

MALARIA IN PREGNANCY: PHAGOCYTOSIS AND HIV

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Resumo

Para milhões de mulheres grávidas em todo o mundo, o parasita *Plasmodium falciparum* representa uma das maiores ameaças que enfrentam. Infecções com este parasita podem resultar numa condição a que se dá o nome de Malária na Gravidez. Os efeitos da Malária na Gravidez podem ser sentidos pela mulher grávida ou pelo feto. Mulheres que experimentam a sua primeira gravidez estão em maior risco de infecção e consequentemente maior risco de efeitos adversos o que é ainda mais agravado se as mesmas estiverem infectadas com VIH. Em mulheres com mais que uma ou duas gravidezes, a existência de anticorpos contra um tipo de *Plasmodium falciparum* que afecta especialmente mulheres grávidas protege-as desses efeitos negativos.

Nesta tese, eu desenvolvi um novo protocolo que permite avaliar a quantidade e função desses anticorpos e medi esses anticorpos em primigrávidas e secundigrávidas, num contexto de infecção por VIH. Isto permitiu-me descobrir que o VIH afecta a aquisição de anticorpos nas primigrávidas e que estes anticorpos estão associados a um aumento de peso dos fetos. Fui também capaz de demonstrar, pela primeira vez, uma possível associação de IgE com a promoção da fagocitose de parasitas *Plasmodium falciparum*.

Summary

For millions of pregnant women all over the world the *Plasmodium falciparum* parasite represents one of the biggest threats they face. Infections with this parasite can result in a condition called Malaria in Pregnancy. The Malaria in Pregnancy effects can be felt by the pregnant women or by the foetus. Women in their first pregnancies are at highest risk of infection and as a result more prone to experience adverse effects, which can be made worse if they also exhibit an HIV infection. In women with already one or more pregnancies, the existence of antibodies against a type of *Plasmodium falciparum* that mainly infects pregnant women protects them from Malaria in Pregnancy.

In this thesis I have developed a new assay that allows the quantification and assessment of phagocytic function of antibodies against those parasites. I have measured these antibodies in primigravidae and secundigravidae in the context of HIV infection. That allowed me to find that in primigravidae HIV primarily impacts on the acquisition of the antibodies and that in secundigravidae these antibodies are associated with a higher birth weight of the babies. I also demonstrated, for the first time, a possible involvement of IgE in the induction of phagocytosis of *Plasmodium falciparum* parasites

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Abbreviations used in this thesis

ADCC	Antibody Dependent Cell Cytotoxicity
ATS	Acidic Terminal Sequence
CD#	Cluster of Differentiation #
CIDR	Cysteine-rich Interdomain Region
CM	Cerebral Malaria
Coeff	Linear Regression Coefficient
CR	complement receptor
CS2 _{VSA}	Variant Surface Antigens of the parasite line CS2
CSA	Chondroitin Sulphate A
CSPG	Chondroitin sulphate proteoglycan
CytoD	Cytochalasin-D
DBL	Duffy-Binding Like
DDT	dichlorodiphenyltrichloroethane
dThp-1	differentiated Thp-1
ELISA	enzyme-linked immuno-sorbent assay
EtBr	Ethidium Bromide
FACS	Flowrescence-activated cell sorting
FBS	Foetal Bovine Serum
Fc	fragment cristallizable
FcBlock	FcR blocking agent
FcR	Fc-receptors

GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
Hb	Haemoglobin
HIV	Human immunodeficiency virus
HP	High phagocytosis
ICAM	Intracellular Adhesion Molecule - 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTp	intermittent preventive treatment during pregnancy
ITN	insecticide-treated bed-nets
LBW	low birth weight
LP	Low phagocytosis
LR	Linear Regression
MDM	monocyte-derived macrophages
MG	Multigravidae
MiP	Malaria in Pregnancy
MIP	macrophage inflammatory protein
NG	Nulligravidae
NK	Natural Killer
P.	Plasmodium
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffer Saline
PfEMP-1	<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein-1

PG	Primigravidae
Phago atb	Phagocytic antibodies
PM	Placental Malaria
PMA	phorbol-12-myristate-13-acetate
PPS	Patient Pooled Serum
pRBC	parasitised RBC
PRR	pattern recognition receptors
PVM	Parasitophorous vacuole membrane
RaH	polyclonal rabbit anti-human RBC IgG antibody
RBC	Red Blood Cell
RESA	ring-infected erythrocyte surface antigen
rif	repetitive interspersed family
RPMI	Roswell Park Memorial Institute Medium
RPMI- HEPES	RPMI-2-N-hydroxyethylpiperazin-N-2-ethane sulphonic acid
RT	Room temperature
SA	Severe Anaemia
SDS	Sodium Dodecyl Sulphate
SG	Secundigravidae
SP	sulphadoxine-pyrimethamine
stevor	subtelomeric variable open reading frame
TBS-T	Tris Buffer Saline – Tween
TLR	Toll-like Receptors
TNF	tumour necrosis factor

TPA	12-O-tetradecanoylphorbol-13-acetate
TTM	TBS-T with Milk
uThp-1	undifferentiated Thp-1
Vitamin D3	1 alpha,25-dihydroxyvitamin D3
VSA	Variant Surface antigens
	Variant Surface Antigens which mediate the binding to
VSA _{CSA}	CSA
WHO	World Health Organisation

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1. Introduction to the work and structure presented in this thesis

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1.1. Background to the study

For millions of people living in tropical and sub-tropical areas, and mainly children under the age of five years old and pregnant women (particularly women experiencing their first pregnancy, *i.e.* primigravidae), malaria is a reality that often results in adverse clinical outcomes and even death. Although women (as do most adults in endemic areas) are able to develop a clinical immunity against this disease, this immunity does not seem to protect them once they become pregnant. This has been an observation that has puzzled malariologists and immunologists for decades. The discovery of malaria-in-pregnancy (MiP) type parasites and specific placental receptors that enable these parasites to bind to that organ generated a burst of information in the last decade or so that is beginning to paint a clearer image of how this parasite, a protozoan of the genus *Plasmodium*, causes the disease in pregnant women. Although efforts are currently in place to develop a vaccine that could prevent the majority of this burden, the development of immunity against the parasite is still poorly understood and that impacts on the success (or lack of it) that these efforts have achieved. This of course takes place in areas where, together with so many other maladies, HIV infection is a major health concern and inevitably influences immune responses.

1.2. Aims and scope

The majority of efforts aiming at understanding the immune response and development of immunity to the malaria parasite focus on two major areas: cellular immunity and humoral immunity. This thesis focuses mainly on the humoral immunity that is developed against the malaria parasite by primigravidae and comprised the following general aims:

- Understand the impact of HIV on the development of immunity in primigravid women from a malaria endemic area according to their MiP status;
- Identify the relative importance of antibodies that contribute to the process of phagocytosis in these women as compared with the total levels of antibodies;
- Evaluate the possible role of IgE as a phagocytosis mediator in primigravidae.

In order to do this, I developed a new method of evaluating the presence of antibodies that contribute to the phagocytosis process in the sera of malaria exposed individuals.

1.3. Overview and structure of the thesis

This thesis is divided into 5 chapters and 2 appendixes. In Chapter 1 (the present chapter) an overview of the rationale and structure behind this work is presented. In Chapter 2 an

introduction to the problem that malaria poses and to its respective pathogen's life cycle is given along with a general introduction to the problem of Malaria in Pregnancy. Chapters 3-5 present the work and results obtained for the development of the phagocytosis assay, the application of it to a cohort of primigravidae and secundigravidae and early work in the search for an evidence for IgE as an immunoglobulin that may contribute to phagocytosis of *Plasmodium-falciparum* infected red blood cells, respectively.

Each one of those chapters is divided into Introduction, Methods, Results, Discussion and References. Finally, Appendix 1 includes the publications that have arisen from the work presented in this thesis and the work done with collaborators during the completion of this thesis. Appendix 2 includes a *curriculum vitae* at the date of publication of this thesis.

2. Malaria: a general introduction

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2.1. A global problem

Malaria is one of mankind's oldest known diseases. The name malaria derives from the Italian 'Mal aria' which literally translates as 'bad air', since the disease was associated with the bad vapours that emanated from swamps and marshes

The agent that causes this disease is a unicellular apicomplexan parasite of the genus *Plasmodium*. There are 5 species of *Plasmodium* that have humans as hosts: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. All of these species share common characteristics in their life cycles and use *Anopheles* mosquitoes as vectors.

According to the World Health Organization, about 50% of the world's population, mainly in tropical and sub-tropical regions of the globe, is exposed to the disease (Figure 1). This resulted, in 2008, in 243 million cases of malaria worldwide and 863,000 deaths; of these deaths >80% occurred in sub-Saharan Africa and they were, almost exclusively, caused by the most virulent species of the parasite, *P. falciparum* [1].

Malaria represents not only a health problem, but it impacts greatly on the social, political and economic components of the endemic countries. That the countries where malaria is endemic and a major health issue have generally poor economies and are politically unstable seems no coincidence. Although major efforts have been put forward in the last 60 years to eliminate this disease, the latest of which representing an enormous investment in both vaccine development and vector elimination, and until a greater international effort is put in place in order to increase political stability and lessen general poverty indexes in endemic countries, I believe *Plasmodium sp.* are here to stay.

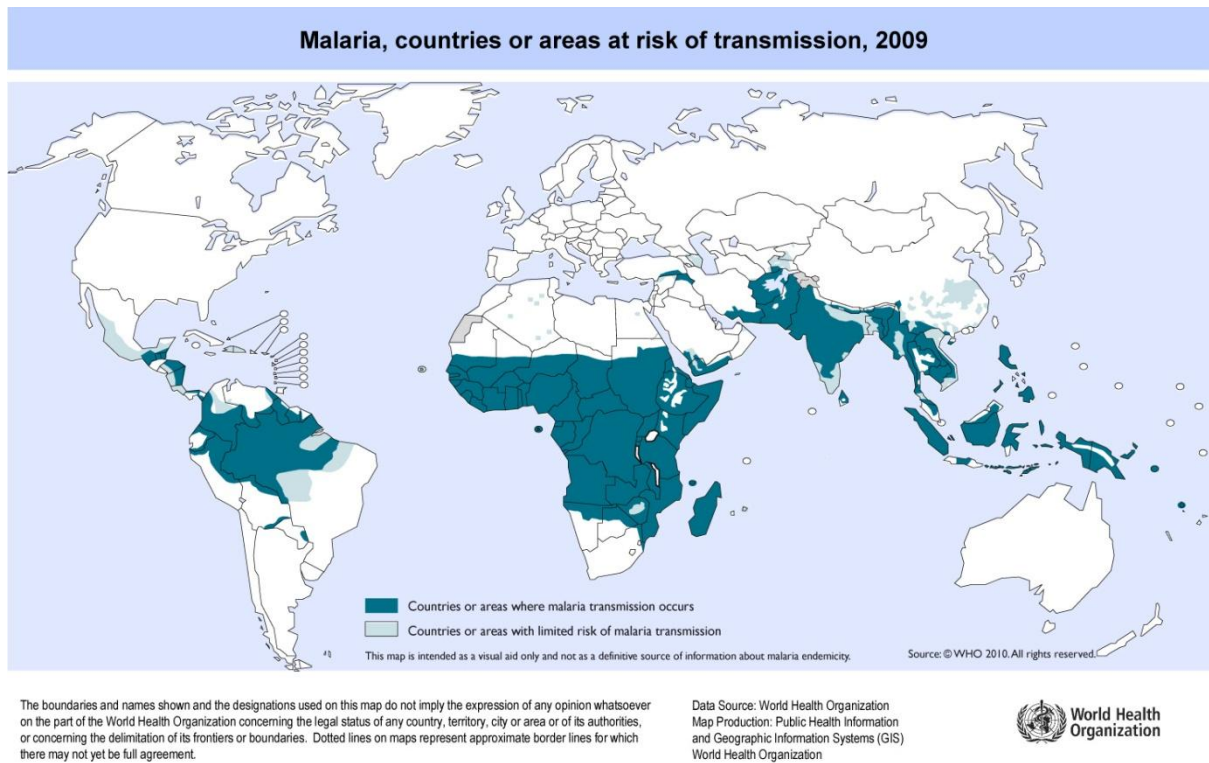


Figure 2-1– World distribution of malaria.

The distribution of malaria is clustered around the tropical and sub-tropical regions of the globe. As shown on (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_ITHRiskMap.JPG) as of November 2010.

2.2. The Plasmodium life cycle

The life cycle of all *Plasmodium* species that infect mammals is very similar and retains extremely conserved morphological and developmental features (minor exceptions are the existence of latent parasite stages, called hypnozoites, in *P. vivax* and, possibly *P. ovale* [2]) (Figure 2-2) . This complex life cycle comprises stages in a vector (mosquito) and a host (mammal) where 2 stages can be identified: an exo-erythrocytic stage, in the liver of the host, and an erythrocytic stage (blood-stage) that follows. This provides two (known) cell types where these parasites can replicate: nucleated hepatocytes and non-nucleated red blood cells. *Plasmodium* species that infect birds and reptiles differ in that they can infect and multiply in multiple tissues and cell types within their host [3, 4].

2.2.1. Human host

The life cycle of *Plasmodium* species that infect humans begins when a female *Anopheles* mosquito, carrying *Plasmodium* in its salivary glands, takes a blood-meal on a human. Upon deposition in the skin, *Plasmodium* sporozoites start migrating in search for and entering blood capillaries [5]. The parasites then travel to (or are most probably taken to) the liver where a unique combination of matrix proteoglycans seems to provide the first signal for their arrest [6, 7]. After their arrest, sporozoites need to find their way into the liver. Studies using mouse models of malaria, suggest that they use Kupffer cells (resident liver macrophages [8]) to do so [9, 10]. Once in the liver, sporozoites traverse several hepatocytes before invading and settling themselves in a final one [11, 12]. Once inside their final hepatocytes, sporozoites start to differentiate and eventually form merozoites. Up to 30,000 merozoites can be generated by a single sporozoite [13]. These merozoites are then released from the liver and into the blood circulation through the budding of parasite-filled vesicles called merozoites (whose membranes are of hepatic origin) [14, 15]. This is believed to allow the parasite's evasion of the host's immune system [16].

Once in the blood stream, merozoites are released from the merozoites and start invading red blood cells (RBC). This invasion process has been extensively studied and involves 4 steps: (1) recognition and reversible attachment to the RBC membrane, (2) reorientation of the apical end of the merozoite with the RBC membrane, formation of a junction (irreversible attachment) and release of rhoptry-microneme molecules with a parasitophorous vacuole membrane formation, (3) Invagination of the RBC membrane, wrapping of the RBC membrane around the merozoite as there is shedding of merozoite surface proteins and (4) release of the parasitophorous vacuole membrane (PVM) and its RBC membrane wrapping, thus completing the invasion process [13, 17-19].. Each invad-

ing merozoite will, over a time period of 48-72h (depending on the species) generate between 16-32 new merozoites [19] which, upon release from the RBC, are able to perpetuate the cycle. It is during the RBC invasion, multiplication and rupture cycle that the clinical symptoms of malaria are felt [13]. Some of these merozoites, after invasion of the RBC, will differentiate into gametocytes [13] that can be taken-up by mosquitoes, during a blood-meal.

2.2.2. The mosquito vector

Once inside the mosquito's midgut, the drop in temperature and pH stimulate the male gametocyte to begin a process of exflagellation leading to the formation of microgametes that are able to fertilize the female macrogamete and originate a zygote. This zygote will then mature into an ookinete that penetrates the epithelial cell layer of the mosquito's midgut and forms an oocyst where consecutive rounds of division and differentiation will result in generation of thousands of sporozoites. These will then travel to the salivary glands, through the mosquito's haemolymph, where they will finish their maturation and be ready to be deposited in another human host's skin, when the female mosquito takes another blood-meal.

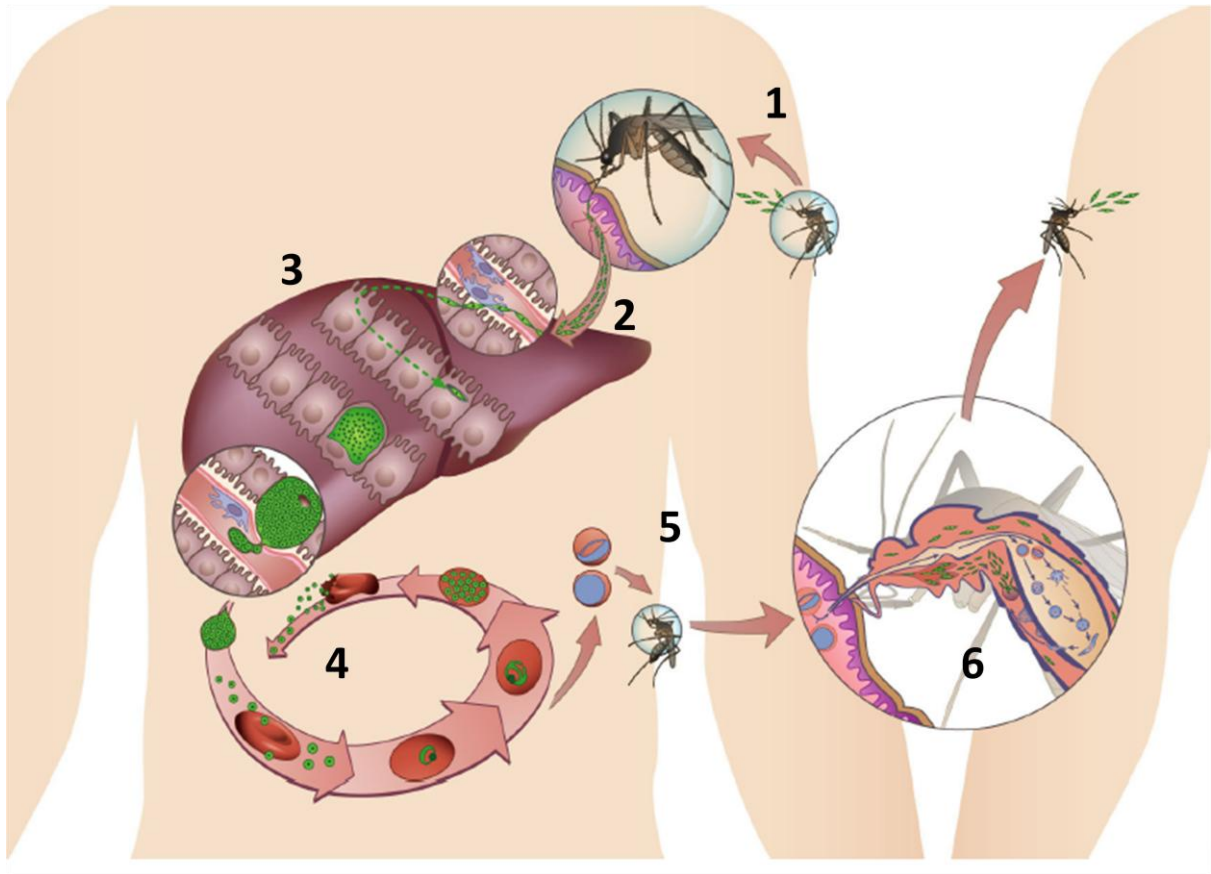


Figure 2-2 - *Plasmodium* life cycle.

The life cycle of *Plasmodium* parasites begins when an infected female *Anopheles* mosquito takes a blood-meal (1). This results in the deposition of sporozoites in the skin of the human host. These sporozoites eventually find their way to the small capillaries that irrigate the skin and use them to reach the Liver, where they use Kupffer cells as their means of entry (2). Once in the Liver, the sporozoites traverse an average of 4 hepatocytes before invading a final cell, where they divide into 10,000 to 30,000 merozoites (3). These are then released, using structures known as merozoites, into the host's blood-stream. Each merozoite invades a red blood cell (RBC) and initiates the intra-erythrocytic stage of the life-cycle. The parasites replicate asexually inside the RBC giving rise to new merozoites that initiate the intra-erythrocytic cycle again (4). Some parasites escape this cycle and mature into gametes that can be taken up by female *Anopheles* mosquitoes during a blood meal (5). Once these gametes are inside the mosquitoes' mid-guts they fuse and form a zygote that matures and invades the epithelia of the mosquitoes' mid-guts. Further divisions and maturation leads to the development of new sporozoites that migrate to the mosquitoes salivary glands, ready to be deposited in a new host (6).

2.3. Clinical features and Susceptibility to Malaria

An infection with malaria parasites can elicit a broad spectrum of responses that range from no symptoms to quickly fatal disease. This is dependent on several host and parasite factors and on the length of exposure of one to the other [20]. In areas where there is intense malaria transmission both children under 5 years old and pregnant women take the major bulk of the burden associated with malaria, while in areas of low malaria transmission, young adults and adults from non-endemic regions can also share the burden [1, 21, 22].

2.3.1. Uncomplicated malaria

Patients that present with uncomplicated malaria often reveal a variety of symptoms that overlaps that of many other diseases. These may include cough, abdominal pain, vomiting, diarrhoea, headache and general body pains [20].

2.3.2. Complicated Malaria

Complicated malaria includes, but is not restricted to, Cerebral Malaria (CM), defined as an unarousable coma that cannot be attributable to any other cause but a *Plasmodium falciparum* infection [23], severe anemia (SA), where patients present haemoglobin values of <7 g/dL for adults or <5 g/dL for children [23], and malaria in pregnancy (MiP).

2.4. Malaria in Pregnancy

2.4.1. The burden of Malaria in Pregnancy

It has been estimated that between 25 to 50 million women/year become pregnant in areas where malaria infections are endemic, resulting in up to 200 000 infant deaths/year [22, 24, 25] and a very recent review increased those numbers to 125 million pregnant women [26]. In malaria endemic areas, pregnant women are particularly more susceptible to malaria infection than their non-pregnant counterparts. Women in their first (primigravidae, PG) and second (secundigravidae, SG) pregnancies are the most affected suffering from severe anaemia and delivering low birth weight (LBW) babies [22, 27-29] although, in areas of low or unstable transmission CM, pulmonary oedema and renal failure may occur [30].

2.4.2. The pathology of Malaria in Pregnancy

Most of the pathology associated with this disease is attributed to *P. falciparum* infections, although in areas where *P. vivax* is the prevalent species, like the Thai-Burmese border or regions of the Indian sub-continent, *P. vivax* malaria can be the causative agent of MiP and is associated with severe maternal anaemia, thrombocytopenia, hypoglycaemia and infant low birth weight (LBW) [31, 32]. In sub-Saharan Africa, where most of the samples used in this thesis were collected, *P. falciparum* infections are the most common causes of MiP [1].

The main characteristic that can be attributed to the occurrence of MiP is the presence of *Plasmodium* material in the placenta, either whole parasites (usually mature forms) or haemozoin [33]. This accumulation of parasite material occurs in the intervillous spaces of the placenta, a region where the maternal blood bathes the syncytiotrophoblast layer of the placenta (see Figure 2-3). Accumulation of maternal immune cells [34, 35] and an increased level of inflammatory cytokines and chemokines [22, 35-38] are also associated with MiP particularly in PG. Several other changes can be observed in infected placentas. These include the excess of syncytial knotting, the thickening of the basement membrane of the trophoblast layer and an occurrence of excessive perivillous fibrinoid deposits [33, 39, 40].

