Fakultät für Medizin
Institut für Medizinische Mikrobiologie, Immunologie und Hygiene

Exploring the impact of environment and infection on fetomaternal immunity

-

A comparative study between Germany and Gabon to assess immunological differences in placental gene expression and T cell responses in fetomaternal dyads

Esther Susanne Ludwig

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades einer Doktorin der Medizinischen Wissenschaft (Dr. med. sci.) genehmigten Dissertation.

Vorsitz: Prof. Dr. Klaus-Peter Janssen
Prüferinnen der Dissertation:
1. Prof. Dr. med. Clarissa Prazeres da Costa
2. Priv.-Doz. Dr. Silvia Lobmaier
3. Prof. Dr. Christine Josenhans
Die Dissertation wurde am 17.03.2021 bei der Technischen UniversitätMünchen eingereicht und durch die Fakultät für Medizin am 11.08.2021 angenommen.

Declaration

I solemnly declare that I have written the dissertation entitled

“Exploring the impact of environment and infection on fetomaternal immunity - A comparative study between Germany and Gabon to assess immunological differences in placental gene expression and T cell responses in fetomaternal dyads”

and submitted it to the Faculty of Medicine of the Technical University of Munich for doctoral examination at the Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Trogerstr. 30, 81675 München, under the guidance and supervision of Prof. Dr. med Clarissa Prazeres da Costa without other help and, while writing it, only used aids in accordance with the academic and examination regulations of the Translational Medicine Program.

[x] I have not involved any organization that offers doctoral advisors for a fee or partially or entirely fulfils examination obligations required of me on my behalf.

[x] I have submitted the dissertation in this or a similar form in no other examination procedure as an examination achievement.

[ ] The complete dissertation was published in _____. The Faculty of Medicine has approved the advance publication.

[x] Parts of this thesis have already been published in the Scientific Reports with the title “Placental gene expression and antibody levels of mother-neonate pairs reveal an enhanced risk for inflammation in a helminth endemic country” in October 2019.
Esther Ludwig, Jutta Harder, Matthew Lacorcia, Yabo Josiane Honkpehedji, Odilon Paterne Nouatin, Govert J. van Dam, Paul L. A. M. Corstjens, Erliyani Sartono, Meral Esen, Silvia M. Lobmaier, Ayola Akim Adegnika & Clarissa Prazeres da Costa

[x] I have not yet acquired the Doctor of Medical Science and I have not definitely failed in a previous doctoral procedure for the Doctor of Medical Science.

[ ] I have already submitted a dissertation with the subject _____at the Faculty of _____ the university _____ on _____ to apply for admission to the doctoral examination with the result: _____

I am familiar with the academic and examination regulations of the Translational Science Program of the Technical University Munich.

Münster, 13.01.2021 _____ _____

Table of Contents

Figures

Tables

Abbreviations

A

AWA	anti-adult worm antigen

B

BF	buffer

C

CAA	circulating anodic antigen
Ca	Calcium
cDNA	copy DNA
CERMEL	Centre de Recherches Médicales de Lambaréné
CMBC	Cord-blood Mononuclear Cells
CYP2R1	Vitamin D-25-hydroxylase
Cyp24a1	24-Hydroxylase
Cyp27b1	1-alpha-hydroxylase
CRP	C reactive protein

D

DBP	Vitamin D Binding Protein
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphate

E

EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia*/ for example
ELISA	Enzyme-linked immunosorbent assay

F

FACS	fluorescence-activated cell sorting
FcRn	neonatal Fc receptor
FCS	fetal calf serum
Foxp3	forkhead box P3

H

hCG	human chorionic gonadotropin
HKG	house-keeping gene
hPL	human prolactin
Hsd3b1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
H2O	water

I

IFN	interferon
IL	interleukin
Ig	immunoglobulin
IQR	interquartile range

L

LPS	lipopolysaccharide

M

MRI	München, Klinikum rechts der Isar

N

NK cells	natural killer cells

P

P4	progesterone
PAS	periodic acid-Schiff
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PCR	polymerase chain reaction
P/I	PMA plus ionomycin
Phosphorus	P

Q

qRT-PCR	quantitative real-time PCR

R

RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute medium
RXR	retinoid X receptor

S

S.	Schistosoma
S. mansoni	Schistosoma mansoni
S. haematobium	Schistosoma haematobium
SD	standard deviation
S/N	Supernatant
Spp.	species

T

Treg	regulatory T cells
Th1	T helper cell 1
Th2	T helper cell 2
Th17	T helper cell 17

U

uNK	uterine natural killer cells

V

VDR	vitamin D receptor
VDRE	vitamin D response elements
VitD	vitamin D

vs.	versus

Others

$1,25(OH)_2D$	1,25-Dihydroxyergocalciferol/Calcitriol
25(OH)D	25-Hydroxycholecalciferol
3βHSD	3β-hydroxysteroid dehydrogenase/isomerase

Abstract

In recent years it has become clear that *in utero* exposures to environmental factors, including toxic and infectious disease agents, can modify the propensity to develop allergies and possibly other immune disorders later in life. The underlying mechanisms of this fetomaternal crosstalk still remain unclear and demand more investigation. First murine studies of our group revealed that inflammatory maternal signals elicited by chronic infection within the placenta imprint a distinct gene expression profile related to the Vitamin-D-receptor (VDR)-inflammation-related axis. Thus, the central hypothesis is that pro- or anti-inflammatory immune responses elicited by environmental factors or maternal infection influence placental function and the regulation of vitamin D metabolism.

Aims and Methods: The main aim of this study and thesis was to answer the question whether VDR and vitamin-D-related gene expression, and particularly pro- and anti-inflammatory immune responses within the fetomaternal interface, differ between women from helminth-endemic and non-endemic areas, and between *Schistosoma haematobium* infected and uninfected pregnant women at the time of birth. The non- endemic area was Munich, Germany, and as a helminth-endemic area, Lambaréné, Gabon, known to be endemic especially for *S. haematobium*, was chosen.

Results and Conclusion: Our preliminary results, from 47 German and 54 Gabonese women including 13 *S. haematobium* infected Gabonese women, indicate that Gabonese mothers are at a higher risk for placental inflammation. Indications that placental inflammation was higher for Gabonese women include lower VDR, Cyp27b1, Foxp3 and IL10 expression compared to German placentae, as well as less strong placental transfer of IgG4. Moreover, living in a non-endemic as compared to a tropical, parasite endemic area in general was associated with altered immune response and susceptibility for helminths, with a significantly higher IgE/IgG4 ratio in the Gabonese cohort. The stimulation and examination of cellular cytokine responses from cord-blood mononuclear cells (CBMCs) as well as peripheral mononuclear cells (PBMCs) worked nicely and fist results revealed lower lymphocytes populations in infected *S. haematobium* Gabonese mothers as well as higher IL10 production. In future, further measurements of placental vitamin D levels and immune activation in newborns could clarify whether infection during pregnancy is indeed related to altered relationships between the VDR-related gene expression and inflammatory or anti-inflammatory immune responses. Due to these finding, we assume that the exposure *in utero* to different environments might play an important role in the early development and modulation of the immune system of the newborn.

Zusammenfassung

In den letzten Jahren ist deutlich geworden, dass die Exposition gegenüber Umweltfaktoren, einschließlich toxischer und infektiöser Krankheitserreger, die Neigung zur Entwicklung von Allergien und möglicherweise anderen Immunstörungen im späteren Leben verändern kann. Die zugrunde liegenden Mechanismen diese fetomaternalen Kommunikation sind noch unklar und erfordern weitere Untersuchungen. Erste Mausstudien unsere Gruppe zeigten, dass entzündliche mütterliche Signale, die durch eine chronische Infektion innerhalb der Plazenta hervorgerufen wurden, das plazentare Genexpressionsprofil prägen, welches im Zusammenhang mit dem Vitamin-D-Rezeptor (VDR) und Entzündung steht. Die zentrale Hypothese ist, dass pro- oder anti-entzündliche Immunantworten, die durch Umweltfaktoren oder mütterliche Infektionen ausgelöst wird, die Funktion der Plazenta und die Regulation des Vitamin-D-Stoffwechsels beeinflussen können.

Ziele und Methoden: Das Hauptziel dieser Studie und Doktorarbeit war es, die Frage zu beantworten, ob sich zum Zeitpunkt der Geburt die VDR- und Vitamin-D-bezogene Genexpressionen und die pro- und entzündungshemmenden Immunantworten innerhalb der Plazenta, zwischen Frauen aus Helminthen endemischen und nicht-endemischen Gebieten, sowie zwischen schwangeren Frauen infiziert und nicht infiziert mit *Schistosoma hämatobium* unterscheiden. Als nicht endemisches Gebiet wurde München, Deutschland, gewählt und als endemisches Lambaréné, Gabun, bekannt für ein hohe Prävalenz von Schistosomiasis.

Ergebnisse und Schlussfolgerungen: Die vorläufigen Ergebnisse, von 47 deutschen und 54 gabunischen Frauen mit einer Untergruppe von 13 *Schistosoma hämatobium*-positiven Frauen, deuteten darauf hin, dass gabunische Mütter ein höheres Risiko für Plazentaentzündungen haben. Anzeichen dafür waren einerseits eine verminderte VDR-, Cyp27b1-, Foxp3- und IL10-Genexpression in gabunischen Plazentas im Vergleich zu deutschen Plazentaproben, sowie ein geringerer plazentarar Übergang von IgG4 in Gabun. Darüber hinaus war das Leben in einem nicht-endemischen Gebiet im Vergleich zu einem Helminthen endemischen Gebiet im Allgemeinen mit einer veränderten Immunantwort und einer geringern Anfälligkeit für Helmintheninfektionen verbunden, was sich mit einem signifikant höheren IgE/IgG4-Verhältnis in der gabunischen Kohorte äußerte. Die Stimulation und Untersuchung zellulärer Zytokinreaktionen von Nabelschnur-mononukleären Zellen (CBMCs) und peripheren mononukleären Zellen (PBMCs) funktionierte gut. Die ersten Ergebnisse zeigten niedrigere Lymphozytenpopulationen bei infizierten gabunischen Müttern, sowie eine höhere IL10-Produktion. Künftig könnten weitere Messungen der plazentaren Vitamin D Spiegel und der Immunaktivierung bei Neugeborenen klären, ob eine Infektion während der Schwangerschaft tatsächlich mit veränderten Beziehungen zwischen der VDR-bezogenen Genexpression und entzündlichen oder entzündungshemmenden Immunantworten zusammenhängt. Aufgrund dieser Ergebnisse gehen wir davon aus, dass die

Exposition im Uterus gegenüber verschiedenen Umgebungen eine wichtige Rolle bei der frühen Entwicklung und Modulation des Immunsystems des Neugeborenen spielen könnte.

1. Introduction

1.1 Schistosomiasis causing chronical inflammation

Parasitic flatworms (blood flukes) of the genus Schistosoma cause the neglected tropical disease schistosomiasis or bilharzia and is responsible for considerable morbidity in parts of the Middle East, South America, Southeast Asia and, particularly, in sub- Saharan Africa. This disease is highly debilitating and is intimately linked to poverty [173].

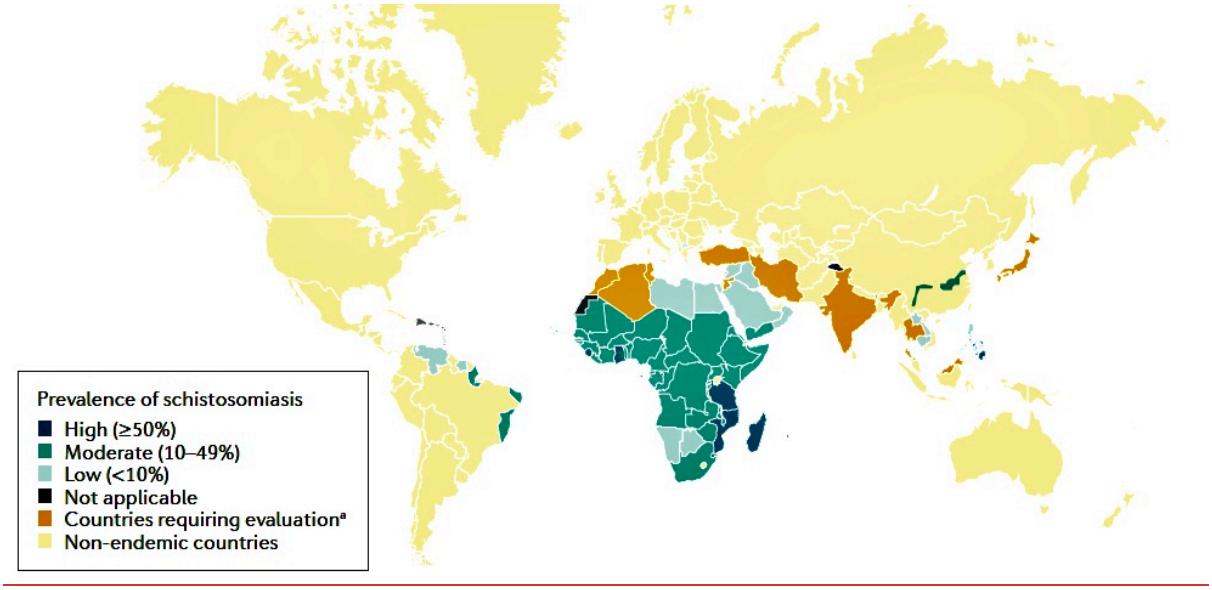


Figure 1.1 Prevalence of Schistosomiasis

Worldwide distribution of schistosomiasis in 2012. Figure adapted from [173].

There are six main species that infect humans, the most commonest being *Schistosoma mansoni, Schistosoma haematobium,* and *Schistosoma japonicum* [275] and other species like *S. mekongi* or *intercalatum* being much less prevalent. Worldwide about 700 million people are at risk of acquiring an infection [41]. *S. haematobium* has been reported in 54 countries and is the most common species, occurring in sub- Saharan Africa and the Middle East [173]. It is highly prevalent in Gabon, Sub-Saharan Africa [6]. The patency of infection varies by age and gender [53] whereas infection rates are highest amongst children, early adolescents and also pregnant women [41].

Figure 1.2 shows the life cycle of *S. haematobium*: 1. Miracidia invade their intermediate host, freshwater snails of the genus *Bulinus* where they multiply, 2. develop into cercariae and are shed by their intermediate host into fresh water in search of a human host. 3. Upon penetration of the intact skin, cercariae lose the tails and are now called schistosomulae. 4. These migrate

via the venous circulation via the lungs and heart, into the mesenteric and portal veins and in the case of *S. haematobium* into the venous plexus of the genito-urinary tract system where they mature to juvenile worms. 5. Next, male and female start to mate, become adults and live in the venules for up to 20 years. 6. Upon mating, the females start to produce eggs, which are then excreted with the stool or urine into the environment. Under suitable conditions, the eggs can hatch and develop the miracidia to restart the life cycle (1.) [9] .

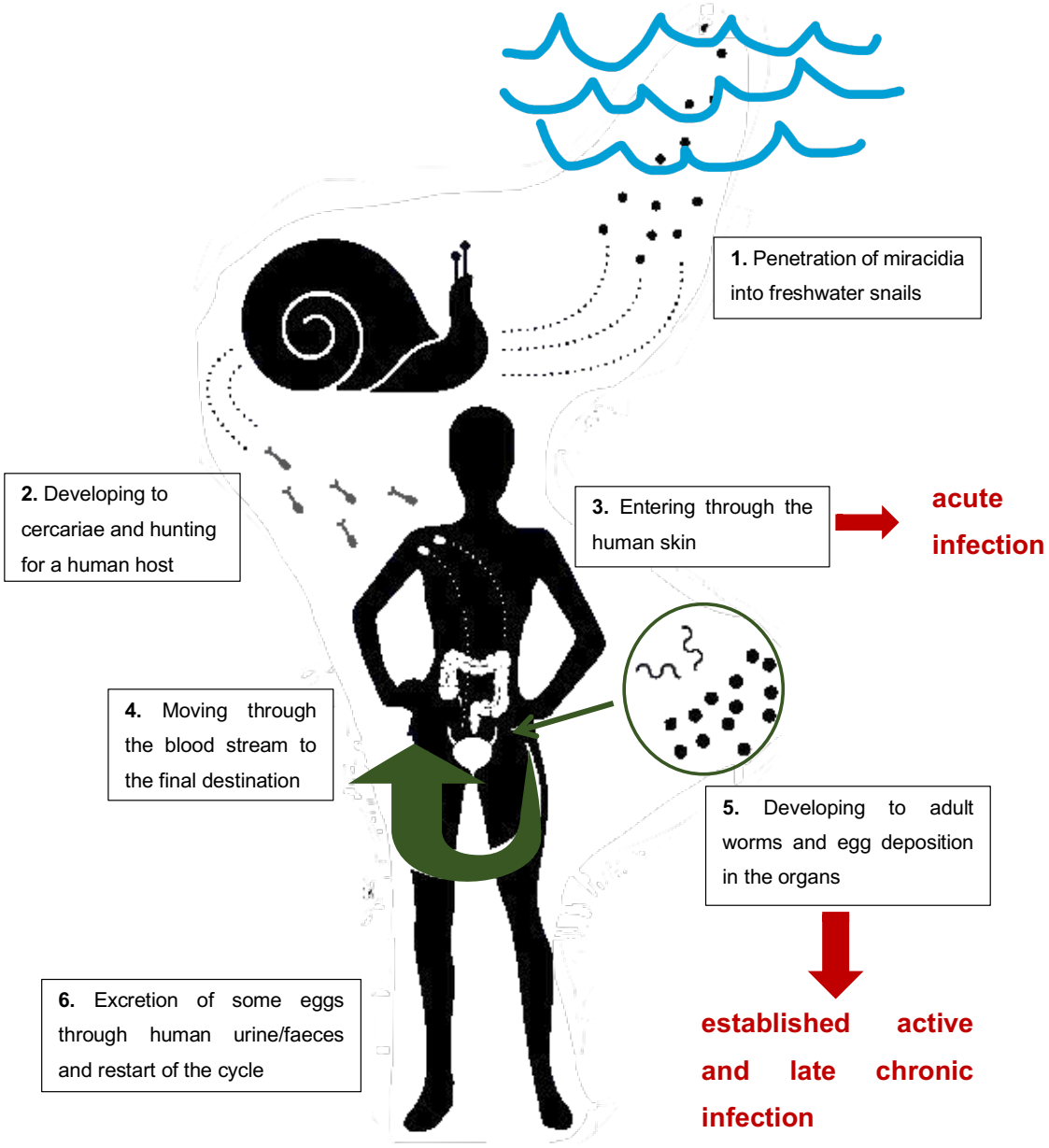


Figure 1.2 The schistosomiasis life cycle.

The figure was sketched single-handed and adapted from [9].

The infection is followed by three different disease stages due to the parasite's development:

1. Acute infection: Cercarial dermatitis after cercarial skin penetration into the venous circulation [173]. Acute infection is also known as Katayama fever and occurs 2 weeks

to 3 months after exposure [227], mainly in travelers to schistosomiasis-endemic areas; the schistosomula migration or initial egg deposition provoke antigen release followed by systemic hypersensitivity reactions and formation of immune complexes [227]. Furthermore, eosinophilia and transient pulmonary infiltrates (as observed on chest radiography) are symptoms of acute infection [50, 227]. The symptoms usually include myalgia, abdominal pain in the right upper quadrant, diarrhea (with or without blood), fatigue, malaise, fever and, in case of *S. haematobium* infection, hematuria [227].

2. Established active infection and late chronic infection: This affects mainly individuals from poor rural areas with long- standing infections [200]. Both, established active and late chronic infections, are characterized by immunopathological reactions against schistosome eggs trapped in host tissues leading to proinflammatory immune responses. The tissues and organs affected depend on the infecting schistosome species, as the worms nest in different preferential anatomical locations [173]. Late chronic infection with *S. haematobium* causes urogenital schistosomiasis mainly involving lesions of the bladder wall [53]. Urogenital schistosomiasis can cause pelvic discomfort and pain, abnormal vaginal discharge, itch and contact bleeding in girls and women [106] or hematospermia and painful ejaculation in men [263]. In both sexes, genital schistosomiasis has been associated with increased risk of HIV infection [131]. Furthermore, *S. haematobium* belongs to the group 1 of carcinogen agents for bladder cancer [117] and "…an estimated 3-4 patients out of 100,000 people infected with *S. haematobium* develop bladder cancer each year [37]. The reasons may be multifactorial and inflammation caused by egg excretion into the bladder wall, a skewed T cell environment, modulation of transcript expression and epigenetic changes or modulations of the urine microbiome are only some explanations which demand for further investigations [117].

Up to date Praziquantel is the only anti-schistosome drug of choice. It is safe and efficacious against adult worms of all the six schistosome species infecting humans. However, it does not prevent reinfection and the emergence of drug resistance is a concern [173]. No schistosomiasis vaccine has yet been accepted for public use.

In most cases, especially in people living in endemic areas, symptomatic acute schistosomiasis is not observed, and the disease reaches a stage of established active infection, with mature adult worms and egg production as well as excretion of eggs containing live miracidia in stool or urine. Mostly, no local inflammation or symptoms are diagnosed [173]. *In utero* sensitization is one possible answer for the absence of susceptibility to acute symptoms in highly endemic areas. Immune responses to schistosome antigens may be less pronounced in infants born from infected mothers [130]. Another explanation is that repeated

exposure to skin-penetrating cercariae induces IL10 producing $CD4^+$ T cells in the skin, resulting in regulated immune response [235].

1.2 Immunobiology of schistosomiasis

The infection with schistosomes is characterized by the development of strong $CD4^+$ T cell and B cell responses. During acute infection parasites survive in the human body by modulation of the innate immune system but concurrent tissue damage provoked by lodging of parasite eggs. The pivotal bottom of the modulation is a strong controlled Th2 response which reinforces furthermore the development of resistance to further infection [84].

During the course of infection, the immune responses progress through at least three phases (Figure 1.3). In the first three to five weeks, during which the host is exposed to migrating immature parasites, the dominant response is T helper 1 (Th1)-like designated by the production of pro-inflammatory cytokines like IFNγ, TNFα or interleukin 6 (IL6) in the plasma or in stimulated peripheral-blood mononuclear cells (PBMCs) [65].

During the following five to six weeks the parasites mature, mate and begin to produce eggs as well as egg antigens. The response alters remarkably: the Th1 component decreases and this is supersedes by a strong Th2 response [207] and cytokine release (e.g. IL4, IL5, IL6 or IL10). The production of IL10 during this period might have a crucial role in downregulation the Th1 immune responses [180].

The ensuing chronic infection status is characterized by a regulatory immune state and regulatory T cells (Treg) producing e.g. IL5, IL10, IL13 or IFNγ.

Straubinger et al. amongst many others showed that rodent schistosomiasis reflects human infections in terms of pathology and schistosome-specific immune phases [141, 252].

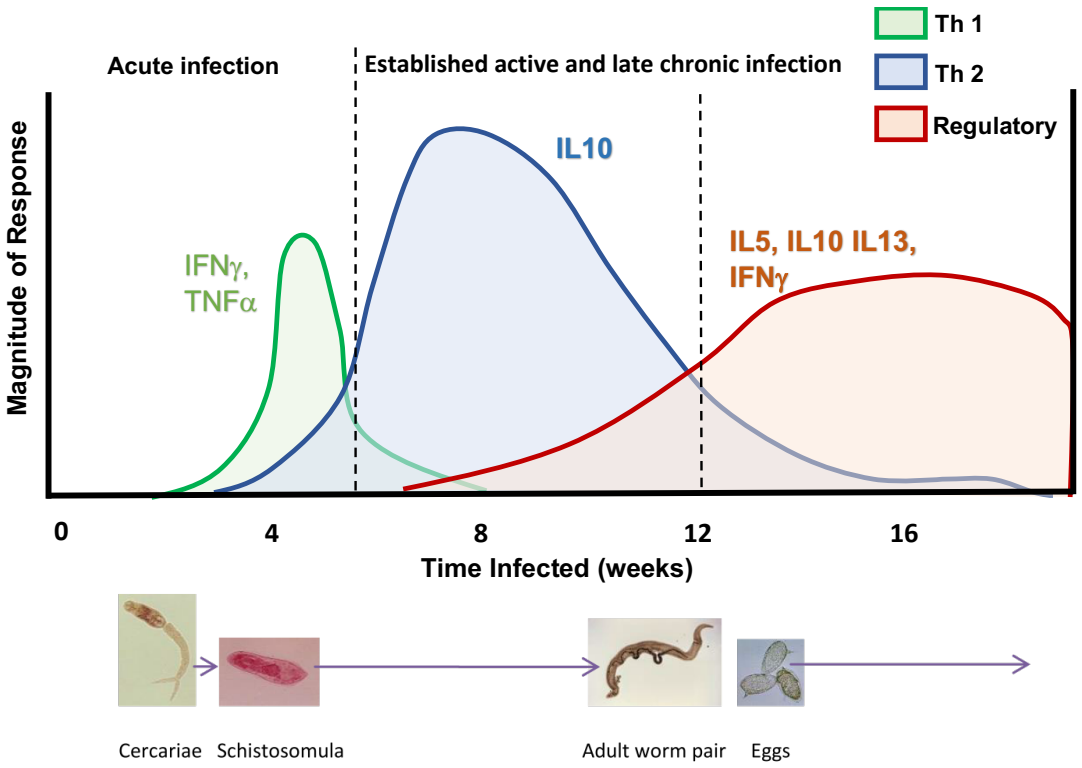


Figure 1.3 Immune phases of infection with *S. mansoni*

Scheme of different immune phases during schistosome infection. The figure was adapted from [153] .

Investigation of cord-blood mononuclear cell (CBMCs) of babies from schistosome-infected mothers showed that both cord blood $CD4^+$ cell and maternal $CD4^+$ cells had similar cytokine production including the ability of IL4 and IL5 secretion [162]. This supports the view that *in utero* sensitization occurs and, moreover, that the fetal response is phenotypically similar to the response of the mother [130]. Thus, a pre-existing Th2 immunity in such children might make them less likely to develop a pro-inflammatory response upon the first infection with schistosomes (see chapter 1.4) [207].

1.3 Detection of circulating anodic antigen (CAA) as a diagnostic tool for Schistosomiasis

As schistosomiasis belongs to the 20 neglected tropical diseases (NTD), the World Health Organization (WHO) set up a strategic plan 2012– 2020 for schistosomiasis which comprises the control of disease morbidity by 2020, the elimination of schistosomiasis as a public health problem and the interruption of transmission in selected areas by 2025 [202]. The strategies

to reach this goal are to increase the frequency of preventive chemotherapy and to improve the access to clean water and adequate sanitation (WASH). They also include control of the intermediate host snails, monitoring of the progress of control programs and rigorous surveillance to identify remaining transmission hotspots and individuals with high infection levels. But there is still a lack of accurate, quick and cheap diagnostics. The common and well proved diagnostic tools (Figure 1.4) are most sensible for moderate and heavy infections only and not useful for low-endemic settings which are to be expected once the control programs succeed.

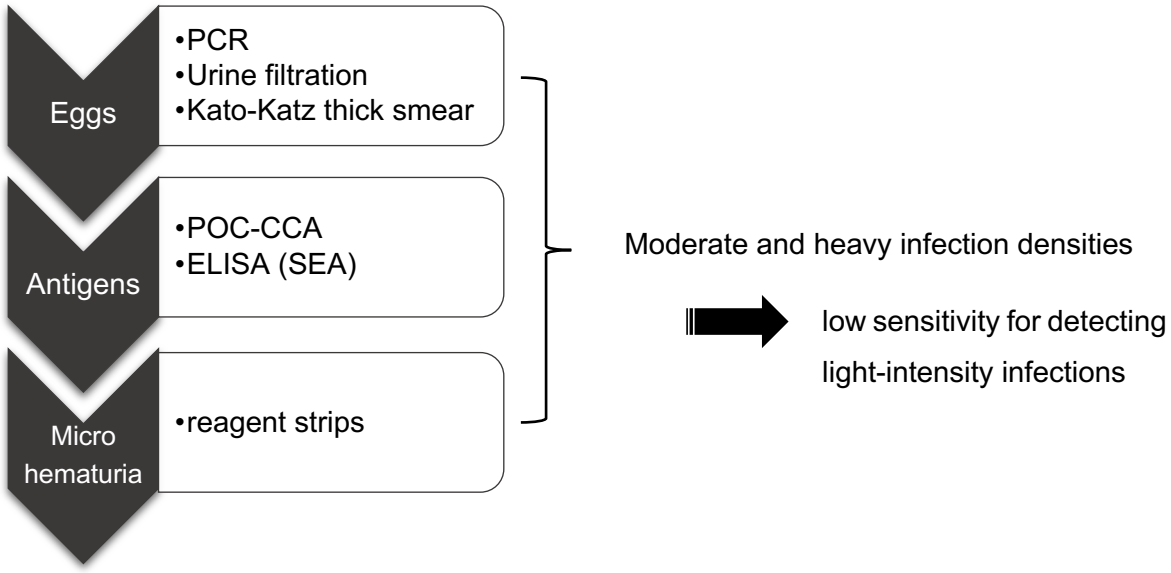


Figure 1.4 Diagnostic tools to detect schistosome infection.

Diagnostic tools are ranked according to their sensitivity with PCR as the most sensitive tool, but still the urine filtration as gold standard. Testing urine for microhematuria is the less sensitive method.

To detect light-intensity infections, the group of Govert van Dam and Paul Corstjens developed a novel assay [52, 58-61, 262, 268]. In principle, adult worm circulating anodic antigen (CAA) in serum and urine is detected using an up-converting phosphor-lateral flow (UCP-LF) assay. CAA is a glycosaminoglycan-like carbohydrate with a unique structure. The antigen is regurgitated by all species of adult schistosome worms in the blood stream and can eventually be detected in any other body fluid.

It has several advantages as compared to other tests stated in Figure 1.4, the most important being its high sensitivity by recognizing a very low number of worms up to even a single worm. It is furthermore sensitive enough to be used in randomly collected single urine samples or any other body fluid. The procedure is described elsewhere [132] and summarized in 2.2.6.

The convenient transport of the dry reagents without a cold chain to third-party laboratories is a big advantage. But a remaining problem for using this tool in the field as a rapid test is the

need of laboratory equipment and the currently still high costs of production. Nevertheless, it is a suitable tool for large-scale monitoring of schistosomiasis in control programs in low-endemic settings targeting elimination and for surveillance in areas that achieved elimination.

1.4 Modulation of the immune system by helminths and its effects through exposure in utero

Chronic inflammatory disorders, such as allergic airway inflammation, are negatively associated with high levels of helminth infection such as schistosomes [216, 267], particularly in low-income countries [86] where prevalence of allergic reactivity like a positive skin test and auto-antibodies (e.g. immunoglobin E) increases after anthelmintic treatment [97, 189, 265]. Approximately 220 million people are infected with schistosomes [55] and thus, approximately 40 million women of childbearing age. Gabon is a high endemic area for helminths and other parasitic infections. Adegnika et al. screened pregnant women in the area of the city Lambaréné and analyzed their infection status: 25 % had an infection with *Plasmodium falciparum*, whereas only 40% had clinical symptoms, 49 % with intestinal helminths, 12% *Schistosoma haematobium*, 65% at least one parasite infection, and 22% at least two parasite infections [6].

Yet little is known about the specific morbidities inflicted on pregnant women and their offspring [91]. Complications such as anemia and low-birth-weight [178], as well as skewed immune responses, which can alter susceptibility for other diseases such as diarrhea [28], are just some examples of the impact of a pregnancy modulated by helminth infection. Helminth infections modulate host immune responses through a variety of mechanisms [269], and thereby allow long-term survival of the parasite within the host, while minimizing damage to host tissues [184]. The helminths do not reproduce in the same host, but only produce eggs or larvae. These are excreted by the host and the life cycle of the helminths continues to infect new hosts (Figure 1.2). By modulating the immune system at almost all levels in their favor and by inducing a kind of immune hypo responsiveness in the host that leads to immunological tolerance, they cause a stable chronic infection in the host that allows them to survive up to 20 years in the host [129, 158, 199].

Recent research indicates that early exposure to such a modified environment during gestation already primes the fetal immune system *in utero* and leads to enhanced immunological maturity at birth [64, 118, 140].

Studies using experimental helminth infection models during pregnancy have shown that offspring have altered immunity to subsequent infection. *In utero* exposure to the nematode parasites *Dipetalonema viteae, Brugia malayi*, or *Acanthocheilonema viteae* enhanced the

offspring's susceptibility to subsequent infection by these parasites and resulted in impaired spleen cell responsiveness to T cell mitogens and filarial antigens [15, 160, 232]. In other studies, female mice infected with *Schistosoma mansoni* during pregnancy gave birth to a decreased number of surviving offsprings. This trend was observed in subsequent pregnancies of these same chronically infected mice. In addition, el-Nahal et al. were able to show that the newborns from infected mothers not only showed a lower worm load upon subsequent infection when compared to the control group, but also that the egg count in the liver and intestinal tissue as well as the size of the hepatic granuloma were significantly reduced [78, 79].

There is considerable evidence from studies in both animal models [147, 181, 204] and humans [80, 142, 147, 174, 192, 250] that *in utero* exposure to helminths such as *Schistosoma spp., Onchocerca volvulus* or geohelminths can influence susceptibility to the same helminth species later in life. "…While increasing susceptibility to consecutive infection, the tolerogenic effects of prenatal exposure may reduce inflammation-induced pathology, resulting in an improved outcome for both helminth and host…" [184]. People from high endemic regions for helminths often experience less severe acute disease and subsequent pathology than migrants or travelers from non-endemic areas [54, 74, 99, 157, 236] and this is likely be due to alterations and modulations of the human immune system done by the helminths and starting already *in utero*.

Multiple reports have demonstrated transplacental transport of soluble egg (SEA) and adult worm antigens (AWA), as well as their presence in the placenta, cord blood or the newborn [44, 88, 100, 116, 139]. Several studies indicate that the transplacental transfer of schistosome antigens results in immune sensitization, including parasite specific antibody- [196] and cytokine production [116] in the fetus. Taken together, there is evidence that schistosome antigens enter the placental compartment and cross the placenta to the fetus [139].

A Kenyan study showed that mononuclear umbilical cord blood cells (CBMC) of infants born in an area endemic to schistosomiasis, filariasis and geohelminth infections produce polyclonal IgE antibodies and helminth specific IgG antibodies after stimulation with soluble lysates of adult *S. haematobium* (SWAP) or *Brugia malayi* worms (BmA) [130]. In line with this hypothesis that exposure leads to neonatal immune priming *in utero* stands a significantly increased percentage of $CD5^+$ B cells in umbilical cord blood collected in the helminthic endemic country Gabon [243]. Other studies investigating the effect of maternal atopic disease on the neonatal immune system also demonstrated an association of maternal disease with increased immunoglobin E levels in neonates [24, 123]. The results suggest that not only chronic helminth parasite infection during pregnancy, but also intrauterine exposure to allergens and genetic factors influence the maturation of the fetal immune system and can already stimulate antigen-specific B-cell immunity and T-cell memory *in utero* [130].

1.5 Antibody class-switch to increase protection against helminth infections starts already *in utero* in high endemic areas.

IgE levels depend not only on the types of diseases or exposure to certain factors, but in the context of helminths, the activity of Treg cells and the production of IL10 have a strong impact the IgE levels. Moreover, IL10 concentrations correlate closely with an isotype switch from the proallergic / inflammatory immunoglobin E (Ig E) to non-inflammatory IgG4 [165].

IgG4 belongs to the smallest fraction of IgG antibodies (<5%). IgG and its subclasses are the only maternal antibodies which are able to cross the placental barrier [14, 26, 231]. Palmeira et al. reviewed this regulated transfer as followed: "…The maternofetal crossing is mediated by neonatal Fc Receptor (FcRn), expressed on syncytiotrophoblasts. This crossing depends on the one hand on maternal levels of total IgG and its specific antibodies as there is an inverse relationship between efficiency of placental transfer to the maternal level. On the other hand, the gestational age is important as there is a sharp increase in cord blood levels after 36th week of gestation. Moreover, the IgG subclass and the nature of antigen play a role. Maternal age, weight, parity, and type of delivery do not influence the placental antibody transfer…". Meaning that FcRn can be saturated by maternal IgG and thus less antibodies are able to cross the placental barrier.

Known disturbance factors for reduced transplacental transfer of Igs are maternal HIV infection [66, 85] or placental injuries, like malaria or inflammation in general [38, 172]. An altered placental transfer of antibodies but also chemokines or nutrition in mothers with primary immunodeficiencies or autoimmune diseases such as myasthenia gravis, bullous pemphigoid, idiopathic thrombocytopenic purpura, and systemic lupus erythematosus has been observed, too [205]. A strong correlation has been demonstrated for elevated IgG4 maternal and neonatal serum levels and the presence of helminthic infections. Significant amounts of IgG4 are found not only in schistosomiasis, but also in other helminth infections or inflammatory diseases such as cholangitis or autoimmune pancreatitis without fully understanding the exact function yet [101]. In contrast to other antibodies, the role of IgG4 in the immune system is more modulatory, as it is inefficient at activating the complement system [259] and in binding to receptors on monocytes and macrophages [101].

Besides a high level of IgG4 antibodies, large amounts of specific and nonspecific IgE are another well recognized feature of the immune response to parasitic helminth infections, including schistosomiasis [101]. Strong anti-parasitic IgE responses are associated with resistance to infection [260], whereas high levels of IgG4 have been associated with susceptibility [87]. It is not just a high IgE expression or just a lower IgG4 expression but rather

the ratio of IgE and IgG4 i.e. a positive IgE/IgG4 ratio, that improves the protection against helminths infection.

The interaction of IgE through specific receptors on eosinophils, macrophages and platelets, and the ability of these inflammatory cells to kill schistosomes *in vitro*, has been established in experimental models [101]. A study conducted in The Gambia demonstrated that reinfection with schistosomes was significantly less likely in those people with high levels of IgE antibody against antigens derived from the adult worm and more likely in those with high levels of IgG4 antibodies against either the worm or egg antigens [101]. This “protection” and antibody switch from a mainly IgG4 response to an IgE response takes time to develop, thus the prevalence of infection amongst children is higher than amongst adults. There are two putative mechanisms whereby preferential expression of IgG4 over IgE could occur leading to the prevention of IgE-mediated immunity and thus improving the survival of the parasite. Firstly, it has been established that antibody-producing plasma cells switch sequentially from IgM via IgG4 to IgE [260]. Secondly, allergen-specific IgG has been described as ’blocking antibody’ owing to its ability to effectively compete for the same epitopes as IgE in both helminth and non-helminth derived allergens. Hence, IgG4 may enhance parasite survival by inhibiting IgE-mediated effector responses [260].

In this context it could be speculated that a lower IgE/IgG4 ratio in newborns from infected women might indicate a higher propensity to develop a subsequent infection and further studies with long term follow-ups of the children could reveal interesting answers.

1.6 The placenta as communicator between mother and fetus.

The tight control of maternal antibody transfer to the neonate is only one crucial task of the placenta. During pregnancy the placenta plays a decisive role in pregnancy maintenance and the development and protection of the fetus. Besides its function as hormone producer (e.g. estrogen, progesterone, human chorionic gonadotropin (hCG) or human placenta lactogen) for pregnancy maintenance, the placenta is responsible for fetal sustenance, such as the supply of oxygen, energy and nutrients. Furthermore, it has very important immunological functions, since it is the only barrier between mother and fetus. As shown in Figure 1.5 the mature, disc-shaped placenta can be divided into three zones. First the basal plate or decidua (consisting of cyto-, syncytio- and extravillous-trophoblast cells) which is predominantly the maternal side of the placenta; the fetal side is composed of the chorionic plate (syncytiotrophoblasts, cytotrophoblasts and the parietal leaf of the extraembryonic mesoderm); between the maternal and the fetal side is the intervillous space with maternal blood and the villous trees containing fetal blood; the fetomaternal zone consist mainly in multinucleated syncytiotrophoblasts, endothelial cells of fetal capillaries, fibroblasts, and Hofbauer cells.

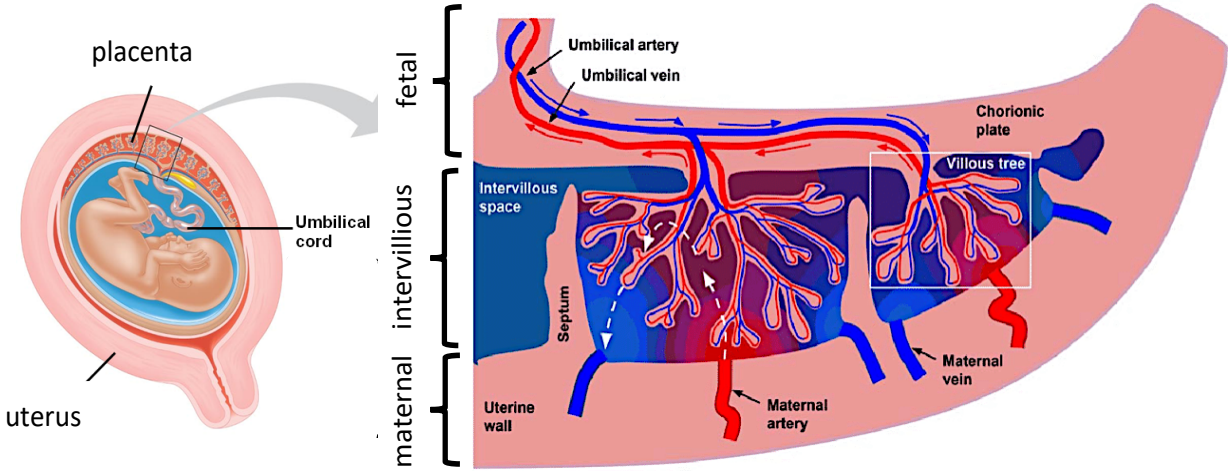


Figure 1.5 Structure of the human placenta

The figure shows the sturcture of a human placneta which is divided into a fetal and a maternal part and an intervillious space which is inbetween. The figure is addapted from [248] and [21].

Up to 30-40% of all decidual stroma cells are leucocytes [81]. The placental leukocyte population is compromised by around 70% uterine natural killer cells (uNKs), about 20% macrophages and 10% T-lymphocytes (with 10-15% regulatory T-cells), but also dendritic cells and mast cells can be found in the early placental bed [40, 83]. All these cells are responsible for a strong anti-inflammatory response. On the one hand, to maintain pregnancy and to avoid a host vs “graft” reaction and the recognition of the fetus as foreign by the mother, which would accidently lead to a rejection and an abortion. On the other hand, this strong anti-inflammatory response should avoid any infection in the placenta which could put the fetus under risk. Progesterone (P4) plays an essential role in maintaining immune responses more tolerogenic and less inflammatory at the fetomaternal interface [255]. At that, together with vitamin D, P4 promotes the profiling of immunosuppressive Foxp3 positive T cells, a subgroup of $CD4^+$ T lymphocytes [46, 122] and suppress the activation and expansion of proinflammatory T cells, such as Th17 cells [143, 144]. This effect could mainly be demonstrated in CBMCs [144]. In cord blood measurements, low vitamin D values correlated with a low number of naive $CD4^+$ T cells, $CD4^+$ T helpers and cytotoxic $CD8^+$ T lymphocytes [285]. Thus, these steroid hormones lead to immunotolerance and make the immune responses within the placenta and here especially on the fetal side more tolerogenic and less inflammatory. In order to avoid inflammatory or cytolytic reactions and to maintain the integrity of the fetomaternal barrier, fetal trophoblasts and maternal leukocytes secrete furthermore predominantly anti-inflammatory cytokines such as IL10 [253]. Thus, both, the mother and the fetus adjust their side of the

placenta according to their individual needs for a healthy and successful pregnancy. Hence, independent gene expressions on the fetal or maternal side of the placenta can be detected. Placental inflammation, e.g. provoked by the parasite *Plasmodium falciparum*, can alter this highly regulated placental transfer of e.g. nutrition, cells or antibodies. Placental *P. falciparum* infections were associated with substantially lower maternofetal antibody transfer efficiency in Papua New Guinean primigravid women and these alterations are discussed to be responsible for protection against malaria during early infancy [172]. Still, the exact mechanism of the modulated antibody transfer is poorly understood. Furthermore, downregulation of the expression of transport receptors for glucose or amino acids and diminished transfer occur in case of pathological changes in the placenta caused by local inflammation, fibrin deposition or syncytial knotting and thickening of the trophoblastic basement membrane [172].

In multivariate models adjusted for geohelminths, a significantly increased amount of inflammatory cytokines like TNFα and IL10 in maternal blood, TNFα, IL1β, TNFα RII or IL6 in placental and also IL1β and TNFα RII in cord blood was detected in pregnant women suffering from schistosomiasis. In addition, the infection was connected with subchorionitis [139]. In the context of schistosomiasis infection some other case reports have identified placental [27] or cervical [284] inflammation, too. This could occur if immature worms or eggs directly become lodged in the placenta and cause inflammation. More probable is that egg or worm antigens access the placenta and cause inflammation [91].

In summary, the environment, including helminth infections, can result in increased levels of proinflammatory cytokines in the placenta, raising the risk of placental inflammation and adverse birth outcomes.

1.7 Role of VDR and vitamin D in general, in immune responses and specifically within the placenta as risk factor for placental inflammation

Our genome is adapted to certain environments and maternal stress is characterized by down- and upregulation of specific genes such as growth hormone releasing hormones or genes involved in the p53 oncogene pathway in the placenta [96]. Indeed, our group recently demonstrated that experimental chronic infection with the helminth *Schistosoma mansoni* during pregnancy influences the outcome of allergic asthma in offspring [252]. This was further associated with downregulation of genes associated with either Vitamin-D-metabolism and –pathways such as the transcription factor Vitamin-D-receptor (VDR) [253], the enzyme 1α-hydroxylase (Cyp27b1), responsible for vitamin D activation, as well as hydroxy-delta-5-steroid dehydrogenase (Hsd3b1) [252], crucial for the biosynthesis of hormonal steroids such

as progesterone [197] and the modulation of the fetal immune system towards more tolerogenic and less inflammatory immune responses [143, 144, 217].
Similarly, other studies investigating the crosstalk between maternal microbial exposures and asthma predisposition in offspring found associations with altered placental gene expression, including down-regulation of placental TLR expression by exposing murine dams to cowshed-derived bacterium *Acinetobacter lwoffii* [57].
The transcription factor VDR is responsible for the transcription of almost three percent of the human genes and expressed in almost all immune cells, including activated $CD4^+$ and $CD8^+$ T cells, B cells, neutrophils, and antigen- presenting cells, such as macrophages and dendritic cells (DCs). VDR and vitamin D metabolizing enzymes are present in almost all tissue types, including the placenta, and in many different cell types, including immune cells indicating its central role in immunity. Additionally, progesterone was found to induce the VDR gene expression in T cells [255], but also vitamin D is known to increase the expression of progesterone receptors on $CD4^+$ T cells [217], emphasizing an important collaboration between VDR and Hsd3b1.
Besides progesterone, vitamin D has an antibacterial and anti-inflammatory function [214]. The general function and role in the immune system of vitamin D and –pathway related genes will be summarized below.

1.7.1 The sun as main source for vitamin D

Vitamin D belongs to the group of fat-soluble vitamins and secosteroids. In the body, the physiologically most important representative is cholecalciferol (= vitamin D3). Although there are some aliments such as fatty fish, egg yolk or food supplemented with vitamin D, cutaneous synthesis of 25(OH)D by exposure to UV-B has been and still remains our principal source of vitamin D [154] and the major cause for the pandemic vitamin D deficiency is the lack of adequate sun exposure [111, 239, 273]. The exposure to the sun above and below approximately 33° of latitude in the winter (approx. October to March) does not result in any significant production of vitamin D, which leads to pandemic vitamin deficiency in Europe during winter [112]. Otherwise, Luxwolda et al. showed that Tanzanian people living near the equator (2-4° of latitude) and having optimal sun radiation during the hole year, do indeed have an adequate vitamin D level of about 40-60 ng/dL [155]. The definition of vitamin D deficiency based on the 25(OH) vitamin D level remains controversial. The American Institute of Medicine continues to list 20 ng/mL as the lower limit. The Endocrine Society, National and International Osteoporosis Foundations and the American Geriatric Society chose to define vitamin D sufficiency as the blood level of 25(OH)D of at least 30 ng/mL [113]. The Endocrine Society recommend a preferred range for “sufficiency” even of 40– 60 ng/mL [112].

1.7.2 The tight regulation of vitamin D and VDR

After the synthesis in the skin, 25(OH)D gets hydroxylated and activated by Cyp27b1, whereby high $1,25(OH)_2D$ concentrations induce Cyp24a1 which leads to $1,25(OH)_2D$ in inactivation (Figure 1.6) [272].

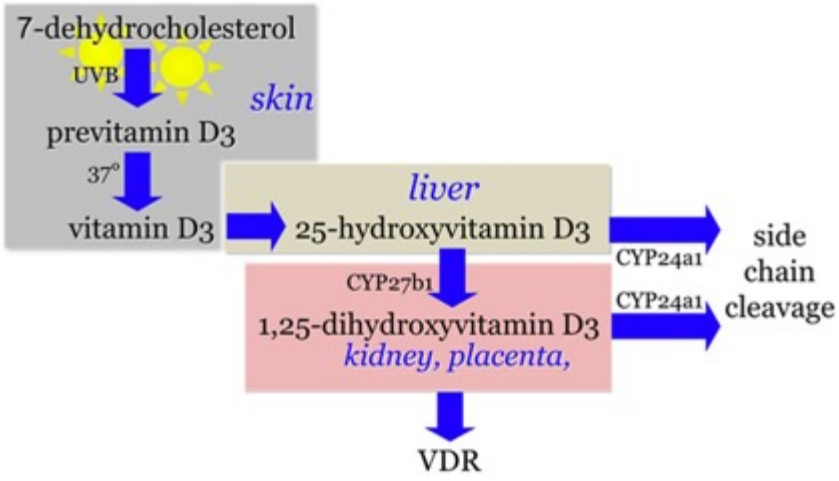


Figure 1.6 The endocrinology of vitamin D.

Figure taken from [5]: "…The left panel depicts the essentials of the endocrine synthesis, metabolism and action of vitamin D. The key enzyme in the synthesis of the active, vitamin D receptor-(VDR) interacting metabolite, 1,25-dihydroxyvitamin D, is the CYP27b1-hydroxylase. In normal human endocrine physiology the CYP27b1-hydroxylase is expressed principally in the kidney but also in the placenta. The catabolic CYP24a1-24-hydroxylase is ubiquitously distributed among human tissues and serves to inactivate 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D..."

To allow transport in peripheral blood vitamin D is bound to the vitamin D binding protein (VDBP). The exact mechanism of 25(OH)D and $1,25(OH)_2D$ entering most non-kidney tissues is still poorly understood [245]. Figure 1.7 demonstrates that after entering the cytoplasm, $1,25(OH)_2D$ dimerizes with VDR and retinoid x-receptor (RXR). After transfer to the nucleus, the heterodimer $1,25(OH)_2D$-VDR-RXR binds to its promoter region (VDRE) of certain gene loci [272].

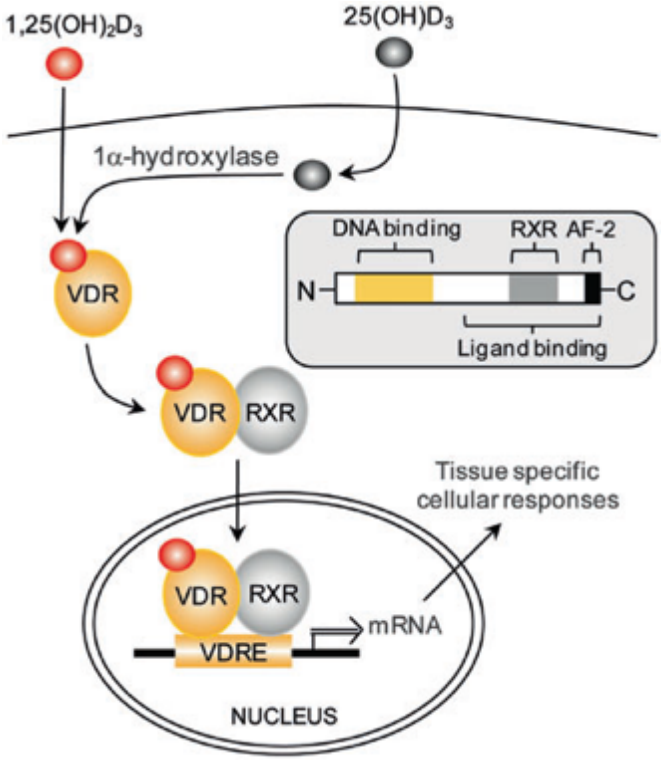


Figure 1.7 Mechanism of intracellular VDR-action

The mechanism of the VDR from top-dwon: (1) binding of $1,25(OH)_2D$ to VDR; (2) dimerization of $1,25(OH)_2D$-VDR with RXR to VDR-RXR complex; (3) binding of the complex to VDRE at the genom; (4) induction or inhibition of gene transcription; *Figure taken from [272]*

The wide-ranging actions of vitamin D include e.g. regulating calcium (Ca) and phosphorus (P) homeostasis, immune system modulation, cellular differentiation, and apoptosis [76] [164]. The function of $1,25(OH)_2D$ as a regulator of Ca and P homeostasis has long been known. Calcium is a vital electrolyte for bone mineralization and deficiency not only leads to insufficient bone formation but also to life-threatening muscle spasms [213]. Hence, a tight regulation of the calcium blood levels by $1,25(OH)_2D$ is of utmost importance. In deficiency both $1,25(OH)_2D$ and VDR stimulate intestinal absorption of Ca and P, mobilize Ca from the bone and reduces Ca excretion through urine. To avoid too high Ca levels, $1,25(OH)_2D$ and VDR induce simultaneously Cyp24a1, a 24-hydroxylase mitochondrial cytochrome p450 enzyme which inactivates 25(OH)D and $1,25(OH)_2D$ [213]. Consequently, it is suggestive to measure Ca levels when looking at peripheral vitamin D levels as the control of both levels is tightly connected.

VDR promotes not only the expression of various genes (see below) but is also responsible for the inhibition of certain genes such as the nuclear factor of activated T-cells (NFAT) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [190]. Overall the

heterodimer 1,25$(OH)_2$D-VDR-RXR is responsible for the control of the expression of about 3 % of all genes [194].

1.7.3 The important role of VDR and vitamin D in the immune system

Alongside its important role in the regulation of calcium levels in the blood and in bone formation, vitamin D metabolizing enzymes and vitamin D receptors are even present in many cell types including various immune cells such as antigen- presenting-cells, T-cells, B-cells, and monocytes. Vitamin D affects the function of both the adaptive and innate immune systems. Thereby it dampens the activity of the innate immune system and strengthens the one of the adaptive immune system [245]. VDR is present in cells of all stages of the immune system (Figure 1.8). The innate immune system is responsible for physical, chemical, biological as well as cellular and humoral defense mechanisms against pathogens. Especially macrophages are important actors of the innate immune system. They are responsible for the phagocytosis of many pathogens. In case of an infection with Mycobacterium tuberculosis for example, VDR and Cyp27b1 are increasingly expressed in the macrophages and thereby induce the production of antimicrobial peptides which lead to the killing of the bacterium [272]. In addition, the innate immune system activates the acquired immune system.

