preterm delivery, fetal growth restriction and stillbirth [287]. PM will be further explained at chapter 4.6.

4.4 Diagnosis and treatment

Apart from the clinical diagnosis of malaria, laboratory diagnosis is essential to confirm the infection, as the clinical features of the disease are non-specific and may result in over-treatment of malaria or non-treatment of other diseases. A rapid

and effective malaria diagnosis is also fundamental for the decrease of community transmission.

There are many diagnostic techniques. The most conventional is microscopic diagnosis by Giemsa-stained thin and thick peripheral blood smears. Thick blood smear allows the detection of 50-100 parasites/μl [71], although thin blood smear allows a better visualization of the parasite that is useful for the species identification.

Rapid diagnostic tests (RDTs) are fast and easy to perform and do not require microscopy or training. They detect *Plasmodium* antigens in blood such as histidine-rich protein II (HRP-II) or lactate dehydrogenase (LDH). However, they showed wide variations in sensitivity and there could be cross-reactions of *P. falciparum* samples with the *P. vivax* LDH or non-*falciparum* samples with the *P. falciparum*-specific test line [72, 73].

Other methods used are serological tests, based on the detection of antibodies usually against asexual blood stage malaria parasites. For example, immunofluorescence antibody (IFA) testing is highly sensitive and specific and has been useful in epidemiological surveys [288]. The limitations of IFA is that it needs fluorescence microscopy and trained technicians, and it is not recommended in the acute diagnosis of malaria as antibodies against malaria parasites are produced about 2 weeks after infection [72].

The polymerase chain reaction (PCR) technique is considered to be one of the most specific and sensitive diagnostic methods for infectious diseases, including malaria [74]. This molecular-based technique consists in the amplification and detection of parasite DNA. It detects as few as 1-5 parasites/μl of blood (≤ 0.0001% of infected red blood cells) and can help drug-resistant parasites and mixed infections [72]. The principal limitation of this technique is that is more expensive, complex and may not be suitable for malaria diagnosis in remote rural areas.

Treatment for malaria include several drugs such as chloroquine, quinine, artemether mefloquine (MQ) or primaquine, among others. Malaria drugs were first used at China, obtained from the leaves of sweet wormwood (*Artemisia annua*). The bark of the cinchona tree, whose active ingredient is quinine, had been used by the Quechuas from Peru and Bolivia [289]. Quinine has been used for centuries to treat malaria successfully and some synthetic derivates, such as chloroquine, demonstrated its efficiency. Currently, artemisinin-combination therapies (ACTs) are recommended for the treatment of malaria and sulfadoxine-pyrimethamine (SP) or MQ has been shown to be also effective against the parasite [75–77]. Those drugs are also used for chemoprophylaxis in vulnerable populations, such as

pregnant women or infants. The administration of IPTp with SP as early as possible at the beginning of the second trimester and up to delivery to prevent malaria during pregnancy is recommended by the WHO guidelines [290]. Although SP in HIV positive pregnant women on cotrimoxazole is contraindicated due to an increased risk of adverse events when both drugs are given in parallel, it was demonstrated that MQ IPTp had similar safety profile and pregnancy outcomes than SP [291]. However, with the emergence of drug-resistances, other malaria control measures are needed, with important interest on vaccine development for malaria prevention.

4.5 Naturally acquired immunity to malaria

Naturally acquired immunity (NAI) to malaria is a process in which individuals living in malaria-endemic areas gradually develop protection in response to repeated infection. This protection can be against clinical disease (anti-disease immunity) or against parasitemia, by suppressing parasite replication (anti-parasite immunity) [78, 292]. In hyperendemic areas with constant exposure and repeated infections, hosts can develop tolerance to the parasite and acquire partial protection against new infections by maintaining a low-grade and asymptomatic parasitemia. This natural defense mechanism is known as premunition [78, 293]. NAI is developed in individuals living in malaria endemic areas with a high parasite transmission, in which repeated *P. falciparum* exposures lead to an acquired protective immunity to the disease. Therefore, in endemic areas NAI is higher and more frequent among older people, while infants and young children less exposed to the parasite are at higher risk of developing severe malaria [79].

Apart from the relation of NAI with parasite exposure, many other factors could affect the acquisition of immunity, such as genetics of the human host. For example, hemoglobinopathies like sickle cell disease, thalassemias or ovalocytosis that are more frequent in malaria endemic areas are associated with protection [294]. Also, polymorphisms present in genes encoding proteins that are important for immune function or pathogenesis may confer resistance to malaria [294]. Other factors influencing NAI could be nutritional status, coinfections and the composition of microbiota [78].

Immune responses against the parasite can be developed for any parasitic stage, although the acquisition of antibodies against merozoite antigens has been associated with protective immunity from clinical malaria in many studies and are the target of numerous vaccines [80, 81]. Antibodies can act against merozoites

before erythrocyte invasion, targeting antigens such as MSP1, AMA1 or EBA175 and blocking their ability to enter the RBC, or can act against infected RBCs through phagocytosis mediation or blocking cytoadhesion of the infected RBC to endothelial surfaces [79]. Infected RBCs expose an array of altered host antigens and parasite antigens such as PfEMP1 that can be recognized by antibodies. Therefore, both antibody mechanisms against "free-merozoites" or infected RBC have been shown to produce effective protection [295]. Specifically, IgG antibodies play a vital role in combating clinical malaria and IgG1 and IgG3 subclasses have been associated with protection, probably because their capacity to fix complement and mediate opsonic phagocytosis [82–84]. However, not all antibodies against merozoite antigens are related with protection, but can be either markers of exposure [296].

Some studies reported that anti-sera raised against recombinant $MSP1_{42}$ inhibit *P. falciparum* growth *in vitro* [297, 298], suggesting that antibodies against this antigen could correlate with protection. Nevertheless, a poor correlation between growth inhibition activity and protection has also been reported [299]. Another study found that antibodies to $MSP1_{42}$ increased with age but were inversely correlated with growth-inhibitory activity, that tended to decline with increasing age and exposure [300]. Also, antibody responses against this antigen decreased after RTS,S immunization and antibody levels correlated with risk of clinical malaria over 1-year follow-up in children participating in the RTS,S/AS01E phase 3 clinical trial [296]. RTS,S vaccination decreases exposure to the parasite and therefore reduces antibody levels against exposure antigens. Thus, anti-$MSP1_{42}$ IgG are not related with protection from clinical malaria but are good detectors of changes in parasite exposure. Instead, in that RTS,S study, antibody responses to EBA140, MSP1 bl2, MSP5 and Rh4.2 were associated with immunity, as IgG levels increased after vaccination and were associated with reduced risk of clinical malaria. IgG targeting EBA140 have also been associated with reduced clinical malaria risk in other studies in individuals in regions where malaria is endemic [276, 301–303]. Similarly, antibodies against the binding domain of PfRh4 can block the binding of this protein to the surfaces of erythrocytes and therefore inhibit the invasion of parasite, preventing high density parasitemia and protecting against clinical malaria [304, 305]. Serum IgG antibodies against MSP1 bl2, specially IgG3, were strongly associated with protection in a study in Ghana, in which lower frequencies of clinical malaria were seen among children who had anti- MSP1 bl2 IgG antibodies [306]. In the same line, MSP5 is also recognized by naturally acquired antibodies, predominantly IgG1 and IgG3, which were associated with reduced incidence of clinical malaria in two endemic Senegalese villages [273] and in the Brazilian Amazon [307].

Other antibodies such as anti-EXP1 or anti-MSP2 can have different behaviors, depending on the circumstances. It has been suggested that both EXP1 and MSP2 IgG levels reflect malaria exposure as they were more affected by previous malaria and did not increase after RTS,S immunization [296]. However, in the same study, malaria-protected children had an increase of IgG against these antigens from birth to month 3 of life, whereas children with malaria cases had a decrease, suggesting a role in protection. Regarding IgG subclasses, IgG3 responses to MSP2 are higher in adults and reduced susceptibility to malaria, while IgG1 responses predominated in children and were associated with risk [308, 309].

Sporozoite antigens such as CSP or TRAP can also be recognized by antibodies, inhibiting their motility or blocking hepatocyte invasion. IgG could also mediate killing of the sporozoite via opsonization and phagocytosis [78]. Such ability also led to the research of sporozoite vaccine candidates that could potentially provide protection against malaria [310].

For an appropriate antibody response against *Plasmodium* antigens, an activation of B cells is required. Thus, NAI also includes cellular immune responses essential for protection. $CD4^+$ T and T_{FH} cells provide help to B cells to produce antibodies. In addition, there are other cellular immune responses involved in immunity: the inhibition of the parasite development and destruction of infected hepatocytes by $CD8^+$ T cells, the activation of macrophages by $CD4^+$ T cells that phagocyte merozoites and the production of Tregs that mediate tolerance to the parasite in order to minimize the damage caused by the pathogen or by the host's immune response [78].

However, during pregnancy NAI is compromised and pregnant women become more susceptible to severe malaria, particularly during first and second gestations [311]. Infected RBCs can accumulate in the intervillous spaces of the placenta or bind to the surface of the syncytiotrophoblast and cause PM (further explained in chapter 4.6). In endemic areas where pregnant women had been more exposed to malaria, acquisition of antibodies that block the binding of infected RBCs to placental receptors enable a better control of the infection. Therefore malaria in subsequent pregnancies is not as severe as in primigravidae women who do not have this acquired immunity [85, 86]. Also, the increased susceptibility of pregnant women to malaria is thought to be related to the physiological immunomodulation that occurs during gestation and the accumulation of infected RBCs in the placenta [292]. This accumulation of infected RBCs has been correlated with an infiltration of monocytes/macrophages in the intervillous space, causing chronic intervillositis related to severe PM [312]. Moreover, increased frequencies of immunoregulatory cells on pregnant women may limit the development of NAI, essential for the

protection against malaria [313]. In addition, placental *P. falciparum* infection can also have a negative effect on the development of immunity in the offspring and on transplacental transfer of antibodies [91]. This is of utmost importance as infants and young children are at higher risk of severe disease, and they are protected during the first 6 months of life principally thanks to maternal IgG antibodies transferred through the placenta [292].

Passive transfer of maternal antibodies against *P. falciparum* has been correlated with protection from symptomatic malaria in the offspring [87–90]. This protection is characterized by low parasite densities, suggesting that maternal antibodies in young infants can control and clear parasitemia [314, 315]. Although it has been discussed that passively transferred maternal antibodies may actually be a biomarker of exposure and risk of infection rather than a correlate of protection [220, 316, 317], it has been observed that children with high initial MSP1 specific antibody levels had a significant period of protection [318].

4.6 Placental malaria

The reduction of NAI during pregnancy increases the risk of complications for both the mother and her fetus, such as severe maternal anemia, abortion, still birth, intrauterine growth retardation, low birth weight and death [319]. The placenta plays an important role in pregnancy outcomes and due to malaria infection different pathophysiological processes occur.

Infected RBCs with VAR2CSA, a member of the PfEMP1 protein family, with active DBL domains can bind to the CSA portion of chondroitin sulfate A proteogylcans, which is the target receptor in the placenta. These infected RBCs attach to the apical lining of the syncytiotrophoblast and infiltrate within the intervillous spaces of the placenta, together with immune cells such as monocytes and macrophages with ingested malaria pigment (hemozoin) [85, 86]. Compared to peripheral blood, the parasite densities in placenta are much higher [92]. All together cause thickening of placental basement membrane, perivillous fibrinoid deposits, syncytial knotting and reduction in the uterine artery blood flow, which results into altered exchange system between mother and fetus [93]. Moreover, cortisol is increased during pregnancy and this hormone directly inhibits NK cell activity against infected RBCs, increasing the susceptibility to malaria [320]. Also, PM enhance non-specific Th1 type pro-inflammatory responses and elevated levels of pro-inflammatory cytokines in the placenta of infected women, especially among

primigravidae, results in placental pathology and adverse pregnancy outcomes [94].

Placental infection can be histologically classified into three stages: active, chronic and past. Active infection is characterized by the accumulation of parasites and pigments in maternal RBCs in the intervillous spaces but no pigment or cells within fibrin. Chronic infections show parasites and pigment in maternal RBCs and pigment and/or cells within fibrin. Finally, in past infections parasites are not present but pigment deposition in fibrin is detected [321]. Chronic infections are associated with the most severe changes, particularly intervillous mononuclear inflammation [93].

Due to the damage caused to the placenta, the maternofetal antibody exchange may be compromised. In fact, some studies observed an association between PM and decreased maternal antibody transfer to the fetus [47, 48, 50, 95], although it is not consistent as many others have shown no impact [47, 219, 221, 322].

5. HIV

Acquired immunodeficiency syndrome (AIDS) was first identified in 1981 in five young homosexual men that had the opportunistic infection *Pneumocystis carinii pneumonia* and a rare and aggressive form of cancer called Kaposi's sarcoma [323]. It was soon speculated that the cause of AIDS was an infectious agent transferred by body fluids and by exposure to contaminated blood [324]. Around the same time, the first human retroviruses, HTLV-I, were discovered in a patient with cutaneous T-cell lymphoma [325]. In 1983, Luc Montagnier and his team at the Pasteur Institute detected a new human retrovirus on cultured T cells from a lymph node biopsy from a young homosexual French patient with AIDS symptoms. This retrovirus belonged to the HTLV family but was different from the previous isolate [326]. Soon after that, Robert Gallo at the National Institutes of Health reported evidences that retroviruses belonging to the HTLV family, named HTLV-III, were the causal agents of AIDS [327]. This theory was strengthened by Levy et. al from the University of California, that isolated AIDS-associated retroviruses and found antibodies against them in AIDS patients [328]. The name of "Human Immunodeficiency Virus" (HIV) was stablished in 1986 by the International Committee on the Taxonomy of Viruses [329]. By the end of that year, 85 countries from Africa, America, Asia, Europe and Oceania had reported 38.401 cases of AIDS [330].

HIV is a lentivirus of the family Retroviridae and subfamily Orthoretrovirinae, composed of two copies of single-stranded RNA molecules that are enclosed within the core of the virus particle. This RNA genome is conversed into double-stranded DNA by reverse transcription, encoding structural and regulatory proteins. Two types of HIV have been characterized on basis of genetics and differences in the viral antigens: HIV-1 and HIV-2 [96].

5.1 Epidemiology

In 2019, the number of people living with HIV was 38 million. Of those, 1.7 million were new HIV infections and about 61% were in sub-Saharan Africa [4]. Since 2010, the number of deaths related to AIDS declined by 33%, accounting for 700,000 in 2019 [331, 332]. This reduction was mainly thanks to the use of antiretroviral therapy (ART) in low- and middle-income countries [333].

HIV-1 is the most common type of HIV and accounts for the majority of infections, whereas HIV-2 is relatively uncommon and less infectious and remains essentially confined to West Africa [97]. HIV is transmitted through sexual fluids, blood, breast milk and vertically (mother to child). Heterosexual transmission remains the dominant mode of transmission and accounts for about 85% of all HIV-1. The rates of new HIV-1 infections are the highest in Southern Africa, where HIV/AIDS is a leading cause of disease burden. Young women accounted for 26% of new HIV infections in 2018, which is specially concerning as it has additional implications for mother-to-child transmission. In fact, the number of children living with HIV in East and Southern Africa was 1.1 million in 2018 and the main route of transmission was through birth [334].

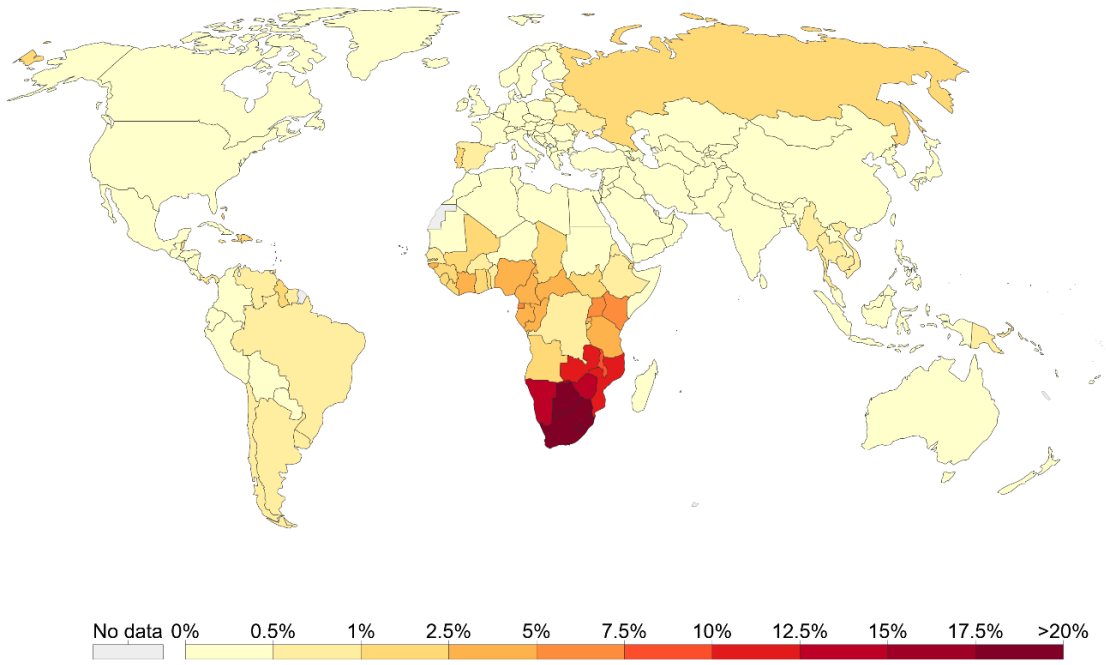

Figure 12: People aged 15 to 49 infected with HIV in 2017. Institute for Health Metrics and Evaluation (IHME).

Mozambique is on the top 10 countries most affected in the world, with 2.2 million people living with HIV in 2019 [98]. Adults aged 15-49 had the highest prevalence [335]. In the general adult population, the prevalence among women was estimated at 15.4% and 10.1% among men [99].

In the south of Mozambique, the Maputo Province has the highest prevalence of HIV with 22.9% of the population estimated to be infected in 2015, among which 29.6% were women and 15.8% men [99]. A cross-sectional community-based study in the Manhiça District, in the Maputo Province, between 2010 and 2012 showed that overall HIV prevalence was 39.9% and higher among women (43.1%) than men (37.6%) [100].

5.2 Structure and biology

HIV virions are spherical structures of approximately 100 nm in diameter with a lipid bilayer membrane envelope enclosing a nucleocapsid (**Figure 13**). The HIV genome encode viral proteins and enzymes. The *env* gene encode two viral proteins, surface glycoprotein gp120 and trimeric transmembrane glycoprotein gp41, that are embedded in the lipid envelope and are responsible for the attachment to the host cell. The *gag* gene encode the matrix protein p17, the core

proteins p24 and p6 and the nucleocapsid protein p7. The *pol* gene encode three enzymes: protease, integrase and reverse transcriptase. In addition to structural proteins, other proteins encoded by HIV genes with regulatory or immune-modulatory functions are viral infectivity factor, virus protein R, transactivator protein, RNA splicing-regulator and negative regulatory factor. Finally, HIV-1 encode the virus protein U that is not present in HIV-2, and HIV-2 encode the viral protein X that is not found in HIV-1 [108].

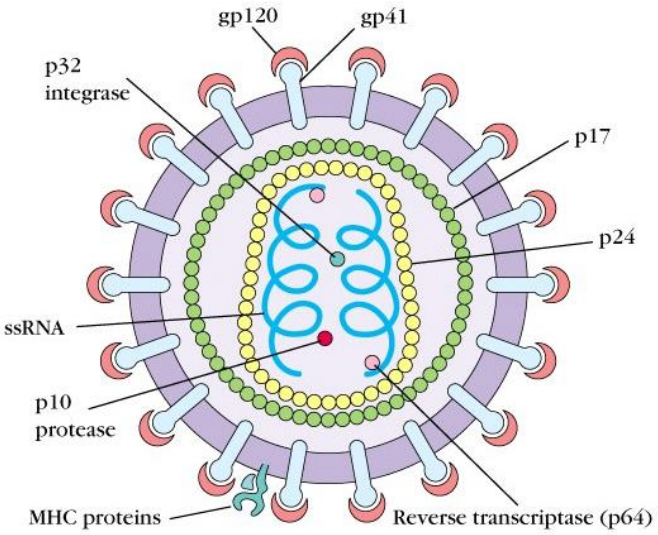

Figure 13: Schematic diagram of HIV virion. © Duane W. Sears

Target cells of HIV-1 are primarily $CD4^+$ T cells and macrophages. First, the HIV-1 envelope protein gp120 recognizes and interacts with the CD4 receptor and the membrane-spanning co-receptor CC-chemokine receptor 5 (CCR) and mediate the viral fusion with the host cell surface. Then, the viral core is released within the host cell cytoplasm. Partial core shell uncoating facilitates reverse transcription of the viral genome into double-stranded DNA. At this point, the pre-integration complex (PIC) is formed and includes the viral DNA, viral proteins (viral protein R, matrix and integrase) and host proteins. Then, viral DNA is imported into the cell nucleus and inserted into the cellular DNA by the integrase. The viral transactivator protein recruits the host RNA polymerase II (RNA Pol II) and positive transcription elongation factor b (P-TEFb), that mediate the transcription of viral DNA to mRNA, that serves as templates for protein production. The retroviral structural proteins are synthesized as part of the Gag precursor polypeptide to assemble virus-like particles at the plasma membrane and bud from cells. Genome RNA is incorporated into virus-like particles, that are released from the cell. Finally, proteases convert immature particles to infectious virions via the proteolysis of Gag precursor polypeptides to yield the structural components of the virus (**Figure 14**) [336].

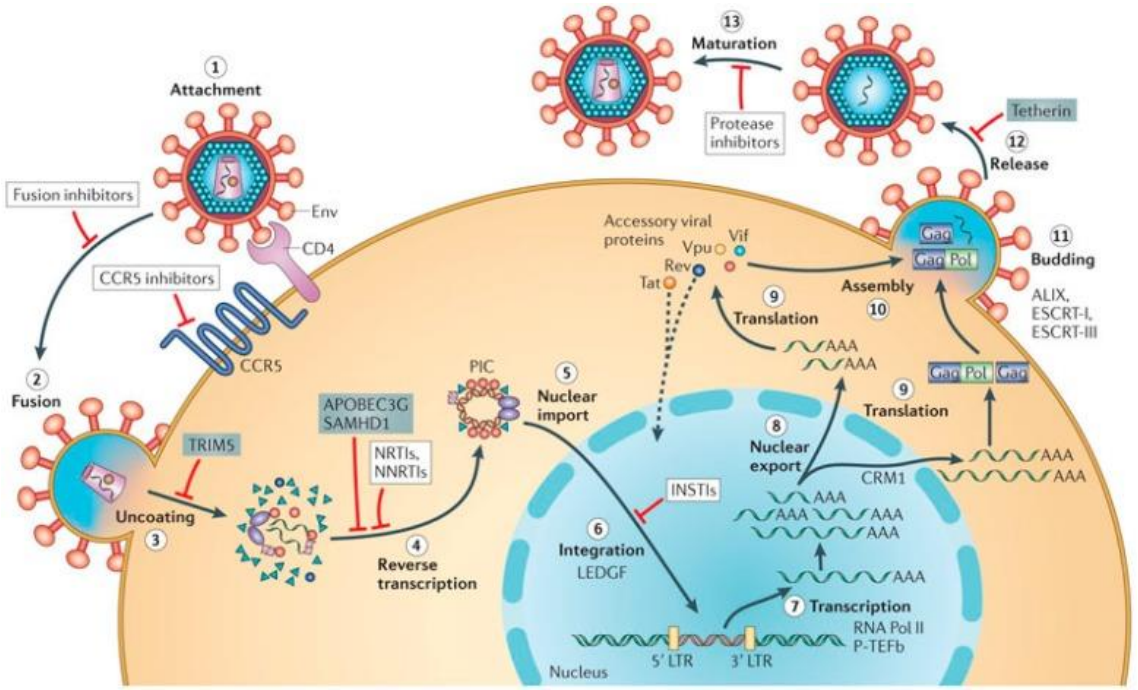

Figure 14: Schematic representation of the replication cycle of HIV. Each step in the HIV-1 lifecycle is a potential target for antiviral intervention. The sites of action of clinical inhibitors (boxed) and cellular restriction factors are indicated with red and green block signs, respectively. Musumeci D, et al (2015). *Molecules* 20(9):17511-17532.

5.3 HIV and the immune system

As mentioned above, HIV targets immune cells expressing CD4, including T cells, macrophages and dendritic cells, and establishes a permanent infection that persists for the lifetime of the host cell. The production of progeny virus causes cell death and leads to the development of AIDS.

The typical time course of HIV-1 infection consists in four phases: eclipse, acute infection, chronic infection and AIDS. The eclipse phase starts one or two weeks after the virus transmission, in which HIV-1 is actively replicating and spreading to different tissues and organs without detectable viremia, symptoms or immune response. The next phase is the acute, characterized by the detection of high levels of viremia (>10^7 copies of viral RNA/mL), large pools of infected CD4+ T cells in blood and lymphoid tissues and acute depletion of $CD4^+$ T cells. During this phase, flu-like symptoms appear and both humoral and cellular immune response are initiated, with antibodies targeting viral proteins and $CD8^+$ cytotoxic T cells targeting antigen-expressing infected cells. This is followed by the chronic infection phase, in which $CD4^+$ T cells levels continue decreasing and there is a chronic immune activation and inflammation. If untreated, the infection advances

to the AIDS phase, that is marked by significantly declining $CD4^+$ T cell numbers, increasing viremia levels and immune exhaustion, characterized by the loss of effector functions and proliferative capacity in memory T cells. This impairment of the immune system leads to opportunistic infections, cancers or death of the infected individual [101].

The innate immune system may contribute to HIV control. TLR activate the synthesis of type I IFN, that inhibits both the early and late steps of the HIV-1 lifecycle, decreases HIV-1 infection on several cell types and impairs HIV-1 transmission from dendritic cells to CD4+ T cells. However, the main producers of natural type I IFN are plasmacytoid dendritic cells (pDC), which are depleted in chronic HIV infection [337]. Regarding the adaptative immune responses, cytotoxic $CD8^+$ T cells (CTLs) are crucial for the control of HIV infection, although HIV possess several mechanisms to escape from the CTL response. For example, HIV has the ability to interfere with antigen presentation and recognition by a number of mechanisms, such as epitope deletion, variance of the sequence of its HLA-I-restricted antigens, or avoiding the display of these HLA-I-associated antigens on the surface of infected cells, that therefore will not be recognized by CTLs [338]. Another evasion mechanism of the virus is the establishment and compartmentalization of non-replicating viruses in $CD4^+$ T cells as latent virus reservoirs during primary HIV infection [101]. Other types of T cells, such as Th17 cells, may also be compromised by immune activation during HIV infection. Within B cell population, HIV infection has been associated with numerous perturbations, including increased frequencies of immature B cells associated with $CD4^+$ T cells lymphopenia, expansion of activated mature B cells associated with chronic HIV viremia, exhausted B cell expansion and loss of resting memory B cells. Moreover, tissue-like memory B cells have decreased proliferative capacity and effector function. It has been also demonstrated that HIV can bind to B cells, facilitating the virus cell-to-cell transmission and increasing B cell apoptosis [339, 340]. Taken together, HIV infection contribute to B-cell dysfunction.

5.4 HIV in pregnancy

In 2015, there was about 1.4 million pregnant women living with HIV worldwide [102]. In the Manhiça District of Mozambique, the percentage of HIV-infected pregnant women was nearly 30% [100]. The global proportion of maternal deaths between HIV-infected women between 1990 and 2005 ranged from 7% to 21% and the maternal mortality ratio increased by more than 40% in all countries in southern

Africa in this period [341]. For treating and preventing HIV infection, Mozambique introduced antiretroviral drugs in 2004, and in 2013 the country adopted the latest WHO guidelines [342, 343]. Despite this, HIV infection continues to be associated with an increased risk of maternal hospital admissions in this area [103].

Pregnant HIV-infected women have a higher risk of adverse pregnancy outcomes such as maternal anemia, malnutrition, concurrent infection, spontaneous abortions and stillbirth, as well as low birth weight and prematurity [103]. Maternal HIV infection has also been associated with altered antibody levels and responses upon infant vaccination, lower numbers of neutrophils and lymphocytes and increased susceptibility to infections in HIV-exposed infants [344], which have higher morbidity and mortality than HIV-unexposed infants [345–347].

Placental cells of HIV-infected pregnant women produce more proinflammatory cytokines and growth factors. This inflammatory status of the placenta has been associated with mother-to-child transmission of HIV and can be enhanced by coinfections such as PM, that has been associated with an upregulation of CCR5 on placental cells that facilitate HIV infection [104, 105].

Due to the negative effects of HIV on the immune system and the placenta functionality, the transplacental transfer of maternal antibodies to the fetus could be impaired. A number of studies have shown a reduction on placental IgG transfer efficiency among HIV-infected women [49, 50, 54, 106], although the knowledge of the mechanisms for this placental IgG transfer impairment is limited, as well as the impact of HIV on the transplacental transfer of IgG subclasses. Apart from the reduction of antibody concentrations in HIV-infected mothers, another explanation for the reduction in transplacental transfer of antibodies could be maternal hypergammaglobulinemia. This condition is common in chronic viral infections [348], and it is associated with poor placental IgG transfer efficiency probably due to a saturation of FcRs expressed in the placenta [41, 107]. This decreased antibody transfer may have negative implications on infants, as suggested in some studies showing that HIV-exposed uninfected infants have up to 4-fold higher rates of morbidity and mortality from diarrheal and respiratory infections compared to unexposed infants [349–351].

5.5 Antiretroviral therapy

Control and management of HIV can be achieved. Since the introduction of antiretroviral drugs in 1987, there has been significant progress in the HIV therapy, especially with the addition of different types of drugs and further drugs being developed [108].

HIV drugs can be grouped into different classes depending on their action mechanism. Reverse transcriptase inhibitors can bind and inhibit the reverse transcriptase enzyme function and, consequently, reduce HIV multiplication. Examples of this drugs include zidovudine, tenofovir or didanosine. Protease inhibitors such as ritonavir or nelfinavir can block protease enzymes that are needed for the maturation of viral particles. Other drugs commonly used are fusion inhibitors of HIV with CD4 cells, CCR5 antagonists that prevents cells to get infected and integrase strand transfer inhibitors that prevent the integration of viral DNA into the host genome [352]. As HIV is highly variable, drug-resistant viral forms constantly emerge in HIV-infected individuals. To avoid this, a combination of different inhibitor groups is usual [353].

Many studies had raised concern about the adverse effects on ART [354, 355], and the general practice was to initiate ART in patients with low $CD4^+$ T cell counts. However, current HIV treatment guidelines recommend ART for all patients with detectable viremia, regardless of the $CD4^+$ T cell count [356].