2.4.3. Plasmodium falciparum pRBC in the placenta

2.4.3.1. The parasitized red blood cell

Plasmodium spp. heavily modify the RBC they invade [13, 41]. As in other forms of severe malaria, MiP is associated with the sequestration (adhesion and immune evasion) of pRBC in the host tissues, in this case the maternal placenta [33]. *Plasmodium falciparum* (as do *P. ovale* and *P. malariae*) [13] pRBC possess protein structures on their surfaces called Knobs which seem to be associated with the capacity that these parasites have of sequestering in the host tissues. Knobs as observed by electron microscopy are electron-dense, cup-shaped protrusions of the pRBC [42]. It is in this complex protein structure that a parasite derived protein family, *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP-1), is exposed to the host and contributes to parasite sequestration by serving as a ligand between the pRBC and a variety of host tissues [43-46]. Besides this role in sequestration, PfEMP-1 proteins have been linked to rosetting (a phenomenon in which pRBC are able to bind to uninfected RBC) [47, 48], to parasite immune evasion through antigenic variability [49, 50] and to the host immune response as targets of phagocytosis and antibody generation [51-53].

PfEMP-1 proteins are encoded by a family of approximately 60 large (6-13 kb) genes known as *var* genes [45]. At any one time point, only the product of a single *var* gene is expressed on the surface of the pRBC [54] although other *var* genes may be simultaneously transcribed [55]. PfEMP-1 molecules contain cysteine-rich domains named Duffy Binding-Like (DBL) domains which are usually 300 amino acids long [56]. These tend to be interspersed by regions of highly variable sequence. There are 5 types of DBL domains (alpha to epsilon) plus

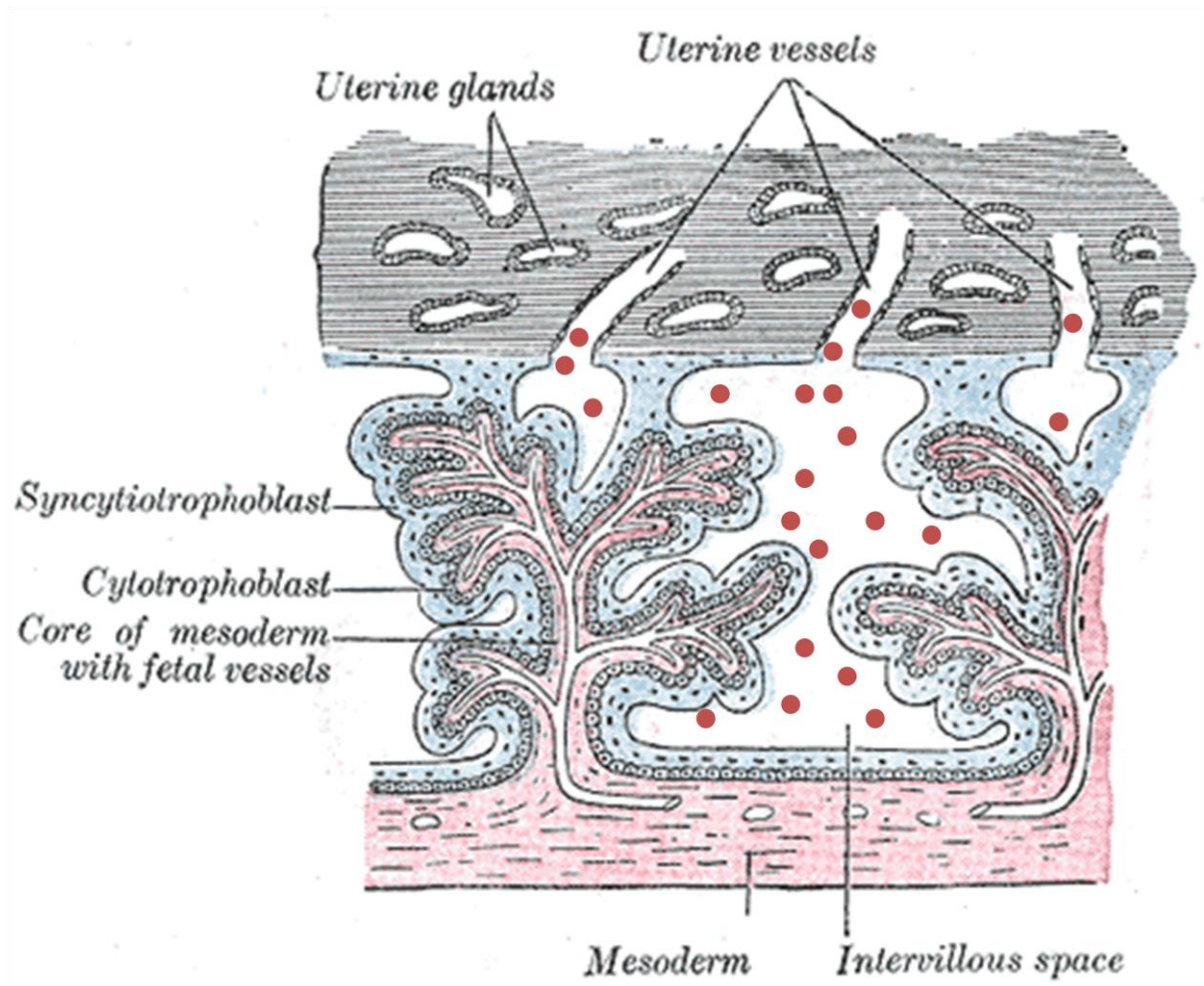


Figure 2-3 – Diagram of the Placenta

A diagram of the placenta showing maternal RBC (red dots) bathing the syncytiotrophoblast. (adapted from [57])

a more heterogeneous DBLX domain. In addition, one more domain can be identified in these proteins and that is the CIDR (Cysteine-rich Interdomain Region) [56]. All the members of the PfEMP-1 family contain a transmembrane region plus a very well conserved intracellular region called the acidic terminal sequence (ATS). Of all the DBL domains, DBL1 α seems to be the most conserved [58], although this only reaches 36-52% amino acid identity [59]. Importantly, though, *var* genes are able to switch and thus change the PfEMP-1 molecule on the surface of the pRBC allowing the parasite to exhibit antigenic variability [49, 60]

Together with PfEMP-1, other parasite-derived protein families have been identified on the surface of the pRBC. Among these are the STEVOR and RIFIN protein families. *rif* (repetitive interspersed family) genes form the largest *Plasmodium falciparum* family of multi-genes and RIFINS can, together with PfEMP-1, be the target of an immune response by the host [61-63]. As for *stevor* (subtelomeric variable open reading frame) genes, of which there are approximately 30 [64], only recently have they been identified on the surface of pRBC as has a possible role for members of this family in the antigenic variability of *Plasmodium falciparum* [65].

2.4.3.2. CSA and VAR2CSA

The binding of pRBC to the placenta of pregnant women has been one of the hallmarks of MiP [29, 66]. After the discovery in 1995 of the capacity of a glycosaminoglycan called Chondroitin Sulphate A (CSA) to serve as a receptor able to mediate the binding of pRBC, not long passed until it was discovered that MiP *Plasmodium falciparum* isolates were able to strongly adhere to it [67, 68].

The involvement of PfEMP-1 in other adhesive processes, namely to Complement Receptor-1 [47] and CD36 [69] made it a natural step for researchers to investigate possible members of the PfEMP-1 family responsible for the CSA-binding phenotype. These are generally referred to as Variant Surface Antigens which mediate the binding to CSA (VSA_{CSA}). The first candidate genes, and respective protein products, reported were *var1csa* (encoding VAR1CSA) and *CS2var* (encoding CS2VAR) (thoroughly reviewed in [70]). Both these PfEMP-1 proteins are able to bind to CSA, but it soon became clear that a few conditions that should be met by MiP specific VSA_{CSA} were not present in pRBC that expressed these proteins [70]. Namely, both *var1csa* and *CS2var* are only rarely transcribed by parasites isolated from the placenta of infected women and neither of them increase when laboratory parasites are selected for CSA-adherence [59, 71].

Another candidate, found to be associated with the CSA-binding phenotype was the *var2csa* gene [72]. This is an unusual *var* gene in that it does not have a DBL1 α domain [70]. This gene was found in isolates from infected placentas [71, 73, 74] and its expression increases with CSA-binding selection [71, 72]. Besides this, a protein associated with the expression of this gene, VAR2CSA, was found on the surface of pRBC and contains CSA-binding domains [74-78]. Serum from pregnant women from malaria endemic areas also proved to contain antibodies that recognised VAR2CSA showing a pregnancy-dependent, parity dependent and sex-dependent pattern [79-85]. So far, *var2csa* and its associated PfEMP-1 protein VAR2CSA remain the best parasite-derived candidate that explain the higher susceptibility of pregnant woman to malaria.

2.4.4. Immune responses to Malaria in Pregnancy

2.4.4.1. Cellular immunity, cytokines and chemokines

A normal pregnancy is characterised by a predominantly type-2 (Th-2) cytokine response. This, in contrast to the inflammatory Th-1 response, enables the mother to “tolerate” the foreign foetus tissues [86] and thus allows for a successful pregnancy to take place.

MiP has been shown to be associated with a shift from the Th-2 to the Th-1 type response with increased levels of inflammatory cytokines and chemokines in the placenta and/or the periphery [36, 87, 88]. Some of these cytokines, have been shown to be associated with adverse outcomes like LBW (tumour necrosis factor (TNF)- α , interferon (INF)- γ) and anemia (TNF- α) [87, 88]. IFN- γ has also been associated with decreased prevalence of placental malaria, although this was done using *in vitro* modelling [89]. Other cytokines and chemokines that have been found increased in MiP (although not always associated with either protection from or severity of disease) include interleukin (IL)-10, IL-2, IL-8, IL-12, macrophage inflammatory protein (MIP)-1 α and MIP-1 β [35, 36, 90, 91].

Studies directed at uncovering the cells responsible for the release of these cytokines have revealed the possible involvement of monocytes/macrophages, NK cells and CD4-T cells [35, 89, 90]. This increased level of cytokines may not be just the responsibility of circulating immune cells, as it has been shown that the placenta itself, through the syncytiotrophoblast layer, is able to secrete biologically active cytokines and chemokines, in response to a *Plasmodium* infection, which in turn can attract circulating immune cells [92].

Although several studies have addressed the direct interaction of immune cells (mainly monocytes/macrophages and NK cells) with *Plasmodium* pRBC of peripheral, non-

pregnancy related origin, no major studies have been done so far for MiP-type parasites. It is known that MiP-type parasites are able to evade the binding to CD36, a molecule able to induce phagocytosis [52, 93] but the effect of this in the activation or inactivation of phagocytic cells is not known.

2.4.4.2. Humoral Immunity

The humoral immune response to MiP (particularly in PG) will be reviewed in detail in Chapter 4.

Fried et al found that the serum from Kenyan pregnant women had the ability to inhibit the adhesion of MiP-type parasites to CSA [94] and that it did so in a parity dependent fashion, with PG failing to inhibit the adhesion of the parasites to CSA. This was the observation that demonstrated the importance of humoral immunity in MiP. Following this study, antibodies that are able to agglutinate placental isolates were also found in pregnant women [95] and a pregnancy-dependent acquisition of antibodies towards a laboratory adapted parasite line expressing VSA_{CSA} was described [96]. These antibodies have been shown to be mainly of the IgG class, with an emphasis on the IgG1 and IgG 3 sub-classes [97-99] which also contribute to the phagocytic clearance of pRBC [99].

2.4.4.3. Effectors of immunity to Malaria in Pregnancy

Several factors have been shown to influence the development of immunity, mainly humoral immunity, to MiP. Among these, environmental factors (endemicity) [100], prevention strategies (use of intermittent preventive treatments) [101, 102] and HIV [80, 103, 104] are the most important.

2.4.4.4. Immunity towards VAR2CSA

As was mentioned, studies have demonstrated the ability of pregnant women to develop antibodies that recognise VAR2CSA [79-85]. Recent studies have additionally shown the conservation between *var2csa* from geographically distant placental isolates with significant antigenic overlap [79]. Significant epitope sharing between pregnant women's antibody responses was also observed [79]. Several domains of the VAR2CSA molecule are recognised by the serum of malaria exposed pregnant women, but it seems that DBL3X and DBL5y are the most important targets of the immune response [76, 79-81].

It has been shown that immune cells that contain pRBC-derived material can be found in the placentas of women exposed to malaria [24, 105, 106]. Nevertheless, to date no stud-

ies have been specifically conducted with the aim of looking at VAR2CSA or its domains and activation of cellular immune responses.

2.4.5. Prevention and treatment strategies for Malaria In Pregnancy

Strategies employed for the prevention and treatment of MiP are, with few exceptions, not distinct from the ones used in the general prevention and treatment of malaria. Although not all of these strategies have been the subject of major studies directed at the specific effects on MiP, prevention strategies include better sanitation, insecticide spraying, use of bed-nets and intermittent preventive treatment during pregnancy (IPTp). Treatment of *Plasmodium* infections is highly dependent on the region and species of parasite present as resistance to drugs is becoming a big concern. Finally, vaccines against *Plasmodium spp.* still reveal a low level of feasibility/efficacy.

2.4.5.1. Environmental strategies and use of insecticides

Sanitation measures eliminate mosquito breeding sites and provide less contact between vector and host providing a high degree of protection, calculated at 88%, to the target populations [107]. The use of insecticides, both outdoors and indoors, has been the subject of long debates. Although the use of dichlorodiphenyltrichloroethane (DDT) virtually eliminated malaria cases in the “western world” [108] several concerns are now raised against its use due to environmental and health issues [109] and its proposition, by the WHO, for use in African countries for indoor residual spraying [110].

2.4.5.2. Bed nets and Intermittent preventive treatment

The use of bed-nets, and more recently, insecticide-treated bed-nets (ITN) have been shown to protect the target populations against malaria symptoms and adverse outcomes [111, 112].

IPTp is a strategy that consists on the receipt of drug concentrations at determined periods during pregnancy in order to maintain an adequate level of protection throughout the duration of the pregnancy. The drug of choice in most African countries is sulphadoxine-pyrimethamine (SP), and despite some observed levels of resistance (and proposed changes in drug use [113]), SP has proved to be able to protect women from the adverse outcomes of MiP [112, 114-116].

2.4.5.3. Drugs

A vast number of drugs have been used with more or less success in the fight against malaria in pregnancy. This has been the subject of recent reviews [117-120]. Drugs used, either for treatment, prophylactically or during IPTp, include Quinine (it is the oldest anti-malarial and it appears to be safe to use for treatment during pregnancy), Chloroquine (more recommended to non-falciparum malarias due to *P. falciparum* resistance to it), Amodiaquine (appears safe to use), SP (first line of anti-malarials for IPTp use due to long half-life, presents some theoretical risks if used in the first trimester of pregnancy), Chlorproguanil dapsone (now withdrawn), Artemeter-lumefantrine (Coartem) and artesunate (not yet proven to be good pregnancy-safe drugs [121]) and Mefloquine (theoretical concerns exist over its use early in pregnancy, but appears safe after that).

2.4.5.4. Vaccines

The development of an effective vaccine against MiP is one of, if not the major, drive behind MiP research. The lack of a vaccine against not only *Plasmodium spp* but also any humanparasitic disease is an indication of the difficulty that such an endeavour represents. Although, for some, VAR2CSA represents the “golden” antigen with which vaccines can be developed [53], there is still a long way to go before that can be achieved. Various studies have shown that immunization of mice, rabbits and goats with domains of VAR2CSA is able to induce the production of antibodies that not only recognise the surface of VSA_{CSA}-expressing pRBC but also function as inhibitors of adhesion [77, 122, 123], but there is still some work to be done in terms of recognition of these domains by pregnant women’s sera, associations of this recognition with protection from MiP, epitope mapping and epitope conservation between geographically distinct regions and ultimately clinical trials that provide hard evidence that a significant and lasting immunity can be achieved at low cost.

2.4.6. HIV and Malaria in Pregnancy

Human immunodeficiency virus (HIV) has become over the past 30 years a major global health concern affecting 40 million people worldwide [124]. This virus infects cells expressing the CD4 receptor on their surfaces and integrates into the genome of the host, making it virtually impossible to eliminate. The interactions between HIV and malaria are well known, with higher rates of malaria disease and higher densities and prevalence of parasitaemia seen among HIV-infected patients compared to those that are uninfected [125-131]. On the other hand, malaria is also responsible for higher HIV-1 viral loads [132, 133] demonstrating that both infections are able to directly affect the outcome of one another.

2.4.6.1. Impact of HIV on immunity to Malaria in Pregnancy

During pregnancy, HIV has profound impacts on the immunity to MiP. Studies have demonstrated that HIV-positive women possess less anti-VSA_{CSA} antibodies when compared to HIV-negative women [103, 134], which may contribute to the increased amount of parasites observed in the placentas of HIV-positive women and lead to a higher impact of MiP. As was mentioned earlier, various cell types are able to secrete chemokines and cytokines that are important effectors during MiP. HIV infections significantly reduce the production of IFN- γ by maternal immune cells stimulated *in vitro* with crude malarial antigens [135], thus depriving the placenta of a cytokine associated with better outcomes [89]. IL-12, a cytokine involved in the regulation of IFN- γ was also found to be reduced [136]. As would be expected the levels of these cytokines were highly associated with the numbers of CD4⁺ T cells [135, 136]. Although no studies have been conducted so far (representing a serious lack of knowledge) on the function of monocytes/macrophages in HIV/MiP co-infected women, it is known that changes in the composition of the monocyte/macrophage populations do occur with an increase in CD16⁺ macrophages carrying HIV-1 visible in HIV/MiP co-infected women [137].

2.4.6.2. Antiretroviral drugs and Malaria in Pregnancy

There is a striking lack of information regarding the interaction between antiretroviral drugs and all aspects of MiP.

What is known is that antiretroviral drugs are becoming widely available in more areas of the globe where antimalarials are also highly used. The potential for interactions between both treatment drugs has not been appropriately investigated and studies call for more information [120, 138].

The role that antiretrovirals can play in the acquisition or maintenance of immunity against MiP, be it cellular or humoral immunity, has also not been the subject of many studies although that promises to change in the near future. Although these studies were all performed outside of the context of MiP, it is worthwhile to mention that Nathoo et al have looked at the CD36-depleting effect that antiretrovirals have on macrophages [139] and that a very exciting field of research is now beginning with the discovery of the potential for antiretroviral drugs to act as antimalarials [140-143].

2.5. Non-falciparum Malaria during pregnancy

This thesis is purposely biased towards the study of *Plasmodium falciparum* parasites and pregnancy due to the low evidences, so far, for the involvement of other *Plasmodium spp* in the occurrence of MiP. *Plasmodium vivax* infections can result in severe anaemia and low birth weight during pregnancy as well as increase the risks of still births and miscarriages [31, 144]. Very recently, *Plasmodium vivax* was shown to also be able to adhere to placental cryo-sections, albeit at extremely low levels, but more evidence will need to be gathered [145]

2.6. Summary

MiP remains a major health problem and presents numerous challenges that need to be addressed in order to improve the life of the millions of women, their offspring and ultimately their communities and countries. Parasites that adhere to the placenta of the women affected elicit an immune response that we need to be able to accurately measure and understand in order to develop the best treatments and ultimately the best vaccines. Interactions of HIV with MiP will remain extremely important in sub-Saharan Africa and understanding how HIV affects the development of immunity to MiP is critical.

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3. Development of a new, high-throughput phagocytosis assay for the measurement of antibody mediated phagocytosis

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3.1. Introduction

The measurement of antibodies towards malaria parasites (both as markers of infection and/or protection) has been one of the most widely used tools in malaria immunology (reviewed in Chapter 2). The discovery that serum antibodies raised in malaria infected patients, when incubated with parasites that were subsequently injected into a different host, protected this host from the infection [1] allied with the growing awareness in the 1960's [2] that anti-malarial immunoglobulins were associated with protection from disease, led to the development of techniques that would allow investigators to look in more detail at these antibodies. The methods employed for the measurement/detection of antibodies towards malaria have been evolving with time, following both the knowledge of the disease itself and the availability of new technologies.

From standard microscopy, to immunoblots, to ELISAs to Flow cytometry a number of different methods have been employed to look at a number of antibody parameters: titre, class (or isotype), sub-class, function. Almost all of these methods can be grouped into four main categories:

Agglutinating assays – where antibodies are measured (functionally and quantitatively) by their capacity to cause pRBC to agglutinate. This usually happens due to antibodies present in the serum binding to more than one antigen, and thus causing the antigens to “clump together” (agglutinate). An example of an early application of this test to malaria can be found in [3].

In total binding assays, the total amount of antibodies, or an antibody class (e.g. IgG) that bind to a specific malarial antigen (e.g. schizont extracts, late-stage whole parasites, specific parasite proteins, etc) are evaluated, with no regards for the functionality of these antibodies. ELISA and Flow cytometry based assays looking at the total IgG to pRBC Variant Surface Antigens (VSA) are a good example of these [4]

In anti-adhesion assays the ability of antibodies (either present in serum or purified) that cover the surface of pRBC to prevent the adhesion of these pRBC to a target tissue/cell/receptor is assessed. For an example of these, see [5].

Phagocytosis assays are assays in which the capacity of antibodies to opsonise the surface of the pRBC and elicit a phagocytic response from leucocytes (either neutrophils, monocytes, macrophages or various cell lines) is evaluated. Although phagocytosis is recognisably an important immune function in malaria (particularly associated with the role

of the spleen in the clearance of the parasites) these antibodies haven't been the subject of many studies at the population level. For an example of one such study see [6].

3.1.1. Use of serology tests in malaria in pregnancy

As reviewed by Beeson and Duffy in 2005 [7], the methods used for the analysis of anti-malarial antibodies and their relationship with MiP, involved only three of the four above-mentioned categories. As can be seen in Figure 3-1 – until 2005, the predominant methods used for the evaluation of antibodies in MiP were anti-adhesion assays, agglutination assays and particularly total IgG binding assays (with flow cytometry based assays replacing old immune-fluorescence based assays). The absence of phagocytosis assays is important if we take into account the relevance that is attributed to phagocytes (from the Greek “phago” eat and “Cite” cell) in the spleen [8, 9]. The review by Beeson and Duffy only gives us the methods employed after 1998 with the discovery of antibodies to VSA involved in the binding to Chondroitin Sulphate A and their association with parity [5], but it is fair to say that even before that there were no large studies looking at the role of phagocytes in MiP.