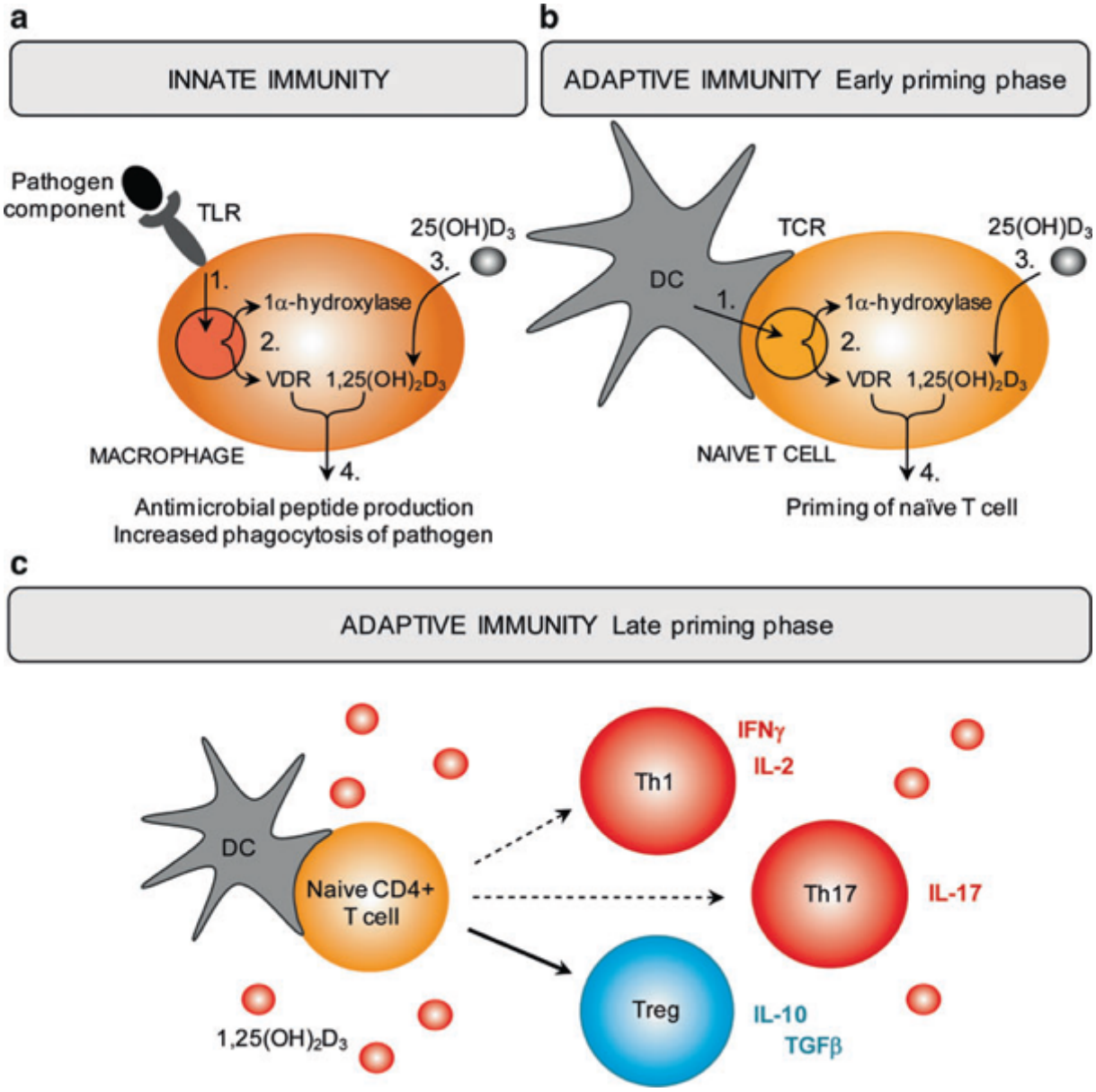


Figure 1.8 Model for the immunomodulatory effects of VDR and 1,25(OH)$_2$D

(a) Innate immunity: expression of 1α-hydroxylase and VDR by macrophages after TLR stimulation; 1,25(OH)$_2$D production and VDR activation; production of antimicrobial peptides and increased phagocytosis activity; **(b)** expression of 1α-hydroxylase and VDR by naïve T cell stimulation; priming of naïve T cells; **(c)** increase of 1,25(OH)$_2$D in secondary lymphoid organs; activation of DCs by 1.25(OH)$_2$D and promotion of differentiation of Treg cells, instead of Th1 and Th17 effector T cells; Dotted lines and text rendered in red indicate inhibitory actions of 1,25(OH)$_2$D and black lines and text rendered in blue increased actions. DC=denditric cell; *Figure taken from [272].*

VDR expression is very low in resting conditions, but upon activation and proliferation, T and B cells upregulate VDR expression significantly [214]. 1,25 (OH)$_2$D has immunomodulatory properties similar to locally active cytokines and multiple effects. Here just some examples for its effect on T cells: 1,25 (OH)$_2$D leads to a suppression of T helper cell proliferation and

differentiation, and to a modulation of its cytokine production. It inhibits Th17 cells, proinflammatory Th1 (IL2, IFNγ, TNFα), Th9 (IL9), and Th22 (IL22) cells and their cytokine expression and promotes the production of anti-inflammatory cells [214]. 1,25 $(OH)_2D$ has been shown to act as a potent modulator of both innate and acquired immune responses, including enhanced macrophage bacterial killing [150, 226], suppression of natural killer (NK) cell function [175], inhibition of dendritic cell (DC) maturation [23, 98, 208, 212], inhibition of T-cell proliferation, and modulation of T-cell phenotype [19, 35, 82, 198]. Moreover, in T cells, VDR is induced by progesterone, making them even more sensitive to vitamin D, which guarantees a highly sensitive regulation of the T cells even in the case of deficient vitamin D levels. This not only increases the activation of Tregs, but also inhibits Th1 and Th17 cells. Due to the significant effects on the effective regulation of T cells of progesterone and vitamin, adverse immune reactions during pregnancy shall be prevented [205, 206, 223, 224, 255]. Hand in hand with VDR goes Cyp27b1, the vitamin D activation enzyme. Various immune cells, including macrophages, DCs, and even B-cells and T-cells were found to express Cyp27b1 [16] and its expression as well as that of VDR is dramatically enhanced upon cell activation [16]. 1,25$(OH)_2D$ blocks B-cell proliferation, plasma-cell differentiation and immunoglobulin production [183].

Thus, hypovitaminosis D is associated with an increased risk of multiple malignancies, metabolic and cardiovascular diseases, and immune disorders such as autoimmune diseases and susceptibility to bacterial, viral or parasite infections. Interestingly the season for upper respiratory tract infections in Europe falls together with the winter season and low levels of vitamin D, and both are associated with each other [16].

1.7.4 The role of vitamin D and -pathway related genes in the placenta

The different components of the vitamin D-VDR-pathway are subject to a strict regulation in order to ensure adequate 1,25$(OH)_2D$ levels. Figure 1.9 roughly shows the pattern of the vitamin D pathway feedback loop.

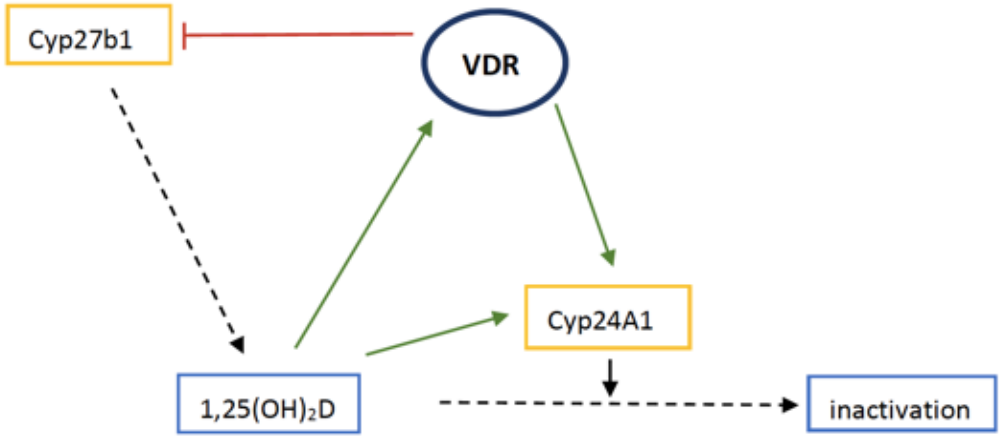


Figure 1.9 Renal regulated vitamin D pathway feedback loop

Feedback loop of the vitamin D activating and inactivation enzymes, vitamin D and VDR. Red arrows represent activation, black and dashed arrow show consequences and red lines stand for inhibition. Enzymes are represented in a yellow box. *The figure was sketched single-handed and adapted from* [1, 283].

Cyp27b1 hydroxylases vitamin D to bioactive 1,25-$(OH)_2$D, which binds to VDR and activates its expression. Cyp24a1 - responsible for the inactivation of 1,25-$(OH)_2$D - gets activated by high levels of VDR and 1,25-$(OH)_2$D itself. As a result, both 1,25-$(OH)_2$D and VDR levels control themselves to avoid excess. Furthermore, high VDR levels lead to a Cyp27b1 inhibition and no more 1,25-$(OH)_2$D will be produced until its level declines below a certain amount and the production and the loop start again [1, 283].

VDR, Cyp24a1 and Cyp27b1 are expressed in both the decidua [69] and trophoblast regions [288], suggesting that the placenta can produce its own activated vitamin D and thus has an autocrine or paracrine function [82, 149]. In pregnancy, the self-control via Cyp24a1 activation by high 1,25$(OH)_2$D levels is uncoupled, and 1,25$(OH)_2$D levels are significantly elevated, pointing out its important a role during pregnancy [195]. In a study Novakovic et al. found "…a methylation CYP24A1 in human placenta, purified cytotrophoblasts, and primary and cultured chorionic villus sampling tissue..." In contrast, in any tested somatic human tissue no methylation was detected [195]. Hence trophoblast cells in the human placenta don't show the feedback control of 1,25$(OH)_2$D present in other VDR-expressing tissues. The evaluation of transplacental 1,25-$(OH)_2$D transfer from mother to fetus is hardly understood and demands further investigations [70, 234]. Certainly, it is well known that 25(OH)D crosses the placental barrier from maternal to fetal circulations [195, 225].

Figure 1.10 gives an overview of the vitamin D pathway related gene expressions in the different placental tissues at the fetal and maternal side and summarizes its role.

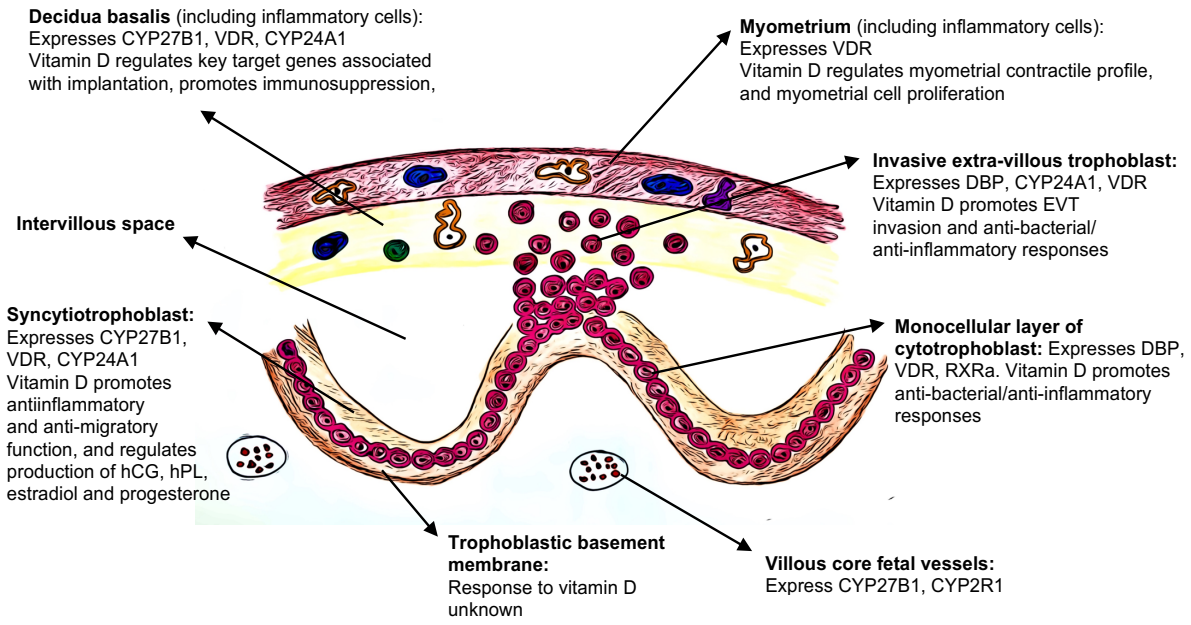


Figure 1.10 Vitamin D pathway components at the fetomaternal interface

Scheme showing cell types expressing components of the vitamin D system: vitamin D-25-hydroxylase (CYP2R1), CYP24A1, CYP27B1, DBP, hCG, human prolactin (hPL), RXR, VDR. *The figure is sketched manually and adapted from* [93].

Vitamin D deficiency has been linked to several adverse pregnancy outcomes, including those associated with placental insufficiency [33] which is thought to play a major role in several adverse pregnancy outcomes. Low 1,25$(OH)_2$D levels and altered Ca metabolism have been implicated in this process of insufficiency [242]. A study conducted by Young et al. showed that placental VDR gene expression, taken from a not specified side, did not differ by neonatal race, ethnicity, or sex [283]. However, at present, very little is known about the consequences of altered environmental, genetic, or epigenetic regulation of vitamin D homeostasis at the feto-maternal interface.

The ability of different tissues and cells to produce activated vitamin D (1,25 $(OH)_2$D) after specific immune signals provides strong evidence of their autocrine and/or paracrine role [16]. The activation of vitamin D (25(OH)D) to 1,25 $(OH)_2$D promotes antibacterial and anti-inflammatory reactions in maternal decidua and fetal trophoblasts [149]. Liu et al. showed that the loss of 1,25 $(OH)_2$D production in the fetal compartment of the placenta provoked generalized dysregulation of placental inflammation [149]. Thus, defective VDR signaling in placental tissue may lead to an increased risk of placental inflammation and expression of inflammatory cytokines or attenuation of anti-inflammatory or tolerogenic cytokines.

Furthermore, a murine study investigating placental gene expression pointed out that the expression of the anti-inflammatory IL10 was significantly lower in $VDR^{-/-}$ placentas relative to $VDR^{+/-}$ or $VDR^{+/+}$ equivalents after treatment with Lipopolysaccharide (LPS) [149]. The same

study also shows elevated IFNγ levels in Cyp27b1$^{-/-}$ placentas relative to Cyp27b1$^{+/+}$ placentas, and this increased even further after treatment with LPS [149]. Therefore, not only VDR and Cyp27b1 interact with each other, but also with IL10 or IFNγ. IFNγ is a potent proinflammatory cytokine that modulates hematopoietic cell maturation, differentiation, activation, and apoptosis. Moreover, it plays important roles in diverse cellular processes, including activating innate and adaptive immune responses and inhibiting cell proliferation. It is crucial in immune responses against pathogens and immunosurveillance of tumors [34, 188]. Especially during pregnancy IFNγ plays a decisive role. The proinflammatory cytokine was already identified in the early placenta in the endometrium, but also later in the trophoblasts, and is thought to prevent a severe infection-mediated inflammation, but rather rejection of the fetus [188]. IL10 is produced by Forkhead-Box-Protein P3 (Foxp3) expressing regulatory T cells alongside other cell types such as the villous cytotrophoblasts within the placenta, where it appears to work as a key facilitator of successful pregnancy [149]. Foxp3 expressing regulatory T cells (also known as natural T regulatory cells (nTregs)) together with peripherally induced Treg cells are associated with human helminths infection [13]. They are more active in helminth-infected subjects and decrease after anthelmintic chemotherapy [159, 276].

In conclusion, vitamin D leads to a shift from a proinflammatory to a more tolerogenetic immune status [16] and a lack of vitamin D or its coplayers such as Hsd3b1, VDR or Cyp27b1 raise the risk of inflammation. Differential placental gene expression evoked by altered environmental or genetic factors influences the probability for placental inflammation and may be affected by the modulatory processes induced by helminths to improve their survival in the host. Thus, helminths do not only have an influence on the cellular immune system but also on alterations in gene expression.

1.8 Main questions and objectives of the study

The causes and consequences of placental inflammation in relation to parasitic infections and associated changes to the placental tissue itself, remain poorly understood. Beyond helminth infections, geographic and genetic factors may influence the susceptibility to placental inflammation, too. To discern the effects of these factors, the objective of my thesis was to conduct and establish a cross-sectional study comparing study cohorts in Germany (Europe), and in Gabon (sub-Saharan Africa) (see Figure 1.11), to examine gene expression in the placenta, maternal fetal antibody transfer as well as inflammation markers in maternal and cord blood.

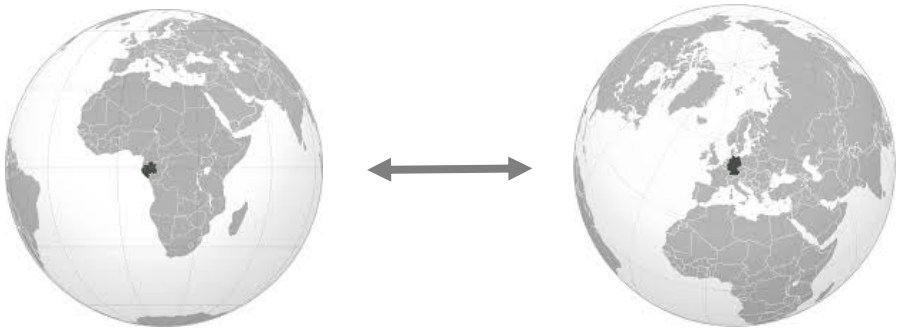


Figure 1.11 World map showing Gabon and Germany

On the left side Gabon is highlighted and on the right side Germany is highlighted. *The figure was sketched single-handed and dapted from* [270, 279].

The following **main questions** within this thesis are listed below:

- Show the study cohorts differences in patients characteristics
- Is placental gene expression influenced by
  a) environmental/genetic background
  b) and/or helminth infection?
- Are placental gene expression levels correlated with vitamin D levels, local or systemic inflammation in the mother and the cord blood?
- Is placental antibody transfer altered by the environment or infection with *S. haematobium*?
- Are altered T cell functions related to placental gene expression?
- Are maternal and cord blood T cell functions influenced by
  a) environmental/genetic backgrounds
  b) peripheral maternal or cord blood vitamin D levels
  c) and/or *S. haematobium* infection?

To answer the questions above, the following **main objectives** are enlisted below:

- Establish a robust sample collection work-flow in Munich, Germany at Klinikum rechts der Isar.
- Transfer methods to CERMEL in Lambaréné, Gabon and establish workflow and SOPs on site.
- Reach a study population of about 50 eligible participants in Germany and Gabon.
- Get a decent subpopulation of participants infected with *S. haematobium.*
- Shipment of all Gabonese samples to Munich, Germany to perform further analysis under same conditions.
- Analysis of CAA in serum of Gabonese maternal and fetal blood in cooperation with the university of Leiden (LUMC).

- Compare placental gene expression between the cohorts.
- Analyze epidemiologic data and biochemical results.
- Analysis of neonatal IgE in CBMCs in cooperation with Professor David Vöhringer, head of the Department of Infection Biology at the University Hospital Erlangen.
- Establish a protocol for stimulation and flow cytometry staining of PBMC and CBMC at MIH.
- Perform PBMC/CMBC stimulation and analyze Facs results.

2. Materials and Methods

2.1 Materials

2.1.1 Equipment

1000Touch™Thermal Cycler	Bio-Rad
Automatic pipettes (2-1000µl)	Gilson®
Centrifuge (Biofuge fresco)	Heraeus
Centrifuge (Eppendorf 5424)	Eppendorf
Centrifuge (Megafuge 3.0R)	Heraeus
Neubauer counting chamber	Assistent®
Freezer (-20°C)	Bosch
Freezer (-80°C)	Thermo Scientific®
Fridge	Bosch
Glassware	Schott
Microscope (Axiovert)	Zeiss
Multichanel pipettes (Acura® 855; 5-350µl)	Socorex
Multipette® plus	Eppendorf
Mr. Frosty™	Thermo Scientific®
NanoDrop® 1000 Spectrophotometer	Thermo Scientific®
Pipetboy (acu)	IBS
Thermocycler (T3000)	Biometra
Vi-CELL® Cell Viability Analyser	Beckman Coulter

2.1.2 Software

GraphPad Prism 5	GraphPad Software
Nanodrop® 1000 V 3.7.0	Kisker
FlowJo v9.5.3 (Flow cytometry analysis software)	TreeStar
Sunrise™ ELISA microplate reader	Tecan

2.1.3 Consumables

BD Luer-Lok™ Access Device	BDBioscience
Blood collection tubes (NH_4 Heparin, EDTA)	Sarstedt
Cryo vials	Alpha Laboratories
Disposable bags	Roth®
Eppendorf tubes (0.5-2ml)	Eppendorf
Falcon tubes (15ml, 50ml)	Greiner
Forceps (sterile, plastic)	Praxisdienst
Gloves	Meditrade®
Leucosep-Tubes	Greiner
Parafilm M®	Pechiney
Pipet tips (0.2-1000µl)	Starlab
Scalpel No.10	pfm medical

2.1.4 Reagents

Bicoll, Ficoll separating solution (density 1.077g/mL)	Biochrom AG
Chloroform	Roth®
Deoxynucleoside triphosphate (dNTPs)	Promega
Dimethylsulfoxid (DMSO)	Sigma®
Ethanol 70%-99.8% (v/v)	MRI Pharmacy
Isopropanol	MRI Pharmacy
Light Cycler® 480 Probes Master	Roche
M-MLV RT 5X Reaction Buffer	Promega
M-MLV RT (H-)Point Mutant	Promega
$Oligo(dT)_{15}$	Promega
RNAlater®	Ambion®
Trypan blue solution 0.4% (v/v)	Sigma®

2.1.5 Medium supplements

Fetal calf serum (FCS)	PAA
β-Mercaptoethanol for cell culture	Gibco®
Non-essential amino acids (100x)	PAA

Penicillin/Streptomycin (100x)	PAA
RPMI 1640 (with L-Glutamine)	PAA
Sodium pyruvate solution (100 mM)	PAA

2.1.6 Kit systems

GenElute™ Mammalian Total RNA Miniprep Kit	Sigma®
Total RNA Kit, peqGOLD	VWR
Tissue homogenizing CKMix - 2mL	Precellys®
QuantiTect Reverse Transcription Kit	Quiagen

2.1.7 Primer sequences

HPRT (reference gene)	
forward primer	5' tgaccttgatttattttgcatacc 3'
reverse primer	5' cgagcaagacgttcagtcct 3'
dual labelled probe	Universal ProbeLibrary Probe #73, Roche
VDR	
forward primer	5’ gaagctgaacttgcatgagga 3’
reverse primer	5’ gtcctggatggcctcaatc 3’
dual labelled probe	Universal ProbeLibrary Probe #15, Roche
Cyp27b1	
forward primer	5' cgcagctgtatggggaga 3'
reverse primer	5'cacctcaaaatgtgttaggatctg 3'
dual labelled probe	Universal ProbeLibrary Probe #53, Roche
Foxp3	
forward primer	5' cttccttgaaccccatgc 3'
reverse primer	5' gagggtgccaccatgacta 3'
dual labelled probe	Universal ProbeLibrary Probe #44, Roche
Hsd3b1	
forward primer	5' tcttcggtgtcactcacagag 3'

reverse primer	5' ggcacactagcttggacaca 3'
dual labelled probe	Universal ProbeLibrary Probe #17, Roche
IL10	
forward primer	5' gatgccttcagcagagtgaa 3'
reverse primer	5' gcaacccaggtaacccttaaa 3'
dual labelled probe	Universal ProbeLibrary Probe #67, Roche
IFNγ	
forward primer	5' ggcattttgaagaattggaaag 3'
reverse primer	5' tttggatgctctggtcatctt 3'
dual labelled probe	Universal ProbeLibrary Probe #21, Roche

2.1.8 Antibodies

Biolegend	Cytoflex S	**Th1 and Th2**	**Th17 and Treg**
FITC	FITC	**GATA3**	
PE	PE	**IL5**	**IL10**
PE/Dazzle	ECD	**TNFα**	CD25
PerCp Cy5.5	PC5.5		
PE/Cy7	PC7		
APC700	APC A 700	CD8	CD8
APC	APC	**IFNγ**	**Foxp3**
APC/Cy7	APC A 750	Zombie NIR	Zombie NIR
BV421	PB 450	CD4	CD4
BV510	KO 525	CD3	CD3
BV605	Violet 610		**IL17**
BV 650	Violet 660		
BV785	Violet 780		

Table 2.1: FACS panel for Th1/Th2 and for Th17/Treg staining

Antibodies with corresponding light response; intracellular markers are highlighted in bold.

2.2 Methods

2.2.1 Study population

The study protocol was approved by the ethics committee of the Institutional ethic committee of CERMEL in BP 242 Lambaréné, Gabon, (CEI-007/2017) and the Ethikkommission der Fakultät für Medizin der Technischen Universität München, Ismaninger Straße 22, 81675 München, Munich, Germany, (22.11.2013, project number 385/13) The study took place at two sites. First, between June and August 2015 and June and August 2017 at the Klinikum Rechts der Isar, Munich, Germany, where 57 pregnant women were screened and samples of 47 participants as European non-endemic control group were collected. All mothers were Caucasians. As the samples were all collected in Germany, I call them “German samples”. The second population consisted of 59 primiparous and multiparous women living in the province of Moyen-Ogooue, Gabon, in central Africa and delivering their newborns between August and November 2017 either at the Hôpital Albert Schweitzer or the Centre Hospitalier Regional Georges Rawiri in Lambaréné. In this case all mothers were Gabonese and live in the region for at least several years. I call these samples “Gabonese samples”. The purpose of the study and the procedures involved were explained and only those mothers granting written informed consent were enrolled as participants.

2.2.1.1 Inclusion criteria

The patients recruited for the study had to fit the following inclusion criteria:

- Age ≥ 18
- Single pregnancy
- Legally competent and mentally fit to understand and follow the instructions of the study personnel
- Signed patient education and informed consent
- Adequate bone marrow reserve with peripheral granulocyte numbers > 1500/µl and thrombocyte numbers > 40.000/µl
- No status post recurrent thrombosis or lung embolism
- No severe salience during physical examinations
- Documentation of known anaphylaxis
- Spontaneous birth at term (≥ 37 gestational weeks)

2.2.1.2 Exclusion criteria

Patients who met any of the following criteria of exclusion were not recruited for the study:

- Knowing of any of the following autoimmune diseases: Anti-Phosphlipid (antibody) syndrome, Goodpasture-Syndrome, rheumatoid arthritis, Lupus erythematodes, Sarcoidosis, ANCA Syndrome, Scleroderma, chronic polychondritis
- Knowing of any of the following immunosuppressive diseases: X-chromosomal A-y-Globulinemia, severe combined immunodeficiency (SCID), common variable immunodeficiency (CVID), selective IG A Deficiency
- Known Hepatitis B and/or C, HIV, HSV, CMV, Syphilis, Toxoplasmosis infection
- Other severe and active infections
- Current treatment with corticosteroids
- Requiring transfusion anemia
- Any other disease or medical treatment, that according to the principal investigator speak against an inclusion (e.g. Preeclampsia, HELLP-Syndrome)
- People with dependent or employment relationship to sponsors or investigators
- Known current or former cancer disease

Finally, a study population as described below was recruited.

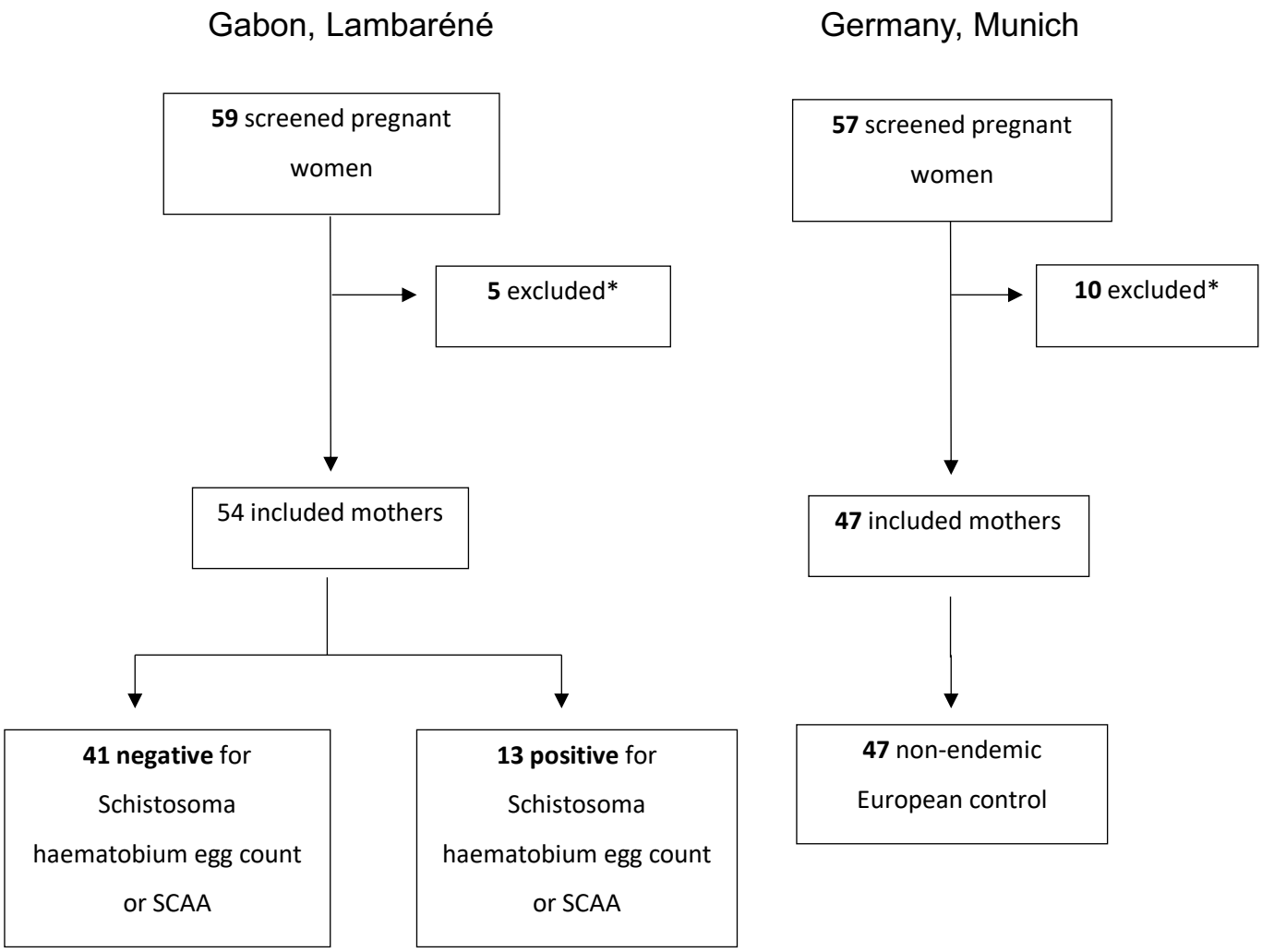


Figure 2.1: Total study population

* Exclusion criteria such as caesarean section, acute severe disease or autoimmune diseases, see above (chapter 2.2.1.2) for more detail;

2.2.2 Maternal and fetal data concerning health and birth taken via standardized questionnaire

- Maternal age, ethnicity, health condition including blood pressure, present diseases or a blood panel
- Chronic diseases of the mother (e.g. allergies, asthma), allergy status of the father and older siblings
- Details of former pregnancies, parity, gravidity and the course of the actual pregnancy including abnormalities
- Supplements (especially vitamin D) and medication during pregnancy
- Gestational age at birth, birth mode and medication or salience during birth
- Child's data: sex, birthweight, size, Apgar score, umbilical cord blood pH (only in Germany)

2.2.3 Work flow of the study

The same study flow was performed as shown below. All was done similar at both sites. Additionally, for Gabonese samples microbiological diagnostic was done and the CAA, as well as AWA specific IgE and IgG4 levels were analyzed.

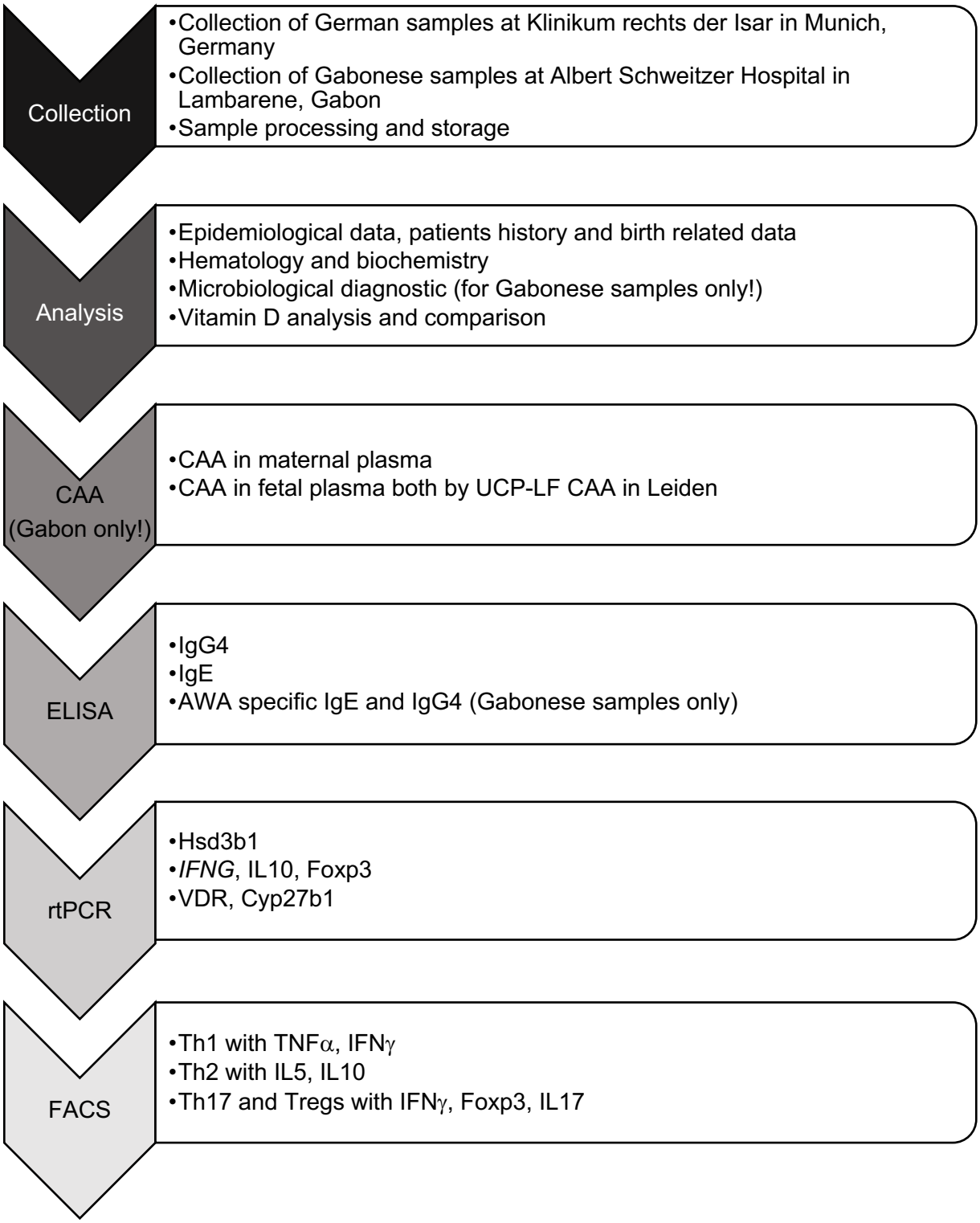


Figure 2.2: Study flow and summary of the data and values gained through the patient's characteristics and analysis of the serum and placenta samples

The figure shows the study flow for both sites or only Gabonese samples where indicated.

2.2.4 Sample collection

Of all patients included in the study, the following sample material was collected. The boxes highlighted in blue indicate samples or analysis, respectively, done only with Gabonese samples.

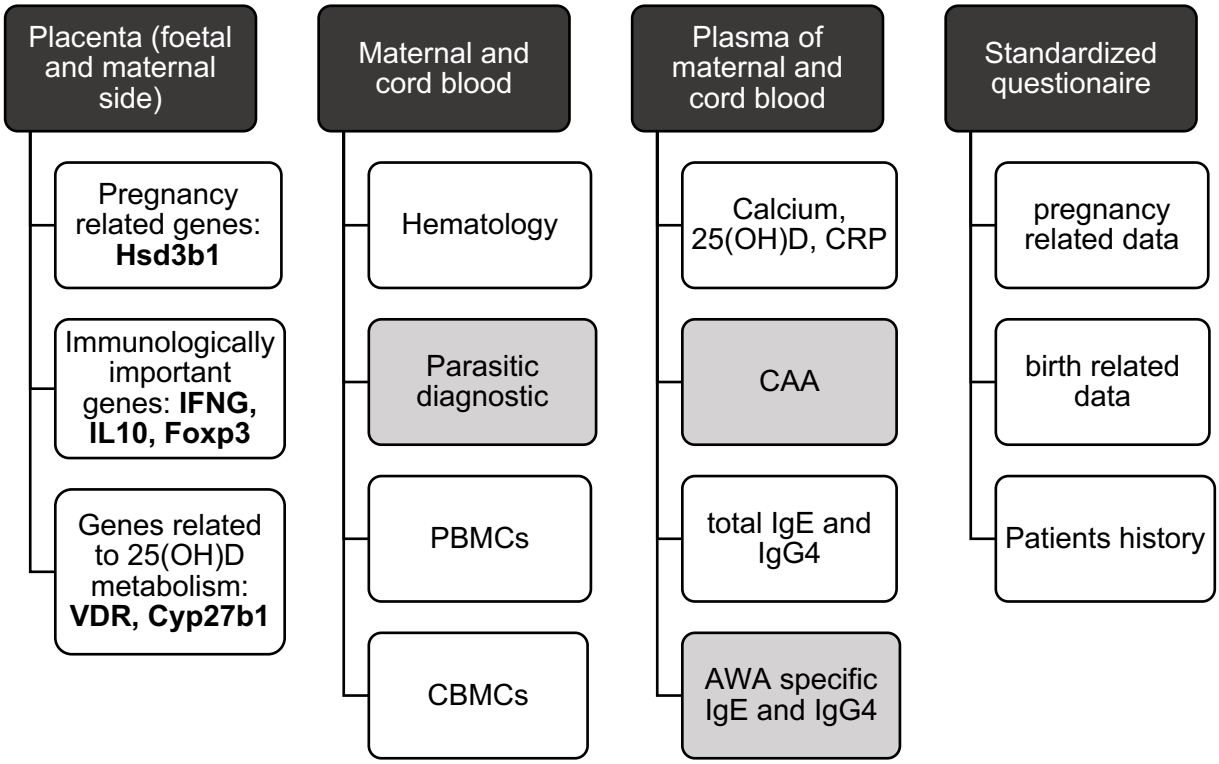


Figure 2.3: Sample processing

Demonstrating the processing of each sample. Protocols only performed at Gabonese samples are highlighted in light blue.

The respective samples were stored and further processed as summarized in Table 2.2.

	sampling timeframe	**processing/storage**
Maternal and fetal placenta samples		• Stored in 700µl RNA later at 4°C for at least 24 hours up to 2 days

	Directly after the delivery of the placenta	• Transferred to freezer (-20°C)
NH_4 Heparinized maternal blood	Preferably before birth while mother was in the delivery room, if not possible directly after birth	• Stored at room temperature for up to 8 hours • Plasma isolation and PBMC/CBMC isolation/cryopreserve-tion, see chapter 2.2.7
NH_4 Heparinized cord-blood	Directly after umbilical cord clamping	• Stored at room temperature for up to 8 hours • Plasma isolation and PBMC/CBMC isolation/cryopreservation, see chapter 2.2.7

Gabonese samples only:

Maternal EDTA blood	Preferably before birth while mother was in the delivery room, if not possible directly after birth	• Immediately transfer to laboratories for parasitic / microbiologic diagnostics, hematology and clinical chemistry
One to three urine samples	First sample preferably before birth, if not possible directly after birth; other samples on two following days	• Immediately transfer to laboratory for *S. haematobium* diagnostic • Stored at -20°C and transfer to Germany
One stool sample	During birth or within 14 days after birth	• Immediately transfer to laboratory for geohelminth diagnostic • Stored at -20°C and transfer to Germany

Table 2.2: Summary of sampling process and storage

2.2.4.1 Blood collection

Paired umbilical cord and maternal peripheral venous blood samples were collected within minutes of delivery. To avoid contamination with maternal blood at sampling, cord blood was taken by direct needle (21 gauge) aspiration from the cord vein after cleaning the umbilical cord. All blood samples collected in 9ml NH_4-Heparin tubes for further PBMC and CBMC processing and for plasma separation. CBMCs and PBMCs were stored in liquid nitrogen. Plasma (2,5 mL) was separated by centrifugation and frozen at – 20°C. Moreover, EDTA blood for parasitology diagnostic and for clinical analyses was taken at the same time point and immediately analyzed.

2.2.4.2 Placenta sample collection

Placenta tissue from both, maternal and fetal side, was collected. For the maternal side about 0,5 cm^3 directly from a macroscopic not calcified region in the middle of the placenta was taken. For the fetal samples, the choric plate was prepared away to take a 1 cm^3 sample from the intervillous space (Figure 2.4). The tissue was put into labelled cryotubes containing 500µl RNAlater® solution to stabilize placental RNA. The samples were stored at 4°C for at least 24 hours and then transferred to -20° for long time storage and shipment on dry ice to Munich, Germany.

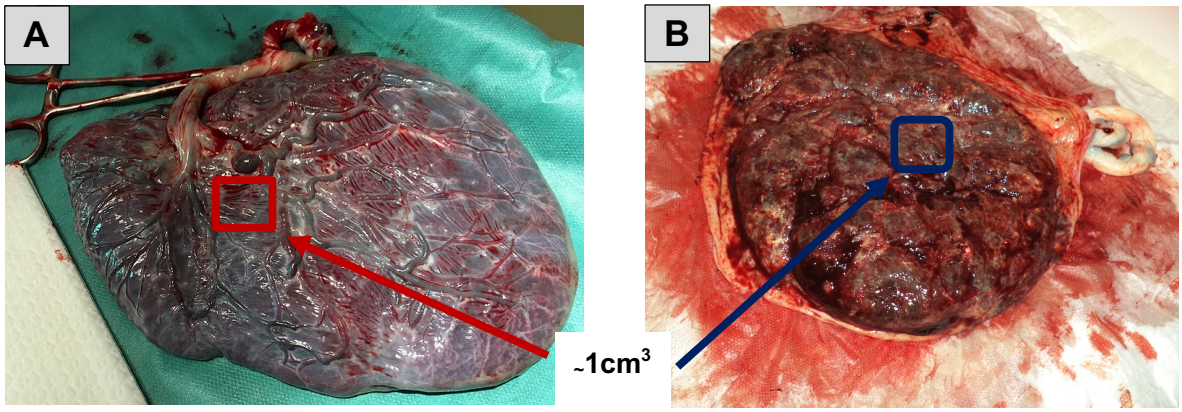


Figure 2.4 Placenta sampling

(A) Fetal side of the placenta; close to the umbilical cord in the the white shining choric plate was prepared away to take a 1 cm^3 sample from the tissue below. **(B)** Maternal side of the placenta; from the middle of the placenta 1 cm^3 tissue, which was macroscopic not calcified, was taken. *Picture addapted from* [56].

2.2.5 Parasitological examinations

Thick blood smears of maternal peripheral blood were stained with Giemsa and examined microscopically for plasmodia and filaria. Gabonese mothers also provided at least one urine

sample for *Schistosoma haematobium* egg-count by microscopy; where possible the samples were frozen at -20°C for shipment and further analysis. Furthermore, levels of schistosome circulating antigen (circulating anodic antigen [CAA]) were measured in cord and maternal peripheral plasma as well as in urine of Gabonese participants by enzyme-linked immunosorbent assay as described [136]. A total number of 13 Gabonese women were positive for *Schistosoma haematobium* by egg count and/or CAA in plasma.

Of the Gabonese participants, 30 women provided stool samples, which were prepared with standard methods for microscopic examination to determine the presence of intestinal helminth infections (Kato Katz, copro culture, McMaster, etc.). Microbiologic examination of the Gabonese mothers was only performed at the time of delivery.

2.2.6 Determination of CAA in maternal and fetal plasma samples

The following describes the detection of Schistosoma adult worm circulating anodic antigen (CAA) in serum or urine using an up-converting phosphor-lateral flow (UCP-LF) assay. The tool is able to recognize very low worm numbers and to be performed with randomly collected single urine samples. It consists in a dry-reagent format, detects up to 0,1 pg/ml CAA and providers claim a sensitivity and specificity of about 100% each [52, 58-61, 262, 268].

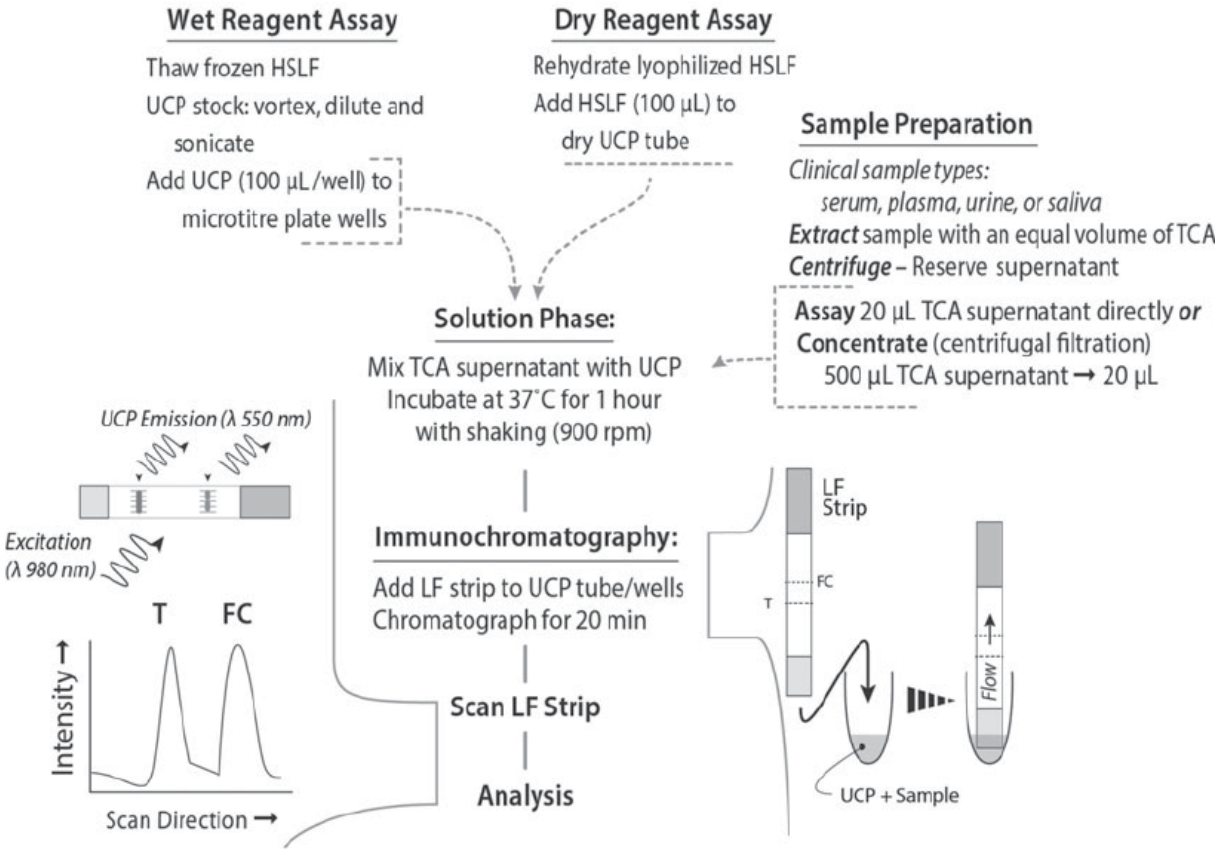


Figure 2.5 Schema of UCP-LF antigen detection (CAA and/or CCA)

The figure summarizes the steps to perform UCP-LF in detail. Figure adapted from [59].

The procedure is furthermore described elsewhere [132] and a workflow as an example for CAA detection on urine is again summarized in the graph below:

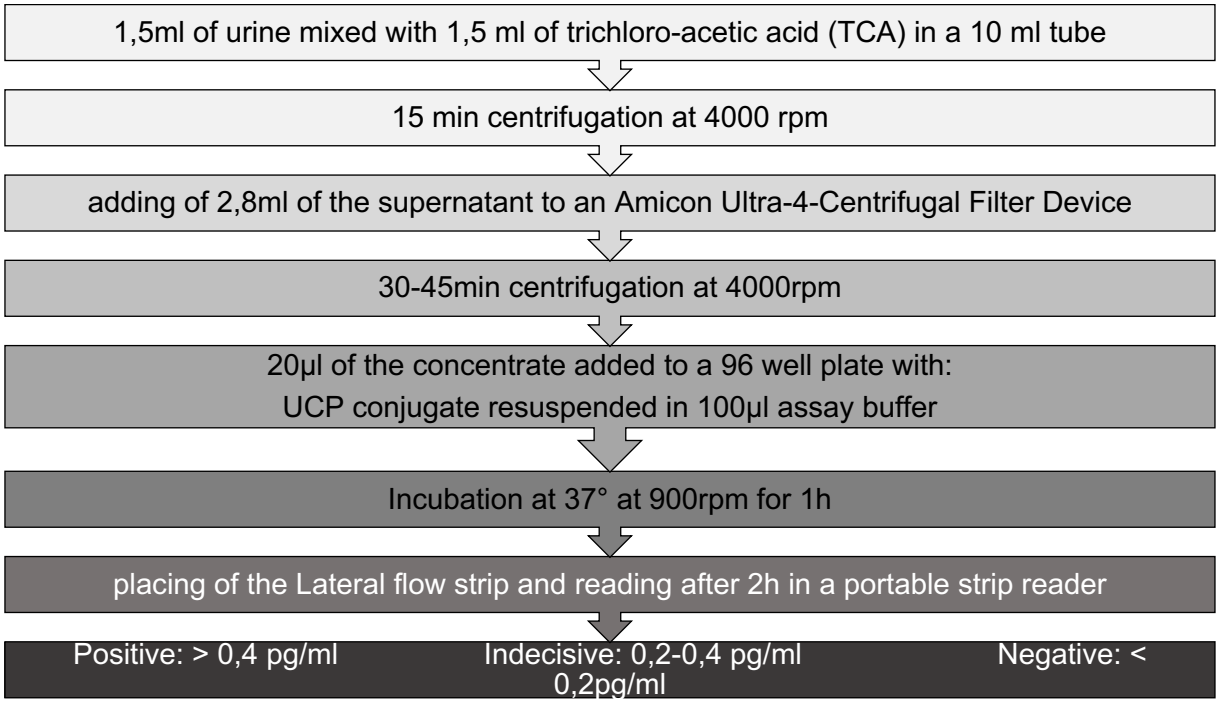


Figure 2.6 UCAA 2000 procedure

The procedure of UCAA as an example of the CAA-UCP-LF. *The figure was sketched single-handed, adaptend from* [132].

For this study levels of schistosome circulating antigen (circulating anodic antigen [CAA]) were measured in cord and maternal peripheral plasma as well as in urine utilizing an immunochromatography based assay [61]. The lateral flow (LF) test applies luminescent upconverting particles (UCP) to quantitatively measure CAA levels with a 10 pg/mL lower limit threshold, analyzing respectively 20 or 10 µL plasma or urine [59].

2.2.7 Isolation and cryopreservation of human peripheral blood mononuclear cells (PBMC)/Cord blood mononuclear cells (CBMC)

For the isolation and following cryopreservation of PBMCs the standard operating procedure of Julia Albrecht, Helmholtz Zentrum München was used. For the isolation heparinized maternal blood and cord blood were collected and handled as described in Table 2.2. Before the actual PBMC/CBMC isolation, plasma was preserved after centrifugation of heparinized

blood and stored at -20°C for further processing. The following isolation was achieved via Ficoll gradient, graphically demonstrated in Figure 2.7. In brief, blood was added in previously prepared leucosep tubes, filled with Ficoll. After centrifugation, the upper plasma layer was carefully removed and only the leukocyte layer, containing the white blood cells, was further used. After two washing steps with PBS and RPMI complete medium and following centrifugation, the cell pellet was re-suspended in 2 ml of RRMI complete medium and cells were counted either manually in a Neubauer hemocytometer or automatically with the Vi-CELL ® Cell viability Analyser (Beckman Coulter) (see chapter 2.2.11.2). After a last centrifugation step the cells were resuspended in 1ml freezing media (10 % DMSO in FCS) per 1x10^7 cells, aliquoted in cryotubes with a maximum of 1x10^7 cells/cryotube and stored in Mr Frosty® at -80°C for slow cooling down for the next 24 hours. Finally, the cryotubes were transferred to liquid nitrogen until further processing. The same procedure was done by the me at both sites. Gabonese CBMCs and PBMCs were transported to Munich, Germany with a dry shipper, to maintain a temperature of maximum -156°C and there again stored in liquid nitrogen until further processing.

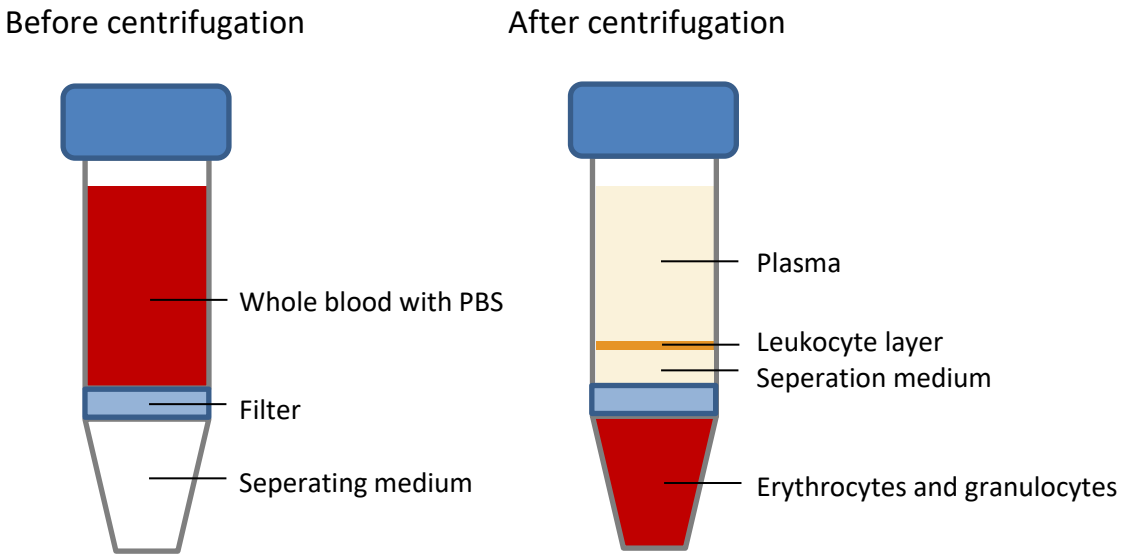


Figure 2.7: PBMC isolation via Ficoll gradient

Through usage of a filter and ficoll as high density medium, Erythrocytes and granulocytes get sucked under the filter after centrifugation whereas the leucocytes as cells of interest form a layer above the filter, inbetween the seperation medium and Plasma.

2.2.8 Biochemical analysis

Concentrations of 25(OH)D were measured by the laboratory for clinical chemistry at the Klinikum rechts der Isar using the Diasorin assay (Diasorin, Stillwater, MN, USA). The same laboratory measured CRP and Calcium levels in all plasma samples.

2.2.9 Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

The workflow presented in Figure 2.8 was followed and the listed material was used for the preparation and implementation of the qRT-PCR.