The implementation of ART is particularly important in sub-Saharan Africa, where the rates of HIV transmission are the highest. Despite the fact that ART coverage in low and middle income countries has increased substantially in recent years, approximately 9 million people remain untreated [109]. It is specially concerning as a large proportion of HIV-infected individuals in these areas are young women. In 2015, infected pregnant women accounted for 1.4 million, and more than 90% of these women lived in sub-Saharan Africa [357]. ART is essential to prevent mother-to-child transmission of HIV, that can occur during three major periods: antenatally, intrapartum and postpartum, although about 80% is believed to occur during the intrapartum period [358]. Despite routine antenatal HIV testing and ART prophylaxis [359], sub-optimal adherence to the treatment has been reported, increasing the risk of vertical transmission of HIV [358]. In Mozambique at the time of the study, ART coverage was low: approximately 50% of the adults were undergoing treatment, the same percentage of HIV-infected pregnant women on ART [110, 111].

6. VACCINATION

Immunization, especially during childhood, has demonstrated to reduce morbidity and mortality related to infectious diseases at a low cost [112]. The EPI represents a major triumph of preventive medicine and public health, with key importance in geographical areas with high rates of infant morbidity and mortality, like the African Region, where children are ›6 times more likely to die before age 1 year than children in high income countries [113]. Mozambique has significant levels of infant malnutrition and infectious diseases, and one of the highest numbers of child deaths. The EPI scheme in Mozambique (**Table 1**) was implemented in 1979 and includes: a Bacille Calmette-Guerin (BCG) and an oral polio vaccine (OPV) dose at birth; an OPV dose plus a tetra/pentavalent (hepatitis B, diphtheria, tetanus, pertussis, plus *Hib* since August 2009) vaccine dose at weeks 6, 10 and 14; and a measles vaccine single dose at month 9. Pneumococcal conjugate vaccine (PCV-10) was recently introduced (April 2013), as well as RV vaccine in 2015. Also, tetanus vaccine is administered to women in childbearing age since several studies have shown the efficacy of maternal immunization with the toxoid in the prevention of neonatal tetanus and in the reduction of neonatal mortality [114]. Since the EPI implementation, immunization coverage in Mozambique improved 19 percentual points (from 47% in 1997 to 57% in 2015) [360].

Table 1. Mozambique Immunization Schedule for Infants and Pregnant women after inactivated polio vaccine, Rotavirus and measles second dose introductions

Immunization for infants			**Immunization for pregnant women and WCBA**		
Age	Visit	Antigen	Visit	Interval	Antigen
Birth	1	BCG, OPV0	1	0 (as earlier as possible	TT1
6 weeks	2	DTP-HepB-Hib1, PCV1, OPV1, Rota1	2	4 weeks after 1st dose	TT2
10 weeks	3	DTP-HepB-Hib2, PCV2, OPV2, Rota2	3	6 months after 2nd dose	TT3
14 weeks	4	DTP-HepB-Hib3, PCV3, OPV3, IPV[1]	4	1 year after 3rd dose	TT4
9 months	5	Measles first dose	5	1 year after 4th dose	TT5
18 months	6	Measles second dose	-	-	-
6-59 months	Every 6m	Vitamin A Supplement		All post-natal mothers	Vit A Supplement

[1] In case the child arrives to the vaccination service after 14 weeks of age, the IPV will be administered at the first encounter after 14 weeks.

IPV, inactivated polio vaccine; WCBA, women of child bearing age; DTP, diphtheria-tetanus-pertussis; OPV1-3, oral polio vaccine doses 1-3; BCG, Bacille Calmette-Guerin; HepB, hepatitis B virus vaccine; PCV1-3, pneumococcal vaccine doses 1-3; Rota1-2, rotavirus vaccine doses 1-2.

6.1 Vaccines

6.1.1 DTP

The diphtheria, tetanus and pertussis (DTP) multidose vaccine was introduced in low-income countries in the 1970s. It has been demonstrated that DTP vaccination has protective effects after the third dose (DTP-3), reinforcing the need of a high immunization coverage that continues to be a challenge in the African Region, with a 69% DTP-3 coverage [115].

Corynebacterium diphtheriae is a gram-positive nonencapsulated bacteria and the causal agent of diphtheria. *C. diphtheriae* produce exotoxins, that are responsible for the clinical manifestations of the disease. The symptoms are similar to the flu and include fever and cervical lymphadenopathy, but commonly they evolve into respiratory manifestations. The disease is due to diphtheria toxin, that is encoded by the *tox* gene, which comes from a lysogenic prophage that integrates its genome with *C. diphtheriae* plasmids. The toxin consists of two major subunits, known as A and B. The A subunit carries the catalytic domain, whereas the B subunit comprises the receptor binding and transmembrane domains. After toxin's binding to cell host surface receptors, it is internalized by endocytosis. Then, the catalytic A subunit is released, which inhibits the cell protein synthesis inactivating the elongation factor required for this function and, consequently, causing cell death [361]. Massive cell death ulcerates the mucosa and induces the formation of an inflammatory pseudomembrane, composed of RBCs, bacteria and debris over the tonsils and throat. If diphtheria toxin disseminates through blood circulation from the respiratory tract, systemic effects can occur, including damage to the myocardium and peripheral nerves. In 2018, the WHO recorded 16.611 cases, although it is under-reported in many regions [362].

Diphtheria control is mainly based on immunization of the population through vaccination with inactivated diphtheria toxin. Immunity against diphtheria is obtained by the induction of neutralizing IgG1 antibodies [116].

Tetanus is an infectious disease caused by the anaerobic gram-positive bacteria *Clostridium tetani*, that produce spores that can survive in soil and cause infection by contaminating wounds. The symptomatology of the infection is caused by tetanus toxin and include muscular rigidity and spasms. The infection can be categorized into generalized, neonatal, local, and cephalic. Generalized and neonatal tetanus cause muscular affectations and backward arching of the column,

and in severe disease may cause respiratory failure and death due to the rigidity of the respiratory muscles. The localized tetanus involves painful spasms of the muscle adjacent to the wound site, whereas cephalic tetanus affects the head and cause paralysis of one or more cranial nerves. Both later cases can develop into the generalized form [363].

The treatment of tetanus is based on antibiotic and antitoxin administration, although muscle symptoms may develop further because tetanus toxin can continue to be transported through the nerve terminals. Prevention is therefore the best strategy, especially because immunity may not develop after a natural tetanus infection [364].

After tetanus inactivated toxin vaccination, a strong protective humoral response against tetanus toxin is induced, with IgG1 and IgG4 antibody responses being predominant [117]. However, antibody titers decrease gradually with age and may fall below the protective threshold, highlighting the need of booster doses [365]. The uptake of the antigen by antigen-presenting cells also induce clonal T-cell responses, mainly $CD4^+$ secreting Th1 cytokines [366]. Although in 1997 the tetanus deaths accounted for 275,000 and the number decreased to 56,743 deaths in 2015, 19,937 of these deaths occurred in neonates, 44% in sub-Saharan Africa, emphasizing the need of maternal vaccination coverage [367].

Pertussis infection is caused by *Bordetella pertussis*, an aerobic gram-negative bacteria that produces an acute respiratory infection marked by severe, spasmodic coughing episodes and may produce severe complications such as bronchopneumonia and acute encephalopathy [368]. *B. pertussis* possess several virulence factors, including pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin and tracheal toxin. Other factors that influence the virulence of this bacteria include surface structures such as filamentous hemagglutinin, fimbriae, pertactin, the type III secretion system and LPS. In 2010, 16 million cases and 195,000 deaths were reported, of which a large number were reported from Africa [369].

Two types of pertussis vaccines were developed, the whole-cell pertussis (wP) and the acellular pertussis (aP). The wP vaccine contain a suspension of killed *B. pertussis* organisms at a high concentration, while the aP vaccine is made from purified antigens of *B. pertussis,* including pertussis toxoid, filamentous agglutinin, pertactin and fimbriae. The aP vaccine has replaced the wP vaccine as it has less adverse reactions than wP vaccine, although wP is still widely used in many low-income countries [370, 371]. In children, both wP and aP induced predominantly IgG1 for all pertussis antigens, and high IgG4 levels were only present in children

that received aP vaccine [118]. Cellular immune responses are also acquired after pertussis vaccination, which induces memory B cells. Regarding T cells, the aP vaccination induces both Th1 and Th2 responses, while the wP vaccination induces only a Th1 response. Both induce a Th17 response and $CD8^+$ T memory cell expansion [372].

6.1.2 Measles

Measles is an infection of the respiratory system and skin caused by measles virus (MV). MV is an enveloped, non-segmented RNA virus that belongs to the genus *Morbillivirus* of the family Paramyxoviridae [373]. The incubation period lasts about 10 days and after this period the first symptoms appear, such as fever and rash. First sites of replication are lymphoid organs and tissues, but MV can also spread to other tissues such as submucosal tissues, tongue, buccal mucosa, trachea, nose and skin. The MV envelope consists of two types of short surface projections: hemagglutinin (H) and fusion (F) proteins. In the respiratory tract, MV attaches to the host epithelial cells through the interaction of the H protein with cellular receptors. [374]. Complications of measles include pneumonia, appendicitis, febrile seizures and complications involving the central nervous system.

In Africa, measles vaccination was first introduced in the 1960s. Thanks to immunization, between 2001 and 2009, measles cases decreased by 93%. However, 869,770 cases were reported in 2019, demonstrating that there is still a need to increase the vaccine coverage [119]. To prevent persisting measles endemicity, a herd immunity of at least 90-95% is required [375] due to its high transmissibility.

As H protein is antigenically stable, it is the target of measles vaccine. Neutralizing antibodies often recognize H protein epitopes and block the binding of MV to host cell receptors [376]. The IgG subclass profiles reported to be induced by measles vaccination is different between studies. For example, one study showed that IgG1 and IgG4 were the dominant isotypes after vaccination [121], whereas another study reported that the predominant IgG subclass in children younger than 3 years was IgG3, but in children older than 4 years and adults, it was IgG2 [120].

6.1.3 *Hib*

In 2006 the WHO recommended routine immunization with the *Hib* conjugate vaccine in all countries and, since then, several studies have demonstrated its impact and effectiveness, especially in middle- and low-income countries. Mozambique introduced *Hib* conjugate vaccine in the EPI in 2009. It has been demonstrated that the vaccine significantly reduced incidence rates of invasive *Hib* disease, meningitis and pneumonia among children in Mozambique, consistently with reports in other low-income and sub-Saharan Africa settings [377].

The vaccine is included in a pentavalent formulation (diphtheria/tetanus/wP or aP, hepatitis B virus vaccine and conjugate *Hib* vaccine) and consists in four doses targeting children aged 2, 3 and 4 months of age. IgG1 and IgG2 antibodies are the IgG subclasses predominantly induced by *Hib* conjugated vaccine [116, 122, 123], and both Th1 and Th2 cytokine responses were detected following glycoconjugate vaccination [378].

6.1.4 Hepatitis B

Hepatitis B virus (HBV) is an enveloped DNA virus of the *Hepadnaviridae* family with unusual features similar to retroviruses. It is classified into eight genotypes, A to H, that have a different geographic distribution. HBV was first discovered at 1966 although it is known that infected humans for at least 500 years [379]. The viral genome encodes surface proteins (HBsAg), viral nucleocapsid or core antigen (HBcAg) and hepatitis B e antigen (HBeAg), whose function is not defined yet although seem to be involved as an immune tolerogen that promote persistent infection [380].

HBV target cells are hepatocytes. Mature virions' surface protein attaches to host cell membranes and after cell entry, the viral particles are uncoated. The viral genome is then transported into the nucleus, and viral DNA is circularized to the covalently closed circular (cccDNA) form, that serves as a template for transcription of RNAs. The RNAs are translated by a polymerase into the different viral proteins. Replication of HBV begins with encapsidation of the genome, followed by reverse transcription of the RNA to DNA. The nucleocapsid then

interacts with the envelope proteins to assemble into mature virions, that are secreted and can infect other hepatic cells [380].

HBV infection has two clinical manifestations: acute and chronic. During acute infection, patients can have subclinical hepatitis, icteric hepatitis, or less commonly fulminant hepatitis. Chronic infection is characterized by an asymptomatic carrier state, chronic hepatitis, cirrhosis and hepatocellular carcinoma. The firsts symptoms include anorexia, nausea, vomiting, abdominal pain and jaundice. In hepatic severe damage, gastrointestinal bleeding or coagulopathy can be developed [381].

There are two modes of transmission for HBV: horizontal transmission, through sexual contact or mucosal surface contact, and vertical transmission, involving maternal-to-newborn perinatal transmission of the virus [381]. The number of people chronically infected by HBV infection range from 240 million to 350 million and caused 887,000 deaths in 2015. High-HBV prevalence is common in sub-Saharan Africa, with up to 12% among adults [382, 383]. Coinfection with HIV increase risk of HBV chronicity and severe liver disease, and it is a major problem in high-transmission settings of HIV [384].

Current therapies suppress viral replication and liver inflammation, although they do not lead to sustained remission and treatment for HBV is rarely available in low-income areas such as sub-Saharan Africa [385]. In 1991, the WHO recommended HBV vaccination in national immunization programs, as infant immunization is considered an effective strategy to prevent HBV infection. Following the WHO guidelines, this vaccine should be given at birth, followed by two or three more doses at least four weeks apart [386].

HBV vaccines containing HBsAg proteins are available as single-antigen formulation and in combination with other vaccines. While single antigen vaccines are recommended for use at birth, the combined vaccines are recommended for individuals aged 6 weeks or more [387].

Children below 5 years of age immunized with recombinant HBsAg develop an IgG1 and IgG3 subclass effective response, although one year after vaccination IgG4 antibodies became the second dominating isotype. Instead, older children show a high variability in specific profiles with a high contribution of IgG4, that is less efficient [124].

Protection against infection is based on the presence of anti-HBsAg IgG $\geq$ 20mIU/mL. Although the long-term protection of this vaccine has been under debate, it has been shown that the proportion of individuals with protective levels

decrease by age and it is correlated with children vaccinated with less doses than recommended [388]. In fact, some studies showed that after three-dose HBV vaccination, about 90% of the subjects remained protected for more than 10 years and up to 30 [389, 390].

6.1.5 Oral polio vaccine

Polyomielitis is an infectious disease caused by a positive strand RNA virus belonging to the *Picornaviridae* family. Poliovirus is classified into three serotypes: poliovirus type 1, poliovirus type 2 and poliovirus type 3 [391]. Wild type 1 poliovirus was the primary cause of the majority of the world's paralytic polio cases until the spread of vaccination. Wild type 2 was eradicated in 2013 and wild type 3 has not been detected since 2012, although wild type 1 cases have been reported in the last years [392].

Transmission occurs primarily via fecal-oral contamination, although oral-oral spread is also possible. Primary infection can lead to viral replication in oropharyngeal and gastrointestinal lymphatic tissues, and from there it can enter the blood and spread into other systemic sites such as the central nervous system [393].

First polio vaccines were developed in the 1950s and were viruses inactivated with formalin [393]. Currently, live attenuated oral polio vaccines (OPV) replaced inactivated vaccines as they were cheaper, more immunogenic, easy to administrate and able to provide passive immunization of unvaccinated persons from viruses shed by vaccines [394].

OPV generates both humoral and mucosal immunity. IgG and IgA antibodies are elicited after vaccination [395, 396] and the trivalent form of OPV is highly effective, especially in industrialized countries. In high-income countries seroconversion is reached in almost 100% of vaccine recipients following three doses of OPV, although in developing countries only about 70% acquire protective antibodies [397]. The reduced response could be due to different factors common in low-income countries such as malnourishment, concurrent infections and intestinal pathologies that may affect mucosal and systemic immunity.

6.1.6 BCG

Mycobacterium tuberculosis is a bacillus and causal agent of tuberculosis, a chronic disease that affects the lungs making pulmonary disease. *M. tuberculosis* is a non-spore forming anaerobic intracellular bacteria that can be neither gram-positive or gram-negative because of very poor reaction with the Gram stain. Apart from causing tuberculosis, this bacterium is also associated with autoimmune diseases and metabolic syndromes [398].

Tuberculosis is one of the top 10 causes of death worldwide. It affected 10 million people in 2019 and caused more than 1 million deaths among HIV-negative people and an additional 208,000 deaths among HIV-positive people [399]. The majority of cases occur in South-East Asia (44%) and Africa (25%) [399]. Drug-resistant strains are widespread and continue to be a public health threat. In 2019, half million people developed resistance to rifampicin, an antibiotic used for the treatment of tuberculosis. Among them, 78% had multidrug-resistant tuberculosis [399].

The principal transmission mechanism is the inhalation of aerosolized droplets from an infected person. About 30-40% of exposed individuals become infected. *M. tuberculosis* can reach the alveolar space and proliferate inside alveolar macrophages, that produce chemokines and cytokines that attract other phagocytic cells, which produce a nodular granulomatous structure known as a tubercle. This tubercle can enter lymph nodes and cause lymphadenopathy [400].

Due to the emergence of drug-resistant tuberculosis, vaccination is the best strategy to control this epidemic, although BCG is the only vaccine available today and its efficacy is controversial. The WHO recommends this vaccine in countries or settings with a high incidence of tuberculosis, mainly from Africa, Asia and South America [401, 402]. Several studies assessed the efficacy of BCG and indicate that, overall, it has 60-80% protective efficacy against severe forms of tuberculosis in children, particularly meningitis, although its efficacy against pulmonary diseases varies geographically [403]. This may be associated with different exposure to *M. tuberculosis* or environmental mycobacteria that may affect the protection induced by BCG.

Currently, several tuberculosis vaccine candidates are in development and can be grouped into mycobacterial whole cell-derived vaccines and subunit vaccines, directed against selected antigens. One of them, the subunit vaccine M72/AS01E candidate is in a phase 2b clinical trial and showed a significant degree of protection, suggesting that protective tuberculosis vaccines are feasible [404].

6.1.7 Rotavirus

RV vaccines Rotarix and Rotateq are currently available. Rotarix was implemented in 2015 in Mozambique, where before vaccine introduction RV infection was the cause of 42.4% of ‹5 years old hospitalized children with acute diarrhea, with the highest prevalence observed in children between 6 and 11 months old [405]. After vaccine introduction, the prevalence was reduced to 13.5% [406]. Protection mostly correlated with specific IgA antibodies, although individuals with IgA deficiency may be protected from severe RV disease by developing compensatory RV-specific IgG antibodies [407]. However, the licensed RV vaccines were found to be less effective in countries of sub-Saharan Africa and Southeast Asia and development of new RV vaccines is a need. A current vaccine candidate targets VP6, a protein that forms the middle layer of RV particles and has been shown to be antigenic and immunogenic [408].

Although several studies showed that protection mostly correlated with specific IgA antibodies [409–411], IgG can also play a role as shown with individuals with IgA deficiency. Studies in mice an non-human primates showed that neutralization by VP6-specific IgG against RV was efficient and protective [412, 413]. It also has been shown that individuals with IgA deficiency may be protected from severe RV disease by developing compensatory RV-specific IgG antibodies [407]. These findings suggest that VP6-specific IgGs may have an important physiological role in neutralizing RV.

6.2 Maternal immunization strategies

Vaccination of pregnant women has the potential to protect pregnant women and their infants from vaccine-preventable diseases. Newborns are protected for the first months of life thanks to the transplacental transfer of maternal IgG antibodies, which provide passive immunity. Thus, the aim of maternal immunization is to increase maternal specific antibody concentrations to be transferred to the fetus and therefore reduce their vulnerability until they can be vaccinated. Currently, influenza, tetanus, diphtheria and pertussis vaccines are recommended during pregnancy in many countries, and others like RSV and group B streptococcus vaccines are under development [125].

The effectiveness of influenza vaccines given to pregnant women for preventing infant hospitalizations by those infections is high. For example, the reduction was

42-92% in the US, 64% in England, 63% in Bangladesh and 50% in South Africa [125]. Maternal immunization with tetanus toxoid has also showed to be effective. Several studies have shown that maternal tetanus vaccination induces IgG1 antibodies that are actively transferred across the placenta, and the 80% of maternal antibodies remain present in infants 1 month after delivery. Pertussis vaccination during pregnancy also induce IgG1 antibodies, which are actively transferred across the placenta. Vaccine effectiveness is high, as it reduces the number of young infant pertussis cases in countries where the vaccine has been implemented, such as Canada, US and UK [414–416], although this vaccine is not implemented in Africa yet. As an example of vaccines in development, RSV immunization has shown to be safe and seems to be effective as it induces IgG1 neutralizing antibodies thought to be protective [149, 417].

In addition of placental transfer, maternal vaccination also can increase the levels of maternal IgA in breastmilk. It has been reported, for example, for pertussis-specific IgA antibodies [418]. Therefore, as IgA acts at the mucosal level, breastfeeding may contribute to the protection of infants against enteric infections and against respiratory illness [419, 420].

Despite the beneficious effects of maternal immunization on the placental transfer of antibodies and subsequent protection of newborns, maternal antibodies could also interfere with infant vaccine responses. For example, in some studies maternal DTP immunization is associated with reduced antibody levels in specific (diphtheria and pertussis) and heterologous (polio and pneumococcal) infant vaccines [421–423]. Maternal antibody interference to measles vaccination response in infants has also been largely studied. Infants are protected during the first year of life thanks to maternal neutralizing antibodies and although maternal immunization against measles has shown to reduce mortality and morbidity of infants, long-lasting immunity after infant vaccination may be not established in the presence of maternal antibodies [424]. This effect is also seen for other infant vaccines such as pneumococcus and tetanus in one study, in which concentrations of IgG after vaccination were higher when the specific maternal IgG levels at birth were lower, although most infants achieved protective levels of antibodies after vaccination [425]. Another study reported that maternal immunization with conjugate pneumococcal vaccines correlated with an increased risk of acute otitis media in young infants and with a reduction of infant antibody responses against the *S. pneumoniae* vaccine serotypes [426]. A reduction of influenza antibodies after infant vaccination has also been reported in infants with high maternal antibodies, although the acquisition of cellular memory responses were not impaired [427].

As previously described, the transplacental transfer of maternal antibodies to the fetus could be impaired due to HIV, resulting in HIV-exposed uninfected infants receiving fewer protective maternal antibodies than infants not exposed to HIV [49, 50, 54, 106]. Thus, maternal immunization could be a good strategy to potentially improve health outcomes among HIV-exposed uninfected infants and HIV-infected pregnant women. However, there is little research exploring the effect of HIV on the effectiveness of vaccines given during pregnancy and the few available reports suggested that HIV infection may limit the immunogenicity of several vaccines [428]. Similarly, PM can impair materno-fetal antibody transfer and potentially reduce the benefit of maternal immunization strategies [126].

Helminth infections may have also negative effects in maternal and child outcomes such as reduced low birth weight, preterm delivery and maternal and infant anemia, and increased the risk of maternal coinfection with malaria [429]. This could have a negative effect in the immune system of infants, as reported in some studies. For example, a study in Kenya showed that infants of mothers infected with malaria and/or helminths had reduced immune responses to Hib and diphtheria vaccines [430]. Another study in Gabon reported lower anti-tetanus toxoid IgG in cord blood of infants born to helminth-infected women that had received tetanus vaccination during pregnancy [127], suggesting that helminth infections could also have a negative effect in maternal immunization.

In summary, maternal antibodies are very effective in protecting neonates and infants against most infectious diseases and maternal immunization is a good strategy to achieve this. Nevertheless, the impact of maternal HIV or other maternal infections in the effectivity of the vaccines should be further assessed, especially in low-income countries where the burden of infectious diseases is higher.

Hypotheses

Effect of maternal HIV and malaria on the transplacental transfer of antibodies against prevalent pathogens and vaccines in Mozambican women

03

HYPOTHESES

The primary hypothesis of this doctoral thesis is that infections with HIV and *P. falciparum* during pregnancy reduce the levels of cord blood and the transfer of maternal antibodies to the newborn. This effect may differ between IgG subclasses and depend on the antigen specificity.

In addition, other maternal factors such as age, gravidity, maternal anemia, malaria treatment, antiretroviral therapy, CD4+ T cell counts and HIV viral load may influence cord blood levels and transfer of antibodies.

Finally, cord blood antibody levels and placental transfer may be diminished due to pregnancy outcomes such as prematurity and low birth weight.

Objectives

Effect of maternal HIV and malaria on the transplacental transfer of antibodies against prevalent pathogens and vaccines in Mozambican women

04

OBJECTIVES

The OVERALL AIM of this doctoral thesis is to determine the impact of maternal HIV infection and malaria during pregnancy on the cord blood antibody levels and transplacental transfer of antibodies against (i) pathogens of high burden and health impact in southern Mozambique, in particular malaria, and (ii) antigens from vaccines used for maternal immunization and included in the Expanded Program of Immunization.

The specific aims of the thesis are:

1. To assess the impact of maternal HIV infection on the cord blood levels and placental transfer of total IgG and IgG subclasses.
2. To assess the impact of maternal malaria on the cord blood levels and placental transfer of total IgG and IgG subclasses.
3. To assess the impact maternal factors (age, gravidity, malaria treatment, antiretroviral therapy, CD4+ T cell counts and HIV viral load), pregnancy outcomes (maternal anemia, prematurity and low birth weight) and seasonality on the cord blood levels and placental transfer of total IgG and IgG subclasses.

Results

Effect of maternal HIV and malaria on the transplacental transfer of antibodies against prevalent pathogens and vaccines in Mozambican women

05

CHAPTER 1

Reduced placental transfer of antibodies against a wide range of microbial and vaccine antigens in HIV-infected women in Mozambique

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Reduced Placental Transfer of Antibodies Against a Wide Range of Microbial and Vaccine Antigens in HIV-Infected Women in Mozambique

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Transplacental transfer of antibodies is essential for conferring protection in newborns against infectious diseases. We assessed the impact of different factors, including gestational age and maternal infections such as HIV and malaria, on the efficiency of cord blood levels and placental transfer of IgG subclasses. We measured total IgG and IgG subclasses by quantitative suspension array technology against 14 pathogens and vaccine antigens, including targets of maternal immunization, in 341 delivering HIV-uninfected and HIV-infected mother-infant pairs from southern Mozambique. We analyzed the association of maternal HIV infection, *Plasmodium falciparum* exposure, maternal variables and pregnancy outcomes on cord antibody levels and transplacental transfer. Our results show that maternal antibody levels were the main determinant of cord antibody levels. Univariable and multivariable analysis showed that HIV reduced the placental transfer and cord levels of IgG and IgG1 principally, but also IgG2 to half of the antigens tested. *P. falciparum* exposure and prematurity were negatively associated with cord antibody levels and placental transfer, but this was antigen-subclass dependent. Our findings suggest that lower maternally transferred antibodies may underlie increased susceptibility to infections of HIV-exposed infants. This could affect efficacy of maternal vaccination, especially in sub-Saharan Africa, where there is a high prevalence of HIV, malaria and unfavorable environmental factors.

Keywords: antibody, maternal antibodies, placental transfer, HIV, pathogens, malaria, IgG, IgG subclasses

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INTRODUCTION

Each year, 2.6 million deaths occur during the neonatal period, with infectious diseases being the leading cause of mortality, particularly in low-income countries (1, 2). Newborns are highly vulnerable to pathogens due to their functional immunological differences from adults as a result of living in a semi-allogeneic sterile environment, where exposure to microbial antigens is limited

(3–6). For example, microorganisms such as respiratory syncytial virus (RSV) cause no or mild disease in adults but induce acute bronchiolitis, viral pneumoniae, and croup in infants, with those between 2 and 6 months of age at the highest risk, especially in low-income countries (7, 8).

Vaccination is among the most cost-effective public health measures worldwide (9), and regions with high rates of infant morbidity and mortality like sub-Saharan Africa benefit from the implementation of the Expanded Program of Immunization (EPI) (10). Nevertheless, acquisition of immunity from vaccination is not immediate and vaccines are not available for all infectious diseases. At present, only three vaccines are being administered at birth in some countries: Bacillus Calmette-Guérin (BCG), hepatitis B virus (HBV), and oral polio vaccine (OPV) (11–13). Therefore, newborns mostly rely on the protection elicited by maternal antibodies transferred across the placenta, which provide passive immunity against common pathogens (14).

Transplacental transfer of antibodies occurs *in utero* and it is facilitated by neonatal fragment crystallizable (Fc) region receptor (FcRn), expressed in the human syncytiotrophoblast (15, 16). Only IgG is transferred across the placenta with the highest rate occurring during the third trimester of pregnancy (17), although some studies suggest that maternal IgE may also be transferred to the fetus as IgG/IgE complexes (18). IgG subclasses have different affinities for the FcRn receptor leading to differences in the efficiency of transfer (19); classically it was stated that the greatest transport occurs for IgG1, followed by IgG4, IgG3, and finally IgG2 (20), although a recent update on transplacental transfer of IgG subclasses showed that it is different depending on the antigen and study populations (21).

To be effective, the transferred IgG must reach protective levels after birth. Maternal immunization is a valuable strategy to prevent newborn infections, ensuring a sufficient transfer of protective antibodies to the neonate (22, 23). Maternal vaccination against tetanus, pertussis, and influenza has been implemented in many populations and has been effective at protecting young infants from these pathogens (24–26), and could be used to protect newborns from RSV (27). However, it has been reported that IgG placental transfer and cord levels could be affected by some factors such as maternal antibody concentrations, gestational age, placental integrity, maternal infections, Fc binding strength, and the antigen specificity (16, 28–32), although the effects are not consistent among studies. Placental malaria (PM) has been shown to reduce transplacental transfer of antibodies against tetanus, measles, *Streptococcus pneumoniae* (*S. pneumoniae*), herpes simplex virus type 1 (HSV-1), RSV and varicella-zoster virus (VZV) (28, 33–35). However, other studies have shown no impact of PM on transplacental transfer of tetanus, *S. pneumoniae, Haemophilus influenzae type b* (*Hib*), diphtheria, measles, or RSV antibodies (16, 33, 35–38). The effect of maternal HIV infection is also controversial. Some studies demonstrated that HIV infection leads to a reduction of the transplacental transfer of *Hib*, diphtheria, pertussis, pneumococcus, measles, tetanus, and *Plasmodium falciparum* (*P. falciparum*) specific antibodies (28, 29, 39–45), but others have shown no effect (29, 33, 38, 41, 42, 46). Those studies had several limitations, as the numbers of antigens tested and HIV-infected women included in the analyses were low. Moreover, the information regarding IgG subclasses is scarce (41, 45, 46). Therefore, the effect of HIV and malaria on transplacental transfer of antibodies, particularly IgG subclasses, is still not clear.