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Table 1. Summary of studies examining associations between antibodies to the surface of placental and/or CSA-binding *P. falciparum*-infected erythrocytes and parity, parasitemia, and/or pregnancy outcomes

Study ¹	Population	Source of <i>P. falciparum</i> ²	Antibody Measure ³	Antibody associations
Fried et al., 1998	Kenya, Malawi, Thailand	Placenta (Kenya)	Adhesion inhibition	Parity ⁴ Negatively associated with placental parasitemia in Kenyan SGs
Beeson et al., 1999	Malawi	Placenta; Peripheral blood	Agglutination	Parity (with placental isolates, but not isolates from children)
Maubert et al., 1999	Cameroon	Peripheral blood; CSA-binding line (RP5/FCR3)	Agglutination	Parity (with CSA-binding isolate, but not isolates from non-pregnant donors; among women in 2 nd trimester and at delivery)
Ricke et al., 2000	Ghana	CSA-binding line (PA/FCR3)	IgG binding	Parity (with CSA-binding line but not CD36-binding line)
Staalsoe et al., 2001	Cameroon	CSA-binding line (FCR3) and isolate (2H3)	IgG binding	Parity Inverse association with placental parasitemia among MGs (but not PGs)
O'Neill-Dunne et al., 2002	Cameroon	CSA-binding line (3D7/NF54)	Adhesion inhibition	Parity (among women in 2 nd trimester, but not at delivery) Not associated with presence of placental infection Inverse correlation with placental parasitemia ⁷
Duffy & Fried, 2003	Kenya	Placental isolates	Adhesion inhibition	Positively associated with birth weight Inversely correlated with placental parasitemia Not associated with anemia
Staalsoe et al., 2004a	Kenya	CSA-binding lines	IgG binding	Positively associated with birth weight Inversely associated with anemia
Beeson et al., 2004	Malawi	CSA-binding line (CS2) Placental isolates	IgG binding Agglutination Adhesion inhibition	Parity (2 nd trimester and at delivery) Placental infection among PGs for all antibody measures; Not associated with placental parasite density or outcomes
Taylor et al., 2004	Cameroon	CSA-binding line (3D7/NF54)	Adhesion inhibition	Inversely correlated with placental parasite density ⁵
Mount et al., 2004	Malawi	CSA-binding line (CS2)	IgG binding Agglutination	Antibodies lower in HIV-1-infected women; Inversely correlated with immunosuppression and HIV viral load; Not correlated with parasite density or associated with pregnancy outcomes
Khatab et al., 2004	Gabon	CSA-selected placental isolates	IgG binding	Parity Placental infection in PGs
Staalsoe et al., 2004b	Kenya	CSA-binding lines (FCR3 and Busua)	IgG binding	Lower among women receiving intermittent presumptive treatment/prophylaxis

Figure 3-1- Antibody methods used in the detection of antibodies during MiP (adapted from [7])

There were, evidently, studies that looked at the presence of antibodies associated with MiP before the 1998 date set by that review [10-19], but as far as MiP is concerned only after that seminal date were antibodies consistently found to be important sources of in-

formation (thoroughly reviewed in Chapter 4). From 1998 until the time of writing of this thesis, 4 studies focused on phagocytosis and MiP [6, 20-22] (see section 1.5.1).

3.1.2. Phagocytosis

3.1.2.1. What is phagocytosis

Phagocytosis is a highly conserved and complex process that has evolved in eukaryotic cells, with the exception of yeasts, for the uptake (either for metabolic processes or for destruction) of particulate matter.

Since first observed by Metchnikoff in 1905 (reviewed in [23, 24]) phagocytosis has been a focus of research. With roles in host defence, ingestion of metabolites, inflammation and tissue homeostasis, phagocytosis is mediated by receptors on the surface of cells [25]. In mammals, these cells can be divided into non-professional phagocytes (epithelial cells, fibroblasts) and professional phagocytes (neutrophils, dendritic cells, monocytes/macrophages) [24, 25].

3.1.2.2. The mechanism and receptors involved in phagocytosis

Groves et al, reviewed the molecular mechanisms behind this process in 2008. In its classical sense, phagocytosis is a receptor mediated zipper-like process [26]. This multi-step process initiates with the recognition of the particle either directly (by pattern recognition receptors, PRR) or indirectly (through agents called opsonins that recognise and cover the surface of the particle, e.g. IgG, complement). The receptors responsible for recognising opsonins include Fc-receptors (FcR) [27] and complement receptors (CR1-4) [28] while those that directly recognise the particles include a series of receptors that recognise molecular patterns previously referred to as PAMPs (Pathogen-Associated Molecular Patterns) [25]. This designation, PAMPs has been criticised as it does not include molecular patterns found on commensal bacteria and necrotic and apoptotic cells. A member of the PRR group is CD36, a glycoprotein expressed by a variety of types of cells [29] and known today to be an important mediator of the phagocytosis of malaria pRBC [30]. Other receptors of the same diverse group are able to mediate phagocytosis, as is the case of SR-A, but still have controversial roles during a malarial infection [31-34]

3.1.2.3. Fc-receptors

Most interactions between cells of the immune system and antibodies are mediated by glycoproteins on the surface of the immune cells called Fc-receptors (FcR). Although both IgA [35] and IgM [36] isotypes have their own receptors (FcαR and FcμR, respectively)

their role as mediators of phagocytosis is not proven, so I will focus on the FcR that recognise IgG (FcγR) and IgE (FcεR).

The structure and homology between both FcγR and FcεR, as well as their roles as immune mediators, have been thoroughly reviewed [27, 37]. Both receptor families have subunits derived from a common ancestral gene. FcR are designated by their affinity towards the immunoglobulin isotype to which they bind. So for FcγR we can find three groups of receptors according to their affinity to IgG (from high to low): FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) [27]; and for FcεR we find also three groups, once again from high to low affinity: FcεRI, FcεRII (CD23) [38] and Galactin-3 [39, 40]. Adding to the complexity of these receptors is the fact that CD32 has 2 isoforms that differ in their role. CD32a is an activating isoform that promotes phagocytosis, while CD32b is an inhibitory receptor that downregulates the phagocytosis activity [37].

It is worthwhile mentioning that, although IgG is present at much higher concentrations in human serum than IgE, the affinity of IgE for its high-affinity receptor FcεRI is two to five orders of magnitude higher than that of IgG to CD64, and that the “low-affinity” receptor CD23 has as much affinity for IgE as CD64 has for IgG [38] (For more on IgE view chapter 5).

3.2. Phagocytosis and Malaria

3.2.1. The beginning

Early on, during the course of assessing the protective effect that antibodies seemed to have on the protection from a malaria infection [2, 41], researchers developed two non-conflicting theories. The first one stated that antibodies (mainly of the IgG isotype [2]) would block the entry of merozoites into RBC, thus preventing the infection from occurring. This was supported by studies showing that serum from patients living in malaria-endemic areas was able to inhibit *in vitro* cultures of *Plasmodium falciparum* [42-44]. The second theory sprang from the observation that enlarged spleens containing immune cells full of malaria pigment were one of the features of a malaria infection [8, 9]. The role that these cells, mainly monocytes/macrophages [9], could have in the clearing of malaria parasites was supported by several studies, both in animal models and using human-derived cells, showing the phagocytic activity of monocytes/macrophages against malaria parasites and the enhancement of that activity promoted by immune sera from individuals from malaria-endemic countries [45-47].

3.2.2. Monocytes and malaria

As was noted above, early observations pointed to the role that monocytes and macrophages, especially of splenic origin, could play in the clearance *Plasmodium* parasites [8, 9]. It was also early understood that although monocytes and macrophages were able to phagocytose pRBC alone, their capacity to do so dramatically increased with the opsonisation of the targets with immune sera [45-47].

3.2.3. Non-opsonic phagocytosis

Immune cells, particularly monocytes/macrophages, dendritic cells and neutrophils are able to recognise particles that have not been opsonised by either complement or antibodies. This is referred to as non-opsonic phagocytosis and it is mediated by receptors on the surface of the immune cells that recognise certain molecular patterns on the surface of pathogens, commensal bacteria and self-apoptotic or -necrotic cells [25]. These receptors include the Toll-like Receptors, Scavenger receptors (of which CD36 is a member) and other Pattern Recognition Receptors (e.g. the macrophage Mannose Receptor) [48].

TLR functions and their modulation during exposure to malaria parasites have revealed these receptors as an important part of the innate immune response. Of particular interest to malaria are TLR2 and TLR4 (capable of binding parasite-derived glycosylphosphatidylinositol (GPI)), and TLR9 (an intracellular receptor capable of interacting with parasite derived hemozoin). These are not, however, essential for the phagocytosis of the pRBC [49-51].

The CD36 receptor, an integral membrane protein of many cell types, is especially important and has been widely studied in malaria [30, 52-59]. Not only has CD36 been shown to be recognised by several natural isolates of *Plasmodium falciparum* and to aid in the sequestration of this parasite in the tissues of the human host [59, 60] but also studies have demonstrated, both in animal models [58] and in human models [30, 57] the ability that macrophages have of using this receptor to phagocytose intact and unopsonised pRBC.

It is interesting to point out that there is some evidence pointing to the independent pathways by which these two sets of receptors (TLRs and CD36) act since monocytes can perform CD36-mediated phagocytosis without eliciting TLR activation and, on the other hand, be activated by parasite-derived TLR ligands in the absence of CD36 [61].

3.2.4. Opsonic-phagocytosis

As was mentioned earlier, both complement proteins and antibodies can attach to, and thus cover, the surface of particles (including pathogens, apoptotic cells or even healthy

cells in the case of immune auto-antibodies) and by doing so have the potential to induce the phagocytosis of these particles by monocytes/macrophages and neutrophils. The process of attaching to, and covering, the surface of an antigen is called opsonisation and so both complement and antibodies can be referred to as opsonins [48].

The complement system, a cascade of proteins and glycoproteins present in the serum, can have very diverse functions, from opsonisation of targets, to chemotaxis, to direct lysis of a pathogen or even as an homeostasis regulator [48, 62]. The role of complement and complement receptors in inducing the phagocytosis of malaria pRBC has been the focus of some interesting research during the last decade. It has been shown that C3b, a component of the complement cascade that induces phagocytosis in macrophages [28, 63, 64] can bind to immune complexes on the surface of pRBC and induce one of two functions: either stripping (a process by which the entire immune complex and associated receptors on the surface of the pRBC are literally stripped off by macrophages, leaving the pRBC surface “free” of complement receptors, like CR1) or phagocytosis [65]. C3b can also bind directly to antigens and stimulate its clearance by liver macrophages (Kupffer cells) although not all the receptors on the Kupffer cells responsible for this have been identified [66]. It does not seem like complement is able to directly induce the lysis of pRBC [67].

Both IgE (reviewed on Chapter 5) and IgG can opsonise and induce immune cells to respond to targets. These responses can be of two sorts: Antibody Dependent Cell Cytotoxicity (ADCC) or phagocytosis [25, 38, 61, 68-73]. In ADCC, the immune cells (monocytes, macrophages, mast cells, NK cells, etc) recognize the Fc' portion of the antibody attached to the antigen and release a focused array of cytokines and enzymes that lead to the death of the target cell [48]. The most important mechanism used by monocytes/macrophages during a malaria infection, though, seems to be the phagocytosis of pRBC [45, 46, 73-77]. Not all the sub-classes of IgG seem to contribute to the process of phagocytosis. Several studies have demonstrated that the sub-classes IgG1 and IgG3 (called cytophilic classes due to their high capacity to induce immune cells to respond) are the most prominent classes among individuals living in malaria endemic areas that have achieved partial immunity [78] and are also the sub-classes that elicit phagocytosis in monocytes and macrophages [20, 72, 73]. It is worthwhile to point out that the remaining sub-classes of IgG, IgG2 and IgG4, have been associated with protection from malaria symptoms and with susceptibility to it, respectively [79-81]. It is thought that these antibodies, especially IgG4 might compete with the cytophilic antibodies for epitopes on the surface of the pRBC.

I would like to point out that in this thesis, whenever phagocytosis assays were used by a method that does not exclusively identify IgG1 and IgG3, the antibodies responsible for that phagocytosis will be referred to as phagocytic antibodies.

3.2.5. Monocytes and phagocytosis in MiP

The realization that pregnant women, (especially primigravidae), in malaria endemic areas frequently developed an accumulation of monocytes/macrophages in the placenta, and that these had evidences of having phagocytosed pRBC or pRBC-derived products (e.g. hemozoin) [82-84] prompted investigators to research on the topic of the role of phagocytosis in pregnancy.

It was soon realised that parasites that were associated with MiP were particular in that they did not bind to CD36 [85] but rather to placental chondroitin sulphate A (CSA) [85, 86] and thus could potentially avoid non-opsonic phagocytosis [87]. This particular feature led investigators to monitor the interaction between monocytes and parasites that exhibited this CSA-binding phenotype.

Only recently have antibodies that promote the phagocytosis of CSA-binding parasites been a focus of research in MiP [6, 20, 21]. They have been shown to be decreased in HIV infected women compared to HIV uninfected women [20, 21], to correlate with levels of T-CD4+ cells in HIV-infected women [21], to predict, at enrolment, anaemia at delivery [6], to be associated with lower incidence of anaemia at delivery [21] and to be associated with a lower incidence of treatment failure [6].

3.2.6. Evaluation of antibodies using phagocytosis assays in malaria

Phagocytosis assays for the evaluation of the presence of phagocytic antibodies can be divided into different categories according to the cell-type used (neutrophils [88], monocytes [22], macrophages [20], laboratory cell-lines [6, 21, 89]), to the activation state of those cells (activated-adherent cells [6, 20, 21], non-activated cells [88]) or to the method employed to quantify it (microscopy [73], colorimetric [6, 21], flow-cytometry [22, 88]) (note: only some examples of studies making use of the methods mentioned are given, but by no means is the list an exhaustive one).

3.2.6.1. Phagocytosis assays in MiP

As was mentioned in section 3.1.1, four studies have been conducted in the research field of MiP, that have made use of phagocytosis assays [6, 20-22]. Due to the limited number of studies an individual discussion of each assay as employed in each study follows.

Chronologically, the study by Keen et al [20] was the first to make use of phagocytosis assays in the context of MiP. In this study, both human and mouse macrophages were used as adherent phagocytic cells and light microscopy was used to quantify the amount of phagocytosis. In contrast with this rather laborious and tedious assay, Tippet et al [22] developed a phagocytosis assay that was able to use both cell-lines (the Thp-1 cell line was used) and whole-blood and that made use of flow-cytometry to measure phagocytosis. This assay still didn't lend itself for use with many samples, but had the great advantage of allowing the use of whole-blood, and thus evaluate monocytic function in their "natural" environment. The next two studies, from Feng et al [6] and Jaworowski et al [21], both made use of a colourimetric assay using the pro-monocytic cell line Thp-1. In this assay Thp-1 cells are plated and stimulated with PMA (phorbol-12-myristate-13-acetate) to differentiate chemically for 3 days. After phagocytosis of pRBC, the Thp-1 are lysed and the haemoglobin released is measured using spectrophotometry. The advantage of this assay is that it lends itself to be used in 96-well plates and not 5-mL glass tubes (as is the case for Tippet et al [22]). On the other hand it relies on chemically differentiated cells and takes 3 days.

3.3. Thp-1 cells

Thp-1 cells are a human pro-monocytic leukaemia cell line derived from a one year old boy with acute monocytic leukemia [90]. The first papers that described this cell line [90-93] were able to show that they were positive for alpha-naphtyl butyrate esterase and negative to alkaline phosphatase and peroxidase. Examination of their karyotype revealed that these cells have a diploid (46) number of chromosomes. These initial studies also show for the first time the ability that Thp-1 cells have of ingesting IgG-covered particles, that this activity peaks at around 30 min and that phagocytosis is much higher for IgG-covered particles than for non-Ig-covered particles. The ability of these cells to differentiate into a more macrophage-like adherent phenotype was also evaluated and several inducers of differentiation can be used (IFN- γ , Vitamin-D3, 12-O-tetradecanoylphorbol-13-acetate (TPA), PMA) [91-95].

It is important to state, at this point, that from this point forward Thp-1 that are in suspension and have not been exposed to differentiating agents will be referred to as undifferentiated Thp-1 (uThp-1) while Thp-1 cells that have been exposed to such agents will be referred to as differentiated Thp-1 (dThp-1).

The differences between uThp-1 and dThp-1 have been the subject of many studies. Of importance for the present work is the fact that dThp-1 cells are adherent, express CD36

on their surface and also express CD16, all contrary to what is seen on uThp-1 cells [29, 91, 92, 96].

Another, and very important, discovery was the presence of both sets of FcR (FcγR and FcεR) on the surface of Thp-1 cells, although CD16 is absent from uThp-1 cells [73, 94, 97, 98]

3.3.1. Thp-1 and Malaria

The first use of Thp-1 cells in malaria studies, that acknowledged the importance that these cells could have in the study of malaria immunity was performed by Kumaratilake et al in 1997 [89] using dThp-1. Tebo et al [73], in 2002, made use of dThp-1, also, and demonstrated that FcγR-mediated phagocytosis could be measured by using these cells. More important was the fact that they used specific blocking antibodies for CD64 and CD32 and showed an equal contribution of both sets of receptors for the phagocytosis performed by these cells.. In a paper by Shwenk et al, 2003 they show that dThp-1 cells recognise and phagocytose sporozoites opsonised with antibody generated by an immune response to the central region of CS (using the RTS,S vaccine). This showed also the potential of these cells to be used readily as tools for vaccine development to the exo-erythrocytic stages of the parasite [99]. Craig et al [63], used dThp-1 to show a process called transfer, that basically is the “stripping” of immune complexes (IC) from the surface of pRBC and that is known to occur both in vivo and in vitro. Following this, came the MiP phagocytosis studies already discussed in section 1.5.1 [6, 21, 22, 73].

3.4. Rationale for developing a new phagocytosis assay

Thp-1 cells had already been used to measure phagocytosis of pRBC using immune sera [73] and had been used also to measure the levels of phagocytic antibodies in small cohorts of pregnant women [6, 21]. None of these assays, though, had the ability to combine the high-throughput, reliability and reproducibility needed to conduct studies using hundreds of samples. With this in mind I decided that by culturing uThp-1 cells in suspension, performing the assays in 96-well plates and measuring phagocytosis by Flow-cytometry, I could develop a more high-throughput, less user-dependent assay.

3.5. Methods

3.5.1. Cell Cultures

3.5.1.1. Plasmodium falciparum

A number of different *P. falciparum* lines adapted for laboratory culture were used. The parasite lines used were the CS2 (known to bind to CSA and an accepted model of MiP parasites [86, 100]), a CS2 transfected with a plasmid containing a green fluorescent protein (GFP) expression cassette (kindly provided by Danny Wilson and James Beeson, WEHI) [101], the CS2 parental line E8B-ICAM (selected for its high binding to ICAM-1 and not CSA [102]) and a genetically distinct parasite line, 3D7, known to be a CD36-binding line, which is the most common parasite phenotype found in non-pregnant individuals [102].

3.5.1.1.1. Culture of Plasmodium falciparum

All parasite lines were cultured according to the method of Trager and Jansen [103], with slight modifications. The culture medium used was RPMI-2-N-hydroxyethylpiperazin-N-2-ethane sulphonic acid (RPMI-HEPES) supplemented with 0.2% w/vol NaHCO₃. CS2 and CS2-GFP were further supplemented with 0.5% Albumax II (GIBCO™) while E8B-ICAM and 3D7 received 0.25% Albumax II (GIBCO™) and 5% vol/vol of pooled human sera (gift from the Australian Red Cross Blood Service). The GFP expression plasmid of CS2-GFP was maintained episomally by the addition of 4µg/ml of Blasticidin-S-HCL (Invitrogen) to the culture medium and GFP expression was confirmed by staining pRBC cultures with 10µg/mL of Ethidium Bromide (EtBr, Bio-Rad Laboratories) for 15 min at room temperature and comparing EtBr fluorescence in the FL-2 channel with that of GFP in the FL-1 channel using a FACSCalibur flow cytometer (BD Biosciences). All parasite lines were maintained at 37°C in a low oxygen atmosphere composed of 1% O₂, 5% CO₂ and 94% N₂. Type O human RBC (provided by the Australian Red Cross Blood Service) were used in all cultures. All parasites were selected for the expression of Knobs by gelatine flotation (5.1.1.4) every 2 weeks and synchronised when needed by the use of Sorbitol (5.1.1.3). Parasitaemia was checked by the use of thin blood smears (5.1.1.2).

3.5.1.1.2. Monitoring cultures by thin blood smears – Giemsa Staining

Both the stage and the parasitaemia of *P. falciparum* cultures were monitored using thin blood smears stained with Giemsa stain, as previously described [104]. Briefly, a drop of parasite culture was dropped onto a glass slide and smeared along it. After allowing it to air-dry, the slide was placed in absolute methanol for 30s and stained in 10% Giemsa stain in tap-water for a minimum of 5-10 min. The slides were then air dried and viewed under a light microscope with the help of immersion oil and a 100x objective. Parasitaemia was calculated by counting the percentage of pRBC relative to total RBC present in ten

microscope fields. All the different blood-stages of the parasite are able to be observed using this technique.

3.5.1.1.3. Synchronising *Plasmodium falciparum* cultures – the Sorbitol Technique

The synchronisation of parasite cultures with Sorbitol by the method of Lambros and Vanderberg [105] (used here with slight modifications) allows us to highly enrich the parasites in one specific stage of their cycle (in this case, early rings). Briefly, parasite cultures exhibiting early-ring stages were centrifuged at 400xg and resuspended in a 5% w/vol solution of D-Sorbitol in water at 10% hematocrit at 37°C for 5 min. The suspension was then centrifuged again and washed with RPMI-HEPES before being put back in culture. This culture would consist of RBC and early-ring stage pRBC only.

3.5.1.1.4. Selecting *Plasmodium falciparum* cultures for knobs – The Gelatin flotation technique

The enrichment of parasite cultures for those parasites expressing knobs at their surface was done, with slight modifications, according to the method described by Goodyer et al [106]. Briefly, parasite cultures exhibiting mid-to late trophozoite stages were centrifuged at 400xg and resuspended in a 0.75% w/vol solution of gelatin in RPMI-HEPES at 10% hematocrit. The cells were left in suspension for 45 min in a low oxygen atmosphere composed of 1% O₂, 5% CO₂ and 94% N₂ resulting in a pellet of RBC and pRBC (rings and knobless mature stages) and in a supernatant of knob-expressing pRBC. This supernatant (usually the top 2/3 of it) were then collected and washed twice with RPMI-HEPES before being put back into culture with fresh RBC.

3.5.1.1.5. Purifying trophozoite stages of *Plasmodium falciparum* The Percoll gradient technique

Parasite trophozoites, for use in the phagocytosis assays, were purified from parasite cultures using the Percoll method as described [107], with minor modifications. Briefly, parasite cultures were overlaid on a gradient of 80%, 60% and 40% Percoll (Amersham) in RPMI-HEPES. After centrifugation at 2,000g x 15 min, cells resting on the 60% layer were collected and washed thrice at RT with RPMI-HEPES (400g for 3 min). After this the cells were resuspended in the appropriate volume of Thp-1 culture medium until exposed to the Thp-1 cells for phagocytosis.

3.5.1.2. Culture of Thp-1 cells

Thp-1 cells were maintained in an humidified 37°C incubator with 5% CO₂ in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS, GIBCO), 1% penicillin-streptomycin (GIBCO), 2 mM Glutamine (GIBCO), 25mM HEPES (GIBCO) and 55x10⁻³ mM 2-Mercapto-Ethanol (SIGMA) at a density below 5x10⁵ cells/mL. Thp-1 cells were obtained from the ATCC (catalog number: TIB-202™). Undifferentiated Thp-1 cells used in our assays had not been exposed to agents such as PMA or TPA (12-O-tetradecanoylphorbol-13-acetate) [92, 93].

3.5.2. The phagocytosis assay

3.5.2.1. A standard phagocytosis assay using uThp-1 cells and CS2-GFP parasites

Mid- to late-trophozoite stage CS2-GFP pRBC (for other parasites, stained with Ethidium Bromide, see below) were purified by density gradient centrifugation, as described in section (5.1.1.5). Cells were then resuspended at 5x10⁷ cells/mL and incubated with defined amounts of either purified antibodies or serum, according to the test to be performed (see below). Typically, in a 96-well u-bottom plate previously coated with FBS, 3.3µL of serum samples and either dilutions of a standard patient pooled serum (pps) or of a polyclonal rabbit anti-human RBC IgG antibody (Abcam) (for use as a standard curve) were incubated with 30µL of pRBC suspension and mixed. This included one well with no serum sample (no-serum control) and one well with no parasite (Thp-1 alone control). pRBC were opsonised by the serum for 1h at RT in the dark and resuspended once, within that hour. The cells were then washed thrice as before and resuspended in 150µL of Thp-1 medium. Following this 50µL were aliquoted into three wells of a final 96-well u-bottom plate.