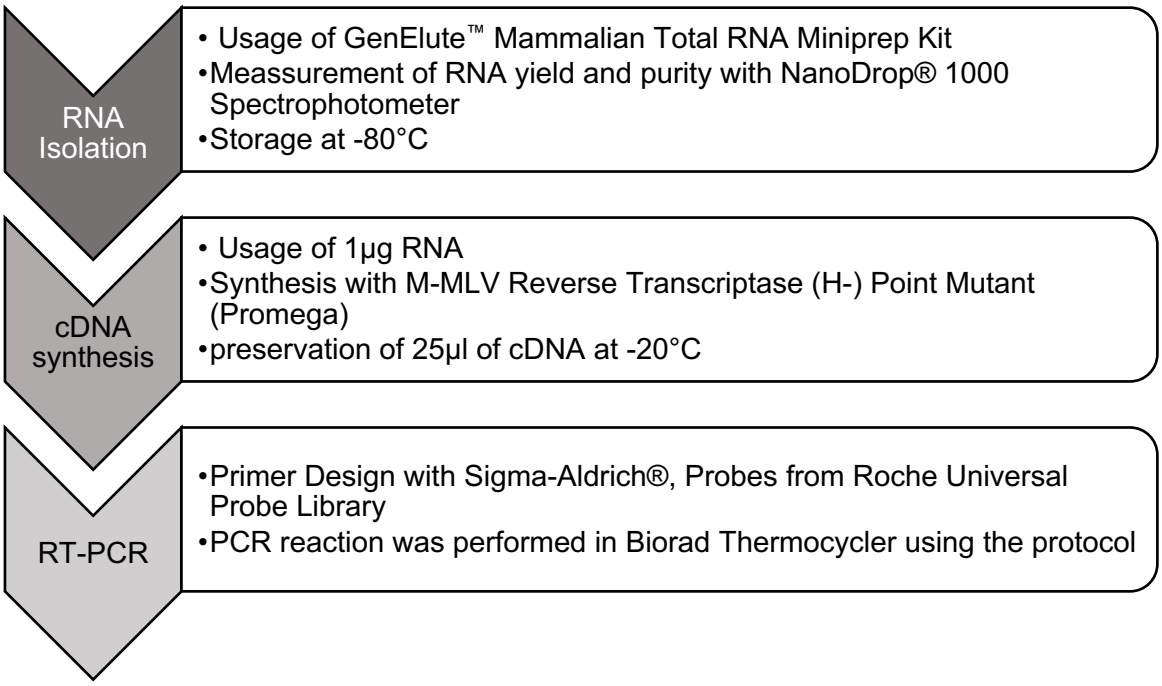


Figure 2.8: Workflow and material for qRT-PCR

2.2.9.1 RNA isolation

Samples were thawed for the first time. RNA was isolated from the fetal and maternal placenta samples using GenElute™ Mammalian Total RNA Miniprep Kit. The method was performed as recommended by the manufacturer. In brief, approximately 40 mg of tissue was cut from the placenta sample and homogenized in 600 µl Lysis buffer using Precellys® Tissue homogenizing CKMix – 2 mL and Precellys®24 tissue homogenizer. The lysate was transferred on a DNA Removing Column. After centrifugation at 12.000*g for 1 minute at room temperature, the flow through was placed in another 1,5 ml Eppendorf tube. After addition of an equal amount of 70% Ethanol and vortexing, the solution was loaded on a Perfect Bind RNA Column. Flow-through was discarded after another round of centrifugation. After two washing steps with 500 µl Wash Buffer I and 600 µl Wash Buffer II, the RNA Column was dried

by centrifugation for two minutes at 10.000*g. Finally, the RNA was eluted with 50 µl of RNase-free water. RNA yield and purity were measured using a NanoDrop® 1000 Spectrophotometer. The resulting RNA was stored at -80°C until further processing.

2.2.9.2 cDNA synthesis

cDNA was prepared following the Promega usage information for first-strand cDNA synthesis with M-MLV RT (H-) Point Mutant. In short, 1 µg of RNA and 1 µl of Oligo$(dT)_{15}$ were mixed with DEPC-treated water to a total volume of 14 µl. The reaction tubes containing the reaction mix were heated in a thermocycler to 70°C for 5 minutes and then quickly cooled on ice for 5 minutes.
A master mix for the subsequent syntheses was prepared as follows:

Component	Volume
reaction buffer	5 µl
nucleotide mix	1,25 µl
reverse transcriptase	1 µl
water	3,75 µl

After cooling, 11 µl of the master mix were added to each tube, resulting in a total volume of 25 µl. The following protocol was used for the synthesis within a thermocycler: 40°C for 10 minutes, 42°C for 50 minutes (incubation), 70°C for 15 minutes (inactivation of reaction) and subsequent cooling to 4°C. The resulting cDNA was stored at -20°C until further processing.

2.2.9.3 qRT-PCR

qRT-PCR was performed to measure the relative concentrations of certain genes, using Roche Universal Probe Library and HPRT as house-keeping gene. The reaction master mix was prepared as follows:

Component	Volume
LightCycler® Probes Master (Roche)	5 µl
forward primer (10µM)	1 µl
reverse primer (10µM)	1 µl
probe	0,3 µl
water	11,7 µl

The LightCycler® Probes Master (Roche) is a ready-to-use reaction mix containing the Taq Polymerase and appropriate buffers.
After addition of 19µl of the respective reaction master mix in each well of a 384 well plate, 1µl of the particular cDNA was added. The following qRT-PCR was performed in a Thermocycler C 1000 (Bio-Rad) using the following protocol:

	Temperature (°C)	time	
Hot start	95	10 min	
Denaturation	95	10 sec	44 cycles
Annealing	60	30 sec	
Elongation	72	15 sec	
cooling	45	10 sec	
storage	12	∞	

2.2.10 Enzyme-linked immunosorbent assay (ELISA) for detection of total and AWA specific IgE and IgG4 levels

2.2.10.1 Total IgE and IgG4

Cord and maternal plasma samples were transported on dry ice to Munich, Germany, where measurements were performed. Levels of total IgE and IgG4 were measured in all European and African maternal and cord plasma samples using the Invitrogen IgE, or IgG4 Human ELISA Kit, respectively, according to manufacturer's instructions (Thermo Fischer). The plasma was diluted 1:10 for IgE measurement and 1:1000 for IgG4. Immediately after stopping the reaction, the plates were measured at 450nm and 570nm using the Sunrise^{TM} ELISA microplate reader. The cytokine concentration within the sample was then calculated according to the standard curve.

2.2.10.2 Anti-adult worm antigen (AWA) specific IgE and IgG4

Plasma samples were transported on dry ice to the Leiden University Medical Center in the Netherlands, for AWA specific IgE and IgG4. *S. haematobium* adult worm antigen (AWA) specific IgG4 and IgE were measured by ELISA modified from previous protocols [264]. Briefly, maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight with 5 mg/ml AWA diluted in carbonate buffer pH 9.6. For IgG4, the plates were blocked with PBS-5%BSA while for IgE with PBS-2%BSA. The plasma was diluted in PBS-0.05%Tween-5% FCS (Fetal calf serum) or Tris-HCl–0.05% Tween for IgG4 and IgE ELISA, respectively. The presence of IgG4 was shown by using HRP-labelled anti human IgG4 (1:3000) (Sanquin, Amsterdam, the Netherlands). For IgE assay, the detection antibodies used were biotinylated goat anti human IgE (1:1000; Vector Laboratories, Burlingame, CA, USA) followed by streptavidin HRP conjugate (1:10000; Sanquin, Amsterdam, the Netherlands). The assays were developed with tetramethylbenzidine (TMB) and stopped with 10% H2SO4. Absorbances were measured at 450 nm. The levels of antibody present in a given sample are expressed in arbitrary units (AU/ml) according to the standard curve of pooled positive *S. haematobium* plasma.

2.2.11 Fluorescence-activated cell sorting

2.2.11.1 Thawing of cryopreserved human PBMCs and CBMCs

The samples were thawed for the first time. After taking the tubes to the laboratory, the tubes were wiped with 70% ethanol. Below a biosafety hood, the cap was quickly twisted a quarter-turn to relieve pressure and then it was retightened again. The temperature in the cryovial that contains the PBMCs and CBMCs was rapidly raised to 37°C by placing it in a water bath for 1-3 min until a tiny ice-crystal was left. The cryovial was wiped again with 70% ethanol. The content was quickly tipped into 50ml falcon tubes, which contained 15-40ml warm (37°C) RPMI Culture Medium. The culture medium consists of 500 ml RPMI, 50ml FCS, 5 ml Penicillin-Streptomycin, 5ml Sodium Pyruvate, 5ml Non-essential Amino Acids and 550µl Beta-Mercaptoethanol.

Special care has been taken to ensure that all tubes are correctly labelled. To recover the residual cells from the cryovial, 0.5 - 1ml of warm (37°C) RPMI Culture Medium was pipetted into each cryovial, aspirated, and added to the rest of the cells.

The cell suspension was centrifuged at room temperature RT (18 - 25°C) at 350 g for 10 min with rapid acceleration and brake on high. The supernatant was decanted, and the cell pellet carefully resuspended by tapping the tube (by avoid pipetting or vortexing). 10 ml of warm RPMI Culture Medium was added and the cells mixed by flipping the tube twice 180° with the cap tightly closed. The cell suspension was centrifuged again at room temperature RT (18 - 25°C) at 350 g for 10 min with rapid acceleration and brake on high. Again, the supernatant

was decanted, and the cell pellet carefully resuspended by tapping the tube (by avoid pipetting or vortexing). 10 ml of warm RPMI Culture Medium was added and the cells mixed by flipping the tube twice 180° with the cap tightly closed. Afterwards the cap was opened slightly for resting in the incubator overnight. Cells were counted and plated the next morning as described below.

2.2.11.2 Cell counting and plating

Cells were counted either manually in a Neubauer hemocytometer or automatically with the Vi-CELL ® Cell viability Analyzer (Beckman Coulter). A small aliquot of the cell suspension was added to Trypan blue solution in a dilution 1.10 or 1:100, to further distinguish between dead and viable cells. Total living cells were then counted in 16 quadrants of the grid. The cell titer (cell/ml) of the original suspension was then calculated using the following formula:
cell/ml = counted cells x dilution factor (Trypan blue dilution) x 10^4 x ml^{-1}.
A cell suspension of 150µl containing 150 000 to 300 000 cells diluted in culture medium were plated in 96 well plate. Special care has been taken, that samples of each group was represented on each plate.

2.2.11.3 Stimulation and staining

Per Th subtype one well was used for stimulation and one without stimulation. For each intracellular antibody one more well was plated with mixed cells and cells were colored with a mastermix not containing the certain intracellular antibody as a control.
For ICS, non-specific stimulation was performed with PMA/Ionomycin for 6 hrs. in presence of BFA for the last 4 of culture: 50µl containing 100ng/ml PMA and 1µg/ml Ionomycin was added to the wells meant to be stimulated. To all the other 50µl culture medium was added. After two hours later 50µl of 10 µg/ml EC of BFA in RPMI culture medium was added to all wells for 4 hours. The plate was centrifuged (5min @ 1300 RPM, 4°C) and the S/N discarded. Then 150µl FACS buffer was added into wells containing cells and washed once (5min @ 1300 RPM, 4°C), S/N was discarded. The hole stimulation was done sterile.
The **live/dead staining** was performed with ZOMBIE NIR. ZOMBIE NIR was prepared in a 1:1000 dilution in PBS and 100µl was added per well. The plate was covered and incubated at 4°C in the dark for 20min. All wells were diluted with additional 100µl FACS BF and centrifuged for 5min at 1300 RPM at 4°C. After discarding the S/N the for **surface marker staining** prepared dilutions of fluorochrome-labelled antibodies in FACS BF (see Table 2.3 and Table 2.4) were added per well (50µl/well). Moreover, one well was left as control and one as Zombie single color. Single colors for further compensations were done with beads.

FITC	FITC	**GATA3**	
PE	PE	**IL5**	**IL10**
PE/Dazzle	ECD	**TNFα**	CD25
PerCp Cy5.5	PC5.5		
PE/Cy7	PC7		
APC700	APC A 700	CD8	CD8
APC	APC	**IFNγ**	**Foxp3**
APC/Cy7	APC A 750	Zombie NIR	Zombie NIR
BV421	PB 450	CD4	CD4
BV510	KO 525	CD3	CD3
BV605	Violet 610		**IL17**
BV 650	Violet 660		
BV785	Violet 780		

Figure 2.9 Colour-panel for flow cytometry

Th1, Th2				
		dilution	**in 50µl**	
Surface	CD3	1:400	0,125	µl
	CD4	1:400	0,125	µl
	CD8	1:400	0,125	µl
	Medium		49,625	
		dilution	**in 100µl**	
Intracellular	GATA3	1:200	0,5	µl
	IL5	1:150	0,67	µl
	TNFα	1:200	0,5	µl
	IFNγ	1:150	0,67	µl
	Medium		97,66	

Table 2.3: Panel and dilution for Th1-, Th2- cells FACS staining

Th17, Treg			
		dilution	**in 50µl**

Surface	CD3	1:400	0,125	µl
	CD4	1:400	0,125	µl
	CD8	1:400	0,125	µl
	CD25	1:50	1	µl
	Medium		48,625	
		dilution	**in 100µl**	
Intracellular	IL10	1:150	0,67	µl
	IL17	1:150	0,67	µl
	Foxp3	1:200	0,5	µl
	Medium		98,16	

Table 2.4: Panel and dilutions for Th17-, Treg cells FACS staining

Plates were incubated for 25 min at 4°C. Afterwards they were diluted with 100µl FACS BF per well, centrifuged for 5min at 1300 RPM and 4°C and the S/N was discarded. The washing was repeated once. For the **permeabilization/fixation** 100µl BD Cytofix/Cytoperm was added to the wells stained with the Th1/Th2 surface master mix and 100µl eBioscience FoxP3 intracellular perm solution was added to the wells stained with the Th17/Treg surface master mix. Parafilm was carefully wrapped around the plates and they were incubated overnight in the fridge.

The next morning the plates were centrifuged for 5min at 1300 RPM and 4°C and the S/N was discarded. One washing step with 100µl was performed with the corresponding wash buffer (BD or eBiocience)

The required dilution of antibodies for **intracellular staining** were prepared in corresponding wash buffer (see Table 2.3 and Table 2.4) and 100µl was added per well. The plates were incubated for 30 min in the fridge and afterwards diluted in 100µl corresponding wash buffer and centrifuged for 5 min at 1300 RPM and 4°C. The S/N was discarded, and the washing step repeated once.

The final cell pellet was resuspended in 200µl FACS BF per well and analyzed immediately with the Cytoflex S (Beckman Coulter).

2.2.12 Statistical analysis.

Statistical analysis was performed using SPSS for Windows version 23.0 (SPSS Inc., Chicago, IL) and with PRISM® 5.01 (GraphPad Software Inc., San Diego, CA, USA). D'Agostino and Pearson omnibus normality tests were performed, and parametrically distributed data was

analyzed with unpaired T test (2 groups) and two-tailed Mann-Whitney-Test was used for non-parametric data. Statistical dependence was analyzed by Spearman's rank correlation coefficient. Categorical data were analyzed by Pearson chi-squared test. Data are presented as median ± interquartile range (IQR) or median ± standard deviation (SD).

In all cases, statistical significance was assumed with a p value < 0.05 and where significance level was reached, p values are indicated in individual graphs.

3. Results

3.1 Differences in maternal and neonatal characteristics between Gabon and Germany

	Gabon	Germany	p value
Number n	54	47	
Mother			
Age [years]	26 ± 7	33 ± 5	**< 0.0001**
Parity	2 ± 2	1.7 ± 0.7	0.51
Gravidity	4 ± 2	2.1 ± 1.5	**< 0.0001**
Abort	0.62 ± 0.86	0.52 ± 1.2	0.15
Gestational age at delivery (weeks)	39.3 ± 2	39.7 ± 1.2	0.20
Hemoglobin [g/dL]	10.6 ± 2.21	12.15 ± 1.11	**0.0011**
White blood cells [10^3/mm^3]	11.81 ± 5.15	13.11 ± 3.82	0.26
Eosinophils [%]	1.91 ± 1.91	Not done	
25-OH-Vitamin D3 concentration [ng/mL]	34.5 ± 8.8	23.9 ± 13.8	**<0.0001**
Calcium [mmol/L]	2.29 ± 0.12	2.31 ± 0.11	0.42
CRP [mg/dL]	1.09 ± [0.63;2.17]	0.63 ± [0.24;1.13]	**0.0022**
Newborn			
Gender (male)	30 (56%)	22 (47%)	0.38
Length [cm]	50 ± 2	53 ± 3	**<0.0001**
Birthweight [g]	3076 ± 510	3331 ± 544	**0.0012**
Low birth weight (<2500g)	8 (15%)	1 (2%)	**0.025**
25-OH-Vitamin D3 concentration [ng/mL]	36.0 ± 8.64	28.94 ± 15.66	**0.0011**
Calcium [mmol/L]	2.66 ± 0.22	2.79 ± 0.15	**0.0004**
CRP [mg/dL]	0.01 ± [0.01;0.02]	0.01 ± [0.01;0.02]	0.56

Table 3.1: Maternal and newborn characteristics in Gabon and Germany

Data are presented as mean ± SD (n), median ± IQR (n) for C-reactive Protein (CRP) and ordinally scaled values, values or as numbers (%) where indicated; t-test was performed where data are normal distributed; for data without normal distribution a two-tailed Mann Whitney U-test and for ordinally scaled values a Chi Square test was performed; blood for plasma parameters was taken from maternal peripheral vein blood or from cord blood, respectively;

For the study, 54 pregnant women in Gabon, and 47 in Germany, fulfilled all inclusion criteria and were successfully included into the study (Table 3.1).

At birth obstetric data related to maternal health, pregnancy, birth, and neonatal health were taken (Table 3.1). Significant differences between the two study populations emerge not only between the mothers but also between the newborns. Gabonese mothers were on average seven years younger than the mothers in the European control cohort. Both cohorts had the same amount of deliveries and no significant difference of total aborts, whereas the number of gravidities was significant higher in Gabon. In Gabon and in Germany, the mothers delivered their newborns on average at 39 weeks of gestation. That Gabonese mothers were more anemic is shown by a significantly lower hemoglobin concentration (1.55g/dL) within the Gabonese cohort, whereas there is no significant difference in the amount of white blood cells between Gabonese and German mothers. As the percentage of eosinophils was measured only in Gabonese plasma samples, no comparison with the German cohort was possible. The 25(OH)vitamin D3 concentration in Gabonese plasma was significantly higher than in German plasma samples, both in the maternal plasma and in cord blood plasma.

The levels of the C-reactive Protein (CRP), which plays an important role in inflammatory responses, were significantly higher in Gabonese maternal plasma samples than in German samples. In cord blood, CRP was almost non-detectable, which was expected, as CRP normally does not cross the placental barrier. Anyhow, the duration of labor may influence CRP levels in maternal blood (see below 3.2).

There was no difference in gender distribution of neonates between both countries. Gabonese newborns were, however, significantly smaller (3cm) and on average 255g lighter than their German counterparts. In Germany only one and in Gabon even 8 (in total 15%) newborns suffered from low birth weight applying WHO definition (birth weight of less than 2500g) [62]. Furthermore, the Calcium concentration in Gabonese cord blood plasma was significantly lower compared to plasma levels in the German cord blood samples, whereas no difference in Ca concentration was observed between plasma samples from Gabonese and German mothers.

Within the Gabonese cohort, where participants with and without infection with *S. haematobium* were compared, the infection status did not influence any of these parameters significantly (see Table 3.2 below).

	Gabon		p value
	Negative for *Schistosoma haematobium*	**Positive for *Schistosoma haematobium***	
Number n	41	13	
Mother			
Age [years]	26 ± 6	27 ± 5	0.66
Parity	2 ± 2	2 ± 2	0.84
Gravidity	4 ± 2.1	4 ± 1.6	0.69
Abort	0.69 ± 0.92	0.36 ± 0.50	0.42
Gestational age at delivery (weeks)	39.4 ± 2	39.0 ± 2	0.66
Hemoglobin [g/dL]	10.64 ± 2.36	10.42 ± 1.71	0.54
White blood cells [10^3/mm^3]	11.77 ± 5.47	11.94 ± 4.15	0.92
Eosinophils [%]	1.74 ± 1.86	2.40 ± 2.03	0.14
25-OH-Vitamin D3 concentration [ng/mL]	33.4 ± 8.7	37.8 ± 8.9	0.19
Calcium [mmol/L]	2.29 ± 0.12	2.26 ± 0.11	0.35
CRP [mg/dL]	1.05 ± [0.66; 2.25]	1.43 ± [0.28; 2.01]	0.76
Anti-helminthic treatment	34 (82%)	13 (100%)	0.11
Infection with other helminths	6 (15%)	2 (15%)	0.95
Antimalaria treatment	11 (26%)	4 (30%)	0.78
Malaria during pregnancy	4 (10%)	2 (15%)	0.57
Newborn			
Gender (male)	24 (59%)	6 (46%)	0.27
Length [cm]	50 ± 2.7	50 ± 2.2	0.56
Birthweight [g]	3054 ± 535	3145 ± 436	0.58
Birth weight (<2500g)	7 (17%)	1 (8%)	0.44
25-OH-Vitamin D3 concentration [ng/mL]	34.8 ± 7.9	39.6 ± 10.0	0.08
Calcium [mmol/L]	2.68 ± 0.15	2.61 ± 0.37	0.92
CRP [mg/dL]	0.01 ± 0.01	0.01 ± 0.01	0.50

Table 3.2: Maternal and neonatal characteristics of the Gabonese cohort

Data are presented as mean ± SD (n), median ± IQR (n) for C-reactive Protein (CRP) and ordinally scaled values, values or as numbers (%) where indicated; t-test was performed where data are normal distributed; for data without normal distribution a two-tailed Mann Whitney U-test and for ordinally scaled values a Chi Square test was performed; blood for plasma parameters was taken from maternal peripheral vein blood or from cord blood, respectively; S. haematobium diagnostics was done by egg count or detection of levels of schistosome specific circulating anionic antigen (CAA) in plasma samples.

Additionally, data about e.g. other helminthic diseases or anti-helminthic were collected in Gabon. In the group of negative tested for *S. haematobium* 34 (82%) mothers took anti-helminthic treatment such as Helmintox®, Mebendazole or Albendazole, which is part of pregnancy prophylaxis. At time of delivery, six mothers were infected with *Strongyloides* (1), *Ascaris* (1) or *Loa loa* (4), and two *S. haematobium* positive mothers were coinfected with *Hookworm* (1) or *Strongyloides* (1), whereas all of these mothers received anti-helminthic treatment. In total 15 mothers reported to have taken anti-malaria drugs (Combimal® (Pyrimethamine / Sulfadoxine 25/500 mg) or Quinine). Some mothers stated to have taken this for the purpose of pregnancy prophylaxis, although six mothers claimed an actual infection with *Plasmodium falciparum* during pregnancy. At delivery all mothers had a negative blood smear as malaria disease was an exclusion criterion.

To detect sickle cell disease, hemoglobin electrophoresis was performed for 43 Gabonese women. 8 were positive for sickle cell hemoglobin (HbS). None of these 8 women were infected with *S. haematobium* but one declared to have suffered from malaria during pregnancy. Regarding anemia and sickle cell disease, no significant difference could be observed.

3.2 Positive correlation between CRP levels and duration of labor in Germany

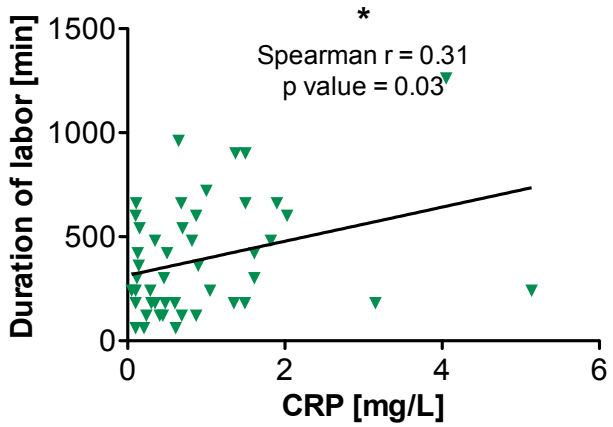


Figure 3.1 Correlation between CRP levels in peripheral plasma and the duration of labor in Germany

Correlation of maternal peripheral plasma CRP levels with the duration of labor in minutes. P values are for Spearman's rank-order correlation. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (Germany) = 46;

C reactive protein is common marker measured in blood to detect inflammatory processes. But its levels may also be influence by the duration of labor [152]. Indeed, there was a strong and significant correlation between the plasma levels and the duration in German women. Unfortunately, in Gabon corresponding data could not be recorded as delivery habits a different and the women came mostly already during labor at the hospital.

3.3 Higher prevalence of anemia in Gabon

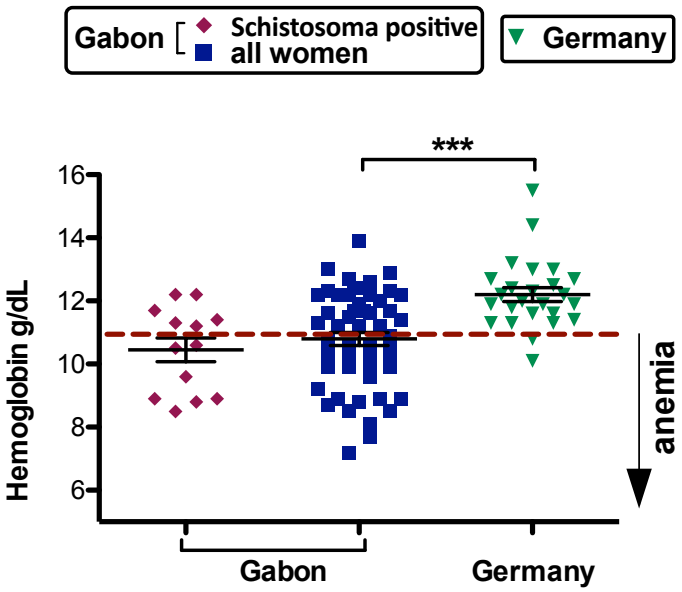


Figure 3.2 Hemoglobin concentrations in maternal peripheral blood

Data are presented as mean ± SD (n); t-test was performed; blood for plasma parameters was taken from maternal peripheral vein blood; S. haematobium diagnostics was done by egg count or detection of levels of schistosome specific circulating anionic antigen (CAA) in plasma samples. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; n (Schistomosa positive, Gabon) = 13; n (Gabon) = 54; n (Germany) = 25. The red line presents the cut off for anemia in pregnant women (11g/dL);

In the Gabonese cohort of all included women 27 from a total of 54 (50%) had hemoglobin levels below 11g/dL and suffered from anemia, independent from *S. haematobium* infection (7 out of 13 (54%) had anemia). In Germany data about hemoglobin concentrations were available from 25 women, of whom 2 had levels below 11g/dL, only 8%.

3.4 Assessment of *Schistosoma haematobium* infection

ID	R=T/FC	pg/mL	Egg count positive
M001	0,020	1	no
F001	0,024	2	
M002	1,691	**832**	**yes**
F002	0,030	3	
M003	0,019	1	no
F003	0,038	4	
M004	0,028	2	no
F004	0,000	0	
M006	0,026	2	no
F006	0,023	2	
M007	0,022	2	no
F007	0,038	4	
M008	0,155	**30**	**yes**
F008	0,021	1	
M009	0,022	2	no
F009	0,009	0	
M010	0,021	1	no
F010	0,019	1	
M011	0,042	5	no
F011	0,024	2	
M012	0,032	3	no
F012	0,017	1	
M014	0,019	1	no
F014	0,023	2	
M015	0,037	4	no
F015	0,025	2	
M013	0,026	2	no
F013	0,018	1	
M016	0,048	6	no
F016	0,029	3	
M017	0,044	5	no
F017	0,020	1	
M018	0,058	8	no
F018	0,000	0	

ID	R=T/FC	pg/mL	Egg count positive
M032	0,025	2	no
F032	0,043	5	
M033	0,024	2	no
F033	0,028	3	
M034	0,041	5	no
F034	0,030	3	
M036	0,255	**59**	**yes**
F036	0,048	6	
M037	0,028	2	no
F037	0,044	5	
M038	0,030	3	no
F038	0,022	2	
M039	0,370	**97**	**yes**
F039	0,020	1	
M040	0,035	4	no
F040	0,037	4	
M041	0,242	**55**	**yes**
F041	0,026	2	
M042	0,024	2	no
F042	0,023	2	
M043	0,031	3	no
F043	0,027	2	
M044	0,024	2	no
F044	0,031	3	
M045	0,022	2	no
F045	0,025	2	
M046	0,249	**57**	no
F046	0,044	5	
M047	0,026	2	no
F047	0,020	1	
M048	2,793	**1966**	no
F048	0,017	1	
M049	0,027	2	no
F049	0,019	1	

M020	0,014	1	no	M050	0,021	2	no
F020	0,020	1		F050	0,026	2	
M021	1,519	**704**	no	M051	0,045	5	no
F021	0,038	4		F051	0,027	2	
M022	0,640	**203**	**yes**	M052	0,029	3	no
F022	0,021	2		F052	0,015	1	
M026	0,126	**23**	no	M053	0,025	2	no
F026	0,024	2		F053	0,021	1	
M025	0,018	1	no	M057	0,076	**11**	**yes**
F025	0,038	4		F057	0,019	1	
M027	0,024	2	no	M054	0,025	2	no
F027	0,022	2		M055	0,018	1	no
M028	0,033	3	no	F055	0,013	1	
F028	0,024	2		M056	0,013	0	no
M029	0,051	7	no	F056	0,018	1	
F029	0,030	3		M071	3,129	**2454**	no
M030	0,028	3	no	F071	0,021	1	
F030	0,039	4		M072	0,706	**233**	no
M031	0,012	0	no	F072	0,015	1	
F031	0,023	2					

Table 3.3 Comparison of CAA concentration and result of egg count by microscopy

The table shows the concentration of CAA measured in peripheral maternal plasma or cord blood plasma in pg/mL. Maternal patients ID is taged with “M” and cord blood ID with “F”. Results were generated at Leiden University Medical Center and personal permission was kindly given.

As described in chapter 1.3 the diagnostics of schistosomiasis via microscopical egg count is sensitive mainly in high infection density settings. Furthermore, as all participants were recruited at time of delivery, getting useful urine samples was difficult in some cases and contaminated with blood. Therefore, a second tool, to verify and detect obscure infections was employed and plasma samples were analyzed with the CAA-UCP-LF assay at Leiden university. Interestingly, they detected six more women infected with Schistosoma. Finally, seven women were diagnosed positive by egg count, all of them also positive for elevated CAA levels. Additional six more women were diagnosed with an active infection at time of birth via the UCP-LF and were thus considered to belong to the cohort of infected women. In the end, a subgroup of 13 women was infected with *Schistosoma haematobium* at the time of screening.

In total 24% of all Gabonese women were infected with *S. haematobium* and using CAA testing increased the detection rate by almost 100%. Interestingly, no CAA was detected in cord blood, even if mothers had high levels of CAA.

3.5 Higher vitamin D plasma levels in the Gabonese cohort

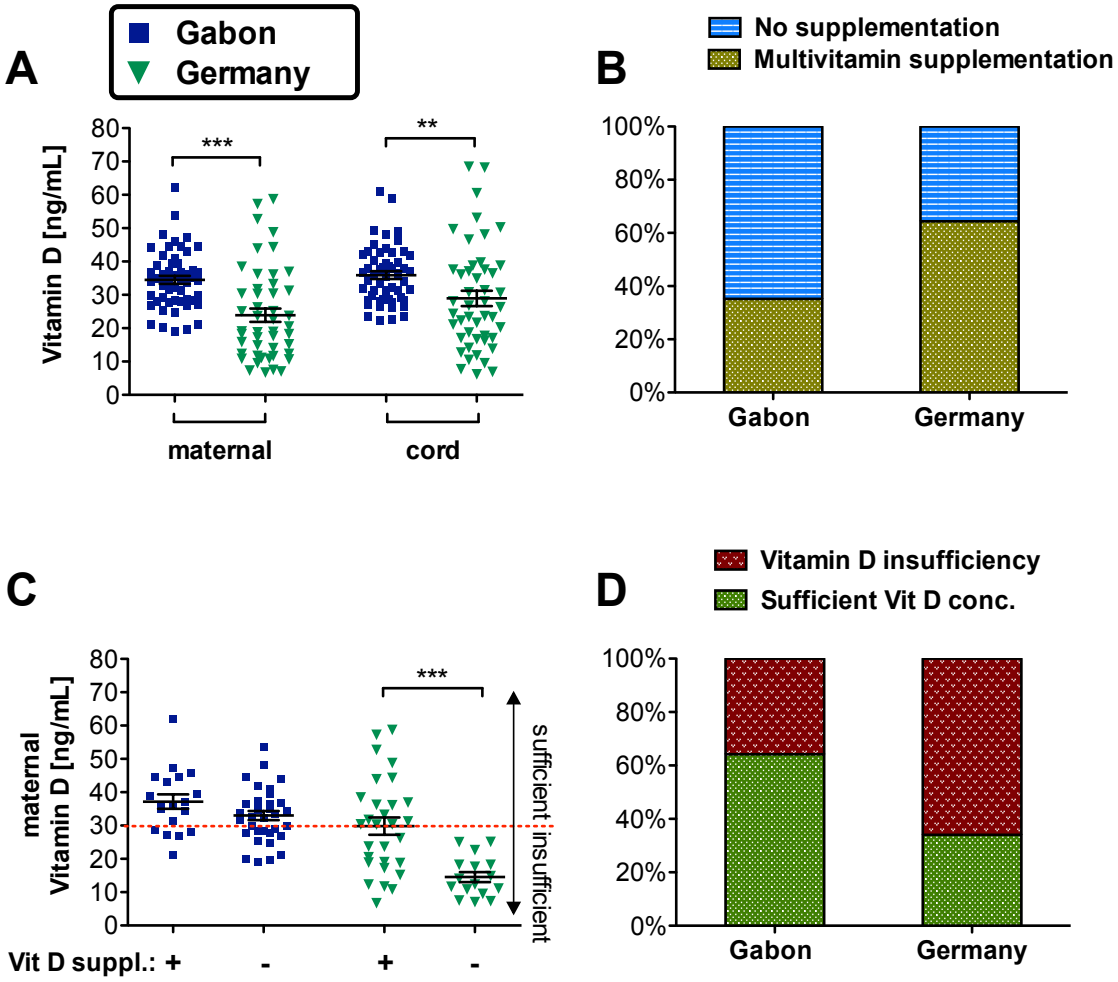


Figure 3.3 vitamin D concentration measured in plasma samples and its association to supplementation

Data are shown with mean and standard deviation. P values are for t-tests. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; n (maternal, Gabon) = 53; n (maternal, Germany) = 47; n (cord, Gabon) = 53; n (cord, Germany) = 47; n (25(OH)D suppl., Gabon) = 19; n (no suppl., Gabon) = 35; n (25(OH)D suppl., Germany) = 29; n (no suppl, Germany) = 16; n (sufficient 25(OH)D conc., Gabon) = 34; n (25(OH)D insufficiency, Gabon) = 53; n (sufficient 25(OH)D conc., Germany) = 16; n (25(OH)D insufficiency, Germany) = 47; **(A)** 25-OH-Vitamin D3 concentration in maternal and cord blood plasma. **(B)** Percentage of multivitamin supplementation containing vitamin D of Gabonese and German participants. **(C)** maternal Gabonese and German plasma 25-OH-Vitamin D3 concentration in association with vitamin D supplementation; the red line presents the cut off for sufficent vitamin D concentration. **(D)** Percentage of vitamin D sufficiency in maternal plasma samples; Insufficiency: < 30ng/mL.

Since sunlight amongst others has a strong influence on vitamin D levels and we furthermore understand that supplementation could affect vitamin D levels, vitamin D concentration in the plasma of mothers and the cord blood of their newborns was assessed. In Gabonese plasma samples the concentration, both, in maternal and cord blood was significantly higher than in German samples (Figure 3.3 A). In Gabon 35.2% took multivitamin supplements such as Ranferon®, Vicombil® or Vitafer® containing approximately 200-800 International Units (IU) of vitamin D, whereas in Germany 64.4% supplemented vitamin D, mainly by taking dietary supplements such as Femibion®, Femibaby® or LaVita® likewise containing 200-800IU vitamin D (Figure 3.3 B). In Germany the supplementation indeed had an effect on the vitamin D concentration in maternal plasma samples which were significantly higher in women who supplemented vitamin D in any manner (Figure 3.3 C). However, in Gabon almost 65% of the mothers had sufficient vitamin D levels in plasma, and in Germany only 34% (Figure 3.3 D). The cut-off for vitamin D insufficiency was set at <30ng/mL following the recommendations of the Endocrine Society.

In summary, even though direct vitamin D supplementation was more common amongst German mothers (64% versus 35%) and moreover 50% of these mothers had sufficient vitamin D levels, in Gabon two-thirds of all the mothers had adequate vitamin D plasma levels (vs. 34% of all German mothers).

Similar to maternal vitamin D levels 70% of plasma cord blood samples showed sufficient levels. In German cord blood this was the case in only 42%.

3.6 Steroid and vitamin D pathway associated gene expression within the maternal and fetal side of the placenta

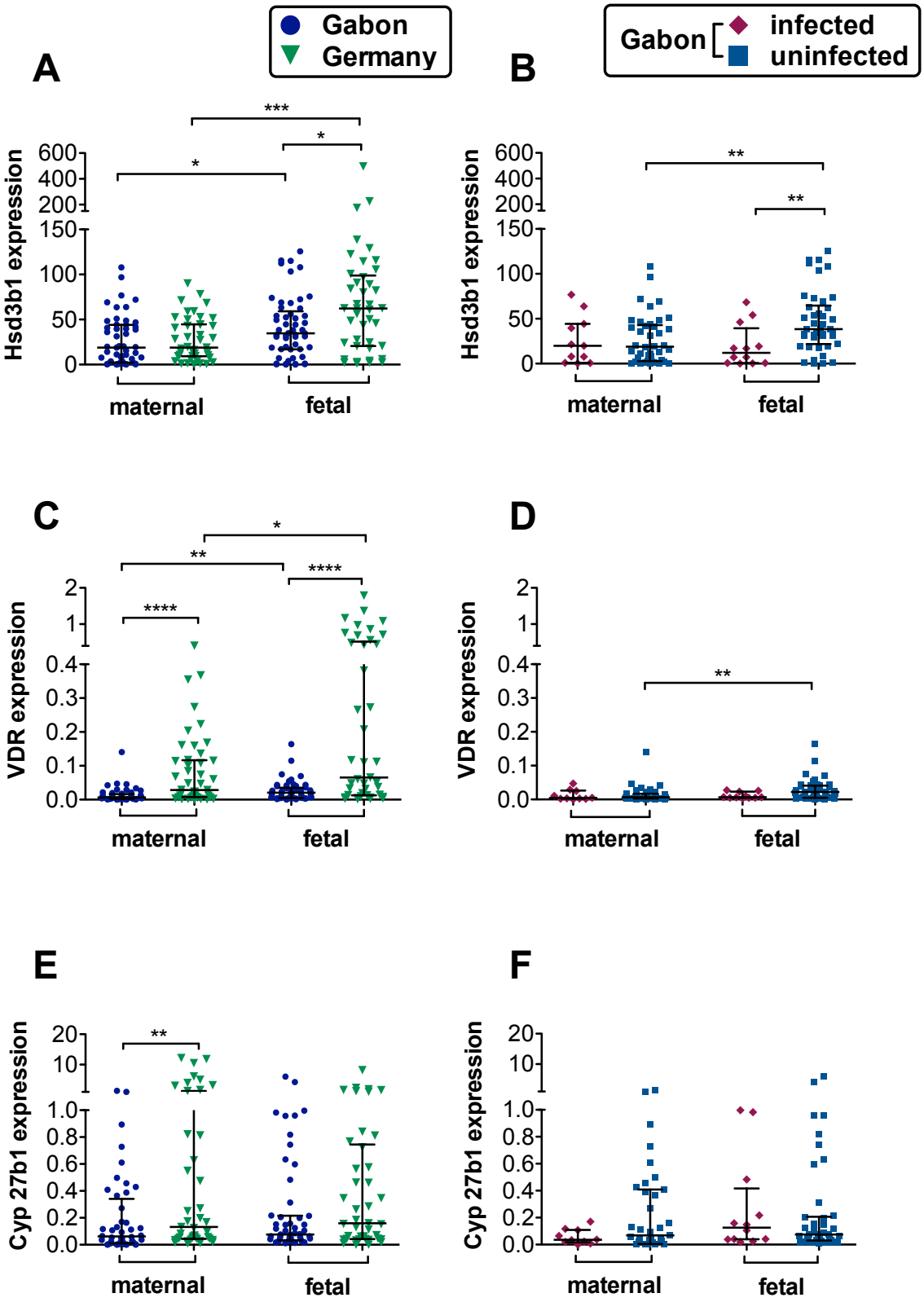


Figure 3.4: Comparison of steroid and vitamin D pathway associated genes

Relative Hsd3b1, VDR and Cyp27b1 expression, normalized to hypoxanthine-guanine phosphoribosyl transferase (HPRT) as house-keeping gene (HKG). All data are shown with median and interquartile range. P values are for Mann-Whitney U-tests. Comparison between the two maternal, cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 50; n (maternal, Germany) = 44; n (fetal, Gabon) = 53; n (fetal, Germany) = 41; n (Gabon, maternal, uninfected) = 40; n (Gabon, maternal, infected) = 10 n (Gabon, fetal, uninfected) = 42; n (Gabon, fetal, infected) = 11; **(A)** expression of Hsd3b1 in Gabonese and German placenta tissue. **(B)** Hsd3b1 gene expression in placentae from infected or uninfected Gabonese mothers. **(C)** expression of VDR in Gabonese and German placenta tissue. **(D)** VDR gene expression in placentae from infected or uninfected Gabonese mothers. **(E)** expression of Cyp27b1 in Gabonese and German placenta tissue. **(F)** Cyp27b1 gene expression in placentae from infected or uninfected Gabonese mothers.

In order to study potential differences in placental gene expression depending on the geographical region as well as maternal helminth infection, samples from the maternal and fetal side of the placenta were investigated for expression of genes known to be important for proper placental function and in the vitamin D pathway. As shown in Figure 3.4Figure 3.4: Comparison of steroid and vitamin D pathway associated genes A, C and E, comparative analyses of gene expression between Gabonese samples revealed a significantly lower expression for VDR and Cyp27b1 on maternal placental side, whereas no German/Gabonese-differences were detected regarding Hsd3b1 expression. Respective the fetal side of the placenta of the total Gabonese and German cohort, significantly lower expression for Hsd3b1 and VDR was found in the Gabonese samples and no differences for Cyp27b1. However, these genes were expressed to a greater extent when compared to their corresponding maternal side (Figure 3.4 A, C). Within the Gabonese cohort, placenta samples of mothers infected with *S. haematobium* to those uninfected were compared (Figure 3.4 B, D, F), and showed a lower expression of Hsd3b1 on the fetal side of placenta from infected mothers. No differences for the VDR and Cyp27b1 expression between the infected and uninfected cohort was detected.

Taken together, lower gene expression for Hsd3b1 (fetal side only), VDR (both sides), and Cyp27b1 (maternal side only) in the Gabonese samples was shown. *S. haematobium* infection lead to reduced Hsd3b1 expression on the fetal placental side but had no influence on VDR and Cyp27b1, whereby overall levels of expression were low.

3.7 Expression of immunologically relevant genes within the maternal and fetal side of the placenta

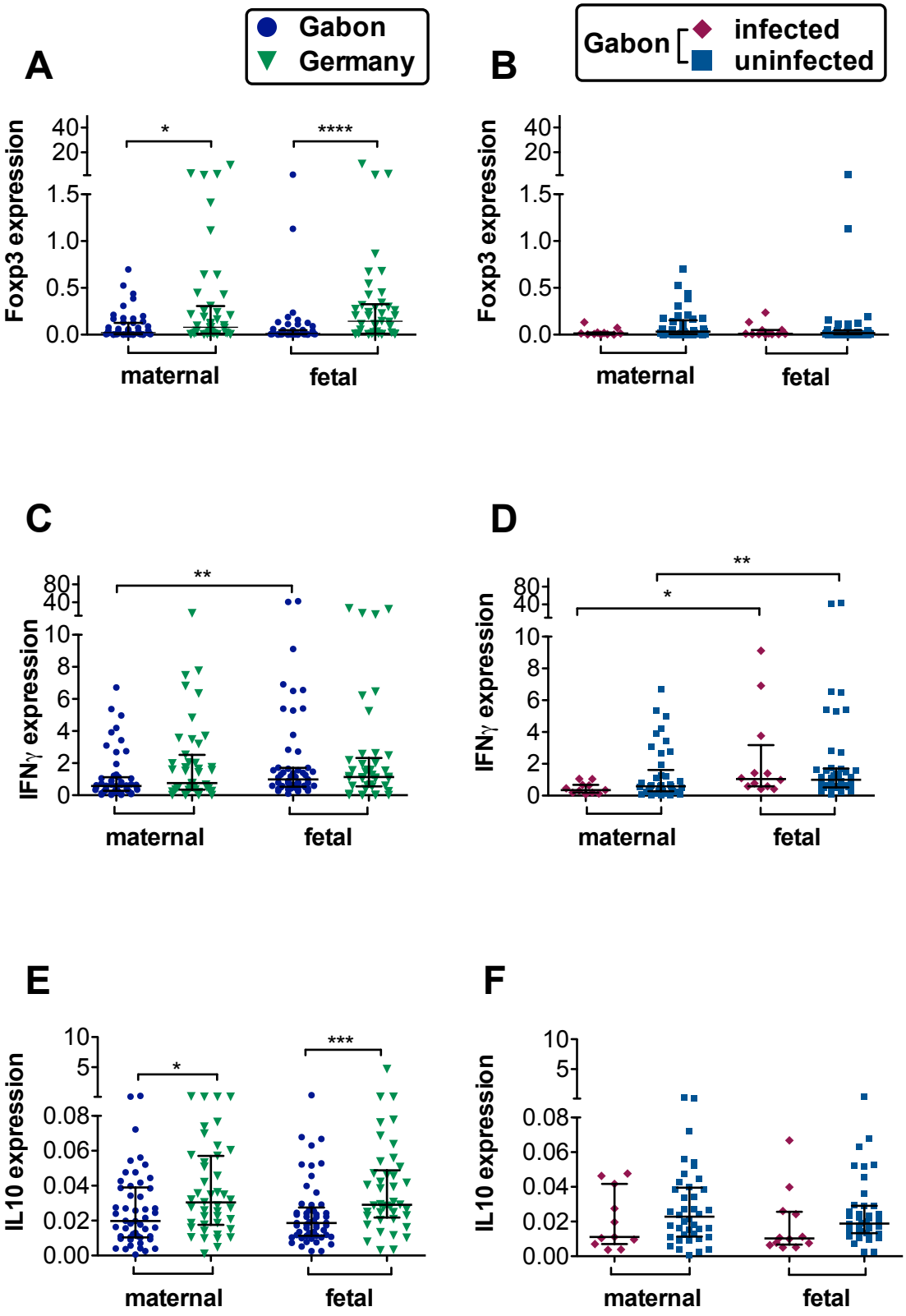


Figure 3.5: Comparison of immunological important genes

Relative Foxp3, IFNγ, and IL10 expression, normalized to HPRT as HKG. All data are shown with median and interquartile range. P values are for Mann-Whitney U-tests. Comparison between the two maternal, cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 50; n (maternal, Germany) = 44; n (fetal, Gabon) = 53; n (fetal, Germany) = 41; n (Gabon, maternal, uninfected) = 40; n (Gabon, maternal, infected) = 10 n (Gabon, fetal, uninfected) = 42; n (Gabon, fetal, infected) = 11; **(A)** expression of Foxp3 in Gabonese and German placenta tissue. **(B)** Foxp3 gene expression in placnetae from infected or uninfected Gabonese mothers. **(C)** expression of IFNγ in Gabonese and German placenta tissue **(D)** IFNγ gene expression in placnetae from infected or uninfected Gabonese mothers. **(E)** expression of IL10 in Gabonese and German placenta tissue. **(F)** IL10 gene expression in placnetae from infected or uninfected Gabonese mothers.

Since the expression of immune-related genes, such as Foxp3, IFNγ or IL10, were compared between the Gabonese and German cohort (Figure 3.5), interestingly, Foxp3 and IL10 were expressed to a lower extent on the maternal and fetal side of the placenta in Gabonese samples when compared to those from the German cohort (Figure 3.5 A, E). However, infection with *S. haematobium* had no influence on the gene expression within the Gabonese cohort (Figure 3.5 B, D, F).

Comparison of the gene expression with the maternal and fetal side revealed a significant difference only for IFNγ in the Gabonese cohort with a higher expression on the fetal side, which was independently of *S. haematobium* infection (Figure 3.5 C, D).

3.8 Distinct differences between Gabon and Germany in correlation analysis of fetal and maternal gene expression

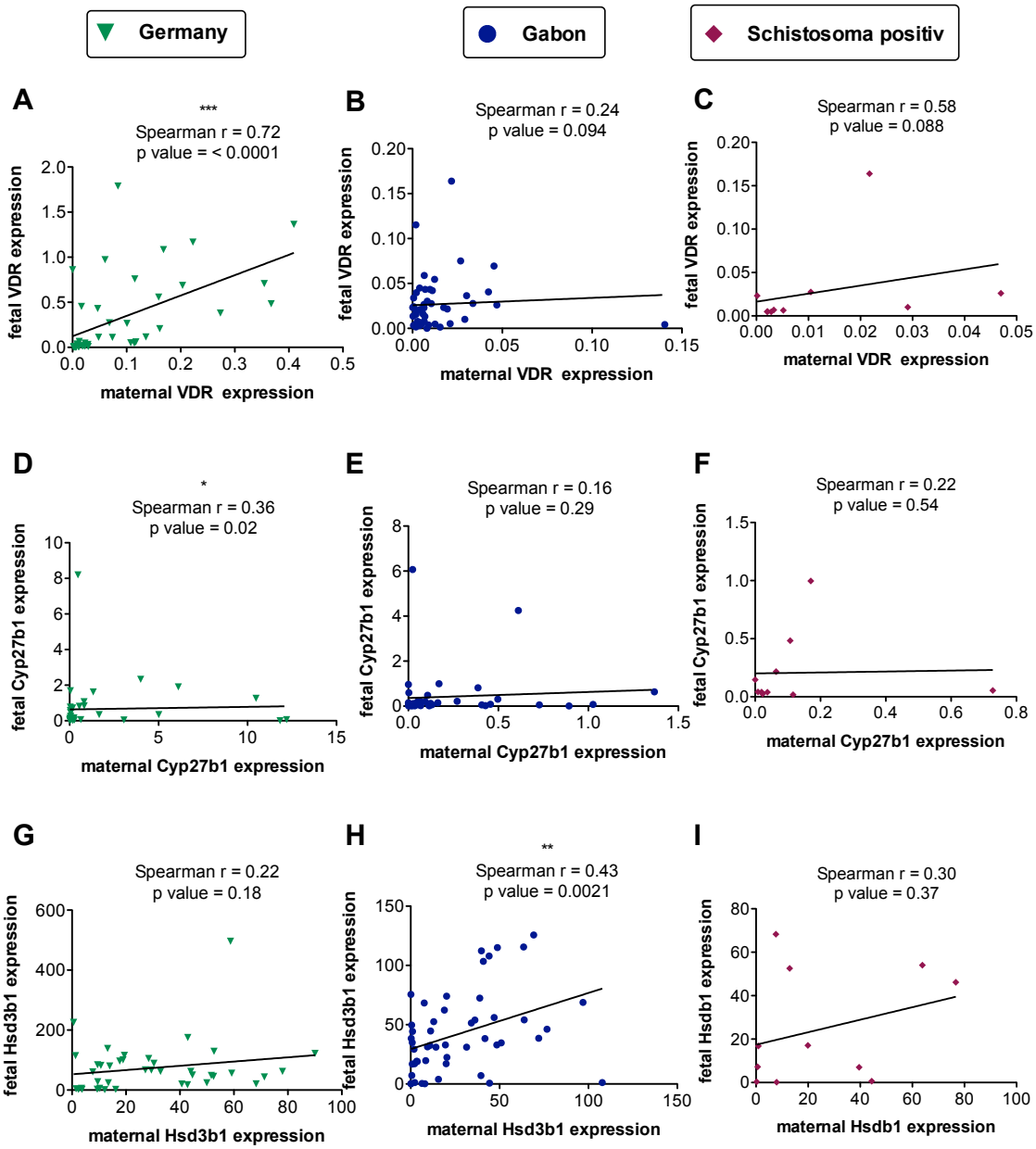


Figure 3.6 Correlation of maternal and fetal gene expression of steroid and vitamin D pathway associated genes

Correlation of relative VDR, Cyp27b1 and Hsd3b1 expression, normalized to HPRT as HKG. P values are for Spearman's rank-order correlation. Correlation of maternal and fetal placental gene expression. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (Germany) = 41; number of pairs (Gabon) = 50; number of pairs (Gabon, Schistosoma positive) = 10; **(A-C)** Correlation of VDR expression. **(D-F)** Correlation of Cyp27b1 expression. **(G-I)** Correlation of Hsd3b1 expression.

Within the placenta we can distinguish between a maternal, intervillous and fetal side. Moreover, the different sides are characterized by different cell types (see chapter 1.6). It is known that $25(OH)_2$ D crosses the placental barrier from the mother to the fetus. But still, not only more research about maternal and fetal vitamin D pathway associated gene expressions in the placenta, but also the impact of one side to the other side is highly needed. A nicely and healthy working interaction might take expression in a positive correlation between maternal and fetal. Thus, confounding factors such as inflammation or infection could destroy or disturb the close collaboration and correlation. Alternatively, both sides could "decouple" from each other in case of a threat to pregnancy maintenance and thus regulate gene expression according to their own needs.

In this thesis the correlation differed between the German and Gabonese cohort with stronger correlations in German samples. A significant correlation between maternal and fetal placental gene expressions for VDR and Cyp27b1 was demonstrated in the German cohort (Figure 3.6 A, D, G). Whereas in Gabonese placenta samples only for Hsd3b1 a significant correlation was found (Figure 3.7 B, E, H) and in the group of infected mothers none significant correlation could be seen (Figure 3.6 C, F, I).