In our study, we aimed to assess the impact of different factors, including maternal HIV infection and malaria in pregnancy on the placental transfer and cord levels of IgG and IgG subclasses to a broad range of highly prevalent microbial and vaccine antigens in a sub-Saharan African country, including: *Corynebacterium diphtheriae* (*C. diphtheriae*), *Clostridium tetani* (*C. tetani*), *Bordetella pertussis* (*B. pertussis*), *Hib, S. pneumoniae, Shigella dysenteriae* (*S. dystenteriae*), *Vibrio cholerae* (*V. cholerae*), hepatitis B virus (HBV), measles, RSV, *Cryptosporidium parvum* (*C. parvum*), *Giardia intestinalis* (*G. intestinalis*), and *P. falciparum*. A better understanding of factors affecting cord IgG levels will help with designing better preventive measures and strategies for maternal and child health.

MATERIALS AND METHODS

Study Design and Sample Collection

A total of 197 HIV-uninfected and 144 HIV-infected women were recruited among those participating in two clinical trials of antimalarial intermittent preventive treatment in pregnancy (IPTp, ClinicalTrialGov NCT00811421 for both) (**Supplementary Figure 1**) in the Manhiça District, Southern Mozambique (47, 48), between May 2011 and September 2012, to perform an immunology ancillary study. The first clinical trial evaluated mefloquine (MQ) as an alternative IPTp drug to sulfadoxine-pyrimethamine (SP) in HIV-uninfected pregnant women. The study arms were (1) SP, (2) single dose MQ (MQ full), and (3) split dose over 2 days MQ (MQ split). The second trial evaluated MQ as IPTp drug in HIV-infected pregnant women in whom SP is contraindicated and who received daily cotrimoxazole (CTX), and women received either three monthly doses of MQ or placebo. All women received bed nets treated with long-lasting insecticide and supplements of folic acid and ferrous sulfate. All women also received tetanus toxoid vaccination during pregnancy. At the time of the study, the intensity of malaria transmission was low/moderate (49). 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 (50). The number of women on ART regime was 116, of which 81 started before pregnancy and 35 at the start of the study recruitment. A total of 24 women were not in an ART regime.

At delivery, peripheral and cord blood samples from women were collected into sodium heparin and EDTA vacutainers to perform the antibody assays. Plasma samples from peripheral blood and cord blood were available for this study from 332 (195 HIV-uninfected and 137 HIV-infected) and 303 women (178 HIV-uninfected and 125 HIV-infected), respectively. There were 294 mother-cord paired samples. The extraction of the maternal blood was done before delivery, when women were admitted to the hospital.

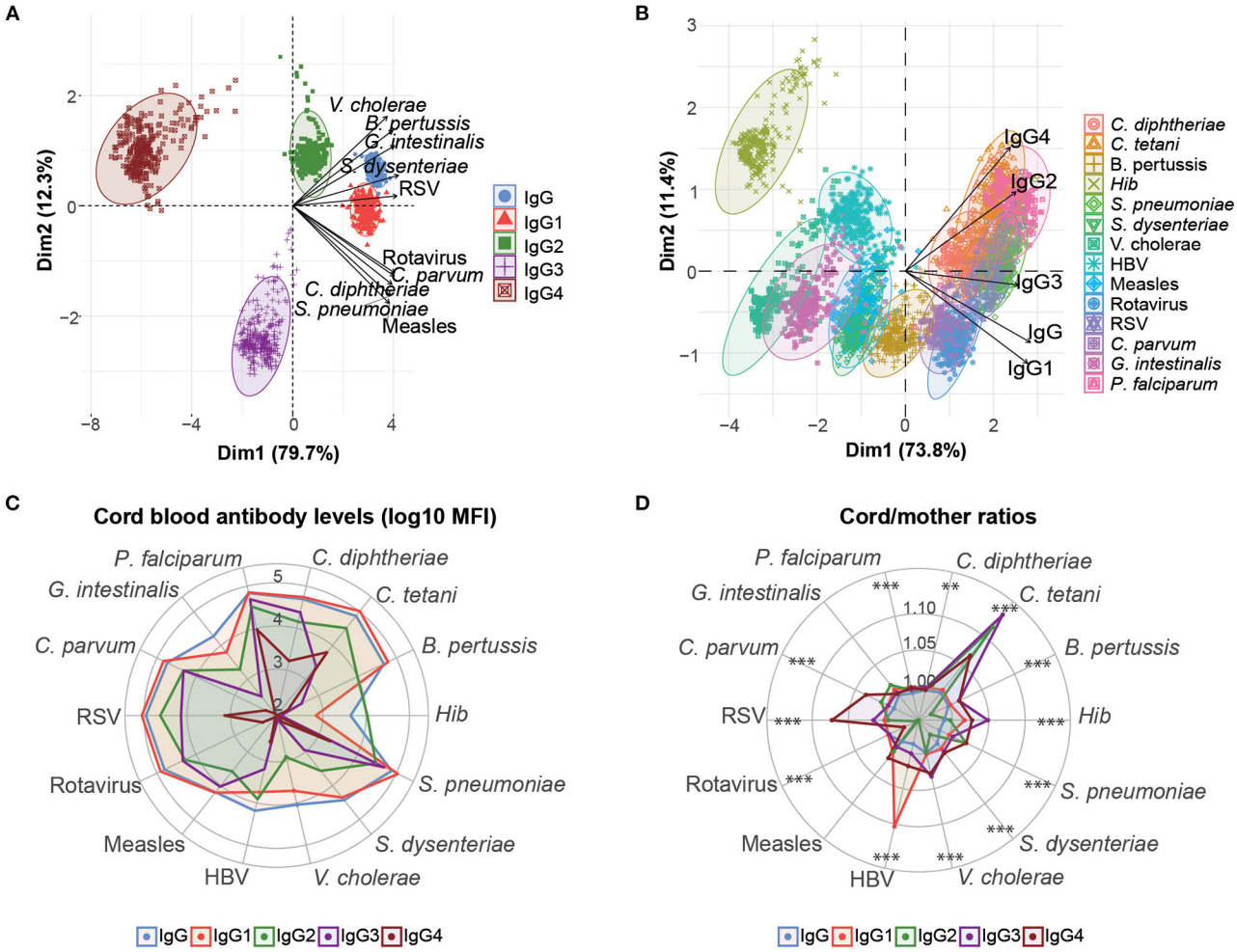

FIGURE 1 | Overview of cord blood levels of IgG and IgG subclasses for all women. Principal component analysis (PCA) plots of cord IgG and IgG subclass levels against all antigens clustered by subclass type **(A)** and antigen type **(B)**. The two principal components (Dim 1, Dim2) that explained the 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. Medians of IgG subclass placental transfer for each antigen were compared by Kruskal-Wallis test and *p*-values were adjusted for multiple testing by the Benjamini-Hochberg approach (False Discovery Rate 5%). ***$p < 0.001$, **$p < 0.01$.

For the detection of *P. falciparum* species, thick and thin blood smears were assessed according to standard procedures (47, 48). Fifty microliter of maternal peripheral, placental, and cord blood samples were collected on Whatman 903™ filter paper during two visits before delivery (one during second trimester and the other during third trimester) and at delivery. Real-time quantitative polymerase-chain-reaction (qPCR) assay targeting the 18S ribosomal RNA was performed (51). Tissue samples from the maternal side of the placenta (decidua) were also collected for the assessment of placental malaria. Microscopy data of peripheral and placental blood smears at delivery were available for 308 and 340 women, respectively. Peripheral and placental blood qPCR data were available for 242 and 236 women, respectively.

Antibody Assays

Quantitative suspension array technology (qSAT) assays applying the xMAP™ technology (Luminex Corp., TX) were used to measure antigen-specific IgG, IgG1, IgG2, IgG3, and IgG4 responses to vaccine and pathogen antigens. A total of 16 recombinant proteins were selected for the analysis: diphtheria toxoid (*Corynebacterium diphtheriae,* Alpha Diagnostic DTOX15-N-500), tetanus toxin (*Clostridium tetani,* Santa Cruz SC222347), pertussis toxin (*Bordetella pertussis,* Santa Cruz SC200837), *Hib* Oligosaccharide (BEI Resources NR12268), pneumococcal surface protein A (PspA, *Streptococcus pneumoniae,* BEI Resources NR33179), shiga toxin (*Shigella dysenteriae,* BEI Resources NR4676), anti-O-specific polysaccharide (OSP, *Vibrio cholerae,* Massachusetts

General Hospital, MA, USA) (52), hepatitis B surface antigen (HBsAg, Abcam ab91276), hemagglutinin (measles, Alpha Diagnostic RP655), viral protein 6 (VP6, rotavirus, Friedzgerald 80-1389), F protein (respiratory syncytial virus, BEI Resources NR31097), 17-kDA surface antigen (Cp17, *Cryptosporidium parvum,* Centers for Disease Control and Prevention, GA, USA) (53), variant-specific surface protein 5 (VSP5, *Giardia intestinalis*) (53), 42 kDA fragment of merozoite surface protein 1 ($MSP1_{42}$, *P. falciparum*, WRAIR) (54), merozoite surface protein 2 (MSP2, *P. falciparum,* University of Edinburgh) (55), and exported protein 1 (EXP1, *P. falciparum,* Sanaria) (56). $MSP1_{42}$ antigen was selected for representing *P. falciparum*. Eight recombinant proteins represented the most prevalent pathogens circulating in the study area (57–59) and six were from the vaccines administrated to the infants through the EPI in Mozambique (60).

qSAT assays were previously standardized and optimized to control for sources of variability (61–63). Briefly, antigens covalently coupled to MagPlex beads were added to a 96-well μClear® flat bottom plate (Greiner Bio-One) in multiplex resuspended in 50 μL of PBS, 1% BSA, 0.05% Azide pH 7.4 (PBS-BN). Fifty microliter of test samples, negative or positive controls (64) were added to multiplex wells and incubated overnight at 4°C protected from light. After incubation, plates were washed three times with PBS-Tween 20 0.05%, and 100 μL of anti-human IgG (Sigma B1140), anti-human IgG1 (Abcam ab99775), anti-human IgG2 (Invitrogen MA1-34755), anti-human IgG3 (Sigma B3523) or anti-human IgG4 (Invitrogen MA5-16716), each at their corresponding dilution, were added and incubated for 45 min. Then, plates were washed three times more and 100 μL of streptavidin-R-phycoerythrin (Sigma 42250) at the appropriate dilution were added to all wells and incubated 30 min for IgG, IgG1, and IgG3. For IgG2 and IgG4, 100 μL of anti-mouse IgG (Fc-specific)–biotin (Merck B7401) were added and incubated for 45 min, followed by another washing cycle and the incubation with streptavidin-R-phycoerythrin for 30 min. Finally, plates were washed and beads resuspended in 100 μL/well of PBS-BN. Plates were read using the Luminex 100/200 analyzer, 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 1/10,000), and IgG1 and IgG3 (1/100 and1/2,500). For IgG2 and IgG4 only 1 dilution was tested (1/50) because of their usual low levels. Twelve serial dilutions (1:3, starting at 1/25) of a positive control (WHO Reference Reagent for anti-malaria *P. falciparum* human serum, NIBSC code: 10/198) were used for QA/QC and to select an optimal sample dilution for data analysis. Two blanks were also added to each plate for quality control purposes. Sample distribution across plates was designed to ensure a balanced distribution of groups. Single replicates of the assay were performed.

Statistical Analysis

To stabilize the variance, the analysis was done on $\log_{10}$-transformed values of the MFI measurements. A positive control curve was used to select the sample dilution for each antigen-isotype/subclass-plate. The dilution nearest to the midpoint between the two positive control curve serial dilutions ranging the maximum slope was chosen. Plates were normalized using the positive control curve in each plate and the average positive control curve from all plates. The MFI values of samples were multiplied by the corresponding normalization factor (MFI value of the chosen dilution from the average positive control curve divided by the MFI value of same dilution in the plate curve).

The Shapiro-Wilk test of normality confirmed that most of the antibody data were not normally distributed. The Chi-square and the non-parametric Wilcoxon-Mann-Whitney tests were used to compare categorical and continuous variables, respectively, between HIV-infected and HIV-uninfected women. Comparisons of crude Ig levels across antigens and subclasses between HIV exposure groups were assessed by Wilcoxon-Mann-Whitney tests, and between ART groups, were assessed by Kruskal-Wallis and Dunn's tests. Kruskal-Wallis and Dunn's tests were also performed in order to compare IgG subclass placental transfer for each antigen, represented as the cord/mother ratios. Univariable linear regression models were fit to determine the effect of variables on the cord blood antibody levels ($\log_{10}$) or the cord blood/mother ratio ($\log_{10}$) (**Supplementary Material 1**). The variables considered in this analysis were $\log_{10}$ maternal antibody levels, maternal HIV infection, *P. falciparum* exposure, PM (acute, defined by the presence of parasites on sections without malaria pigment; chronic, by presence of parasites and pigment; or past, by the presence of pigment alone), age, gravidity (defined as *primigravidae* and *multigravidae*), maternal anemia (defined as hemoglobin level <11 g/dL), low birth weight (defined as <2,500 g at birth), prematurity (defined as delivery before 37 weeks of gestational age), gestational age [measured by Ballard score (65)], treatment (defined as MQ or placebo in the HIV-infected women ancillary study and MQ full, MQ split or SP in HIV-uninfected women ancillary study), ART received before pregnancy or at recruitment, $CD4^+$ T cell counts (<350 or ≥350 cells/μL), HIV viral load (<400, 400–999, 1,000–9,999, and >9,999 copies/mL), and seasonality (dry or rainy). Exposure to *P. falciparum* was computed as the sum of the maternal IgG antibody levels (MFI) for the following immunogenic *P. falciparum* antigens: $MSP1_{42}$, MSP2, and EXP1, as antibody levels to these antigens have been shown to reflect exposure to malaria (66, 67). Seasonality was computed for each woman based on the pregnancy period—if at least four of the pregnancy months fell under the category of rainy period (November through April), the season was defined as such. In any other case, the season was defined as dry. A base multivariable model including maternal antibody levels, maternal HIV infection and *P. falciparum* exposure was established for each antigen and IgG or IgG subclass. Base model for $MSP1_{42}$ did not include *P. falciparum* exposure as this variable includes antibodies to this antigen. We performed additional regression models testing exhaustively all possible combinations of predictor variables (added to our base model) and selected the models based on the Akaike information criterion (AIC), Bayesian information criterion (BIC) and

TABLE 1 | Characteristics of study participants.

	All ***N* = 341**	**HIV-uninfected** ***N* = 197**	**HIV-infected** ***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 hemoglobin (*n*, %)				0.025
Anemia (<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*, %)				NS
Low (<2,500 g)	29 (8.5)	17 (8.6)	12 (8.33)	
Normal (≥2,500 g)	312 (91.5)	180 (91.4)	132 (91.7)	
Prematurity (*n*, %)				NS
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)	
(1,000–9,999)	48 (14.6)	–	48 (36.6)	
>9,999	21 (6.4)	–	21 (16.0)	
Placental malaria[b] (*n*, %)				NS
No	321 (94.1)	184 (93.4)	137 (95.1)	
Yes	20 (5.9)	13 (6.6)	7 (4.9)	
Peripheral malaria[c] (*n*, %)				NS
No	290 (85.0)	165 (83.8)	125 (86.8)	
Yes	51 (15.0)	32 (16.2)	19 (13.2)	
P. falciparum exposure (log_{10} MFI IgG)	5.27 [5.19; 5.34]	5.29 [5.21; 5.35]	5.26 [5.18; 5.33]	0.011

For numerical variables, the median and first and third quartile, 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 Wilcoxon-Mann-Whitney 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 parasitemia by any method.

[c]Peripheral malaria was considered positive if there was any evidence of P. falciparum peripheral parasitemia by any method.

The statistical significance was considered when p < 0.05; MQ, mefloquine; NS, not significant; NP, not-performed tests; SP, sulfadoxine-pyrimethamine.

adjusted r-square parameters. All *p*-values were considered statistically significant when <0.05 after adjusting for multiple testing through Benjamini-Hochberg. All data collected were pre-processed, managed, and analyzed using the R software version 3.6.3 and its package *devtools* (68). The *ggplot2* package was used to perform boxplot graphs (69). The *FactoMineR* and *factoextra* packages were used to perform Principal Component Analysis (PCA) (70, 71).

RESULTS

Description of Participants

A total of 341 women (197 HIV-uninfected and 144 HIV-infected) participated in the study (**Table 1**). HIV-infected women were older than the HIV-uninfected and there were more *primigravidae* among the HIV-uninfected. HIV-infected women had significantly more anemia than the HIV-uninfected. There

were no significant differences in birth weight or prematurity between infants born to HIV-infected and those born to HIV-uninfected women. Among the 155 infants born from HIV-infected women, eight tested HIV-positive at 6 weeks of age by polymerase chain reaction (PCR) analysis performed following national guidelines. Placental histology was performed on 307 samples from study participants, of which three had acute PM and eight past PM. In total, 20 women had PM (positive in placental blood, by microscopy or PCR at delivery, or acute or past PM by histology), but there were no differences by HIV infection. Peripheral malaria (positive in peripheral blood by microscopy or PCR at any of the visits during pregnancy) was detected in 51 women, but there were no differences by HIV infection. Finally, *P. falciparum* exposure was lower among HIV-infected women.

Profile of Antibody Levels in Cord Blood

PCA of the cord antibody levels and maternal antibody levels separately, including IgG, IgG1, IgG2, IgG3, and IgG4 to all antigens tested, were performed to reduce the dimensionality of the data and get insights into the overall antibody patterns. Cord and maternal PCA looked very similar (data not shown). Cord antibody responses clearly clustered by IgG subclasses (**Figure 1A**). The distribution of the IgG subclass clusters across the PCA dimensions was influenced by the antigens. Specifically, IgG and IgG1 clusters were closer, showing similar responses, whereas IgG4 and IgG3 were the most distant. Cord antibody responses also clustered by antigens (**Figure 1B**). *Hib* cluster was clearly separated from the rest, indicating a different antibody profile (**Figure 1B**). Overall, PCA results suggested different antibody profiles depending on the antigen specificity. Consistently, median IgG and IgG1 levels were higher than the rest of IgG subclasses and were both similar between them for most of the antigens with the exception of *Hib* (**Figure 1C**). IgG2 had lower median levels than IgG1, followed by IgG3 and the lowest levels were shown for IgG4.

We also determined the placental transfer of antibodies, measured as the ratio of cord blood levels to the maternal levels. The efficiency of placental transfer was different depending on the IgG subclass in all antigens with the exception of measles and *G. intestinalis* (**Figure 1D**). The transfer efficiency was greatest for IgG1, IgG3, or IgG4 depending on the antigen. IgG1 placental transfer was significantly higher for *C. diphtheriae, P. falciparum,* HBV, and rotavirus. IgG3 placental transfer was significantly higher for *B. pertussis, C. tetani, Hib,* and *V. cholerae*. IgG4 placental transfer was significantly greater for *C. parvum, S. dysenteriae,* and RSV. The least efficiently transferred subclass was IgG2 for most of the antigens, with the exception of *S. pneumoniae* for which IgG2 and IgG4 were the greatest.

Altered Maternal and Cord Blood Antibody Levels in HIV-Infected Women

We first compared total IgG levels and IgG subclasses in maternal and cord blood from HIV-infected and HIV-uninfected women. Significant differences in maternal antibody levels were only detected for *C. tetani* (IgG and IgG1), *S. pneunomiae* and RSV (IgG2), and *C. diphtheriae* and *P. falciparum* (IgG4), with lower antibody levels in HIV-infected compared to HIV-uninfected women (**Figure 2**). In contrast, higher *G. intestinalis* and HBV IgG levels were found in HIV-infected women (**Figure 2A**).

Total IgG cord blood levels were lower in HIV-infected than HIV-uninfected women for *C. tetani, B. pertussis, S. pneumoniae,* measles, rotavirus, and *C. parvum*. Similarly, HIV-infected women had lower IgG1 cord levels for *C. diphtheriae, C. tetani, B. pertussis, Hib, S. dysenteriae, V. cholerae,* and measles for IgG1 (**Figure 2B**). No differences were observed between groups for IgG2 and IgG3 (**Figures 2C,D**), whereas lower *C. diphtheriae and P. falciparum* IgG4 levels were also found in cord blood of HIV-infected than HIV-uninfected women (**Figure 2E**).

We also compared cord blood levels of IgG and IgG subclasses between HIV-infected women taking ART before pregnancy, at recruitment or without ART regime during pregnancy. The only significant differences were observed for *S. dysenteriae* and RSV IgG4 (**Supplementary Figure 2E**). HIV-infected women taking ART before pregnancy had higher cord blood levels of IgG4 against S. *dysenteriae* and RSV than those women taking no ART ($p < 0.05$ in Dunn's test adjusted by Benjamini-Hochberg, False Discovery Rate 5%), but no differences were found among women taking ART at recruitment than those not taking ART. Similarly, no differences were found between women taking ART before pregnancy or at recruitment.

Multivariable models were also performed to assess the factors associated with cord blood levels of total IgG and IgG subclasses. Maternal antibodies, HIV infection and *P. falciparum* exposure were included in the models. Maternal antibody levels had the strongest positive correlation with cord antibody levels for all the antigens and subclasses (**Figure 3A**). However, the effect of maternal antibody levels was more variable for IgG3-4 than for total IgG and IgG1 subclass. A 10% increase in maternal total IgG levels was associated with increases from 8.1 to 9.7% in total IgG cord blood levels, depending on the antigen. For IgG subclasses, a 10% increase in maternal antibody levels was associated with increases of cord blood levels from 7.6 to 10.9% for IgG1, 5.4 to 9.6% for IgG2, 5 to 9.9% for IgG3, and 5.3 to 9.3% for IgG4.

Maternal HIV infection (**Figure 3B**) had a negative effect on total IgG cord blood levels to all antigens, except for *C. diphtheriae, Hib,* and *V. cholerae*. HIV infection was associated with a reduction ranging from 1.83 to 4.13% in the IgG cord blood levels. For IgG1, HIV infection negatively impacted cord blood levels against *C. diphtheriae, B. pertussis, S. dysenteriae*, HBV, measles, *C. parvum,* and *G. intestinalis* (from 2.97 to 7.17% reduction). For IgG2, a reduction was observed against *B. pertussis, S. dysenteriae*, HBV, and *G. intestinalis* (from 2.98 to 7.02% reduction), whereas HIV was associated with an increase of 3.19% of IgG2 to RSV. Finally, we only detected a negative effect of HIV infection on IgG3 levels to *C. tetani* (10.90% reduction) and IgG4 to *P. falciparum* (1.85% reduction).

P. falciparum exposure was negatively associated with cord blood IgG levels against *S. dysenteriae* and HBV, IgG1 against *S. pneumoniae* and rotavirus, IgG2 against HBV and IgG3 against *C. diphtheriae* and rotavirus (**Figure 3C**). Depending on the IgG sublcass and antigen, 10% increase in *P. falciparum* exposure reduced the cord blood levels ranging from 0.32 to 0.80%.

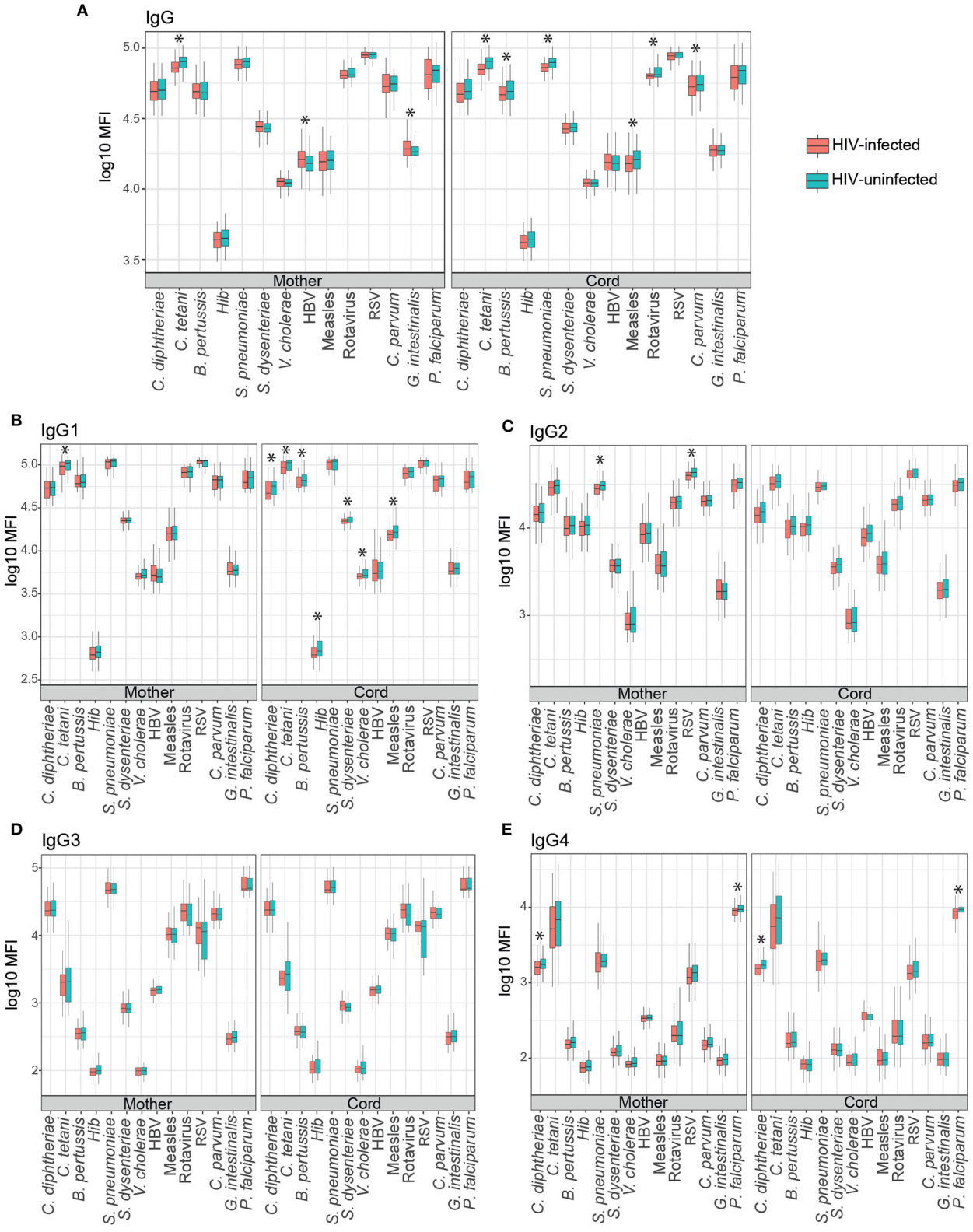

FIGURE 2 | Mother and cord blood antibody levels (log_{10} MFI) in HIV-positive and HIV-negative women. Boxplots illustrate the medians and the interquartile range for IgG **(A)**, IgG1 **(B)**, IgG2 **(C)**, IgG3 **(D)**, and IgG4 **(E)** subclasses in HIV-positive (red) and HIV-negative women (blue). Levels between groups were compared by Wilcoxon-Mann–Whitney test and *p*-values were adjusted for multiple testing by the Benjamini-Hochberg approach (False Discovery Rate 5%). Statistically significant differences are highlighted with an asterisk.