While the pRBC were being opsonised, Thp-1 cells were collected, centrifuged at 300g for 5 min at RT and resuspended at 5x10⁵/mL in Thp-1 medium.

One hundred microliters of the Thp-1 suspension were dispensed into each well of the final plates which contained 50 µL pRBC suspension. The cells were mixed and left to phagocytose for 40 min at 37°C in a 5% CO₂ humidified incubator. Phagocytosis was stopped by centrifugation at 4°C, 350 g for 3min. The supernatant was discarded and the cells were resuspended at 37°C in 150 µL of FACS Lysing solution (BD Biosciences) at a 1/10 dilution in distilled water, and left at RT for 10 min in order to eliminate unphagocytosed pRBC. Lysis was stopped by the addition of 50 µL of cold PBS (-Ca²⁺, -Mg²⁺) 2% FBS and 0.02% NaNO₃ (FACS Buffer). The plates were centrifuged at 4°C, 350 g x 3 min. After

3 washes with 150 μ L of cold FACS Buffer at 4°C, 350 g x 3 min, the cells were fixed in cold 2% Paraformaldehyde in PBS and left on ice before acquiring.

3.5.2.1.1. Flow-cytometry

Cells were acquired in a FACSCalibur flow cytometer. Thp-1 cells alone were first gated on the basis of their forward- and side-scatter properties. Their level of auto-fluorescence in the FL-1 channel (for CS2-GFP parasites) was set to always be less than 5% (for the procedure when using EtBr stained parasites, see below). Sample triplicates were then acquired, usually at a rate of 250-350 cells/s until 10,000 Thp-1 cells were acquired.

3.5.2.1.2. Analysis of the results

All results were analysed using BD CellQuest™ software version 5.2.1. Triplicate results for samples and standard curves were obtained after subtraction of the “Thp-1 alone” results. Using GraphPad Prism version 4.2 for Windows (GraphPad Software, San Diego California USA), the means of the triplicate standard curve phagocytosis results were plotted against their dilution. The result was a saturation curve. After natural log transformation of the x-axis, a standard curve was obtained. Samples were then transformed in the same way, plotted on the standard curve and transformed back in order to obtain their relative titre.

Two parameters were obtained when using this assay (Figure 3-2):

1. The phagocytic antibodies' function, measuring the percentage of Thp-1 cells that phagocytosed pRBC compared to the maximum percentage obtained in the standard curve (e.g. a result of 30% Thp-1 cells positive on a sample compared to 60% Thp-1 cells positive using positive control serum would give a 50% phagocytic activity);
2. The phagocytic antibodies' titre, which, like an ELISA, compares the test sample to the standard curve of the positive control to derive a relative titre of antibody.

Phagocytosis results higher than the standard curve, by the nature of the assay, were discarded from the analysis.

3.5.2.2. A standard phagocytosis assay using uThp-1 cells and Ethidium Bromide-stained parasites

Not all parasites used in this study had the advantage of having been transfected with GFP. Ethidium Bromide (EtBr) is a DNA intercalating dye that is commonly used for the

separation (in a flow cytometer) of pRBC populations from uRBC [100, 108]. This dye fluoresces highly on the FL2 and FL3 channels.

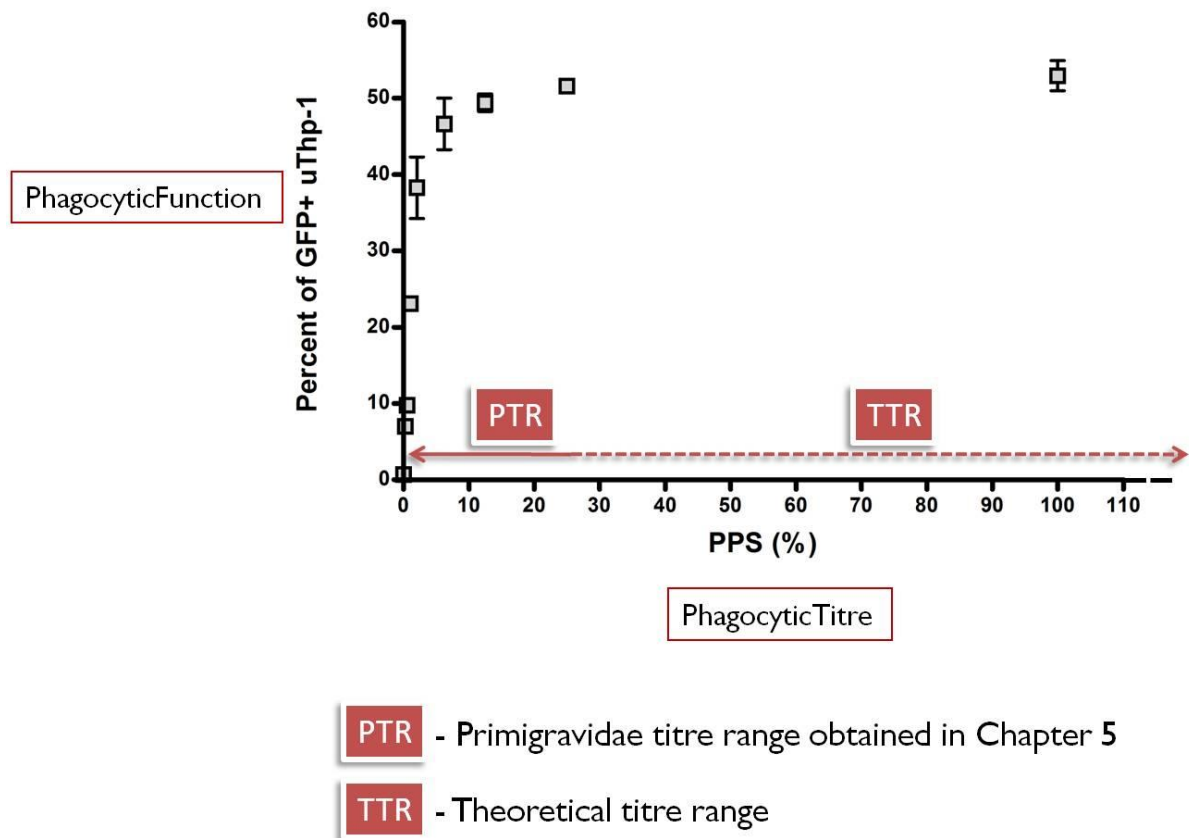


Figure 3-2 – Diagram of the parameters function and titre obtained with the uThp-1 assay

Typical standard curve plot of percentage of patient pooled serum (PPS) used for pRBC opsonisation against percent of GFP-positive uThp-1 cells acquired by FACS. Indications of absolute phagocytosis values used for phagocytic antibodies' function, titre and both the titre range obtained in the study conducted on Chapter 4 of this thesis (PTR) and a theoretical titre range (TTR) (extrapolated from the patient pooled serum titre) are given.

All the steps for the phagocytosis assay using EtBr stained pRBC are the same as mentioned in section (5.2.1) with the following exceptions:

Before opsonisation with the serum or control antibodies the purified pRBC are stained with EtBr. This was done by resuspending the purified pRBC at a density of $1-3 \times 10^7$ in a RPMI-HEPES solution containing 10 $\mu\text{g/mL}$ of EtBr. This was followed by 15 min incubation in the dark at RT and 3 washes with RPMI-HEPES.

Due to the nature of the dye, uThp-1 alone (no contact with parasite) will always have a much lower MFI than any of the other Thp-1 which contacted EtBr-stained parasites. This means that the proper control for this should be uninfected RBC subjected to the same EtBr staining process (and thus giving us an idea of the amount of “free” EtBr in the solution and that has been taken up by the uThp-1) or the use of the non-opsonised pRBC control to set the minimum amount for phagocytosis (see results section for a rationale for this).

3.5.2.3. Phagocytosis assay using human CD14+ monocytes

3.5.2.3.1. Isolation of CD14+ cells

CD14 cells for use in phagocytosis assays were isolated from Buffy coats (BC) provided by the Australian Red Cross Blood Bank.

2:1 volume of room temperature PBS without Ca^{2+} and Mg^{2+} (PBS-) was added to the BC. After calculating 5 mL of Ficoll-Hypaque to each 10 mL of BC/PBS mixture, the volume calculated of Ficoll-Hypaque (15 mL) was added to new 50 mL centrifuge tubes. An overlay, of the corresponding volume of BC/PBS mixture (30 mL) was done. Tubes were centrifuged 30 min 900xg at 18°-20°C without brake. Using a sterile transfer pipette all but 5 mL of supernatant were removed, and kept in a separate 50 mL tube. The remaining 5 mL were vacuum aspirated, thus removing the upper layer containing mostly of the platelets. This was discarded.

Using a sterile transfer pipette the cloudy interface (PBMCs) was removed and placed in new 50-mL centrifuge tubes. This was followed by a wash by adding 3x volume of PBS- and centrifuging 15' at 500xg, 18°-20°C. After tipping off the supernatant, a new wash was done with chilled PBS- and centrifuging at 4 °C. (This discarded most platelets and remaining Ficoll). Five mL of sterile NaCl 0.2% were added. After 3 min 20 mL of chilled PBS- with 2mM EDTA (PBS-E) were added and the wash repeated. Cells were resuspended in 20 mL chilled PBS-E and counted using 100x dilution in Trypan-blue (0.04%).

After this the cells were passed through a filter before starting MACS procedure so as to avoid cell aggregates.

The isolation of specific cells populations was achieved by resuspending the cell pellet in 80µL MACS-Buffer: PBS- with 0,5% FBS and 2mM EDTA (MB) per 10^7 PBMC. After, 20µL of CD14 MicroBeads per 10^7 total cells were added, mixed well and incubated for 15 min at 4-8 °C. Cells were then washed by adding 2 mL of buffer per 10^7 cells and centrifuging at 300xg for 10 min. The supernatant was discarded completely and the cells resuspended, up to 10^8 cells, in 500 µL of MB.

A separation column was then placed in the magnetic field of a suitable MACS Separator (I have used LS columns). The preparation of the column involves rinsing with 3 mL of MB to extract bubbles. The cell suspension was then applied onto the column and the unlabeled cells which pass through were collected, and the column washed with 3x3 mL of MB with every wash flow-through collected. This constituted the CD14neg cell population.

The column was then removed from the separator and placed onto a suitable collection tube. Five mL of MB were then applied into the column and immediately flushed out. The fraction with the magnetically labelled cells is dislodged by firmly applying the plunger supplied with the column. This constituted the CD14pos population.

The cells were then counted and frozen in 10%DMSO, 90% FBS (filter Sterile)

3.5.2.3.2. The new phagocytosis assay adapted to CD14+ cells

For the use of CD14+ cells, the new phagocytosis assay is performed as described in section 3.5.2.1 with the following modifications:

The assay should start by the isolation of CD14+cells. These are plated at 5×10^4 cells/well in Thp-1 medium in 96-well U-bottom plates coated with FBS and left to 'rest' for 1h-2h before the assay is performed. This is done to ensure that cells will not be used under stress from the isolation procedure and that during the 'resting' period the cells remain in suspension and do not begin attachment to the well wall.

3.5.2.4. Minute-by-minute time course of phagocytosis

This was performed in exactly the same way as a regular CS2-GFP phagocytosis assay, but after bringing together the Thp-1 cells and the pRBC, the plate was left at room temperature and the cells were analysed in the FACSCalibur machine without prior lysis of unphagocytosed RBC or fixation.

3.5.2.5. The use of Inhibitors of phagocytosis

In this work two inhibitors of phagocytosis were used.

Cytochalasin-D (C8273, SIGMA) is a potent inhibitor of actin polymerization and thus known to affect active processes like phagocytosis. One milligram of Cytochalasin-D was received, and diluted in 100 μ L of DMSO, after which 900 μ L of PBS. This resulted in a 100x stock solution that was added to Thp-1 cells one hour before the phagocytosis.

FcR blocking agent (Miltenyi Biotec) consists of the Fc' portions of human IgG antibodies and efficiently blocks any Fc γ R induced phagocytosis. This was added to the Thp-1 cells 5 min before the phagocytosis at a 1/40 dilution from the original stock.

3.5.3. Human serum samples used in this chapter

All the samples used in this study derived from studies conducted in Malawi with pregnant women, specifically women enrolled in the UNC and LAIS study cohorts, which have been described already [6, 109]. Both Gaoqian Feng's and Elizabeth H. Aitken's work was re-analysed by me in order to get comparisons between their results and those of my assay and I am very grateful to them for agreeing to give me their results.

Ethical clearance for the UNC study was provided by the College of Medicine Research Ethics Committee, University of Malawi and the Human Research Ethics Committee of Melbourne Health, and ethical clearance for the LAIS study was provided by the College of Medicine Research Ethics Committee and the human research ethics committee of the Walter and Eliza Hall Institute, Melbourne.

3.6. Results

3.6.1. uThp-1 are able to perform phagocytosis of serum-opsonised pRBC

In order to make use of uThp-1 cells as phagocytic cells it was first required to show that these cells were able to perform phagocytosis of *Plasmodium falciparum* infected RBC.

Using CS2-GFP pRBC opsonised with PPS or non-opsonised I was able to show that over 90 minutes uThp-1 could perform phagocytosis and that this phagocytosis could be measured by FACS (Figure 3-3). Early attempts at demonstrating that this phagocytosis was Fc-receptor mediated, using Cytochalasin D showed that Cytochalasin D was not able to completely inhibit the phagocytosis of PPS-opsonised pRBC (Figure 3-3).

3.6.2. Time course of phagocytosis using uThp-1 cells

In order to verify what would be the minimum amount of time required for uThp-1 cells to perform phagocytosis of opsonised pRBC, a time-course of phagocytosis was performed. Four time points were chosen: 15 min, 45 min, 70 min and 90 min, using PPS opsonised CS2-GFP pRBC (Figure 3-4A). The results showed that 40 min was a good time to obtain maximum phagocytosis.

In order to understand the kinetics of the process, a minute by minute phagocytosis assay was also performed, showing that phagocytosis is likely to start occurring after 10 min of contact between uThp-1 cells and opsonised pRBC (Figure 3-4B).

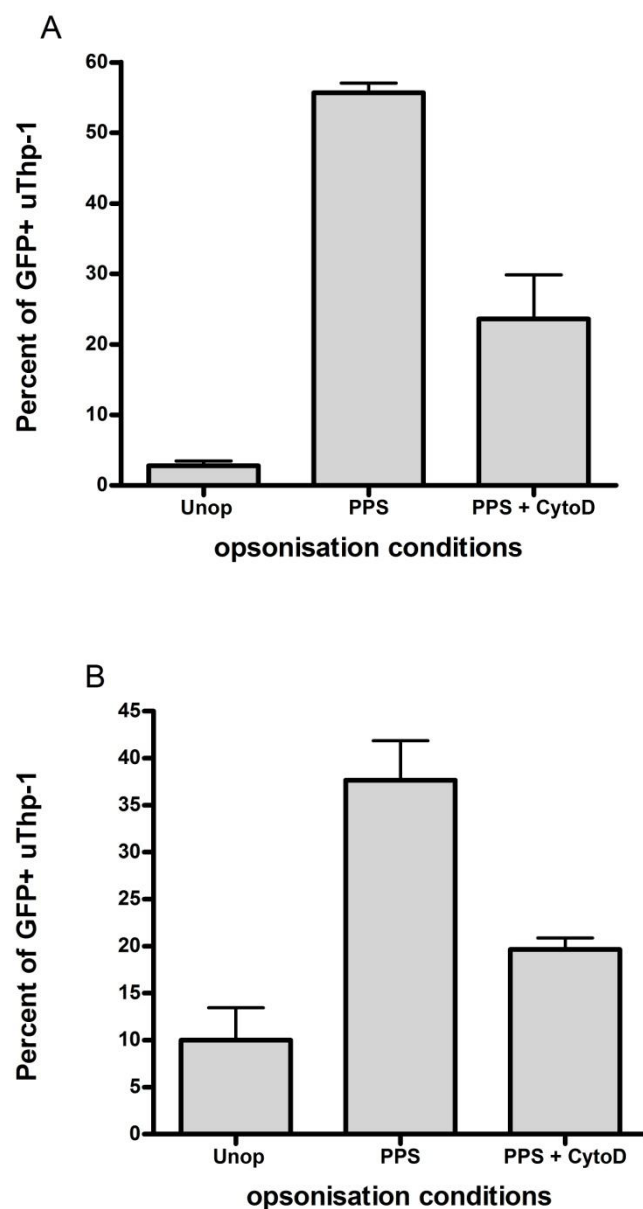


Figure 3-3 – First results obtained using a FACS-based phagocytosis assay with uThp-1 cells

Two examples of results obtained with an earlier version of the phagocytosis assay are plotted. The assay was performed by the method described in section 3.5.2.1 with the following modifications: phagocytosis occurred over 90 minutes and a 3% solution of NaCl applied for 3 min replaced FACS Lysing Solution as the lysing agent. (Unop) unopsonised CS2-GFP pRBC, (PPS) CS2-GFP pRBC opsonised for 1h with patient pool serum with known high reactivity to VSA_{CSA}, (PPS + CytoD) same as PPS except uThp-1 were exposed to 1μ/mL of Cytochalasin D for 1h before phagocytosis.

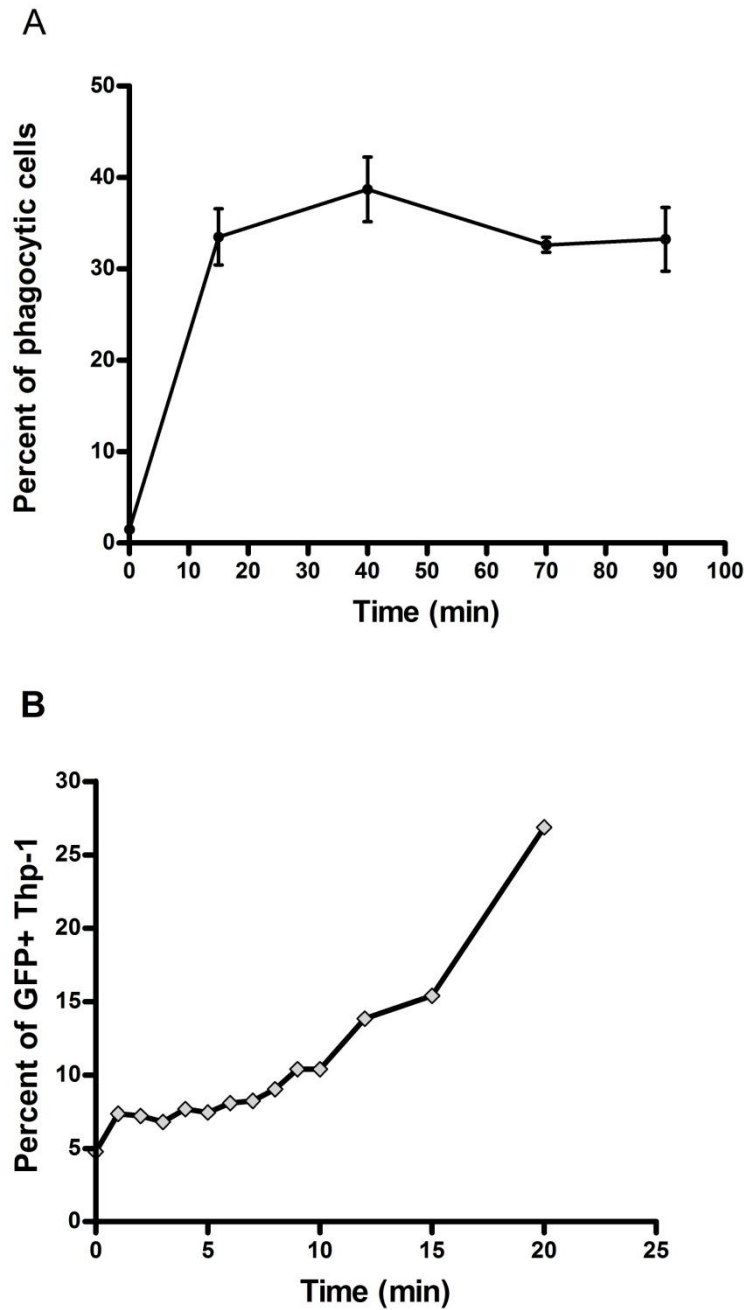


Figure 3-4- Time-course of phagocytosis by uThp-1 cells

The phagocytosis of CS2-GFP pRBC opsonised with PPS was evaluated using FACS over 4 different time periods (A) and in a minute by minute assay (B) in order to understand the kinetics of phagocytosis and choose the best time point to ensure maximum phagocytosis. Graphs represent examples of at least 2 experiments.

3.6.3. uThp-1 are able to detect different amounts of opsonising antibody

When analysing the levels of antibodies present in the sera samples to be tested it is important that assays are able to detect differences in the amount of antibody present.

CS2-GFP pRBC were exposed to different concentrations of human sera with known high amounts of IgG to CS2_{VSA}. These were then mixed with uThp-1 cells for 40 min and phagocytosis was measured by FACS. Undifferentiated Thp-1 were able to detect different amounts of opsonising antibody and did so in a manner that was consistent, providing the possibility of using standard curves to standardise assays (Figure 3-5A and 5B). Consistency of results between different assays, on different days was high: intra-assay variability was minimal, with linear correlation between triplicates of serum samples run in the same day always high ($r^2 > 0.98$) (Figure 3-5C and 5D).

3.6.4. uThp-1 do not phagocytose unopsonised pRBC and seem to mainly perform Fc-receptor mediated phagocytosis

Several *Plasmodium falciparum* parasite lines were used to assess the ability that uThp-1 might have of performing non Fc-mediated phagocytosis. The parasite line E8B-ICAM (selected for its binding capability to Intercellular adhesion molecule 1 (ICAM-1)) and the parasite line 3D7 (known to bind to CD36) were exposed to uThp-1 cells. Positive controls were, in both cases, pRBC opsonised with a polyclonal Rabbit anti-human RBC antibody. Inhibiting phagocytosis of IgG-opsonised pRBC, by inhibiting the process (using Cytochalasin D) or the receptors (using Fc-Block) revealed that uThp-1 cells are semi-refractory to inhibition by Cytochalasin D, since a certain degree of phagocytosis was still observed (Figure 3-3 – First results obtained using a FACS-based phagocytosis assay with uThp-1 cells and Figure 3-7 – Phagocytosis by uThp-1 cells seems to be Fc-receptor dependent). When Fc-Block was used, phagocytosis was completely abrogated (Figure 3-7 – Phagocytosis by uThp-1 cells seems to be Fc-receptor dependent).