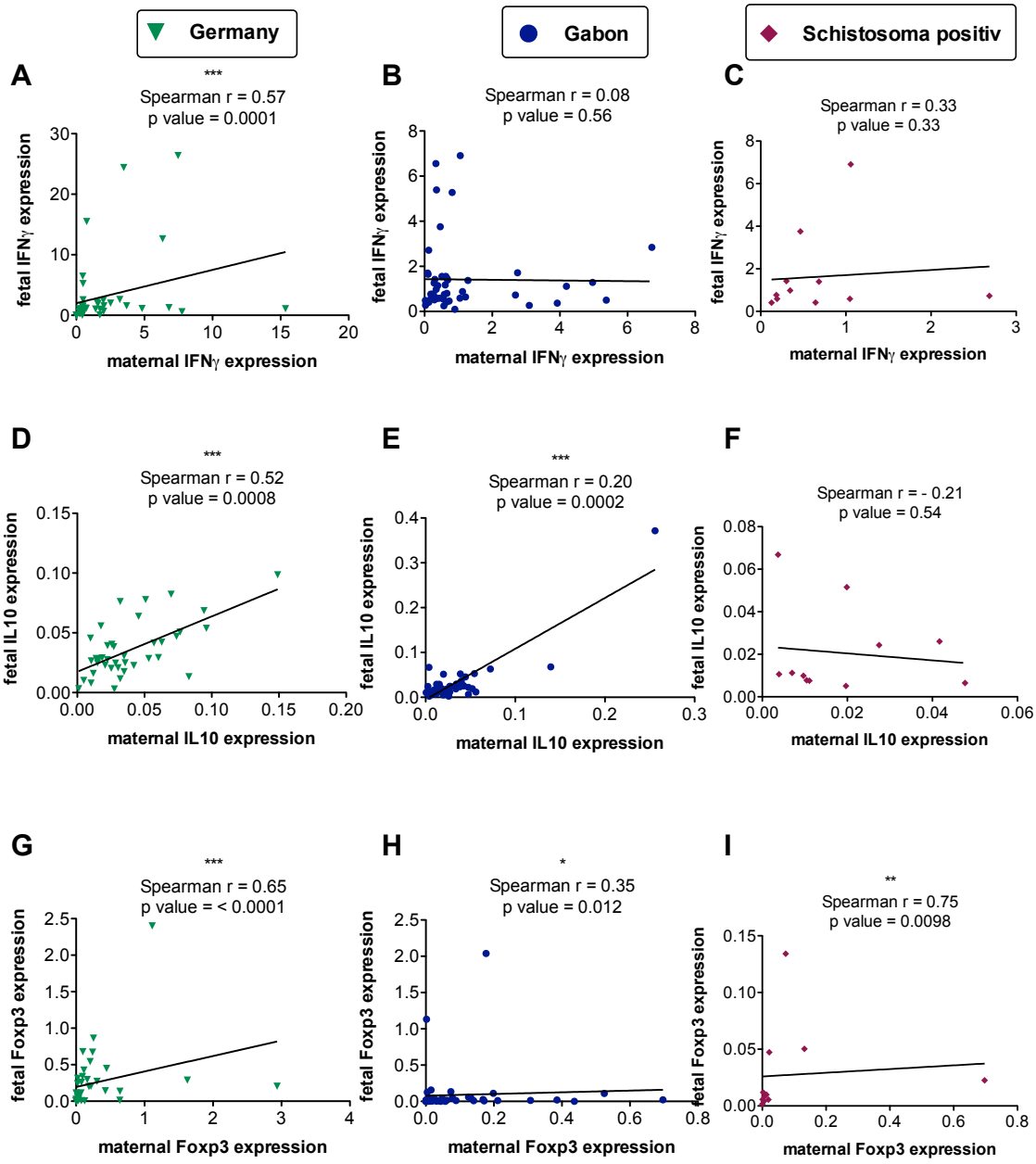


Figure 3.7 Correlation of maternal and fetal gene expression of immunological important genes

Correlation of relative IFNγ, IL10 and Foxp3 expression, normalized to HPRT as HKG. P values are for Spearman's rank-order correlation. Correlation of maternal and fetal placental gene expression. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (Germany) = 41; number of pairs (Gabon) = 50; number of pairs (Gabon, Schistosoma positive) = 10; **(A-C)** Correlation of IFNγ expression. **(D-F)** Correlation of IL10 expression. **(G-I)** Correlation of Foxp3 expression.

Since differences for vitamin D pathway related genes were shown Figure 3.6, it was on a special interest, to look at some immunological relevant genes (IL10, IFNγ and Foxp3) in terms

of correlation between maternal and fetal side. Amongst others immunological genes modulated by helminths or different environments.
In German placenta samples was a strong correlation between maternal and fetal side for all measured genes, which were IL10, IFNγ and Foxp3 (Figure 3.7 A, D, G). In the Gabonese cohort the correlation stayed significant for IL10 and Foxp3 (Figure 3.7 B, E, H), and in the *S. haematobium infected* group, only a correlation for Foxp3 could be shown (Figure 3.7 C, F, I). All genes correlated positively between maternal and fetal side in the German cohort. But in Gabon this was not the case for IFNγ and in the infected group for IL10 and IFNγ, indicating the presence factors disturbing the fetomaternal interface in Gabonese placenta samples.

3.9 Almost independent vitamin D regulation pathway

As described in chapter Figure 1.7 the VDR expression depend on a feedback loop together with vitamin D and Cyp27b1. This feedback loop is described for peripheral vitamin D concentrations and gene expressions. It is already shown that the placenta does not underlie these feedback regulations to satisfy its needs. Here, the maternal placental gene expression of VDR, Cyp27b1 and the peripheral maternal 25(OH)D concentration in venous blood were compared with each other.

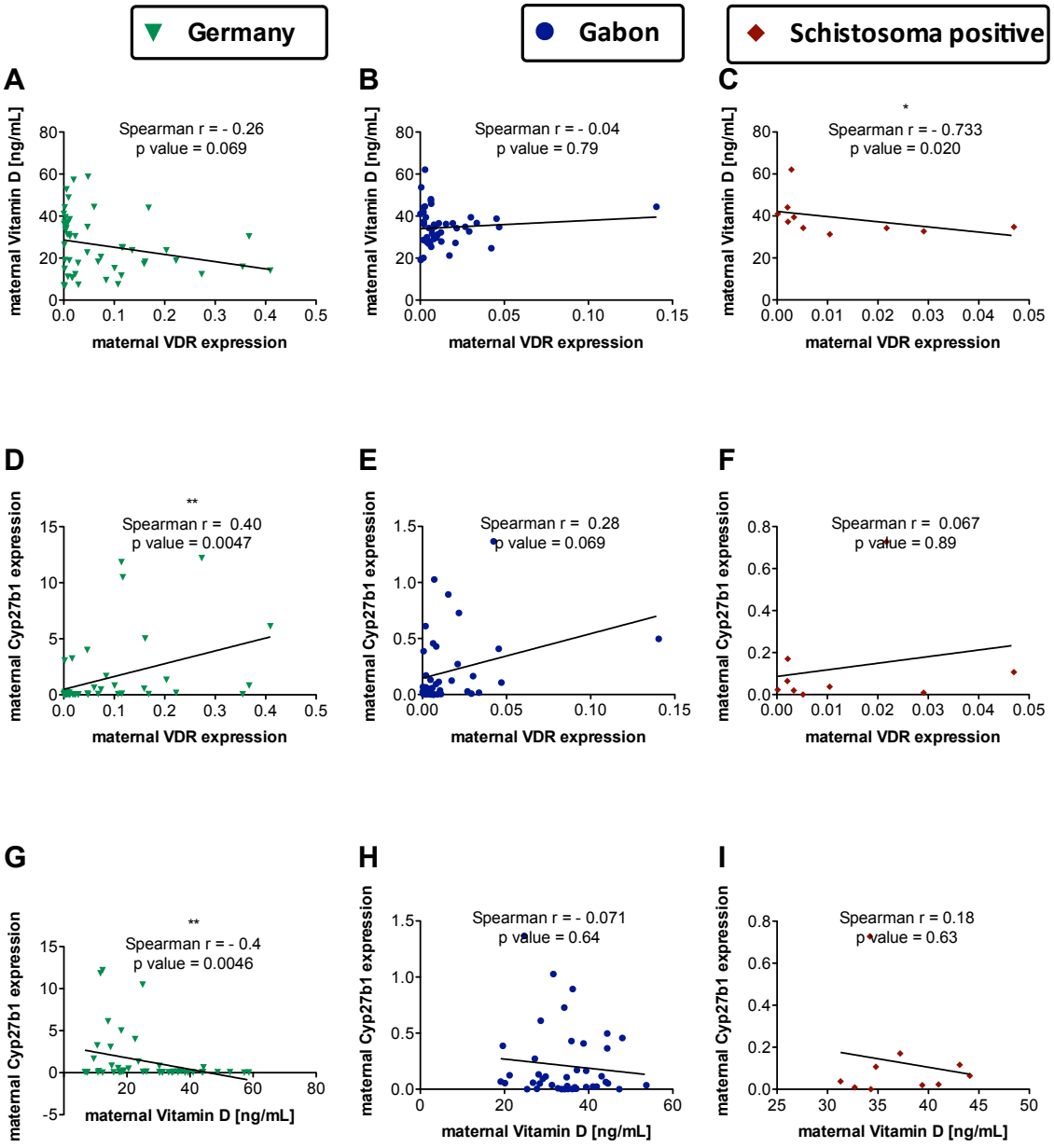


Figure 3.8 Correlation of vitamin D and pathway associated gene expressions in maternal samples

Correlation of maternal peripheral vitamin D concentration, VDR and Cyp27b1 expression, normalized to HPRT as HKG. P values are for Spearman's rank-order correlation. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (maternal, Germany) = 44; number of pairs (maternal, Gabon) = 50; number of pairs (Gabon, maternal, Schistosoma positive) = 10; **(A-C)** Correlation of vitamin D concentration with VDR expression. **(D-F)** Correlation of Cyp27b1 with VDR expression. **(G-I)** Correlation of Cyp27b1 expression with vitamin D concentration.

In the German cohort indeed, a significant correlation could be seen between VDR and Cyp27b1 and between Cyp27b1 and 25(OH)D concentration (Figure 3.8 A, D G). VDR

expression did not correlate with the 25(OH)D concentration. In the Gabonese cohort no correlation at all could be shown (Figure 3.8 B, E, H). And within the group of infected participants the only significant correlation was demonstrated for VDR expression and 25(OH)D concentration (Figure 3.8 C, F, I). Therefore, it seems that vitamin D and VDR are acting independently from each other in absence of *S. haematobium* infection.

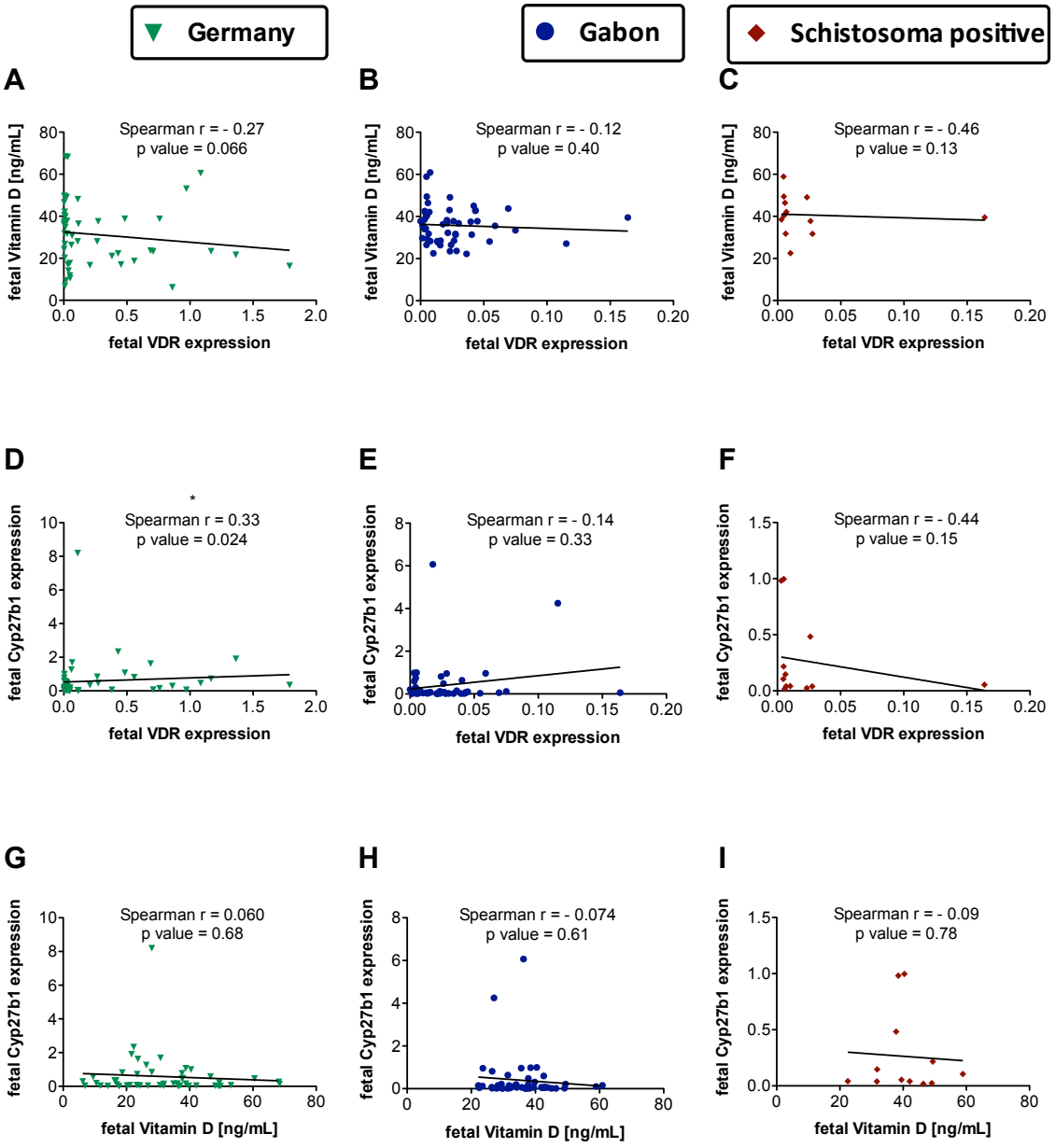


Figure 3.9 Correlation of vitamin D and pathway associated gene expressions in neonatal samples

Correlation of fetal peripheral vitamin D concentration, VDR and Cyp27b1 expression, normalized to HPRT as HKG. P values are for Spearman's rank-order correlation. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (fetal, Germany) = 41; number of pairs (fetal, Gabon) = 53; number of pairs (Gabon, fetal, Schistosoma positive) = 11; **(A-C)** Correlation of vitamin D concentration with VDR expression. **(D-F)** Correlation of Cyp27b1 with VDR expression. **(G-I)** Correlation of Cyp27b1 expression with vitamin D concentration.

Maternal 25(OH)D can easily pass the placental barrier. Anyway, it was of a special interest, to see whether the fetus maintains the known feedback loop, if it acts similarly to the maternal side or independently. Therefore, fetal placental gene expression of VDR, Cyp27b1 and the 25(OH)D concentration in cord blood were compared with each other. In the German cohort the only significant correlation could be seen between VDR and Cyp27b1. In the Gabonese cohort nor in the cohort of *S. haematobium* positive mothers any correlation between VDR and Cyp27b1 or vitamin D, respectively, or between Cyp27b1 and 25(OH)D was found, indicating an uncoupled vitamin D feedback loop at the fetal side of the placenta.

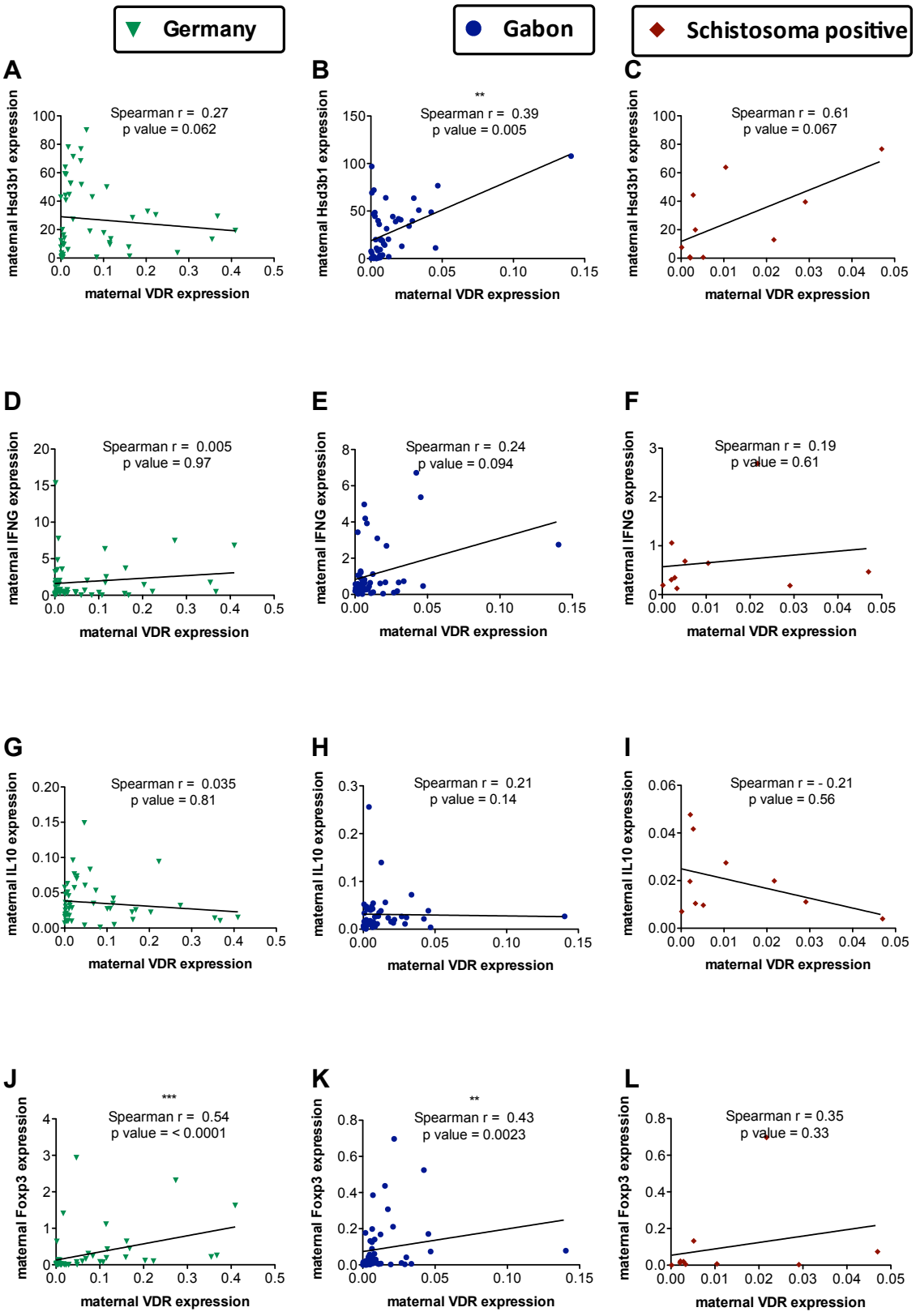


Figure 3.10 Correlation of VDR expression with immunological important genes in the maternal placenta

Correlation of maternal VDR expression with Hsd3b1, IFNγ, IL10, and Foxp3 expression, normalized to HPRT as HKG. P values are for Spearman's rank-order correlation. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (maternal, Germany) = 44; number of pairs (maternal, Gabon) = 50; number of pairs (Gabon, maternal, Schistosoma positive) = 10; **(A-C)** Correlation of vitamin D concentration with VDR expression. **(D-F)** Correlation of Cyp27b1 with VDR expression. **(G-I)** Correlation of Cyp27b1 expression with vitamin D concentration.

In chapter 1.7 the important role of vitamin D and its ability to modulate the immune system are described. Furthermore, the important role of VDR was pointed out. It acts not only as a transcription factor but is also present in almost all kinds of immune cells and different layers of placenta tissue. To reveal possible coherences between VDR gene expression and immunological important genes, maternal VDR expression was compared with the Hsd3b1, IL10, IFNγ and Foxp3 expression. In the German cohort the only strong correlation could be seen between VDR and Foxp3. In the Gabonese cohort there was a significant correlation between VDR and Hsd3b1 as well as Foxp3. In the infected cohort there was no correlation at all of VDR with neither Hsd3b1, IL10, IFNγ nor Foxp3 (Figure 3.10).

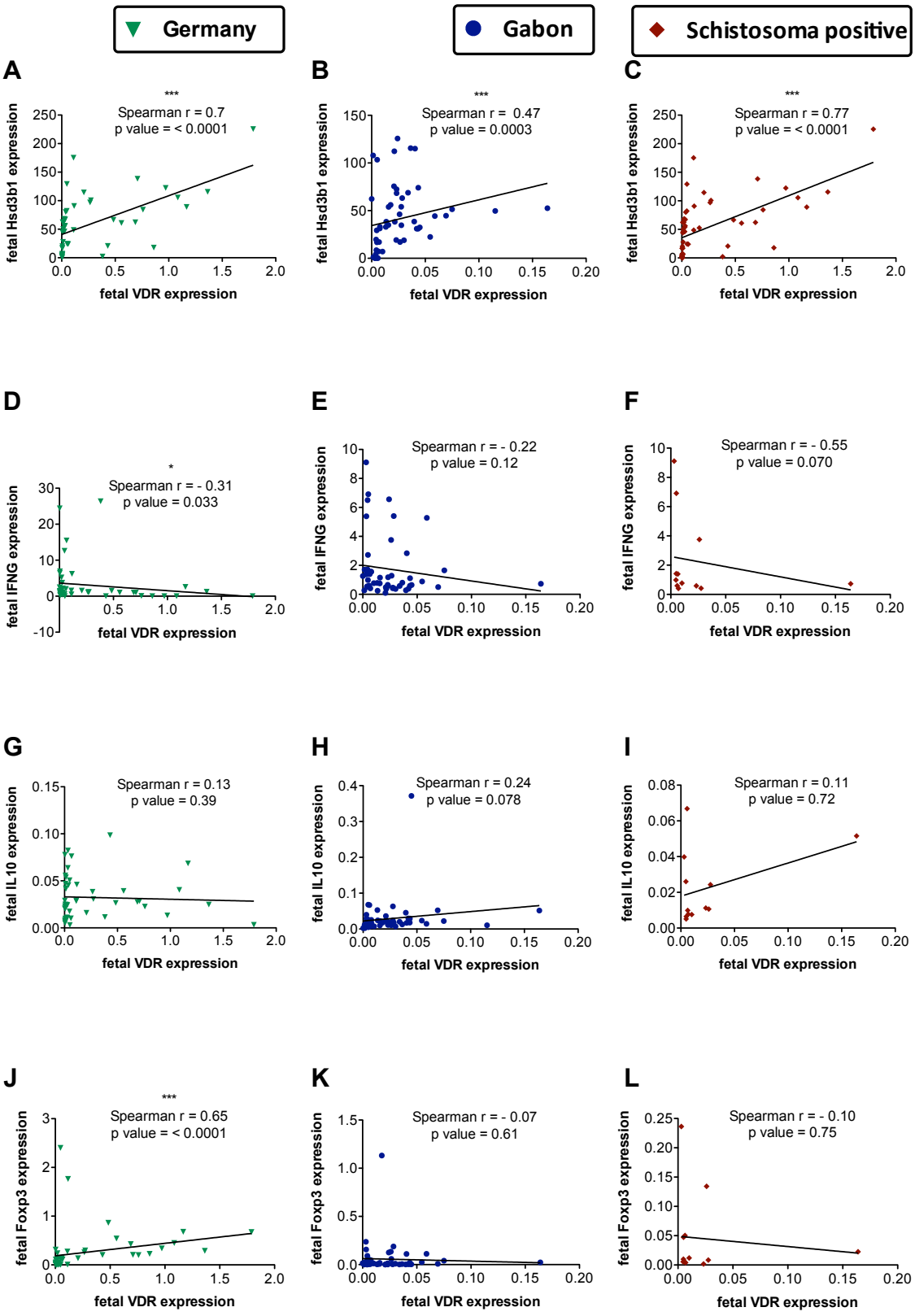


Figure 3.11 VDR expression correlated with immunological important genes in the fetal placenta

Correlation of fetal VDR expression with Hsd3b1, IFNγ, IL10, and Foxp3 expression, normalized to HPRT as HKG. P values are for Spearman's rank-order correlation. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (fetal, Germany) = 41; number of pairs (fetal, Gabon) = 53; number of pairs (Gabon, fetal, Schistosoma positive) = 11; **(A-C)** Correlation of vitamin D concentration with VDR expression. **(D-F)** Correlation of Cyp27b1 with VDR expression. **(G-I)** Correlation of Cyp27b1 expression with vitamin D concentration.

Once more to reveal gene expression differences between the fetal and the maternal placental side, similarly the expression of fetal VDR was correlated with fetal Hsd3b1, IL10, IFNγ or Foxp3 respectively. In the German cohort a significant correlation could be shown for VDR correlated with Hsd3b1, IFNγ and Foxp3. In the Gabonese cohort, as well as in the cohort of *S. haematobium* infected mothers a strongly significant correlation of VDR with Hsd3b1 could be shown, whereas VDR did not correlate with IL10, IFNγ or Foxp3. The strong correlation of VDR with Hsd3b1 gene expression on the fetal side of the placenta was present in all three groups (Figure 3.11).

Again, in German placenta samples most genes (Hsd3b1, IFNγ and Foxp3) correlate strongly with VDR. In Gabon only a correlation between VDR and Hsd3b1 could be seen, suggesting a modulation of the fetal placental gene expression due to any factors.

3.10 Summary of gene correlations

As I did a lot of correlations, I will sum up the results in the tables below. Arrows will show significant correlations and genes written in bold, red and italic stand for a positive correlation of this gene expression between the maternal and the fetal side of the placenta.

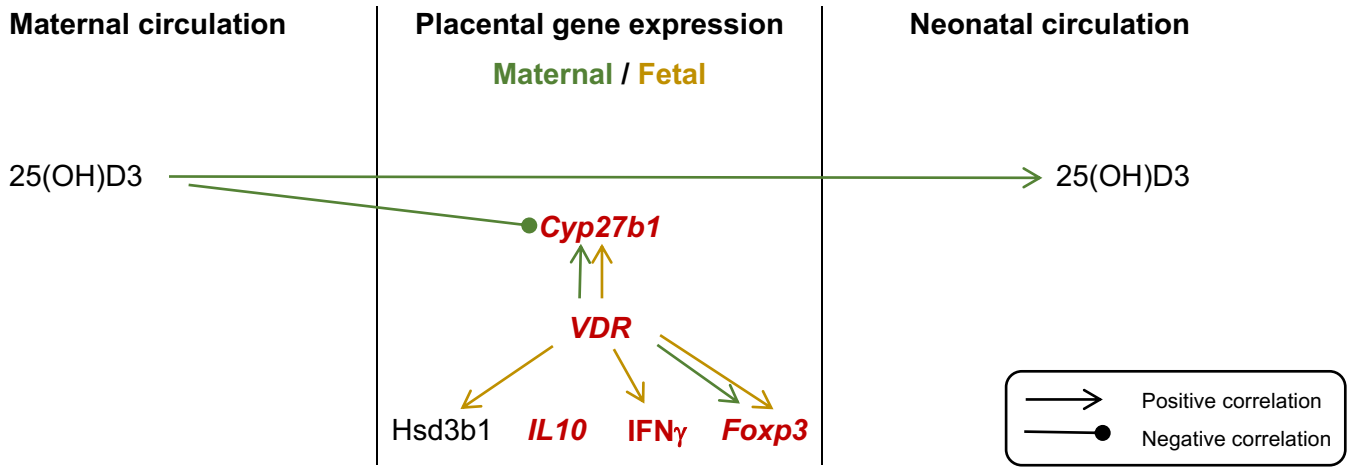


Figure 3.12 Correlation of placental gene expressions or vitamin D concentration, respectively, in Germany

Correlation of peripheral vitamin D concentration and placnetal gene expressions as well as gene expression between the maternal and fetal side in German samples. Genes in bold, red and italic show a significant correlation of the respective gene expression between the maternal and fetal side of the placenta.

In the German cohort I could observe the highest number of gene correlations (Figure 3.12 Correlation of placental gene expressions or vitamin D concentration, respectively, in Germany. On the one side significant correlations could be shown within the 25(OH)D feedback loop and of the VDR expression with the other genes. Merely VDR and 25(OH)D seemed to act independently. On the other side likewise, gene expressions between the maternal and fetal side correlated strongly with each other. This was the case for Cyp27b1, VDR, IL10, IFNγ and Foxp3.

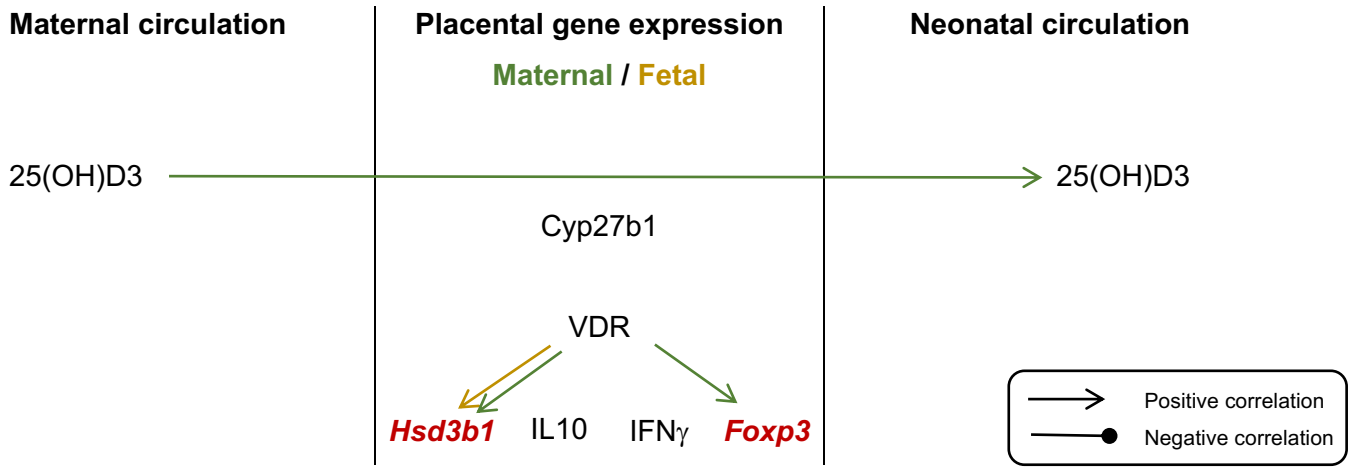


Figure 3.13 Correlation of placental gene expressions or vitamin D concentration, respectively, in Gabon

Correlation of peripheral vitamin D concentration and placental gene expressions as well as gene expression between the maternal and fetal side in Gabonese samples. Genes in bold, red and italic show a significant correlation of the respective gene expression between the maternal and fetal side of the placenta.

In the Gabonese cohort the 25(OH)D feedback loop (Vitamin D, VDR, Cyp27b1) seemed to be completely uncoupled, at the maternal side as well as at the fetal side. Maternal VDR correlated only with Hsd3b1 and Foxp3 and fetal VDR only with Hsd3b1. The gene expression of Hsd3b1 and Foxp3 correlated significantly between the maternal and fetal side of the placenta (Figure 3.13).

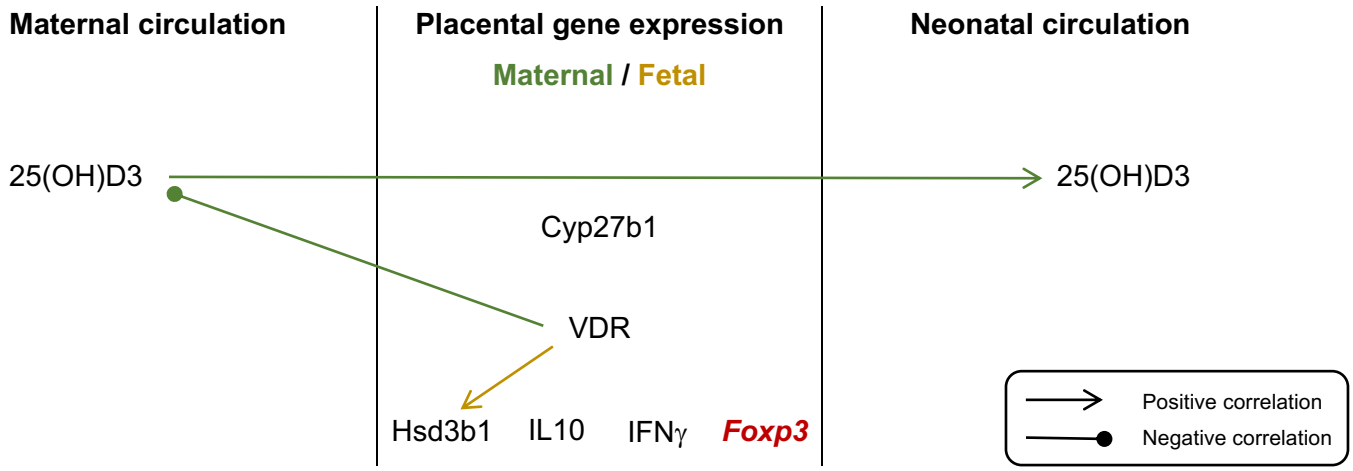


Figure 3.14 Correlation of placental gene expressions or vitamin D concentration, respectively, in the Schistosoma positive cohort

Correlation of peripheral vitamin D concentration and placnetal gene expressions as well as gene expression between the maternal and fetal side in samples of the Schistosoma infected cohort. Genes in bold and italic show a significant correlation of the respective gene expression between the maternal and fetal side of the placenta.

In the cohort of Gabonese mothers infected with *S. haematobium* at time of delivery I found even less correlations than in the other two groups, suggesting that not only environmental factors but even infection influences the placental gene expressions strongly. On the maternal side, there was only a significant correlation between VDR expression and 25(OH)D concentration, whereas on the fetal side VDR correlated significantly with Hsd3b1. Foxp3 was the only gene which's expression correlated significantly between maternal and fetal side of the placenta (Figure 3.14).

In summary, In Germany and Gabon, the only positive significant correlation between maternal and fetal gene expression in common was found for Foxp3. In Germany most significant (positive and negative) correlations were found, whereas in the infected Gabonese group both placental sides seemed to act differently and to be regulated less tightly. Moreover, the $25(OH)_2D$ concentration in maternal peripheral venous blood correlated with the concentration in cord blood, which was expected since it is known to cross the placental barrier.

3.11 Significantly higher total IgG4 plasma levels in German plasma samples

Transfer across the placenta is tightly regulated to protect the developing fetus against infections and toxins, while also providing adequate nutrition and factors for optimal growth. In

this context, IgG, upregulated during helminth infections, is the only antibody subclass which is able to cross the placental barrier.

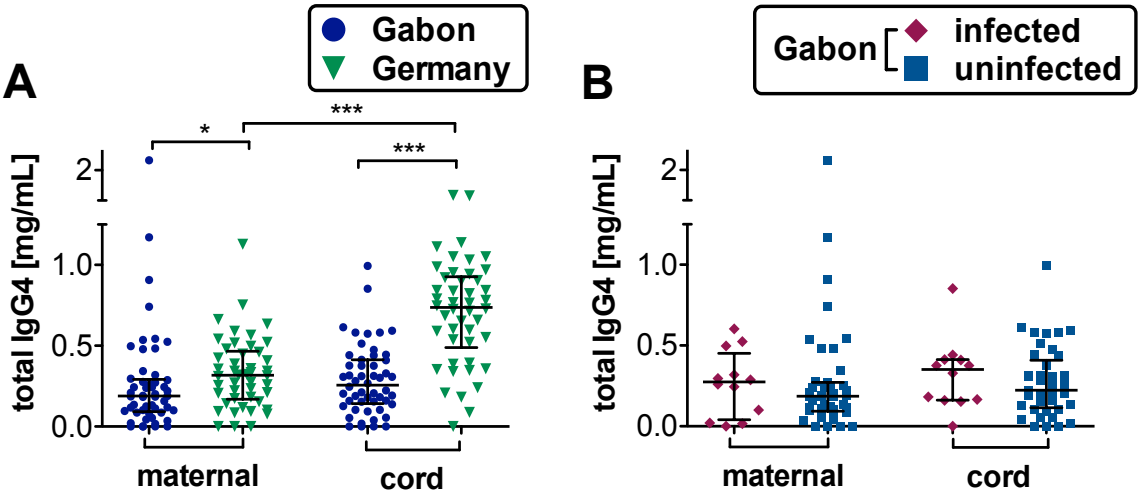


Figure 3.15: **total IgG4 levels**

IgG4 levels were measured in plasma via ELISA. All data are shown with median and interquartile range. P values are for Mann-Whitney U-tests. Comparison between the two maternal and cord blood groups, or maternal with corresponding cord to show transplacental transfer, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; n (maternal, Gabon) = 54; n (maternal, Germany) = 47; n (cord, Gabon) = 53; n (cord, Germany) = 47; n (Gabon, maternal, uninfected) = 42; n (Gabon, maternal, infected) = 12 n (Gabon, cord, uninfected) = 42; n (Gabon, cord, infected) = 12; **(A)** total IgG4 levels in all German maternal all Gabonese plasma samples **(B)** total IgG4 levels in Gabonese plasma samples devided into infected and uninfected group.

This placental transfer can be disturbed e.g. through placental inflammation. Therefore, in addition to measuring genes related to inflammation, I assessed antibody transfer itself by measuring IgG4 levels in cord blood plasma and comparing it to maternal levels. In German maternal and cord blood plasma samples, the general IgG4 levels were significantly higher compared to those from Gabon (Figure 3.15 A). Additionally, there was significantly higher general IgG4 level in German cord blood samples compared to their mothers. This was not seen within the Gabonese cohort irrespective of *S. haematobium* infection (Figure 3.15 B).

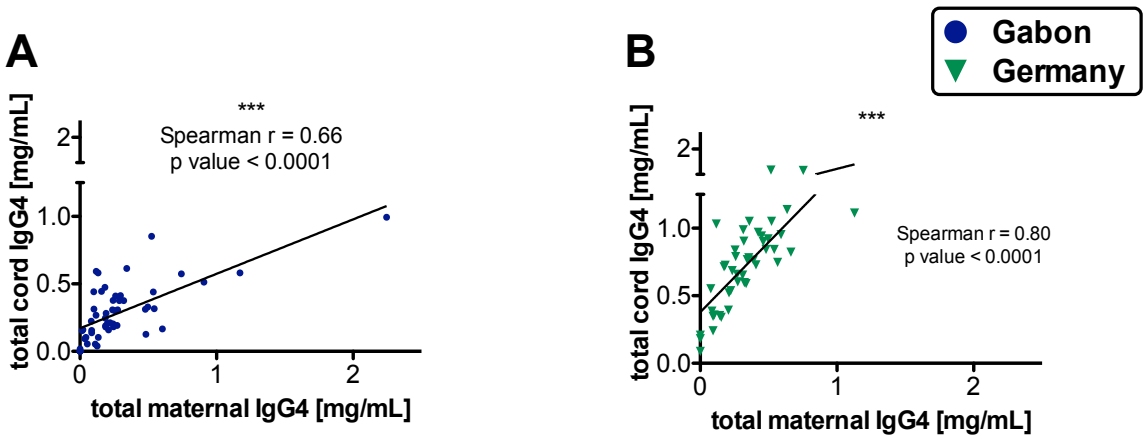


Figure 3.16: Correlation of cord and maternal IgG4 plasma levels

IgG4 levels were measured in plasma via ELISA. P values are for Spearman's rank correlation. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; number of pairs (Gabon) = 53; number of pairs (Germany) = 45; **(A)** correlation between general maternal and cord blood IgG4 levels in Gabonese plasma samples; spearman r = 0,66, p value = < 0,0001. **(B)** correlation between general maternal and cord blood IgG4 levels in German plasma samples; r = 0.80; p value = <0,0001.

I further correlated maternal with cord blood levels to examine transfer more closely. A strong correlation between maternal and cord blood concentrations of the levels of general IgG4 could be seen in Gabonese samples as well as in German samples (Figure 3.16 A, B), strongest in the German cohort (Spearman r = 0.66 in Gabonese vs. Spearman r = 0.80 in German samples).

Taken together IgG4 plasma levels were significantly higher in German samples. In German cord blood levels are significantly higher compared to the corresponding mother, while in Gabonese samples maternal and fetal levels are more or less equal, suggesting a disturbed placental transport here.

3.12 No correlation between IgG4 levels and placental gene expression

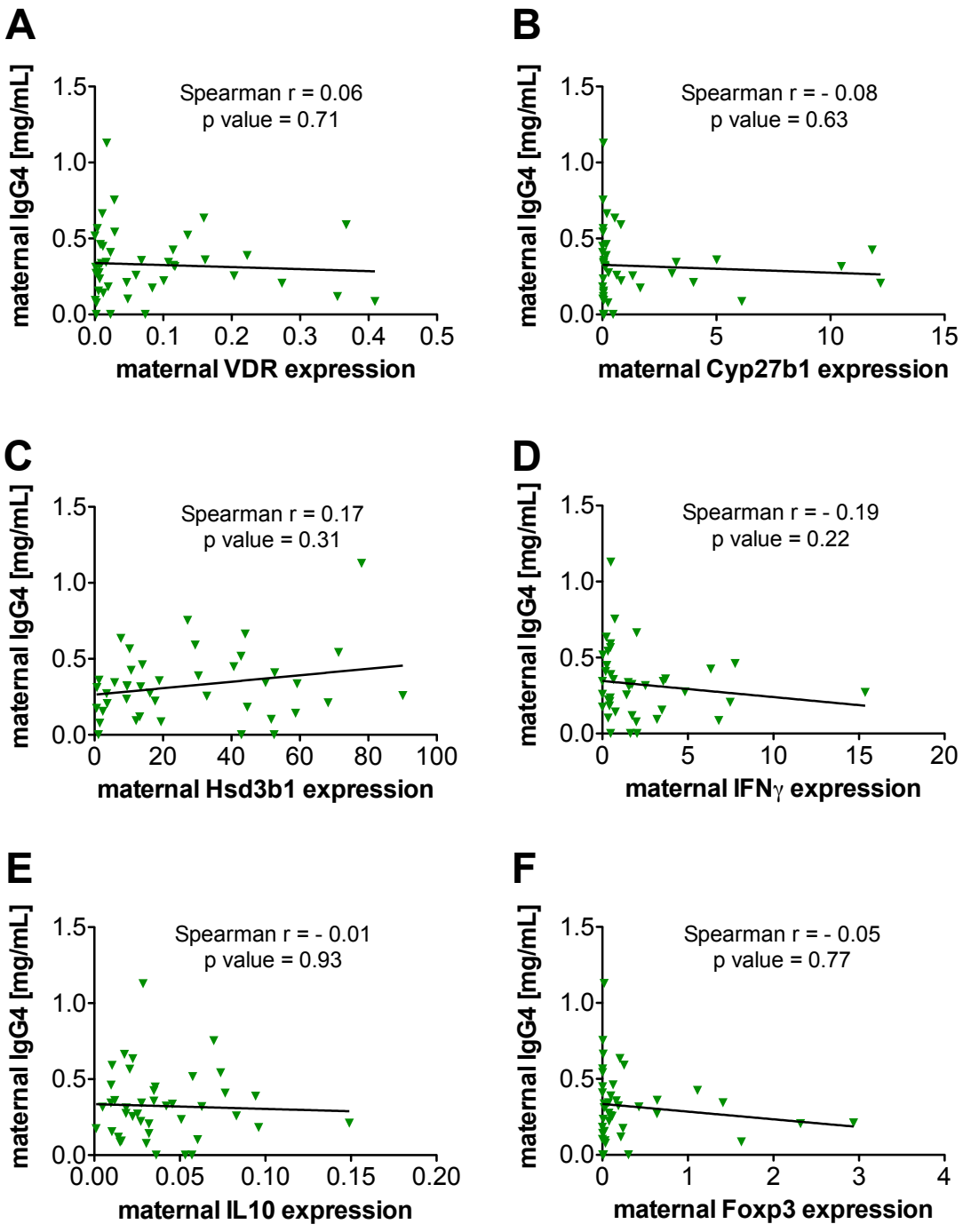


Figure 3.17 Correlation of total maternal IgG4 levels with placental maternal gene expression in German samples

Correlation of total maternal IgG4 levels measured in plasma samples with gene expression of the maternal side of the placenta within the German cohort. (A) Correlation between maternal total IgG4 levels and VDR gene expression. (B) Correlation between maternal total IgG4 levels and Cyp27b1 gene expression. (C) Correlation between maternal total IgG4 levels and Hsd3b1 gene expression. (D) Correlation between maternal total IgG4 levels and IFNG gene expression. (E) Correlation between maternal total IgG4 levels and IL10 gene expression. (F) Correlation between maternal total IgG4 levels and Foxp3 gene expression.

The Ig subtype IgG4 is positively related to infection level of helminths [260] and is able to regulate the immune system as reviewed in [259] and mentioned above. IgG4 as well as IgE are regulated by Th2 cytokines such as IL4, IL13 or IL10 [260]. IgG4 is mainly produced by B cells acts directly together with T cells. The cytokine IL10 is strongly involved into its regulation. IgG4 can be enhanced by IL10 whilst down-regulating IgE [260]. An early production of specific IgG4 and later production of specific IgE raises the possibility that development of protection against schistosomes and other helminth infections and depends on changes in the lymphocyte populations involved in cytokine production [101].

Figure 3.17 shows the correlation of total maternal IgG4 levels measured in plasma samples with gene expression of the maternal side of the placenta within the German cohort. No significant correlation between IgG4 and one of the six measured genes (VDR, Cyp27b1, Hsd3b1, IFNG, IL10 and Foxp3) could be found.

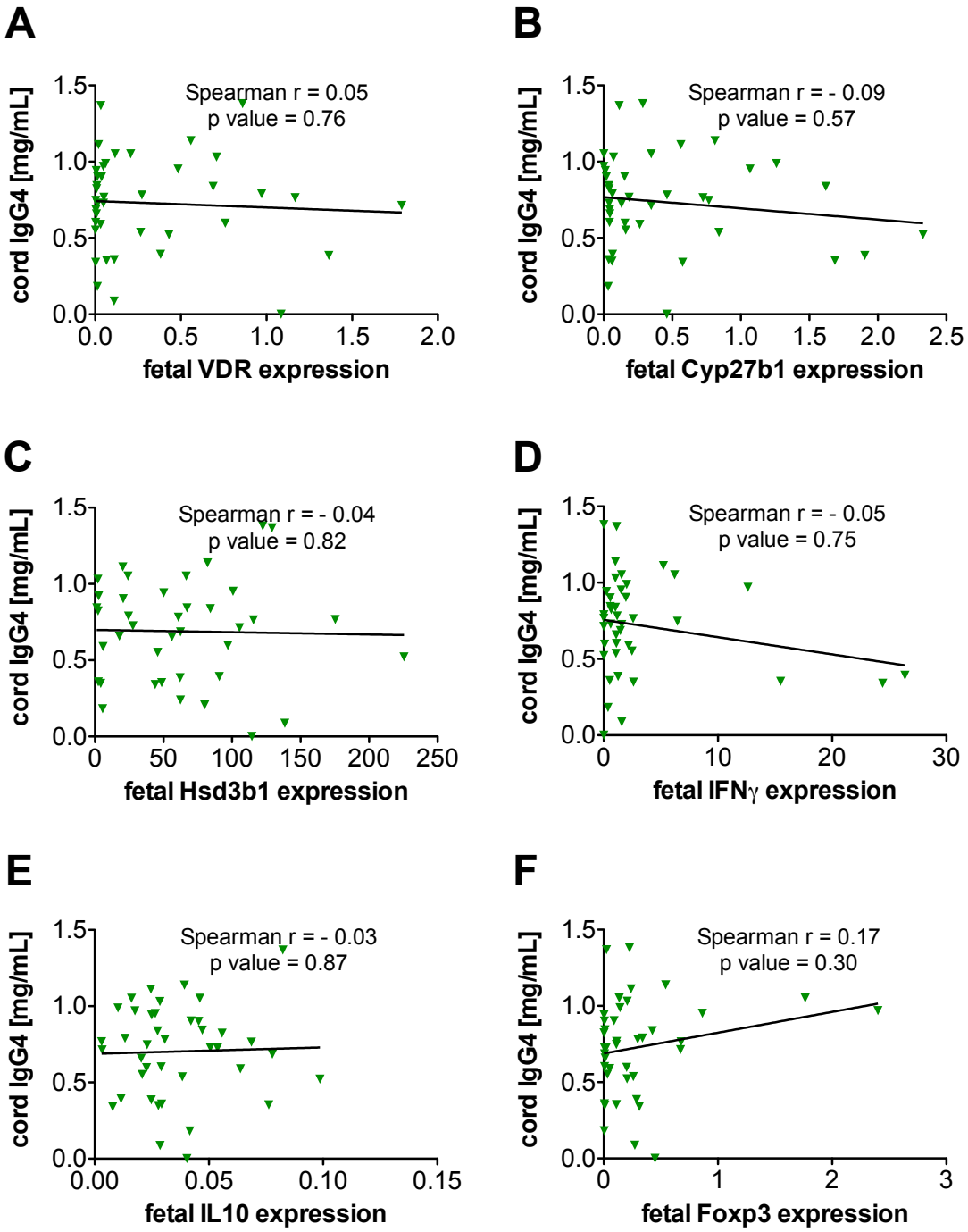


Figure 3.18 Correlation of total cord blood IgG4 plasma levels with placental fetal gene expression in German samples

Correlation of total cord blood IgG4 plasma levels with gene expression of the fetal side of the placenta within the German cohort. (A) Correlation between cord blood IgG4 plasma levels and VDR gene expression. (B) Correlation between cord blood IgG4 plasma levels and Cyp27b1 gene expression. (C) Correlation between cord blood IgG4 plasma levels and Hsd3b1 gene expression. (D) Correlation between cord blood IgG4 plasma levels and IFNG gene expression. (E) Correlation between cord blood IgG4 plasma levels and IL10 gene expression. (F) Correlation between cord blood IgG4 plasma levels and Foxp3 gene expression.

Figure 3.18 pictures the correlation of total cord blood IgG4 plasma levels with gene expression of the fetal side of the placenta within the German cohort. Similar to the maternal side, again no significant correlation could be found between IgG4 and VDR, Cyp27b1, Hsd3b1, IFNG, IL10 or Foxp3.

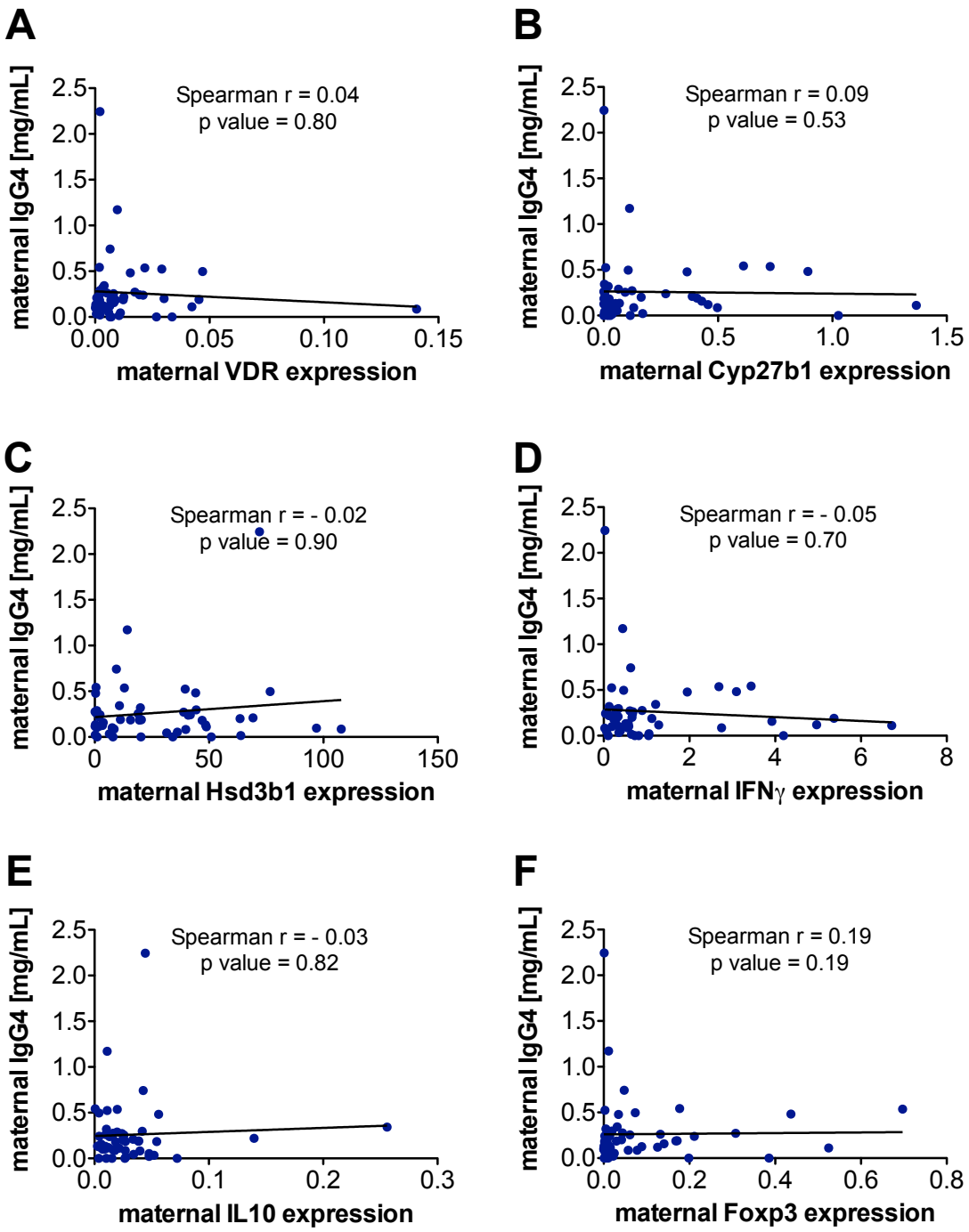


Figure 3.19 Correlation of total maternal IgG4 levels with placental maternal gene expression in Gabonese samples

Correlation of total maternal IgG4 levels measured in plasma samples with gene expression of the maternal side of the placenta within the Gabonese cohort. (A) Correlation between maternal total IgG4 levels and VDR gene expression. (B) Correlation between maternal total IgG4 levels and Cyp27b1 gene expression. (C) Correlation between maternal total IgG4 levels and Hsd3b1 gene expression. (D) Correlation between maternal total IgG4 levels and IFNG gene expression. (E) Correlation between maternal total IgG4 levels and IL10 gene expression. (F) Correlation between maternal total IgG4 levels and Foxp3 gene expression.

Likewise to the German cohort, the Gabonese cohort did not show any significant correlation between total IgG4 levels and placental gene expression, neither for maternal samples (Figure 3.19) nor for cord blood/ fetal samples (Figure 3.20).

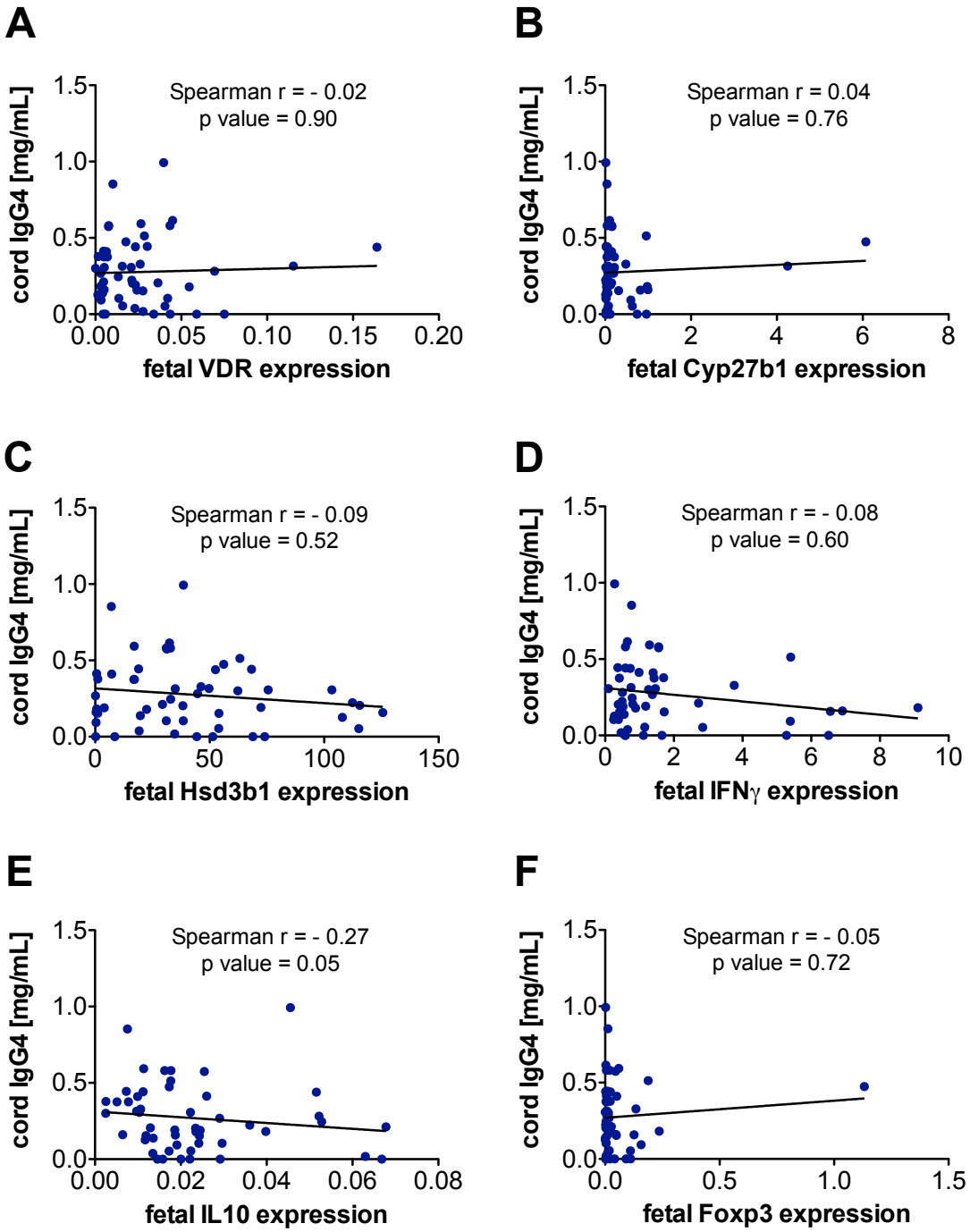


Figure 3.20 Correlation of total cord blood IgG4 plasma levels with placental fetal gene expression in Gabonese samples

Correlation of total fetal IgG4 levels measured in cord blood plasma samples with gene expression of the fetal side of the placenta within the Gabonese cohort. (A) Correlation between cord blood IgG4 plasma levels and VDR gene expression. (B) Correlation between cord blood IgG4 plasma levels and Cyp27b1 gene expression. (C) Correlation between cord blood IgG4 plasma levels and Hsd3b1 gene expression. (D) Correlation between cord blood IgG4 plasma levels and IFNG gene expression. (E) Correlation between cord blood IgG4 plasma levels and IL10 gene expression. (F) Correlation between cord blood IgG4 plasma levels and Foxp3 gene expression.