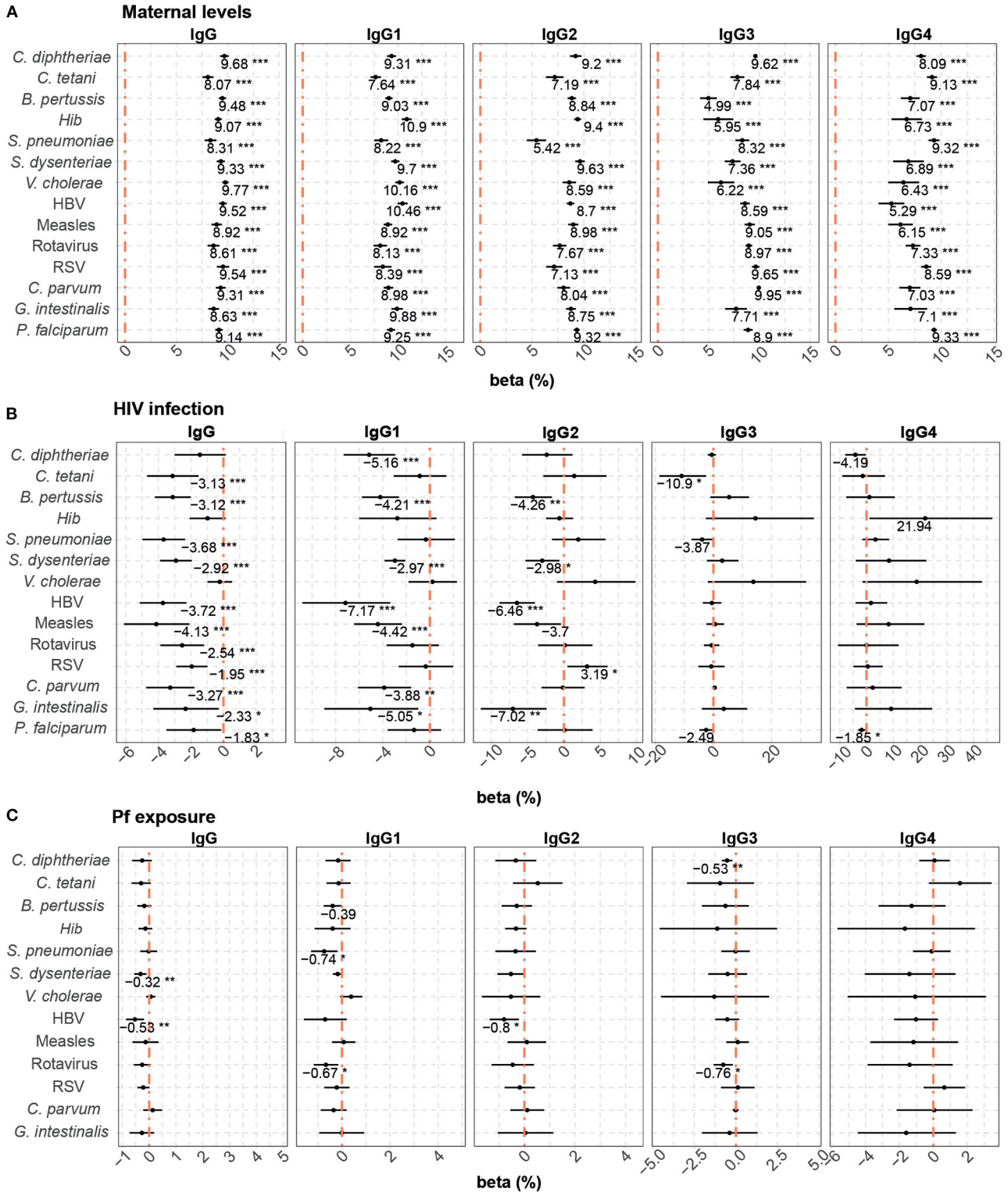

FIGURE 3 | Difference of IgG and IgG subclass levels in cord blood by study factors. Forest plots show the effect (in percentage) of maternal antibody levels **(A)**, HIV infection **(B)** and *P. falciparum* exposure (Pf exposure) **(C)** on cord blood levels of IgG and IgG subclasses for all the antigens tested. The differences in percentage correspond to beta-transformed values (%) 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 (False Discovery Rate 5%). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

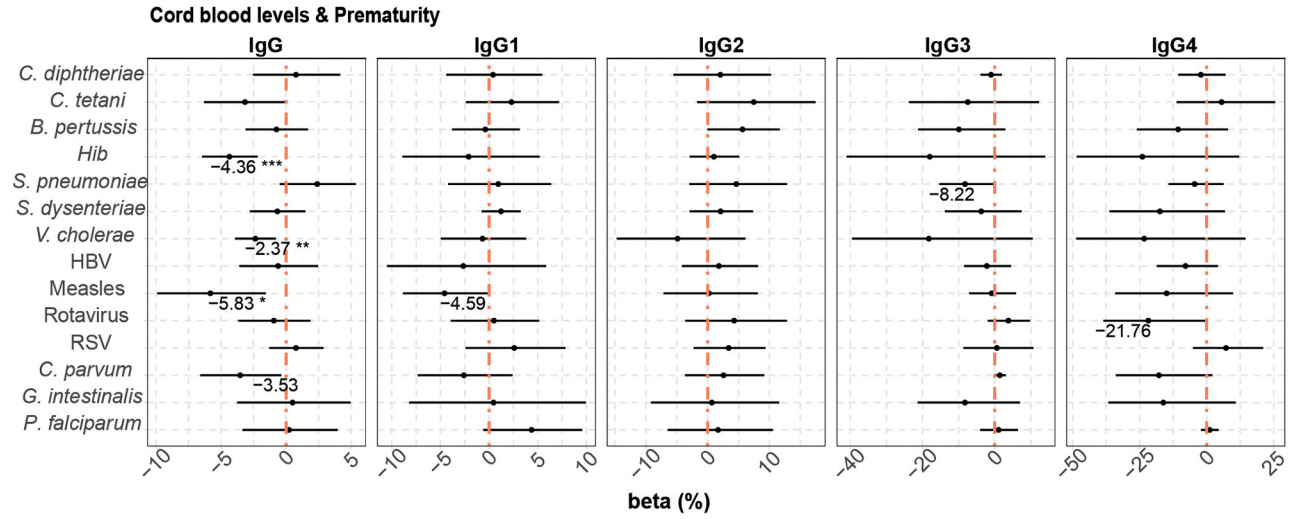

FIGURE 4 | Difference of IgG and IgG subclass levels in cord blood by prematurity. Forest plots show the effect (in percentage) of prematurity on cord blood levels of IgG and IgG subclasses, for all the antigens tested. The differences in percentage correspond to beta-transformed values (%) that were calculated from the beta values in the multivariable models. This beta value was obtained when prematurity was included in multivariable models with maternal antibody levels, HIV infection and *P. falciparum* exposure. Beta transformed values (%) are displayed when raw *p*-values are significant. Asterisks are shown when adjusted *p*-values by Benjamini-Hochberg are significant (False Discovery Rate 5%). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Previous studies suggest that PM rather than peripheral malaria may affect cord blood levels and transplacental transfer of antibodies and lead to adverse outcomes due to the damaged placental tissue (37, 72, 73). Therefore, we explored the effect of PM on cord blood levels in multivariable models without *P. falciparum* exposure despite the low number of women with any evidence of PM. PM had no significant associations with antibody cord blood levels (data not shown). When analyzing HIV-infected women only, PM was associated with lower *B. pertussis* IgG1, *C. diphtheriae* IgG2 and HBV IgG3 levels in cord blood (**Supplementary Figure 3**).

Prematurity, previously shown to have a detrimental effect on placental transfer of antibodies (74), was added to the multivariable model, which included the variables maternal antibody levels, HIV infection and *P. falciparum* exposure, as it increased the quality (AIC) of some of the models. Prematurity (**Figure 4**) was associated with lower cord blood total IgG levels against *Hib* (4.36% reduction compared with term cord blood levels), *V. cholerae* (2.37% reduction), measles (5.83% reduction), and *C. parvum* (3.53% reduction without statistical significance after adjusting for multiple testing).

The rest of the variables (age, maternal anemia, gravidity, low birth weight, IPTp treatment, seasonality; and $CD4^+$ T cell counts, ART and viral load for HIV-infected women) did not provide any added value to the multivariable models and were not included.

Altered Placental Transfer of Antibodies in HIV-Infected Women

Placental transfer of total IgG and IgG1 was significantly lower in HIV-infected women for all antigens except for *Hib* and *V. cholerae* (total IgG) and *C. tetani, S. pneumoniae, V. cholera,* and RSV (IgG1) (**Figures 5A,B**). For IgG2, only *G. intestinalis, B. pertussis,* and HBV had significantly lower transfer in HIV-infected women, while *S. pneumoniae* and RSV had higher transfer in HIV-infected women (**Figure 5C**). For IgG3, only *C. tetani* had a significantly lower transfer in HIV-infected compared to HIV-uninfected women (**Figure 5D**). No significant differences in placental transfer between the two groups were found for IgG4 (**Figure 5E**).

We compared placental transfer of IgG and IgG subclasses between HIV-infected women taking ART before pregnancy, at recruitment or without ART regime. No significant differences were found, with the exception of *C. parvum* IgG4 (**Supplementary Figure 4E**), for which HIV-infected women taking ART before pregnancy or at recruitment had higher placental transfer than those not taking ART ($p < 0.05$ in Dunn's test adjusted by Benjamini-Hochberg, False Discovery Rate 5%). There were no differences between taking ART before pregnancy or at recruitment in the *C. parvum* IgG4 placental transfer.

In multivariable models including HIV infection and *P. falciparum* exposure, HIV infection (**Figure 6A**) in general was associated with reduced placental transfer of total IgG and IgG1 (from 1.99 to 6.75% reduction depending on the antigen). HIV infection was also associated with a reduced transfer of IgG2 against *B. pertussis*, HBV, and *G. intestinalis*, but an increase in IgG2 RSV transfer (5.42% increase). Although adjusted *p*-values were not significant, a similar trend of positive correlation was found for *S. pneumoniae* IgG2 and *Hib* and *V. cholerae* IgG3 and IgG4.

P. falciparum exposure (**Figure 6B**) had a negative effect on the placental transfer of antibodies for some antigens. An

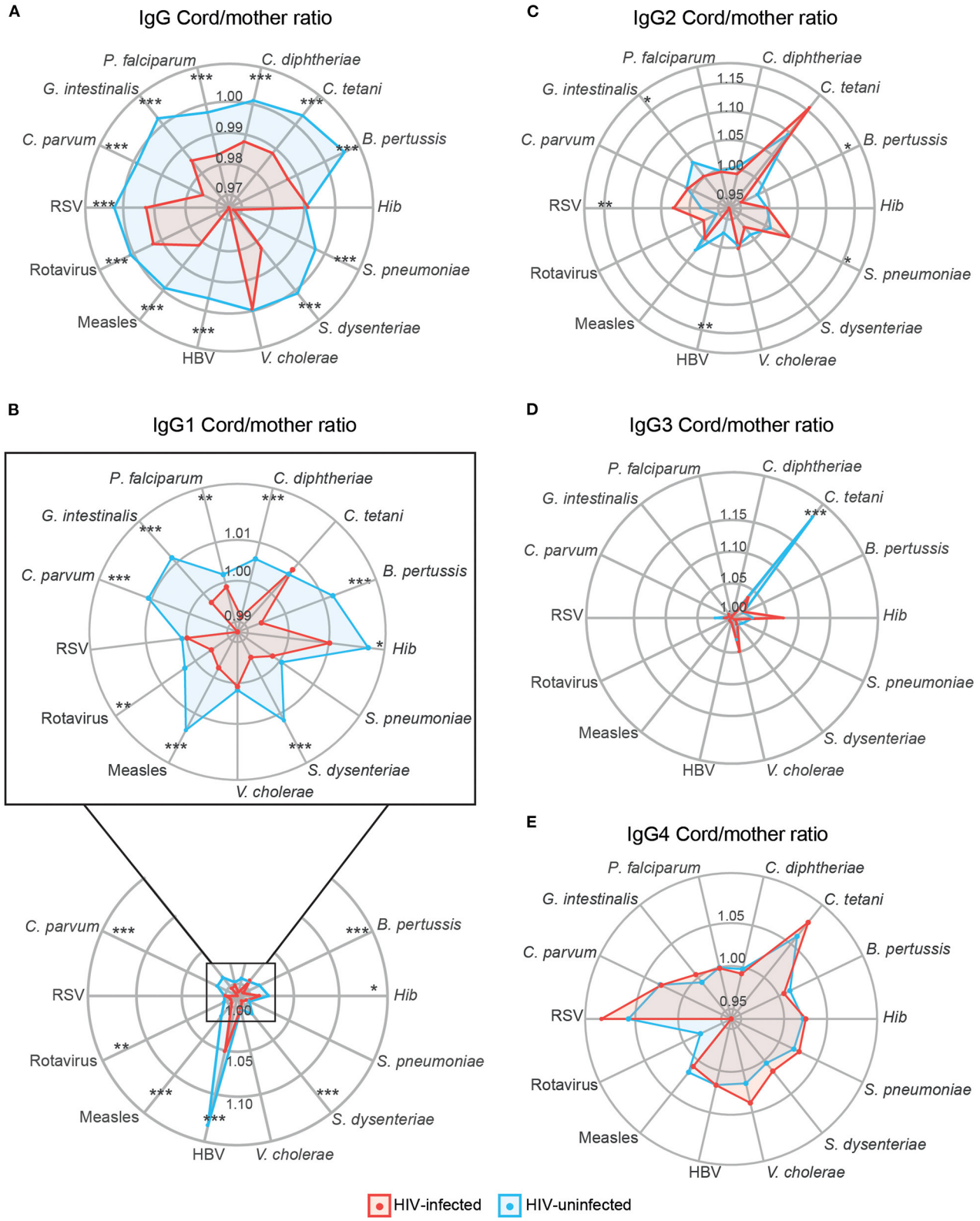

FIGURE 5 | Antibody placental transfer in HIV-positive and HIV-negative women. Radar charts representing the medians of each antigen antibody cord/mother ratio in HIV-positive and HIV-negative women for IgG **(A)**, IgG1 subclass **(B)**, IgG2 **(C)**, IgG3 **(D)**, and IgG4 **(E)**. Ratios between HIV-positive and negative women were compared by Wilcoxon-Mann-Whitney test and *p*-values were adjusted for multiple testing by the Benjamini-Hochberg approach (False Discovery Rate 5%). Statistically significant differences between HIV-positive and negative women ratios are highlighted with an asterisk. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. HIV-positive women are represented in red and HIV-negative women in blue.

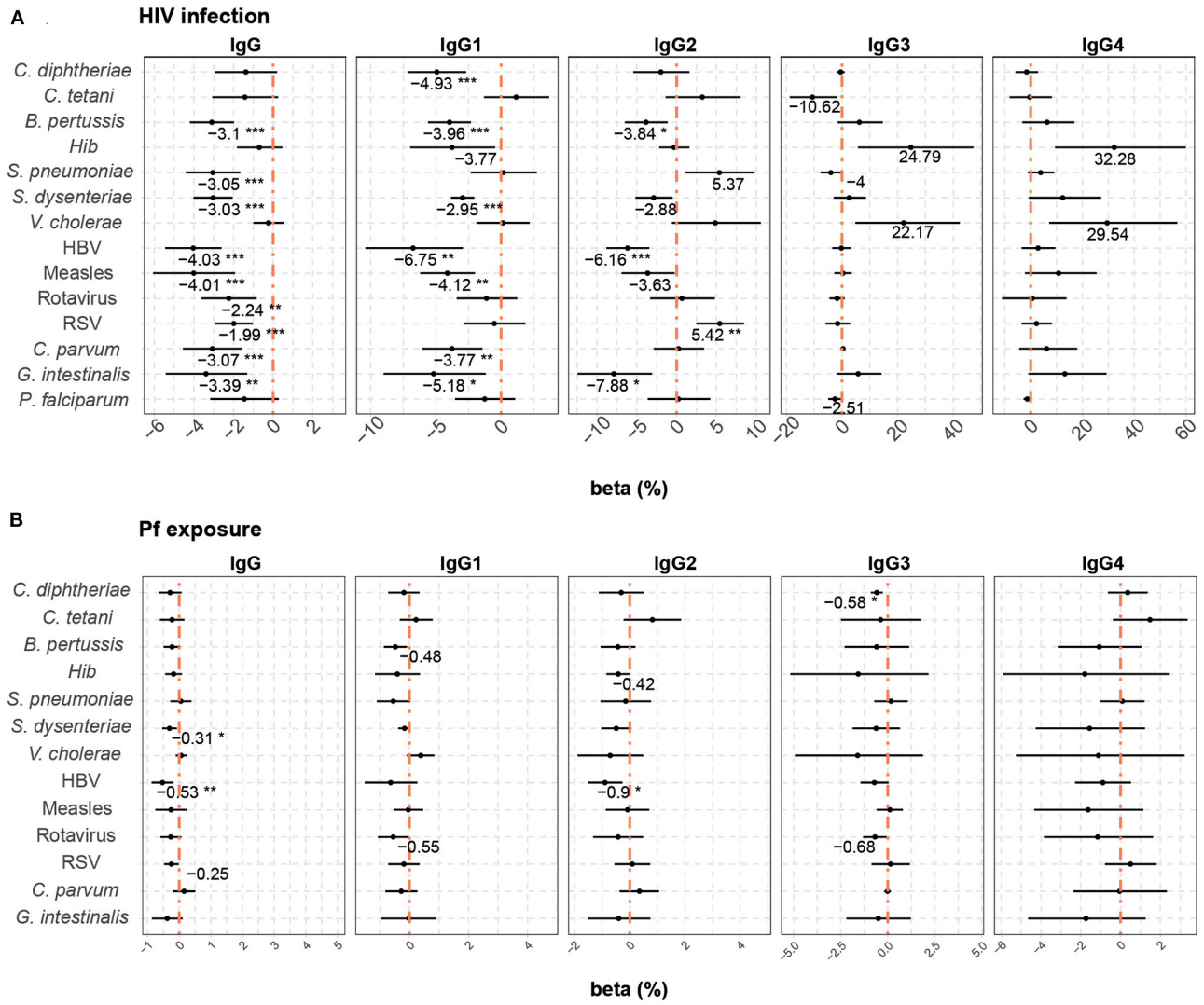

FIGURE 6 | Difference of IgG and IgG subclass placental transfer by study factors. Forest plots show the effect (in percentage) of HIV infection **(A)** and *P. falciparum* exposure (Pf exposure) **(B)** on transplacental transfer of IgG and IgG subclasses for all the antigens tested. The differences 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 (False Discovery Rate 5%). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

increase of 10% in *P. falciparum* exposure reduced the placental transfer of total IgG against *S. dysenteriae* and HBV by 0.31 and 0.53%, respectively, IgG2 against HBV by 0.90% and IgG3 against *C. diphtheriae* by 0.58%.

PM, in contrast to *P. falciparum* exposure, did not have any impact on transplacental transfer of antibodies in exploratory analyses and did not improve any of the models, although it had similar correlation trends for total IgG. Nevertheless, PM was associated with a diminished placental transfer of IgG1 *B. pertussis* in HIV-infected women (**Supplementary Figure 5**).

When prematurity was added to the multivariable models that included HIV infection and *P. falciparum* exposure, this additional covariable had a negative effect on placental transfer of only *Hib* and *V. cholerae* total IgG antibodies (4.43 and 2.38% reduction in premature vs. term newborns, respectively) (**Figure 7**).

No additional variables were included in the multivariable analysis as they did not provide any added value to the model.

DISCUSSION

Our comprehensive analysis of maternal and cord plasma total IgG and IgG subclasses against a wide range of microbial and vaccine antigens allowed a deep immunoprofiling, that is essential to decipher the mechanisms affecting antibody placental transfer and maternal and newborn immunity in women chronically exposed to pathogens. We confirmed that the main determinant of cord total IgG and IgG

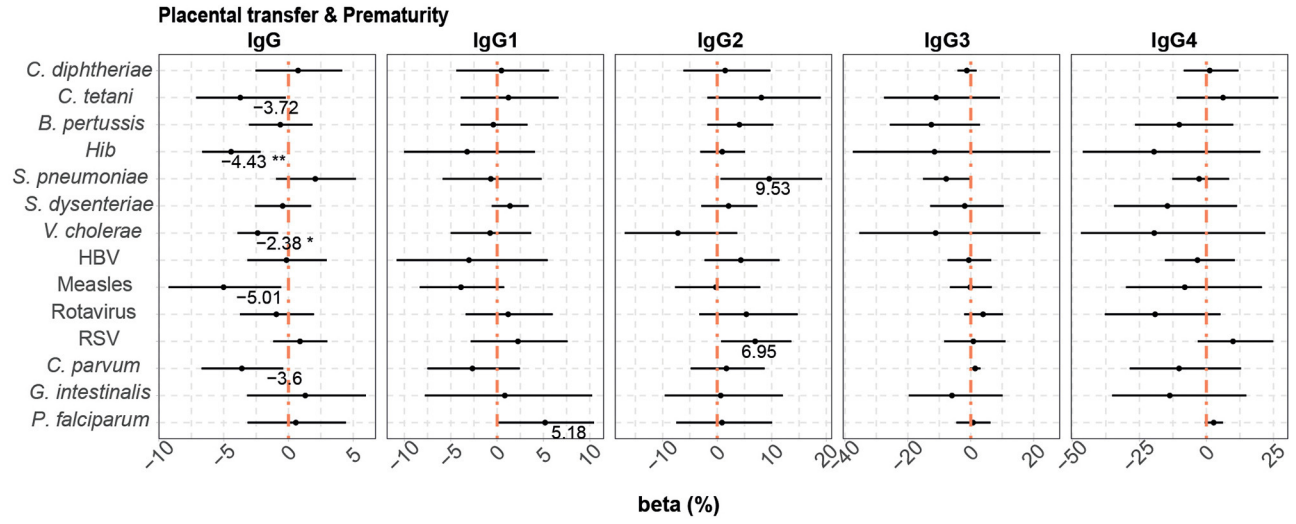

FIGURE 7 | Difference of IgG and IgG subclass placental transfer by prematurity. Forest plots show the effect (in percentage) of prematurity on transplacental transfer of IgG and IgG subclasses, for all the antigens tested. The differences in percentage correspond to beta transformed values (%) that were calculated from the beta values in the multivariable models. This beta value was obtained when prematurity was included in multivariable models with HIV infection and *P. falciparum* exposure. Beta transformed values (%) are displayed when raw *p*-values are significant. Asterisks are shown when adjusted *p*-values by Benjamini-Hochberg are significant (False Discovery Rate 5%). $^{**}p < 0.01$, $^{*}p < 0.05$.

subclass levels are the corresponding maternal antibody levels, and that maternal HIV infection is associated with a reduction of total IgG levels in the cord due to low maternal levels, but also to a reduction of IgG and IgG1 placental transfer.

Maternal and cord blood antibody levels are usually correlated in many studies, suggesting that maternal levels are the main determinant for transfer efficiency (14, 75, 76). However, the effect of HIV infection on placental transfer is not consistent among studies and prior analyses of the effect of HIV on maternal and cord blood levels have mainly focused on total IgG (16, 28, 29, 33, 39, 41–43, 46). Our results showed that HIV infection reduced the total IgG maternal levels for some antigens, the cord blood levels overall, and also had a negative effect on transplacental transfer of total IgG antibodies. It is interesting that although we found higher maternal HBV and *G. intestinalis* IgG levels among HIV-infected women, cord blood levels and transplacental transfer were lower than in HIV-uninfected women. Higher maternal antibody levels against these pathogens in HIV-infected women may be due to an increased susceptibility to co-infections with these pathogens, as described before (77–79), but it seems that they are not being transferred as efficiently as in HIV-uninfected women. This could be due to hypergammaglobulinemia, demonstrated to be common among HIV-infected individuals (80) and previously shown to impair transplacental transfer of antibodies (14, 16, 35), or due to an impairment of the Fc receptors caused by maternal HIV infection (16).

Our results are consistent with previous studies reporting that HIV infection led to a reduction of the cord blood levels and transplacental transfer of total IgG against *B. pertussis* (29, 42), *C. tetani* (28, 29, 40, 42), *S. pneumoniae* (33, 40, 42, 44, 81), RSV (82, 83), and measles (39, 40). Some studies also found a negative effect on *Hib* (29, 81) that is not appreciated in our study, although we found reduced IgG1 levels in cord in univariable analyses. However, our results differ from other studies that did not find any effect of HIV status on IgG levels against *C. diphtheriae* (38), *C. tetani* (33, 38), *S. pneumoniae* (46, 83), HBV (38), and measles (33, 38). These discrepancies could be due to the different geographical areas, statistical methods used, small sample sizes in the previous studies, type of antigen tested and the serological method applied.

IgG subclasses may be elicited differently depending on the pathogen, the antigen or the epitope (84), and the differences in Fc region between IgG subclasses, which mediates effector functions, confers them different roles during infection and pathogen clearance (84–86). The efficiency of the antibody placental transfer varies for each subclass due to differential affinity of the receptors FcRn (19). We found that the efficacy of IgG placental transfer also depended on the antigen. IgG1, IgG3, or IgG4 transferred better than IgG2, except for *S. pneumoniae,* for which IgG2 transfer was significantly higher. This was unexpected as classically it has been described that the greatest transport occurs for IgG1, followed by IgG4, IgG3, and finally IgG2 (20), although a recent report showed a different hierarchy of subclass transfer and identified a number of other studies that also observed different transfer efficiencies, depending on the antigen tested (21). However, IgG1 levels were the highest for almost all antigens in cord blood, probably because of the overall higher levels of this IgG subclass in maternal blood. One exception was *Hib* that presented higher IgG2 cord levels than IgG1, although IgG1 transplacental transfer was higher than IgG2 consistently with previous studies (87). The mothers had an IgG2-predominant response to *Hib*, and consequently higher

IgG2 than IgG1 levels were found in cord blood as previously described (88, 89).

In our multivariable models, we observed that HIV infection reduced mainly IgG1 cord levels due to a diminished transplacental transfer, similarly to IgG. Interestingly, maternal HIV infection increased the placental transfer of IgG2 to RSV and had a positive effect on RSV IgG2 cord blood levels, although IgG2 maternal levels were lower in HIV-infected women. To our knowledge, an increased placental transfer by HIV infection has not been described before. This may have implications for maternal immunization with RSV vaccines under development. Natural RSV infection seems to elicit an age-dependent IgG1, IgG2, and IgG3 response against the F protein, the major target of the host's immune response (90, 91), and of some RSV vaccines (92). Antibodies binding to the F protein are protective (93), and IgG1 is the IgG subclass mainly produced in response to RSV infection (94, 95) and an IgG1 monoclonal antibody against RSV F protein with neutralizing function, has shown to be effective (96), suggesting that the protective response is predominantly IgG1. Here, we found that total IgG and IgG1 against RSV F protein had the highest levels in cord blood compared to other subclasses, but HIV infection reduced total IgG cord blood levels and placental transfer. Therefore, HIV infection could compromise the levels of RSV neutralizing antibodies transferred to the newborn and, consequently, diminish the effectivity of an RSV vaccine.

Regarding other variables, our data did not show any significant association between $CD4^+$ T cell counts or HIV viral load on cord blood levels and transplacental transfer of antibodies. Even though these results agree with previous studies that did not find any associations (29, 81, 97), other reports describe that lower $CD4^+$ T cell counts and higher HIV viral load lead to a reduction of the transfer of some pathogen-specific antibodies and vaccines such as measles and *S. pneumoniae* (37, 43, 98). Some studies describe that HIV-infected women receiving ART transferred higher pathogen-specific antibodies than those who were not under ART (97) or who initiated it during pregnancy (99). However, in our cohort we did not find any significant associations in regards to ART. The discrepancies between studies could be explained by the different ART drug regime type.

At the time of the study, malaria transmission intensity was very low in the area and only a few women had active malaria during pregnancy. Nonetheless, we found a negative correlation between *P. falciparum* exposure and both placental transfer and cord blood antibody levels for some antigens and IgG subclasses. Previous studies are contradictory, as some found that PM led to a reduction of the transplacental transfer of some pathogen-specific IgG to *C. tetani* (34), measles (35, 39), RSV (37) and *S. pneumoniae* (33), but others did not find any effect for IgG against *C. diphtheriae* (37, 38), *C. tetani* (28, 35, 38), *Hib* (37), HBV (38), measles (38) RSV (36), and *S. pneumoniae* (37). Discrepancies between studies could be due to differences in study sites, prevalence of malaria, sample sizes, type of antigens, sensitivities of serological methods, exposure to the pathogens tested, and other co-infections. We also found that *P. falciparum* exposure was lower among HIV-infected women. Although all HIV-infected women were on prophylactic treatment with CTX, a broad-spectrum antibiotic that has been suggested to be protective against malaria (100, 101), HIV-negative women were also on IPTp with SP or MQ that has been demonstrated to be effective (47). Thus, this lower exposure could be more related to the fact that exposure to *P. falciparum* was computed as the sum of the maternal IgG antibody levels against three immunogenic *P. falciparum* antigens, and maternal HIV infection could reduce the IgG levels against them.

We found prematurity to be associated with lower cord blood IgG levels and placental transfer for some antigens, as previous studies have shown (74, 102, 103), although the effect was not consistent among subclasses. It has already been reported that the greatest transport occurs in the third trimester of gestation (17), and due to this fact, preterm infants may have lower amounts of transplacental IgG than term infants (74, 103, 104).

Our results are important for maternal immunization implementation in settings with a high prevalence of HIV infection. Here, the only vaccine given during pregnancy was tetanus. Although HIV infection was associated with lower maternal and cord blood tetanus toxoid IgG and IgG1 levels in univariable models, HIV did not affect cord blood IgG1 levels in multivariable models adjusted by maternal levels. Systemic tetanus vaccination during pregnancy has been implemented in Africa and has demonstrated a high efficacy (105). Pertussis vaccination in pregnancy has also been implemented in some countries such as Canada, US, and UK (106, 107), but not in Africa. Acellular pertussis vaccine induces mainly IgG and IgG1 responses that are thought to confer protection (108–110). We found lower cord blood levels and a reduced placental transfer of IgG and IgG1 against *B. pertussis* among HIV-infected women and those exposed to *P. falciparum*. These results highlight the need for further studies assessing the impact of these infections on pertussis vaccine efficacy and antibody placental transfer when implemented in pregnant women from African countries.

As study limitations, we could not establish the threshold of protection of antibody levels, therefore it is difficult to infer the clinical relevance of the reductions in antibodies detected in cord blood from the HIV-infected women. We could not measure hypergammaglobulinemia, which has been associated with a reduced transplacental transfer of antibodies (35–37), and demonstrated to be induced by chronic infections such as HIV, malaria or helminthiasis (80, 111, 112). Also, diabetes may have an impact on placental transfer of antibodies (31), but this diagnosis was not available in our cohort. Another limitation is that this study was only performed in a Mozambican cohort, therefore results obtained may not be representative of other populations. Specifically, IgG allotype diversity, or ethnic/geographic differences in FcRn, may impact the effect of HIV and malaria on placental transfer. As HIV-infected mothers also receive CTX, it could potentially limit antigen exposure and, therefore, antibody responses. Finally, we had to assess malaria exposure instead of PM due to the low number of PM cases and consequently our results are not comparable with those assessing PM.

In conclusion, our results demonstrate that maternal HIV infection was associated with reduced levels of antibodies

against a broad range of pathogens and vaccine antigens in cord blood. Part of this reduction in antibody levels was due to altered antibody levels in the mother, which are the main determinants of cord blood levels, but HIV-infection also diminished transplacental transfer of antibodies. Importantly, IgG1 was the most affected by maternal HIV infection but, depending on the pathogen, other subclasses were also affected. *P. falciparum* exposure also reduced the levels and transfer of some antibodies, although overall the effect was lower than HIV infection. In future studies, the clinical impact of the reduced placental transfer of antibodies caused by HIV infection and *P. falciparum* exposure will be assessed. Our findings are important for effective maternal immunization strategies and for newborn and infant's health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitè Ètic d'Investigació Clínica (CEIC, Hospital Clínic, UB), Spain, Comité Nacional de Bioética (CNBS), Mozambique. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SA, CD, and GM wrote the first draft of the manuscript, conceived the immunological study, 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. SA and MC produced antigens. EA provided the $MSP1_{42}$ protein. GR-O and MV-S performed the statistical analysis. AM, CD, and GM designed the immunology study ancillary to the clinical trials. MM, RB, and CJ processed the samples. PC and LF-S performed the PCR. RG, MR, JA, EM, AV, ES, and CM designed and enrolled participants in the clinical trials. AN was the clinical trial data manager. JA was the clinical trial statistician. RA, MC, RG, CM, GR-O, and MV-S contributed to the write up of the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.614246/full#supplementary-material

Supplementary Figure 1 | IPTp trial profile.

Supplementary Figure 2 | Cord blood antibody levels in HIV-positive women taking ART. Radar charts representing the medians of each analyte antibody cord levels in HIV-positive women who started ART before pregnancy, at recruitment or were not taking ART for IgG **(A)**, IgG1 subclass **(B)**, IgG2 **(C)**, IgG3 **(D)**, and IgG4 **(E)**. Cord blood levels were compared by Kruskal-Wallis test and p-values were adjusted for multiple testing by the Benjamini-Hochberg approach (False Discovery Rate 5%). Statistically significant differences between ART before pregnancy, ART at recruitment and no ART are highlighted with an asterisk. $^{**}p < 0.01$, $^{*}p < 0.05$. ART at recruitment is represented in green, ART before pregnancy is represented in blue and no ART is represented in red.

Supplementary Figure 3 | Difference of IgG and IgG subclass levels in cord blood levels of HIV-infected women. Forest plots show the effect (in percentage) of placental malaria on cord blood levels of IgG and IgG subclasses, for all the antigens tested, when placental malaria was included in multivariable models with maternal antibody levels. 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 (False Discovery Rate 5%). $^{**}p < 0.01$, $^{*}p < 0.05$.