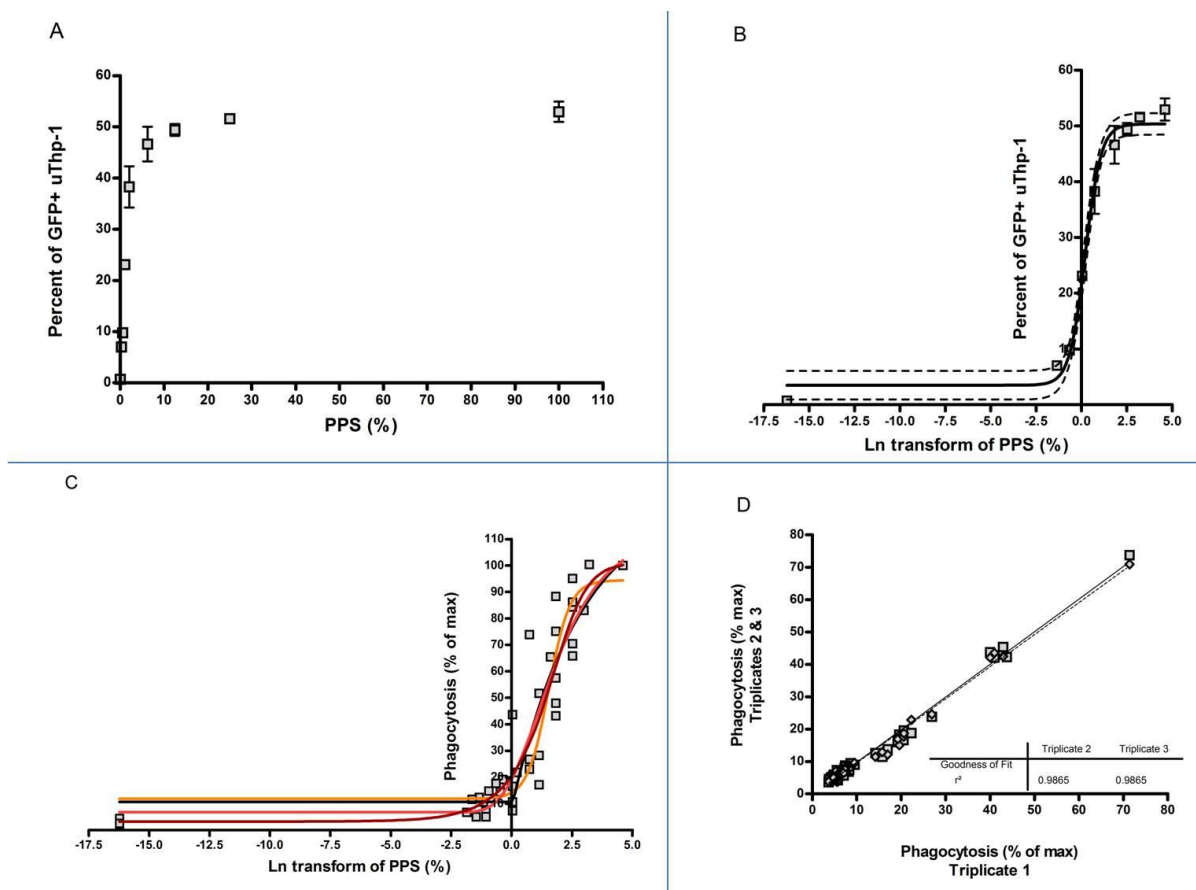


Figure 3-5 – Phagocytosis by uThp-1 using CS2-GFP pRBC: titration of PPS and correlation between intra and inter assays

uThp-1 cells are able to detect different amounts of opsonising antibody on CS2-GFP pRBC. (A) A typical example of several experiments using titrations of PPS opsonised CS2-GFP pRBC is shown. (B) After logarithmic transformation the results can be expressed as a standard curve. Best-fit curve and 95% confidence intervals are plotted ($r^2 = 0.98$). (C) The analysis of several standard curves reveals the similarity of the exponential phase between assays performed on different days. (D) Intra assay variability is practically negligible as reveals the analysis of triplicates of a typical experiment using human serum samples for opsonisation of CS2-GFP pRBC (in C and D, the phagocytosis is expressed as the percentage of phagocytosis obtained with the lowest dilution of each standard curve). (PPS) patient pooled serum.

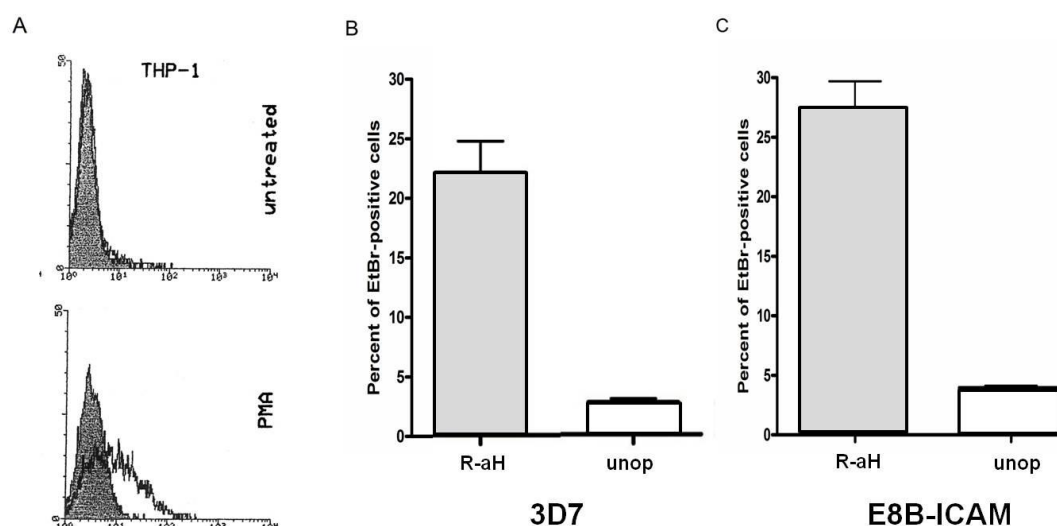


Figure 3-6 – uThp-1 cells do not phagocytose unopsonised pRBC

(A) Flow cytometry analysis of CD36 expression on thp-1 cells showing that uThp-1 cells (untreated) do not express the scavenger receptor CD36, contrary to dThp-1 (PMA) (figure adapted from [29]. Y-axis: number of cells, X-axis: Mean fluorescence intensity). uThp-1 were mainly restricted to phagocytosis of antibody opsonised pRBC failing to phagocytose both (B) unopsonised 3D7 pRBC and (C) E8B-ICAM parasites. Phagocytosis was performed using EtBr-stained pRBC. (R-aH) Rabbit anti-human RBC antibody opsonised pRBC, (unop) unopsonised pRBC. Assays were done in triplicate.

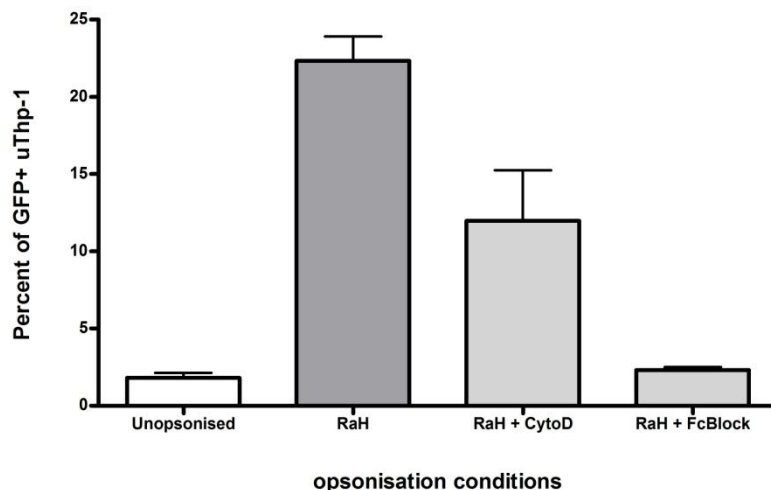


Figure 3-7 – Phagocytosis by uThp-1 cells seems to be Fc-receptor dependent

Phagocytosis of CS2-GFP pRBC either unopsonised (white) or opsonised with Rabbit anti-human RBC antibody (RaH) (grey). uThp-1 were left to phagocytose either alone (white and dark grey bars) or in the presence of phagocytosis inhibitors (light grey bars). Cytochalasin D (cytoD) was added to uThp-1 cells 1h before the assay. Fc-receptor blocking agent (FcBlock) was added 5 min before the assay. See 3.5.2.5.

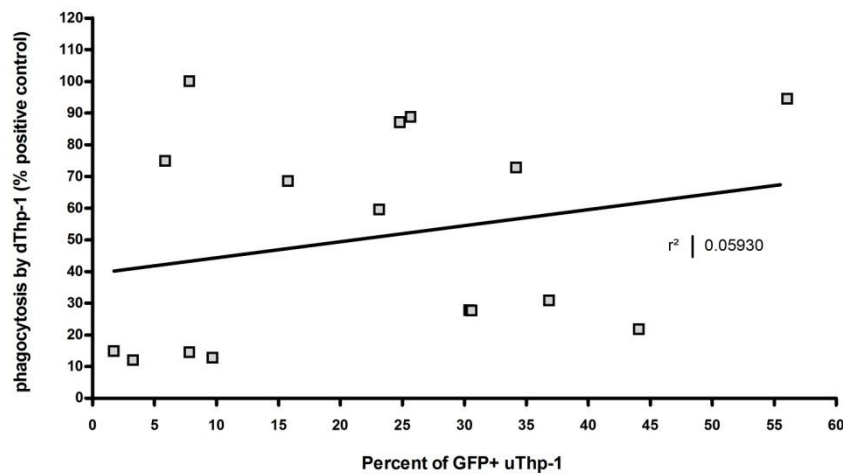
3.6.5. Phagocytosis by uThp-1 cells does not correlate completely with phagocytosis by dThp-1 cells

In order to see how the new phagocytosis assay using uThp-1 assay correlated with the assay that was more commonly performed, using PMA-differentiated Thp-1 cells, I used a few samples from a study cohort that had been studied by Feng, et al using the dThp-1 assay [6]. The results obtained with the new assay were not correlated with those obtained obtained using the dThp-1 method, (Spearman's rank ($\rho = 32$, $P = 0.231$), linear regression ($r^2 = 0.06$, $P = 0.383$) (Figure 3-8A)). The results were better correlated with the ones obtained by the use of donor monocyte-derived macrophages (MDM) also used in the same study (Spearman's rank ($\rho = 79$), linear regression ($r^2 = 66.8$) (Figure 3-8B)). It should be noted that these results were obtained with a small number of samples ($n = 16$).

3.6.6. uThp-1 are a feasible way of detecting patterns of hummoral response to pRBC VSA_{CSA}

When applied to a larger number of serum samples from pregnant women ($n = 141$), the new uThp-1 assay was able to retrieve important and coherent results. As would be expected from known patterns of immunity to pRBC VSA_{CSA}, the new assay was able to show the gravidity dependence of antibody production (Figure 3-9A), the decrease in antibody levels associated with HIV infection (Mann-Whitney ranksum: $z = -3.12$, $P = 0.002$, Figure 3-9B) and the association of antibody levels with anaemia (Mann-Whitney ranksum: $z = -2.0$, $P = 0.045$, Figure 3-9C), all of which published by Feng, et al using the dThp-1 phagocytosis assay [6].

A



B

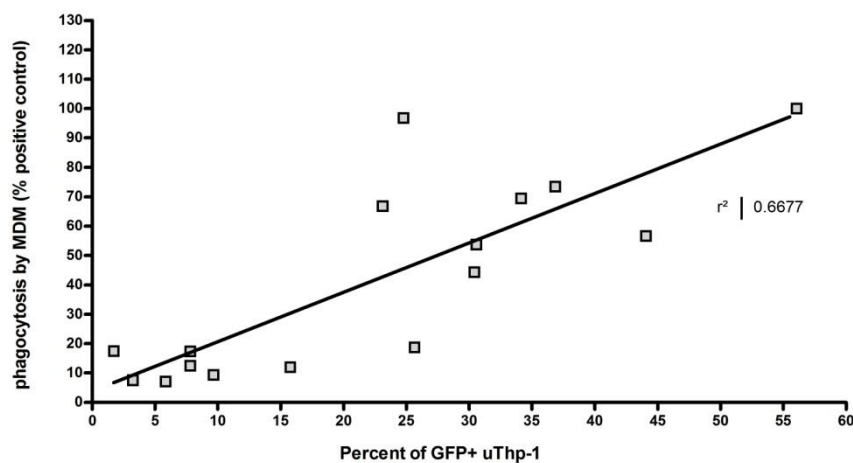


Figure 3-8 – Correlation of new uThp-1 phagocytosis assay with those using dThp-1 cells and MDM (using results obtained by Feng, et al [6])

The new uThp-1 assay was applied to 16 random samples from a cohort of pregnant women being studied by Feng, et al [6]. **(A)** Results obtained with the new uThp-1 assay, in this set of samples had little correlation to those obtained using dThp-1 cells, Spearman's rank ($\rho = 32$), Linear regression ($r^2 = 0.06$). **(B)** Results obtained with the new uThp-1 assay correlated with those obtained using MDM, Spearman's rank ($\rho = 79$), Linear regression ($r^2 = 66.8$). For a detailed description of the dThp-1 and MDM assays refer to the reference given above. Each point represents the mean of duplicates.

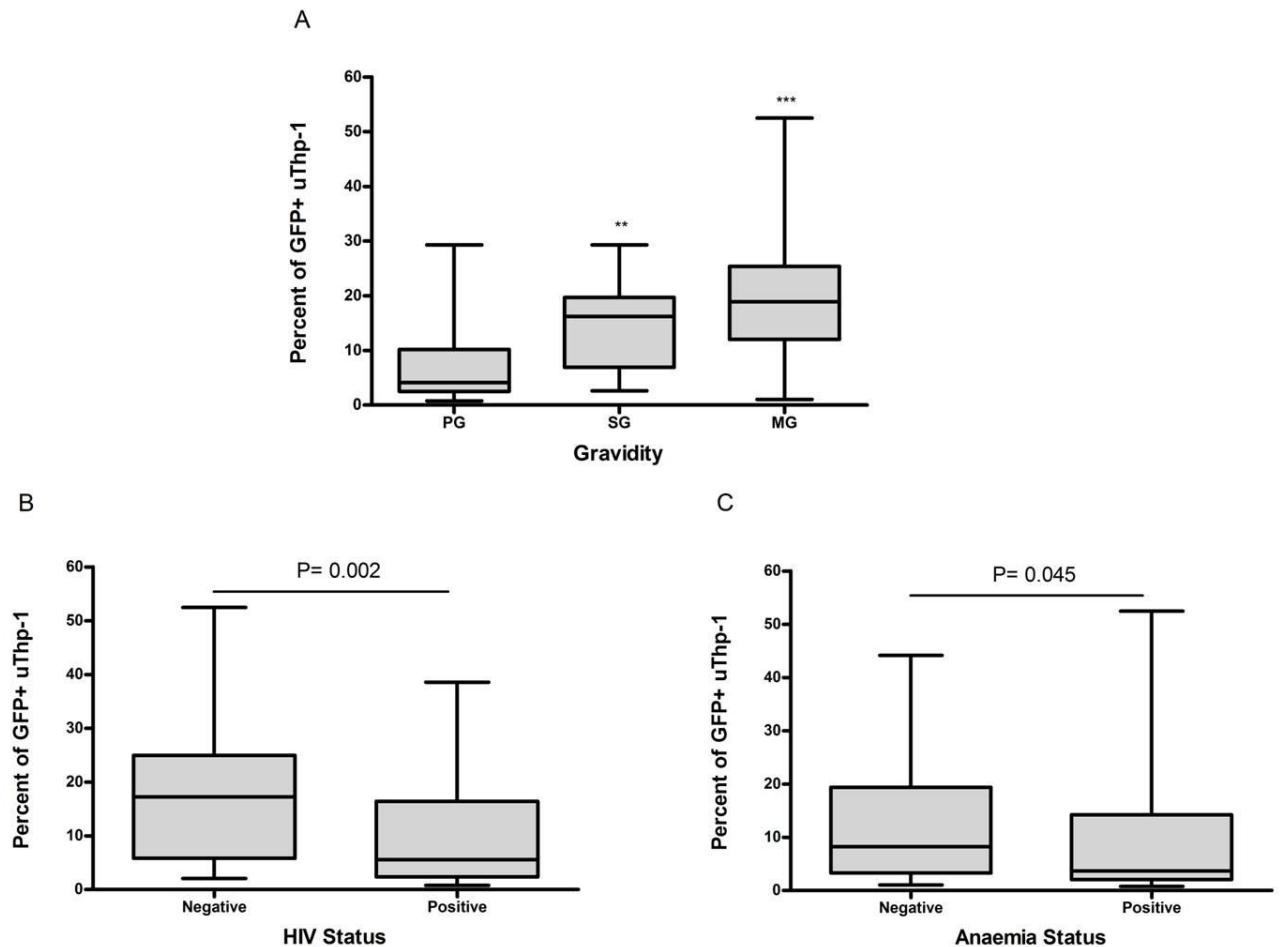


Figure 3-9 – Applying the uThp-1 phagocytosis assay to large study cohorts

The new uThp-1 phagocytosis assay was applied to a cohort of pregnant women for whom studies were already being conducted (see [6]) in order to verify the capability of the assay of detecting clinically relevant information. **(A)** The percentage of uThp-1 cells that had phagocytosed CS2-GFP pRBC opsonised with different patient samples correlated well with the gravidity status of the women. Mann-Whitney ranksum tests detected significant differences between PG and SG (** $P < 0.01$) and between PG and MG (*** $P < 0.001$). **(B)** Phagocytosis of CS2-GFP pRBC opsonised with patient serum samples by uThp-1 cells was able to detect a decrease in the levels of phagocytic antibodies in HIV positive women when compared to HIV negative women. P-value for Mann-Whitney ranksum is shown. **(C)** Phagocytosis of CS2-GFP pRBC opsonised with patient serum samples by uThp-1 cells was able to detect an association between phagocytic antibodies at time of enrolment and maternal anaemia status at delivery. P-value for Mann-Whitney ranksum is shown

3.6.7. Donor derived CD14+ cells can be kept in suspension and used in the assay, replacing uThp-1 cells: Proof of concept.

CD14+ cells from a single Melbourne donor were isolated and used in a phagocytosis assay in replacement of uThp-1 cells. Cells were left to phagocytose for 40min and 90min, EtBr-stained CS2 pRBC either unopsonised or opsonised with PPS were used as targets for phagocytosis. The results obtained suggest that, as for uThp-1, 40min are enough for the measurement of phagocytosis and, at least for CS2 pRBC, there is no phagocytosis of unopsonised parasites (Figure 3-10)

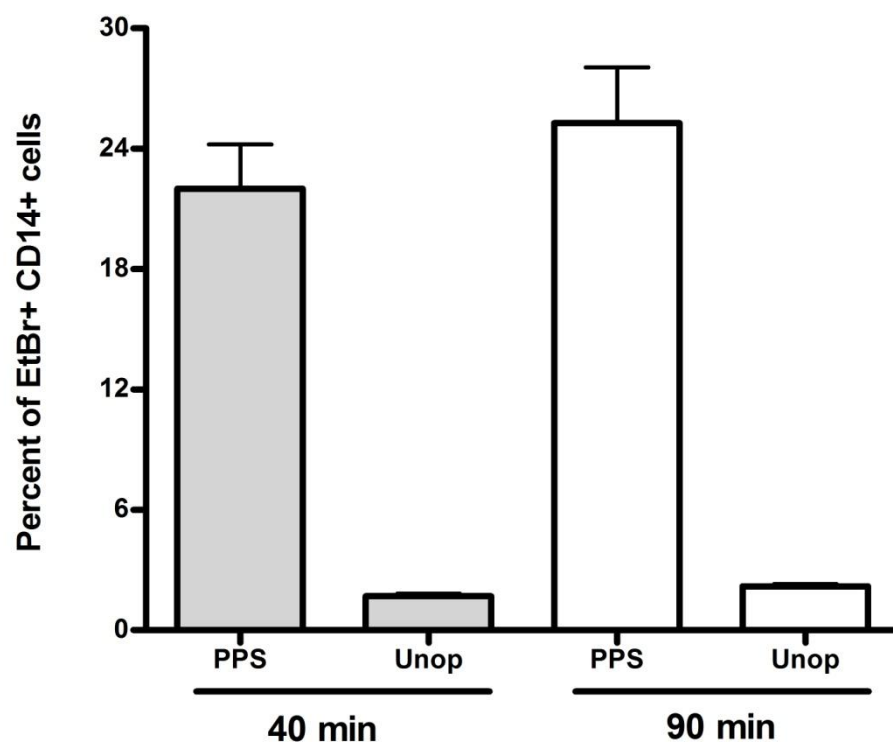


Figure 3-10 – Phagocytosis assay performed by CD14+ cells: proof of concept.

Donor derived CD14+ cells were isolated from a Melbourne donor and used in the new phagocytosis assay replacing uThp-1 cells. Cells were allowed to phagocytose for 40min or 90min EtBr-stained CS2 pRBC opsonised with positive serum (PPS) or unopsonised (Unop). Assay was conducted in triplicates.

3.7. Discussion

3.7.1. uThp-1 cells can perform phagocytosis and represent an alternative over the use of differentiated cells for the measurement of Fc-Receptor mediated phagocytosis

Undifferentiated Thp-1 cells had previously been shown to be useful in measuring erythrophagocytosis [110], but no high-throughput assay concerning Fc-mediated phagocytosis that could actually be used consistently on large sample sets existed.

In this chapter a new, high-throughput method has been developed and tested in order to accurately and rapidly estimate Fc-receptor mediated phagocytosis induced by *P. falciparum* malaria-specific antibodies.

This new method facilitates the measurement of functional antibodies to malaria VSA in large sample sets. Based on the use of undifferentiated Thp-1 cells that grow in suspension, this assay lends itself to various small modifications that might be used to study both the effector cell response to pRBC expressing specific ligands, and the antibody responses to these pRBC. Although this method and the bulk of the work presented in this chapter was not designed to directly compare the dThp-1 assay [6, 21, 111] with the new uThp-1 assay, I believe it is important to make comparisons, where comparisons can be made. There are several pertinent differences between the two assays, as can be seen in Table 3-1.

Table 3-1 – Main differences between the new uThp-1 assay and the commonly used dThp-1 assay

New assay		Commonly used assay	
Undifferentiated Thp-1 assay (uThp-1)		Differentiated Thp-1 (dThp-1)	
Cells plated and used in the same day		Cells plated, stimulated and used 3 days later	
Cells in suspension		Cells are adherent	
56 Samples per 1 day/Assay (triplicate)		36 Samples per 3 day/Assay (triplicate)	
Fluorescence based Assay – FACS		Colorimetric based assay- Plate reader	
Read-out: Percentage of phagocytic cells that have ingested labelled pRBC		Read-out: Amount of haemoglobin released by lysis of phagocytic cells	
Possibility of including other parameters in the study (e.g. cell surface markers)*		Only one parameter measured	
Phagocytic cells readily accessible for further studies*		Phagocytic cells destroyed	
Phagocytosis almost exclusively via Fc-receptors		Phagocytosis through various receptors/mechanisms*	

Comparison between phagocytosis assay done using undifferentiated Thp-1 cells (used in this study) and chemically- differentiated Thp-1 cells. * these parameters were not evaluated in these thesis, but can logically be derived from characteristics of both cell types/assays.

The new uThp-1 assay is able to run 168 samples (in triplicates) over the time period that takes the dThp-1 assay to run 36 samples (in triplicates), thus providing a much more time-saving method not to mention a more suitable one to use with large sample sets. The high consistency of intra-assay results (Figure 3-5) permits us to run the assay in duplicates instead of triplicates thus increasing even more the amount of samples that can be measured. The use of FACS allows us to have an immediate perception of the amount of cells present in the wells when acquiring the results (through the rate at which cells are acquired, which is relative to their concentration), something that cannot be done in the dThp-1 assay where each well has to be observed under a light microscope if we want to verify an even distribution of dThp-1 cells in the well (not to mention that no feasible cell count can be made).