3.13 Significantly higher levels of total IgE in Gabonese plasma samples

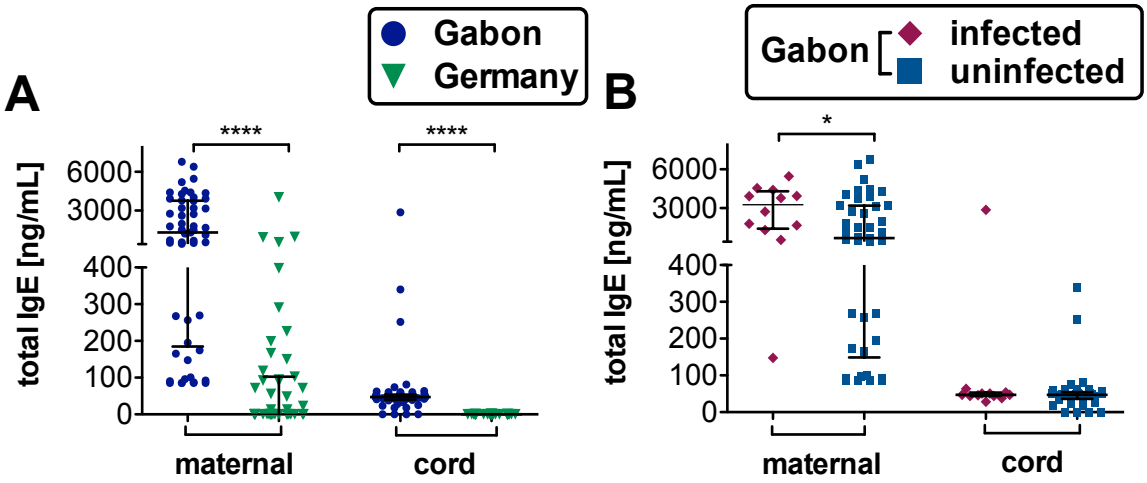


Figure 3.21: **Total IgE levels**

IgE levels were measured in plasma via ELISA. All data are shown with median and interquartile range. P values are for Mann-Whitney U-tests. Comparison between the two maternal or cord blood groups, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 54; n (maternal, Germany) = 47; n (cord, Gabon) = 53; n (cord, Germany) = 47; n (Gabon, maternal, uninfected) = 42; n (Gabon, maternal, infected) = 12 n (Gabon, cord, uninfected) = 42; n (Gabon, cord, infected) = 12; **(A)** total IgE levels in all German maternal all Gabonese plasma samples **(B)** total IgE levels in Gabonese plasma samples devided into infected and uninfected group.

IgE is known to interact with IgG4 and to be equally important during helminth infections. Both influence susceptibility and protection against helminth infections. However, in contrast to IgG4, IgE is not able to cross the placental barrier and cord blood levels must be produced by the fetus itself, so its presence in cord blood is evidence of fetal immune system priming already *in utero*. Comparing total IgE levels in maternal plasma between Gabon and Germany yielded strong significant differences, with higher levels in Gabon (Figure 3.21 A). This significant difference could be seen for the cord blood samples too. In almost 98% of the Gabonese cord blood plasma samples total IgE levels were detectable, whereas in the German

cord blood plasma samples IgE was not detectable (Figure 3.21 A). Within the Gabonese cohort, the total IgE levels were significantly higher in the group of mothers infected with *S. haematobium*, whereas the levels in cord blood were similar despite apparent infection status (Figure 3.21 B).

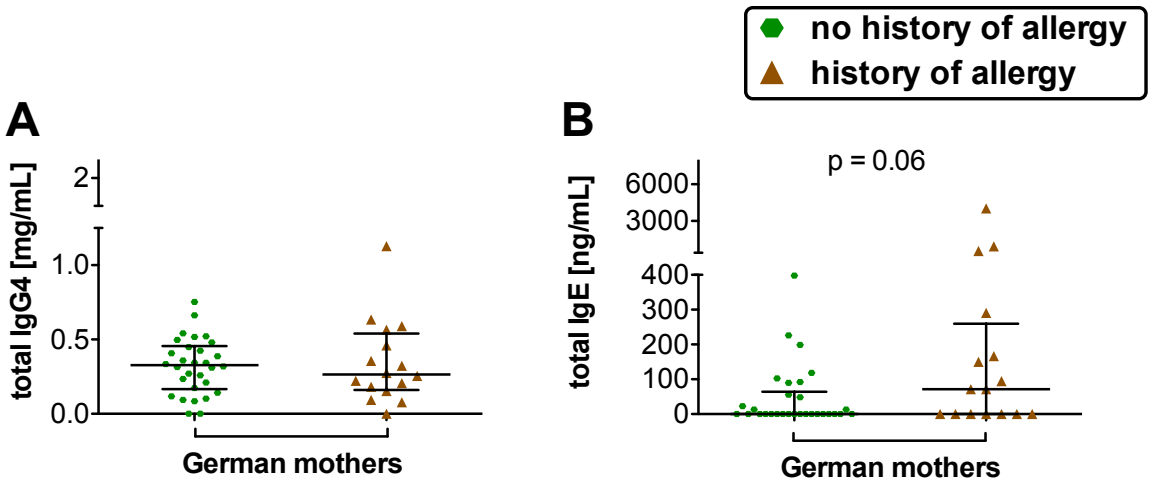


Figure 3.22: Total IgG4 and IgE levels in German mothers with and without history of allergy

Total IgG4 and IgE levels were measured in plasma via ELISA. All data are shown with median and interquartile range. P values are for Mann-Whitney U-tests. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Germany) = 47; n (cord, Germany) = 47; **(A)** total IgG4 levels in German mothers with and without history of allergy. **(B)** total IgE levels in German mothers with and without history of allergy.

Germany is a helminth and parasite non-endemic area. Therefore, high IgE levels must be due to further reasons such as allergies, which were part of the questionnaire but no exclusion criteria. Thus, I looked at IgG4 levels (Figure 3.22 A) as well as at IgE levels depending on history of allergy in German mothers (Figure 3.22 B). For both IgG4 and IgE there was no significant difference in antibody concentration between mothers with and without history of allergy, whereas for IgE a trend of higher IgE levels in case on allergy was seen clearly. Hay fever and asthma was the main allergy stated by German mothers. Only two Gabonese mothers stated to suffer from allergy (penicillin).

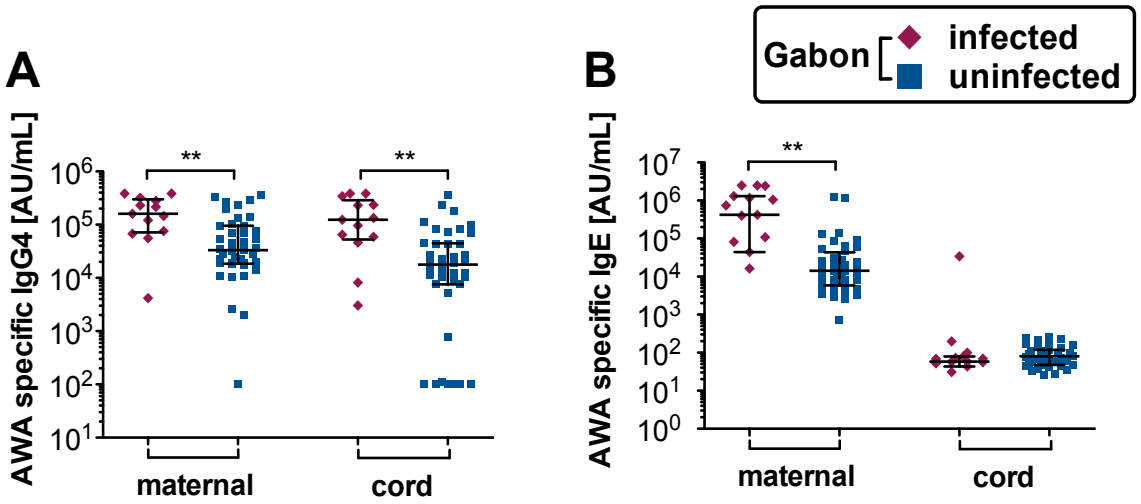


Figure 3.23: AWA specific IgG4 and IgE levels in Gabon

AWA specific IgG4 and IgE levels were measured in plasma via ELISA. All data are shown with median and interquartile range. P values are for Mann-Whitney U-tests. Comparison between the two maternal, or cord blood groups, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (Gabon, maternal, uninfected) = 42; n (Gabon, maternal, infected) = 12; n (Gabon, cord, uninfected) = 42; n (Gabon, cord, infected) = 12; **(A)** AWA specific IG4 levels in in Gabonese plasma samples devided into infected and uninfected group. **(B)** AWA specific IgE levels in Gabonese plasma samples devided into infected and uninfected group.

To get a clearer hint about active infection in Gabonese mothers, AWA specific IgG4 and IgE levels were measured (Figure 3.23). The levels of AWA specific IgG4 were indeed higher in maternal and cord blood samples from *S. haematobium* infected mothers (Figure 3.23 A). The same results were found for AWA specific IgE levels (Figure 3.23 B). In German samples, no AWA specific antibodies were measured.

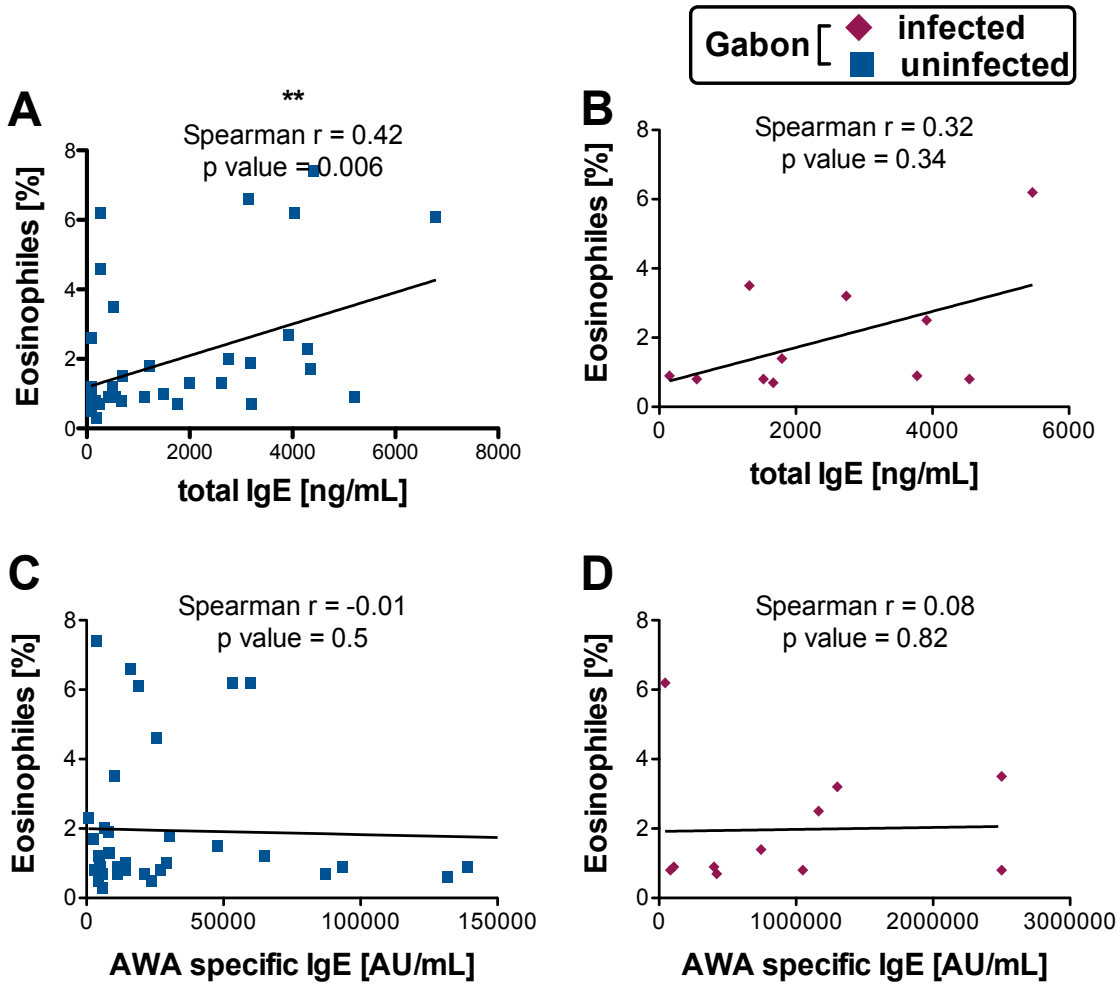


Figure 3.24 Correlation between IgE plasma levels and eosinophils in Gabonese maternal samples

Total and AWA specific IgG4 and IgE levels were measured in maternal Gabonese plasma samples via ELISA. P values are for correlationa analyisis Spearman r. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (Gabon, maternal, uninfected) = 42; n (Gabon, maternal, infected) = 12; n (Gabon, cord, uninfected) = 42; n (Gabon, cord, infected) = 12; **(A)** total maternal IgE plasma levels correlatied with percentage of eosinophils **(B)** total maternal IgE plasma levels correlatied with percentage of eosinophils within the S. haematobium infected subgroup **(C)** AWA specific maternal IgE plasma levels correlatied with percentage of eosinophils s **(D)** AWA specific maternal IgE plasma levels correlatied with percentage of eosinophils within the S. haematobium infected subgroup.

Not only antibodies such as IgE, but also eosinophil granulocytes, part of the white blood cells, play an important role in the body's response to allergic reactions, asthma and parasite infections. Many parasites, especially those that penetrate tissue, can cause eosinophilia. To fight an infection both IgE and eosinophils are coplayers. Interestingly, there actually was a strong significant correlation between the number of eosinophils and total IgE levels in Gabonese mothers, but not in maternal samples collected from women infected with *S.*

haematobium (Figure 3.24 A and B), showing a possible failure of the cooperation between IgEs and eosinophils due to S. haematobium infection. This got even stronger by looking at the correlation analysis between AWA specific maternal IgE plasma levels and eosinophils (Figure 3.24 C and D), where no correlation could be observed.
Since in German samples no eosinophils were measured correlation could only be done for Gabonese samples.

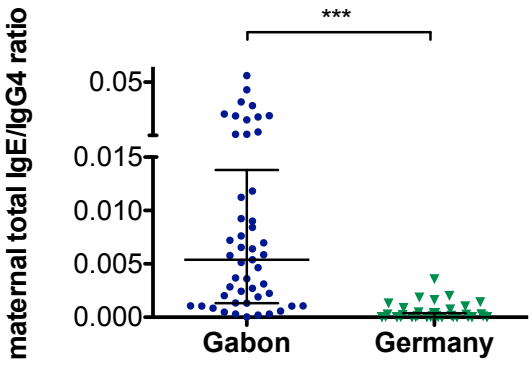


Figure 3.25: Maternal total IgE/IgG4 ratio

IgE/IgG4 ratio in maternal plasma samples; IgE and IgG4 levels were measured in plasma via ELISA. Data are shown with median and interquartile range. P value is for Mann-Whitney U-test. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 54; n (maternal, Germany) = 47;

As strong anti-parasite IgE responses are associated with resistance to helminth infection, and high levels of IgG4 have been associated with susceptibility, and since both interact, the IgE/IgG4 ratio was of special interest. The ratio of total maternal IgE/IgG4 was significantly higher in Gabonese samples than in German samples (Figure 3.25). For cord blood no comparison could be done, as there were no IgE antibodies detectable in German cord blood.

3.14 No detectable IgE production in CBMCs

It is already known that IgE has been identified in the umbilical cord blood of newborns born of mothers infected with *Schistosoma*. In this study, IgE was not exclusively found in the cord blood of neonates of with *S. haematobium* infected mothers, but also in mothers with negative CAA tests. However, we do not know whether IgE was transported directly from mother to child via the placenta, whether antigens were able to cross the placental barrier and thus the fetus itself produced IgE, or whether maternal B cells even crossed the placental barrier and then released IgE on the fetal side.

Therefore, it was necessary to investigate whether the fetal cells could react to antigens with IgE production, whether these antibodies had somatic hypermutation indicating a germinal center reaction and possibly an already functional adaptive immune response, or whether whole maternal B cells were transported from mother to fetus. The latter can be determined by the similarity between maternal and fetal IgE sequences.

The experiments, as well as the analysis of the associated data, were kindly performed and provided by Paul Haase and Professor David Vöhringer, head of the Department of Infection Biology at the University Hospital Erlangen:

Number in experiment	ID Mother	ID Child
#1	M003	F003
#2	M020	F020
#3	M032	F032
#4	M037	F037
#5	M043	F043
#6	M049	F049
#7	M051	F051

Table 3.4 Sample ID for this experiment

RNA was isolated via the column free TRIsure™ method of named PBMCs and CBMCs. "…RNA was transcribed into cDNA with Superscript™ III and oligo-dT primers. From the cDNA immunoglobulin sequences were amplified via PCR. Primer for the VH1, VH3 and VH4 family were used together with primer for the Cα, Cε, Cγ and Cμ regions respectively. For amplification a semi nested PCR was used and products of the first PCR were purified using the AMPure-XP system.

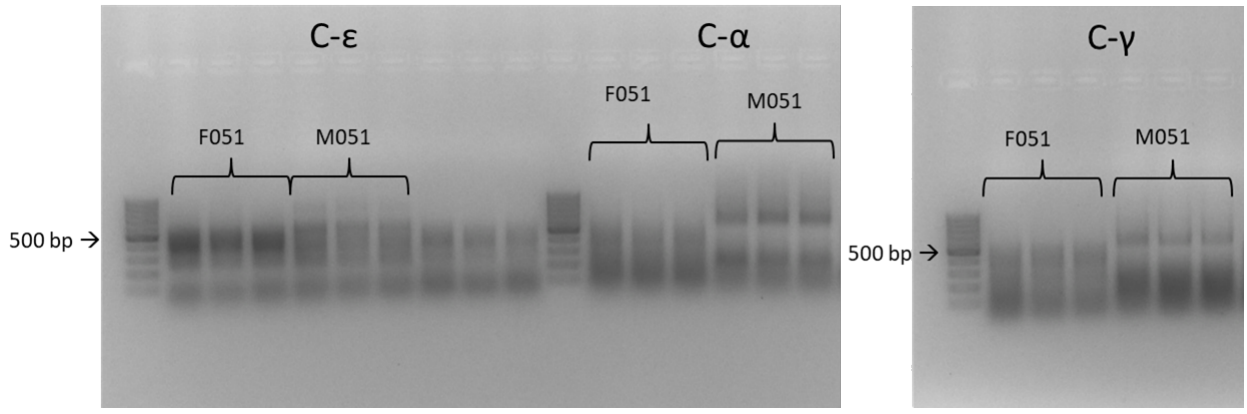


Figure 3.26 First trial amplification of one mother child pair

The cDNA of mother child pair M/F051 was amplified using primer specific for the different constant regions. 1.5% agarose gel with 100 bp DNA-ladder added. Expected product size C-α: 630~650 bp; C-γ: 630~650 bp; C-ε: 580~630 bp. *Figure with personal permission of Prof. Vöhringer*.

First the mother #7 (ID: M051; amplified from 3.6 µg RNA) was used to optimize PCR conditions and to test the primers. After successful amplification of maternal DNA, the cDNA of the corresponding child (ID: F051; amplified from 3.8 µg RNA) was amplified. The products for C-ε of both mother and child were sequenced and IgE sequences were identified for the mother, whereas no IgE sequences were found for the child. Figure 3.26 shows exemplary final amplifications for M051 and F051.

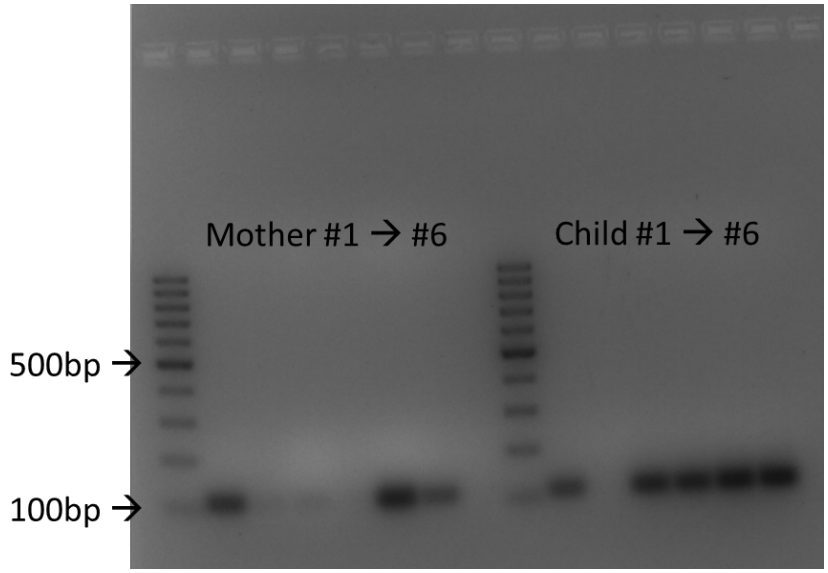


Figure 3.27 PCR for housekeeping gene HPRT

The cDNA of six mother child pairs was amplified with primers for HPRT. 1.5% Agarose gel with 100 bp DNA-ladder added. Expected product size 109 bp. *Figure with personal permission of Prof. Vöhringer*

Next the RNA from the remaining mother child pairs was amplified. The cDNA was generated from 2-5 µg RNA (exceptions: Mother #6 with 0.8 µg and Child #6 with 6.5 µg RNA). The cDNA was controlled via amplification of the housekeeping gene HPRT, as shown in Figure 3.27.

Except for Mother #4 and child #2 HPRT specific bands are visible for al samples.

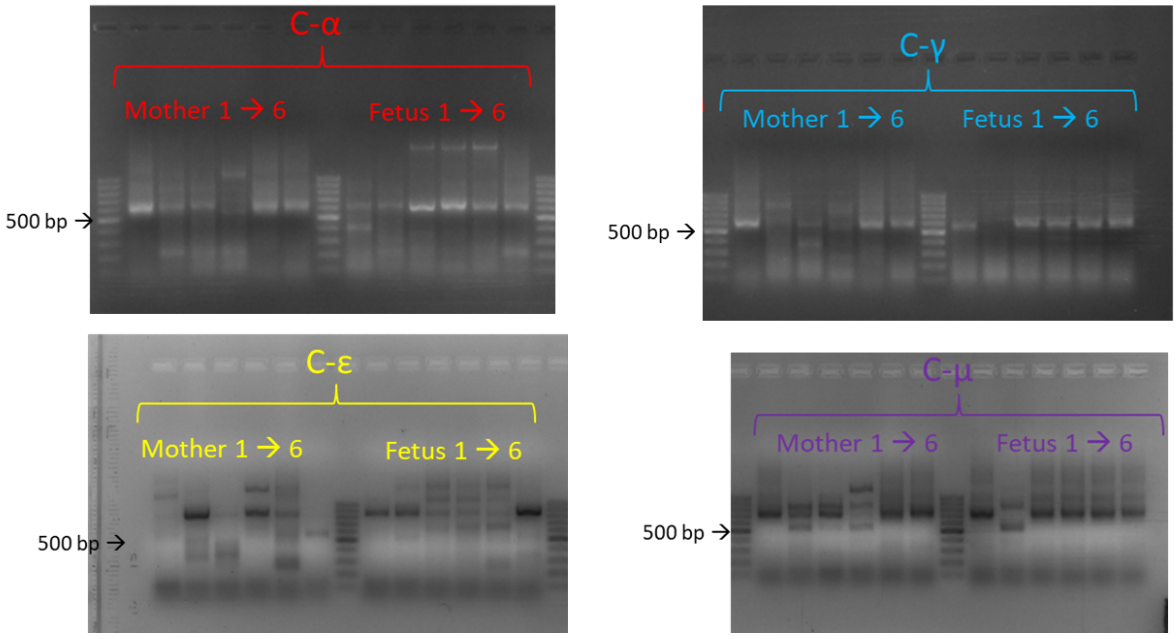


Figure 3.28 Amplification of remaining mother child pairs

The cDNA of the remaining six mother child pairs was amplified using primer specific for the different constant regions. 1.5% agarose gel with 100 bp DNA-ladder added. Expected product size C-α: 630~650 bp; C-γ: 630~650 bp; C-ε: 580~630 bp; C-μ: 700~800 bp. *Figure with personal permission of Prof. Vöhringer.*

Finally, the cDNA of the remaining mother child pairs was amplified under the previously adjusted optimized conditions, shown in Figure 3.28. Products for C-α, C-γ and C-μ could be successfully amplified for most mothers and children and the product quality aligned with the previously determined HPRT-PCR. For the C-ε specific PCR no products of expected size were observed for the children and only in some mothers. C-ε samples were sequenced for mother #5 and #6 as well as children #3-5. For mother #6 IgE sequences could be confirmed whereas no IgE was found in the children's sequences. For the children, only PCR artifacts created by primer multimerization and non-recombined VH sequences, missing a J- and D-region, were found.

After extensive testing and adjusting of the PCR condition sequences could be successfully amplified out of RNA from PBMCs of infected mothers. In contrast to the RNA out of CBMCs of corresponding newborn children where only C-α, C-γ and C-μ sequences could be amplified but no C-ε sequences…" In conclusion, only partial IgE-specific sequences could be detected for the mothers, whereas in the infants a complete absence of the template seems probable and thus either none or not enough IgE is present in the cells of the umbilical cord blood at the cellular level. However, C-α, C-γ and C-μ sequences could be amplified, suggesting a fetal production of IgA and IgM.

3.15 No profound differences in cell composition and peripheral immune cell responses between Gabon and Germany

To analyze whether German and Gabonese blood samples differed in terms of basic cell-composition as well as reaching after unspecific mitogen stimulation, PBMCs and CBMCs were stimulated with PMA/Iono and analyzed them via fluorescence-activated cell scanning (FACS).

3.15.1 Almost similar patients' characteristics between the Gabonese and German subgroup

Since 13 Gabonese women were found positive for *S. haematobium* infection, a further subgroup of 13 Gabonese and German participants with similar characteristics in terms of age was chosen for further analysis. This allows the comparison between two groups of the same size and thus the minimization of errors caused by different group sizes.

	Gabon	**Germany**	**p value**
Number n	12	13	
Mother			
Age [years]	30 ± 2	32 ± 1.3	0.31
Parity	3 ± 0.5	1.5 ± 0.5	**0.007**
Gravidity	4.8 ± 0.7	1.7 ± 0.3	**0.0003**
Gestational age at delivery (weeks + days)	39+3 ± 3	39+3 ± 3	0.89
Hemoglobin [g/dL]	11.2 ± 0.3	12.5 ± 0.3	**0.0099**
White blood cells [10^3/mm^3]	12 ± 1.6	14.4 ± 1.3	0.26
Eosinophils [%]	1.9 ± 0.6	Not done	
25-OH-Vitamin D3 concentration [ng/mL]	31.2 ± 2	27.3 ± 4.8	0.48
Calcium [mmol/L]	2.36 ± 0.03	2.3 ± 0.02	0.35
CRP [mg/dL]	0.98 ± [0.5;2.1]	0.96 ± [0.38;1.58]	0.51
Newborn			
Gender (male)	6 (50)	3 (77)	0.19
Length [cm]	50 ± 0.4	53 ± 0.9	**0.0053**
Birthweight [g]	3243 ± 91	3420 ± 162	0.35
25-OH-Vitamin D3 concentration [ng/mL]	32.8 ± 2.3	29.4 ± 5.1	0.56

Calcium [mmol/L]	2.7 ± 0.05	2.8 ± 0.04	0.54
CRP [mg/dL]	0.01 ± [0.01;0.02]	0.01 ± [0.01;0.02]	0.98

Table 3.5 Maternal and newborn characteristics of the subgroup in Gabon and Germany

Data are presented as mean ± SD (n), median ± IQR (n) for C-reactive Protein (CRP) and ordinally scaled values, values or as numbers (%) where indicated; t-test was performed where data are normal distributed; for data without normal distribution a two-tailed Mann Whitney U-test and for ordinally scaled values a Chi Square test was performed; blood for plasma parameters was taken from maternal peripheral vein blood or from cord blood, respectively;

Eventually two Gabonese cohorts (noninfected and infected with *S. haematobium)* consisted of 12 mother-newborn pair in each group. Data of 13 German pairs could be successfully analyzed, too (Table 3.5 and Table 3.6).

Looking at the noninfected Gabonese and German subgroup, only some significant differences revealed. As requested, both mothers had on average the same age (30 or 32 years). Significant differences between the two study populations emerged in regard to parity (Gabonese mothers beared on average 1.5 more children), but also the gravidity was significantly higher in Gabon (4.8 vs. 1.7), suggesting more pregnancy loss in Gabon. Similar to all participants, also the subgroups showed accouchement on average at 39 weeks of gestation. Furthermore, the hemoglobin concentration remained significantly lower (1.3g/dL) within the Gabonese cohort, whereas there is no significant difference in the amount of white blood cells between the Gabonese and German subgroup. Interestingly, measurements of 25(OH)D-Vitamin D3 as well as CRP or calcium plasma levels resulted in on average equal levels in Gabonese and German maternal and cord blood plasma samples. And still, in cord blood, CRP was almost non-detectable.

There was no difference in gender distribution of neonates between both countries, with 6 out of 12 male newborns in Gabon and 3 out of 13 in Germany. Even though birth weight did differ in both subgroups, the Gabonese newborns were, however, significantly smaller (3cm).

Thus, in summary despite the numbers of parity and gravidity (significantly higher in the Gabonese subgroup) and the lower maternal hemoglobin levels and the smaller birth size in Gabon, the Gabonese and German subgroups had both similarities and differed only slightly in terms of patients' characteristics.

Looking at all participants of the Gabonese cohort the infection with *S. haematobium* did not influence birth related data significantly (see Table 3.2 above). Anyhow, as shown in Table 3.6, the infected mothers are on average six years younger than the uninfected chosen for the subgroup. Moreover, the concentration of maternal and cord blood 25-OH-Vitamin D3 was around 7.3ng/mL (maternal) or 7.9ng/mL (cord blood) significantly higher in plasma samples from infected participants. Here, maternal calcium plasma levels were likewise significantly lower (0.11mmol/L). In the infected group all mothers took antihelminthic treatment during

pregnancy as prophylaxis, whereas in the uninfected group only 83%. At birth on mother negative for *S. haematobium* was infected with *Loa loa* and two *S. haematobium* positive mothers were coinfected with *Hookworm* (1) or *Strongyloides* (1). Anti-malaria drugs (Combimal® or Quinine) were taken by 3 mothers from the uninfected subgroup and 4 from the infected, whereas only two mothers stated to have suffered from malaria during pregnancy. Anti-malaria drugs are also taken for the purpose of pregnancy prophylaxis in Lambaréné. At delivery all mothers had a negative blood smear as malaria disease was an exclusion criterion.

	Gabon		**p value**
	Negative for *Schistosoma haematobium*	**Positive for *Schistosoma haematobium***	
Number n	12	12	
Mother			
Age [years]	30 ± 2	24 ± 1.4	**0.03**
Parity	3 ± 0.5	2 ± 0.5	0.13
Gravidity	4.8 ± 0.7	3.5 ± 0.5	0.19
Gestational age at delivery (weeks + days)	39+3 ± 3	39+0 ± 0	0.68
Hemoglobin [g/dL]	11.2 ± 0.3	10.5 ± 0.6	0.33
White blood cells [10^3/mm^3]	12 ± 1.6	13.1 ± 1.8	0.64
Eosinophils [%]	1.9 ± 0.6	1.9 ± 0.5	0.99
25-OH-Vitamin D3 concentration [ng/mL]	31.2 ± 2	38.5 ± 2.5	**0.034**
Calcium [mmol/L]	2.36 ± 0.03	2.25 ± 0.03	**0.03**
CRP [mg/dL]	0.98 ± [0.5;2.1]	1.38 ± [0.26; 2.04]	0.97
Anti-helminthic treatment	10 (83%)	12 (100%)	0.14
Infection with other helminths	1 (8%)	2 (17%)	0.53
Antimalaria treatment	3 (25%)	4 (33%)	0.65
Malaria during pregnancy	3 (25%)	2 (17%)	0.62
Newborn			
Gender (male)	6 (50)	6 (50)	1
Length [cm]	50 ± 0.4	49 ± 0.5	0.36
Birthweight [g]	3243 ± 91	2990 ± 119	0.10
25-OH-Vitamin D3 concentration [ng/mL]	32.8 ± 2.3	40.7 ± 2.8	**0.04**
Calcium [mmol/L]	2.7 ± 0.05	2.6 ± 0.1	0.30
CRP [mg/dL]	0.01 ± [0.01;0.02]	0.01 ± [0.01;0.02]	0.83

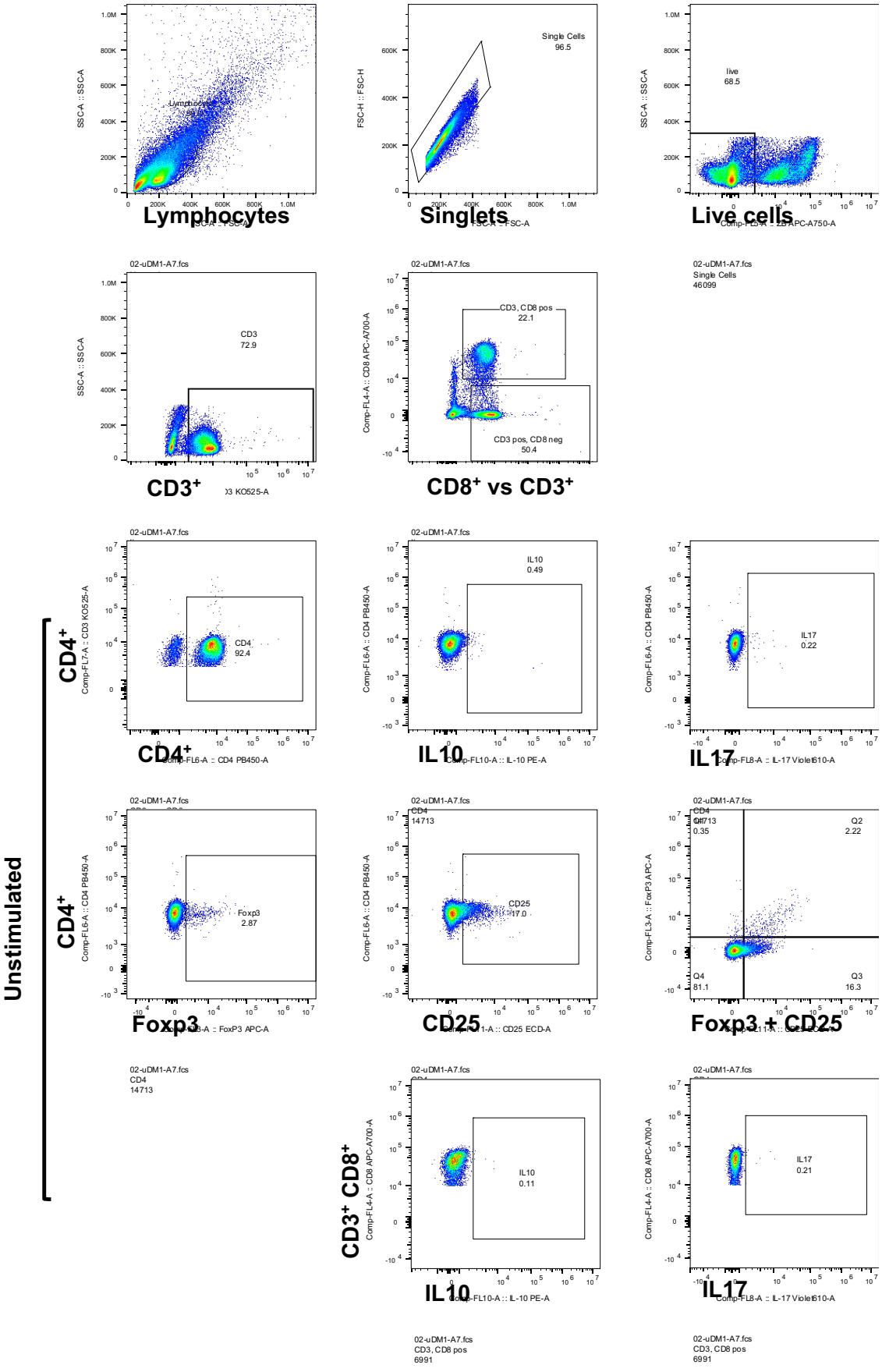
Table 3.6 Maternal and neonatal characteristics of the Gabonese subgroups

Data are presented as mean ± SD (n), median ± IQR (n) for C-reactive Protein (CRP) and ordinally scaled values, values or as numbers (%) where indicated; t-test was performed where data are normal distributed; for data without normal distribution a two-tailed Mann Whitney U-test and for ordinally scaled values a Chi Square test was performed; blood for plasma parameters was taken from maternal peripheral vein blood or from cord blood, respectively; S. haematobium diagnostics was done by egg count or detection of levels of schistosome specific circulating anionic antigen (CAA) in plasma samples.

Hence, choosing participants to create subgroups leads to less significant differences between the Gabonese and German subgroups, but spawned few significant differences (age, parity, gravidity or vitamin D plasma levels) within the Gabonese subset.

3.15.2 Gating strategy

Two panels were generated to detect Th17 and Treg cells and the cytokines IL10 and IL17 (Figure 3.29) and Th1 and Th2 cells and the cytokines IL5, TNFα and IFNγ (Figure 3.30). Each group consisted of twelve to 13 samples and every samples was individually analyzed with FlowJo®. Below I show an example of one sample for the gating strategy of each panel. The compensation matrix was done with compensation beads.


Unstimulated
Lymphocytes
Singlets
Live cells
CD3+
CD8+ vs CD3+
CD4+
IL10
IL17
CD4+
Foxp3
CD25
Foxp3 + CD25
CD3+ CD8+
IL10
IL17
Lymphocytes
68.5
Single Cells
96.5
live
68.5
CD3
72.9
CD3, CD8 pos
22.1
CD3 pos, CD8 neg
50.4
02-uDM1-A7.fcs
Single Cells
46099
CD4
92.4
IL10
0.49
IL17
0.22
Foxp3
2.87
CD25
17.0
Q2
2.22
Q4
81.1
Q3
16.3
02-uDM1-A7.fcs
CD4
14713
IL10
0.11
IL17
0.21
02-uDM1-A7.fcs
CD3, CD8 pos
6991

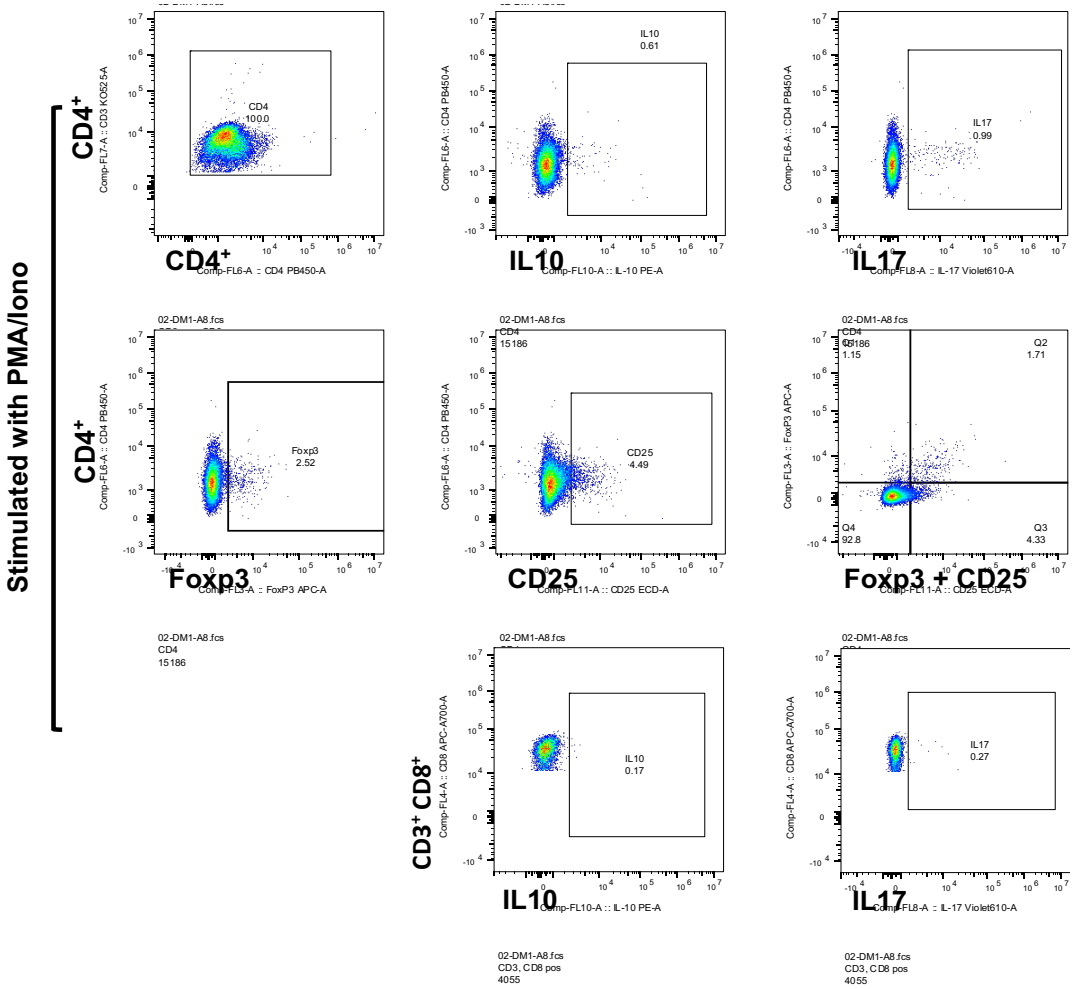
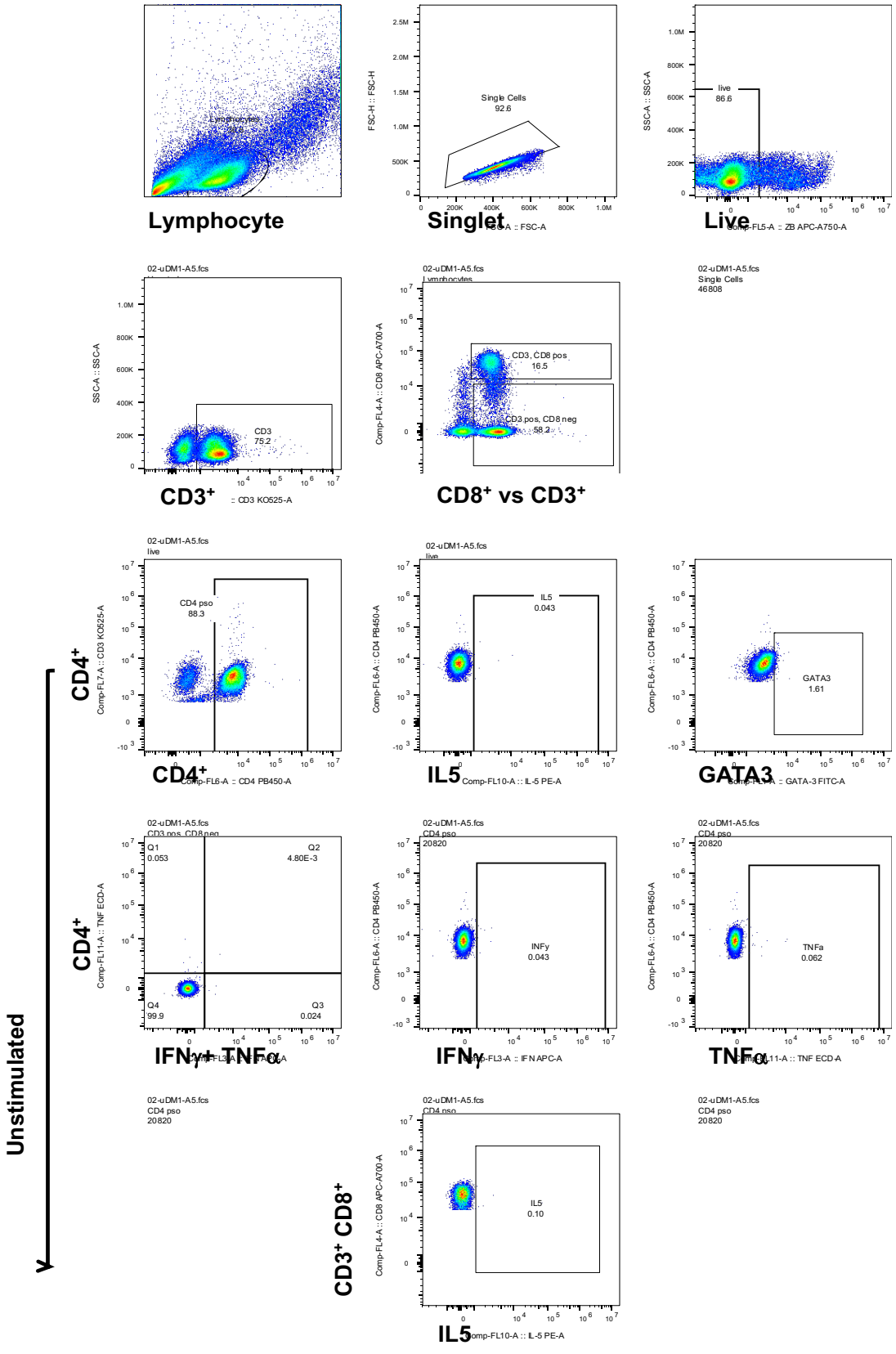


Figure 3.29: Flow cytometry gating strategy for the Th17/Treg panel

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stimulated with PMA plus ionomycin (P/I) for six hours and stained with fluorescently labeled antibodies specific to surface markers (Zombie, CD3, CD4, CD8, CD25) and with intracellular markers (FOXP3, IL10, IL17). Here I show an example of the gating strategy used for the identification of stimulated and unstimulated $CD4^+CD8^-$ and $CD3^+/CD8^+$ cells. First cells were gated for lymphocytes (FSC-A vs SSC-A) and for singlets (FSC-A vs FSC-H). Next, live cells stained with Zombie NRI were selected, following by $CD3^+$ and $CD8^+$ or $CD8^-$ cells, respectively; either $CD4^+$, followed by either IL10, IL17 or Foxp3 and CD25 (for $CD3^+CD8^-$), or IL10 or IL17 for $CD3^+/CD8^+$ was applied.

First, a gate was set on lymphocytes, afterwards on single cells and then on live cells. To see the amount of $CD3^+$ cells were gated on SSC-A vs. CD3. As well $CD3^+/CD8^-$ and $CD3^+/CD8^+$ cells were separated. Afterwards the $CD3^+/CD8^-$ cells were gated for $CD4^+$ cells, followed by IL10, IL17, Foxp3 or CD25, respectively (CD4 vs IL10/Foxp3/CD25). Moreover, to see Foxp3/CD25 double positive cells, Foxp3 was gated vs CD25. $CD3^+/CD8^+$ cells were gated for

the cytokines IL10 and IL17 (CD8 vs IL10/IL17). Same gating strategy was performed for with PMA/Iono stimulated and for unstimulated cells.



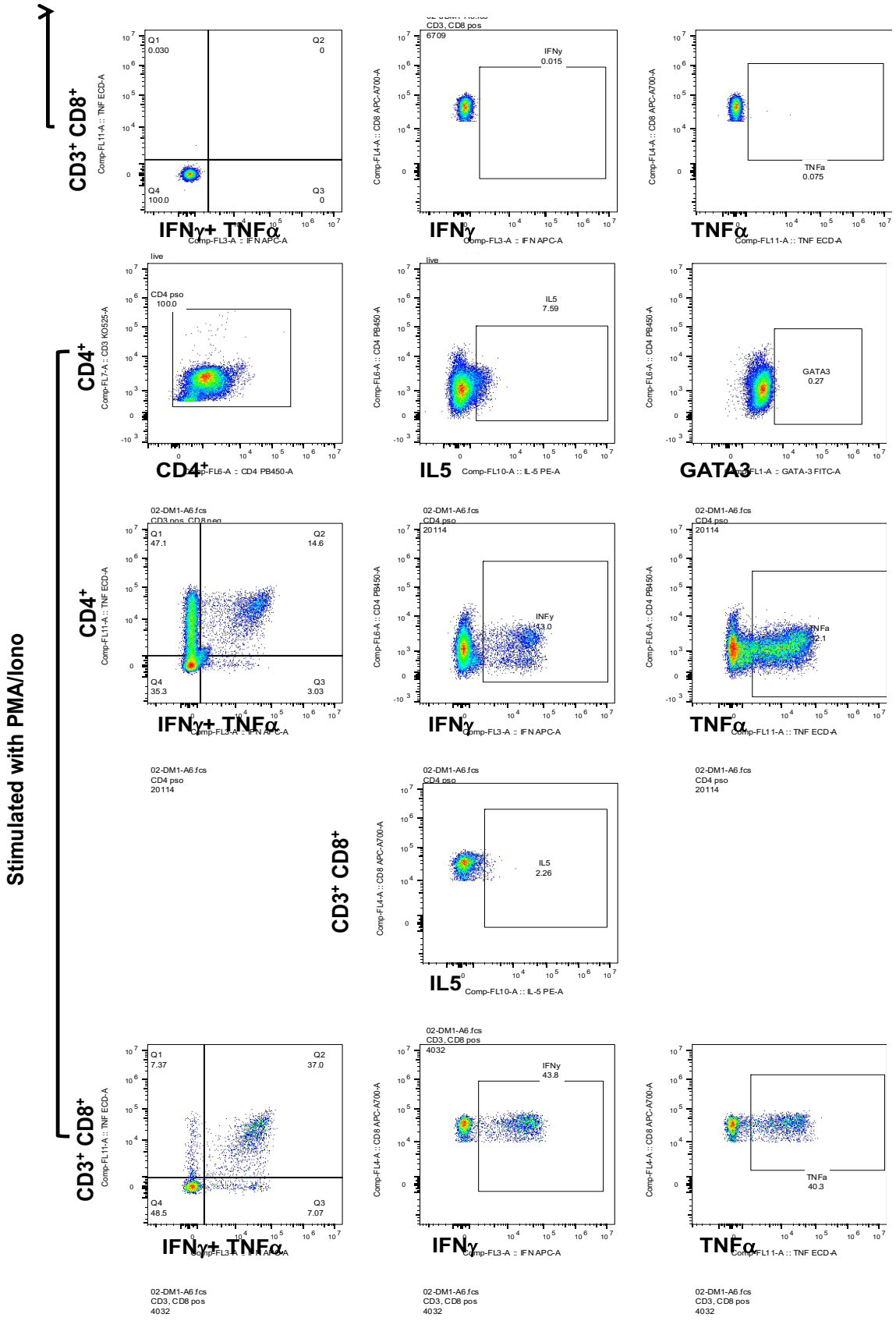


Figure 3.30: Flow cytometry gating strategy for the Th1/Th2 panel

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stimulated with P/I for six hours and stained with fluorescently labeled antibodies specific to surface markers (Zombie, CD3, CD4, CD8) and intracellular markers (GATA3, IL5, TNFα, IFNγ). Here I show an example of the gating strategy used for the identification of stimulated and unstimulated $CD4^+CD8^-$ and $CD3^+/CD8^+$ cells. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and for singlets (FSC-A vs FSC-H). Next, live cells stained with Zombie NRI were selected, following by $CD3^+$ and $CD8^+$ or $CD8^-$ cells, respectively; either $CD4^+$, followed by either GATA3, IL5, TNFα, and IFNγ for $CD3^+CD8^-$ or IL5, TNFα, and IFNγ for $CD3^+/CD8^+$ was applied.

For the Th1/Th2 panel the same procedure was performed as described above to get either $CD4^+$ or $CD3^+/CD8^+$ cells. In the $CD4^+$ cells the expression of GATA3, IL5, TNFα and IFNγ was investigated. Within the the $CD3^+/CD8^+$ cell population IL5, TNFα and IFNγ were on special interest. In both groups, TNFα vs. IFNγ was gated to see double positives. Same gating strategy was performed for with PMA/Iono stimulated and unstimulated cells.

3.15.3 Less lymphocytes and live cells in PBMCs from *S. haematobium* infected Gabonese mothers

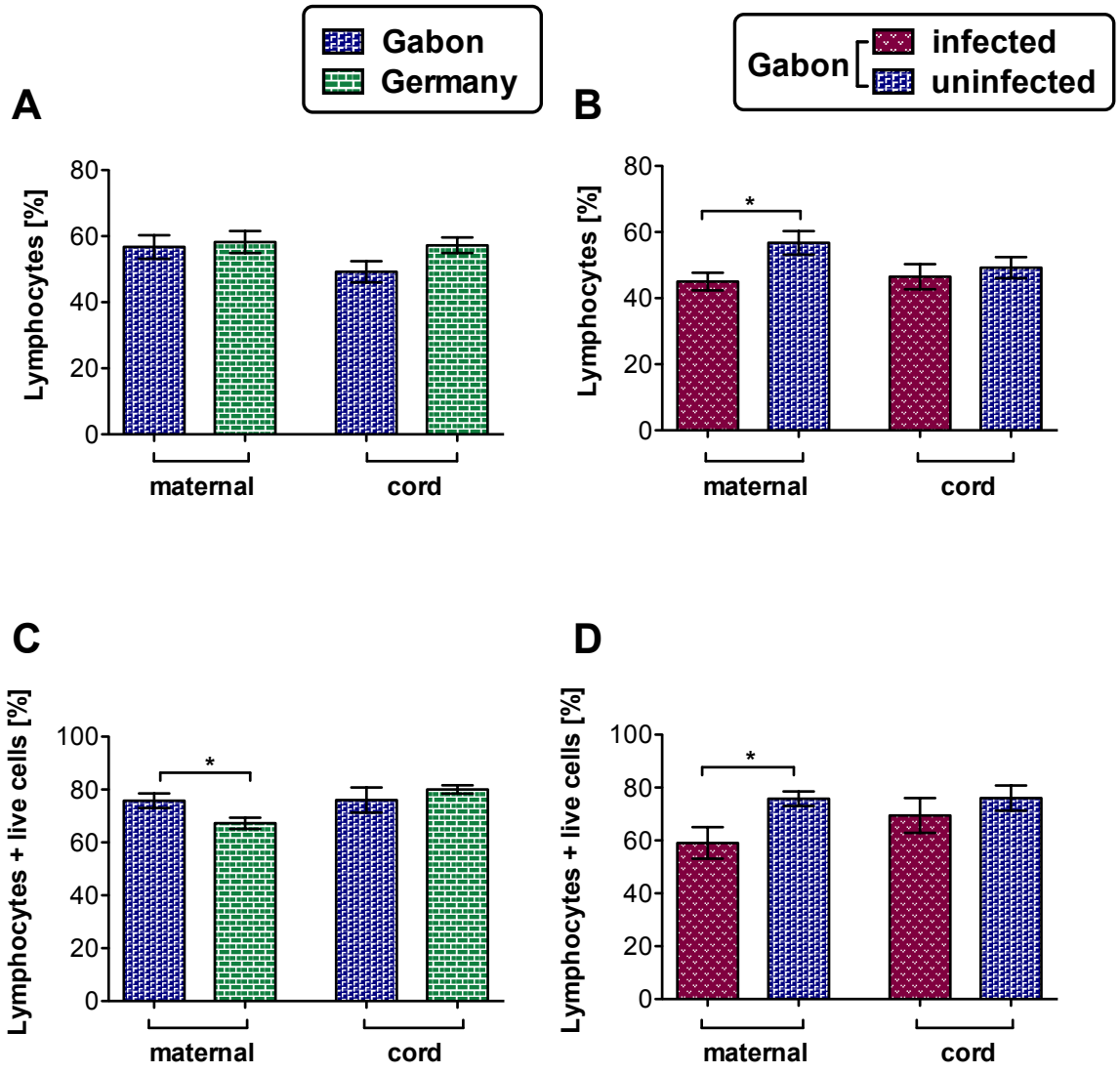


Figure 3.31 Percentage of lymphocytes and live cells in CBMCs and PBMCs.