Supplementary Figure 4 | Antibody placental transfer in HIV-positive women taking ART. Radar charts representing the medians of each analyte antibody cord/mother ratio in HIV-positive who started ART before pregnancy, at

recruitment or were not taking ART for IgG **(A)**, IgG1 subclass **(B)**, IgG2 **(C)**, IgG3 **(D)**, and IgG4 **(E)**. Ratios were compared by Kruskal-Wallis test and *p*-values were adjusted for multiple testing by the Benjamini-Hochberg approach (False Discovery Rate 5%). Statistically significant differences between ART before pregnancy, ART at recruitment and no ART are highlighted with an asterisk. $*p < 0.05$. ART at recruitment is represented in green, ART before pregnancy is represented in blue and no ART is represented in red.

Supplementary Figure 5 | Difference of IgG and IgG subclass placental transfer in HIV-infected women. Forest plots show the effect (in percentage) of placental malaria on transplacental transfer of IgG and IgG subclasses, for all the antigens tested, when placental malaria was included in multivariable models. 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 (False Discovery Rate 5%). $*p < 0.05$.

Supplementary Material 1 | Cord blood levels and placental transfer of antibodies univariable models.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 2

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

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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.

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

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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 de-

fined 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^{TM} technology (Luminex Corp., TX) was performed.

Eight *P. falciparum* recombinant proteins were selected for our analysis: Duffy-binding like domains 3-4 (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 (PfEMP1 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 4°C 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).

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 methodStatistical significance was considered when *p–value <0.05;* MQ, mefloquine; NP, not−performed tests; SP, sulfadoxine-pyrimethamine.

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*

The PCA analysis of antibody levels in 303 cord and 332 maternal blood samples showed very similar patterns (maternal antibody PCA analyses not shown). Clusters showing similarity of responses were detected in cord antibody levels by IgG subclasses (Fig. 1a) and antigens (Fig. 1b). While dimension 1 explained the majority of the variance and contributed to the separation of IgG4, IgG2 and IgG/IgG1/IgG3, dimension 2 contributed to the separation of the IgG1 and IgG3 responses (Fig. 1a) and MSP1 bl2, MSP2 from the rest of the antigens (Fig. 1b). DBL3-4 greatly contributed to IgG1 whereas MSP1 bl2 and MSP2 contributed more to the IgG3

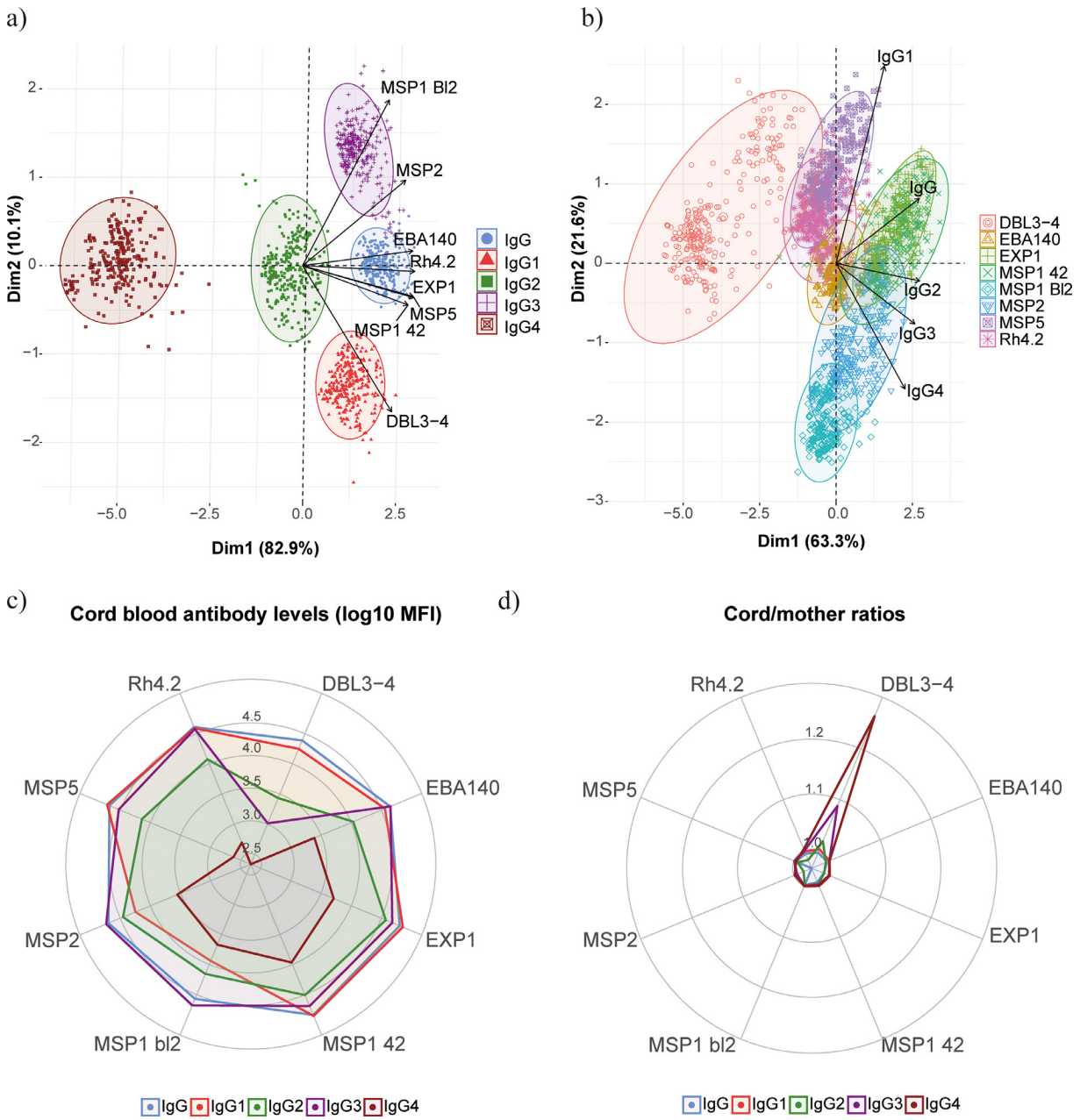

Fig. 1. Overview of cord blood levels of IgG and IgG subclasses to *P. falciparum* antigens for all women. a) Principal component analysis (PCA) plots of cord IgG and IgG subclass levels against all antigens clustered by subclass type. b) PCA plots of cord IgG and IgG subclass levels clustered by antigen 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.

responses (Fig. 1a). DBL3-4 was clearly separated from the rest indicating a different antibody profile (Fig. 1b). Consistently, DBL3-4 had lower IgG3 levels and MSP1 bl2 and MSP2 had lower IgG1 levels than the other antigens (Fig. 1c). Overall, IgG2 had lower median levels than IgG1 and IgG3 for most antigens, except for MSP1 bl2, MSP2 and DBL3-4. The lowest levels were shown for IgG4 in all antigens, with especially very low responses for DBL3-4, MSP5 and Rh4.2 (Fig. 1c).

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

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

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

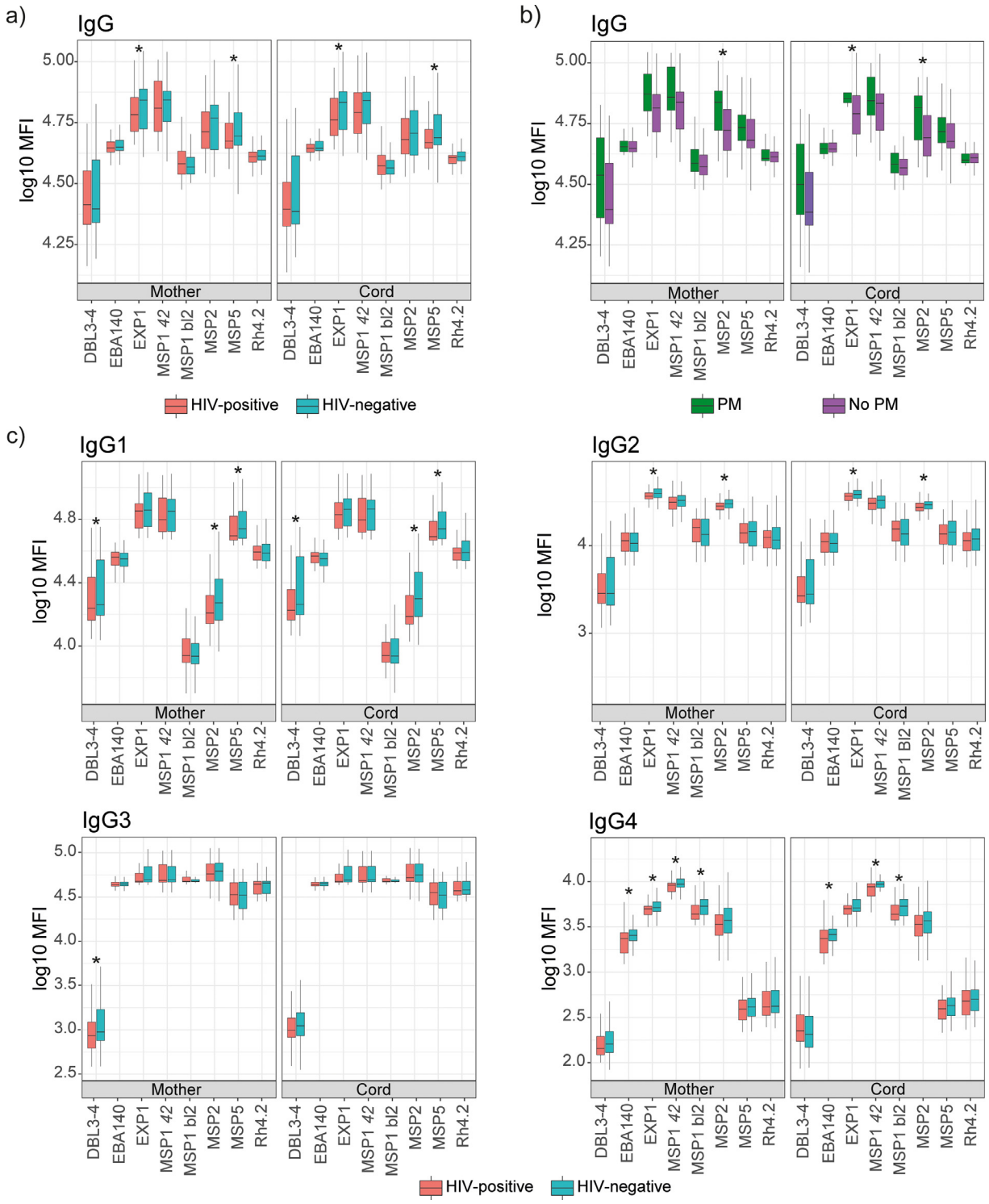

Fig. 2. Mother and cord blood antibody levels (log_{10} MFI) in HIV-positive and HIV-negative women and women with PM and without PM. Boxplots illustrate the medians and the interquartile range for IgG in HIV-positive and HIV-negative women (a), IgG in women with PM and women without PM (b), and IgG1, IgG2, IgG3 and IgG4 subclasses in HIV-positive and HIV-negative women (c). Levels between groups were compared by the non-parametric Mann–Whitney U test and p-values were adjusted for multiple testing by the Benjamini–Hochberg approach. Statistically significant differences are highlighted with an asterisk. HIV-positive women are represented in red, HIV-negative women in blue, women with PM in green and women without PM in purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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

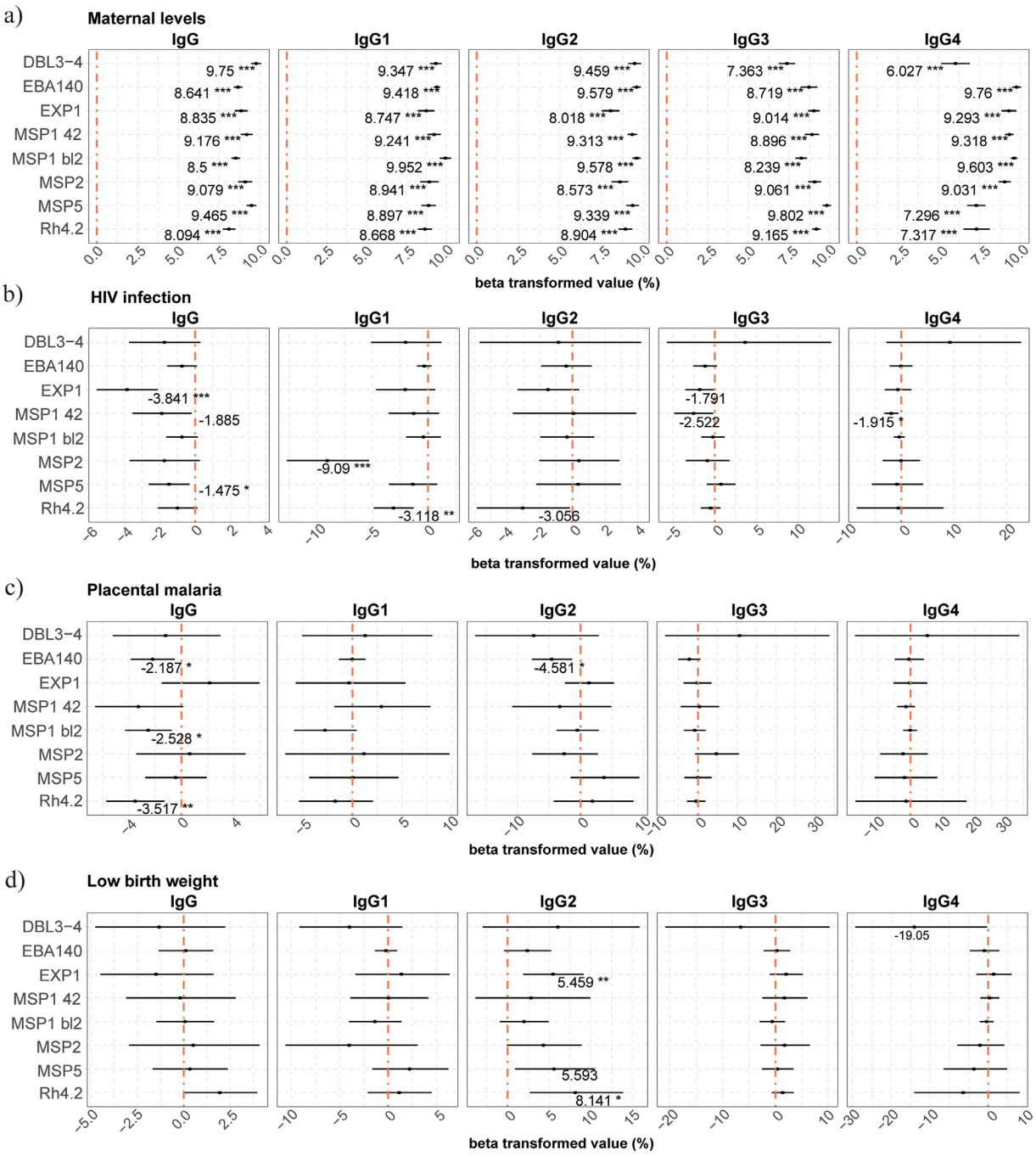

Fig. 3. Difference of IgG and IgG subclass levels in cord blood by study factors. Forest plots show the effect (in percentage) of a) maternal antibody levels, b) HIV infection, c) placental malaria and d) low birth weight, on cord blood levels of IgG and IgG subclasses for all the antigens tested. The 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 ≤ 0.001, ** = p-value ≤ 0.01, * = p-value ≤ 0.05.

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

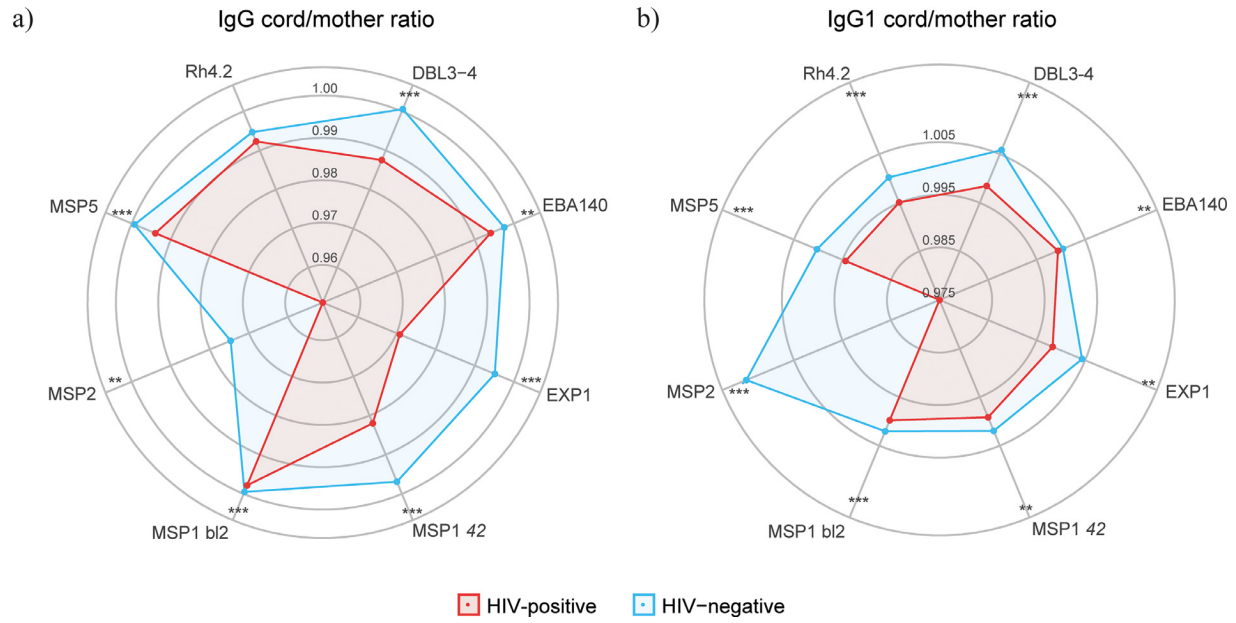

Fig. 4. Antibody placental transfer in HIV-positive and HIV-negative women. Radar charts representing the medians of each analyte antibody cord/mother ratio in HIV-positive and HIV-negative women for IgG (a) and IgG1 subclass (b). Ratios between HIV-positive and negative women were compared by the non-parametric Mann-Whitney U test and p-values were adjusted for multiple testing by the Benjamini–Hochberg approach. Statistically significant differences between HIV-positive and negative women ratios are highlighted with asterisks. *** = p-value ≤ 0.001, ** = p-value ≤ 0.01, * = p-value ≤ 0.05. HIV-positive women are represented in red and HIV-negative women in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$MSP1_{42}$. 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 ART 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 on 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 in this cohort and will be addressed in 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.

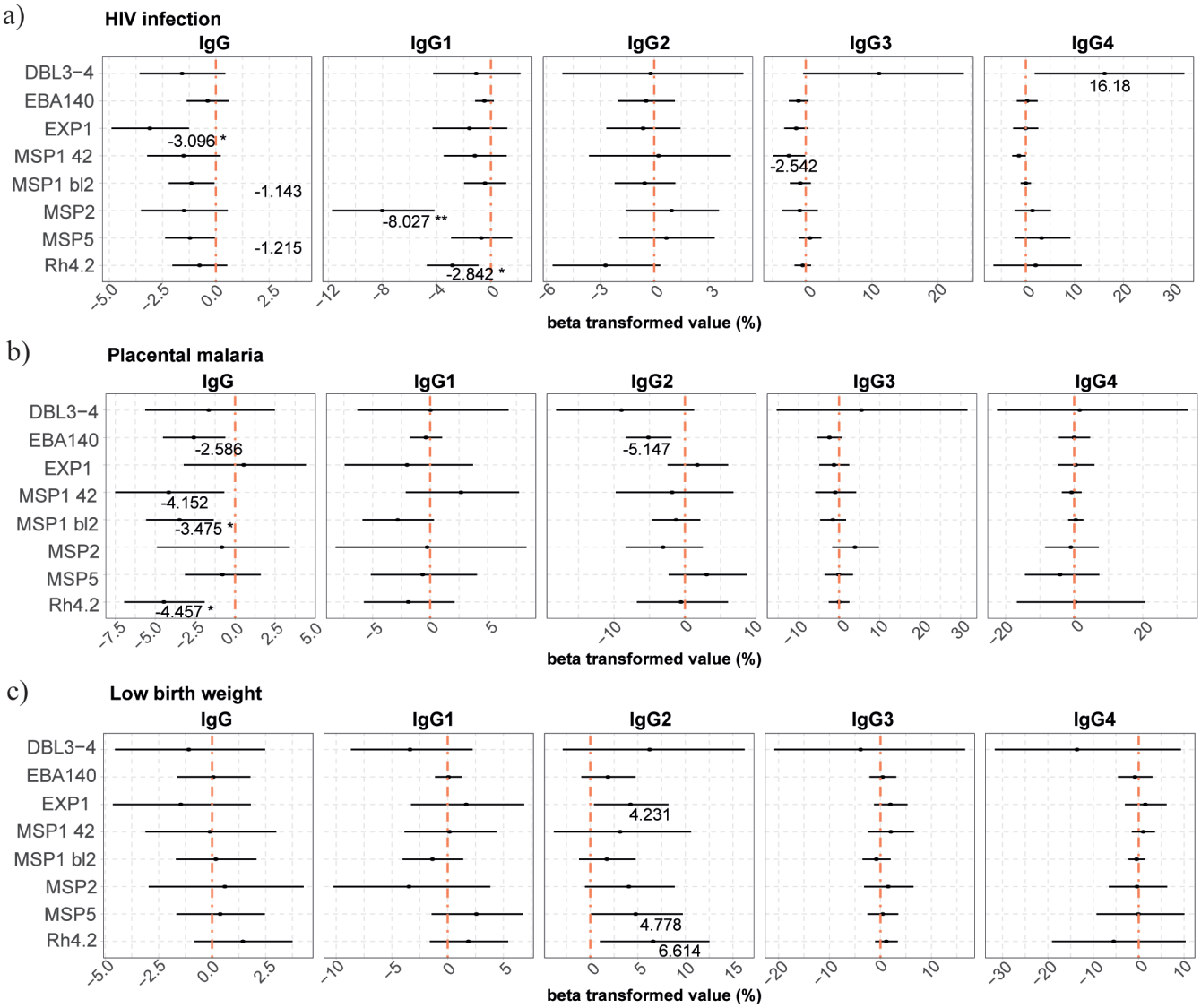

Fig. 5. Difference of IgG and IgG subclass placental transfer by study factors. Forest plots show the effect (in percentage) of a) HIV infection, b) placental malaria and c) low birth weight, on placental transfer of IgG and IgG subclasses for all the antigens tested. The differences in percentage correspond 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 ≤ 0.001, ** = p-value ≤ 0.01, * = p-value ≤ 0.05.

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.

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Declaration of Competing Interests

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2021.02.024.

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Discussion

Effect of maternal HIV and malaria on the transplacental transfer of antibodies against prevalent pathogens and vaccines in Mozambican women

06

DISCUSSION

In this doctoral thesis, we measured maternal and cord blood total IgG and IgG subclasses against 21 antigens, as well as the placental transfer. The antigens selected represent the most prevalent pathogens in the Manhiça district, in Mozambique, divided in respiratory pathogens, gastrointestinal pathogens and malaria, and vaccines administered to the infants through the EPI at the time of the study. Overall, we confirmed that cord total IgG and IgG subclass levels positively correlate with their corresponding maternal antibody levels, suggesting that maternal levels are the main determinant for cord levels. We also observed that the efficiency of placental transfer was different depending on the antigen, and that maternal HIV infection and malaria had a negative effect on maternal and cord antibody levels and transfer.

Infectious respiratory diseases are one of the leading causes of children and neonatal deaths in Mozambique and are caused by pathogens such as *S. pneumoniae* and RSV [10, 128]. IgG subclasses may be elicited differently depending on the pathogen or the antigen and they have different roles on pathogen clearance [29, 199]. We found that cord IgG1 levels against *S. pneumoniae* PspA were higher than the rest of subclasses, consistently with previous reports that showed a predominant response of IgG1 after PspA immunization in mice and pregnant women [431, 432]. However, the transplacental transfer of IgG1 was lower than that of other IgG subclasses such as IgG2, which had the highest transfer efficiency. This could be explained by the fact that IgG1 levels were the highest in maternal blood and may saturate the Fc receptor of the placenta, while other IgG subclasses with lower maternal concentrations may be more actively transferred. This was initially unexpected as it has been classically described that the greatest transport occurs for IgG1, followed by IgG4, IgG3, and finally IgG2 [38]. Nevertheless, our results are in line with a recent report showing a different hierarchy of subclass transfer that was also dependent on the antigen [39]. Similar to *S. pneumoniae*, anti-RSV IgG1 cord blood levels were higher than the rest of subclasses. RSV is one the most prevalent respiratory virus among hospitalized young children with severe pneumonia in Mozambique [433, 434]. IgG1 is the subclass produced in a natural infection and induced by vaccines targeting the F protein [129, 130], and although in this case IgG4 was more efficiently transferred through the placenta, the levels in cord were still the highest for IgG1. IgG1 was also predominant in maternal blood, while the rest of subclasses were lower, reinforcing our hypothesis that the transfer of these antibodies could be more efficient as the Fc receptor is not saturated.

Diarrheal diseases are the second leading cause of death among children under the age of 5 globally [10]. For the gastrointestinal pathogens rotavirus, *C. parvum*, *G, intestinalis*, *S. dysenteriae* and *V. cholerae,* IgG1 subclass had the highest levels in cord blood, which correlated with their corresponding maternal antibody levels, as shown in previous studies [41, 45, 95, 435, 436]. Indeed, IgG1 was also the predominant antibody response induced by these gastrointestinal infections in the mother [131–134], although the placental transfer was more variable between them.

Malaria is another leading cause of mortality among infants, with the highest prevalence in the WHO African region [58], and infection during pregnancy increases the risk of severe complications. Maternal antibodies may confer protection to their newborns against malaria for the first 3-6 months of life. However, they may interfere with the acquisition of protective antibodies after malaria vaccination. This has been suggested previously with RTS,S/AS01E immunization against CSP and with non-CSP protection-related antigens, and seen with other vaccines included in maternal immunization strategies [60, 296, 437]. To assess the placental transfer of antibodies against *P. falciparum* antigens, we included a selection of antigens considered to be pregnancy-specific markers (DBL3-4) [438], markers of exposure (EXP1, $MSP1_{42}$ and MSP2) and immunity (EBA140, MSP1 bl2, MSP5 and Rh4.2), as we defined previously in our RTS,S malaria vaccine studies [296].

We observed that IgG1 and IgG3 levels in cord blood against *P. falciparum* antigens were the highest and IgG4 the lowest, while the transplacental transfer was more efficient for IgG4, especially for DBL3-4. Interestingly, IgG4 cord levels against DBL3-4 were the lowest, and this highly effective placental transfer could be explained by lower maternal antibody concentrations having an active placental transfer [439]. As IgG1 and IgG3 are cytophilic antibodies considered to be protective [135–137], high cord levels could protect newborns during their first months of life, especially those against the antigens of the PfRh, EBA invasion ligand families and MSP5 that have been correlated with immunity to malaria [440–443]. Also, IgG2 and IgG3 antibodies against MSP2 could be protective as suggested in some studies [308, 309], whereas IgG4 antibodies have been associated with risk of infection [444]. Our results showed high anti-MSP2 IgG2 and IgG3 levels in the cord and low IgG4 levels that could be associated with malaria protection in infants.

Vaccination is a major triumph in public health and immunization programs are essential for the prevention of infectious diseases. This is of utmost importance in the African Region, where the rates of infant morbidity and mortality are

higher than in high-income countries [113]. Not only infant immunization has demonstrated to be effective for preventing infant deaths, but also vaccination of pregnant women can confer protection to their newborns for the first months of life [37]. In the case of the vaccine antigens *C. diphtheriae*, *C. tetani, B. pertussis*, *Hib,* HBV and measles, we found that although placental transfer was antigen-dependent, cord IgG1 levels were higher than the rest of subclasses for most of the antigens, except for HBV and *Hib*. For HBV, IgG1 and IgG2 cord levels were similar and correlated with the maternal antibody concentrations. Our results are in line with a previous study that found an elicitation of both IgG1 and IgG2 responses after HBV vaccination [138]. For *Hib,* IgG2 cord blood levels were clearly higher than IgG1, and this could be the explained as maternal responses are predominantly IgG2 [116, 122]. The rest of the vaccine antigens induced IgG1 responses that are known to be correlated with protection [117, 118, 121, 124], therefore increased concentrations of IgG1 in cord blood may be protective for the newborn against these vaccine-preventable diseases.

An important component of this thesis was the understanding of factors that may affect the transfer of antibodies to the pathogens and vaccines related above. Thus, we evaluated the effect of maternal HIV, malaria and pregnancy outcomes on the cord blood total IgG and IgG subclass levels, as well as the placental transfer of these antibodies.

Maternal HIV infection is known to have a negative impact on the immune system of the mother and their offspring. This is particularly concerning in low-income countries where infectious diseases are prevalent, and Mozambique is among the most affected countries with a high prevalence among pregnant women [100]. Due to the HIV-related immunodeficiency, the efficacy of vaccines could be impaired. It has been suggested that HIV-infected women might mount poorer responses to vaccines as they had lower antibody levels to measles, tetanus, influenza and *Hib* than HIV-uninfected women [445]. As a result, placental antibody transfer could be diminished and consequently, HIV-exposed uninfected infants could have less maternal antibodies. Having lower antibodies against vaccine-preventable diseases could lead to a lack of protection, which might explain the higher incidence of severe infections, hospitalizations and deaths among HIV-exposed uninfected infants [347, 446–451]. However, the effect of HIV on placental transfer of antibodies and cord blood levels is not consistent among studies and most of them mainly focused on total IgG [45, 46, 49–53, 107, 452].

Our results showed that HIV infection was associated with a reduction of maternal and cord antibody levels and placental transfer of antibodies for the

majority of antigens. For respiratory pathogens, we found lower cord blood levels and placental transfer of anti-*S. pneumoniae* and anti-RSV total IgG in HIV-infected women. This result was in line with previous studies reporting a reduction of total IgG cord levels and placental transfer against *S. pneumoniae* due to HIV infection [49, 51, 54, 453, 454], although others did not see any effect [452, 455]. Interestingly, we found that HIV reduced IgG2 maternal levels against both *S. pneumoniae* and RSV, but increased the placental transfer of IgG2 to RSV and had a positive effect on RSV IgG2 cord blood levels. The negative effect of HIV infection shown in RSV and *S. pneumoniae* antibodies suggest that maternal HIV could reduce the effectivity of maternal vaccines against RSV and *S. pneumoniae* that are under development. Many RSV vaccines target the F protein [9, 456–460], and antibodies binding this protein, principally IgG1, have been demonstrated to be protective [417, 461]. For *S. pneumoniae*, maternal immunization with PspA has been tested in mice and showed a strong response of anti-PspA specific IgG, predominantly IgG [431, 462]. Thus, HIV infection may have negative implications for maternal immunization against these pathogens.