Using this assay on a large number of serum samples from pregnant women proved that this is a fast, reliable and sensitive method for the detection of phagocytic antibodies in human cohorts. Nevertheless, from the work presented in this thesis, it is difficult to compare the efficacy of both the uThp-1 and the dThp-1 methods in the detection of clinically relevant information with regards to antibodies towards *P. falciparum* VSA since these assays were conducted by different people, using different controls and different methods of analysis of results [6].

3.7.2. Standard curves

The use of standard curves in the new assay allows the immediate comparison of the behaviour of the uThp-1 cells across different experimental days and as such is an assurance of inter-assay consistency of results. Standard curves, should ideally be made with the use of an antibody that will opsonise the surface of the pRBC, irrespective of the amount of parasite VSA present, or of the parasite line used. It was with this in mind that I started using the Rabbit polyclonal anti-human RBC antibody.

3.7.3. Fc-Receptor mediated phagocytosis

Undifferentiated Thp-1 cells look to be of particular interest in the study of phagocytic antibodies since it is clear that the IgG-mediated phagocytosis is performed through Fc γ receptors I and II, the only Fc γ receptors present in these cells [97]. Of note, uThp-1 cells do not express CD36 [29] a known cell-membrane receptor that can promote phagocytosis of unopsonised pRBC [30]. Undifferentiated Thp-1 cells were unable to phagocytose unopsonised pRBC of the CD36-binding parasite line 3D7, and also, although uThp-1 express ICAM-1, the ICAM-binding parasite line E8B-ICAM did not trigger a phagocytic re-

sponse in the absence of immune serum, demonstrating that cytoadherence *per se* does not trigger uThp-1-mediated phagocytosis.

Although exposed to CytoD concentrations shown to be able to inhibit the phagocytic activity of macrophages [112], uThp-1 cells were still able to perform some phagocytosis of opsonised pRBC. This may represent a poor uptake of CytoD by the cells or a lack of effect of this compound on these particular cells. Since no studies of the effect of this drug on uThp-1 cells were done so far, it is difficult to dissect the reasons why phagocytosis was still performed. It was not the goal of this thesis, though, to dissect molecular mechanisms behind the phagocytic process of Thp-1 cells. That the uptake of pRBC opsonised with IgG was mediated by Fc-Receptors, was clearly demonstrated by the use of Fc-Block. This blocking agent is nothing more than the Fc' portion of human IgG and thus binds to Fc γ receptors preventing the binding of subsequent IgG (Figure 3-7).

3.7.4. Assay plasticity and future studies

The assays reported here were performed using GFP expressing parasites and EtBr stained parasites thus allowing for the study of virtually every parasite line. Although not shown in this thesis, with the proper controls and parasite lines (for instance a parasite line that is transfected with GFP and one with Red fluorescent protein, RFP), it is even possible to conduct studies using 2 parasite lines at the same time. This could be used, for example, to verify how the presence of a specific parasite type may affect the phagocytic response to a second type.

Positively selected donor monocytic cells can ultimately also be used (Figure 3-10), which will permit the large scale study of variations in host phagocytic cell responses. If, for instance, other parameters like cell surface molecules (e.g. CD80, CD86, CCR5) are included in the assay and together with this, analysis of Fc-receptor polymorphisms [37] is conducted, then it is possible to start getting an idea of the potential that this assay carries for characterising function and phenotype of donor monocytes on a large scale.

The development of a fast, accurate and sensitive method for the measurement of antibody responses to VSA_{CSA} provided the perfect tool for the study of PG and SG women performed in the following chapter.

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4. Impact of HIV on the acquisition of antibodies: Studies on Primigravidae and Secundigravidae

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4.1. Summary

As was reviewed in Chapter 3 of this thesis, in malaria endemic areas, pregnant women, that would otherwise be protected from clinical malaria after acquiring a degree of immunity over the years, suddenly become susceptible to it and can suffer from severe clinical outcomes like maternal anaemia [1-3], infant low birth weight [2, 4] and miscarriages [1]. Primigravidae (PG) are widely recognised as the most susceptible group of pregnant women [1-3]. Malaria in pregnancy (MiP) has been linked to the appearance of *Plasmodium falciparum* (*P. falciparum*) parasites with the capacity to bind to the placenta of these women [3] by expressing a Variant Surface Antigen (VSA) that is able to bind Chondroitin Sulphate A (CSA) (VAR_{CSA}) [5, 6]. Susceptibility to MiP tends to decrease over successive pregnancies [1-3] in part because the development of antibodies towards these VAR_{CSA} confers a certain immunity. However, development of immunity to MiP in PG and SG, specifically, is still poorly understood [7, 8]. In those areas where HIV and *P. falciparum* co-exist, higher rates of malaria infection, higher densities and prevalence of parasitaemia and lower levels of antibodies to pregnancy-specific VSA are associated with HIV infection making the combined presence of both HIV and malaria particularly deleterious for the health of both mothers and newborns [9-13]. Also, antibodies that specifically contribute to the phagocytosis of opsonised pRBC were shown to be decreased in the serum of HIV-positive MG women but reported missing in PG [14].

Using the new phagocytosis assay I developed (see Chapter 3), I decided to look at PG and SG from the same cohort separately and to determine the presence of phagocytic antibodies in these two groups of women and to evaluate the impact that a co-infection with HIV would have. The relationship between the presence and level of these antibodies with the occurrence or protection from adverse clinical outcomes was also assessed. I was able to show that PG are able to respond quickly to infection and to develop phagocytic antibodies and that the acquisition of these antibodies is severely impaired by HIV. In PG, phagocytic antibodies were not associated with protection from complications of MiP but I hypothesised that the presence of phagocytic antibodies would be more important during a second pregnancy. With that in mind I analysed the group of SG in the same cohort and was able to show that, indeed, phagocytic antibodies at delivery protect from infant birth weight, independently of age, maternal weight or HIV status.

4.2. Literature Review

4.2.1. Primigravidae and Secundigravidae: humoral immunity to malaria in pregnancy

4.2.1.1. The early years

Until the second half of the last decade of the 20th century almost nothing more was known about how MiP affected PG and SG women than was already known for almost 40 years before that [1-3]. This knowledge was based on observations that could not easily be explained. It had been observed that pregnant women were more susceptible to malaria than their non-pregnant counterparts and that, among these pregnant women, PG were the most affected, suffering from severe anaemia and delivering low birth weight (LBW) babies [1-3]. Antibodies generated towards malaria during pregnancy had been the focus of some interest and since the late 1960s, early 1970s, were even known to be mainly of the IgG class [15, 16], but at this time the field of antibody-mediated immune response during MiP was non-existent and would remain so for a few years more. During the 1980's, although the puzzle over what made PG so much more susceptible continued, few studies addressed this question. Investigations regarding the possibility that decreased antibody levels could explain susceptibility were hindered due to the lack of knowledge of the unique parasite phenotypes infecting these women against which antibodies could be tested, (this would be available in subsequent years) The few studies that looked into antibodies towards MiP, and especially in PG, failed to detect any consistent associations between an antibody response, or even the lack of one, with MiP and its gravity-dependent severity [17-19].

4.2.1.2. First measures of levels of antibody: before CSA.

The observation by Adele et al [20] that IgG was present in the sera of Caucasian patients experimentally infected with *Plasmodium vivax* (*P. vivax*) prompted investigators to search for these anti-plasmodia antibodies in populations resident in malaria endemic countries. Pregnant women were the study population chosen by Williams et al [15] to show that indeed these IgG antibodies were present.

Early studies that specifically addressed the antibody response of PG mainly used antigens derived from peripheral-type parasites and/or ring-stage parasites, like the ring-infected erythrocyte surface antigen (RESA). It is not clear why researchers at this point had not taken into account the earlier studies of Williams et al [21] showing how a malarial antigen derived from the placenta could be obtained, but one might speculate that re-

searchers at that time did not consider placental parasites to be any different from the ones found in the periphery. It is also worthwhile mentioning that this search for anti-malarial antibodies in pregnant women was not so much focused on the process of MiP but instead on understanding how maternal immunity resulted in conferred immunity for the newborn [22].

Deloron et al [18], interested in the high prevalence of MiP in PG compared to multigravidae (women with 3 or more pregnancies, MG), compared the levels of anti-*P. falciparum* IgG antibodies in Nigerian nulligravidae (no pregnancies, NG), PG and MG and found fewer PG had anti-*P. falciparum* IgG compared to MG or NG, but these anti-*P. falciparum* IgG levels did not predict protection from infection, either individually or as a group. A lack of protection and a lack of association between antibodies towards whole pRBC or RESA peptides and gravidity was then shown in subsequent studies [19, 23-27]. At the time, as cortisol, a known immune-suppressor, was shown to be higher in PG it was proposed that cellular immune-suppression was responsible for the PG susceptibility. At this point SG were not the object of any particular study as an individual group, but were always included in the MG group. The only relevant study that looked at SG was conducted by Fievet et al [28] in which they looked at cellular responses to several antigens (of malarial and non-malarial origins) during first pregnancy, after delivery and during a second pregnancy (always following the same women). They also looked at antibody levels but once again, and due to the use of RESA and a periphery-type parasite line, no major differences between groups were found as some antigens were higher in PG than SG, others were higher in SG, and others still outside of the context of pregnancy.

4.2.1.3. Development of antibodies: the post CSA-adhesion era

As was already mentioned, the discovery of CSA as an adhesion molecule for *P. falciparum* pRBC and its role in placental adhesion [5, 6] brought some light into an otherwise “shot in the dark” affair.

Fried et al, in 1998, showed how antibodies towards CSA-binding parasite isolates from placentas could inhibit the CSA-binding phenotype and were more prevalent in MG and SG than in PG where, as it was reported in that paper, they were absent. They also found that serum from Asian women were able to inhibit the binding of African-derived parasite isolates to CSA and vice-versa, thus pointing to the existence of a geographically conserved ligand in these parasites [29]. After this work, studies were able to find agglutinating [30, 31] and anti-adhesion antibodies [32] in PG, using both parasite isolates and a CSA-binding parasite line. Also, the gravidity-dependence demonstrated for susceptibility

to MiP could now be associated with the levels of these antibodies [31, 32] and thus bring an almost century year old question to rest.

4.2.1.4. The kinetics of antibody production

Now that the existence of antibodies towards CSA-binding *P. falciparum* isolates/lines seemed established, the question became: at what time during pregnancy did these antibodies develop?

O'Neil-Dunne et al [33] published a study concerning the kinetics of production of antibodies both in primigravidae and multigravidae from Yaounde in Cameroon, where the transmission of malaria is perennial. They used a laboratory line, 3D7-CSA parasites, panned on placental CSPG (Chondroitin sulphate proteoglycan). Strangely, in this study, no antibodies that inhibit adhesion of parasites to CSPG were found in multigravidae before 12-16 weeks of gestation but high levels of antibodies ensued. Primigravidae only had antibodies coming up at 20-24 weeks of gestation. After the 20-24 weeks of gestation period there were no differences between both groups of women in terms of the inhibitory antibody level. This seemed to be confirmed by other studies that also set the onset of antibody acquisition in PG at 20 weeks gestation [34]. Later studies have raised the possibility of these antibodies developing as early as the 10th week of gestation and a recent study by Aitken et al that made use of ultra-sound technology for the precise assessment of gestational age seems to point to 14 weeks of gestation, although this can actually be earlier as 14 weeks was the minimal gestational age of enrolment for the study in question [35].

4.2.1.5. Var2CSA and its domains: recognition by Primigravidae and Secundigravidae

The discovery of the *var2csa* parasite gene and its encoded VAR2CSA surface protein [36, 37], which seems to mediate the binding of placental-type parasites to CSA [36-39] led researchers to the identification of specific domains in this protein [40, 41] which not only mediate the binding to CSA but also towards which antibodies could be generated. The search for such antibodies and the specific domains to which they could bind showed that two domains in particular seem to be the targets of the immune response: DBL3X [42, 43] and DBL5ε [43-45].

Primigravidae were shown to have antibodies that were able to bind to motifs in the DBL3X domain. The number of motifs recognised was inferior to the number of motifs recognised by MG [42]. This matched also the results obtained when looking at the rec-

ognition of all the domains of VAR2CSA, where it was shown that PG had lower levels of antibodies to all of the domains when compared to MG [43]. It seems, though, that DBL5 ϵ may constitute an important domain for PG. Guitard et al have shown that at delivery, uninfected PG had higher levels of IgG3 towards DBL5 ϵ than infected PG [44] while Brodin et al showed that DBL5 ϵ is recognised in a parity-dependent pattern, contrary to DBL3X. Also, DBL5 ϵ has been recently shown to possess epitopes that are recognised at different levels between PG and MG [46].

4.2.1.6. Antibodies and clinical outcomes: a protective role or a marker of infection?

There are a panoply of results obtained in the studies conducted so far regarding the protective role of antibodies during MiP and it seems that no particular pattern can be discerned for either PG or SG.

Depending on the study, antibodies towards CSA-binding parasites were found to be associated with decreased placental parasitaemia or parasite density in SG [29, 31], in PG and SG [8], just in MG [34], in all gravid women [33], or even not associated at all [47].

Both maternal haemoglobin and infant birth weight can be negatively affected during MiP [1-4]. Antibodies directed at pRBC expressing VSA_{CSA} and that can protect pregnant women from these deleterious effects were first described in SG where they were associated with protection from low birth weight (LBW) babies [8], although this study was cross-sectional and thus did not allow for a true protective verdict to be obtained. A relationship between antibody and protection was reported to be absent in both PG and MG, although the absence of an association in MG was suggested to be due to the presence of these antibodies in all MG women. Besides this study though, no other study was able to demonstrate any protective effect of antibodies towards VSA_{CSA} in SG or PG as individual groups.

What seems clear is that, at least for PG (for SG it is harder to conclude since not many studies consider this population independently), antibodies towards pRBC expressing VSA_{CSA} are a sound marker of placental *Plasmodium* infection. This has been observed in many studies [7, 31, 47, 48].

4.2.1.7. Determinants of humoral immunity

4.2.1.7.1. Anti-malarial drugs and the use of Bed-Nets

Early observations led researchers to conclude that the use of anti-malarial drugs had the capacity to decrease a pregnant woman's levels of anti-malarial antibodies, presumably due to a reduced amount of exposure to antigen [15, 18, 22, 49].

When antibodies towards VAR_{CSA} expressing parasites were investigated in PG receiving Intermittent Preventive Treatment (IPTp) with sulphadoxine pyrimethamine (SP) (refer to Chapter 3 for IPTp definition), it was shown that women receiving SP had lower levels of anti-VSA_{CSA} antibodies than women receiving placebo [50]. Interestingly, this study also showed that the antibody levels to non-CSA binding VSA were not affected by the receipt of IPTp-SP. A later study contradicted this result showing that no difference existed in the levels of antibodies to VSA_{CSA} in PG but this later study looked at a very small number of women [51]. Another recent study seems to point to PG having a low antibody response to VSA_{CSA} in the presence of IPTp, although this study also revealed low levels of *P. falciparum* infection [35].

No studies have, so far, looked specifically at the effect of insecticide-treated nets (ITNs) on the levels of antibodies towards VSA_{CSA} in PG or even in the general pregnant women population. One study, though, reports on the levels of antibodies to other antigens during pregnancy in a malaria-endemic area. This study, by Kariuki et al [52], analysed the antibody levels to merozoite and sporozoite antigens in pregnant women that reported using ITNs and found that some of these antibodies were decreased in women using ITNs while others were increased. The study failed to analyse the effect on PG and SG individually.

4.2.1.7.2. HIV impact on levels of antibodies

In areas where HIV and malaria co-exist, higher rates of malaria disease and higher densities and prevalence of parasitaemia are seen among HIV-infected patients compared to those that are uninfected [9-11, 53].

In pregnant women, the first study that looked at antibody levels in pregnant women infected with HIV used several non-pregnancy-specific *P. falciparum* antigens and determined that for only one of those antigens (a peptide from the circumsporozoite protein) were antibody levels impaired in those women [54]. This led the authors to conclude that "increased susceptibility to malaria among HIV-infected pregnant women may not be ex-

plained on the basis of their reduced antibody response to malaria antigens” [54]. Although other studies seemed to point in this direction also, and reporting no association of HIV with a change in antibody levels even when looking at VSA_{CSA} antigens [7], it later became clear that HIV impair the antibody response of pregnant women. It was reported by Mount et al [55], using levels of agglutinating antibodies and total IgG to VSA_{CSA}, that pregnant women and particularly HIV-infected PG had lower levels of antibodies than HIV uninfected PG. In agreement with these results, Brolin et al [45] have shown that HIV-positive PG seem not only to have less antibodies towards the VAR2CSA domain DBL5 ϵ , but also that those antibodies have less affinity to their target. Nevertheless, others have reported that HIV-infected MG had a more pronounced humoral immune suppression than HIV-infected PG and SG [56] although it should be noticed that this study was done with very few samples (n=20) and using women that were all extremely immune-suppressed (only one woman out of 20 had a CD4 T cell count above 300x10⁶ cells/L).

4.2.2. Aims of this Chapter

In view of the known literature and of the limited knowledge that existed on the development of phagocytic antibodies in PG and SG, the main aims of this part of the study were to:

- Evaluate the presence of phagocytic antibodies in PG and SG;
- Compare the sensitivity of the phagocytosis assay to measure antibodies when compared to total IgG to VSA_{CSA};
- Determine the relationship between an HIV co-infection and both the total IgG to VSA_{CSA} and phagocytic antibodies according to the different stages of placental infection;
- Determine the presence of associations between antibody levels and clinical outcomes;

4.3. Methods

4.3.1. Ethics statement

The work presented in this chapter used samples from the MHP study cohort conducted in Blantyre, Malawi, which received ethical clearance from the College of Medical Research

and Ethics Committee, University of Malawi and the Melbourne Health Human Research Ethics Committee.

1.1 Cell Cultures

4.3.1.1. Plasmodium falciparum

The parasite line CS2, which is an accepted model of MiP parasites [5, 47]), was used. This particular CS2 line was transfected with a plasmid containing a green fluorescent protein (GFP) expression cassette (kindly provided by Danny Wilson and James Beeson, WEHI) [57]. All parasite cultures were as described on Chapter 3, section 3.5.1.1.

4.3.1.2. Culture of Thp-1 cells

Culture of uThp-1 cells used in this part of the work was conducted exactly as described on Chapter 3, section 3.5.1.2.

4.3.2. The phagocytosis assay

4.3.2.1. A standard phagocytosis assay using uThp-1 cells and CS2-GFP parasites

Both the PG samples and the SG samples were studied using the exact same phagocytosis method, with the exception that PG samples were run in triplicate and SG samples were run in duplicate.

The phagocytosis assay and the analysis of the results obtained, as used in this part of the work was performed exactly as described on Chapter 3, section 3.5.2.1.

4.3.3. Total IgG to CS2_{VSA} assay

For PG samples this assay was performed by Wina Hasang.

Flow cytometry was used to measure the total IgG levels to VSA expressed on CS2 pRBC (total IgG to CS2_{VSA}) using a method described by Mount [55] with minor modifications. In summary, in a 96 well plate heat inactivated human sera (1:20 dilution) were co-incubated with 0.1% haematocrit of 3-8% parasitemia mid to late stage trophozoites PRBC in a total volume of 50µl PBS with 1% Foetal Bovine Serum (PBS/FBS) for 30 min (note parasites used in flow cytometry experiments were cultured and synchronized as described in (3.1.1.1). The PRBC were then washed thrice with PBS/FBS and co-incubated with 25 µl of polyclonal rabbit antihuman IgG antibody (DakoCytomation, Denmark) diluted 1:100 in

PBS/FBS for 30 min, washed again, and incubated with Alexafluor® 488 donkey anti-rabbit IgG (Invitrogen, Eugene, Oregon USA), 1:500 in PBS/FBS with 10µg/ml ethidium bromide (EtBr) for 30 minutes. PRBC were then washed again and resuspended in PBS. Cells were gated based on forward and side scatter characteristics, and plotted by fluorescence in channel 1 (Alexafluor® 488) and channel 2 (EtBr). The adjusted-geometric mean fluorescence intensity (MFI) for each sample was calculated by subtracting the MFI of non-infected RBC from that of gated EtBr-positive cells (ie PRBC): Adjusted MFI = MFI right quadrant – MFI left quadrant

Sera from six individual malaria unexposed adults from Melbourne (Australian Red Cross Blood Service) were used as negative controls. The positive control was pooled serum from women with known high IgG levels to CS2_{VSA}. The relative MFI (designated as the % of positive control) was then calculated using negative and positive controls. Disagreeing duplicates, defined as having an adjusted MFI variance greater than 20% from their mean and a relative MFI variance >10, were rerun (if possible) or excluded. Results were also discarded if flow cytometry plots did not show distinct cell populations.

4.4. Results

4.4.1. Study samples and definitions used

The serum samples used in this chapter came from a cohort which has previously been described [12, 55]. In brief, women in late third trimester of pregnancy gave written consent to studies including HIV testing, and samples of peripheral blood were collected. Both PG and SG for whom results of placental histology sections had been obtained were included in the present study, resulting in a total of 263 PG samples and 187 SG analysed.

Placental tissue, collected at delivery, was fixed in formalin and Giemsa stained sections were examined histologically as previously described [65]. Placental malaria (PM) was considered to be present if there was evidence of parasites or pigmented monocytes or pigment in fibrin deposits in the placenta, irrespective of the presence of parasites in the peripheral blood (unless stated otherwise).

For PG, and taking advantage of the numbers of samples obtained we were able to combine histology findings with results of microscopy of Giemsa-stained placental and peripheral blood thick films to allocate women to one of four groups: *No Malaria* (no evidence of past or current malaria, either in blood films or placental histology), *Acute Infection* (pRBC on placental histology, without malaria pigment deposition), *Chronic Infection* (both pRBC and malaria pigment deposits on histology) and *Past Infection* (placental malaria pigment

deposits, without pRBC on histology or on blood films). We excluded from the grouping analysis women who presented only with peripheral parasitaemia. In all cases where parasites were present, all infections were with *P. falciparum*.

Maternal anaemia (anaemia) was defined as a haemoglobin measurement of less than 11.0 g/dL. Infant low birth weight (LBW) was considered to be a baby weight of less than 2,500g

4.4.2. Primigravidae results

4.4.2.1. HIV and *Plasmodium* infections: associations with maternal age, maternal haemoglobin and infant birth weight.

Of the 263 women analysed in this study, 156 (60%) were negative for HIV, while 104 (41%) revealed no evidence of a PM infection (Table 4-1).

In a univariate analysis, using student's t-test (Table 4-1 – Characteristics of the PG study group and the associations of HIV and MiP with clinical outcome), both HIV and PM infection significantly decreased maternal haemoglobin levels and infant birth weight. The age of the women was significantly associated with both PM infection (being higher in uninfected women) and HIV (being higher in infected women). Women that were both infected with HIV and PM had lower birth weight babies and presented with lower levels of haemoglobin than other groups (Table 4-1).

When age, HIV status and PM status were included in a multivariate analysis using multilinear regression analysis, both haemoglobin levels (HIV: coef= -1.1, $P<0.0001$; PM: coef= -0.8, $P=0.002$) and birth weight (HIV: coef= -238.7, $P<0.0001$; PM: coef= -193.7, $P=0.002$) were associated with both infections, independently of maternal age.

Since both maternal haemoglobin levels and infant birth weight can be expressed as the categorical clinical outcomes anaemia and LBW, respectively, the association of these with maternal age, HIV and PM infection was evaluated. Each of the infections, independently of maternal age, increased the prevalence of anaemia and LBW (Table 4-2).