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stained with fluorescently labeled antibodies specific to surface markers (Zombie NRI). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group “Gabon, uninfected” in graph B, D, F corresponds to “Gabon” in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** Cells were gated for lymphocytes (FSC-A vs. SSC-A). **(C, D)** Live cells were gated negative for Zombie NIR;

As the immune system and especially T cells, which play a substantial role in the defense against helminth infections on the one side, but also in the development of allergies, were at a

special interest for this thesis, the first gate was done for lymphocytes. On average there were between 40 and 60 percent of lymphocytes. Within the Gabonese cohort, less lymphocytes as well as less alive lymphocytes could be found in PBMCs of *S. haematobium* infected mothers (Figure 3.31 B and D). Figure 3.31 C shows less alive lymphocytes in unstimulated German PBMCs compared to the Gabonese. All in all, there was no significant difference between stimulated and unstimulated cells, meaning a satisfying cell-survival during stimulation (data not shown). Having 60 to 80% live cells in all three groups was indicative that the freezing and thawing process as well as the shipment from Gabon to Germany worked well.

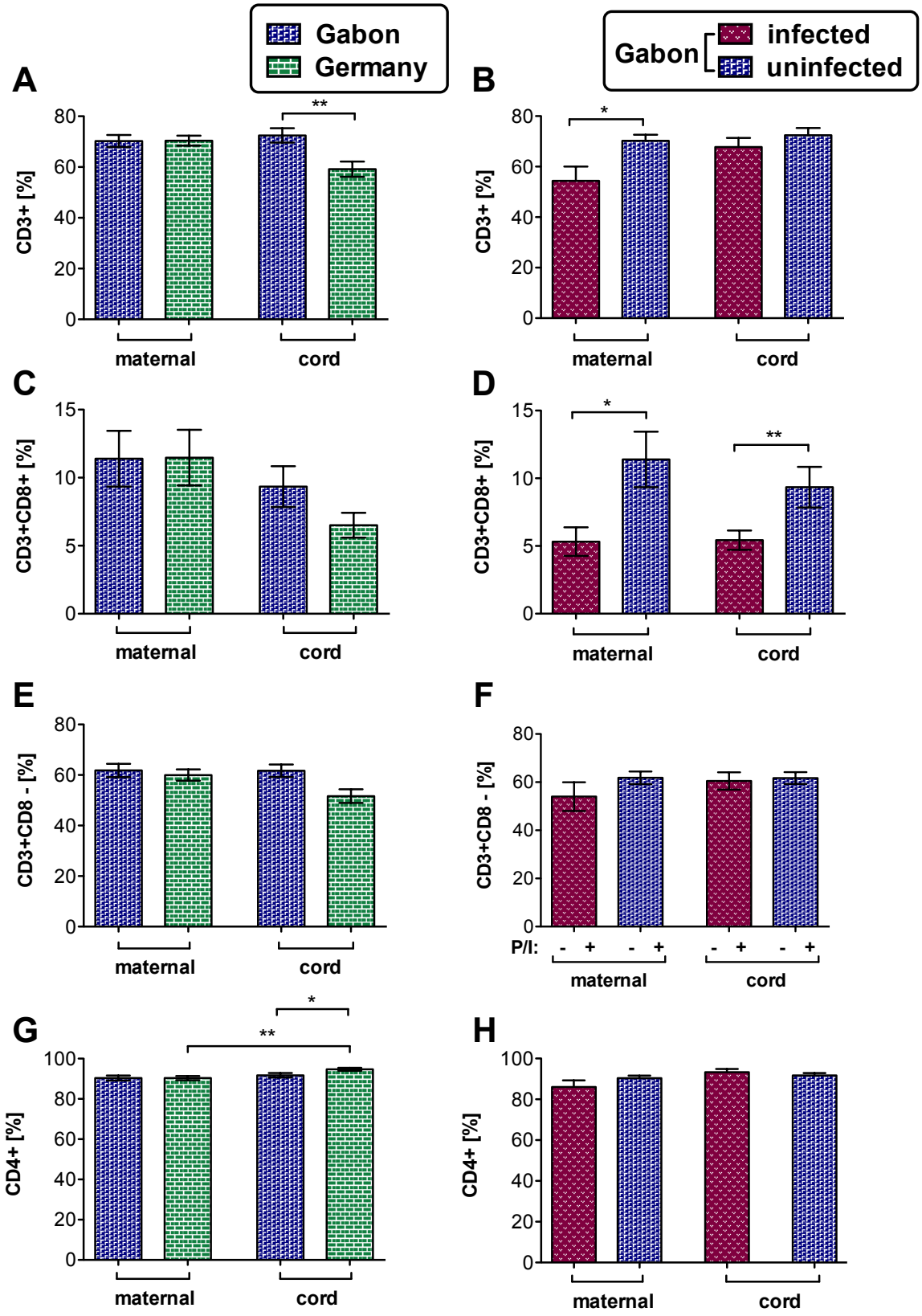


Figure 3.32 Percentage of CD3, CD8 positive, CD8 negative and CD4 cells

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stained with fluorescently labeled antibodies specific to surface markers (Zombie NIR, CD3, CD8, CD4). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group "Gabon, uninfected" in graph B, D, F corresponds to "Gabon" in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** percentage of CD3 positive cells; **(C, D)** percentage of CD3, CD8 positive cells; **(E, F)** percentage of CD3 positive, CD8 negative cells. **(G, H)** percentage of CD4 positive cells within the CD3 positive, CD8 negative cell population;

CD3 is a surface marker for T lymphocytes. The surface marker CD8 is representative for cytotoxic T cells, which are responsible for the direct killing of infected, damaged, or dysfunctional cells. CD4 is expressed on T helper cells such as Th1 and Th2 cells. They recognize exogenous peptide antigens and activate further cells of the immune system by secreting cytokines and direct cell-cell contact. T-helper cells type 1 support the cell-mediated immune response by e.g. activating macrophages or cytotoxic T-cells. T-helper cells type 2 induce the humoral immune response by interacting with B-cells. Normally per definition all $CD3^+/CD8^-$ cells should also be positive for CD4. Comparing the Gabonese with the German cohort (Figure 3.32 A, C, E) almost no significant difference between the percentage of $CD3^+$, $CD3^+/CD8^+$, $CD3^+/CD8^-$ and $CD4^+$ cells was found. Round about 60-80% $CD3^+$, 12% $CD3^+/CD8^+$ and 55-60% $CD3^+/CD8^-$ cells were detected in each cohort. As expected on average almost 90% of the $CD3^+/CD8^-$ cells were also positive for $CD4^+$. Thus, the biggest cell $CD4^+$ population could be found in German cord blood samples, whereas the percentage of total $CD3^+$ cells was less compared to the Gabonese CBMCs. Comparing the cells form Gabonese with *S. haematobium* infected participants with uninfected significantly less T cells ($CD3^+$) in PBMCs as well as less $CD3^+/CD8^+$ cells in PBMCs and CBMCs from infected mothers could be seen (Figure 3.32 B, D, F). The percentage of $CD3^+/CD8^-$ and $CD4^+$ cells remained comparable between the two groups.

Taken together, German cord blood samples showed significantly less $CD3^+$ cells but the biggest percentage of $CD4^+$ cell populations. Furthermore, within the Gabonese group, the infection status with *S. haematobium* seemed to have an impact on the T cells ($CD3^+$) and $CD3^+/CD8^+$ cells, by lowering their amount.

3.15.4 Successful stimulation and cytokine production in Th1 and Th2 cells

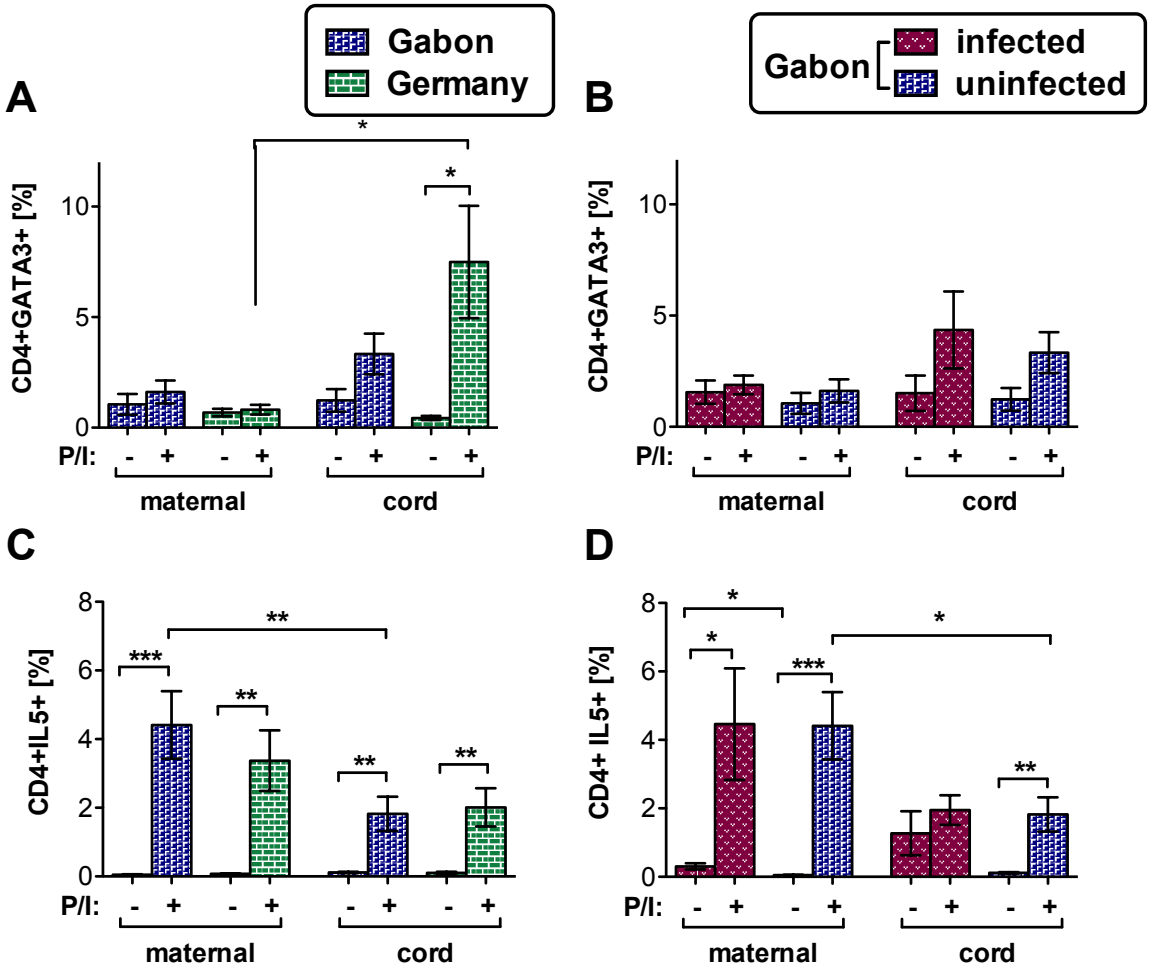


Figure 3.33 Percentage of GATA3 and IL5 expression in CD4 positive cells

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stimulated with P/I for six hours and stained with fluorescently labelled antibodies specific to intracellular markers (GATA3, IL5). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group “Gabon, uninfected” in graph B, D, F corresponds to “Gabon” in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** percentage of $CD4^+$GATA3 positive cells; **(C, D)** percentage of $CD4^+$IL5 positive cells.

The embryonic development and ensuring the correct function of various tissues is regulated by the transcription factor GATA3, among others. At the same time GATA3 has a decisive role in inflammatory and humoral immune responses [18]. GATA3 is required for the development and/or function of innate lymphoid cells as well as for the development of T helper cells (Th cells), particularly Th2 cells [291]. Furthermore, it promotes the secretion of IL4, IL5 and IL13 from Th2 cells [281]. IL5 is produced by Th2 cells and mast cells. Besides its role as key

mediator in eosinophil activation by binding to its receptor, it stimulates B cells and activates antibody secretion. Comparing the Gabonese with the German cohort (Figure 3.33 A, C), there were no significant differences in GATA3 or IL5 expression. In stimulated cells about 2% GATA3 expression and 4.4% IL5 production could be detected. Though GATA3 expression was significantly higher in stimulated German CBMCs compared to their mothers. Furthermore, it was evident that the stimulation for IL5 indeed did work properly (Figure 3.33 C, D) and a trend of higher expression in maternal CD4 cells could be seen. Within the Gabonese cohort (Figure 3.33 B, D) this trend was even significant in the uninfected cohort.

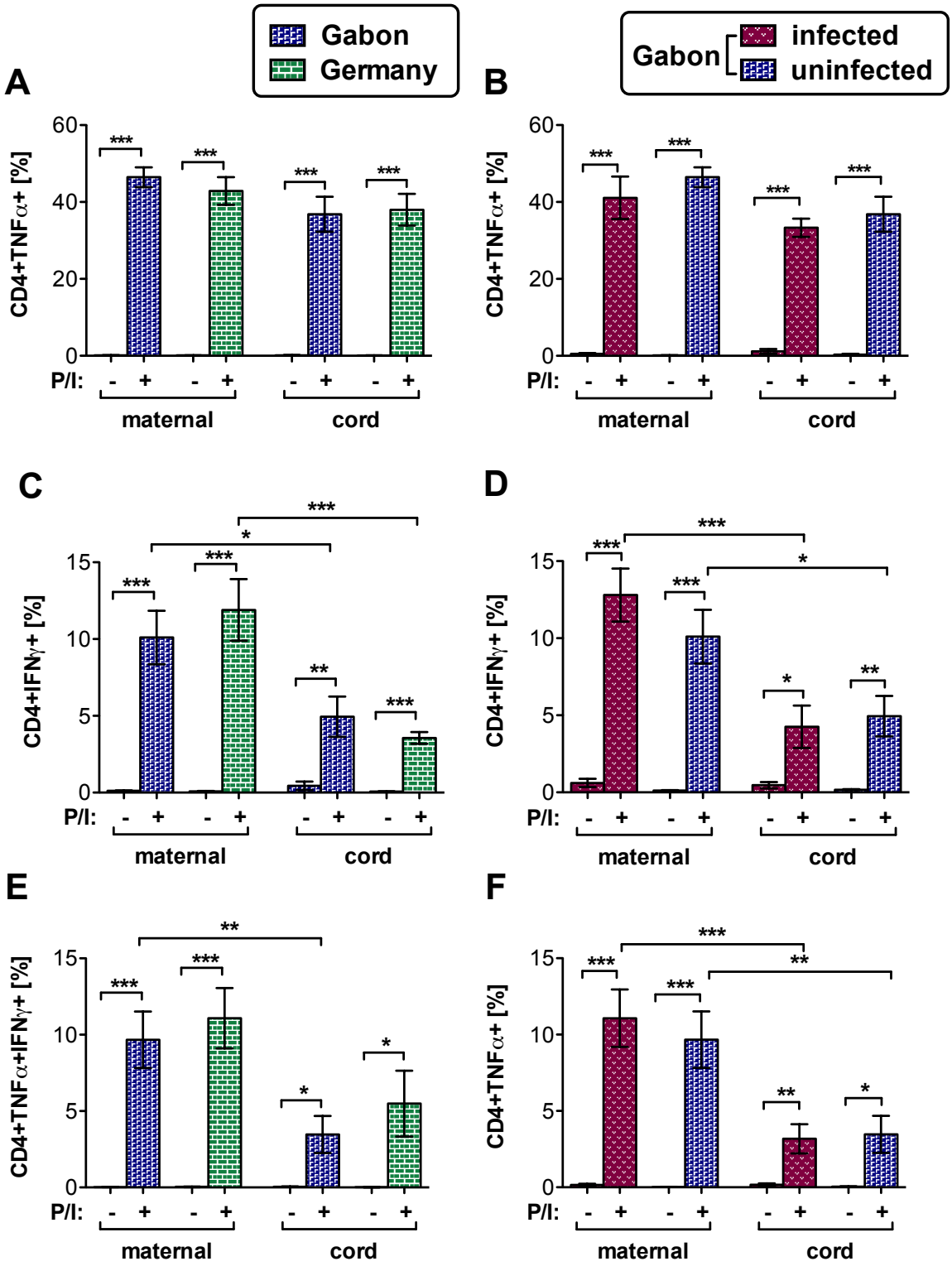


Figure 3.34 Percentage of TNFα and IFNγ expression in CD4 positive cells

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stimulated with P/I for six hours and stained with fluorescently labelled antibodies specific to intracellular markers (TNFα, IFNγ). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group "Gabon, uninfected" in graph B, D, F corresponds to "Gabon" in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** percentage of $CD4^+$TNFα positive cells; **(C, D)** percentage of $CD4^+$IFNγ positive cells; **(E, F)** percentage of $CD4^+$ TNFα and IFNγ double positive cells.

The cytokine TNFα is involved in systemic inflammation and plays a role in the acute phase reaction. It is produced by many different cell types such as $CD4^+$ T cells to regulate immune responses. IFNγ is critical for the innate and adaptive immune system. It is mainly produced by natural killer cells as well as by Th1 and cytotoxic T cells once the antigen specific immunity is developed.

Again, the stimulation worked nicely in PBMCs as well as CBMCs. Stimulated cells produced about 45% TNFα and 12% IFNγ. 11% showed signal for both, TNFα and IFNγ. Figure 3.34 A, C and E shows the comparison between the Gabonese and the German subgroup. Whereas both groups seemed to produce a similar amount of TNFα and IFNγ, for IFNγ the percentage was significantly higher in PBMCs compared to the corresponding CBMCs in both cohorts and for TNFα and IFNγ double positive CD4 cells only in the Gabonese subgroup. These findings were independent from *S. haematobium* infection (Figure 3.34).

In sum, the Gabonese subgroup did not differ in regard of TNFα and IFNγ expression from the German, nor the infection with *S. haematobium* had a significant influence on cytokine production (Figure 3.34). Anyway, the cytokine expression was significant higher in maternal Cd4 cells for only IFNγ in all subgroups and for TNFα and IFNγ double positive in the Gabonese subgroup.

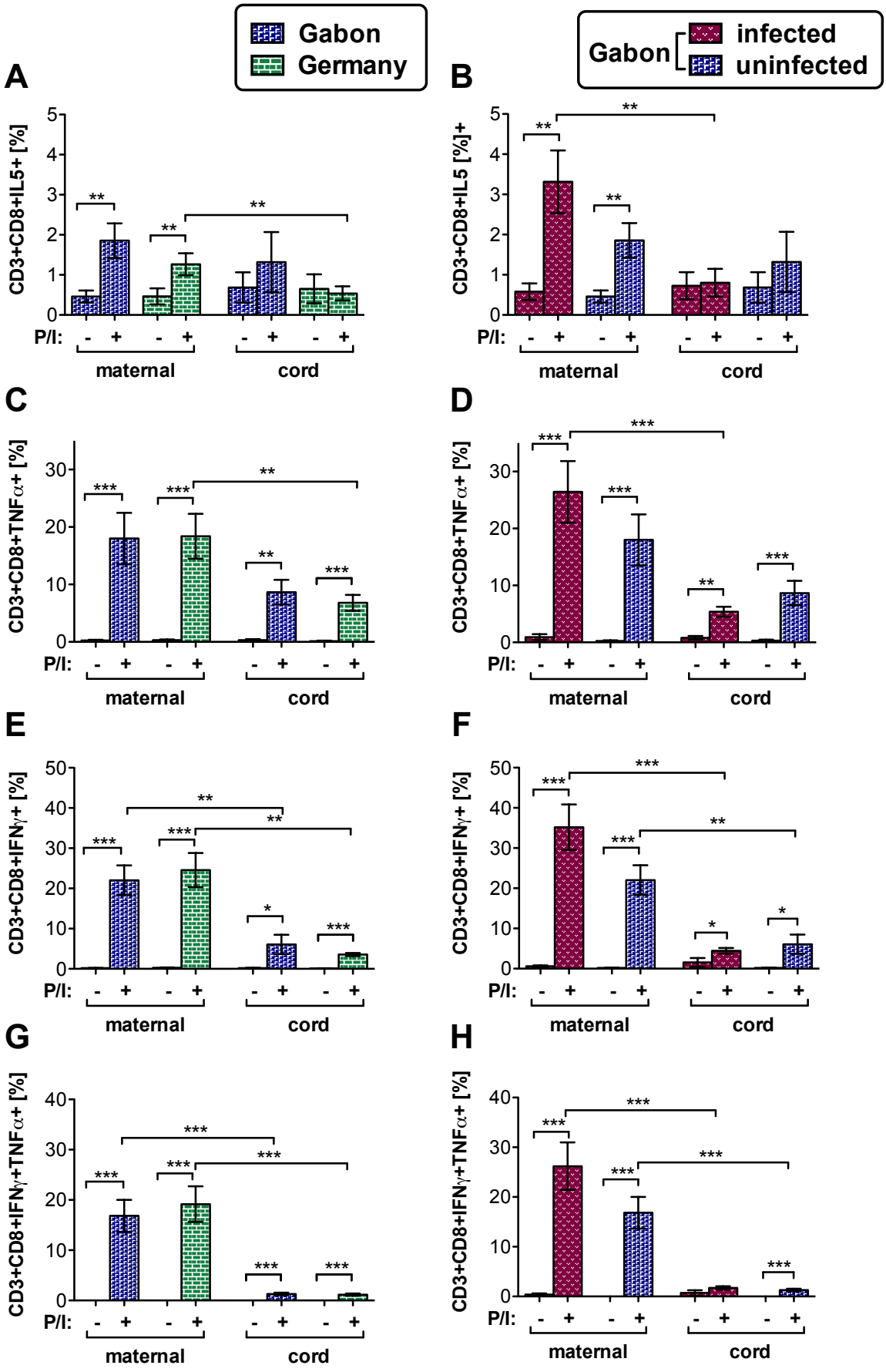

A
Gabon
Germany
CD3+CD8+IL5+ [%]
B
Gabon
infected
uninfected
CD3+CD8+IL5 [%]+
C
CD3+CD8+TNFα+ [%]
D
CD3+CD8+TNFα+ [%]
E
CD3+CD8+IFNγ+ [%]
F
CD3+CD8+IFNγ+ [%]
G
CD3+CD8+IFNγ+TNFα+ [%]
H
CD3+CD8+IFNγ+TNFα+ [%]
P/I:
maternal
cord

Figure 3.35 Percentage of IL5, TNFα and IFNγ expression in CD8 positive cells

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stimulated with P/I for six hours and stained with fluorescently labelled antibodies specific to intracellular markers (IL5, TNFα, IFNγ). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group "Gabon, uninfected" in graph B, D, F corresponds to "Gabon" in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** percentage of IL5 positive cells; **(C, D)** percentage of TNFα positive cells; **(E, F)** percentage of IFNγ positive cells; **(G, H)** percentage of TNFα and IFNγ double positive cells.

Similar to the $CD4^+$ cells, also in the $CD8^+$ cells no difference between the groups in cytokine expression could be found (Figure 3.35). Once some PBMCs produced more cytokines after stimulation than CBMCs. This was true for IL5 and TNFα in the German and S. haematobium infected subgroup and for TNFα and TNFα/IFNγ double positive cells in all subgroups. Nevertheless, the Gabonese and German CMBCs did not yet appear to be able to produce IL5 (Figure 3.35 A, B), as no significant difference between unstimulated and stimulated cells could be detected. For TNFα and IFNγ stimulation worked successfully in both, PBMCs and CBMCs. Stimulated cells produced about 2% IL5, 20% TNFα and 25% IFNγ. 18% showed signal for both, TNFα and IFNγ.

All in all, CD4 positive as well as CD8 positive cells could be stimulated successfully showing mostly significantly higher rates of cytokine production in maternal cells.

3.15.5 Higher IL10 production in T cells form with *S. haematobium* infected mothers

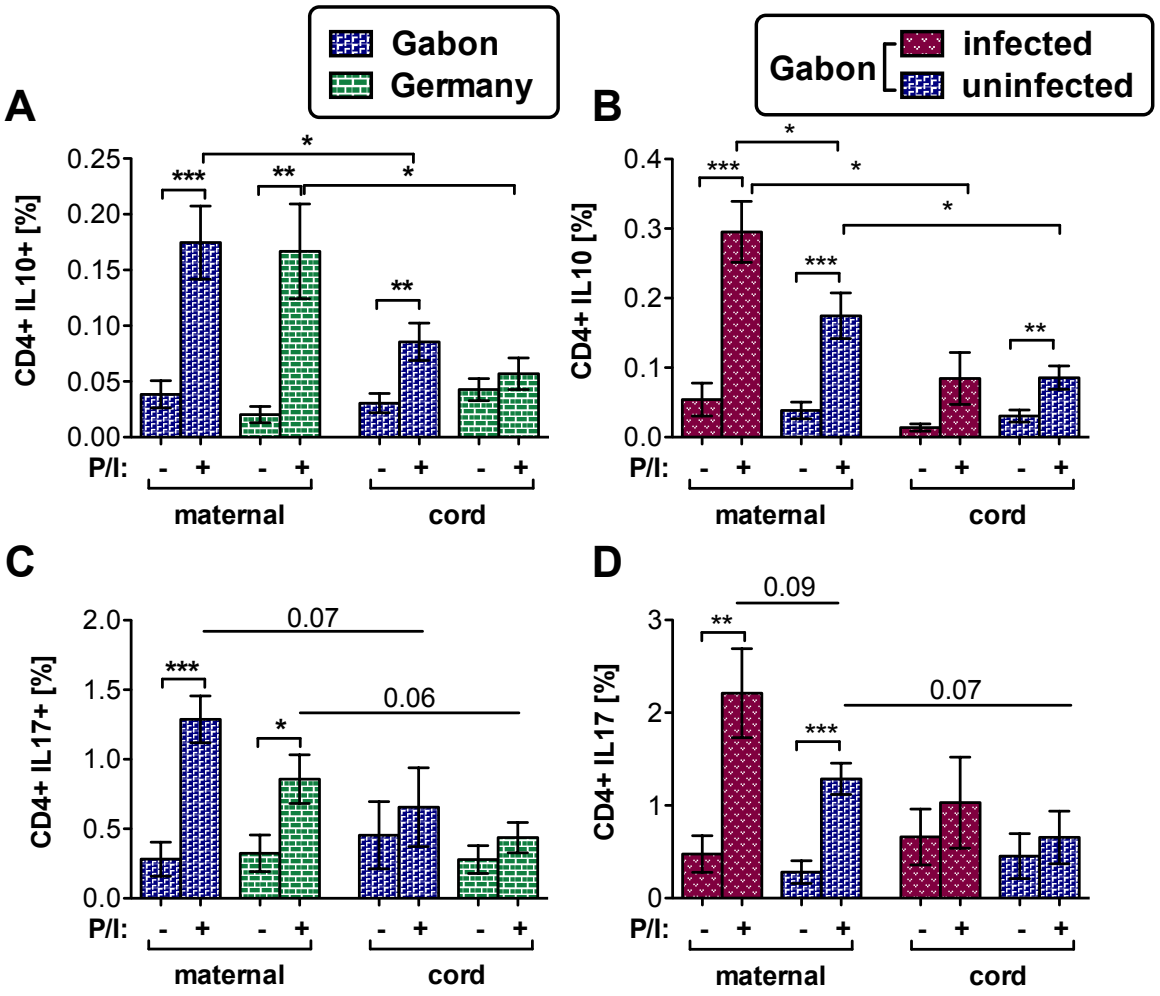


Figure 3.36 Percentage of IL10 and IL17 expression in CD4 positive cells

CBMCs and PBMCs from all participants were isolated, fixed and after thawing stimulated with P/I for six hours and stained with fluorescently labelled antibodies specific to intracellular markers (IL10, IL17). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group "Gabon, uninfected" in graph B, D, F corresponds to "Gabon" in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** percentage of $CD4^+$IL10 positive cells; **(C, D)** percentage of $CD4^+$IL17 positive cells.

The anti-inflammatory cytokine IL10 is mainly produced by monocytes, but also by Th2 or Treg cells. The pro-inflammatory cytokine IL17 is produced by Th17 cells and involved in inducing and mediating pro-inflammatory immune responses, playing a major role in allergic reactions. Contrasting the Gabonese with the German cohort (Figure 3.36 A, C) no differences in IL10 or

IL17 expression between the two countries could be seen. After stimulation maternal cells produced significantly more IL 10 than the cord blood cells. Same trend could be demonstrated for IL17. Stimulated cells produced about 0.17% IL10 and 1.3% IL17. Looking at the Gabonese group the IL10 expression in PBMCs from with *S. haematobium* infected mothers was significantly higher compared to the uninfected group as well as to their corresponding CBMCs. Same trend could be seen for IL17 (Figure 3.36 B). Anyhow, IL10 and IL17 cytokine production was not very large in all CBMCs.

In summary, the stimulation resulted in more IL10 and IL17 production in PBMCs than in CBMCs, with the significantly highest in PBMCs of infected mothers.

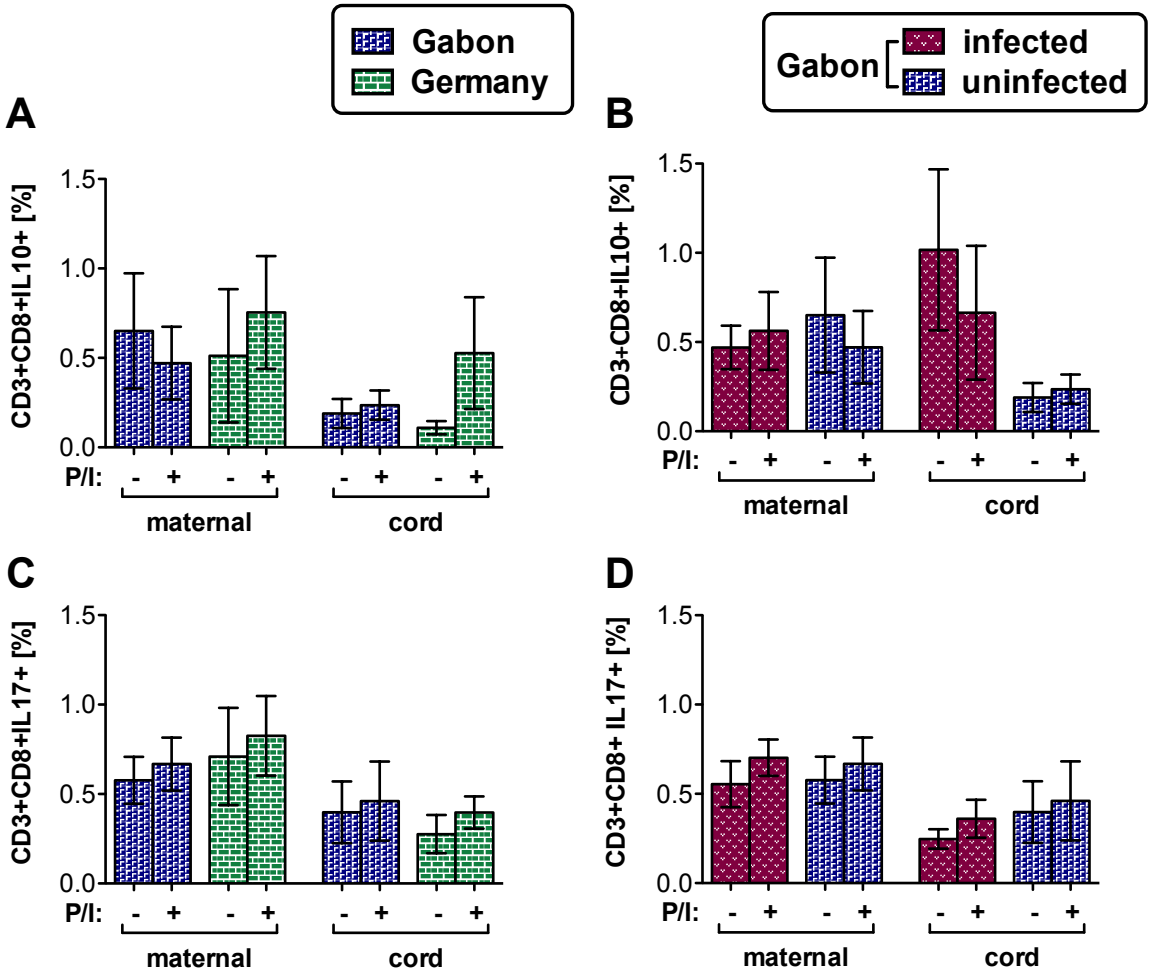


Figure 3.37 Percentage of IL10 and IL17 expression in CD8 positive cells

CBMC and PBMCs from all participants were isolated, fixed and after thawing stimulated with P/I for six hours and stained with fluorescently labelled antibodies specific to intracellular markers (IL10, IL17). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group “Gabon, uninfected” in graph B, D, F corresponds to “Gabon” in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** percentage of $CD8^+$IL10 positive cells; **(C, D)** percentage of $CD8^+$IL17 positive cells.

Within the $CD8^+$ cell population, the cytotoxic T cells, no difference between the German and Gabonese subgroup, independent of *S. haematobium* infection, could be seen Furthermore, the percentage of IL10 and IL17 always stayed under one percent for all groups and the stimulation did not have a significant impact on the IL10 or IL17 cytokine production in none of the groups (Figure 3.37).

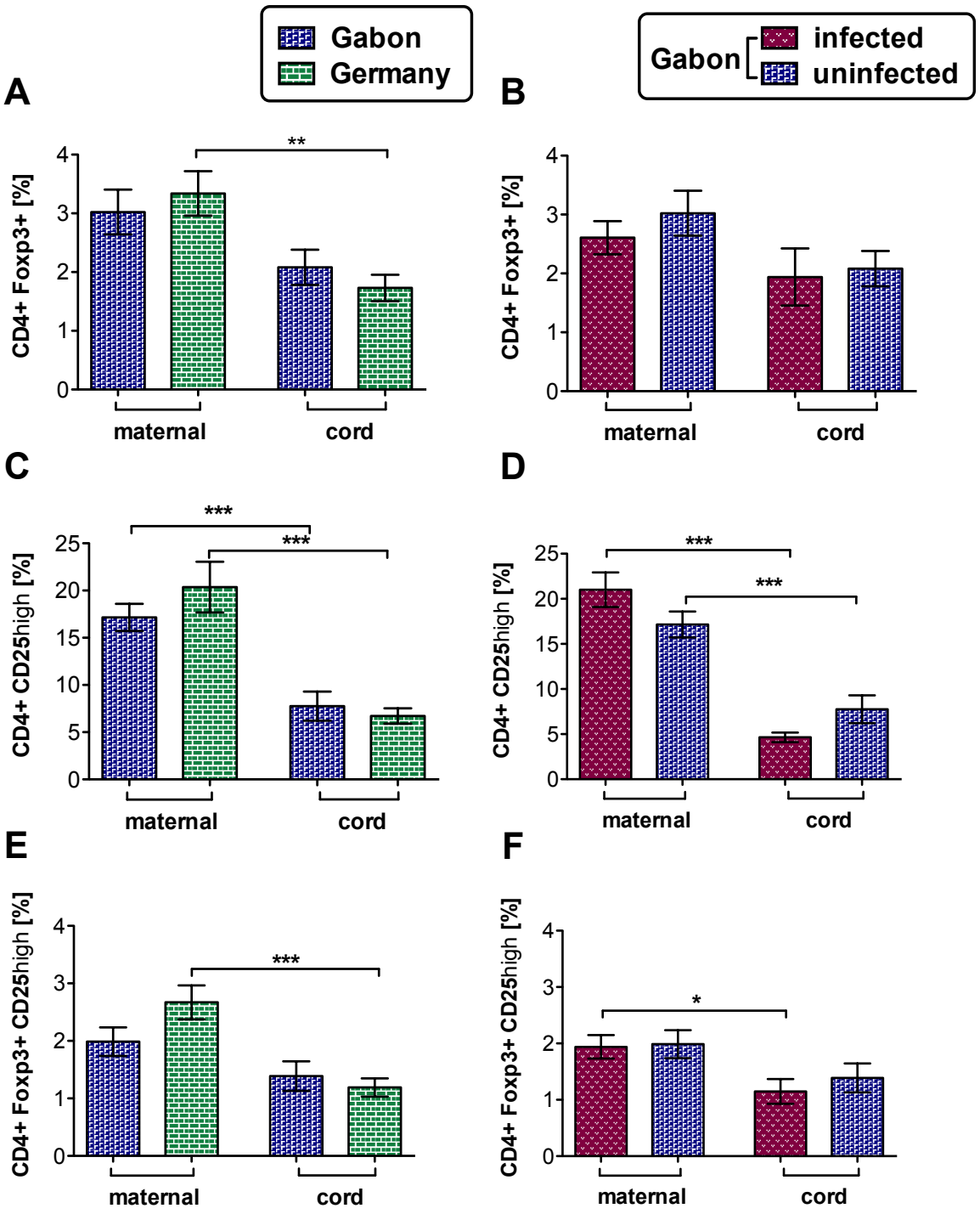


Figure 3.38 Percentage of Foxp3 and CD25 expression in CD8 negative cells

CBMC and PBMCs from all participants were isolated, fixed and after thawing stimulated with P/I for six hours and stained with fluorescently labelled antibodies specific to surface or transcription factor markers, respectively (Foxp3, CD25). All data are shown with mean and standard deviation. P values are for t-tests. Comparison was done between the two maternal and cord blood groups, or maternal with corresponding cord, respectively. P value: * = < 0,05; ** = < 0,01; *** = < 0,001; **** = < 0,0001; n (maternal, Gabon) = 12; n (maternal, Germany) = 13; n (cord, Gabon) = 12; n (cord, Germany) = 13; the group "Gabon, uninfected" in graph B, D, F corresponds to "Gabon" in graph A, C, E; n (Gabon, maternal, infected) = 12; n (Gabon, cord, infected) = 12; **(A, B)** percentage of Foxp3 positive cells; **(C, D)** percentage of CD25 positive cells; **(E, F)** percentage of Foxp3 and CD25 double positive cells.

The surface marker CD25 is mainly expressed on activated B and T cells, especially Tregs. Likewise, the transcription factor Foxp3 is one of the main regulators in the development of regulatory T cells. Both markers are present in $CD4^+$ ($CD3^+/CD8^-$) cells. The function of Tregs consist in suppressing the activation of the immune system and thereby regulating the self-tolerance of the immune system. Figure 3.38 shows that there was no significant difference of the percentage of $Foxp3^+$, $CD25^{high}$ or $CD25^{high}/Foxp3^+$ cell population between Gabon and Germany. Anyway, cell populations are significant bigger in PBMCs than in CBMCs, especially in the German subgroup ($Foxp3^+$, $CD25^{high}$ and $CD25^{high}/Foxp3^+$. Within the Gabonese subgroups maternal cells had significant higher rates of $CD25^+$ cells, independent of *S. haematobium* infection. Whereas significantly higher percentage of maternal $CD25^{high}/Foxp3^+$ cells in comparison to CBMCs were detected only in the infected subgroup.
All in all, in both, the German and the Gabonese subgroups, there were more or less three percent $Foxp3^+$, 17-20% $CD25^{high}$ and two percent $CD25^{high}/Foxp3^+$ T cells with higher rates in PBMCs.

To sum up, T cell populations and cytokine expressions in Gabon and Germany didn't seem to differ significantly. Nevertheless, significantly less lymphocytes, as well as CD3+ and $CD3^+/CD8^+$ cells were detected in PBMCs and CBMCs (only $CD3^+/CD8^+$) from infected mothers. The percentage of CD4 positive cells was significantly lower in Gabonese CMBCs, independent of maternal infection. Stimulation with PMA/Iono lead predominantly to a significant increase of cytokine production with mostly higher levels in maternal cells. A very interesting finding however was the higher expression of IL10 in PBMCs from with *S. haematobium* infected Gabonese women. A similar trend was observed for IL17 which was also expressed to a greater extent in CBMCs from mothers infected with *S. haematobium*.

3.16 Correlation between T cell populations and vitamin D concentration or placental gene expressions

Already early in pregnancy VDR, the 1α-hydroxylase Cyp27b1 and Hsd3b1, which is required for placental progesterone production, are expressed in the placental tissue [245]. This emphasizes the importance of the two steroid hormones vitamin D and progesterone (P4) for a successful pregnancy. Recently, various studies have shown immunomodulatory activity in the terms of an induction of maternal tolerance to the semi-allogenic fetus by vitamin D and P4 [127]. At that, vitamin D and P4 regulate mainly in CBMCs [144] T cell-mediated immune reactions by activating Tregs [46, 122] and suppressing Th1 and Th17 cells [143, 144], thus influencing the outcome of pregnancy. Correlations of in cord blood vitamin D levels naive $CD4^+$ T cells, $CD4^+$ T helpers and cytotoxic $CD8^+$ T lymphocytes were shown [285]. Furthermore, Thangamani et al. showed that P4 induces VDR gene expression in Tregs and strengthens the function of vitamin D [255], whereas Rafiee et al. showed an increase in the expression of P4 receptors on CD4+ T cells by vitamin D [217]. Thus, these steroid hormones lead to immunotolerance and make the immune responses within the placenta more tolerogenic and less inflammatory.
In the following correlations between the results of the flow cytometry performed for this thesis and either the peripheral vitamin D concentration or the placental gene expression of VDR, Hsd3b1 and Foxp3, will investigate whether there is a connection of placental gene expression and cell populations or cytokine production, respectively. For example: Does fetal Hsd3b1 expression influence the rate of Foxp3 positive cell population or IL10 levels in the umbilical cord blood? Is there a significant correlation between vitamin D plasma concentrations and T cell populations?

Correlations were performed between cord blood cell populations or cytokine levels and fetal placental PCR results or maternal FACS results with the corresponding PCR analysis, respectively. The data present the subgroup of twelve to 13 participants in each group (see chapter 3.15.1). The Gabonese cohort presents the group of not infected with *S. haematobium* as results from flow cytometry analysis almost don't differ a lot from each other.

An example of how correlations were performed is shown in the figure below. Spearman r was calculated to analyze the correlation and the line was drawn by using linear regression model. All data are summarized in the table followed.

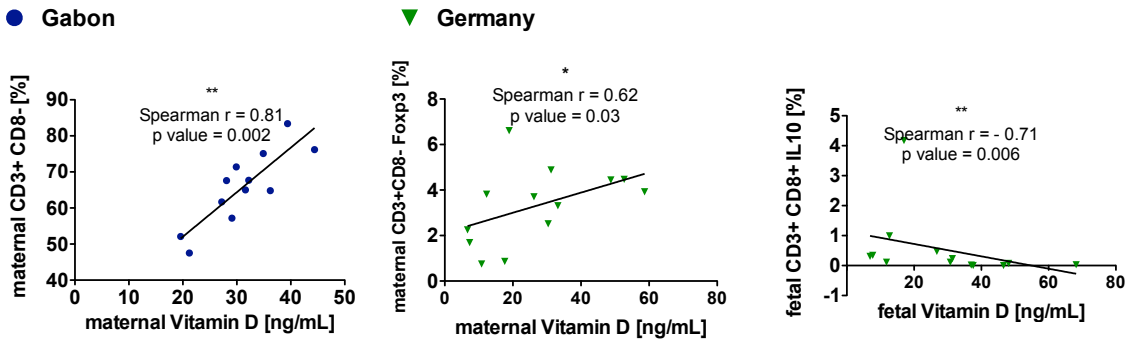


Figure 3.39 Correlation of maternal/fetal vitamin D concentration and T cell populations and their cytokine expressions in Gabonese/German samples

		Vitamin D				VDR				Hsd3b1				Foxp3			
		Gabon		Germany		Gabon		Germany		Gabon		Germany		Gabon		Germany	
		m	f	m	f	m	f	m	f	m	f	m	f	m	f	m	f
$CD3^+$																	
$CD4^+$		++						-									
	$GATA3^+$										++						
	IL5								+								
	TNFα																
	IFNγ												-				
	TNFα+ IFNγ																
$CD4^+$	IL10									-					- -		
	IL17																
	Foxp3			+			-								- -		
	$CD25^{high}$																-
	Foxp3CD 25^{high}																
$CD3^+CD8^+$																	
	IL5																
$CD3^+$ $CD8^+$	TNFα									- -							
	IFNγ																

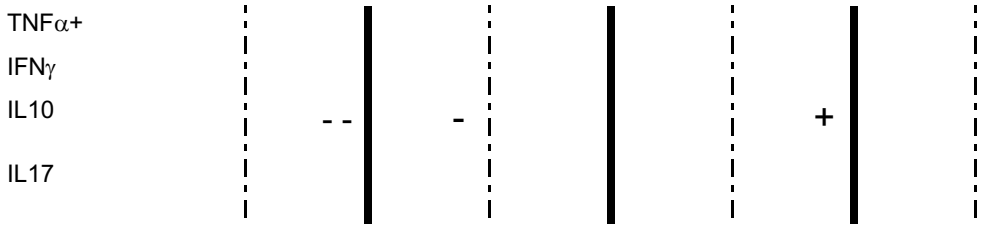


Table 3.7 Summarize of correlation between T cell populations and vitamin D concentration or placental gene expressions

All in all, some significant correlations within the maternal samples could be found.

The analysis of maternal samples revealed a positive correlation of vitamin D plasma concentrations with $CD3^+/CD8^-$ cells for Gabonese mothers and with Foxp3 positive cells for German mothers. Furthermore, German placental VDR expression correlated significantly negative with $CD3^+/CD8^-$ cells. Gabonese maternal placental Hsd3b1 expression correlated each time negative with IL10 from CD4 cells and with TNFα from CD8 positive cells.

Maternal Foxp3 expression did not show any correlation with investigated cell populations and cytokines.

But indeed, several significant negative correlations of fetal Foxp3 expression could be observed. In the Gabonese cohort it correlated strongly with Foxp3 presenting cells as well as with IL10 expression of $CD4^+$cells. In the German fetal subgroup placental Foxp3 expression correlated negatively with $CD25^{high}$ cells.

Additionally, in the German subgroup a significant negative correlation between cord blood plasma vitamin D levels and IL10 percentage from $CD8^+$ cells could be demonstrated, whereas the fetal VDR expression positive with IL5 rates from CD4 cells correlated. In the Gabonese subgroup fetal VDR correlated negatively with Foxp3 expressing cells as well as with IL10 rates from $CD8^+$ cells.

Additionally, German IL10 rates from $CD8^+$ cells in CBMCs correlated positively with fetal Hsd3b1 expression in the placenta, whereas IFNγ from $CD4^+$ cells positively with Hsd3b1 correlated. Gabonese GATA3 expression CBMCs also correlated positively with Hsd3b1 fetal placental gene expression.

In sum, cell populations did not correlate as strong with corresponding placental gene expression as expected (e.g. a strong positive correlation for Hsd3b1 or vitamin D with foxp3 expression cells on the fetal side of the placenta would have been expected), but still, few interesting correlations on the maternal side (e.g. vitamin D concentration with Foxp3+ cells in Germany or IL10 rate with Hsd3b1 expression in Gabon) and even more on the fetal side (vitamin D concentration with IL10 rates in Germany or Foxp3 placental gene expression with

IL10 rates in Gabon) could be observed. In total, most significant correlations could be seen for the expression of the cytokine IL10 and the expression of the transcription factor Foxp3.

3.17 Summary of most important findings

In summary, the following results were obtained in brief and main finding highlighted in bold:

The Gabonese and the German cohorts differed significantly in terms of patients' characteristics from each other. Based on maternal data, Gabonese mothers were younger, had more unsuccessful pregnancies, lower hemoglobin and higher vitamin D and CRP concentrations. Based on newborn's data, Gabonese newborns were significantly smaller and lighter but had higher vitamin D and calcium levels when compared to the German newborns. Gabonese patients' characteristics were not influenced by infection with *S. haematobium*.

The Gabonese and German subgroups were more similar in regard to patients' characteristics. Even though both groups were on average the same age, Gabonese mothers had more (successful and unsuccessful) pregnancies in total and lower hemoglobin levels.
In Gabon, the *S. haematobium* infected group differed from the uninfected group. Infected mothers were younger, and mothers and newborns had lower vitamin D levels.

Assessment of *Schistosoma haematobium* infection: For seven mothers the microscopical egg count and CAA-UCP-LF was positive. Additionally, six more were detected via CAA-UCP-LF **resulting in a total of 13 participants infected with *S. haematobium.***

25-OH-Vitamin D3 concentrations were higher in Gabonese participants: more than 60% of the mothers and newborns had sufficient (>30ng/mL) levels vs. 34% in Germany, and in 2/3 of German mothers and in Gabon only in 1/3 of the mothers vitamin D supplementation was rising vitamin D plasma levels.

Placental gene expression differed between Gabon and Germany.
Expression of anti-inflammatory and immune-modulatory markers such as Hsd3b1 and VDR was stronger on the fetal side of the placenta than on the maternal side, possibly pointing towards the importance of progesterone synthesis in the fetus during pregnancy.
VDR was expressed to a lesser extent in Gabonese placenta samples irrespective of infection with *S. haematobium*. Additionally, Hsd3b1 expression was lower in Gabonese than in German fetal samples and even lower in samples from the *S. haematobium* infected group, suggesting a higher risk of developing an inflammatory milieu in the placenta in Gabon or in case of

infection. Cyp27b1 was less expressed in Gabonese maternal placentas, indicating less vitamin D activation and thus potentially less anti-inflammatory immune modulation in Gabon. Both Foxp3 and IL10 were also expressed less in Gabon, which again highlights the reduced presence of regulatory and anti-inflammatory immune responses and thus the higher risk of an inflammatory milieu in the placenta. **Lower expression of anti-inflammatory and immune-modulatory markers in Gabonese placenta samples suggest higher risk of inflammation.**

In Germany most of the maternal and fetal gene expression correlated with each other. In Gabon this was only the case for Foxp3 and Hsd3b1 and in the infected group, only for Foxp3. **The strong positive correlation between VDR and Hsd3b1 in all three groups** emphasizes their close relationship and is possibly an indication for co-regulation.

Overall, total IgG4 levels were higher in the German cohort when compared to the Gabonese cohort and furthermore higher in German cord blood plasma than in maternal plasma samples. Total IgG4 levels in Gabon did not differ between mother and the newborn. **Total IgE was observed to be higher in all Gabonese** maternal and cord blood plasma samples. Moreover, not only total IgE, but also AWA specific IgE and IgG4 levels were higher in plasma samples from infected mothers, but were nevertheless **present in all uninfected Gabonese plasma samples** indicating previous exposure to schistosomes

Flow cytometric analysis revealed that most of the $CD4^+$, $CD3^+/CD8^+$ and Treg cell populations as well as cytokines were more frequent in PBMCs than in CBMCs. Nevertheless, **PBMCs from the *S. haematobium* infected subgroup had less lymphocytes, CD3 and CD8 positive cells.** Almost all cells responded with cytokine production to an antigen independent stimulus and **significantly more IL10 production was observed in PBMCs from infected mothers.** The same trend was observed for IL17.

4. Discussion

4.1 Lower placental expression of anti-inflammatory genes in Gabon potentially increase the risk of placental inflammation

4.1.1 The influence of Hsd3b1 and VDR gene expression on inflammation in the placenta

In utero exposure to helminths [252], other environmental factors such as poor maternal nutrition [39], exposure to folic acid [137], air pollution [119] or organic pollutants [124] and genetic variants [68] have recently been shown to alter placental gene expression and raise the risk of adverse pregnancy outcomes such as fetal growth restriction, preterm birth or preeclampsia [4]. Besides, other parasites such as *P. falciparum*, causing malaria in humans, is well known to have a strong impact on the placental gene expression and the placental immune milieu [89] [182], and furthermore cause adverse birth outcomes.

Though not only maternal infections, but also maternal hormone levels have strong effects on proinflammatory immune reactions in the placenta. The early expression of VDR, the 1α-hydroxylase Cyp27b1 and Hsd3b1 in the placenta [245] highlights their importance for a successful pregnancy. Several associations between vitamin D deficiency and pregnancy complications [148] such as preeclampsia [30], fetal growth restriction, small-for-gestational-age fetus [31], bacterial vaginosis [32] and gestational diabetes mellitus [290] were already observed. Thus, the hormone vitamin D is a decisive factor associated with placental inflammation most likely by modulating several kinds of T cell populations and responses [93, 271].

Hand in hand with vitamin D goes progesterone (P4). P4 makes T cells more sensitive for vitamin D by inducing VDR in T cells [255]. In naïve $CD4^+$ T cells from cord blood, VDR was one of the major genes induced by P4 as analyzed by microarray technology [144]. This renders T cells to become sensitive to regulation by vitamin D in the placenta even in peripheral vitamin D sufficiency. Progesterone was shown to be significantly increased in cord blood and the fetal circulation [144]. Our study strengthens the clear synergy between Hsd3b1 and VDR through the significant correlation between VDR and Hsd3b1 on the fetal placental side within all groups. Another study investigated the effect of P4 on gene expression in T cells in CBMCs and PBMCs with a genome-wide microarray study. Whilst no effect in PBMCs could be observed, in CBMCs P4 downregulated certain genes and activated the differentiation of naive T cells into immune suppressive Foxp3 expressing Tregs [144] alongside suppressed production of IFNγ or IL17 by Th1 cells [143, 144, 176]. This emphasizes the important role of P4 in suppression of immune responses at the fetomaternal interface [144].

Here, indeed, Hsd3b1, the only isoform expressed in the placenta, was expressed to a significantly lower level within the fetal side of Gabonese placentas, and even lower within the sub-group from *S. haematobium* infected mothers, enhancing the risk of placental inflammation in Gabon. Hsd3b1 is required for the biosynthesis of P4 and is therefore essential for a successful pregnancy [197, 246]. During the first 6 weeks of human pregnancy, the ovary is the source of the progesterone, required for implantation of the embryo and maintenance of pregnancy. After a period of six weeks, the main site of the production of progesterone is the placenta, mainly the fetal side as progesterone levels are greatly increased in cord blood [144]. Since progesterone is one of the most important hormones for pregnancy maintenance, and since low vaginal progesterone levels are well known to correlate with preterm birth causing perinatal morbidity and mortality worldwide [206, 223, 224], the lower expression in the Gabonese cohort may be one of the reasons for the higher number of previous pregnancy losses and the smaller size of the children in this cohort. Furthermore, progesterone functions as an anti-inflammatory hormone and a decreased expression might thereby contribute to placental inflammation.