Another important vaccine administered to pregnant women in which we have seen a reduction in antibody levels and transfer by maternal HIV is tetanus. Systemic tetanus vaccination during pregnancy has been implemented in Africa and has demonstrated to decline neonatal tetanus cases, although the goal of eliminating maternal and neonatal tetanus by 2015 was not achieved in all countries [463]. Tetanus was the only vaccine given during pregnancy in our cohort and we found that HIV-infected pregnant women had reduced total IgG and IgG1 maternal levels. Although HIV did not affect cord blood IgG1 levels in multivariable models adjusted by maternal levels, cord blood total IgG levels and cord IgG3 and placental transfer were diminished in HIV-infected women. However, protective levels in mothers and their newborns could probably be still achieved as maternal and neonatal tetanus was considered to be eliminated in Mozambique in 2010 by the WHO [464], encouraging maternal immunization strategies.

For the rest of vaccine antigens tested, HIV infection led to a reduction of the cord blood levels and transplacental transfer of total IgG and IgG1 against *B. pertussis,* measles and HBV, and IgG2 against *B. pertussis* and HBV. IgG1 against *Hib* was also reduced in univariable analysis. Our results are consistent with previous studies reporting a reduction of total IgG cord blood levels and placental transfer against these vaccine antigens in HIV-infected women [46, 50–52, 453, 454], but differ with other studies that did not find any effect of HIV infection [49, 322]. One explanation for the discrepancies between studies could be the different geographical areas. For example, some studies were

performed in high-income countries such as UK, or in urban settings of low-income countries. However, the two studies that did not find any effect of HIV were performed in rural areas of Kenya and Malawi. Thus, another explanation could be limited statistical power due to small sample sizes in these two studies, which included less than 50 HIV-infected women. Our study provides a better scope with an HIV-positive cohort almost three times bigger. Moreover, the method used to evaluate total IgG responses in these two studies was a commercial enzyme-linked immunosorbent assay kit that differ with our multiplex suspension array technology assay.

Interestingly, HBV, as well as the gastrointestinal pathogen *G. intestinalis*, had higher maternal total IgG levels among HIV-infected women, although cord blood levels and transfer were lower than in HIV-uninfected women. The higher maternal IgG levels against these pathogens could be due to the increased susceptibility to co-infections as reported in many studies [139–141], although maternal HIV was associated with a reduced placental transfer efficiency of IgG against them and consequently lower cord blood IgG levels. Hypergammaglobulinemia, common in HIV-infected women [348], could be a good explanation for this as it has been associated with impaired transplacental transfer of antibodies [41, 47, 107]. Also, the inflammatory status of the placenta reported in HIV-infected pregnant women could be associated with this impairment [104, 105].

Reaching protective levels of antibodies against malaria is essential for the newborn. We found that maternal total IgG, as well as cord blood levels and placental transfer were lower against *P. falciparum* antigens in HIV-infected women [49, 322]. Maternal HIV infection reduced IgG levels against EXP1 and MSP5 in the mother and cord, and impaired the transplacental transfer of total IgG against all the antigens except Rh4.2. Our results are consistent with previous studies showing a reduction in maternal and cord IgG levels and transplacental transfer against some antigens related to malaria exposure and protection in HIV+ women [45, 55, 322]. However, these studies showed some discrepancies depending on the antigen tested. Within IgG subclasses, we showed that HIV-infected women had a reduction of IgG1 cord levels against MSP2 and Rh4.2 due to an HIV impairment of the IgG1 transplacental transfer, and lower IgG4 cord levels against $MSP1_{42}$. As cytophilic antibodies have been associated with malaria protection and IgG4 subclass may also have a protective role [84, 465, 466], diminished levels of these antibodies could increase the risk of infection in newborns. However, no correlates of protection have been established and therefore the implications of such decreases on malaria protection are unknown.

ART implementation is of special importance in areas of high HIV transmission such as sub-Saharan Africa, and it could play a role in preventing the negative effects of HIV infection on transplacental transfer of antibodies. A study in Belgium showed that HIV-infected mothers who initiated ART before pregnancy had higher antibody transfer than those who initiated ART during pregnancy [467], and another in Kenya showed that receiving optimal ART treatment was associated with higher placental transfer of antibodies [468]. However, in our cohort we did not find any association with ART, similarly to other studies that showed no differences between taking ART or not, or only found a reduction on transplacental transfer against some antigens but not against others [55, 322, 469]. The only study that found differences between starting ART before or during pregnancy was performed in a high-income country, which is not comparable with a rural setting with suboptimal hygiene conditions, malnutrition and a high prevalence of pathogens such as Mozambique. The benefits of ART in Africa are unquestionable with the reduction of HIV-related morbidity and mortality [142], with a higher extent in regards to maternal and child health [470]. Thus, ART implementation should be improved in low-income countries, even if there is lack of evidence that ART could enhance placental transfer of antibodies in HIV-infected African women.

Malaria in pregnancy could also have an impact on the transplacental transfer of antibodies, probably due to the damage caused to the placenta, but the effect is not yet clear. It has been reported that PM is associated with reduced transplacental transfer of IgG against several pathogen antigens in some studies [47, 48, 50, 95], although others have shown no impact [47, 219, 221, 322]. Therefore, we assessed the impact of *P. falciparum* exposure and PM on cord blood antibody levels and placental transfer of antibodies against the antigens of our study. Malaria transmission intensity was very low in Manhiça at the time of the study and only a few women had malaria during pregnancy, many of them also under IPTp. Despite this, we found a negative correlation between *P. falciparum* exposure and PM and both placental transfer and cord blood antibody levels for some vaccine and pathogen antigens and IgG subclasses. We also found that *P. falciparum* exposure was lower among HIV-infected women. A plausible explanation could be that maternal HIV infection reduced the IgG levels against the three immunogenic *P. falciparum* antigens used to compute malarial exposure.

Despite the low number of women with any evidence of PM in the study, PM had a negative impact on some anti-malarial IgG transplacental transfer, such as MSP1 bl2 or Rh4.2, that resulted in lower cord blood antibody levels against these antigens. This, together with the reduction of protective antibody levels

and placental transfer observed in HIV-infected women, could increase the susceptibility of newborns to severe malaria. In contrast to *P. falciparum* exposure, PM did not have any impact on transplacental transfer of antibodies against the rest of the pathogens and vaccine antigens tested. We only found an association of PM with a diminished placental transfer of IgG1 *B. pertussis* in HIV-infected women. Although pertussis vaccination in pregnancy has still not been implemented in Africa, acellular pertussis vaccine induces mainly IgG and IgG1 responses that are thought to confer protection [118, 471, 472]. Therefore, our results highlight the need for further studies assessing the impact of these infections on pertussis vaccine efficacy and antibody placental transfer in settings with endemic malaria and high HIV infection prevalence.

Malaria infection among HIV-infected women is very common in Africa and lower $CD4^+$ T cell counts and higher viral load has been reported in co-infected individuals [473]. Both lower $CD4^+$ T cell counts and higher viral load have been associated to a reduction of the transfer of some vaccine and pathogen-specific antibodies vaccines such as measles and *S. pneumoniae* in some studies [53, 219, 474]. This impairment could be due to the higher grade of immunodeficiency caused by HIV infections that may lead to a diminished antibody response, which could be even lower in malaria co-infections that worsen the clinical outlook of HIV-infected persons [475]. Although we have not evaluated co-infections specifically, we did not find any significant association between $CD4^+$ T cell counts or HIV viral load on cord blood levels and transplacental transfer of antibodies.

Other pregnancy outcomes such as prematurity and low birth weight have been shown to have a detrimental effect on cord blood levels and placental transfer of antibodies [227–229, 476, 477]. Preterm infants may have lower amounts of IgG than term infants as the greatest transport occurs in the third trimester of gestation [214, 224, 227, 476]. However, we only found prematurity associated with lower cord blood total IgG levels against *Hib, V. cholerae,* measles and *C. parvum,* but not to any *P. falciparum* antigen. Similarly, we did not observe any association of LBW with lower cord levels or placental transfer, except for IgG2 anti-EXP1 and anti-Rh4.2 *P. falciparum* antigens that were surprisingly higher in cord blood. These increased IgG2 levels could enhance the risk of malaria complications in low weight infants as a positive association of severe malaria and IgG2 levels has been found in some studies [84, 135, 478, 479].

Our study was subjected to some limitations. Specifically, we could not evaluate the impact of maternal HIV and the concentration of anti-*P. falciparum* antibodies in cord blood on the infant's disease risk. Also, we could not measure

hypergammaglobulinemia, that has been demonstrated to be induced by chronic infections such as HIV, malaria or helminthiasis [348, 480, 481] and has been associated with a reduced transplacental transfer of antibodies [47, 219, 221]. Another limitation is that our results may not be representative of other populations as this study was assessed only in a Mozambican cohort, in which HIV was highly prevalent and the adherence to treatments is lower than in high-income countries. Other conditions such as co-infections with helminths or other pathogens, malnutrition and the high *P. falciparum* transmission could also modulate the placental transfer of antibodies, thus the response could differ between different settings. On the other hand, the low number of PM cases in our study may result in low statistical power to detect significant associations and we had to assess malaria exposure instead of PM for evaluating the effect of malaria during pregnancy in vaccine and non-*P. falciparum* pathogen antigens. In addition, qPCR data were not available from all women and we may have missed some cases of submicroscopic PM, which has been associated with higher inflammation markers and could have an effect on the placental transfer of antibodies [482, 483]. Another limitation is that all HIV-infected mothers also receive CTX and it could potentially limit antigen exposure and therefore antibody responses.

In summary, we have provided evidence that maternal HIV infection and malaria have negative implications in maternal and infant health, with special importance in sub-Saharan Africa where the prevalence and transmission of these pathogens are high. Our results point out that these infections should be further considered during vaccine development and evaluation, in particular with regards to maternal immunization strategies that are key in public health. In future work, we will determine the antibody threshold of protection to assess if the impairment caused by HIV and PM reduces the protection in newborns. To further evaluate the clinical impact of our results, we will evaluate the risk of malaria and other infectious diseases in HIV-exposed uninfected infants and in those born from mother with PM by analyzing the frequency and causes of infant hospitalizations in the following two years after delivery. In upcoming studies, we will also assess the effect of maternal HIV and malaria on infant's total IgG and IgG subclass responses against the antigens included in this doctoral thesis to assess their impact on the development of antibody responses to vaccines and natural exposure. Finally, maternal antibody interference with the acquisition of a protective immune response after infant immunization and naturally acquired immunity to malaria is also of utmost interest and will be studied in subsequent studies.

Conclusions

07

CONCLUSIONS

- Maternal antibody levels were the main determinants for cord blood antibody levels, and the efficacy of IgG subclass transfer was different depending on the antigen and may vary with maternal antigen exposure.

- Maternal HIV infection was associated with reduced cord blood levels and placental transfer, principally IgG1, against a broad range of pathogens, including *P. falciparum,* and vaccine antigens. This may in part explain the higher morbidity of HIV-exposed uninfected infants.

- Malaria exposure was negatively associated with cord blood levels and transfer of antibodies for some non-*P. falciparum* pathogens and vaccine antigens in an antigen-antibody dependent manner.

- Placental malaria was also associated with a reduction in total IgG cord levels to some malaria protection-related antigens, but it had no effect on IgG subclasses. This reduction may increase the risk of infants to malaria infections.

- Prematurity was associated with lower cord blood total IgG levels and placental transfer for some non-*P. falciparum* antigens, although the effect was not consistent among subclasses.

- Low birth weight was associated with increased anti-*P. falciparum* IgG2 cord blood levels, which are associated with higher risk of malaria complications.

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APPENDIX

Supplementary material

Reduced Placental Transfer of Antibodies Against a Wide Range of Microbial and Vaccine Antigens in HIV-Infected Women in Mozambique

Selena Alonso, Marta Vidal, Gemma Ruiz-Olalla, Raquel González, M. Nelia Manaca, Chenjerai Jairoce, Miquel Vázquez-Santiago, Reyes Balcells, Anifa Vala, María Rupérez, Pau Cisteró, Laura Fuente-Soro, Marta Cova, Evelina Angov, Arsenio Nhacolo, Esperança Sevene, John J. Aponte, Eusebio Macete, Ruth Aguilar, Alfredo Mayor, Clara Menéndez, Carlota Dobaño* and Gemma Moncunill*.

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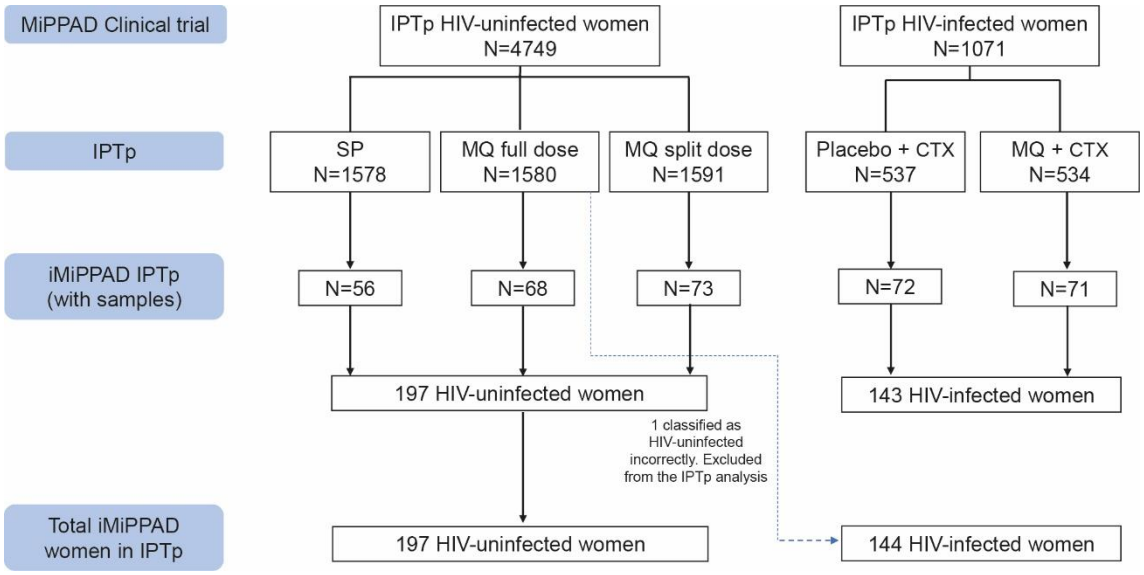
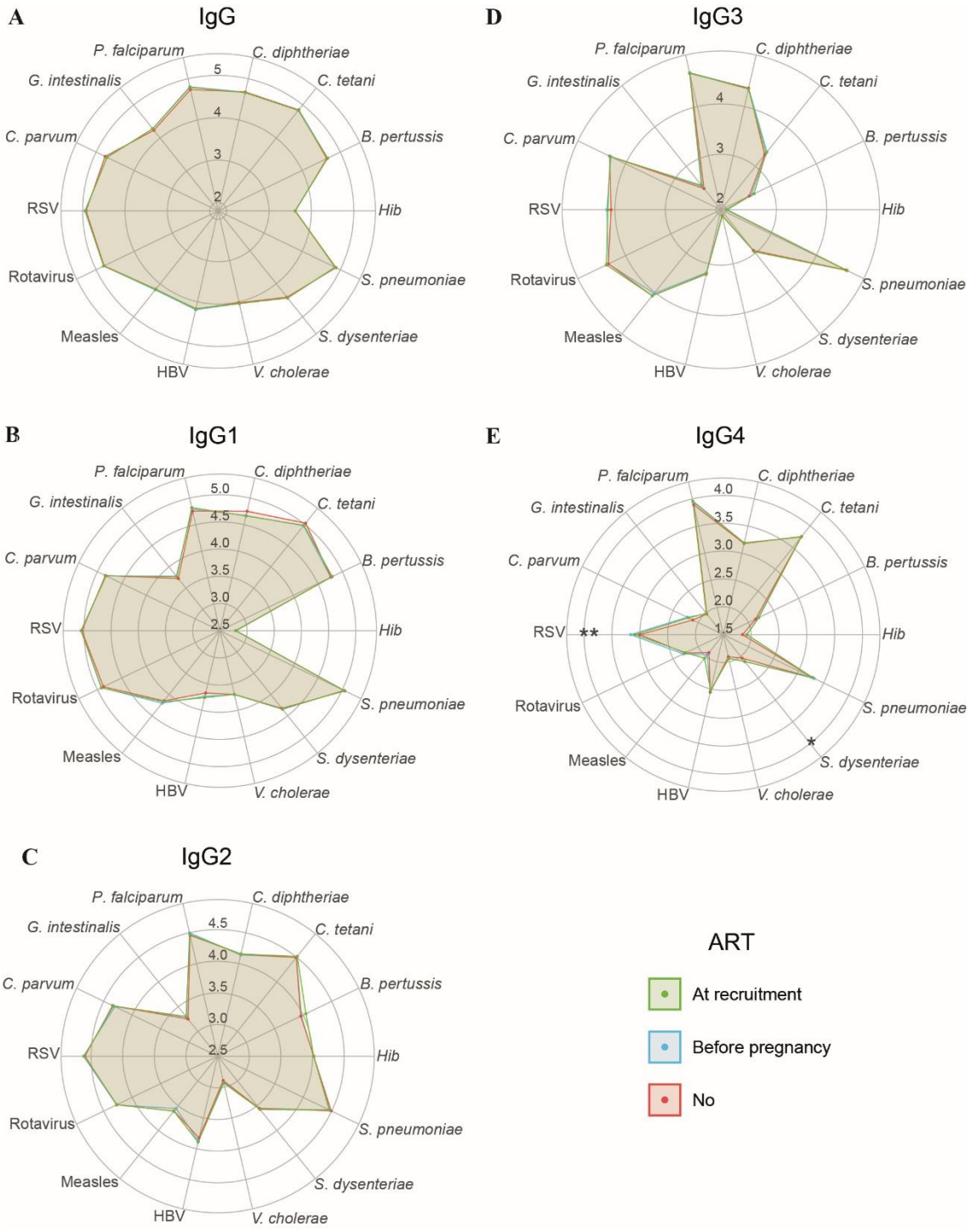
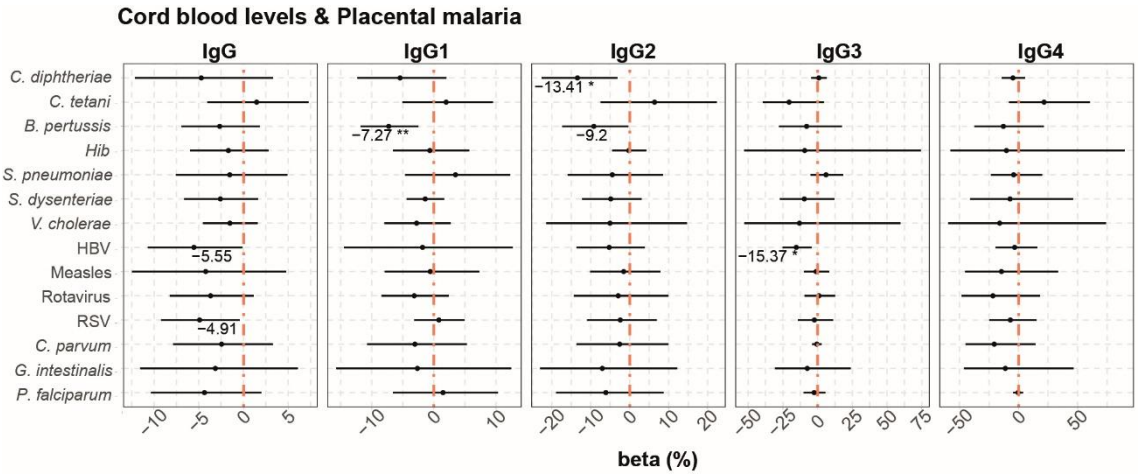
SUPPLEMENTARY FIGURES



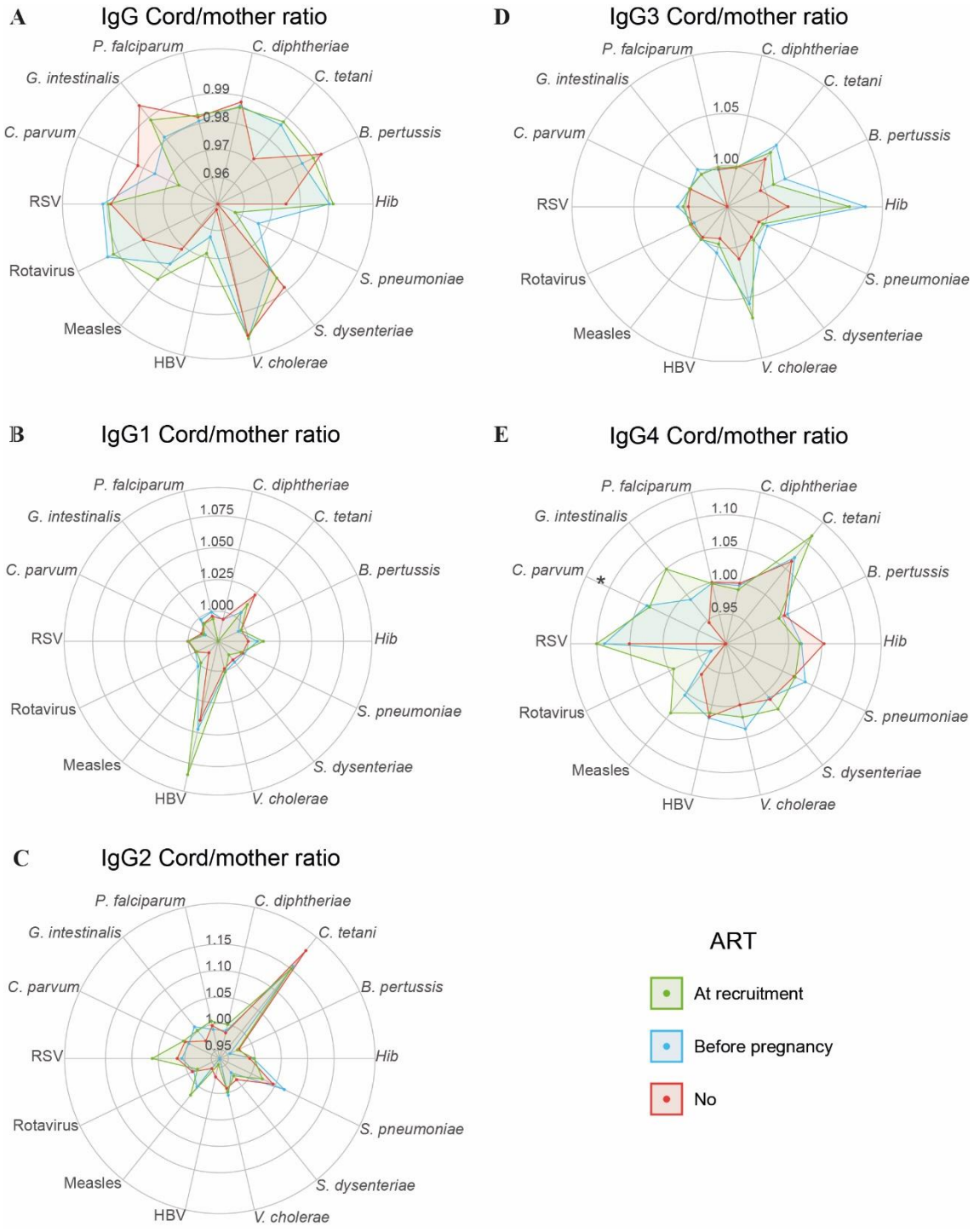
Supplementary Figure 1. IPTp trial profile.



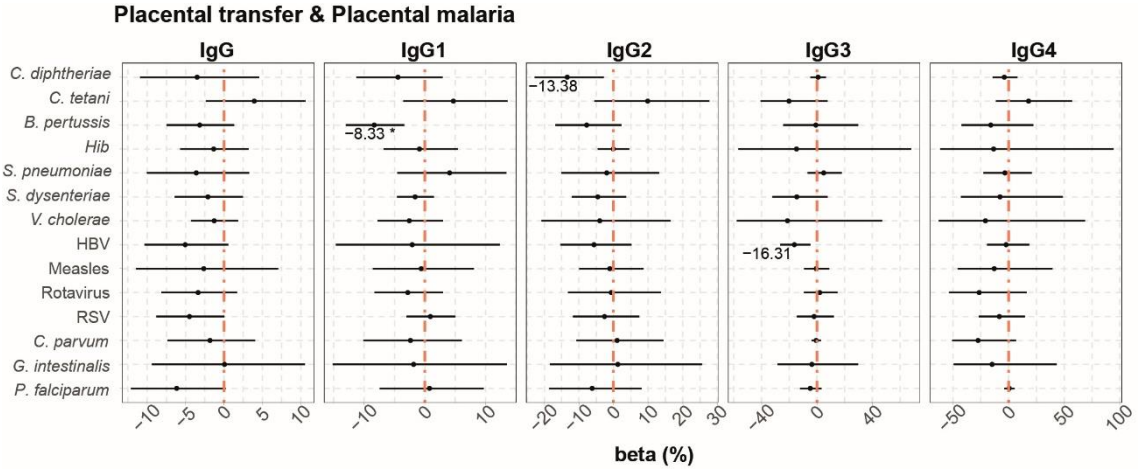
Supplementary Figure 2. Cord blood antibody levels in HIV-positive women taking ART. Radar charts representing the medians of each analyte antibody cord levels in HIV-positive women who started ART before pregnancy, at recruitment or were not taking ART for IgG **(A)**, IgG1 subclass **(B)**, IgG2 **(C)**, IgG3 **(D)**, and IgG4 **(E)**. Cord blood levels were compared by Kruskal-Wallis test and p-values were adjusted for multiple testing by the Benjamini-Hochberg approach (False Discovery Rate 5%). Statistically significant differences between ART before pregnancy, ART at recruitment and no ART are highlighted with an asterisk. $^{**}p < 0.01$, $^{*}p < 0.05$. ART at recruitment is represented in green, ART before pregnancy is represented in blue and no ART is represented in red.



Supplementary Figure 3. Difference of IgG and IgG subclass levels in cord blood levels of HIV-infected women. Forest plots show the effect (in percentage) of placental malaria on cord blood levels of IgG and IgG subclasses, for all the antigens tested, when placental malaria was included in multivariable models with maternal antibody levels. 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 (False Discovery Rate 5%). $^{**}p < 0.01$, $^{*}p < 0.05$.



Supplementary Figure 4. Antibody placental transfer in HIV-positive women taking ART. Radar charts representing the medians of each analyte antibody cord/mother ratio in HIV-positive who started ART before pregnancy, at recruitment or were not taking ART for IgG **(A)**, IgG1 subclass **(B)**, IgG2 **(C)**, IgG3 **(D)**, and IgG4 **(E)**. Ratios were compared by Kruskal-Wallis test and *p*-values were adjusted for multiple testing by the Benjamini-Hochberg approach (False Discovery Rate 5%). Statistically significant differences between ART before pregnancy, ART at recruitment and no ART are highlighted with an asterisk. $^{*}p < 0.05$. ART at recruitment is represented in green, ART before pregnancy is represented in blue and no ART is represented in red.



Supplementary Figure 5. Difference of IgG and IgG subclass placental transfer in HIV-infected women. Forest plots show the effect (in percentage) of placental malaria on transplacental transfer of IgG and IgG subclasses, for all the antigens tested, when placental malaria was included in multivariable models. 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 (False Discovery Rate 5%). $^{*}p < 0.05$.

Supplementary material

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

Selena Alonso, Marta Vidal, Gemma Ruiz-Olalla, Raquel González, Chenjerai Jairoce, M. Nelia Manaca, Miquel Vázquez-Santiago, Reyes Balcells, Anifa Vala, María Ruperez, Pau Cisteró, Laura Fuente-Soro, Evelina Angov, Ross L. Coppel, Benoit Gamain, David Cavanagh, James G. Beeson, Arsenio Nhacolo, Esperança Sevene, John J. Aponte, Eusébio Macete, Ruth Aguilar, Alfredo Mayor, Clara Menéndez, Carlota Dobaño*, Gemma Moncunill*.

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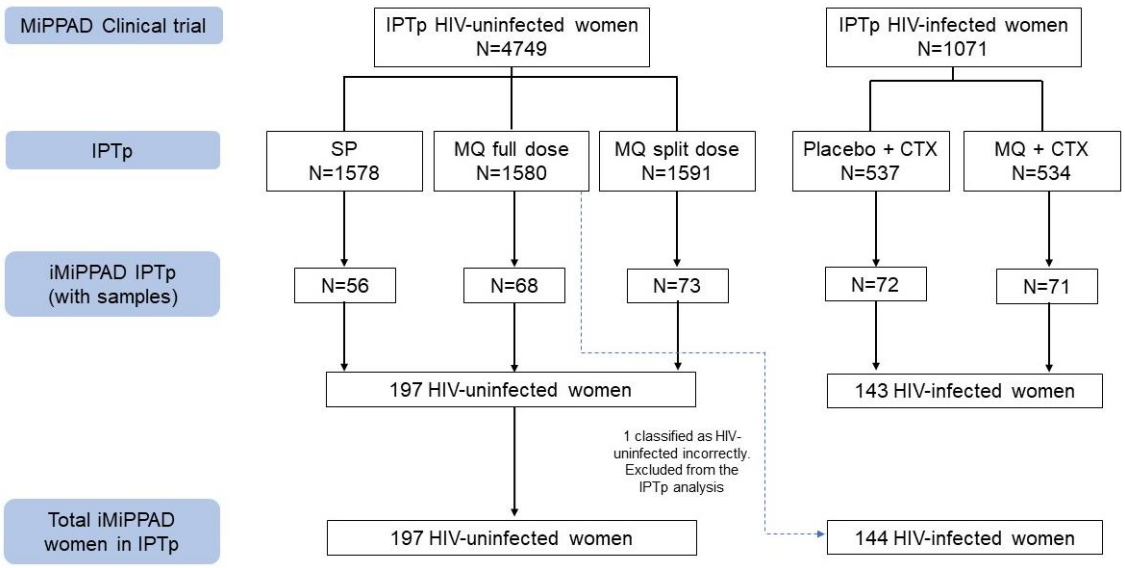
SUPPLEMENTARY MATERIALS AND METHODS

Statistical analysis. Data pre-processing.