4.4.2.2. Levels and spread of phagocytic antibodies and total IgG to CS2_{VSA}

As was discussed in Chapter 3 (see chapter 3) two parameters can be derived from the results obtained with the phagocytosis assay used in this study: the function and the titre of the antibodies. In this group of PG, both the titre and the function of phagocytic antibodies correlated with the levels of IgG to CS2_{VSA} (Spearman's rank correlation $\rho=0.48$ and

0.50 respectively, $p < 0.0001$) but, when a linear regression was performed, correlation between function of phagocytic antibodies and IgG to CS2_{VSA} ($r^2 = 0.56$) was stronger than the one found between titres and IgG to CS2_{VSA} ($r^2 = 0.41$) owing to their spread over a larger range of values (Figure 4-1). For further analysis, only the function of the phagocytic antibodies was used. I make use of the phagocytic antibodies' titre only where significant differences between the two parameters occurred.

Table 4-1 – Characteristics of the PG study group and the associations of HIV and MiP with clinical outcome

		Age			Maternal Hb level (g/dL) ¹			Infant Bw (g) ²		
		n [%]	Mean (SD)	p-value	n [%]	Mean (SD)	p-value	n [%]	Mean (SD)	p-value
Entire Cohort		263 [100]	20.0 (3.3)		258 [100]	11.3 (1.98)		224 [100]	2904 (444)	
HIV	negative	156 [60]	19.5 (2.7)		155 [60]	11.6 (1.96)		123 [55]	2982 (413)	
	positive	106 [40]	20.7 (3.9)	0.007	102[40]	10.8 (1.93)	0.002	100 [45]	2808 (466)	0.003
Malaria infection	negative	104 [41]	20.7 (3.6)		152 [60]	11.5 (1.91)		101 [45]	2978(416)	
	positive	151 [59]	19.6 (2.7)	0.0075	98 [40]	10.9 (2.00)	0.013	121[54]	2845(464)	0.016
	Histology not present	8 [3]								
HIV and Malaria:										
	DOUBLE NEGATIVES ³	43 [16]	19.6 (2.8)		43 [16]	12.1 (1.66)		40 [18]	3158 (402)	
	DOUBLE POSITIVES ⁴	42 [16]	19.8 (3.0)	0.77	42 [16]	10.3(1.96)	<0.0001	41 [18]	2726 (559)	0.0001

Data represents means (standard deviation from means), and numbers of subjects [percent of total for each parameter]. ¹Maternal haemoglobin levels. ²Birth weight of babies at delivery. ³DOUBLE NEGATIVES are women with neither HIV nor malaria. ⁴DOUBLE POSITIVES are women with both HIV and malaria infection. Variables are normally distributed, so Student's *t*-tests were applied. Variables were analysed for all subjects for whom the relevant data were recorded.

Table 4-2 – Association of HIV and PM status with LBW and Anaemia

	LBW¹ [OR, (<i>P</i> -value), CI] ²	Anaemia¹ [OR, P-value, CI]
HIV	2.24, (0.059), 0.97-5.18	2.35, (0.003), 1.32-4.16
PM	2.69, (0.030), 1.10-6.59	2.60, (0.001), 1.45-4.66
Maternal age	1.03, (0.527), 0.93-1.16	1.02, (0.540), 0.95-1.11

Logistic regression between HIV and PM status and the clinical outcomes LBW and Anaemia.

¹ For low birth weight (LBW) and Anaemia definitions see section 4.4.1.

² OR – Odds ratio, CI – 95% confidence interval

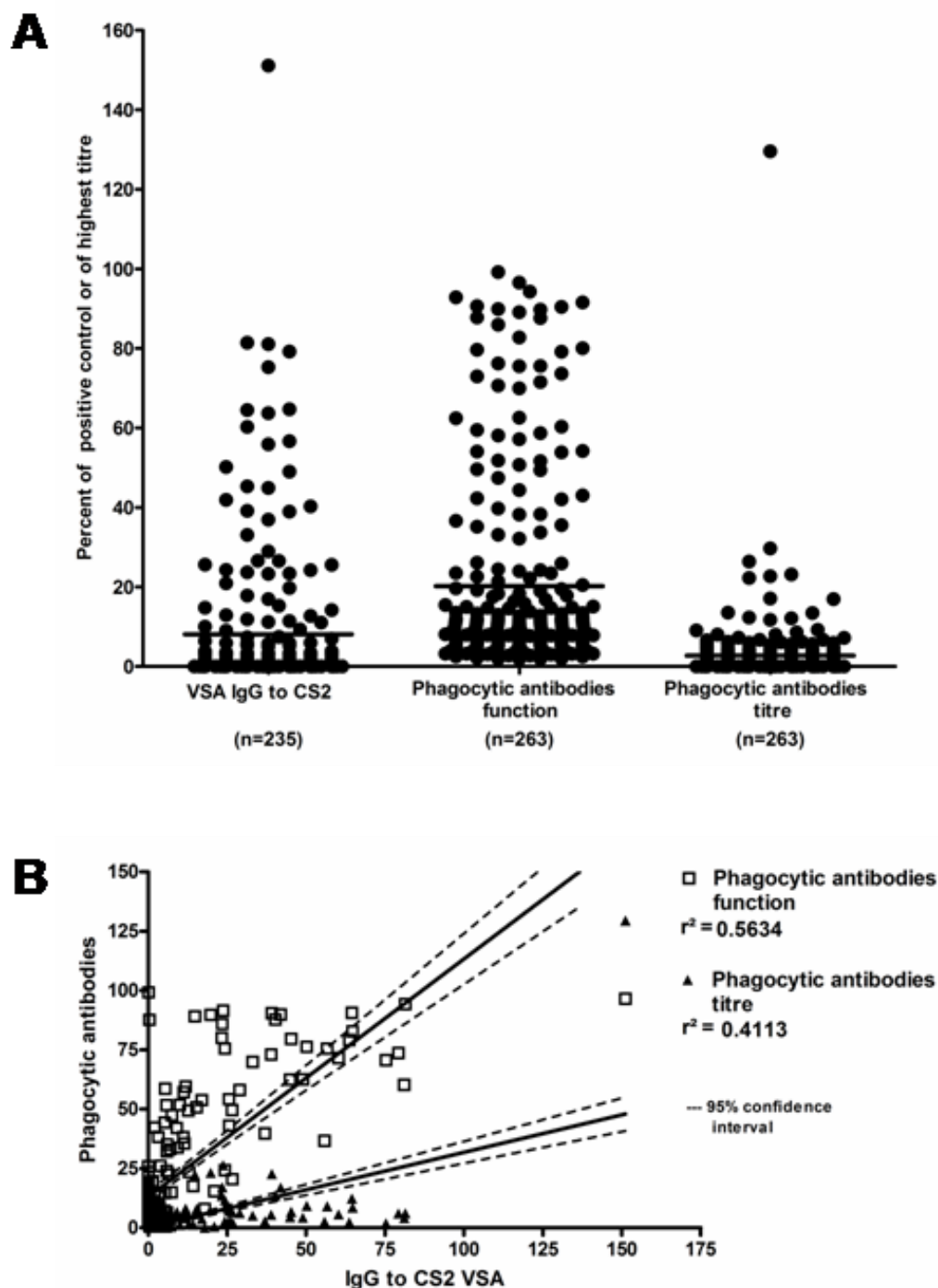


Figure 4-1 - Distribution of, and correlation between, the antibody parameters measured.

A) Dot plot representation of the distribution of values obtained for total IgG to CS2_{VSA}, and both phagocytic antibodies' parameters: function and titre. n= number of samples analysed in each group. B) linear regression analysis of IgG to CS2_{VSA} and both function (open squares) and titre (full triangles) of phagocytic antibodies. Analysis performed on GraphPad Prism v4.0

4.4.2.3. Phagocytic antibodies and Total IgG to CS2_{VSA} are increased in *P. falciparum* infected women and decreased in HIV-infected women.

In a univariate analysis (Mann-Whitney ranksum), both phagocytic antibodies (Phago atb - Figure 4-2) and IgG to CS2_{VSA} (Total IgG - Figure 4-3) were higher in women with evidence of PM infection (Phago atb: $z = 6.08$, $P < 0.0001$; Total IgG: $z = 4.83$, $P < 0.0001$) and lower in HIV infected women (Phago atb: $z = -3.75$, $P = 0.0002$; Total IgG: $z = -2.70$, $P = 0.007$)

4.4.2.4. Phagocytic antibodies and total IgG to CS2_{VSA} are not associated with changes in Infant birth weight, maternal haemoglobin or the clinical outcomes LBW and maternal anaemia.

In a univariate analysis (Mann-Whitney ranksum), phagocytic antibodies were not associated with the presence of neither LBW infants ($z = -0.692$, $P = 0.489$) nor with the occurrence of maternal anaemia ($z = -0.769$, $P = 0.442$) (Figure 4-4 A and B). Phagocytic antibodies also showed no associations, with infant birth weight (Spearman's correlation tests (ρ) = -0.102 , $P = 0.121$) nor with maternal haemoglobin levels ($\rho = 0.097$, $P = 0.117$). Linear regression methods (Figure 4-4, C and D) were used to investigate causality between these parameters and also failed to detect any associations (infant birth weight: $r^2 = 0.0065$, $P = 0.227$; maternal anaemia: $r^2 = 0.035$, $P = 0.342$).

Similarly, the levels of total IgG against CS2_{VSA} were not associated with the presence of LBW infants ($z = -0.149$, $P = 0.881$) or maternal anaemia ($z = -0.538$, $P = 0.591$) (Figure 4-5, A and B), nor were they associated with infant birth weight ($\rho = -0.054$, $P = 0.440$) or maternal haemoglobin levels ($\rho = 0.035$, $P = 0.592$).

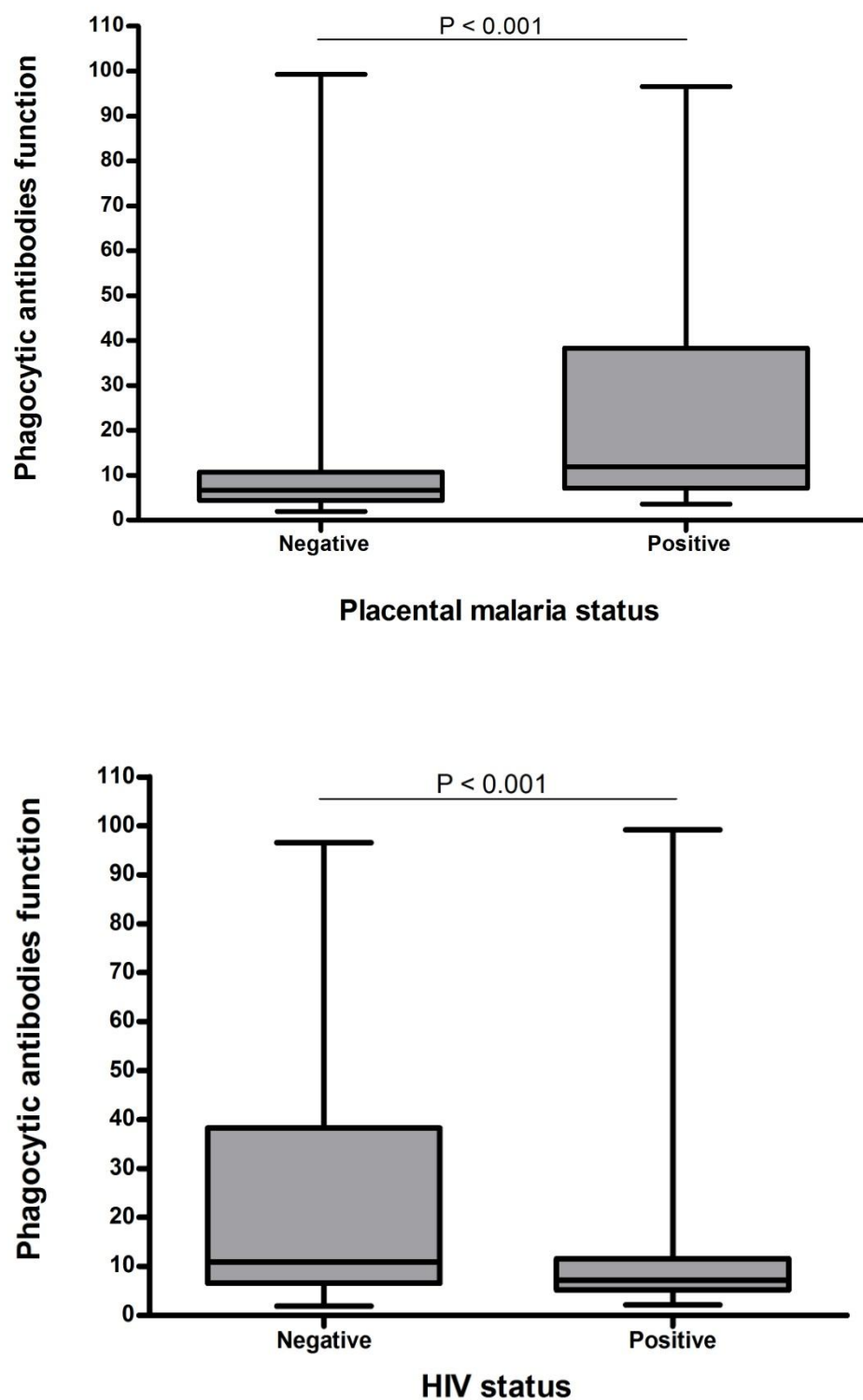


Figure 4-2 – Effect of infections on phagocytic antibodies function

Both the placental malaria status (upper graph) and the HIV status (lower graph) of the women affected the function of phagocytic antibodies towards the *P. falciparum* parasite line CS2-GFP. Mann-Whitney rank sum tests were applied to quantify the differences observed.

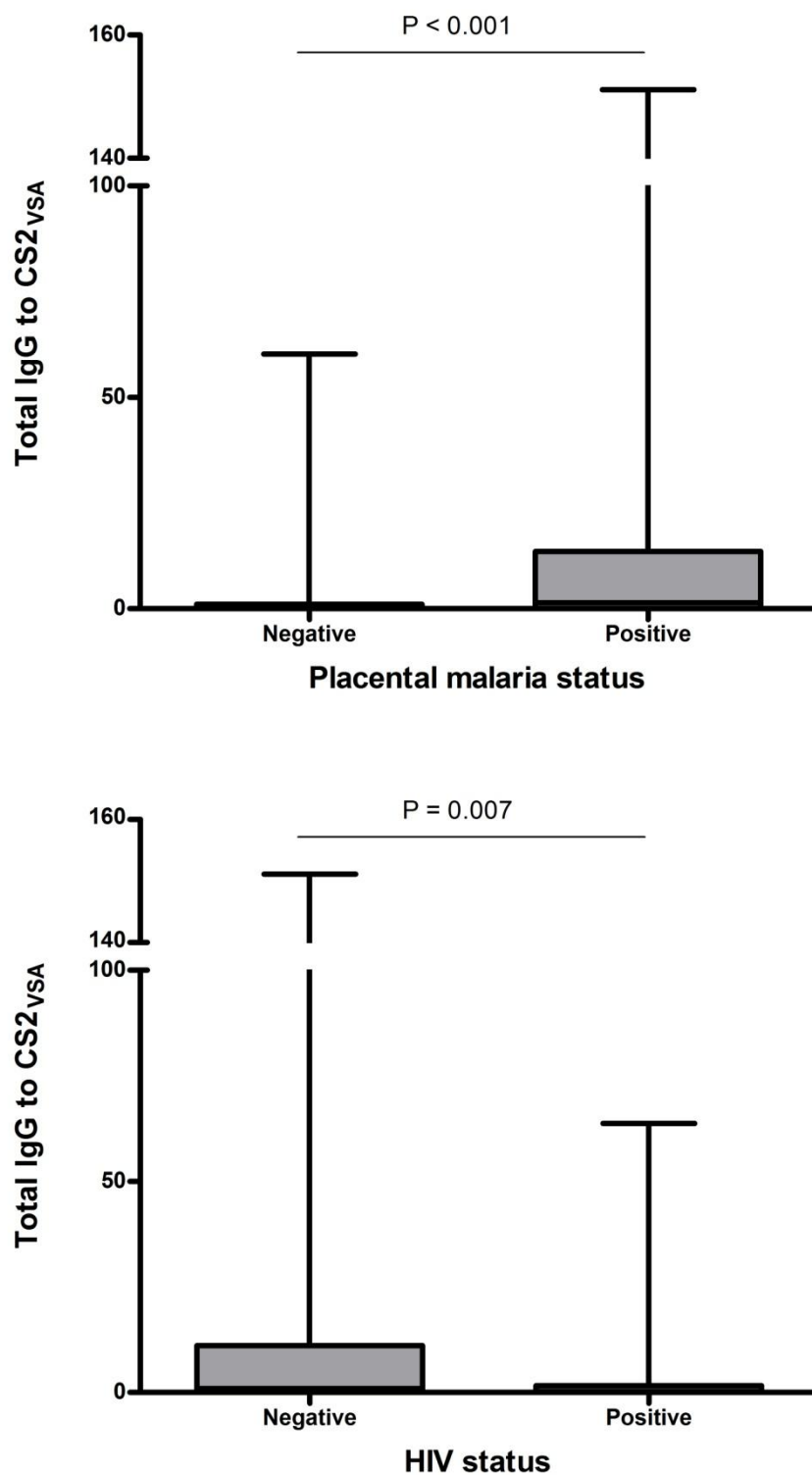


Figure 4-3 – Effect of infections on Total IgG to CS2_{VSA}

Both the placental malaria status (upper graph) and the HIV status (lower graph) of the women affected the total IgG to CS2_{VSA}. Mann-Whitney rank sum tests were applied to quantify the differences observed.

Once more, linear regression analysis did not detect any correlation between phagocytic antibodies (infant birth weight: $r^2 = 0.0035$, $P = 0.400$; maternal anaemia: $r^2 = 0.0026$, $P = 0.437$) nor total IgG to CS2_{VSA} (infant birth weight: $r^2 = 0.0036$, $P = 0.406$; maternal anaemia: $r^2 = 0.0022$, $P = 0.508$) (Figure 4-5, C and D).

4.4.2.5. Multivariate analysis highlights the impact of HIV on phagocytic antibodies.

In a multivariate analysis, using multiple linear regression methods, on *P. falciparum* positive women alone, HIV infection was associated with decreased levels of phagocytic antibodies. Neither maternal haemoglobin levels, infant birth weight nor HIV were associated with the levels of total IgG to CS2_{VSA} (Table 4-3).

4.4.2.6. The levels phagocytic antibodies and total IgG to CS2_{VSA} are correlated with the MiP histopathology groupings identified and are differently modulated by HIV infection

When analysis of antibody measurements was made according to the PM status of the women, similar levels of both total IgG to CS2_{VSA} and phagocytic antibodies were observed in the groups with current or previous *P. falciparum* infection and no HIV (Figure 4-6, light grey bars).

When the relationship between placental malaria status and antibody measurements was analysed within the group of HIV positive women (Figure 4-6, dark grey bars), women in the acute infection group had significantly lower levels of total IgG to CS2_{VSA} than women in the combined chronic and past groups (Mann-Whitney ranksum: $p = 0.018$ and $p = 0.038$, respectively, Figure 4-6A) and significantly lower phagocytic antibodies function than women in the chronic group (Mann-Whitney ranksum: $p = 0.034$, Figure 4-6B).

The comparison between antibody measurements in HIV positive and HIV negative women in the different histopathology groups revealed that HIV positive women in the acute group failed to generate antibodies to pRBC, having low levels of total IgG to CS2_{VSA} (Mann-Whitney ranksum: $p = 0.028$, Figure 4-6A) and low levels of phagocytic antibodies (Mann-Whitney ranksum: $p = 0.013$, Figure 4-6B), when compared to the HIV negative women. HIV positive and negative women in the chronic infection group had similar levels of antibodies. HIV infected women in the past infection group had lower levels of phagocytic antibodies than did HIV negative women in this group (Mann-Whitney ranksum: $p = 0.07$).

Among HIV infected women, peripheral blood CD4 T-cell counts were found to be associated with levels of antibody to pRBC. HIV positive women who presented with a CD4-T cell count less than 350×10^6 cells/L had lower levels of IgG to CS2 VSA (coef: -6.15, $P=0.007$) and less phagocytic antibodies (coef: -11.95, $P=0.002$) than did HIV infected women with CD4 T-cell counts above 350×10^6 cells/L

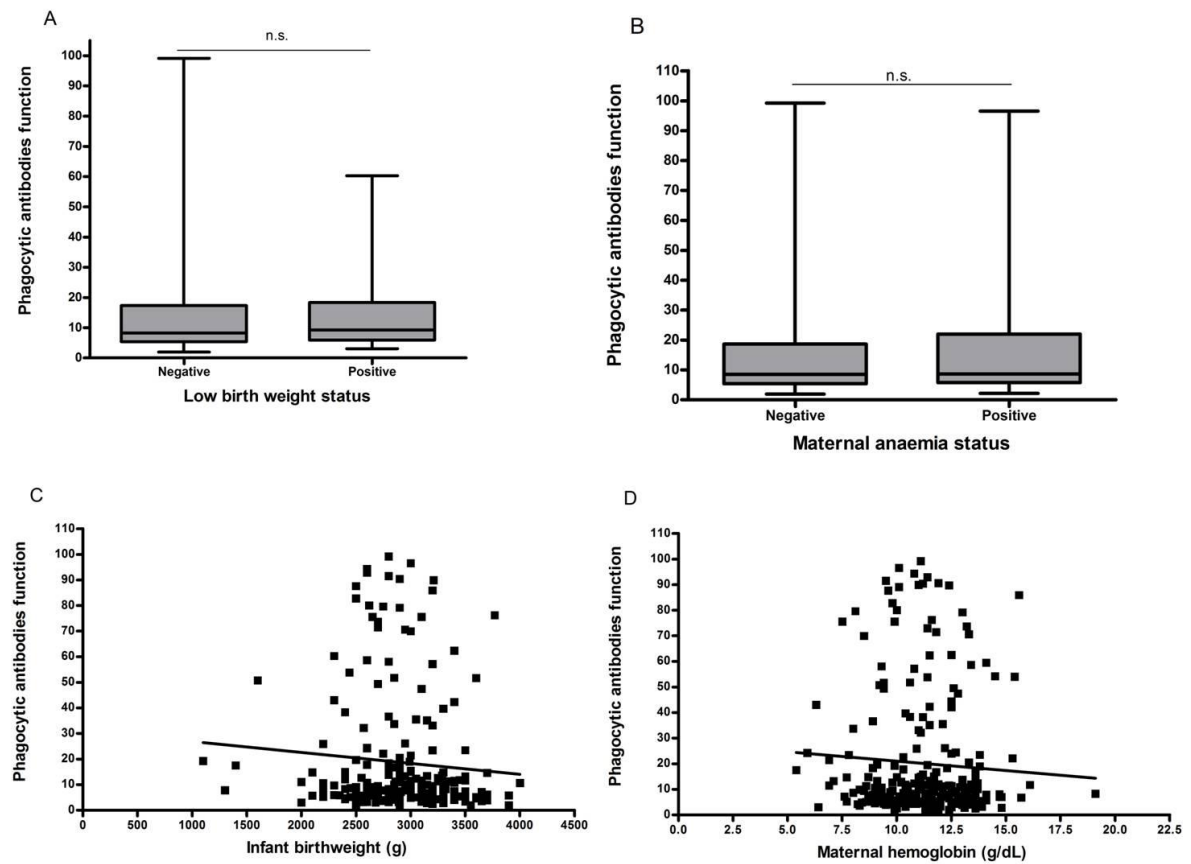


Figure 4-4 – Univariate analysis of phagocytic antibodies to CS2_{VSA} and clinical outcomes in PG

Box plots of phagocytic antibodies' function against the occurrence of infant low birth weight (A) and maternal anaemia (B). Mann-Whitney Ranksum tests were performed and no statistical significance was detected (see values in text). Scatter plots of phagocytic antibodies against infant birthweight (C) and maternal haemoglobin levels (D) showed no correlation between the parameters analysed after spearman's correlation tests and linear regression analysis (see values in text).