Likewise, a significantly reduced expression of VDR as well as Cyp27b1 on the maternal and fetal (for VDR only) side of the Gabonese placenta samples were detected in this study. Here, neither progesterone nor activated placental vitamin D levels in cord blood but rather the gene expressions were measured and still, the results corresponded to previous studies. German mothers, not exposed to parasites or chronic inflammation, showed higher Hsd3b1 on the fetal side and higher VDR expression on both sides, suggesting a balanced fetomaternal immune tolerance. The significantly lower fetal Hsd3b1 expression in the *S. haematobium* infected group emphasizes the overall impact of helminths on the placental immune milieu by altering gene expressions.

Furthermore, the effect of vitamin D on the gene expression should not be ignored. In chapter 1.7.2 we already described a peripheral (positive) regulatory feedback loop. Nevertheless, no correlation between placental VDR expression and peripheral vitamin D levels could be observed (see results and further discussion chapter 3.9and 4.6). The Gabonese cohort had significantly higher peripheral 25(OH)D3 plasma levels, but lower placental VDR and Cyp27b1 expression. Similar results were found by Young et al where placental VDR was significantly negatively associated with neonatal 25(OH)D3, and positively associated with neonatal $1,25(OH)_2D3$ [283]. In contrast to our results, a study conducted in India demonstrated that in the placental tissue of women with 25(OH)D3 deficiency, VDR, RXR, and Foxp3 were downregulated, and Cyp24a1, Cyp2r1 and Cyp27b1 expression were also altered [271]. Such differences could potentially be explained due to different point of sample collection. As we distinguished between maternal and fetal side, the authors took their samples midway between the chorionic and basal plates [271]. Furthermore, participants were included at Gandhi

hospital, located in the center of the megapolis Hyderabad, India [271] and thus exposed to completely different living conditions than the Gabonese participants, coming from mainly rural homes with a high exposure to parasites. To clarify these discrepancies, further studies incorporating several environments or genetic backgrounds are needed.

4.1.2 Interference-prone immune tolerance at the fetomaternal interface in Gabon is reflected by reduced gene expression of IL 10 and Foxp3

Not only the lower expression of the anti-inflammatory, immune modulating acting genes VDR, Cyp27b1 and Hsd3b1 but also of Foxp3 and IL10 in the Gabonese placentas could indicate a greater susceptibility to an inflammatory immune milieu in the placenta.

Altered placental cytokine gene expression have already been linked to parasitic diseases. Moorman and colleagues demonstrated in a study conducted in Malawi a lower expression of IL6 and transforming growth factor b1 (TGFb1) and a higher expression of IL1b, IL8 and TNFα in malaria-infected placentas compared with uninfected. The latter two were also associated with intrauterine growth retardation [182]. MRNA for *IFNG*, IL2, IL4, IL5 or IL12 could not be detected, whilst taking one cm^3 biopsy from the maternal side of the placenta in an off-center position [182]. Other studies investigating placental cytokines found proinflammatory responses in placentas from malaria-infected women, too, with higher TNFα and lower IL6 levels [89]. Thus, infection with parasites like *P. falciparum* during pregnancy may induce a potentially harmful proinflammatory response in the placenta.

IL10 and Foxp3 are crucial for induction of peripheral tolerance and have several anti-inflammatory effects, including suppression of pro-inflammatory Th2 cells and other effector cells of allergic inflammation, such as mast cells, basophils and eosinophils [259]. The transcription factor Foxp3 is expressed in Tregs, which contribute due to their anti-inflammatory effects to the maintenance of immune tolerance and the prevention of placental rejection [233]. This mechanism has been studied in mice as well as in humans [10, 107]. Foxp3 gene expression is present in trophoblasts, in decidua as well as in cytotrophoblasts [73, 115]. Based on the strong impact of Foxp3 on Tregs, it can be assumed that the immunosuppressive effect on trophoblasts should not be underestimated and must be included in the study of the mechanism of maternal-fetal tolerance and the maintenance of maternal immune tolerance. Not only a reduction of Tregs in the placenta can promote inflammation and lead to complications such as miscarriage of premature labor [133, 191, 237, 289] but also decreased levels of Foxp3 mRNA and protein in trophoblasts were associated with recurrent pregnancy loss, preeclampsia or preterm delivery [48, 49, 115]. Moreover, soluble factors from human placental tissue induced the Foxp3 expression and the production of IL10 in CD4+ T cells in vitro [254]. Dimova and colleagues found an accumulation of decidual $CD4^+$ $CD25^{low}$

Foxp3$^+$ cells and concluded that an additional reservoir of Foxp3$^+$ naive Tregs must be present in the uterus to ensure a sufficient number of "classical" Treg cells [73]. This further highlights the central role of the fetal placenta and Foxp3 expression in regulating homeostasis and maintaining pregnancy. The significantly lower expression of Foxp3 in Gabonese placenta samples again indicates an altered or possibly even disturbed immune modulation and an increased risk of a placental pro-inflammatory milieu in Gabon.

Besides Foxp3, IL10 is well known to be expressed in human cytotrophoblasts, too [228]. Not only the placenta itself, but also placental uNK cells, monocytes and Tregs produce high amounts of IL10 [257]. Mouse studies have shown that although IL10 is not essential for pregnancy, it is greatly involved in the inhibition of excessive inflammation and acts as a protective agent during infection [47]. For example, pregnant IL10$^{-/-}$ mice responded more strongly to LPS and CpG challenge (a TLR 9 agonist) [187, 256]. In human placental tissue of neonates born between 24 and 26 weeks of gestation, the anti-inflammatory cytokine IL10 was less expressed in children suffering from bronchopulmonary dysplasia compared to the control group [170]. Similar to vitamin D, progesterone or Foxp3 positive Tregs, IL10 levels were also decreased in women with pre-eclampsia [48], spontaneous miscarriage, recurrent miscarriage, small babies in gestational age or infertility [47].

Since IL 10 was less expressed in Gabonese placentae, we assume that an anti-inflammatory immune response may be less effective and inflammation more likely to occur.

The proinflammatory cytokine IFNγ is present mainly in the early placenta and gestational endometrium [182] and at later stages the trophoblasts dampen IFNγ signaling strongly to avoid rejection of the fetus and possibly strong infection-mediated inflammation [87]. One of the main targets of IFNγ are polymorphic MHC class II genes by increasing its expression [42]. However, these are hardly expressed in human or rodent trophoblast cells [266]. Due to the lack of MHC class II antigens, trophoblast cells attenuate IFNγ signal transduction [188] and contribute to the prevention of conceptus rejection. Furthermore, both anti-inflammatory hormones, vitamin D and progesterone, were found to dampen proinflammatory T cells producing IFNγ within the placenta [255] [63].

Interestingly, no differences in placental *IFNG* gene expression, as a marker of inflammation, were found between Gabon and Germany and Gabon, independent of *S. haematobium* infection status. This is similar to other reports, which found either no *IFNG* gene expression in the placenta at all [182] or a very selective [188] or even a lack of response from trophoblast cells to soluble IFNγ. Thus, rather pregnancy and related hormones themselves than maternal environment or infection seem to influence its expression.

Nevertheless, some studies have demonstrated the presence of IFNγ in placentas from parasite-infected women [89] [182]. In placentas form women with malaria, a known risk factor

for placental inflammation, not only lower concentrations of anti-inflammatory IL10, but also increased IFNγ, IL2 and TNFα levels were detected, indicating a shift of the immune balance towards type 1 cytokines [182]. The main difference here was however that in these studies cytokine protein levels rather than their gene expression were analyzed indicating a different, possibly systemic source of the latter.
Genetic background, too, is a possible confounder for different gene expression in Gabonese and German placenta samples [68]. An impact of maternal stress on genetic variants on the placental landscape by analyzing the methylation and expression quantitative trait loci was observed before [68], whilst others postulated no effect of neonatal race, ethnicity, or sex on, for example, placental VDR expression [283].

Summarizing chapter 4.1and 4.1.2, Gabonese placenta revealed lower gene expressions of several anti-inflammatory immune modulators such as Hsd3b1, VDR, Cyp27b1, Foxp3 and IL10 than in German samples. Consequently, inflammation at the fetomaternal interface may be less controlled and Gabonese women may be at higher risk to suffer from placental inflammation and adverse pregnancy outcomes. Indeed, significantly elevated CRP levels, an inflammation parameter, in most Gabonese women support this assumption. Nevertheless, it needs to be kept in mind that increased levels of CRP are not only found in infection, but also in prolonged duration of labor [152]. In the German cohort CRP levels perfectly correlated with duration of labor, whereas in Gabon no data about labor time was available.
Except for Hsd3b1, infection with *S. haematobium* did not have an influence on vitamin D pathway associated (VDR, Cyp27b1) or immunological (IL10, *IFNG*, Foxp3) genes. Within this study however, the schistosomiasis-infected group was small and furthermore not the primary objective in this study. In conclusion, environmental and perhaps genetic backgrounds might suppress anti-inflammatory signals and alter the regulation and activation of vitamin D.

4.2 Helminths and distinct environments disrupt the alignment of maternal and fetal gene expression within the placenta

Helminth infection or distinct environments may not only impact the fetal immune system *in utero* or gene expressions in organs but may also uncouple the mutual interference of gene expression on the maternal and fetal side of the placenta [4, 139, 271]. Previous investigations of gene expression within the placental tissue distinguished between different placental regions (proximal, intermediate and peripheral region) [261], but have rarely considered

potential expression differences between the fetal and maternal side of the placenta or possible differences in environmental exposure.

Tzschoppe et al. was able to demonstrate that in human placenta samples the mRNA expression of insulin-like growth factor-binding protein (IGFBP-1), prolactin, corticotropin releasing hormone (CRH) and leptin from the proximal part of the placenta didn't differ between different participants, whereas same genes were elevated in samples from the middle placenta region in neonates suffering from intrauterine growth restriction compared to the controls [261]. For other genes specifically expressed in trophoblasts, such as cytokeratin-7 transcripts, a significant difference within and between the placenta samples could be observed [211]. Vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) were higher expressed in the subchorionic lateral margin compared to the medial basal region, too, whereas the expression of adipophilin and human placental lactogen were expressed equally and independent of placental region [280]. Furthermore, genes like paternally expressed gene PEG3 and PEG10 were resistant to differences in the site of sample collection [119], showing the variability in consistence of each placental gene. Depending on the gene, its expression may or may not differ from its location in the placenta.

In this study, all samples were collected in a standardized manner in the proximal region of the placenta, close to the umbilical cord. Thus, the differences due to the sampling site should be minimized. However, further studies on regional differences in gene expression are clearly needed.

Indeed, the human placenta is not only divided into proximal and distal regions, but also into fetal, maternal and intervillous parts (chapter 1.6). In rodents and guinea pigs, the placenta is organized into predominantly two zones, the labyrinthine zone and the connecting/interlobial zone. Many studies have demonstrated different responses to various environmental factors and in a review, Sferruzzi-Perri and Camm summarized how maternal and fetal placental and fetal growth is clearly influenced by the maternal environment [244].

In rats high-fat nutrition led to a reduced formation of the junctional zone, whereas malnutrition in mice and guinea pigs led to a reduction of the junctional/interlobial zone compared to the labyrinthine zone [51, 163, 220, 241]. This possibly indicates an influence of the environment on the placental development to such an extent that a supply of the fetus is always ensured, accepting a possible loss of endocrine function of the placenta. In this context, changes in the vascular reactivity of the uterus and trophoblast remodeling of the maternal spiral arteries through various environmental manipulations have already been shown to modulate the supply of substrates to the placenta [104, 105, 229, 247].

Nevertheless, there are still only few studies distinguishing the two different sides of the human placenta. Comparing the maternal with the fetal side in the German cohort showed lower maternal expression of Hsd3b1 as well as of VDR. However, for all genes except Hsd3b1 a

positive correlation of the respective genes between the two sides was observed, suggesting that there may be a considerable amount of regulated crosstalk associated with pregnancy-related hormones and immunological genes.
Similar to the German group, there was also a significantly higher fetal gene expression of Hsd3b1 and VDR in the non-infected Gabonese, and in addition there was a higher expression of IFNG. However, correlation analyses between maternal and fetal expression of the same gene revealed that only Hsd3b1 and Foxp3 correlated positively, and in the subgroup infected with *S. haematobium* this was even true only for Foxp3.
Moreover, no difference between maternal and fetal Hsd3b1 and VDR expression in the infected subgroup could be observed, only *IFNG* was higher expressed in fetal placental samples.
This encourages the hypothesis of a strong influence of helminths and the environment on placental gene expression and is indicative for independent regulation across these distinct tissue niches. Of course, the role of genetic backgrounds as confounding factors should be taken into consideration, too.

4.3 Influence of environment, genetic background and inflammation on hemoglobin concentrations

Helminths provoke a subtle inflammation in their hosts and cause anemia, also known as anemia of infection or of chronic disease caused by proinflammatory cytokine mediators (e.g. TNFα or IL6) that are produced in response to infections, cancer or autoimmune diseases such as arthritis [90, 92]. Mild anemia during pregnancy is physiologic. The World Health Organization (WHO) defined a hemoglobin concentration under 11 g/d for pregnant women (as compared to 12 g/dL for non-pregnant women) as cut-off for anemia and showed in their report about worldwide anemia from 1993-2005 that the highest prevalence was found in Africa (57.1%) and prevalence in the Eastern Mediterranean was 44.2% [201]. A study performed in the United States of America (USA) by Perry et al showed that hematocrit, hemoglobin, MCV, transferrin saturation, and white blood cell counts of African-Americans were significantly lower than those of Caucasian-American women. The difference in hemoglobin however was not due to iron deficiency [209]. Although the cause for the differences remains unclear, it is suggested not to standardize the cut-off but rather consider the levels individually [25]. Friedmann et all summarized the main reasons for anemia in Africa [90]: a) dietary and especially due to iron insufficiency; b) intestinal blood loss because of *hookworm*, *Trichuris* or schistosomiasis infection; c) hemolysis caused by malaria and hemoglobinopathies; d) anemia of inflammation and chronic diseases such as HIV, tuberculosis and, possibly, malaria and

schistosomiasis. Indeed, according to the definition given by the WHO, the Gabonese, in contrast to the German, cohort in our study suffered from anemia. In 2016 in Germany, 17% of women of reproductive age suffered from anemia whilst in Gabon the prevalence was 59% [185]. Prevalence of anemia among women of reproductive age refers to the combined prevalence of both non-pregnant with hemoglobin levels below 12 g/dL and pregnant women with hemoglobin levels below 11 g/dL. Thus, Gabonese women are in general at a higher risk to suffer from anemia. 85% of people with schistosomiasis live in sub-Saharan Africa [91] and schistosomiasis is known to [6] cause anemia possibly through inflammation [90, 92, 145, 169]. Findings from another study with 998 pregnant women in Gabon demonstrated that 50% of the participants were anemic [178]. The causes of anemia in this geographical region may be multiple including malaria, soil transmitted helminths, nutritional deficiencies and pregnancy itself. In their study population urogenital schistosomiasis was even strongly associated with placental malaria infection, also a well-known high-risk factor for maternal anemia [178]. Here, information about exact dietary habits were not available. In the Gabonese cohort, infections with *S. haematobium* as well as with other helminths were detected. Furthermore, six mothers also reported malaria infection during pregnancy and eight others suffered from sickle cell anemia. In this pilot study, the respective subgroup of schistosome infected women was too small, since the main objective was the comparison between Germany and Gabon. In view of the numerous factors associated with an increased risk of anemia, careful control of confounding variables and good experimental design is required for further studies to quantify the association between schistosome infection and anemia. But still, the mild anemia, the lower expression of anti-inflammatory genes or the elevated levels of the inflammation-marker CRP in the Gabonese cohort support the hypothesis of low-grade inflammation in Gabonese mothers leading to a higher risk of placental inflammation.

4.4 Lighter newborns and higher risk of low birth weight in Gabon

Placental inflammation and pro-inflammatory cytokines might also contribute to fetal hypoxia [90, 91] and adverse birth outcomes, such as low birth weight [178]. Certainly, the neonates in Gabon were significantly smaller (by 3cm) and lighter (by 255g) than those born in Germany, with even 8 of total 55 newborns suffering from low birth weight (<2500g [62]) amongst whom only one was born from a *S. haematobium* infected mother. The observed proportion of underweight newborns in Gabon (15%) corresponds exactly to the report of the WHO and UNICEF, which estimated the prevalence of low birth weight in Gabon at 14.2% in 2015 [218]. The lighter newborns could likewise depend on differences in environmental exposure during pregnancy or a general influence of helminths. In a clinical study with 1115 pregnant women

in Gabon, Mombo-Ngoma et al. pointed out that low birth weight was more common amongst infants of *S. haematobium* infected mothers. This association was unaffected by controlling for demographic characteristics, gestational age and *Plasmodium* infection status [178]. Experimental evidence from animal models indicates that both acute and chronic schistosomiasis may result in decreased birth weight as reviewed by Friedman et al. (2005, 2007). Possible mechanisms of schistosomiasis-mediated adverse birth outcomes are hypoxia due to pro-inflammatory cytokines and placental inflammation and maternal iron deficiency due to chronic bleeding or chronic anemia [90, 91].

Interestingly, absence of VDR, too, is associated with low birth weight and smaller size of the neonate. In a murine study pups of VDR $^{-}$ / $^{-}$ dams have been shown to be smaller whereby skeletally normal at birth [283]. Last but not least, low calcium concentration in the cord blood has also been shown to be associated with reduced birthweight [75, 283] and indeed, calcium levels in the Gabonese cohort were significantly lower than in the German cohort. Like 25(OH)D, Ca is able to cross the placenta barrier. So far, research on the effects of maternal Ca nutritional status during pregnancy has yielded controversial results [75]. While some reported not only higher birth weight after Ca supplementation but also the prevention of pregnancy induced hypertension [215], others have found no association between Ca supplementation in pregnant women and fetal somatic growth, skeletal growth, or size at birth [2]. It should not be overlooked that during pregnancy not only vitamin D metabolism but also Ca uptake and excretion are physiologically altered [134, 135] and the exact regulation is still unknown.

Kurth et al. demonstrated in a cross-sectional study in Central Africa consistently lower birth weights for newborns of adolescent mothers compared to adults [138]. Comparing the participating mothers in terms of their residence, on average the cohort of Gabonese mothers were seven years younger than the German cohort. This difference could even be bigger, taking into account that one of our inclusion criteria was a minimum age of 18 years and other studies performed in Lambaréné, Gabon, suggest that there are indeed significant numbers of women younger than 18 years giving birth [179] and prevalence of 23.7% adolescent pregnancy is reported in Gabon [125]. Different access to antenatal care or contraceptives is only one potential explanations for the higher number of gravidities in Gabon, in addition to a higher proportion of women becoming pregnant at younger ages. Further investigation into these factors would clarify the underlying source of these differences. Another explanation for the age gap could be that the German cohort of mothers was based at a university hospital, which is known to support high-risk pregnancies which occur more frequently in mothers above the age of 35 years.

Thus, younger mothers, low Ca levels, placental inflammation, differences in environmental exposure during pregnancy could all contribute to the birth of smaller babies in our Gabonese cohort.

4.5 Dependence of vitamin D levels on sunlight and diet, but also on environmental and genetic factors

Besides skeletal functions, vitamin D plays a crucial role during immune development, inflammation or infection. All in all, vitamin D leads to a shift from a proinflammatory to a more tolerogenic immune status [16] and a lack of vitamin D or its co-players such as VDR or Cyp27b1 raise the risk of inflammation which could furthermore be associated with placental insufficiency and adverse birth outcomes [30, 148].

The reasons for the higher 25(OH)D concentration (10.6 ng/mL higher) in the Gabonese cohort may be multifactorial. In Gabon about 64% of women had sufficient vitamin D plasma levels which were unaffected by schistosome infection, whereas in Germany, only about 34% of women reached sufficient levels (defined as 30ng/mL).

Concerning our results, it needs to be considered that vitamin D levels were measured in plasma. DiaSorin®, the provider of the in vitro diagnostics reagent kit, however recommended to measure 25(OH)vitamin D3 in serum rather than plasma samples because it can deviate by up to 22% bidirectionally. Thus, this needs to be taken into account when discussing the vitamin D results and especially the interpretation of insufficiency. However, since all samples were measured by the same system and in the same run, the deviation – if at all – applies to all samples in a similar manner. We thus consider that comparability is still given.

As Lambaréné, Gabon, where more than 60% of the participants had sufficient vitamin D levels, is near the equator, sun radiation, which is necessary to produce active vitamin D in the skin, is ensured during the whole year [154]. Munich in Germany belongs to the countries which suffers from insufficient sun radiation between the months of October through March [112]. Indeed, German participants were included only during the summer months to ensure some extent of comparability to the Gabonese cohort [112], but anyway only about 34% reached sufficient levels. At a similar latitude to Munich is Warsaw, Poland. A study to examine seasonal vitamin D content in maternal and umbilical cord blood concluded that both maternal and cord blood concentrations of vitamin D were higher in the summer group than in the winter group. However, even in the summer group, only 16% of pregnant women reached the optimal vitamin D concentration (>30 ng/mL) [278]. Vitamin D levels not only depend on UV-B radiation, but also on alimentation. Luxwolda et al. demonstrated that high fish intake of Tanzanian people may influence the blood concentration positively [155]. Lambaréné, Gabon,

located only a few kilometers south of the equator in the middle of the Central African rainforest on the river Ogooué, has its main economic sector in fishing [193], resulting in fish-enriched diet. Besides food, dietary supplements may be a source of vitamin D. Of our two study populations, more than 60% of the German study population supplemented vitamins by regular intake of multivitamin compounds especially designed for pregnant women and which contain 200 to 800 IU vitamin D per tablet. In Gabon only about 35% of the pregnant women took such dietary supplements. It is noteworthy that because of the high temperatures in Gabon, most people consume milk in the form of milk powder (mainly Nido by Nestle®), which is supplemented with vitamin D and other vitamins. This probably leads to yet unrecognized intake or unknowing supplementation of vitamin D in Gabon. In the future, a detailed questionnaire about eating habits should bring more clarity about milk or fish consumption. In Germany the dietary supplement intake had as expected [177] a positive effect on the vitamin D levels as levels were higher by 10,6ng/mL than the mothers who did not supplement.

Due to its role during inflammation, vitamin D is considered by some scientists to act like an anti-inflammatory cytokine. Accordingly, high levels would signify a kind of protection against inflammation and not a risk factor. Since significantly higher values were measured in Gabonese plasma, this might argue against our theory of having an inflammatory immune milieu in maternal, placental and fetal blood and tissue samples from helminthic endemic areas. However, vitamin D contributes not only to anti-inflammatory immune responses, but rather leads to a more tolerogenetic immune status [16], a possible condition helping helminths to not only survive within their human hosts but also to control tissue damage and disease sequelae such as liver fibrosis. Helminth endemic areas are countries close to the equator or crossed by it (such as sub-Sahara Africa) and the principal source of vitamin D, the adequate exposure to UV-B [154], is guaranteed during the whole year in these countries. Thus, sufficient vitamin D levels in people living in sub-Sahara Africa can be expected. The role of vitamin D in case of infection with *Mycobacterium tuberculosis* or HIV has been described [226]. Findings of a study conducted in Lima, Peru, investigating vitamin D status and risk of incident tuberculosis disease suggest that "…vitamin D predicts TB disease risk in a dose-dependent manner and that the risk of TB disease is highest among HIV-positive individuals with severe vitamin D deficiency. …" [8]. Still randomized control trials are needed to evaluate the role of vitamin D during helminth infections. Here, the vitamin D levels between the infected and uninfected cohort in Gabon did not differ. However, it is noteworthy that the infected group consisted of only 13 participants, and furthermore of a subgroup, namely young, pregnant women. Thus, further investigations are necessary to draw final conclusions. It is highly probable, that the vitamin D levels for achieving a perfect anti-inflammatory and antibacterial effect differ significantly between Europeans and Africans.

Furthermore, vitamin D levels were measured in the peripheral blood and not in blood, squeezed out of the placenta itself. The placenta not only synthesizes its own vitamin D, but also regulates vitamin D activation and downstream action, e.g. by expressing VDR and Cyp27b1 independently [195]. Whether a direct effect or a connection between peripheral vitamin D plasma levels and a possible inflammatory milieu within the placenta could be established in this study is explained in more detail in the following chapter.

4.6 No significant feedback loop between placental gene expression and peripheral 25(OH)Vitamin D concentrations

Normally, in peripheral organs (except the placenta), vitamin D underlies feedback regulation together with VDR and Cyp27b1. As mentioned above, the placenta is likely to act like an autonomous organ to satisfy the needs of the fetus and to guarantee the maintenance of pregnancy [20, 71, 82, 251, 277]. Historically, the placenta was one of the first extra-renal tissues shown to be capable of synthesizing $1,25(OH)_2D$ [82], with Cyp27b1 activity detectable in both maternal decidua and fetal trophoblast [93]. Along this line, Young et al. showed that the placenta does not only produce its own bioactive vitamin D, but also that the well-known feedback loop (see chapter 1.7.4) holds only true for placental $1,25(OH)_2D$ and placental gene expression of VDR and Cyp27b1 [283]. Taking these findings into consideration no clear statements about a possible local dysregulation of the 25(OH)D feedback loop can be made from this work due to chronic maternal infection since local placental 25(OH)D and $1,25(OH)_2D$ should then be investigated together with placental gene expressions. Nevertheless, in this study in German placenta samples VDR expression indeed correlated positively with Cyp27b1, which is in line with previous reports [1, 283]. In the Gabonese cohort this correlation was abolished, suggesting a modulation/disruption of the placental milieu and vitamin D feedback loop possibly by environmental factors.

The rapid induction of VDR and Cyp27b1 expression in the placenta early in pregnancy [287] suggests that vitamin D may play a fundamental role in the process of conception, implantation and development of the placenta itself [20, 93]. Vitamin D deficiency in pregnant women has been shown to be associated with increased risk for pregnancy complications [148]. These include preeclampsia [29], fetal growth restriction, small-for-gestational-age fetus [32], bacterial vaginosis [32] and gestational diabetes mellitus [156]. Maternal vitamin D deficiency has also been linked to adverse effects in offspring, including reduced bone density [121] and childhood rickets [274], as well as increased risk of asthma [43] and schizophrenia [171]. However, a recent meta-analysis and systematic review concluded that vitamin D deficiency is not associated with increased risk of spontaneous recurrent abortion [12]. Furthermore,

progesterone induces not only the VDR expression but enhances the anti-inflammatory regulatory T cell response to calcitriol by three to five times [206, 223, 224, 255] and the importance of progesterone may not be underestimated and included into the classical vitamin D regulation loop. Whether the reduced expression of VDR and Hsd3b1 in Gabonese as compared to German placentae leads to a higher risk of placental inflammation and thus adverse birth outcomes or whether a distinct modulated immune milieu in mothers from helminth endemic areas leads to the downregulation of Hsd3b1 and VDR claims for further investigations. In the future, it would be interesting to measure progesterone, vitamin D and also active vitamin D levels in placental blood and compare it to inflammatory markers.

4.7 Reduced placental transfer of total IgG antibodies and detection of IgE antibodies in cord blood in Gabon

Another consequence of placental inflammation is disorder of the placental barrier with a disruption of the active Fc receptor mediated antibody transfer [172, 205]. Cord blood IgG4 compared to maternal levels was significantly higher in Germany, whereas Gabonese cord and maternal IgG4 levels did not differ from each other, indicating a more efficient mother-to-child transfer in the German cohort [94]. Since this isotype represents maternally-derived antibodies, these results could indicate disturbances of the processes involved in the transfer from the maternal circulation in Gabonese mother-child pairs. Because of the small number of schistosome infected mothers (23%, n=13) in our study, no additional information can be provided on the possible influence of concurrent schistosome infection. Six Gabonese women (11%) stated to have had malaria during pregnancy and 15 (28%) had taken antimalaria drugs. These six women had significantly higher IgG4 levels in peripheral plasma. However, the IgG4 levels in to these six mothers corresponding cord blood did not differ from the others (IgG4 levels in cord blood from mothers who did not claim to have had malaria during pregnancy), suggesting a less efficient placental transfer. This finding however did not correlate with inflammation markers such as CRP most likely because malaria had been successfully treated. Malaria in general can contribute to disturbed placental transfer of e.g. IgG [172] which is important when taking into consideration that infections may be asymptomatic and thus undiagnosed in highly endemic areas for malaria [6]. All Gabonese participants included into this study had a negative blood smear at time of delivery.

Overall levels of total IgG4 were significantly lower in Gabonese plasma when compared to German plasma samples. Furthermore, AWA-specific IgG4 levels were significantly higher in cord and maternal blood of the schistosome-infected group as compared to samples from the non-infected group where antibodies were detected despite no evidence of active infection. This was expected in a population endemic for helminth infections possibly due to the

substantial cross reactivity between helminth antigens [101]. Once schistosome-specific IgG4 and IgE antibodies are produced as part of an immune reaction, they usual remain detectable for a long period of time even after successful anti-helminthic treatment [203]. In addition, as with total IgG4, there was no difference in the level of antigen-specific IgG4 antibodies between maternal and fetal samples.

In general, significant amounts of IgG4 are found not only in helminth infections or schistosomiasis [101], but also in autoimmune diseases with chronic inflammation such as pemphigus vulgaris, myasthenia gravis, bullous pemphigoid and idiopathic membranous glomerulonephritis [259], emphasizing the role of IgG4 during chronic inflammation. However, chronic diseases were part of the exclusion criteria in this study and we assume that these diseases were no confounder. It would be interesting to compare the proportion of AWA-specific antibodies with total IgG4 to assess whether they originate from other diseases mentioned above. Since most of the studies focused on groups with same environmental backgrounds, little is known about the impact of the environment or the race on antibody levels in general. The reasons for higher total IgG4 levels in Germany may thus be multifactorial.

IgG4 predominantly supports an immune-tolerant environment for helminths by counteracting helminth-specific IgE responses and has been shown, for example, to be induced by IL10 released from circulating regulatory T cells [7]. In this study population total IgE levels were significantly higher in the Gabonese cohort when compared to German maternal samples and even higher in the maternal plasma samples from the infected group. The high levels in some maternal German plasma samples correlated with the women's history of allergy. As mentioned in chapter 1.5, high IgE levels are associated with resistance to helminth infection [260], whereas high levels of IgG4 have been associated with susceptibility [87] and thus a positive ratio can provide better insight into protection against helminth infections. Indeed, in Gabon the maternal total IgE/IgG4 ratio is significantly higher than in Germany and lifelong exposures might induce protection against reinfection [101, 260] [87].

In contrast to IgG4, IgE can normally not cross the placental barrier in healthy pregnancies. Thus, it was surprising to detect IgE antibodies in cord blood. The presence of IgE may be for example because of a) contamination with maternal blood; b) production by the fetus in itself *in utero*; c) ability of maternal IgE to cross the placenta.

Contamination with maternal blood is very unlikely, because the blood sampling from the umbilical cord was done in exactly the same way both in Germany and in Gabon and in case of contamination IgE antibodies would be found in German samples, too, which was not the case. If significant mixing of maternal and fetal blood did occur, the proportion of parasite-specific IgE in the cord sera should be equal to that observed in maternal sera which was not the case (to avoid admixture of maternal and cord blood, cord blood was obtained not by squeezing the umbilical cord but instead by direct needle aspiration, taking care to clean the

cord of maternal blood beforehand). Similar to IgE the protein CRP is not able to cross an intact placental barrier and nor in German nor in Gabonese cord blood samples CRP could be detected in those samples where mothers were positive for CRP, indicating a clean collection and no contamination.

There are however some arguments in favor of production *in utero* by the fetus itself. Unlike the German cohort, about 98% Gabonese cord blood samples had low, but detectable levels of IgE antibodies when compared to their mothers. In a study conducted in the Coast Province, Kenya, cord blood lymphocytes were stimulated *in vitro* with soluble lysates of adult *S. haematobium* or *Brugia malayi* worms and about 36% of healthy newborns spontaneously produced polyclonal IgE and IgG [130]. Furthermore, Malhotra et al. showed that polyclonal and parasite-specific IgE antibodies were present in many Kenyan cord and maternal sera whilst remaining undetectable in cord blood sera from North American infants [162]. Likewise, Seydel et al. detected schistosome soluble egg antigen (SEA)-specific IgE antibodies in Gabonese cord blood of those born to mothers infected with schistosomes [243]. However, in this study, even though levels of AWA-specific IgE were high in maternal blood, none could be detected in the cord blood. It might be that adult worm antigens may not cross the placenta in the same quantity as egg-related antigens [243], or that the concentration in cord blood was too low to be detected and that the signals were simply background reactions. However, no German samples were included in the assay as negative controls to generate a cut off. Moreover, the reaction to AWA is less strong than the antibody responses to SEA in general [77, 109]. Worms are able to survive for many years in the human host due to their ability to mask their surface tegument with host antigens [238] and they excrete only small amounts of antigenic molecules [238, 249]. Taken together, the presence of parasite specific IgE in cord blood might be evidence of *in utero* sensitization and production by the fetus itself.

Last but not least, the presence of antibodies other than IgG could indicate a disorder of the tightly regulated placental barrier and the ability of other antibodies to cross it. The lack of IgE specific RNA in the subgroup of seven Gabonese samples in this study indicates that the fetal IgE levels might be indeed maternal IgE which crossed the placental barrier. In another study investigating 18 placentas with moderate inflammation in the birth canal and 14 placentas with acute inflammation of unknown origin IgG and IgA was found in all cases studied and in 45% IgM within the group with moderate infection, and a transfer of all three types of antibodies (IgG, IgA and IgM) in the group with massive inflammation [22]. Infection with *Cytomegalovirus*, *Toxoplasma gondii* and *Rubella* was related with IgA transport from mother to fetus [120]. Further results suggest not only that IgA might be available for the embryo from the first trimester on [36, 120], but also general IgE was detected in cord blood of neonates whose mothers suffered from allergies [128]. Placental inflammation associated with malaria, for example, alters the materno-fetal antibody transfer on the one hand [172], but is also

associated with dysregulated autophagy, which impairs the transplacental amino acid transport on the other hand [72]. Thus, the modulation of the placental barrier in case of infection and inflammation could contribute to the protection of the embryo from congenital infections early in pregnancy [120].

In summary, our results indicate that pregnant women in Gabon might have a higher risk for placental inflammation, with a lower anti-inflammatory milieu when compared to German women. This is associated with reduced transplacental IgG4 transfer whereby the detection of IgE in offspring could be a hint for early priming events such as the de novo antibody class-switching due to *in utero* exposure to helminthic antigens on the one hand. On the other hand the placental barrier might be modulated or disturbed to allow the transfer of maternal IgE to the fetus. The higher maternal IgE/IgG4 ratio in Gabon might prevent severe symptoms in case of infection and evoke protection against reinfection [101, 260] [87].

4.8 Alteration of T cell function and placental gene expression

The immune system is a defense system that includes many biological structures and processes within an organism and protects against diseases. In order to eliminate pathogens such as bacteria, mycobacteria, viruses, fungi or parasites, it must distinguish between foreign and the organism's own healthy tissue. In addition to organs such as the skin, mucous membranes, lymph nodes, spleen or bone marrow, cells are another big part of the immune system. These include granulocytes, macrophages or leucocytes (=B and T lymphocytes). When the human body is infected with pathogens such as bacteria, viruses or parasites, the population of lymphocytes is activated and expands in order to eliminate the "intruder". A low number of lymphocytes can indicate the absence of inflammation but also the inability to achieve an adequate immune response. B lymphocytes are the only cells capable of generating plasma cells and secreting antibodies. In doing so, they recognize foreign antigens and are furthermore activated by T cells [186]. T cells are divided into several subgroups, including the $CD8^+$ T killer cells, which directly destroy sick cells, the $CD4^+$ T helper cells, which can secrete cytokines and activate further components of the immune system, and regulatory $Foxp3^+$ T cells, which are responsible for preventing excessive attacks on healthy body cells and hence contribute to self-tolerance [258].

Moreover, regulatory T cells (Tregs) are of particular importance during pregnancy due to their strong suppressive activity and crucial role in containing the potential pro-inflammatory reaction of the immune system [63].

During implantation, active immune suppression is required to prevent an immune reaction targeted against the embryo. Tregs play a central role in this context. They suppress cytotoxic

T cells, Th1 cells, macrophages, DC and NK cells [221] and lead to a unique immunotolerant milieu at the fetomaternal interface. At that, the expression of Treg cells has been considered to be essential for successful pregnancy outcome. Women with impaired Treg cell proportions suffer from spontaneous abortion and infertility [11, 114]. Moreover, a low percentage of Tregs correlated significantly with unexplained recurrent pregnancy loss (URPL), whereas the percentage of Th17 cells was significantly higher in URPL patients compared to the control group [3]. This immunotolerant milieu in the placenta is maintained by other soluble molecules such as cytokines, chemokines, hormones and prostaglandins [95], whereby increased expression in favor of pro-inflammatory cytokines has been associated with poor pregnancy outcomes in both human and animal models [102, 108, 110, 182].

The dysregulation of Tregs and the associated consequences are further aggravated by vitamin D deficiency [63]. Likewise, hypovitaminosis D is a risk factor for infertility and several adverse pregnancy outcomes [126, 146] and correlation between fertility and season, as well as geographical regions could be established. These variations were attributed to changes in vitamin D levels as a function of UV exposure [222]. In women with vitamin D deficiency and term or preterm births, significant positive correlations were found between serum levels of 25(OH)D and the percentage of $CD4^+CD25^{high}Foxp3^+$Tregs [286]. Vitamin D deficient pregnant women showed not only low circulating Tregs in both maternal and cord blood, but even placental Foxp3 gene expression was impaired in placenta [271]. In placenta samples from 25(OH)D insufficient and deficient pregnant women Foxp3 mRNA expression was down-regulated and significantly different from the sufficient participants. [271] Another study showed that after supplementation with $1,25(OH)_2D$ in women with URPL, the percentage of Tregs increased significantly from baseline [3] concluding that adequate levels of vitamin D can protect against unwanted pregnancy complications [3], by regulating differentiation and activation of $CD4^+$ T cells, leading to a more balanced Th1/Th2 response and thereby limiting the development of self-reactive T cells and preventing inflammation and autoimmunity [45, 282]. Vijayendra Chary et al. compared in a study conducted in India amongst women at birth with sufficient and insufficient vitamin D levels and correlated regulatory T cell populations and vitamin D pathway related placental gene expressions [271]. They demonstrated that the maternal as well as cord blood Treg cell populations were lower in 25(OH)D-deficient pregnant women compared to insufficient and sufficient pregnant women. Furthermore, regulatory cytokines such as TGFβ and IL10 were lower in 25(OH)D insufficient and deficient subjects [271].

In our subgroups (see chapter 3.15.1) for FACS analysis peripheral maternal vitamin D levels correlated significantly positively with $CD4^+$ cells in the Gabonese subgroup and with $Foxp3^+$ cells in the German subgroup, indicating a modulating role of 25(OH)D for T cells. Placental Foxp3 gene expression correlated significantly negative with IL10 expression as well as with

Foxp3 positive cells in Gabonese CMBCs, suggesting an influence on fetal placental gene expressions on cellular immune reactions in cord blood.

The strong and sensitive regulation of Tregs by vitamin D is contingent with an increased expression of VDR due to progesterone [255]. Indeed, some significant correlations between placental gene expressions and our FACS results could be seen. These were for example a significant negative correlation was seen for fetal VDR gene expression and both, Foxp3 and IL10 from $CD8^+$ Gabonese CBMCs and for maternal VDR and German maternal peripheral $CD4^+$ cells. But admittedly, FACS analysis was done for a subgroup of only 12 to 13 feto-maternal and furthermore the 25(OH)D levels were measured in peripheral blood and comparable in both groups. Whether and how placental gene expression has an effect on peripheral T cells, or vice versa, is not yet fully understood and requires further and more in-depth studies.

Lastly, Vijayendra Chary et al. found alterations in gene expressions of vitamin D regulating enzymes in women with 25(OH)D deficiency: Upregulation of Cyp24a1, Cyp2r1 and VDBP expression [271] and downregulation of Foxp3, VDR and RXR expression [271]. In contrast to recent studies stand the results here, comparing the Gabonese placenta samples from women with sufficient peripheral 25(OH)D levels with German samples from mothers with insufficient vitamin D levels, with a higher expression of VDR, Cyp27b1 and Foxp3 in Germany. In fact, within the German cohort, even a significant upregulation of Cyp27b1 and Foxp3 could be observed in the vitamin D deficient and insufficient group compared to the sufficient group. In the Gabonese cohort no vitamin D insufficiency was observed (25(OH)D < 20ng/mL), only vitamin D deficiency (20ng/mL < 25(OH)D < 30ng/mL). Thus, these intermediate levels might not have had such a strong effect on placental gene expression as in Germany. Noteworthy is the small study population investigated by Vijayendra Chary and collegues with only eight participants per group, whereas we included data of placental gene expresssion and vitamin D levels from 54 Gabonese and 47 German women.

In summary, maternal 25(OH)D levels during pregnancy indeed may influence a spectrum of gene expression and impair immune cells in the mother, the placenta and the fetus. However, in future data and correlation analysis between placental active vitamin D levels and placental gene expressions would give a better hint about local regulations within the placenta itself (chapter 4.6).

4.9 Comparable T cell populations and functionality in German and Gabonese PBMCs and CBMCs

During successful pregnancies, fetal trophoblasts and maternal peripheral leukocytes secrete predominantly Th2-type cytokines, e.g. IL10, to prevent initiation of inflammatory and cytolytic-type responses that might damage the integrity of the fetomaternal placental barrier [253]. Schistosomes typically induce a pronounced Th2 and regulatory response. The development of a balanced T cell response is important to prevent disease progression [207] and leads to a Treg cell dominant immune response in the chronic phase of infection [252]. Already in the early stages of fetal development in humans, the maturation of $CD4^{+}CD25^{high}$ thymocytes [210] begins in the thymus, suggesting that immune modulation begins *in utero* and could thus be influenced by maternal infection during pregnancy. During an infection with helminths, such as schistosomes, these modulate the human immune system and possibly simultaneously the placental immune milieu in a manner that ensures their own survival. Control of the T cell response plays a crucial role in this process [84]. Regulatory T cells dominate the immune response and suppress inflammatory cytokines [240] [207].

Recent research also by our group indicates that early exposure to parasites during gestation already primes the fetal immune system *in utero* and leads to enhanced immunological maturity at birth [17, 139, 140, 168]. *In utero* exposure to helminths [252], other environmental factors such as poor maternal nutrition [39], exposure to folic acid [137], air pollution [119] or organic pollutants [124] and genetic variants [68] have recently been shown to alter placental gene expression and to probably influence the offspring's propensity to develop allergies later in life [103, 140, 199, 219, 230].

Further data from analyses of CBMCs taken from the newborns of infected with helmiths such as *S. haematobium*, *Wuchereria bancrofti*, *Trichuris trichuris*, or *Ascaris lumbricoides* and uninfected mothers support the view that *in utero* sensitization does occur and, moreover, indicates that the fetal response is phenotypically similar to the response of the mother [130, 162]. The pre-existing Th2 response in such children might make them less likely to develop a pro-inflammatory response on first infection with schistosomes. [207] In mothers infected with *S. japonicum*, not only increased concentrations of pro-inflammatory cytokines such as IL1β or TNF were found in placental and cord blood [139], but infected women also exhibited an increased risk of placental inflammation or acute subchorionitis, confirmed by histopathological evidence [4]. Furthermore, an increase in pro-inflammatory endotoxin levels in placental blood and peripheral maternal blood has been reported in pregnant women with *S. japonicum* infection. The elevated endotoxin levels were additionally related to acute chorioamnionitis and increased proinflammatory cytokines [168]. Similarly, malaria alters the feto-maternal immune milieu in the form of a Th2 bias towards a pro-inflammatory microenvironment [182]. Hookworm

infection was found to influence significantly cytokine concentrations at the maternal-fetal interface, but no impact on cytokine levels in peripheral maternal peripheral or cord blood could be seen [4].Furthermore, even no association between maternal and cord blood cytokines could be observed [4].

Less lymphocytes and $CD3^+$ (T cells) as well as $CD3^+/CD8^+$ cell populations were found in PBMCs from *S. haematobium* infected Gabonese mothers compared to uninfected Gabonese PBMCs. After *in vitro* stimulation, PBMCs and CBMCs of infected mothers and their offspring produced more IL10 than the uninfected Gabonese pairs. The immune-regulatory cytokine IL10 has a crucial role during helminth infection to facilitate among others the parasite survival [139, 161]. Thus, higher IL10 concentration in Gabonese maternal peripheral blood could indicate an immune activation towards a T cell hyporesponsiveness provoked by the *S. haematobium* infection. No significant differences in cell populations or cytokine production between the German and the non-*S. haematobium* infected Gabonese PBMCs or CBMCs respectively could be detected, while PBMCs expressed significantly more IL5, IL10, IFNγ and TNFα than the corresponding CBMCs. Notably, it was a small subgroup of 12 to 13 participants in each group and therefore differences might only crystalize in bigger cohorts. A study comparing PBMCs from healthy Europeans and Indonesians from a urban as well a rural region (high endemic for helminth infections) using mass cytometry to analyze type 2 and regulatory immune cells observed that Tregs (characterized as $FOXP3^+CD25^{high}CD127^{low}$) did not differ between the three groups [67]. Nevertheless, there were significant differences at closer look at the subgroups of regulatory T cells. Rural Indonesians had more effector CD45RO positive Tregs, while Europeans had a significantly larger population of CD45RA+ Tregs [67]. As urban Indonesians resembled Europeans more than rural Indonesians in their immune profile, the authors concluded that environmental factors in particular contribute to the priming of the immune system rather than ethnic variation. Lambaréné in Gabon is mostly considered as rural area, but similar cell and cytokine rates in uninfected Gabonese and German participants, but not in *S. haematobium* infected mothers (e.g. higher IL10 levels) claim for an influence of an active infection on immune responses. Detailed T cell-subgroup analysis was not performed in our study in order to draw further conclusions. Moreover, the PMA/Ionomycin is a strong but an unspecific stimulus and may not be able to reproduce the full picture of parasite/helminth specific cytokine production. Indeed, in a study conducted in Kenia by Malhotra et al., cord blood samples produced more IL5, IL10 and IFNγ than the American control group only when stimulated with helminth antigens, but not when stimulated with PMA/Ionomycin [162]. However, upon stimulation with helminth antigens, many CBMCs did not produce detectable levels of cytokines at all. The failure of CBMCs from many newborns of infected mothers to produce detectable cytokines may have several reasons. It is

possible that these neonates have become exposed to antigens *in utero* and have become tolerized as shown before for maternal filariasis [17]. Or, even after in utero sensitization, cytokine production is below the limit of detection with the current assays.

Bal and colleagues observed no significant difference in susceptibility to infection between children born with circulating filarial antigen (CFA) -positive and CFA-negative cord blood from mothers with filariasis infected, speculating that not the transplacental transfer of filarial antigens holds the pivotal role in susceptibility to disease, but rather modulation of the fetal immune system of the by exposure *in utero* [17]. Moreover, trophoblasts stimulated in vitro with SEA reacted by a pro-inflammatory immune response, indicating that no direct infection of the placenta is required to change the immune milieu at the fetomaternal interface [167]. In contrast, Malhotra et al. suggest, that the helminth antigen-specific hypo-responsiveness of individuals living in areas endemic for helminth infections may have less to do with prenatal exposure but rather with other factors such as host genetic differences, chronicity of infection, or repeated exposure to infective stages of the parasite [162]. Indeed, acute infection and strong immune reactions are mainly seen in travelers to schistosomiasis-endemic areas, people from helminth non endemic areas with no former exposure [227] (see also chapter 1.1). Taken together, analysis of the subgroup of 12 to 13 PBMC/CBMC pairs reveal similar T cell und Treg populations between healthy German and uninfected Gabonese participants, but in-depth subgroup investigations could reveal yet undetected differences between the immune system of German and Gabonese mothers and their newborns. However, infection with *S. haematobium* had a significant influence by lowering maternal lymphocytes, $CD3^+$ and maternal and cord $CD3^+/CD8^+$ cells as well as on maternal IL10 by amplifying its expression. Nevertheless, cytokine and cell examination at the fetomaternal interface, the placenta, itself were not part of the study and the exact mechanism of immune modeling in different regions has to be analyzed in future.

4.10 Summary

The fetomaternal crosstalk remains one of the most intriguing and underexplored areas of immunological research. Disruption of the evolutionary finely balanced coexistence of mother and fetus can have profound effects not only for the success of pregnancy itself but also potentially for the development of the newborns immune system. Such disruptions could result from environmental impact such as infections. The aim of this thesis was to investigate whether geographical and environmental factors such as exposure to helminths influence development at the feto-maternal interface. Furthermore, to explore whether exposure *in utero* to the

helminth *S. haematobium* is associated with altered outcomes in the placental gene expression. Overall the results revealed that pregnant women in Gabon might have a higher risk for placental inflammation with a lower anti-inflammatory milieu when compared to German women. The susceptibility to named placental inflammation points out in a lower expression of important anti-inflammatory genes such as Hsd3b1, VDR, Cyp27b1, Foxp3 or IL10 in Gabonese placenta samples. Furthermore, this was associated with reduced transplacental IgG4 transfer whereby the detection of IgE in offspring could be a hint for early priming events such as the de novo antibody class-switching due to *in utero* exposure to (helminthic) antigens or a modulated placental barrier due to livelong maternal exposure to helminths. Living in a non-endemic area compared to a helminth endemic area leads to a different immune response and susceptibility for helminths, demonstrated by the significant higher IgE/IgG4 ratio in the Gabonese cohort. The staining and non-specific stimulation of the PBMCs and CBMCs worked nicely and the flowcytometry results of a subgroup showed already strong trends for differences in IL10 expression and lymphocyte populations between with S. haematobium infected and uninfected Gabon participants.

As one of the first studies to compare inflammatory genes between geographically distinct populations, including subgroups based on helminth infection, as well as localized gene expression to placental and fetal placental sub-regions, this thesis provides an initial setup for further investigation into how infection status can modify the complex crosstalk between placental inflammatory responses and healthy fetal development.

4.11 Limitations of the study

Firstly, it should be mentioned that this is a pilot study and it has not been stratified in any way from the figures. Limitations were already mentioned and discussed during the section above and the following list should complement some more important points:

- Helminth diagnostic was only performed for Gabonese mothers, but not for Germans. Anyhow, using a detailed questionnaire about travelling habits and possible exposure to helminths the probability of helminth infecting among the German cohort could be reduced to a minimum.
- Neonates in Gabon were weighed with another scale than in Germany;
- The style of measuring the birth length of the newborns was different as it was performed by different midwives and different styles.
- Maternal hemoglobin concentrations were measured in two different laboratories.
- CRP levels may be influenced by the duration of labor [152], which could not be recorded in Gabon.

- Since placenta samples were not washed gene expression analyses do not entirely represent tissue expression only but possibly also contain RNA derived from circulating cells.
- Only one single sample was taken from each side, always from a similar area near the umbilical cord, but some gene expressions were already found differ depending on the placental location [119].
- PBMC/CBMC stimulation was performed with the unspecific stimulant PMA/Iono and no helminth specific stimulation was done.

5. Résumé and Outlook

The impact of *in utero* exposure to helminth infections on the development of health and disease in the next generation belongs to the emerging research field of "developmental origins of health and disease" or DOHaD and has recently been investigated in several trials [184]. In general, helminth infection leads to chronic low-grade inflammatory immune reactions dominated by regulatory responses suppressing strong pro-inflammatory cytokines [173]. Maternal infection with helminths not only changes the autologous immune responses of the newborn resulting in increased susceptibility to subsequent helminth infections [17] but also heterologous responses with reduced risk to develop atopy and potentially allergies [151, 271]. The underlying mechanisms how maternal helminth infections affect the fetus are not understood and thus focus of the research presented here. We paid particular attention on the main communicator between fetus and child, the placenta as key component of the fetomaternal crosstalk and found that immunosuppressive genes (e.g. VDR, Cyp27b1, Hsdb31, Foxp3 and IL10) are expressed to a lesser extent in Gabonese placental samples and thus, potentially, a higher risk to develop placental inflammation, independent of *S. haematobium* infection, which could emphasize the impact of environmental factors rather than active infection. Furthermore, the surprising presence of IgE in Gabonese cord blood could imply either transfer of maternal or de novo synthesis and in utero B cell class switching in newborns due to maternal schistosome infection.

Taken together, although helminths themselves are usually unable to cross the placental barrier, they appear to modulate the barrier in a manner that allows the transfer of not only antigens but rather other than IgG maternal antibodies. Furthermore, chronic helminth infection alters the placental immune milieu in a pattern that could not only lead to fetal growth retention but also to further adverse pregnancy outcomes [172]. However, it is not yet clear whether it is the infection itself and prenatal exposure or rather other factors such as genetic differences in the host, chronicity of infection or repeated exposure to infectious stages of the parasite that cause a cellular hypo-responsiveness in people living in helminth endemic areas [4, 162].

The soluble immunosuppressive hormones vitamin D and progesterone, a strong coplayer of vitamin D [255] and one of the most important hormones during pregnancy, also play a decisive role in the tight modulation and control of the immune system as a central element for a successful pregnancy [143, 144]. Hence, in addition to the placental expression of the vitamin D receptor (VDR) and Hsd3b1, which had previously proved to be key factors in regulation, these hormones were also included in this study [149].

Indeed, peripheral 25(OH) vitamin D3 levels were much higher in Gabonese women, which is expected due to year-round sun exposure [154]. Nevertheless, since nutrition is another important source for vitamin D [155], the contribution of diet-related variations of vitamin D levels need to be taken into account and could be assessed for example by questionnaires on eating habits. Furthermore, it would be of interest to measure active 1,25(OH) vitamin D levels in peripheral maternal and cord blood, but also in placental blood. The comparison of placental and peripheral hormone levels would reveal a better insight into the placenta as an exocrine and independent organ.

Furthermore, this study provides specific information on the maternal and the fetal side of the placenta as distinct immunological regions. Along these lines, tissue samples from not only one but several different sites of the placenta as well as cell type specific analyses (e.g. trophoblast or syncytiotrophoplast) may provide information on local differences in gene expression profile. Furthermore, performing immune histology of placenta could give more precise information not only about local inflammation foci, but also, for example, about Fc-receptor expression, which is crucial for materno-fetal antibody transfer. Concerning the surprising detection of total IgE in the Gabonese cord blood, clarification of its origin – fetal or maternal – is worthwhile and could involve IgE repertoire and B and plasma cell analyses. Also, the cellular source of increased immunoregulation in the placenta and fetal blood due to infection should be analyzed in greater detail, to discern whether helminths cause such alterations in an antigen-specific or unspecific manner [166]. For example, do fetal Gabonese immune cells produce antibodies independently of maternal antigen presentation or effectively secrete antigen-specific cytokines upon stimulation, or is even the stem cell compartment be changed? This would undermine our preliminary observations that the fetal immune system matures differently *in utero* due to maternal infection.

Based on the findings of this pilot study with its limited number of participants, a cross sectional study with adequate epidemiological sample size calculation is warranted to substantiate the observations and to gain further mechanistic insights.

Currently, a larger cross-sectional study called “HelmVit” is being undertaken in Lambaréné, Gabon, to clarify some of the outstanding queries mentioned above as well as to substantiate the findings comparing three mother-child cohorts: one infected with *S. haematobium* only one infected with other geohelminths only and one healthy group from the same endemic area as the other two. A detailed questionnaire will provide further information about dietary habits, possible sources of vitamin D, but also about allergic diseases. There will be follow-up visits of the children, providing more detailed information in the future about susceptibility to helminth infections or the relationship between maternal infection and immune status as well as gene expression profiles in the placenta. These future investigations will hopefully contribute to a

better understanding of the missing link between infectious diseases and their impact on the fetus *in utero* and its subsequent immune reactions later on in life.

6. Acknowledgements

I am grateful to all pregnant women who participated in the study.