To select the sample dilution for each antigen-isotype/subclass-plate, the dilution nearest to the midpoint between the two positive control curve serial dilutions ranging the maximum slope was chosen. If the MFI of the first sample dilution was lower than the MFI of the second dilution, the second one was chosen. Plates were normalized using the positive control curve in each plate and the average positive control curve from all plates. The MFI values of samples were multiplied by the corresponding normalization factor (MFI value of the chosen dilution from the average positive control curve divided by the MFI value of same dilution in the plate positive control curve).

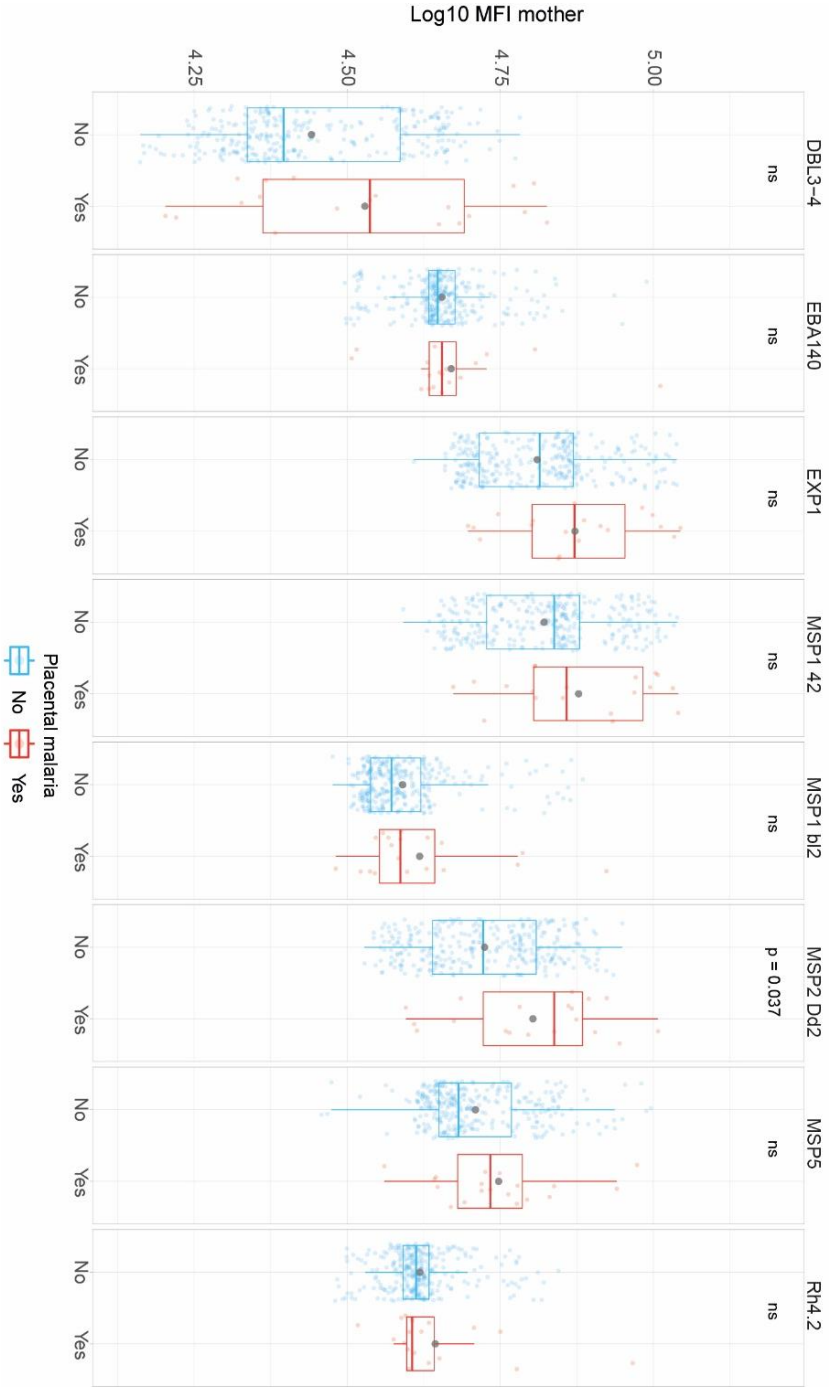
SUPPLEMENTARY FIGURES

Supplementary Fig 1. IPTp trial profile.

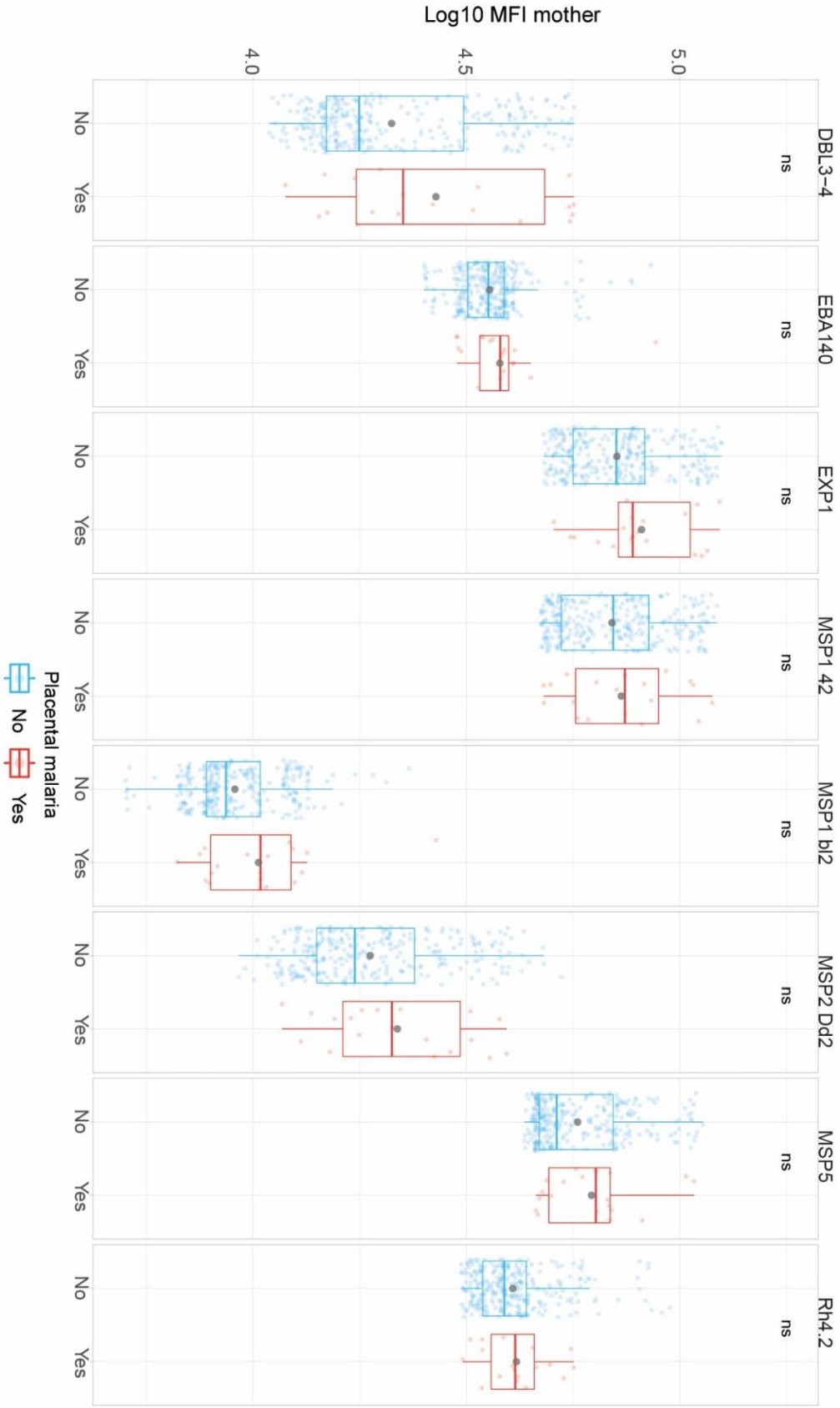


Supplementary Fig 2. Maternal blood antibody levels (log_{10} MFI) in women with placental malaria (PM) and women without PM. Boxplots illustrate the medians, the interquartile range (IQR), the outlier points that are further 1.5*IQR and black dots show the arithmetic means for IgG (a), IgG1 (b), IgG2 (c), IgG3 (d) and IgG4 (e) subclasses. Levels between women with PM and women without PM were compared by Wilcoxon test and p-values were adjusted for multiple testing by the Benjamini-Hochberg approach. ns = not significant. Red represents women with PM and blue women without PM.

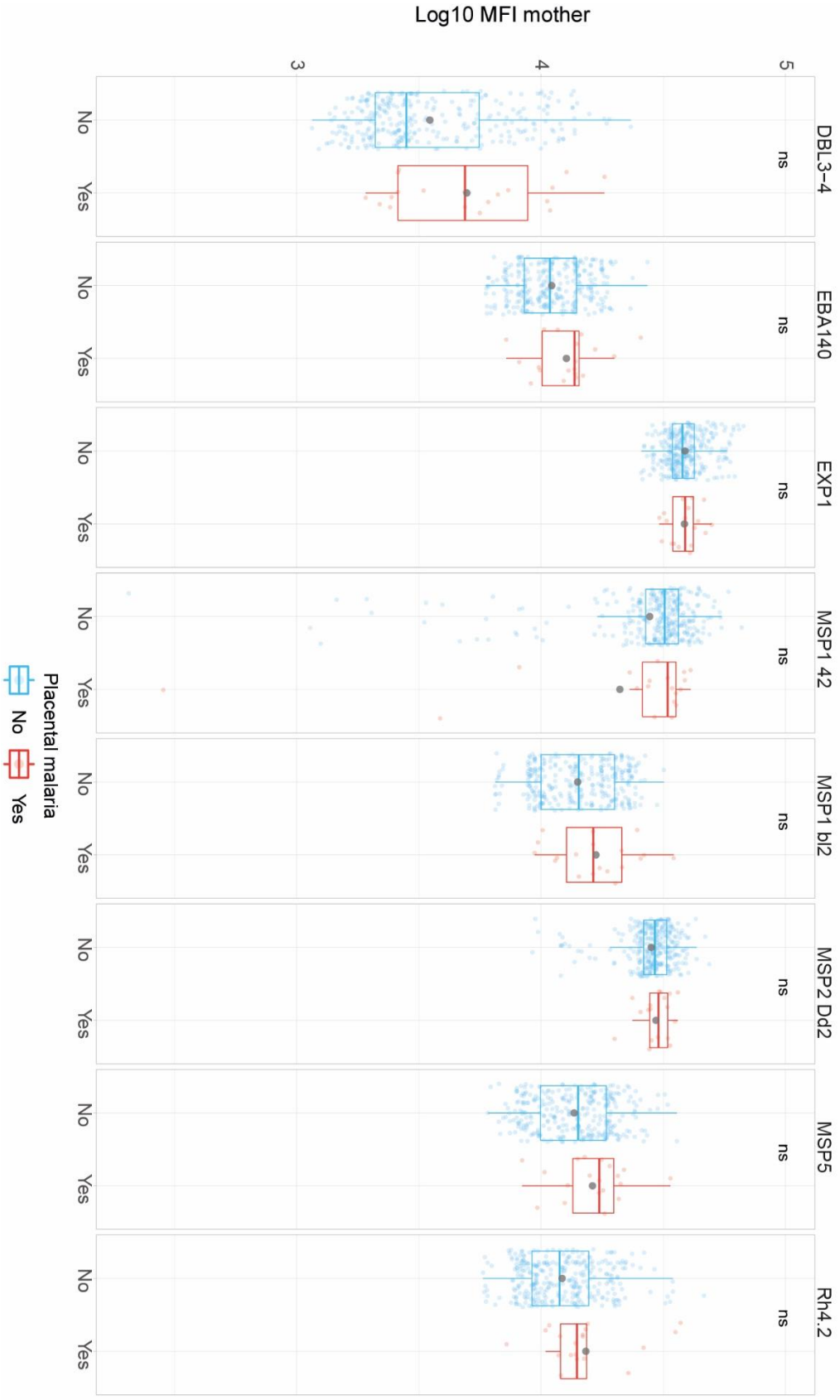
a) Maternal blood IgG



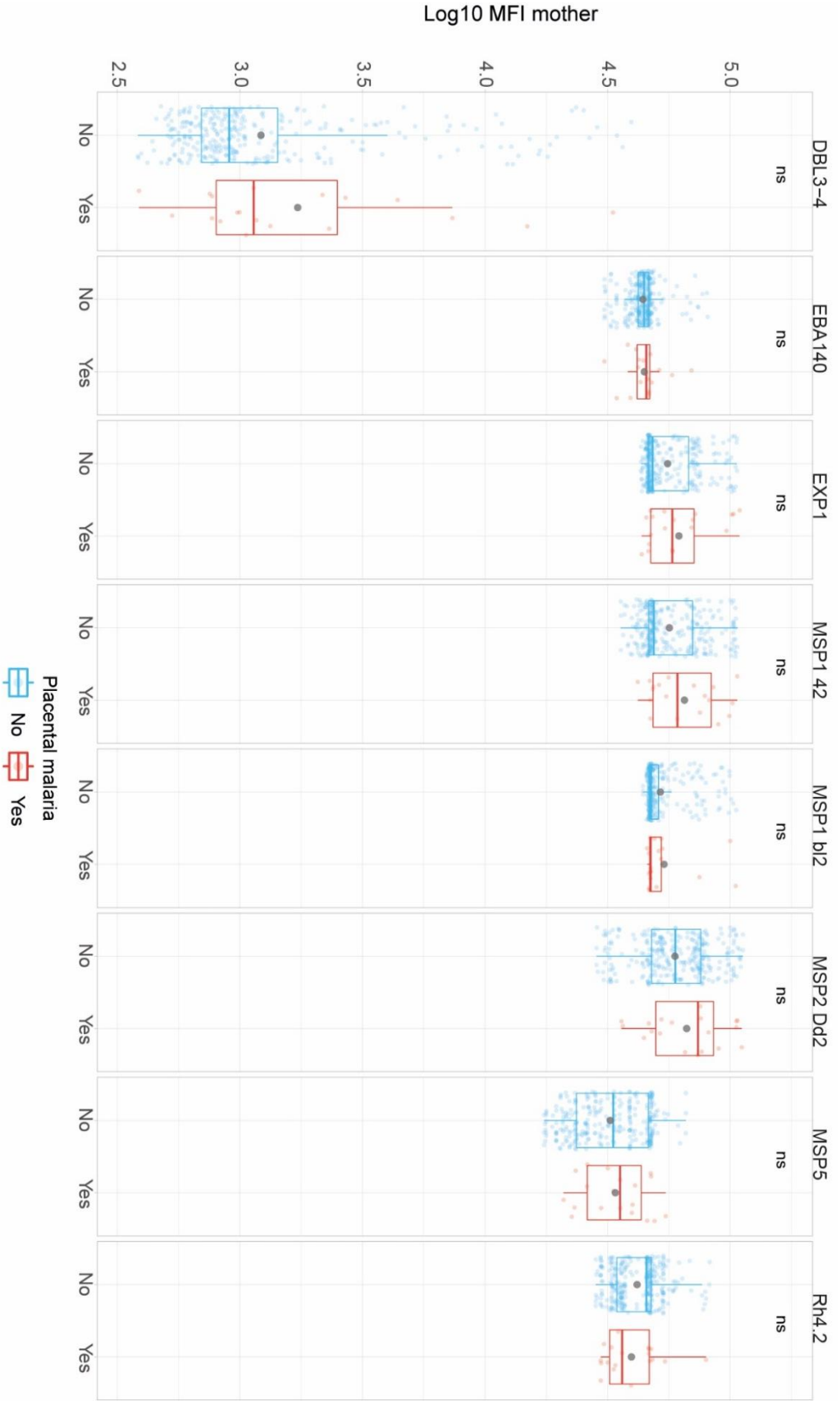
b) Maternal blood IgG1



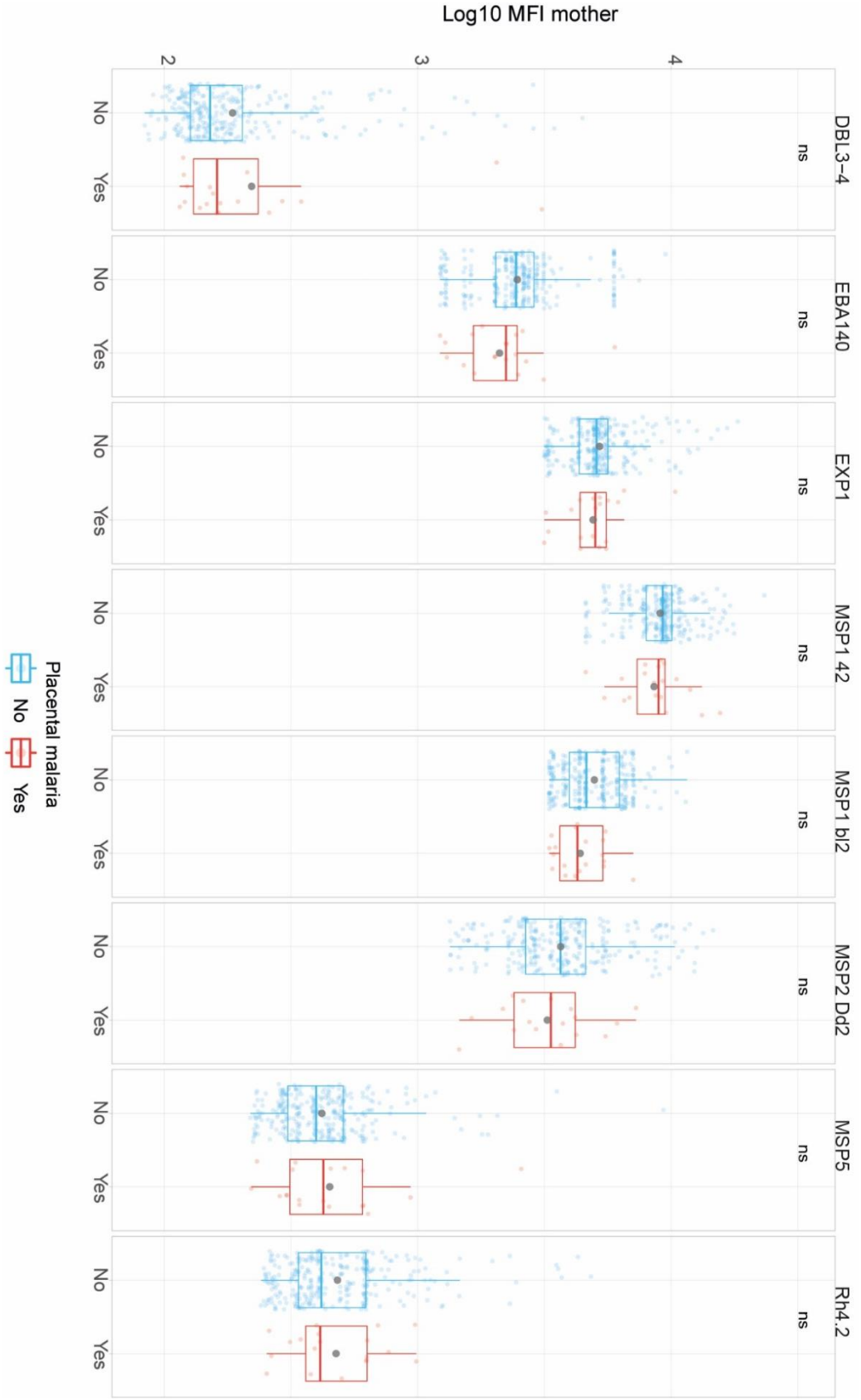
c) Maternal blood IgG2



d) Maternal blood IgG3

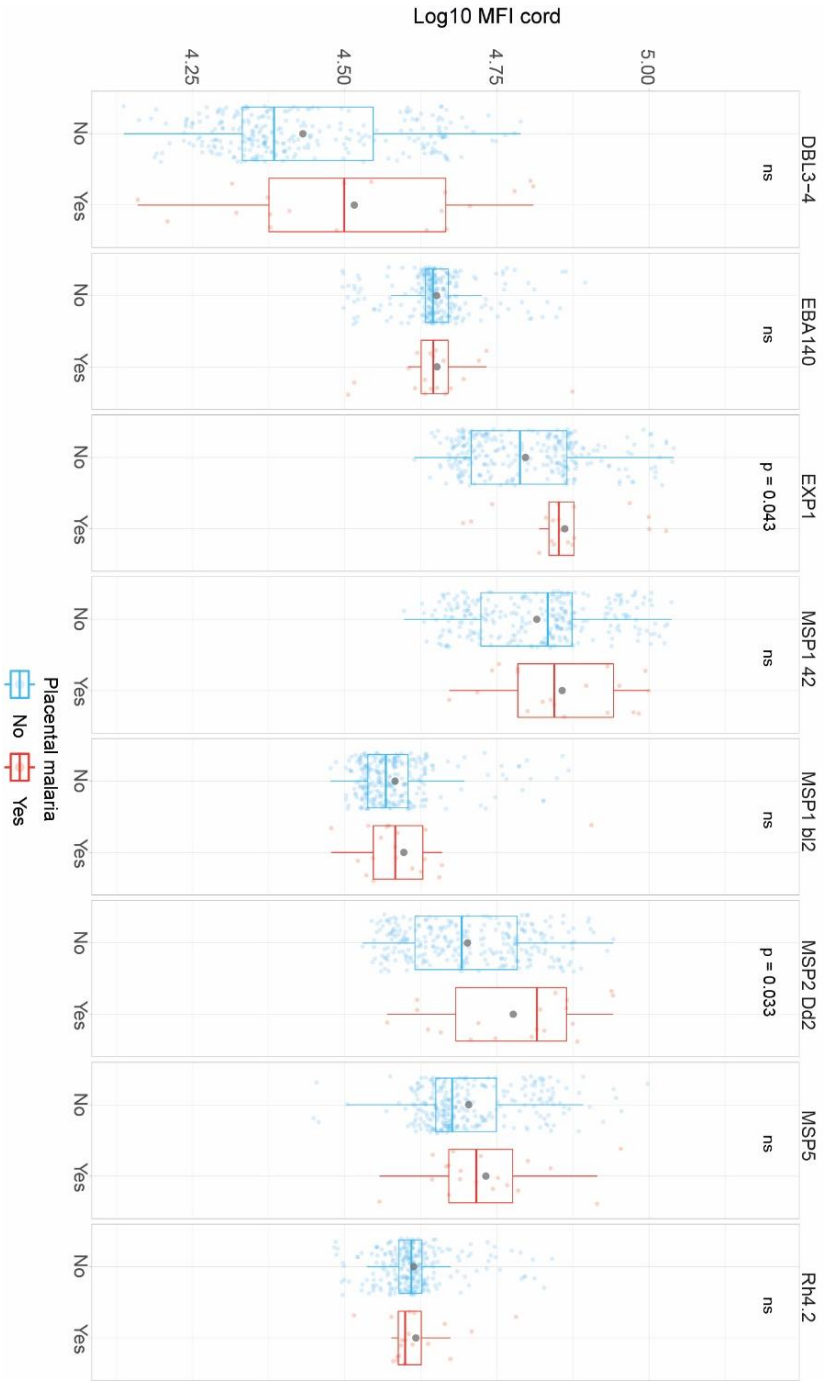


e) Maternal blood IgG4

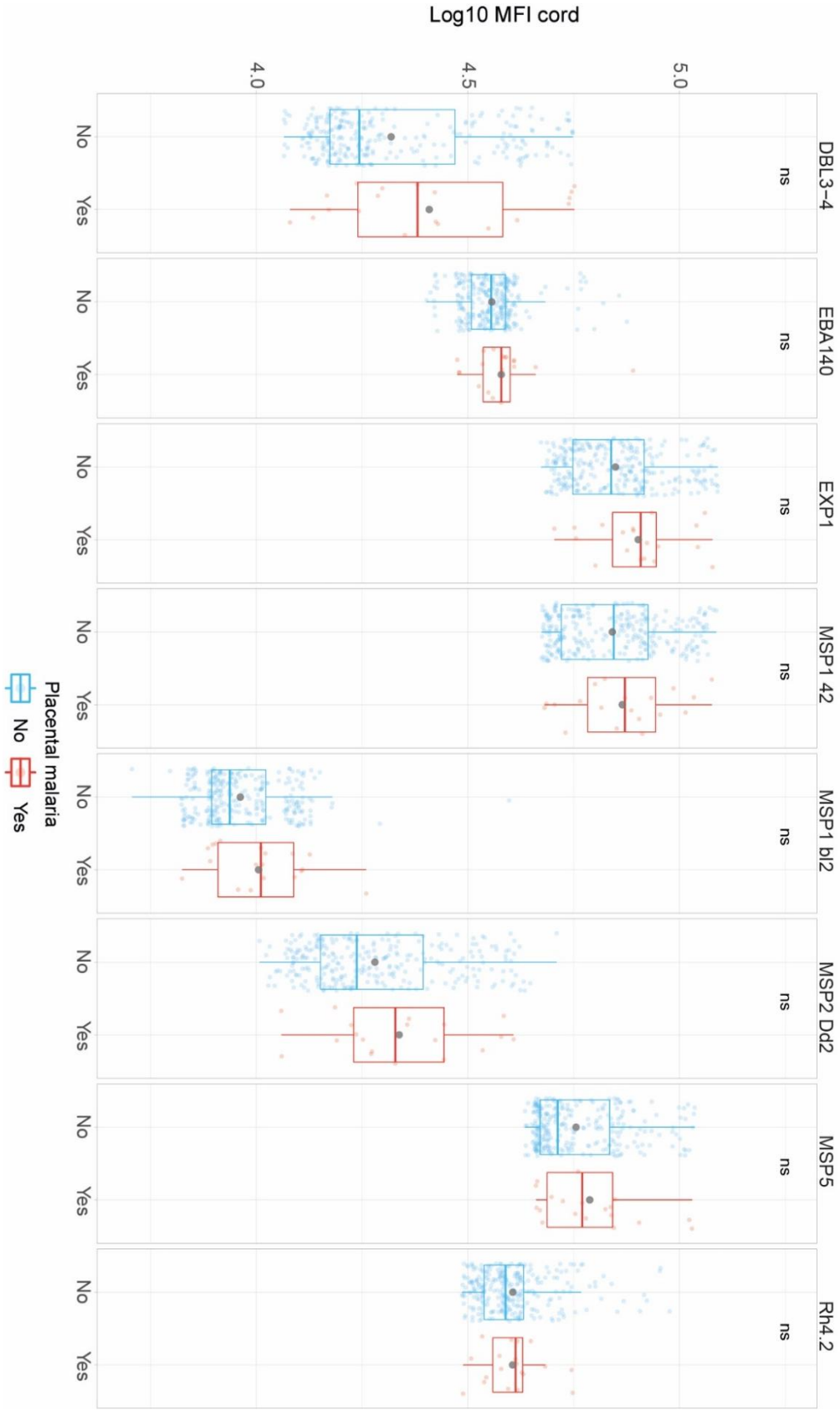


Supplementary Fig 3. Cord blood antibody levels (log_{10} MFI) in women with placental malaria (PM) and women without PM. Boxplots illustrate the medians, the interquartile range (IQR), the outlier points that are further 1.5*IQR and black dots show the arithmetic means for IgG (a), IgG1 (b), IgG2 (c), IgG3 (d) and IgG4 (e) subclasses. Levels between women with PM and women without PM were compared by Wilcoxon test and p-values were adjusted for multiple testing by the Benjamini-Hochberg approach. ns = not significant. Red represents women with PM and blue women without PM.