This dissertation would not have been possible without the help of so many people in so many ways. Firstly, I owe my thank my advisor Professor Clarissa Prazeres da Costa for not only giving me the opportunity to work on this extremely interesting project, but also to but also for arousing my enthusiasm and fascination for science. I would like to thank her for her continuous support, patience, motivation, and instilling in me the qualities of being a good scientist. Without her guidance, encouragement and persistent help this dissertation would not have been possible.

I would like to thank Prof. Ayola Akim Adegnika for allowing me to become part of his research group at CERMEL in Lambaréné and for all his great support and advice at all times, which allowed a successful stay in Gabon and the successful realization of the project. I thank all CERMEL personnel for their exceptional assistance as well as the co-workers at CERMEL. My thank goes especially to Dr. Josiane Honkpehedji for not only organizing the project in Lambaréné, but also for always having the door open to listen to my worries and becoming a friend.

I also want to give thanks to Dr. Silvia Lobmaier for being a supervisor and for making the sample collection at Klinikum rechts der Isar possible. I am indebted to the midwives and nurses at the Klinikum rechts der Isar in Munich, Germany and at Albert Schweitzer Hospital and General Hospital in Lambaréné, Gabon, for their help in collecting the samples.

Another warm thank you goes to Dr. Jutta Harder who initiated the pilot study in Munich, developed the PCR protocol and was responsible for the sample collection and analysis of half of the German participants. Thank you for providing your data, your experience and your knowledge! I would like to thank Prof. Dirk Busch for giving me the possibility to do my medical thesis at the Institute of Medical Microbiology, Immunology and Hygiene. In addition, my sincere thanks goes to my colleagues in our laboratory, especially Sabine Paul and Marija Ram and to Dr. Sonakshi Bhattacharjee, Dr. Fabien Prodjinoto and especially to Matthew Lacorcia for excellent technical assistance and the discussion.

Furthermore, I am grateful to our cooperation partners in Tübingen, especially to Dr. Meral Esen, without whom a successful journey to Gabon would not have been possible.

I would like to thank the collaborators Paul Corstjens and Govert van Dam at Leiden University, as well as David Vöhringer at University Erlangen. Claudia de Dood (LUMC, Cell and Chemical Biology department) is acknowledged for preparing the UCP-LF CAA test materials and performing the CAA assays on the plasma and urine samples and Paul Haase (Department of

Infection Biology at the University Hospital Erlangen) for RNA and DNA sub analysis to detect fetal IgE.

I owe the deepest gratitude to my family, especially to my parents for the trust and never-ending support and encouragement.

7. References

1. *Vitamin D Receptor Gene: Ways to Activate it, Calcitriol, Benefits*. 2019 08.05.2019]; Available from: https://selfhacked.com/blog/natural-ways-to-increase-calcitrol-and-vitamin-d-receptor-gene-expression/.

2. Abalos, E., et al., *Effects of calcium supplementation on fetal growth in mothers with deficient calcium intake: a randomised controlled trial.* Paediatr Perinat Epidemiol, 2010. **24**(1): p. 53-62.

3. Abdollahi, E., et al., *Evaluation of the effects of 1,25 vitamin D3 on regulatory T cells and T helper 17 cells in Vitamin D-deficient women with unexplained recurrent pregnancy loss.* Curr Mol Pharmacol, 2020.

4. Abioye, A.I., et al., *Maternal, placental and cord blood cytokines and the risk of adverse birth outcomes among pregnant women infected with Schistosoma japonicum in the Philippines.* PLoS Negl Trop Dis, 2019. **13**(6): p. e0007371.

5. Adams, J.S., et al., *Redefining Human Vitamin D Sufficiency: Back to the Basics.* Bone Res, 2013. **1**(1): p. 2-10.

6. Adegnika, A.A., et al., *Epidemiology of parasitic co-infections during pregnancy in Lambarene, Gabon.* Trop Med Int Health, 2010. **15**(10): p. 1204-9.

7. Adjobimey, T. and A. Hoerauf, *Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation.* Annals of tropical medicine and parasitology, 2010. **104**(6): p. 455-464.

8. Aibana, O., et al., *Vitamin D status and risk of incident tuberculosis disease: A nested case-control study, systematic review, and individual-participant data meta-analysis.* PLoS medicine, 2019. **16**(9): p. e1002907-e1002907.

9. Aljazeera. *Life cycle: Schistosomiasis*. 05 March 2014 23.05.2019]; Available from: https://www.aljazeera.com/programmes/lifelines/2014/03/life-cycles-schistosomiasis-20143283710740763.html.

10. Aluvihare, V.R., M. Kallikourdis, and A.G. Betz, *Regulatory T cells mediate maternal tolerance to the fetus.* Nat Immunol, 2004. **5**(3): p. 266-71.

11. Aluvihare, V.R., M. Kallikourdis, and A.G. Betz, *Regulatory T cells mediate maternal tolerance to the fetus.* Nature Immunology, 2004. **5**(3): p. 266-271.

12. Amegah, A., M. Klevor, and C. Wagner, *Maternal vitamin D insufficiency and risk of adverse pregnancy and birth outcomes: a systematic review and meta-analysis of longitudinal studies.* PLoS ONE, 2017. **12**: p. e0173605.

13. Ateba-Ngoa, U., et al., *CD4+CD25hiFOXP3+ cells in cord blood of neonates born from filaria infected mother are negatively associated with CD4+Tbet+ and CD4+RORgammat+ T cells.* PLoS One, 2014. **9**(12): p. e114630.

14. Avrech, O.M., et al., *Efficacy of the placental barrier for immunoglobulins: correlations between maternal, paternal and fetal immunoglobulin levels.* Int Arch Allergy Immunol, 1994. **103**(2): p. 160-5.

15. Babu, S., et al., *Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells.* PLoS Negl Trop Dis, 2009. **3**(4): p. e420.

16. Baeke, F., et al., *Vitamin D: modulator of the immune system.* Curr Opin Pharmacol, 2010. **10**(4): p. 482-96.

17. Bal, M., et al., *Maternal Filarial Infection Influences the Development of Regulatory T Cells in Children from Infancy to Early Childhood.* PLoS neglected tropical diseases, 2016. **10**(11): p. e0005144-e0005144.

18. Barnes, P.J., *Targeting cytokines to treat asthma and chronic obstructive pulmonary disease.* Nat Rev Immunol, 2018. **18**(7): p. 454-466.

19. Barrat, F.J., et al., *In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines.* J Exp Med, 2002. **195**(5): p. 603-16.

20. Barrera, D., et al., *Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts.* Reprod Biol Endocrinol, 2008. **6**: p. 3.

21. Base, S. *Placental circulation*. 2008 2019, November 21]; Available from: http://www.sliderbase.com/spitem-1503-10.html.

22. Ben-Hur, H., et al., *Transport of maternal immunoglobulins through the human placental barrier in normal pregnancy and during inflammation.* Int J Mol Med, 2005. **16**(3): p. 401-7.

23. Berer, A., et al., *1,25-Dihydroxyvitamin D(3) inhibits dendritic cell differentiation and maturation in vitro.* Exp Hematol, 2000. **28**(5): p. 575-83.

24. Bergmann, R.L., et al., *Determinants of cord-blood IgE concentrations in 6401 German neonates.* Allergy, 1995. **50**(1): p. 65-71.

25. Beutler, E. and C. West, *Hematologic differences between African-Americans and whites: the roles of iron deficiency and alpha-thalassemia on hemoglobin levels and mean corpuscular volume.* Blood, 2005. **106**(2): p. 740-5.

26. Billington, W.D., *The normal fetomaternal immune relationship.* Baillieres Clin Obstet Gynaecol, 1992. **6**(3): p. 417-38.

27. Bittencourt, A.L., et al., *Placental involvement in schistosomiasis mansoni. Report of four cases.* Am J Trop Med Hyg, 1980. **29**(4): p. 571-5.

28. Blackwell, A.D., *Helminth infection during pregnancy: insights from evolutionary ecology.* Int J Womens Health, 2016. **8**: p. 651-661.

29. Bodnar, L., et al., *Prepregnancy obesity predicts poor vitamin D status in mothers and their neonates.* Journal of Nutrition, 2007. **137**: p. 2437-2442.

30. Bodnar, L., et al., *Maternal vitamin D deficiency increases the risk of preeclampsia.* Journal of Clinical Endocrinology and Metabolism, 2007. **92**: p. 3517-3522.

31. Bodnar, L., et al., *Maternal serum 25-hydroxyvitamin D concentrations are associated with small-for-gestational age births in white women.* Journal of Nutrition, 2010. **140**: p. 999-1006.

32. Bodnar, L., M. Krohn, and H. Simhan, *Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy.* Journal of Nutrition, 2009. **139**: p. 1157-1161.

33. Bodnar, L.M., et al., *Maternal vitamin D deficiency increases the risk of preeclampsia.* J Clin Endocrinol Metab, 2007. **92**(9): p. 3517-22.

34. Boehm, U., et al., *Cellular responses to interferon-gamma.* Annu Rev Immunol, 1997. **15**: p. 749-95.

35. Boonstra, A., et al., *1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells.* J Immunol, 2001. **167**(9): p. 4974-80.

36. Borte, S., et al., *Placental transfer of maternally-derived IgA precludes the use of guthrie card eluates as a screening tool for primary immunodeficiency diseases.* PLoS One, 2012. **7**(8): p. e43419.

37. Botelho, M.C., H. Alves, and J. Richter, *Halting Schistosoma haematobium - associated bladder cancer.* Int J Cancer Manag, 2017. **10**(9).

38. Brair, M.E., et al., *Reduced transfer of tetanus antibodies with placental malaria.* Lancet, 1994. **343**(8891): p. 208-9.

39. Brown, A.S. and E.S. Susser, *Prenatal nutritional deficiency and risk of adult schizophrenia.* Schizophr Bull, 2008. **34**(6): p. 1054-63.

40. Bulmer, J.N., P.J. Williams, and G.E. Lash, *Immune cells in the placental bed.* Int J Dev Biol, 2010. **54**(2-3): p. 281-94.

41. Bustinduy, A.L., J.R. Stothard, and J.F. Friedman, *Paediatric and maternal schistosomiasis: shifting the paradigms.* Br Med Bull, 2017. **123**(1): p. 115-125.

42. Butticè, G., et al., *Interferon-gamma induces major histocompatibility class II transactivator (CIITA), which mediates collagen repression and major histocompatibility class II activation by human aortic smooth muscle cells.* Circulation research, 2006. **98**(4): p. 472-479.

43. Camargo, C., et al., *Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 years of age.* American Journal of Clinical Nutrition, 2007. **85**: p. 788-795.

44. Camus, D., et al., *Sensitization to Schistosoma mansoni antigen in uninfected children born to infected mothers.* J Infect Dis, 1976. **134**(4): p. 405-8.

45. Cantorna, M.T., et al., *Vitamin D and 1,25(OH)2D regulation of T cells.* Nutrients, 2015. **7**(4): p. 3011-21.

46. Chambers, E.S. and C.M. Hawrylowicz, *The impact of vitamin D on regulatory T cells.* Curr Allergy Asthma Rep, 2011. **11**(1): p. 29-36.

47. Chatterjee, P., et al., *Regulation of the Anti-Inflammatory Cytokines Interleukin-4 and Interleukin-10 during Pregnancy.* Frontiers in Immunology, 2014. **5**(253).

48. Chen, J., et al., *Contribution of regulatory T cells to immune tolerance and association of microRNA210 and Foxp3 in preeclampsia.* Mol Med Rep, 2019. **19**(2): p. 1150-1158.

49. Chen, X.M., et al., *[Placental Foxp3 expression in patients with preeclampsia and correlation of Foxp3 gene locus 924 (rs2232365) polymorphism with preeclampsia].* Nan Fang Yi Ke Da Xue Xue Bao, 2015. **35**(1): p. 77-82.

50. Clerinx, J. and A. Van Gompel, *Schistosomiasis in travellers and migrants.* Travel Med Infect Dis, 2011. **9**(1): p. 6-24.

51. Coan, P.M., et al., *Dietary composition programmes placental phenotype in mice.* The Journal of physiology, 2011. **589**(Pt 14): p. 3659-3670.

52. Colley, D.G., et al., *A five-country evaluation of a point-of-care circulating cathodic antigen urine assay for the prevalence of Schistosoma mansoni.* Am J Trop Med Hyg, 2013. **88**(3): p. 426-432.

53. Colley, D.G., et al., *Human schistosomiasis.* Lancet, 2014. **383**(9936): p. 2253-64.

54. Colley, D.G., et al., *Immune responses during human schistosomiasis. XII. Differential responsiveness in patients with hepatosplenic disease.* Am J Trop Med Hyg, 1986. **35**(4): p. 793-802.

55. Combat, U.t. *Bilharzia*. 2017; Available from: https://unitingtocombatntds.org/ntds/schistosomiasis/.

56. Commons, W. *Placenta human newborn.jpg*. [Photo] 2016 2016 September 26 [cited 2020 2020 April 20]; Available from: https://commons.wikimedia.org/wiki/File:Placenta_human_newborn.jpg.

57. Conrad, M.L., et al., *Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe Acinetobacter lwoffii F78.* J Exp Med, 2009. **206**(13): p. 2869-77.

58. Corstjens, P., et al., *Utilizing the ultrasensitive Schistosoma up-converting phosphor lateral flow circulating anodic antigen (UCP-LF CAA) assay for sample pooling-strategies.* Infect Dis Poverty, 2017. **6**(1): p. 155.

59. Corstjens, P.L., et al., *Tools for diagnosis, monitoring and screening of Schistosoma infections utilizing lateral-flow based assays and upconverting phosphor labels.* Parasitology, 2014. **141**(14): p. 1841-55.

60. Corstjens, P.L., et al., *Improved sensitivity of the urine CAA lateral-flow assay for diagnosing active Schistosoma infections by using larger sample volumes.* Parasit Vectors, 2015. **8**: p. 241.

61. Corstjens, P.L., et al., *Up-converting phosphor technology-based lateral flow assay for detection of Schistosoma circulating anodic antigen in serum.* J Clin Microbiol, 2008. **46**(1): p. 171-6.

62. Cutland, C.L., et al., *Low birth weight: Case definition & guidelines for data collection, analysis, and presentation of maternal immunization safety data.* Vaccine, 2017. **35**(48 Pt A): p. 6492-6500.

63. Cyprian, F., et al., *Immunomodulatory Effects of Vitamin D in Pregnancy and Beyond.* Frontiers in immunology, 2019. **10**: p. 2739-2739.

64. Davidson, R., et al., *Influence of maternal and perinatal factors on subsequent hospitalisation for asthma in children: evidence from the Oxford record linkage study.* BMC Pulm Med, 2010. **10**: p. 14.

65. de Jesus, A.R., et al., *Clinical and immunologic evaluation of 31 patients with acute schistosomiasis mansoni.* J Infect Dis, 2002. **185**(1): p. 98-105.

66. de Moraes-Pinto, M.I., et al., *Placental transfer and maternally acquired neonatal IgG immunity in human immunodeficiency virus infection.* J Infect Dis, 1996. **173**(5): p. 1077-84.

67. de Ruiter, K., et al., *Helminth infections drive heterogeneity in human type 2 and regulatory cells.* Science Translational Medicine, 2020. **12**(524): p. eaaw3703.

68. Delahaye, F., et al., *Genetic variants influence on the placenta regulatory landscape.* PLoS genetics, 2018. **14**(11): p. e1007785-e1007785.

69. Delvin, E.E., et al., *Influence of calcitriol on prolactin and prostaglandin production by human decidua.* Mol Cell Endocrinol, 1990. **71**(3): p. 177-83.

70. Delvin, E.E., et al., *Control of vitamin D metabolism in preterm infants: feto-maternal relationships.* Arch Dis Child, 1982. **57**(10): p. 754-7.

71. Diaz, L., et al., *Identification of a 25-hydroxyvitamin D3 1alpha-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells.* J Clin Endocrinol Metab, 2000. **85**(7): p. 2543-9.

72. Dimasuay, K.G., et al., *Impaired placental autophagy in placental malaria.* PLoS One, 2017. **12**(11): p. e0187291.

73. Dimova, T., et al., *Maternal Foxp3 expressing CD4+ CD25+ and CD4+ CD25- regulatory T-cell populations are enriched in human early normal pregnancy decidua: a phenotypic study of paired decidual and peripheral blood samples.* Am J Reprod Immunol, 2011. **66 Suppl 1**: p. 44-56.

74. Doetze, A., et al., *Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift.* Int Immunol, 2000. **12**(5): p. 623-30.

75. Doi, M., et al., *Association between calcium in cord blood and newborn size in Bangladesh.* Br J Nutr, 2011. **106**(9): p. 1398-407.

76. Dusso, A.S., A.J. Brown, and E. Slatopolsky, *Vitamin D.* Am J Physiol Renal Physiol, 2005. **289**(1): p. F8-28.

77. El Ridi, R., et al., *Differential responsiveness of humans with early-stage schistosomiasis haematobium to Schistosoma haematobium soluble adult-worm and egg antigens.* Parasitol Res, 1997. **83**(5): p. 471-7.

78. el-Nahal, H.M., et al., *Mutual effect of Schistosoma mansoni infection and pregnancy in experimental C57 BL/6 black mice.* J Egypt Soc Parasitol, 1998. **28**(1): p. 277-92.

79. el-Nahal, H.M., et al., *Effect of Schistosoma mansoni infection on offsprings born from infected mothers.* J Egypt Soc Parasitol, 1998. **28**(2): p. 523-38.

80. Elson, L.H., et al., *In utero exposure to Onchocerca volvulus: relationship to subsequent infection intensity and cellular immune responsiveness.* Infect Immun, 1996. **64**(12): p. 5061-5.

81. Erlebacher, A., *Immunology of the maternal-fetal interface.* Annu Rev Immunol, 2013. **31**: p. 387-411.

82. Evans, K.N., et al., *Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells.* Biol Reprod, 2006. **75**(6): p. 816-22.

83. Faas, M.M. and P. De Vos, *Innate immune cells in the placental bed in healthy pregnancy and preeclampsia.* Placenta, 2018. **69**: p. 125-133.

84. Fairfax, K., et al., *Th2 responses in schistosomiasis.* Semin Immunopathol, 2012. **34**(6): p. 863-71.

85. Farquhar, C., et al., *High maternal HIV-1 viral load during pregnancy is associated with reduced placental transfer of measles IgG antibody.* J Acquir Immune Defic Syndr, 2005. **40**(4): p. 494-7.

86. Feary, J., J. Britton, and J. Leonardi-Bee, *Atopy and current intestinal parasite infection: a systematic review and meta-analysis.* Allergy, 2011. **66**(4): p. 569-78.

87. Figueiredo, J.P., et al., *Adult worm-specific IgE/IgG4 balance is associated with low infection levels of Schistosoma mansoni in an endemic area.* Parasite Immunol, 2012. **34**(12): p. 604-10.

88. Forsthuber, T., H.C. Yip, and P.V. Lehmann, *Induction of TH1 and TH2 immunity in neonatal mice.* Science, 1996. **271**(5256): p. 1728-30.

89. Fried, M., et al., *Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes.* J Immunol, 1998. **160**(5): p. 2523-30.

90. Friedman, J.F., H.K. Kanzaria, and S.T. McGarvey, *Human schistosomiasis and anemia: the relationship and potential mechanisms.* Trends Parasitol, 2005. **21**(8): p. 386-92.

91. Friedman, J.F., et al., *Schistosomiasis and pregnancy.* Trends Parasitol, 2007. **23**(4): p. 159-64.

92. Friedman, J.F., et al., *Praziquantel for the treatment of schistosomiasis during human pregnancy.* Bull World Health Organ, 2018. **96**(1): p. 59-65.

93. Ganguly, A., et al., *Vitamin D, the placenta and early pregnancy: effects on trophoblast function.* J Endocrinol, 2018. **236**(2): p. R93-r103.

94. Garty, B.Z., et al., *Placental transfer of immunoglobulin G subclasses.* Clin Diagn Lab Immunol, 1994. **1**(6): p. 667-9.

95. Gaynor, L.M. and F. Colucci, *Uterine Natural Killer Cells: Functional Distinctions and Influence on Pregnancy in Humans and Mice.* Front Immunol, 2017. **8**: p. 467.

96. Gheorghe, C.P., et al., *Gene expression in the placenta: maternal stress and epigenetic responses.* Int J Dev Biol, 2010. **54**(2-3): p. 507-23.

97. Greenwood, B.M., *Autoimmune disease and parasitic infections in Nigerians.* Lancet, 1968. **2**(7564): p. 380-2.

98. Griffin, M.D., et al., *Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6800-5.

99. Grogan, J.L., et al., *Elevated proliferation and interleukin-4 release from CD4+ cells after chemotherapy in human Schistosoma haematobium infection.* Eur J Immunol, 1996. **26**(6): p. 1365-70.

100. Grogan, T.M., D.D. Broughton, and W.F. Doyle, *Graft-versus-host reaction (GVHR). A case report suggesting GVHR occurred as a result of maternofetal cell transfer.* Arch Pathol, 1975. **99**(6): p. 330-4.

101. Hagan, P., et al., *Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium.* Nature, 1991. **349**(6306): p. 243-5.

102. Hahn-Zoric, M., et al., *Aberrations in placental cytokine mRNA related to intrauterine growth retardation.* Pediatr Res, 2002. **51**(2): p. 201-6.

103. Hartgers, F.C., et al., *Lower expression of TLR2 and SOCS-3 is associated with Schistosoma haematobium infection and with lower risk for allergic reactivity in children living in a rural area in Ghana.* PLoS Negl Trop Dis, 2008. **2**(4): p. e227.

104. Hayes, E.K., et al., *Adverse fetal and neonatal outcomes associated with a life-long high fat diet: role of altered development of the placental vasculature.* PLoS One, 2012. **7**(3): p. e33370.

105. Hayes, E.K., et al., *Trophoblast invasion and blood vessel remodeling are altered in a rat model of lifelong maternal obesity.* Reprod Sci, 2014. **21**(5): p. 648-57.

106. Hegertun, I.E., et al., *S. haematobium as a common cause of genital morbidity in girls: a cross-sectional study of children in South Africa.* PLoS Negl Trop Dis, 2013. **7**(3): p. e2104.

107. Heikkinen, J., et al., *Phenotypic characterization of regulatory T cells in the human decidua.* Clin Exp Immunol, 2004. **136**(2): p. 373-8.

108. Hennessy, A., et al., *A deficiency of placental IL-10 in preeclampsia.* J Immunol, 1999. **163**(6): p. 3491-5.

109. Hillyer, G.V., L.L. Nieves-Frau, and G. Vazquez, *Identification of a genus-specific Schistosoma mansoni soluble egg antigen reactive with the serum of infected patients.* Am J Trop Med Hyg, 1986. **35**(6): p. 1198-204.

110. Holcberg, G., et al., *Increased production of tumor necrosis factor-alpha TNF-alpha by IUGR human placentae.* Eur J Obstet Gynecol Reprod Biol, 2001. **94**(1): p. 69-72.

111. Holick, M.F., *Biological Effects of Sunlight, Ultraviolet Radiation, Visible Light, Infrared Radiation and Vitamin D for Health.* Anticancer Res, 2016. **36**(3): p. 1345-56.

112. Holick, M.F., *The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention.* Rev Endocr Metab Disord, 2017. **18**(2): p. 153-165.

113. Holick, M.F., et al., *Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline.* J Clin Endocrinol Metab, 2011. **96**(7): p. 1911-30.

114. Hori, S., T. Nomura, and S. Sakaguchi, *Pillars Article: Control of Regulatory T Cell Development by the Transcription Factor Foxp3. Science 2003. 299: 1057-1061.* J Immunol, 2017. **198**(3): p. 981-985.

115. Hu, X., et al., *Forkhead box P3 is selectively expressed in human trophoblasts and decreased in recurrent pregnancy loss.* Placenta, 2019. **81**: p. 1-8.

116. Hussain, R., et al., *IgE responses in human filariasis. I. Quantitation of filaria-specific IgE.* J Immunol, 1981. **127**(4): p. 1623-9.

117. Ishida, K. and M.H. Hsieh, *Understanding Urogenital Schistosomiasis-Related Bladder Cancer: An Update.* Front Med (Lausanne), 2018. **5**: p. 223.

118. Jaakkola, J.J. and M. Gissler, *Maternal smoking in pregnancy, fetal development, and childhood asthma.* Am J Public Health, 2004. **94**(1): p. 136-40.

119. Janssen, A.B., et al., *Placental expression of imprinted genes varies with sampling site and mode of delivery.* Placenta, 2015. **36**(8): p. 790-795.

120. Jauniaux, E., et al., *Materno-fetal immunoglobulin transfer and passive immunity during the first trimester of human pregnancy.* Hum Reprod, 1995. **10**(12): p. 3297-300.

121. Javaid, M., et al., *Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study.* Lancet, 2006. **367**: p. 36-43.

122. Jeffery, L.E., et al., *1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3.* J Immunol, 2009. **183**(9): p. 5458-67.

123. Johnson, C.C., D.R. Ownby, and E.L. Peterson, *Parental history of atopic disease and concentration of cord blood IgE.* Clin Exp Allergy, 1996. **26**(6): p. 624-9.

124. Kappil, M.A., et al., *In utero exposures to environmental organic pollutants disrupt epigenetic marks linked to fetoplacental development.* Environmental epigenetics, 2016. **2**(1): p. dvv013.

125. Kassa, G.M., et al., *Prevalence and determinants of adolescent pregnancy in Africa: a systematic review and Meta-analysis.* Reproductive Health, 2018. **15**(1): p. 195.

126. Kiely, M.E., et al., *Vitamin D status is associated with uteroplacental dysfunction indicated by pre-eclampsia and small-for-gestational-age birth in a large prospective pregnancy cohort in Ireland with low vitamin D status.* Am J Clin Nutr, 2016. **104**(2): p. 354-61.

127. Kim, C.H., *A functional relay from progesterone to vitamin D in the immune system.* DNA Cell Biol, 2015. **34**(6): p. 379-82.

128. Kimpen, J., et al., *Cord blood IgE and month of birth.* Arch Dis Child, 1987. **62**(5): p. 478-82.

129. King, C.L., et al., *Immunologic tolerance in lymphatic filariasis. Diminished parasite-specific T and B lymphocyte precursor frequency in the microfilaremic state.* J Clin Invest, 1992. **89**(5): p. 1403-10.

130. King, C.L., et al., *B cell sensitization to helminthic infection develops in utero in humans.* J Immunol, 1998. **160**(7): p. 3578-84.

131. Kjetland, E.F., et al., *Association between genital schistosomiasis and HIV in rural Zimbabwean women.* Aids, 2006. **20**(4): p. 593-600.

132. Knopp, S., et al., *Sensitivity and Specificity of a Urine Circulating Anodic Antigen Test for the Diagnosis of Schistosoma haematobium in Low Endemic Settings.* PLoS Negl Trop Dis, 2015. **9**(5): p. e0003752.

133. Koucky, M., et al., *Low levels of circulating T-regulatory lymphocytes and short cervical length are associated with preterm labor.* J Reprod Immunol, 2014. **106**: p. 110-7.

134. Kovacs, C.S., *Vitamin D in pregnancy and lactation: maternal, fetal, and neonatal outcomes from human and animal studies.* Am J Clin Nutr, 2008. **88**(2): p. 520s-528s.

135. Kovacs, C.S. and H.M. Kronenberg, *Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation.* Endocr Rev, 1997. **18**(6): p. 832-72.

136. Kremsner, P.G., et al., *Circulating anodic and cathodic antigen in serum and urine from Schistosoma haematobium-infected Cameroonian children receiving praziquantel: a longitudinal study.* Clin Infect Dis, 1994. **18**(3): p. 408-13.

137. Kulkarni, A., et al., *Effects of altered maternal folic acid, vitamin B12 and docosahexaenoic acid on placental global DNA methylation patterns in Wistar rats.* PLoS One, 2011. **6**(3): p. e17706.

138. Kurth, F., et al., *Adolescence as risk factor for adverse pregnancy outcome in Central Africa--a cross-sectional study.* PLoS One, 2010. **5**(12): p. e14367.

139. Kurtis, J.D., et al., *Maternal Schistosomiasis japonica is associated with maternal, placental, and fetal inflammation.* Infect Immun, 2011. **79**(3): p. 1254-61.

140. Lacorcia, M. and C.U. Prazeres da Costa, *Maternal Schistosomiasis: Immunomodulatory Effects With Lasting Impact on Allergy and Vaccine Responses.* Front Immunol, 2018. **9**: p. 2960.

141. Lamb, E.W., et al., *Blood fluke exploitation of non-cognate CD4+ T cell help to facilitate parasite development.* PLoS Pathog, 2010. **6**(4): p. e1000892.

142. Lammie, P.J., et al., *Maternal filarial infection as risk factor for infection in children.* Lancet, 1991. **337**(8748): p. 1005-6.

143. Lee, J.H., J.P. Lydon, and C.H. Kim, *Progesterone suppresses the mTOR pathway and promotes generation of induced regulatory T cells with increased stability.* Eur J Immunol, 2012. **42**(10): p. 2683-96.

144. Lee, J.H., et al., *Progesterone promotes differentiation of human cord blood fetal T cells into T regulatory cells but suppresses their differentiation into Th17 cells.* J Immunol, 2011. **187**(4): p. 1778-87.

145. Leenstra, T., et al., *Schistosoma japonicum reinfection after praziquantel treatment causes anemia associated with inflammation.* Infect Immun, 2006. **74**(11): p. 6398-407.

146. Lerchbaum, E. and B. Obermayer-Pietsch, *Vitamin D and fertility: a systematic review.* Eur J Endocrinol, 2012. **166**(5): p. 765-78.

147. Lewert, R.M. and S. Mandlowitz, *Schistosomiasis: prenatal induction of tolerance to antigens.* Nature, 1969. **224**(5223): p. 1029-30.

148. Lewis, S., et al., *Vitamin D deficiency and pregnancy: from preconception to birth.* Molecular Nutrition and Food Research, 2010. **54**: p. 1092-1102.

149. Liu, N.Q., et al., *Vitamin D and the regulation of placental inflammation.* J Immunol, 2011. **186**(10): p. 5968-74.

150. Liu, P.T., et al., *Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response.* Science, 2006. **311**(5768): p. 1770-3.

151. Lockett, G.A., J. Huoman, and J.W. Holloway, *Does allergy begin in utero?* Pediatric Allergy and Immunology, 2015. **26**(5): p. 394-402.

152. Logan, C.A., et al., *Delivery Mode, Duration of Labor, and Cord Blood Adiponectin, Leptin, and C-Reactive Protein: Results of the Population-Based Ulm Birth Cohort Studies.* PloS one, 2016. **11**(2): p. e0149918-e0149918.

153. Lundy, S.K. and N.W. Lukacs, *Chronic schistosome infection leads to modulation of granuloma formation and systemic immune suppression.* Front Immunol, 2013. **4**: p. 39.

154. Luxwolda, M.F., et al., *Traditionally living populations in East Africa have a mean serum 25-hydroxyvitamin D concentration of 115 nmol/l.* Br J Nutr, 2012. **108**(9): p. 1557-61.

155. Luxwolda, M.F., et al., *Vitamin D status indicators in indigenous populations in East Africa.* Eur J Nutr, 2013. **52**(3): p. 1115-25.

156. Maghbooli, Z., et al., *Correlation between vitamin D3 deficiency and insulin resistance in pregnancy.* Diabetes/Metabolism Research and Reviews, 2008. **24**: p. 27-32.

157. Mahanty, S., et al., *High levels of spontaneous and parasite antigen-driven interleukin-10 production are associated with antigen-specific hyporesponsiveness in human lymphatic filariasis.* J Infect Dis, 1996. **173**(3): p. 769-73.

158. Maizels, R.M. and R.A. Lawrence, *Immunological tolerance: The key feature in human filariasis?* Parasitol Today, 1991. **7**(10): p. 271-6.

159. Maizels, R.M. and H.J. McSorley, *Regulation of the host immune system by helminth parasites.* J Allergy Clin Immunol, 2016. **138**(3): p. 666-675.

160. Maizels, R.M. and K.A. Smith, *Regulatory T cells in infection.* Adv Immunol, 2011. **112**: p. 73-136.

161. Malhotra, I., et al., *Can prenatal malaria exposure produce an immune tolerant phenotype? A prospective birth cohort study in Kenya.* PLoS medicine, 2009. **6**(7): p. e1000116-e1000116.

162. Malhotra, I., et al., *In utero exposure to helminth and mycobacterial antigens generates cytokine responses similar to that observed in adults.* J Clin Invest, 1997. **99**(7): p. 1759-66.

163. Mark, P., et al., *A maternal high-fat diet in rat pregnancy reduces growth of the fetus and the placental junctional zone, but not placental labyrinth zone growth.* Journal of Developmental Origins of Health and Disease, 2011. **2**(1): p. 63-70.

164. Masuda, S. and G. Jones, *Promise of vitamin D analogues in the treatment of hyperproliferative conditions.* Mol Cancer Ther, 2006. **5**(4): p. 797-808.

165. Matsui, E.C., et al., *Mouse allergen-specific immunoglobulin G and immunoglobulin G4 and allergic symptoms in immunoglobulin E-sensitized laboratory animal workers.* Clin Exp Allergy, 2005. **35**(10): p. 1347-53.

166. Mauri, C. and M. Menon, *The expanding family of regulatory B cells.* International immunology, 2015. **27**(10): p. 479-486.

167. McDonald, E.A., et al., *Schistosome egg antigens elicit a proinflammatory response by trophoblast cells of the human placenta.* Infect Immun, 2013. **81**(3): p. 704-12.

168. McDonald, E.A., et al., *Schistosomiasis japonica during pregnancy is associated with elevated endotoxin levels in maternal and placental compartments.* J Infect Dis, 2014. **209**(3): p. 468-72.

169. McGarvey, S.T., et al., *Schistosomiasis japonica and childhood nutritional status in northeastern Leyte, the Philippines: a randomized trial of praziquantel versus placebo.* Am J Trop Med Hyg, 1996. **54**(5): p. 498-502.

170. McGowan, E.C., et al., *Placental IL-10 Dysregulation and Association With Bronchopulmonary Dysplasia Risk.* Pediatric Research, 2009. **66**(4): p. 455-460.

171. McGrath, J., *Does 'imprinting'with low prenatal vitamin D contribute to the risk of various adult disorders?* Medical Hypotheses, 2001. **56**: p. 367-371.

172. McLean, A.R.D., et al., *P. falciparum infection and maternofetal antibody transfer in malaria-endemic settings of varying transmission.* PLoS One, 2017. **12**(10): p. e0186577.

173. McManus, D.P., et al., *Schistosomiasis.* Nat Rev Dis Primers, 2018. **4**(1): p. 13.

174. Mehta, R.S., et al., *Maternal geohelminth infections are associated with an increased susceptibility to geohelminth infection in children: a case-control study.* PLoS Negl Trop Dis, 2012. **6**(7): p. e1753.

175. Merino, F., et al., *Regulation of natural killer cytotoxicity by 1,25-dihydroxyvitamin D3.* Cell Immunol, 1989. **118**(2): p. 328-36.

176. Miyaura, H. and M. Iwata, *Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids.* J Immunol, 2002. **168**(3): p. 1087-94.

177. Mojibian, M., et al., *The effects of vitamin D supplementation on maternal and neonatal outcome: A randomized clinical trial.* Iranian journal of reproductive medicine, 2015. **13**(11): p. 687-696.

178. Mombo-Ngoma, G., et al., *Urogenital schistosomiasis during pregnancy is associated with low birth weight delivery: analysis of a prospective cohort of pregnant women and their offspring in Gabon.* Int J Parasitol, 2017. **47**(1): p. 69-74.

179. Mombo-Ngoma, G., et al., *Young adolescent girls are at high risk for adverse pregnancy outcomes in sub-Saharan Africa: an observational multicountry study.* BMJ Open, 2016. **6**(6): p. e011783.

180. Montenegro, S.M., et al., *Cytokine production in acute versus chronic human Schistosomiasis mansoni: the cross-regulatory role of interferon-gamma and interleukin-10 in the responses of peripheral blood mononuclear cells and splenocytes to parasite antigens.* J Infect Dis, 1999. **179**(6): p. 1502-14.

181. Montesano, M.A., et al., *Neonatal idiotypic exposure alters subsequent cytokine, pathology, and survival patterns in experimental Schistosoma mansoni infections.* J Exp Med, 1999. **189**(4): p. 637-45.

182. Moormann, A.M., et al., *Malaria and pregnancy: placental cytokine expression and its relationship to intrauterine growth retardation.* J Infect Dis, 1999. **180**(6): p. 1987-93.

183. Mora, J.R., M. Iwata, and U.H. von Andrian, *Vitamin effects on the immune system: vitamins A and D take centre stage.* Nat Rev Immunol, 2008. **8**(9): p. 685-98.

184. Mpairwe, H., R. Tweyongyere, and A. Elliott, *Pregnancy and helminth infections.* Parasite Immunol, 2014. **36**(8): p. 328-37.

185. mundi, I. *Gabon - Prevalence of anemia*. [cited 2020 2020 April 10]; Available from: https://www.indexmundi.com/facts/gabon/prevalence-of-anemia.

186. Murphy, K. and C. Weaver, *Janeway's immunobiology*. 2017.

187. Murphy, S.P., et al., *Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice.* J Immunol, 2005. **175**(6): p. 4084-90.

188. Murphy, S.P., et al., *Interferon gamma in successful pregnancies.* Biol Reprod, 2009. **80**(5): p. 848-59.

189. Mutapi, F., et al., *Schistosome infection intensity is inversely related to auto-reactive antibody levels.* PLoS One, 2011. **6**(5): p. e19149.

190. Nagpal, S., S. Na, and R. Rathnachalam, *Noncalcemic actions of vitamin D receptor ligands.* Endocr Rev, 2005. **26**(5): p. 662-87.

191. Nakashima, A., et al., *The balance of the immune system between T cells and NK cells in miscarriage.* Am J Reprod Immunol, 2012. **67**(4): p. 304-10.

192. Nash, T.E., et al., *Schistosome infections in humans: perspectives and recent findings. NIH conference.* Ann Intern Med, 1982. **97**(5): p. 740-54.

193. news, G. *Présentation du Moyen-Ogooué : Lambaréné, ville mondialement connue à travers l'hôpital du célèbre Docteur Albert Scweitzer*. 2010 2010 March 10 [cited 2020 2020 April 11]; Available from: http://www.bdpgabon.org/articles/2010/03/10/presentation-du-moyen-ogooue-lambarene-ville-mondialement-connue-a-travers-lhopital-du-celebre-docteur-albert-scweitzer/#.XpF32G6kI_V.

194. Norman, A.W. and R. Bouillon, *Vitamin D nutritional policy needs a vision for the future.* Exp Biol Med (Maywood), 2010. **235**(9): p. 1034-45.

195. Novakovic, B., et al., *Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface.* J Biol Chem, 2009. **284**(22): p. 14838-48.

196. Novato-Silva, E., G. Gazzinelli, and D.G. Colley, *Immune responses during human schistosomiasis mansoni. XVIII. Immunologic status of pregnant women and their neonates.* Scand J Immunol, 1992. **35**(4): p. 429-37.

197. Ntostis, P., et al., *Genetic variation in the HSD3B1 gene and recurrent spontaneous abortions.* J Matern Fetal Neonatal Med, 2012. **25**(4): p. 408-10.

198. Nunn, J.D., et al., *Regulation of human tonsillar T-cell proliferation by the active metabolite of vitamin D3.* Immunology, 1986. **59**(4): p. 479-84.

199. Nutman, T.B., V. Kumaraswami, and E.A. Ottesen, *Parasite-specific anergy in human filariasis. Insights after analysis of parasite antigen-driven lymphokine production.* J Clin Invest, 1987. **79**(5): p. 1516-23.

200. Olveda, D.U., et al., *The chronic enteropathogenic disease schistosomiasis.* Int J Infect Dis, 2014. **28**: p. 193-203.

201. Organisation, W.H. *Worldwide prevalence of anaemia1993–2005*. 2008 [cited 2019 2 of June 2019]; Available from: https://apps.who.int/iris/bitstream/handle/10665/43894/9789241596657_eng.pdf?sequence=1.

202. Organisation, W.H. *Schistosomiasis: Progress report 2001 - 2011, strategic plan 2012 - 2020*. 2013 [cited 2019 14.05.2019]; Available from: https://apps.who.int/iris/handle/10665/78074.

203. Osada, Y., et al., *The antibody responses to adult-worm antigens of Schistosoma haematobium, among infected and resistant individuals from an endemic community in southern Ghana.* Ann Trop Med Parasitol, 2003. **97**(8): p. 817-26.

204. Othman, A.A., et al., *Congenital exposure to Schistosoma mansoni infection: impact on the future immune response and the disease outcome.* Immunobiology, 2010. **215**(2): p. 101-12.

205. Palmeira, P., et al., *IgG placental transfer in healthy and pathological pregnancies.* Clin Dev Immunol, 2012. **2012**: p. 985646.

206. Patel, B., et al., *Role of nuclear progesterone receptor isoforms in uterine pathophysiology.* Human reproduction update, 2015. **21**(2): p. 155-173.

207. Pearce, E.J. and A.S. MacDonald, *The immunobiology of schistosomiasis.* Nat Rev Immunol, 2002. **2**(7): p. 499-511.

208. Penna, G. and L. Adorini, *1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation.* J Immunol, 2000. **164**(5): p. 2405-11.

209. Perry, G.S., et al., *Iron nutrition does not account for the hemoglobin differences between blacks and whites.* J Nutr, 1992. **122**(7): p. 1417-24.

210. Peterson, R.A., *Regulatory T-Cells: Diverse Phenotypes Integral to Immune Homeostasis and Suppression.* Toxicologic Pathology, 2012. **40**(2): p. 186-204.

211. Pidoux, G., et al., *Large variability of trophoblast gene expression within and between human normal term placentae.* Placenta, 2004. **25**(5): p. 469-73.

212. Piemonti, L., et al., *Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells.* J Immunol, 2000. **164**(9): p. 4443-51.

213. Plum, L.A. and H.F. DeLuca, *Vitamin D, disease and therapeutic opportunities.* Nat Rev Drug Discov, 2010. **9**(12): p. 941-55.

214. Prietl, B., et al., *Vitamin D and immune function.* Nutrients, 2013. **5**(7): p. 2502-21.

215. Purwar, M., et al., *Calcium supplementation and prevention of pregnancy induced hypertension.* J Obstet Gynaecol Res, 1996. **22**(5): p. 425-30.

216. Qiu, S., et al., *Schistosoma japonicum infection downregulates house dust mite-induced allergic airway inflammation in mice.* PLoS One, 2017. **12**(6): p. e0179565.

217. Rafiee, M., et al., *Vitamin D3 induces the expression of membrane progesterone receptors (mPRs) on naive CD4(+) T lymphocyte cells in women of reproductive age.* Int Immunopharmacol, 2019. **72**: p. 55-61.

218. report, G.n. *Gabon Nutrition Profile*. Global nutrition report 2020 [cited 2020 2020 April 10]; Available from: https://globalnutritionreport.org/resources/nutrition-profiles/africa/middle-africa/gabon/.

219. Reynolds, L.A. and B.B. Finlay, *Early life factors that affect allergy development.* Nat Rev Immunol, 2017. **17**(8): p. 518-528.

220. Roberts, C.T., et al., *Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig.* Placenta, 2001. **22**(2-3): p. 177-85.

221. Robertson, S.A. and L.M. Moldenhauer, *Immunological determinants of implantation success.* Int J Dev Biol, 2014. **58**(2-4): p. 205-17.

222. Rojansky, N., A. Brzezinski, and J.G. Schenker, *Seasonality in human reproduction: an update.* Hum Reprod, 1992. **7**(6): p. 735-45.

223. Romero, R., et al., *Vaginal progesterone for preventing preterm birth and adverse perinatal outcomes in singleton gestations with a short cervix: a meta-analysis of individual patient data.* American journal of obstetrics and gynecology, 2018. **218**(2): p. 161-180.

224. Romero, R., et al., *Progesterone to prevent spontaneous preterm birth.* Seminars in fetal & neonatal medicine, 2014. **19**(1): p. 15-26.

225. Ron, M., et al., *Transfer of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 across the perfused human placenta.* Am J Obstet Gynecol, 1984. **148**(4): p. 370-4.

226. Rook, G.A., et al., *Vitamin D3, gamma interferon, and control of proliferation of Mycobacterium tuberculosis by human monocytes.* Immunology, 1986. **57**(1): p. 159-63.

227. Ross, A.G., et al., *Katayama syndrome.* Lancet Infect Dis, 2007. **7**(3): p. 218-24.

228. Roth, I., et al., *Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10.* J Exp Med, 1996. **184**(2): p. 539-48.

229. Rueda-Clausen, C.F., et al., *Effect of prenatal hypoxia in transgenic mouse models of preeclampsia and fetal growth restriction.* Reprod Sci, 2014. **21**(4): p. 492-502.

230. Rujeni, N., et al., *Atopy is inversely related to schistosome infection intensity: a comparative study in Zimbabwean villages with distinct levels of Schistosoma haematobium infection.* Int Arch Allergy Immunol, 2012. **158**(3): p. 288-98.

231. Saji, F., et al., *Dynamics of immunoglobulins at the feto-maternal interface.* Rev Reprod, 1999. **4**(2): p. 81-9.

232. Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance.* Cell, 2000. **101**(5): p. 455-8.

233. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.

234. Salle, B.L., et al., *Perinatal metabolism of vitamin D.* Am J Clin Nutr, 2000. **71**(5 Suppl): p. 1317s-24s.

235. Sanin, D.E., et al., *Helminth Infection and Commensal Microbiota Drive Early IL-10 Production in the Skin by CD4+ T Cells That Are Functionally Suppressive.* PLoS Pathog, 2015. **11**(5): p. e1004841.

236. Sartono, E., et al., *Specific T cell unresponsiveness in human filariasis: diversity in underlying mechanisms.* Parasite Immunol, 1995. **17**(11): p. 587-94.

237. Sasaki, Y., et al., *Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia.* Clin Exp Immunol, 2007. **149**(1): p. 139-45.

238. Saunders, N., R.A. Wilson, and P.S. Coulson, *The outer bilayer of the adult schistosome tegument surface has a low turnover rate in vitro and in vivo.* Mol Biochem Parasitol, 1987. **25**(2): p. 123-31.

239. Schleicher, R.L., et al., *The vitamin D status of the US population from 1988 to 2010 using standardized serum concentrations of 25-hydroxyvitamin D shows recent modest increases.* Am J Clin Nutr, 2016. **104**(2): p. 454-61.

240. Schmiedel, Y., et al., *CD4+CD25hiFOXP3+ Regulatory T Cells and Cytokine Responses in Human Schistosomiasis before and after Treatment with Praziquantel.* PLoS neglected tropical diseases, 2015. **9**(8): p. e0003995-e0003995.

241. Schulz, L.C., et al., *Leptin and the placental response to maternal food restriction during early pregnancy in mice.* Biol Reprod, 2012. **87**(5): p. 120.

242. Seely, E.W., *Calciotropic hormones in preeclampsia: a renewal of interest.* J Clin Endocrinol Metab, 2007. **92**(9): p. 3402-3.

243. Seydel, L.S., et al., *Association of in utero sensitization to Schistosoma haematobium with enhanced cord blood IgE and increased frequencies of CD5- B cells in African newborns.* Am J Trop Med Hyg, 2012. **86**(4): p. 613-9.

244. Sferruzzi-Perri, A.N. and E.J. Camm, *The Programming Power of the Placenta.* Frontiers in physiology, 2016. **7**: p. 33-33.

245. Shin, J.S., et al., *Vitamin D effects on pregnancy and the placenta.* Placenta, 2010. **31**(12): p. 1027-34.

246. Simard, J., et al., *Molecular biology and genetics of the 3 beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family.* J Endocrinol, 1996. **150 Suppl**: p. S189-207.

247. Skeffington, K.L., et al., *Hypoxia, AMPK activation and uterine artery vasoreactivity.* J Physiol, 2016. **594**(5): p. 1357-69.

248. Slator, P.J., et al., *Placenta microstructure and microcirculation imaging with diffusion MRI.* Magn Reson Med, 2018. **80**(2): p. 756-766.

249. Snary, D., M.A. Smith, and J.A. Clegg, *Surface proteins of Schistosoma mansoni and their expression during morphogenesis.* Eur J Immunol, 1980. **10**(7): p. 573-5.

250. Steel, C., et al., *Long-term effect of prenatal exposure to maternal microfilaraemia on immune responsiveness to filarial parasite antigens.* Lancet, 1994. **343**(8902): p. 890-3.

251. Stephanou, A., R. Ross, and S. Handwerger, *Regulation of human placental lactogen expression by 1,25-dihydroxyvitamin D3.* Endocrinology, 1994. **135**(6): p. 2651-6.

252. Straubinger, K., et al., *Maternal immune response to helminth infection during pregnancy determines offspring susceptibility to allergic airway inflammation.* J Allergy Clin Immunol, 2014. **134**(6): p. 1271-1279 e10.

253. Suguitan, A.L., Jr., et al., *Malaria-associated cytokine changes in the placenta of women with pre-term deliveries in Yaounde, Cameroon.* Am J Trop Med Hyg, 2003. **69**(6): p. 574-81.

254. Svensson-Arvelund, J., et al., *The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages.* J Immunol, 2015. **194**(4): p. 1534-44.

255. Thangamani, S., et al., *Cutting edge: progesterone directly upregulates vitamin d receptor gene expression for efficient regulation of T cells by calcitriol.* J Immunol, 2015. **194**(3): p. 883-6.

256. Thaxton, J.E., R. Romero, and S. Sharma, *TLR9 activation coupled to IL-10 deficiency induces adverse pregnancy outcomes.* J Immunol, 2009. **183**(2): p. 1144-54.

257. Thaxton, J.E. and S. Sharma, *Interleukin-10: a multi-faceted agent of pregnancy.* Am J Reprod Immunol, 2010. **63**(6): p. 482-91.

258. ThoughtCo. *The Role of T Cells in the Body* 2019 August 06, 2019 [cited 2019 December 11, 2019]; Available from: https://www.thoughtco.com/t-cells-meaning-373354.

259. Trampert, D.C., et al., *On the role of IgG4 in inflammatory conditions: lessons for IgG4-related disease.* Biochim Biophys Acta Mol Basis Dis, 2018. **1864**(4 Pt B): p. 1401-1409.

260. Turner, J.D., et al., *Allergen-specific IgE and IgG4 are markers of resistance and susceptibility in a human intestinal nematode infection.* Microbes Infect, 2005. **7**(7-8): p. 990-6.

261. Tzschoppe, A.A., et al., *Differences in gene expression dependent on sampling site in placental tissue of fetuses with intrauterine growth restriction.* Placenta, 2010. **31**(3): p. 178-85.

262. van Dam, G.J., et al., *A robust dry reagent lateral flow assay for diagnosis of active schistosomiasis by detection of Schistosoma circulating anodic antigen.* Exp Parasitol, 2013. **135**(2): p. 274-82.

263. van Delft, F., et al., *Cough and alterations in semen after a tropical swim.* Neth J Med, 2007. **65**(8): p. 304-6.

264. van den Biggelaar, A.H., et al., *Immune responses induced by repeated treatment do not result in protective immunity to Schistosoma haematobium: interleukin (IL)-5 and IL-10 responses.* J Infect Dis, 2002. **186**(10): p. 1474-82.

265. van den Biggelaar, A.H., et al., *Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren.* J Infect Dis, 2004. **189**(5): p. 892-900.

266. van den Elsen, P.J., et al., *Lack of CIITA expression is central to the absence of antigen presentation functions of trophoblast cells and is caused by methylation of the IFN-gamma inducible promoter (PIV) of CIITA.* Hum Immunol, 2000. **61**(9): p. 850-62.

267. van der Vlugt, L., et al., *Schistosome-induced pulmonary B cells inhibit allergic airway inflammation and display a reduced Th2-driving function.* Int J Parasitol, 2017. **47**(9): p. 545-554.

268. van Lieshout, L., A.M. Polderman, and A.M. Deelder, *Immunodiagnosis of schistosomiasis by determination of the circulating antigens CAA and CCA, in particular in individuals with recent or light infections.* Acta Trop, 2000. **77**(1): p. 69-80.

269. van Riet, E., F.C. Hartgers, and M. Yazdanbakhsh, *Chronic helminth infections induce immunomodulation: consequences and mechanisms.* Immunobiology, 2007. **212**(6): p. 475-90.

270. viajarseguro.org. *Gabón*. 2019 January 2019 [cited 2019 01-December-2019]; Available from: http://fundacionio.org/viajar/paises/africa/gabon%20alertas.html.

271. Vijayendra Chary, A., et al., *Vitamin D deficiency in pregnant women impairs regulatory T cell function.* J Steroid Biochem Mol Biol, 2015. **147**: p. 48-55.

272. von Essen, M.R. and C. Geisler, *VDR, the Vitamin D Receptor*, in *Encyclopedia of Signaling Molecules*, S. Choi, Editor. 2018, Springer International Publishing: Cham. p. 5907-5914.

273. Wacker, M. and M.F. Holick, *Sunlight and Vitamin D: A global perspective for health.* Dermatoendocrinol, 2013. **5**(1): p. 51-108.

274. Wagner, C. and F. Greer, *Prevention of rickets and vitamin D deficiency in infants, children, and adolescents.* Pediatrics, 2008. **122**: p. 1142-1152.

275. Walker, A.J., *Insights into the functional biology of schistosomes.* Parasit Vectors, 2011. **4**: p. 203.

276. Watanabe, K., et al., *T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment.* Am J Trop Med Hyg, 2007. **77**(4): p. 676-82.

277. Weisman, Y., et al., *1 alpha, 25-Dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 in vitro synthesis by human decidua and placenta.* Nature, 1979. **281**(5729): p. 317-9.

278. Wierzejska, R., et al., *Vitamin D Concentration in Maternal and Umbilical Cord Blood by Season.* Int J Environ Res Public Health, 2017. **14**(10).

279. Wikipedia. *Index of Germany-related articles*. 2019 02-November-2019 [cited 2019 01-December-2019]; Available from: https://en.wikipedia.org/wiki/Index_of_Germany-related_articles.

280. Wyatt, S.M., et al., *The correlation between sampling site and gene expression in the term human placenta.* Placenta, 2005. **26**(5): p. 372-9.

281. Yagi, R., J. Zhu, and W.E. Paul, *An updated view on transcription factor GATA3-mediated regulation of Th1 and Th2 cell differentiation.* Int Immunol, 2011. **23**(7): p. 415-20.

282. Yang, C.Y., et al., *The implication of vitamin D and autoimmunity: a comprehensive review.* Clin Rev Allergy Immunol, 2013. **45**(2): p. 217-26.

283. Young, B.E., et al., *Placental vitamin D receptor (VDR) expression is related to neonatal vitamin D status, placental calcium transfer, and fetal bone length in pregnant adolescents.* FASEB J, 2014. **28**(5): p. 2029-37.

284. Youssef, A.F. and F.H. Abdine, *Bilharziasis of the pregnant uterus.* J Obstet Gynaecol Br Emp, 1958. **65**(6): p. 991-3.

285. Youssef, M.A.M., et al., *In neonates with vitamin D deficiency, low lymphocyte activation markers are risk factors for infection.* Paediatr Int Child Health, 2019. **39**(2): p. 111-118.

286. Zahran, A.M., K.M. Zharan, and H.F. Hetta, *Significant correlation between regulatory T cells and vitamin D status in term and preterm labor.* J Reprod Immunol, 2018. **129**: p. 15-22.

287. Zehnder, D., et al., *The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua.* American Journal of Pathology, 2002. **161**: p. 105-114.

288. Zehnder, D., et al., *The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua.* Am J Pathol, 2002. **161**(1): p. 105-14.

289. Zenclussen, A.C., et al., *Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model.* Am J Pathol, 2005. **166**(3): p. 811-22.

290. Zhang, C., et al., *Maternal plasma 25-hydroxyvitamin D concentrations and the risk for gestational diabetes mellitus.* PLoS One, 2008. **3**: p. e3753.

291. Zhu, J., *GATA3 Regulates the Development and Functions of Innate Lymphoid Cell Subsets at Multiple Stages.* Front Immunol, 2017. **8**: p. 1571.