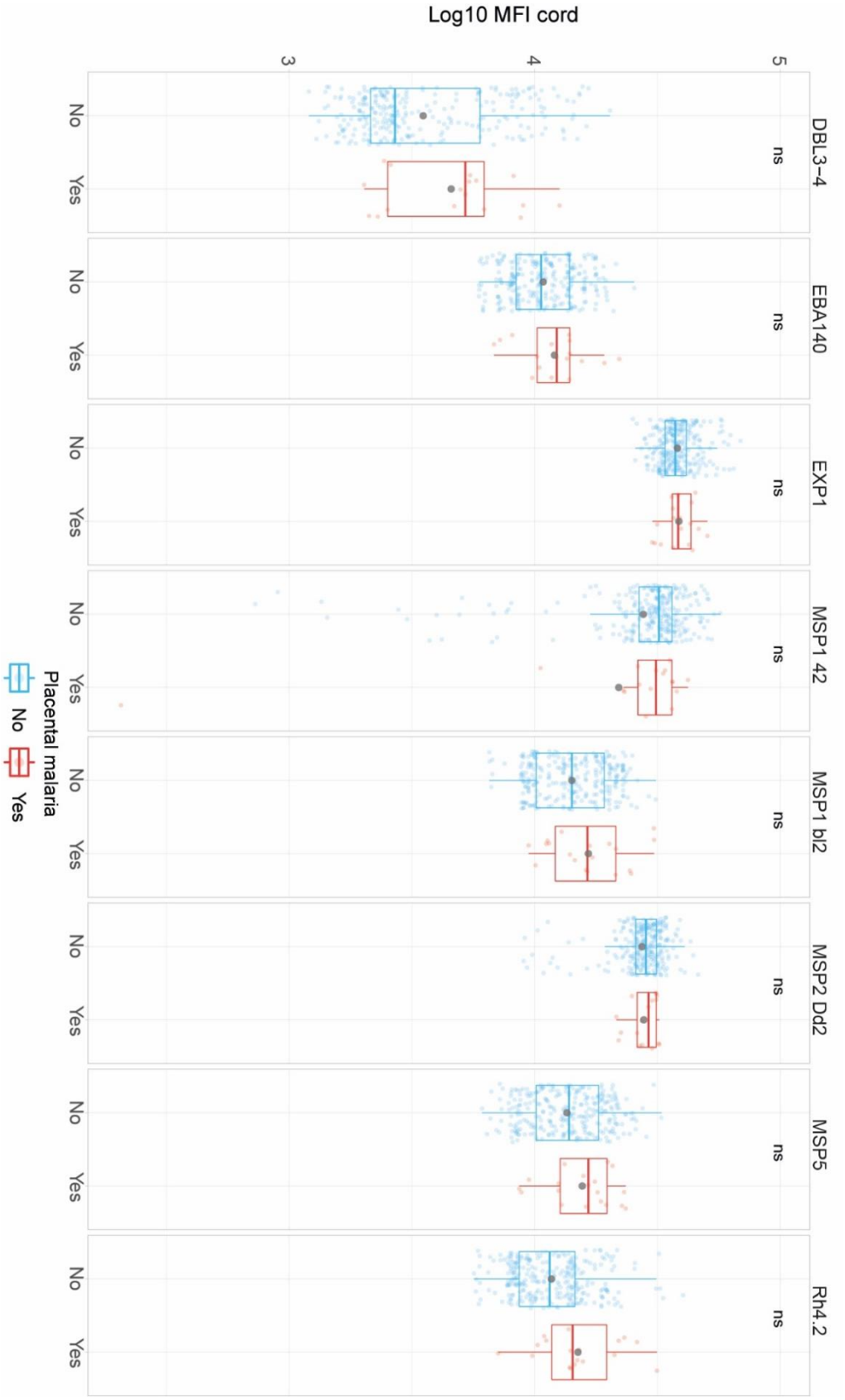
a) Cord blood IgG



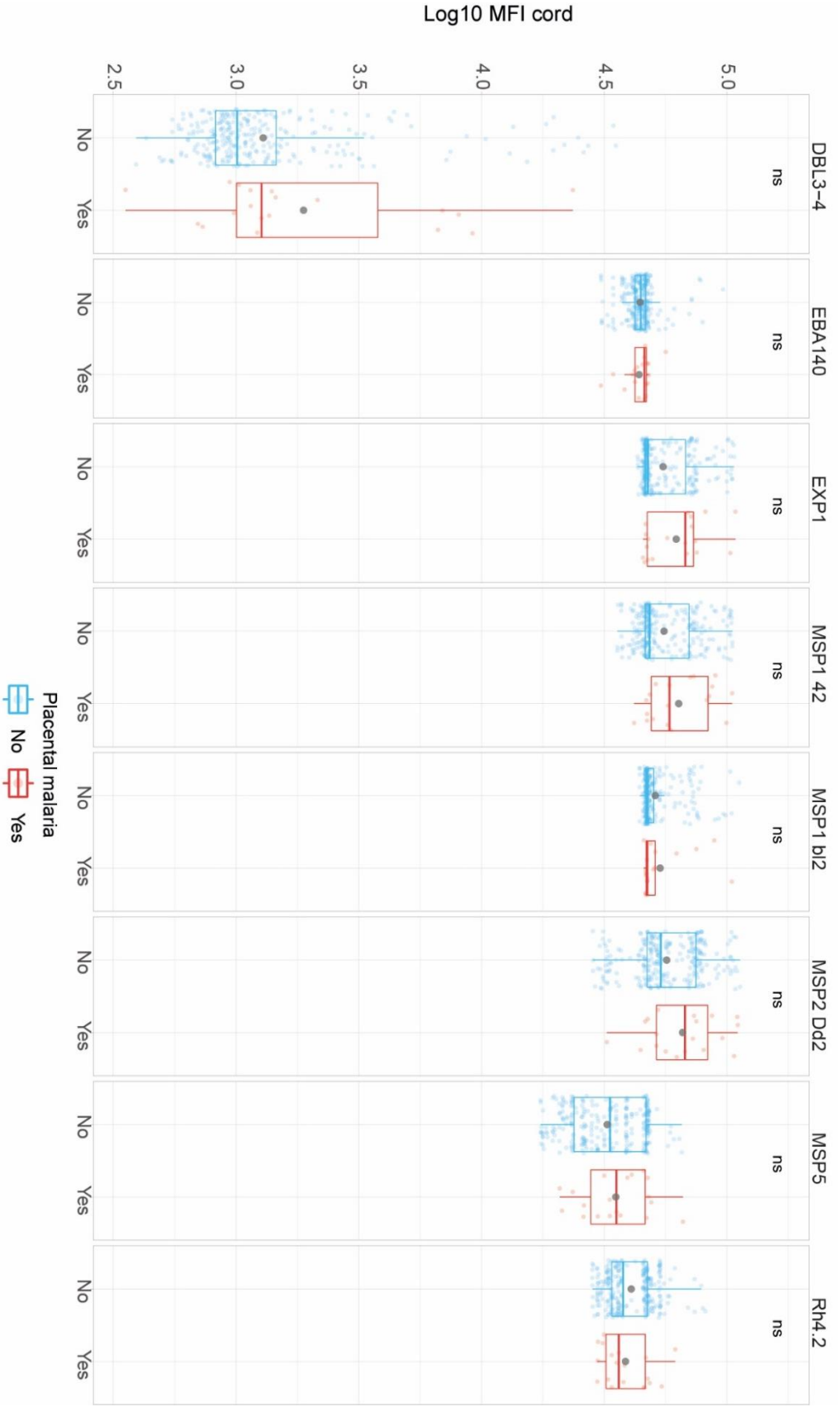
b) Cord blood IgG1



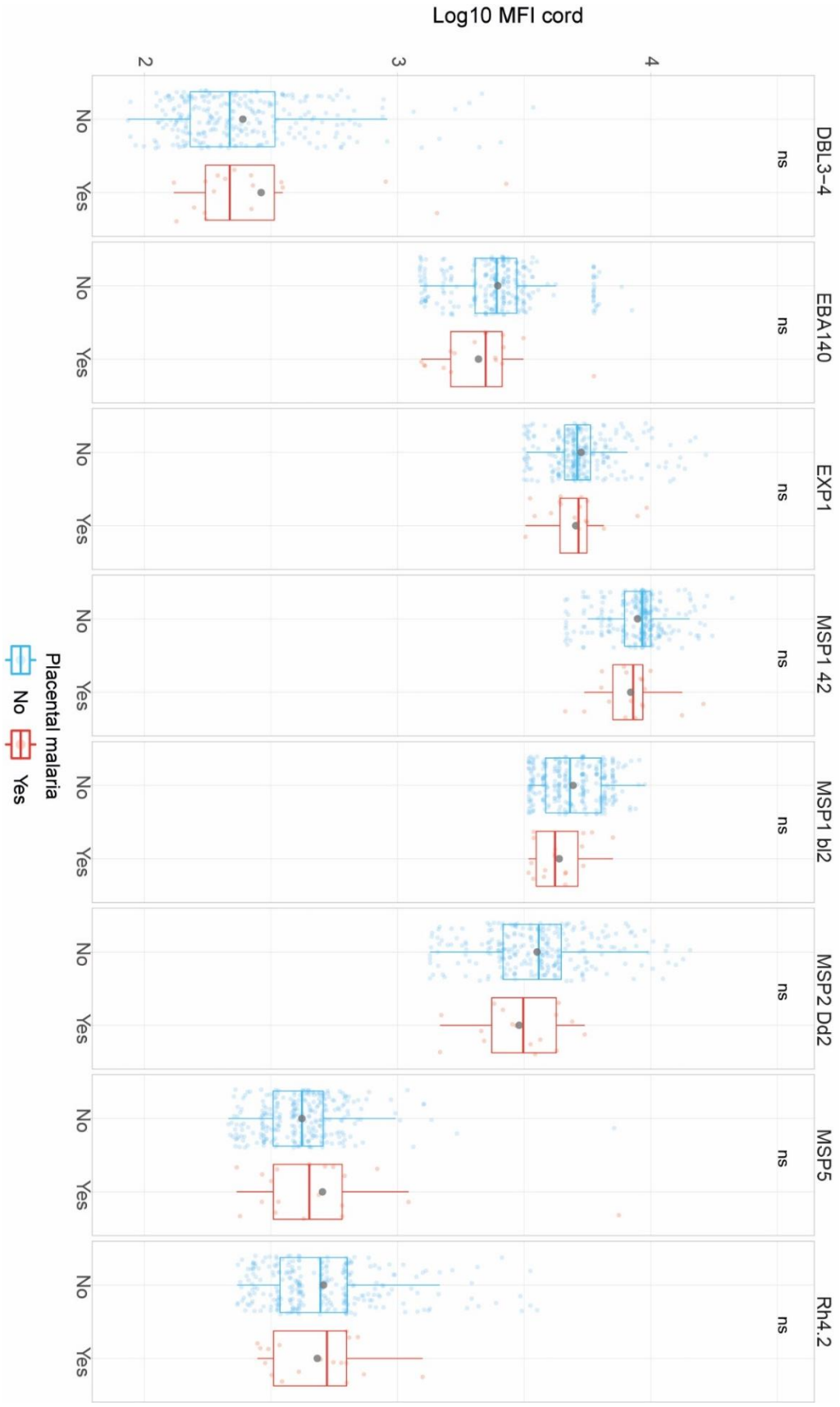
c) Cord blood IgG2



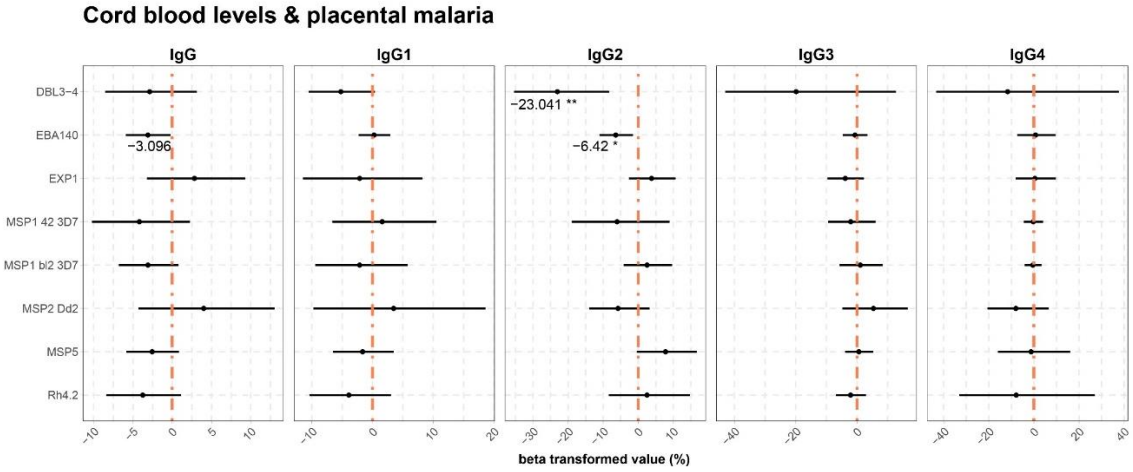
d) Cord blood IgG3



e) Cord blood IgG4

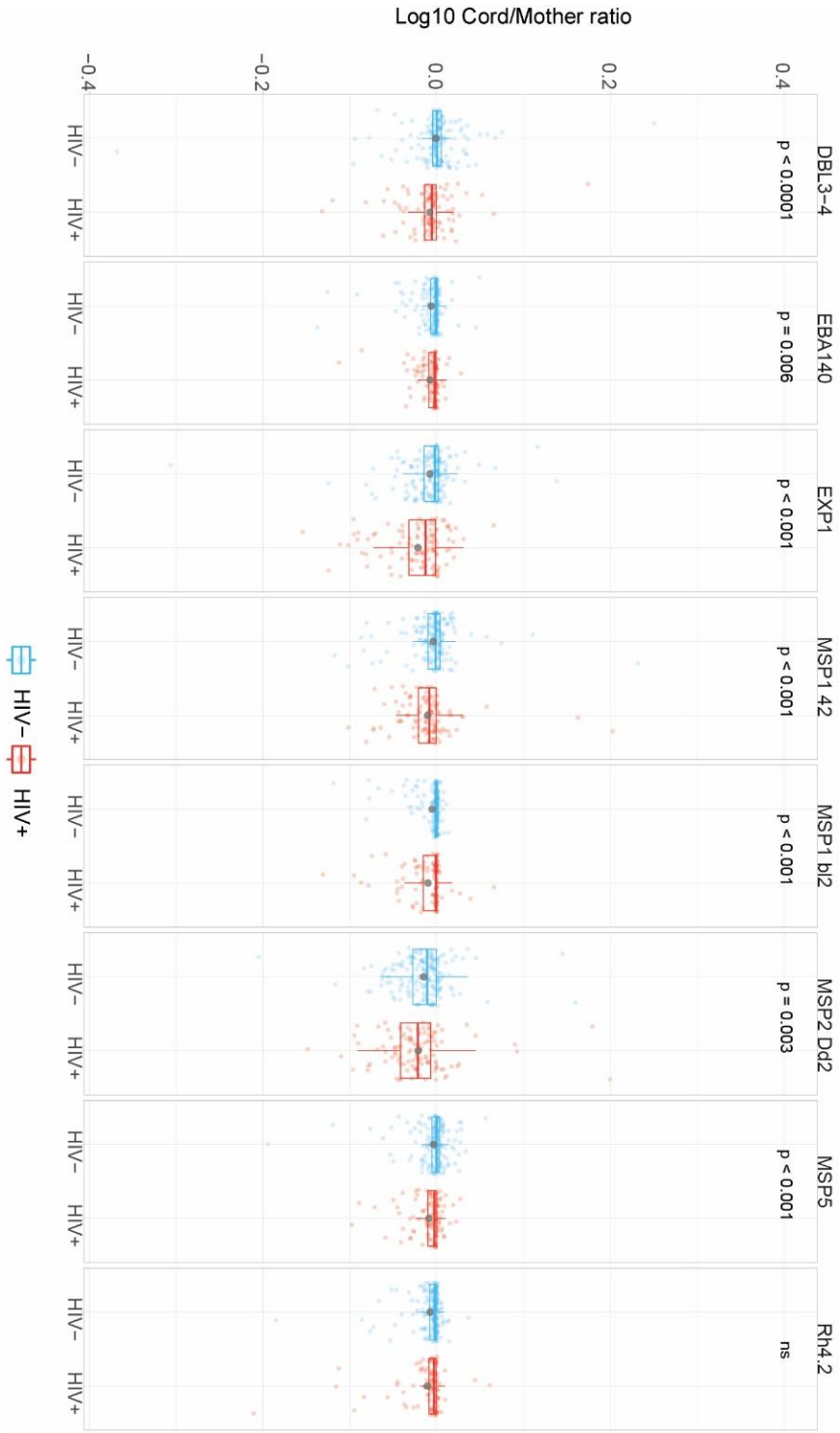


Supplementary Fig 4: Difference of IgG and IgG subclass levels in cord blood by placental malaria in HIV+ women. Forest plots show the effect (in percentage) of placental malaria on cord blood levels of IgG and IgG subclasses for all the antigens tested. The differences in percentage correspond to beta 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 ‹ 0.001, ** = p-value ‹ 0.01, * = p-value ‹ 0.05.

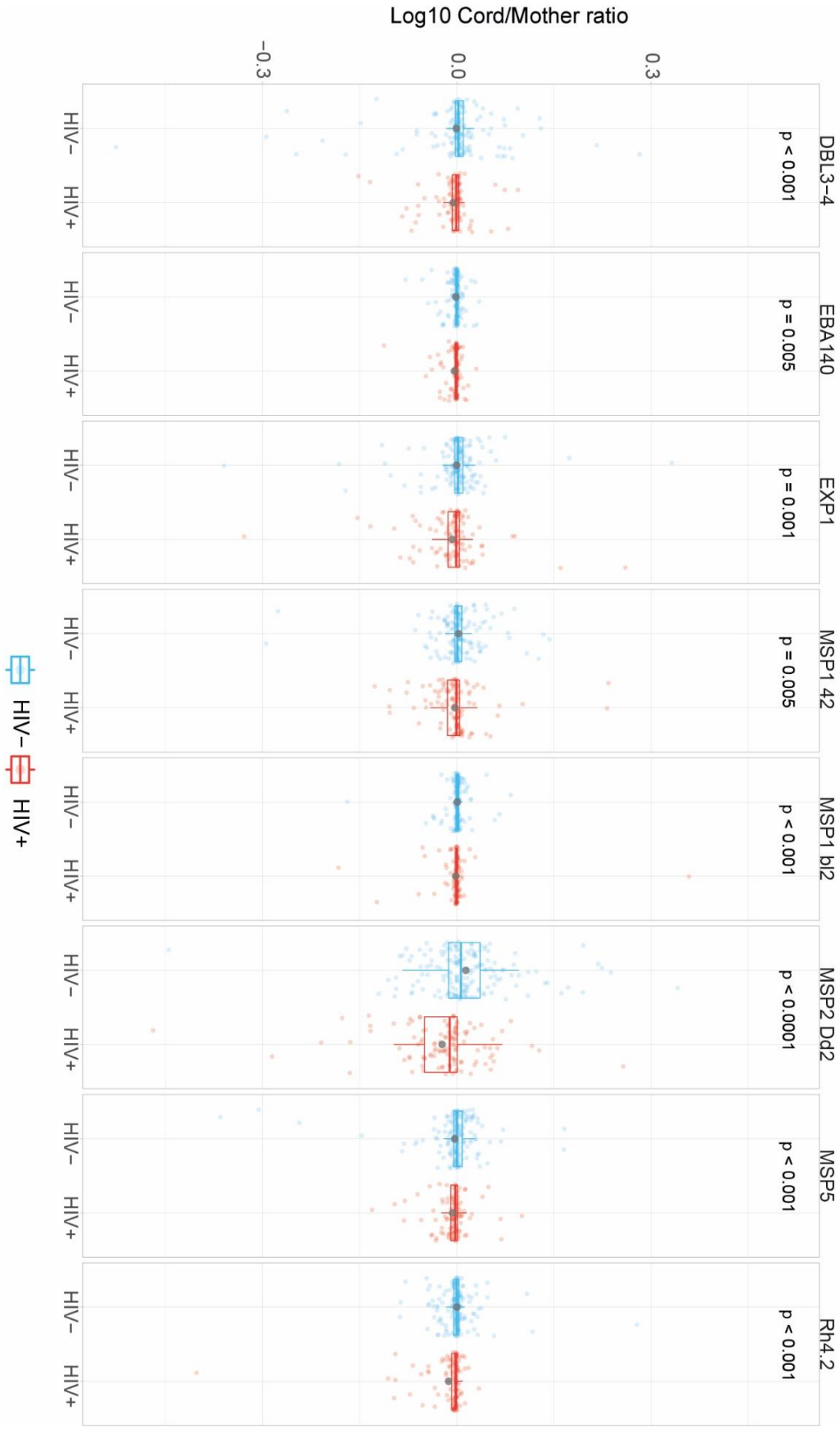


Supplementary Fig 5. Cord/mother log_{10} antibody ratios in HIV-positive and HIV-negative women. Boxplots illustrate the medians, the interquartile range (IQR), the outlier points that are further 1.5*IQR and black dots show the arithmetic means for IgG (a), IgG1 (b), IgG2 (c), IgG3 (d) and IgG4 (e) subclasses. Levels between HIV-positive and negative women were compared by Wilcoxon test and p-values were adjusted for multiple testing by the Benjamini-Hochberg approach. ns = not significant. Red represents HIV-positive women and blue HIV-negative women.

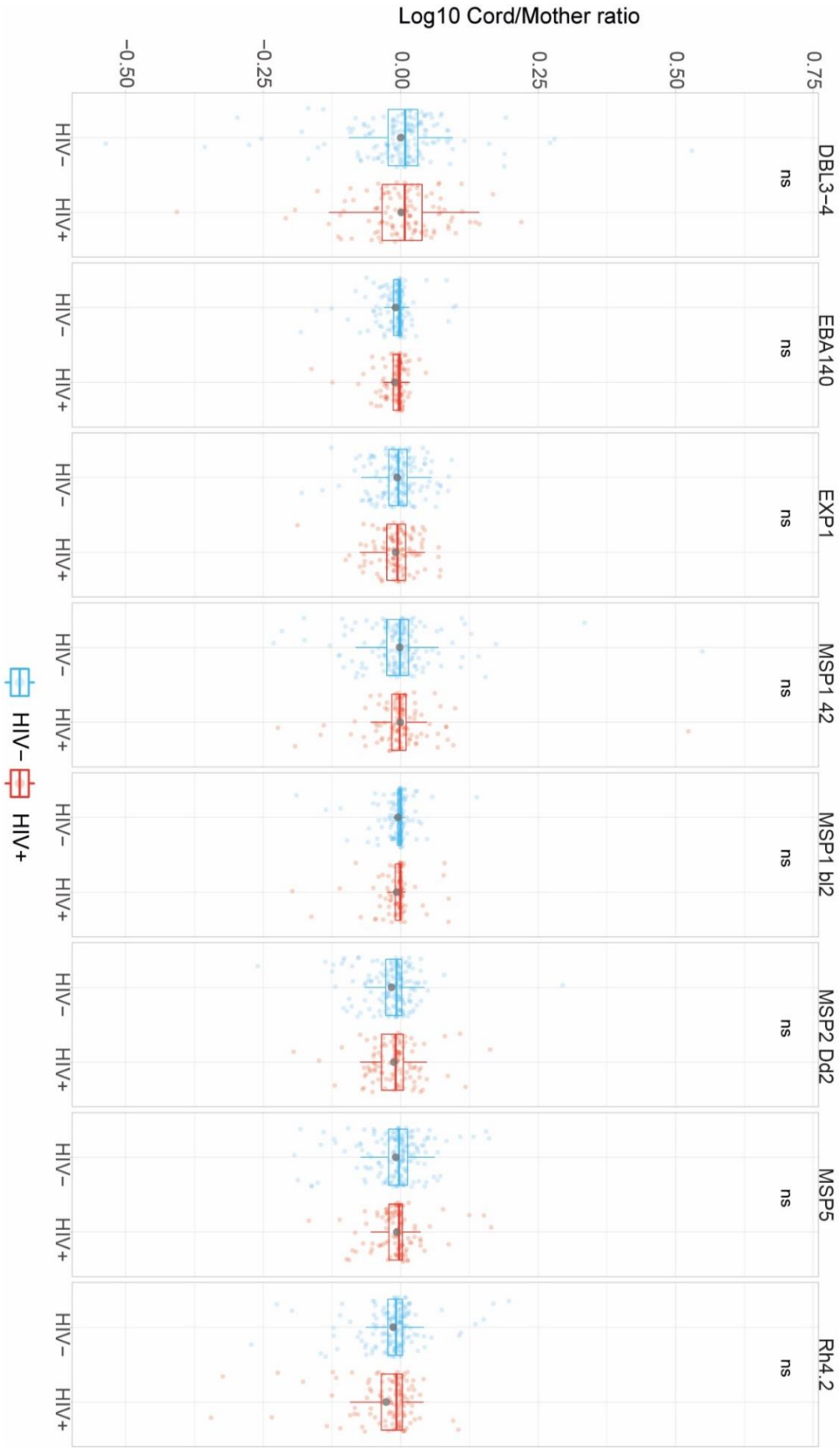
a) IgG ratio



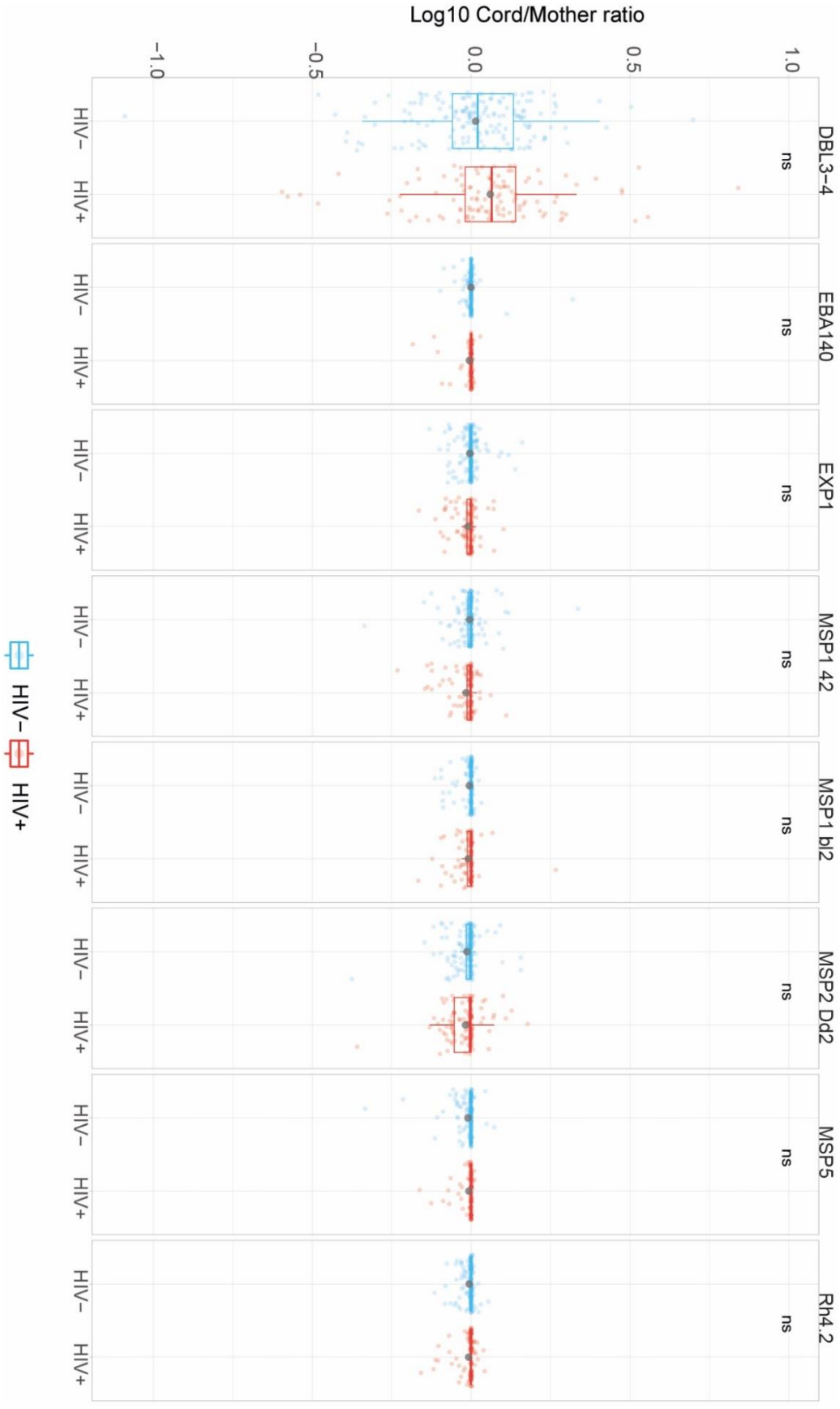
b) IgG1 ratio


Log10 Cord/Mother ratio
−0.3
0.0
0.3
DBL3−4
EBA140
EXP1
MSP1 42
MSP1 bl2
MSP2 Dd2
MSP5
Rh4.2
p < 0.001
p = 0.005
p = 0.001
p = 0.005
p < 0.001
p < 0.0001
p < 0.001
p < 0.001
HIV−
HIV+

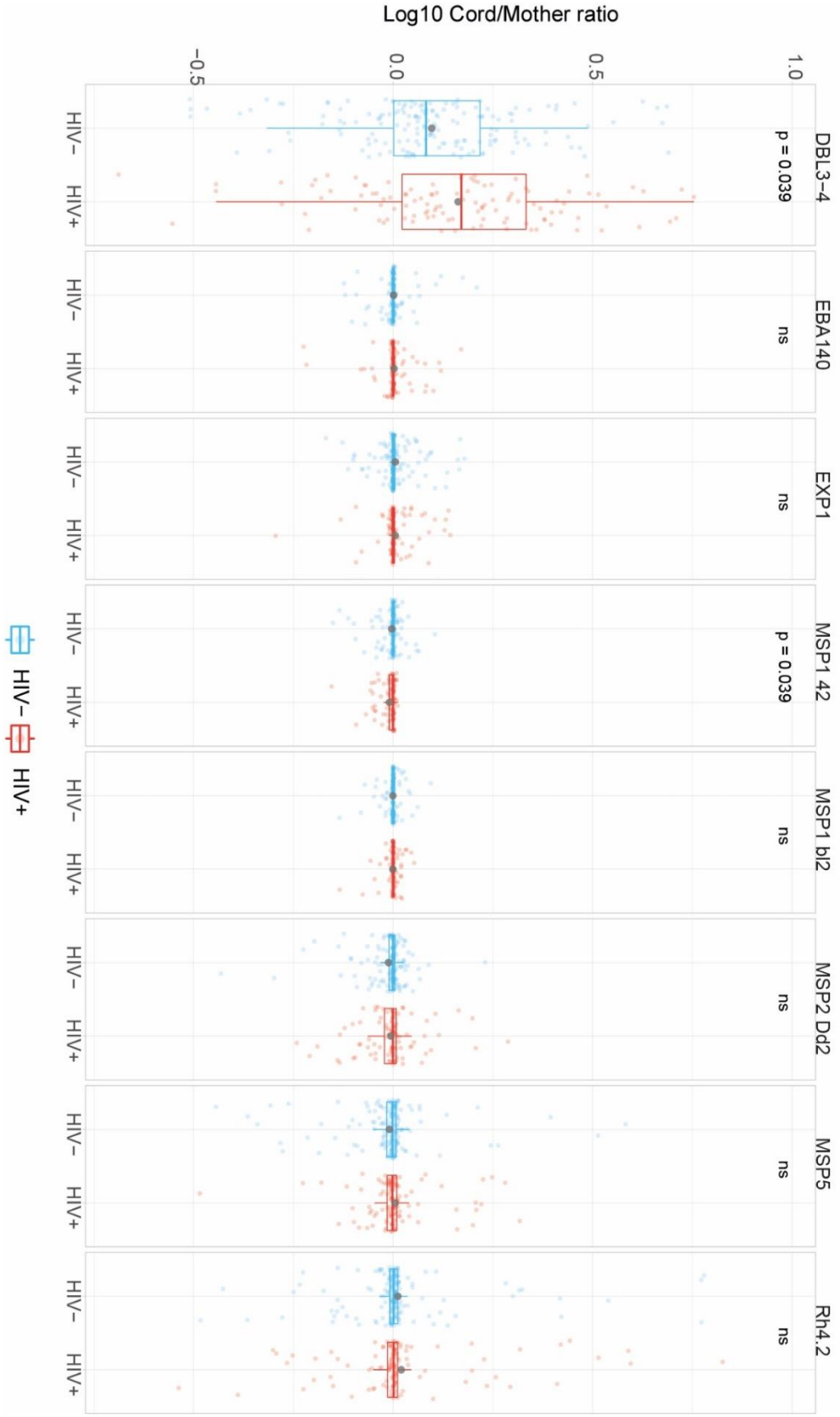
c) IgG2 ratio


Log10 Cord/Mother ratio
−0.50
−0.25
0.00
0.25
0.50
0.75
DBL3−4
EBA140
EXP1
MSP1 42
MSP1 bl2
MSP2 Dd2
MSP5
Rh4.2
ns
HIV−
HIV+

d) IgG3 ratio


Log10 Cord/Mother ratio
−1.0
−0.5
0.0
0.5
1.0
DBL3−4
EBA140
EXP1
MSP1 42
MSP1 bl2
MSP2 Dd2
MSP5
Rh4.2
ns
HIV−
HIV+

e) IgG4 ratio


Log10 Cord/Mother ratio
−0.5
0.0
0.5
1.0
DBL3−4
p = 0.039
EBA140
ns
EXP1
ns
MSP1 42
p = 0.039
MSP1 b12
ns
MSP2 Dd2
ns
MSP5
ns
Rh4.2
ns
HIV−
HIV+
HIV−
HIV+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