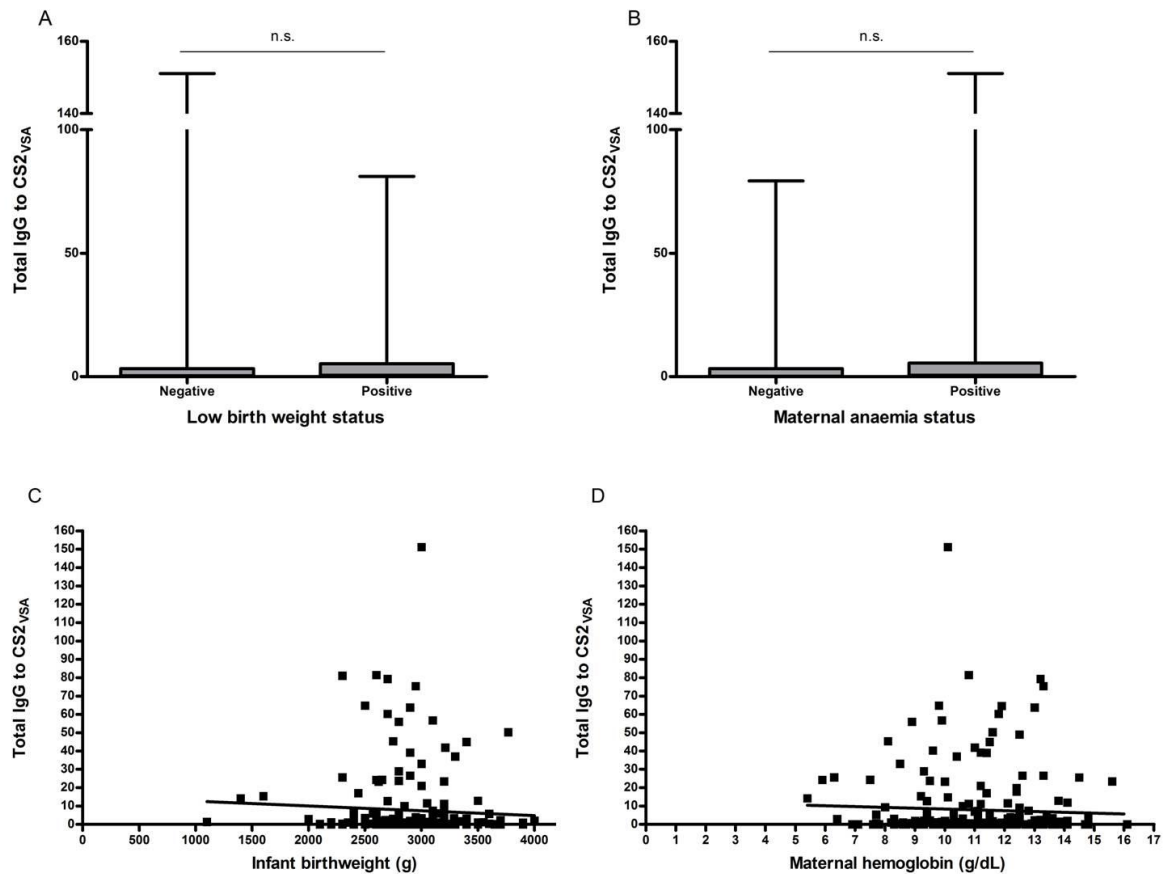


Figure 4-5 - Univariate analysis of total IgG to CS2_{VSA} and clinical outcomes in PG

Box plots of phagocytic antibodies' function against the occurrence of infant low birth weight (A) and maternal anaemia (B). Mann-Whitney Ranksum tests were performed and no statistical significance was detected (see values in text). Scatter plots of phagocytic antibodies against infant birthweight (C) and maternal haemoglobin levels (D) showed no correlation between the parameters analysed after spearman's correlation tests and linear regression analysis (see values in text).

Table 4-3 – Multivariate analysis of antibodies measured and clinical parameters analysed in PG

Variables	Phagocytic function		Total IgG to CS2	
	Multivariate analysis ¹		Multivariate analysis ¹	
	Coeff (95% CI)	P	Coeff (95% CI)	P
Plac malaria		NA		NA
HIV	-10.97 (-21.33, -0.61)	0.038	-7.60 (-16.9, 1.68)	0.107
Hemoglobin levels²	-0.99 (-3.36, 1.39)	0.412	-0.56 (-2.90, 1.78)	0.637
Infant Birthweight²	0.001 (-0.009, 0.012)	0.834	0.002 (-0.008, 0.012)	0.708

¹ Adjusted estimate for malaria positive women only (Multi linear model correlation coefficients and respective 95% confidence intervals and *P*-values are shown). (NA) non-applicable, (Coeff) regression coefficient (95% CI) 95% confidence intervals. A negative coefficient implies a decrease of antibody levels. A positive coefficient implies an increase in antibody levels.

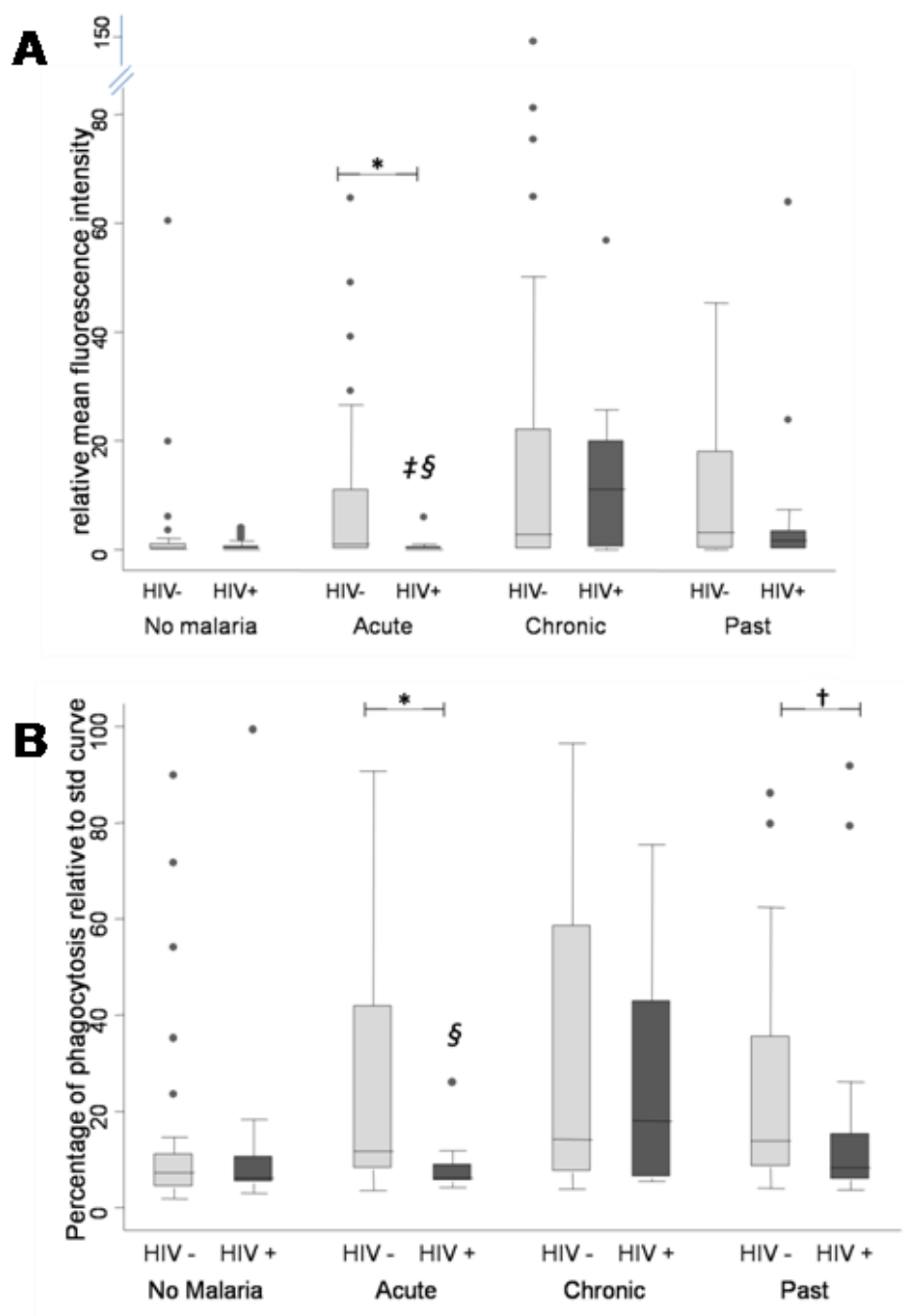


Figure 4-6 – Antibody measurements across MiP histopathology groups in PG

Light grey bars – HIV negative (HIV-), dark grey bars – HIV positive (HIV+). **A)** Total IgG to CS2VSA. Mann-Whitney ranksum tests performed on HIV- women: §acute vs chronic $p=0.018$, ‡ acute vs past: $p=0.038$. Mann-Whitney ranksum tests performed between HIV- and HIV+ women: * acute group: $p=0.028$. **B)** phagocytic antibodies' function. Mann-Whitney ranksum tests performed on HIV- women: § acute vs chronic $p=0.034$. Mann-Whitney ranksum tests performed between HIV- and HIV+ women: * acute group: $p=0.013$, † past group: $p=0.07$. Bars: median and interquartile ranges. Dots: outliers.

4.4.3. Secundigravidae results

4.4.3.1. HIV and PM: associations with maternal age, maternal haemoglobin and infant birth weight in SG

One hundred and eighty seven samples were available for this study to be conducted. Forty two percent of the SG had placental malaria and 65% were HIV infected (Table 4-4). Most women (62%) were anaemic, and the mean haemoglobin concentration $\pm$ standard deviation (SD) was 10.6 ± 2.1 g/dL. Haemoglobin levels were not associated, in a univariate analysis using Student's T-tests, with placental malaria infection or with HIV status in this study group (Table 4-4). Infant birth weight did not vary with PM infection, but was significantly lower among HIV-positive women ($P < 0.001$, Table 4-4). Women with evidence of PM infection were significantly younger than uninfected women ($P = 0.002$; Table 4-4)

When age, HIV status and PM status were included in a multivariate analysis using multilinear regression, infant birth weight was still associated with HIV infection and not PM infection (HIV: coef= -369, $P < 0.001$; PM: coef= -27.5, $P = 0.721$) while maternal haemoglobin levels remained not associated with either infection (HIV: coef= -0.11, $P = 0.815$; PM: coef= -0.52, $P = 0.205$) independently of maternal age.

Analysis of the relationship between HIV infection and PM infection and the outcomes LBW and maternal anaemia, using logistic regression methods, did not reveal any associations that differed from the ones obtained with the continuous parameters (all P values > 0.05).

Table 4-4 - Characteristics of the secundigravidae study group and the associations of HIV and MiP with clinical outcome

		Age			Maternal Hb level (g/dL) ¹			Infant Bw (g) ²		
		n [%]	Mean (SD)	p-value	n [%]	Mean (SD)	p-value	n [%]	Mean (SD)	p-value
Entire Cohort		187 [100]	22.5 (3.1)		129 [100]	10.6 (2.14)		181 [100]	2852 (518)	
HIV	negative	65 [35]	22.1(2.3)		30 [23]	10.7 (2.1)		61 [34]	3091 (452)	
	positive	122 [65]	22.7 (3.4)	0.197	99 [77]	10.6 (2.2)	0.934	120 [66]	2731 (510)	<0.001
Malaria infection	negative	89 [48]	23.1 (3.5)		58 [45]	10.9 (2.2)		87 [44]	2852 (561)	
	positive	79 [42]	21.6 (2.3)	0.002	61 [47]	10.5 (2.0)	0.252	78 [43]	2865 (438)	0.875
	Not available	19 [22]			10 [8]			16 [9]		
HIV and Malaria:										
DOUBLE NEGATIVES³		27 [14]	22.3 (2.0)		9 [7]	11.1 (2.4)		26 [14]	3112 (501)	
DOUBLE POSITIVES⁴		47 [25]	21.5 (2.1)	0.119	42 [33]	10.5 (2.0)	0.404	46 [25]	2714 (390)	<0.001

The data represents numbers of patients (percentages of total for each parameter) and means [standard deviations]. ¹Maternal haemoglobin levels. ²Birth weight of babies at delivery. ³DOUBLE NEGATIVES are women with neither HIV nor malaria. ⁴DOUBLE POSITIVES are women with both HIV and malaria infection. Variables are normally distributed, so Student's t-tests were applied

4.4.3.2. Levels and spread of phagocytic antibodies and total IgG to CS2_{VSA} in secundigravidae

The correlation between the levels of total IgG to CS2_{VSA} and the phagocytic function in this cohort of SG women was similar, although slightly higher, to the one found in the PG group ($r^2=0.67$, Figure 4-7A). The value for Spearman's rank correlation was also slightly higher ($\rho=0.61$, $P<0.001$). The spread of values for total IgG to CS2_{VSA} was larger than that for phagocytic antibodies (Figure 4-7B).

4.4.3.3. Associations between antibody levels and HIV, Placental malaria status, maternal haemoglobin and infants' birth weight in Secundigravidae

We compared antibody levels in women with current placental malaria infection (PM+) and without infection (PM-) using Mann-Whitney ranksum tests. For phagocytic antibodies, levels were higher in PM+ women than in PM- women ($z = 4.54$, $P<0.001$, Figure 4-8B). Similarly, total IgG to CS2_{VSA} was higher in PM+ women than PM- women ($z = 4.03$; $p<0.001$, Figure 4-8D). Phagocytic antibodies were significantly decreased in HIV infected women compared to HIV uninfected women ($z = -2.55$, $P=0.011$, Figure 4-8A), whereas total IgG to CS2_{VSA} showed a non-significant difference between HIV-infected women and women who were HIV-negative ($z = -1.76$, $P=0.079$; Figure 4-8C).

Using linear regression methods, neither phagocytic antibodies nor total IgG to CS2_{VSA} were correlated with maternal haemoglobin levels. Infant birth weight was correlated with phagocytic antibodies (coef=3.34, $p=0.034$) (Figure 4-9A) but not associated with total IgG to CS2_{VSA} (coef= 0.007, $p=0.223$) (Figure 4-9B).

A multivariate analysis was performed using HIV status, antibodies and maternal age and weight against infant birth weight and maternal haemoglobin concentration. Women were divided into PM+ and PM-. Total IgG to CS2_{VSA} was not associated with significant changes in infant birth weight in either group (data not shown). Phagocytic antibodies were not associated with infant birth weight in PM- women (LR: coef= 0.42, $p=0.902$). In contrast, in PM+ women, the function of phagocytic antibodies was found to be significantly associated with birth weight (LR: coef= 3.57, $p=0.025$), independently of HIV status, maternal age and weight. It is important to point out that both HIV status and maternal weight were independently associated with infant birthweight in PM- and PM+ women. Neither antibody measurement was associated with maternal haemoglobin levels in multivariate analyses (Table 4-5).

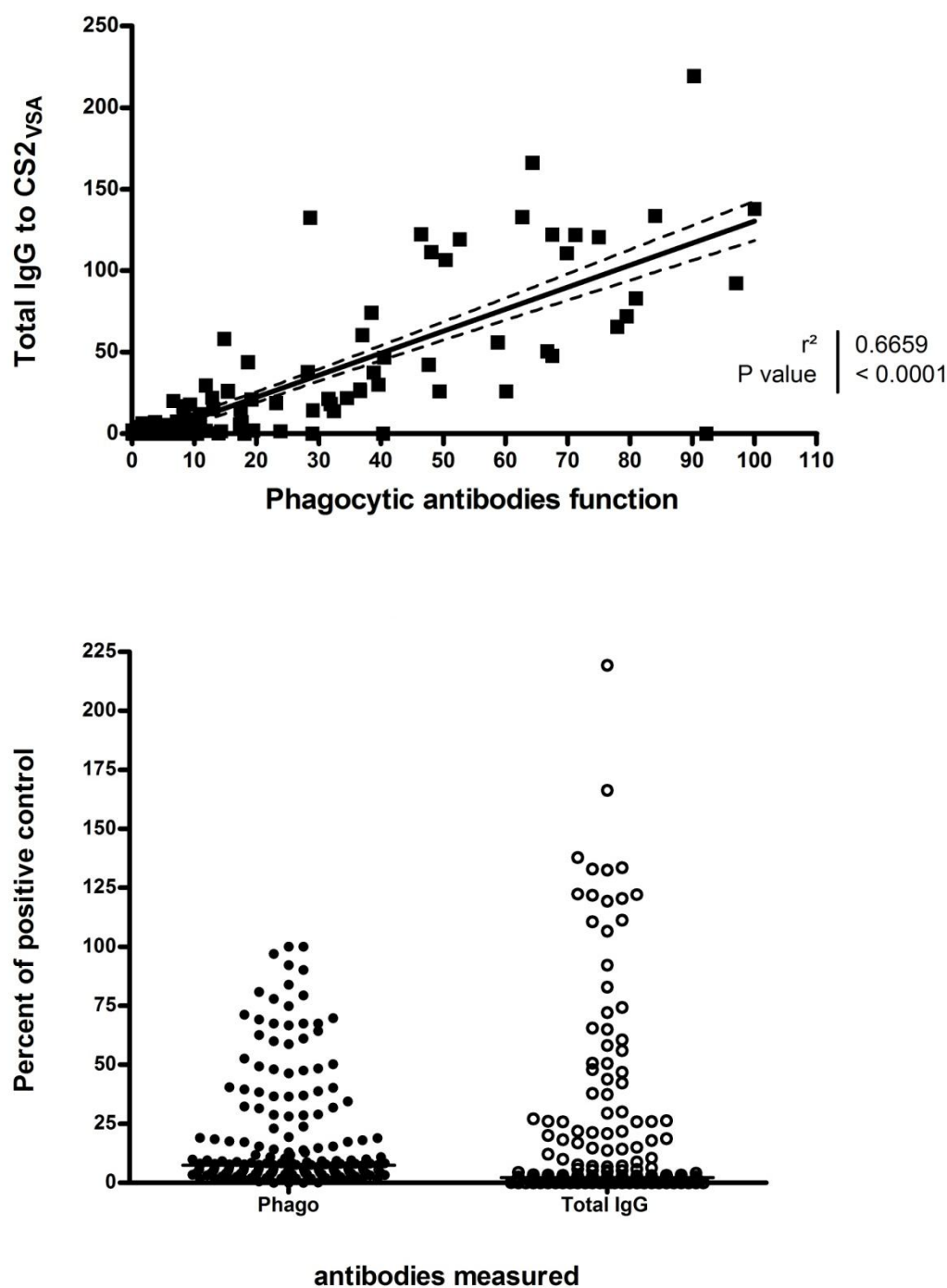


Figure 4-7 – Correlation and spread of antibodies parameters measured in SG

The correlation (top graph) between phagocytic antibodies and total IgG to CS2_{VSA} was evaluated by linear regression and Spearman's rank correlation (values in text). Both the linear regression and the 95% confidence interval are plotted. The dotplot spreads of the two parameters measured (lower graph), phagocytic antibodies (phago) and total IgG to CS2_{VSA} (total IgG), are plotted alongside the medians.

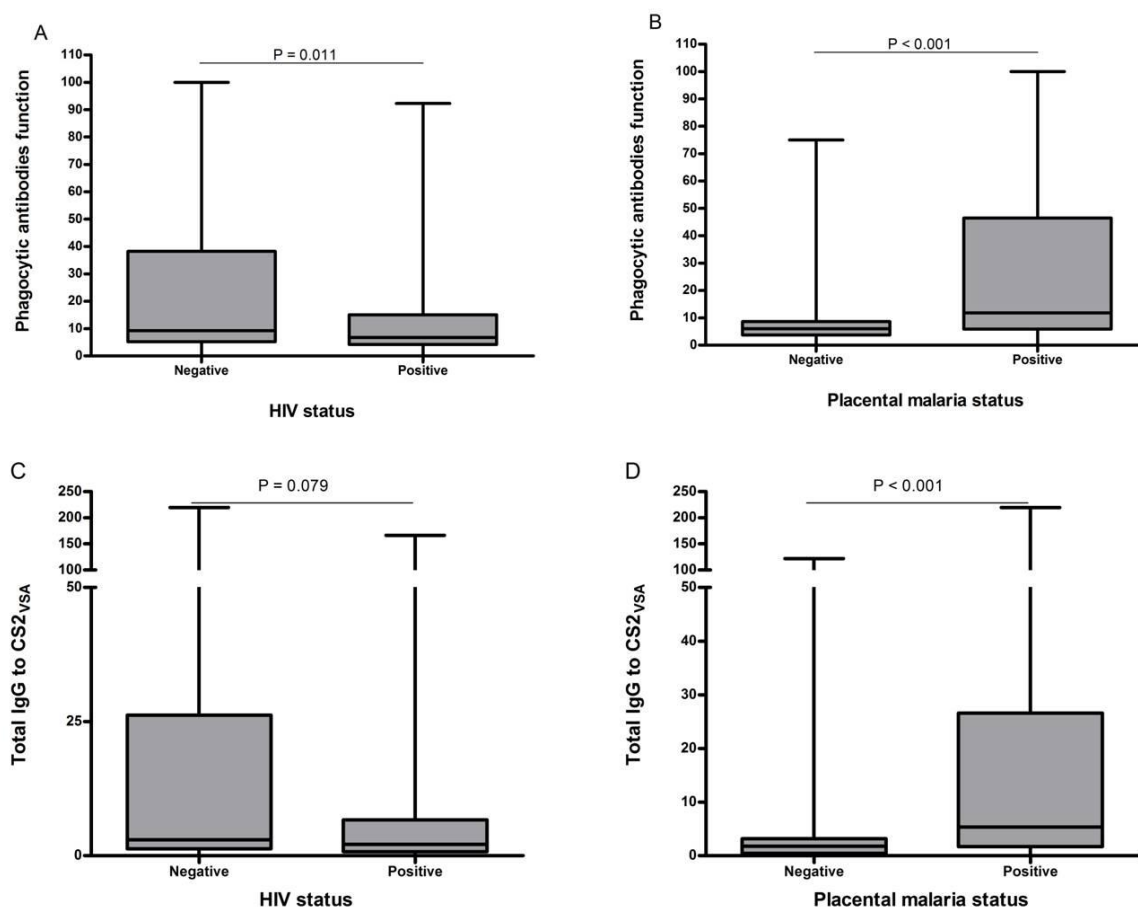


Figure 4-8 – Univariate analysis of antibodies measured versus HIV and placental malaria in Secundi-gravidae

Both phagocytic antibodies (A and B) and total IgG to CS2_{vsa} (C and D) were significantly modulated by HIV (left panels) and PM (right panels). A univariate analysis was performed using Mann-Whitney rank sum tests. Box, median and interquartile range; whiskers, lowest and highest values. P values are shown.

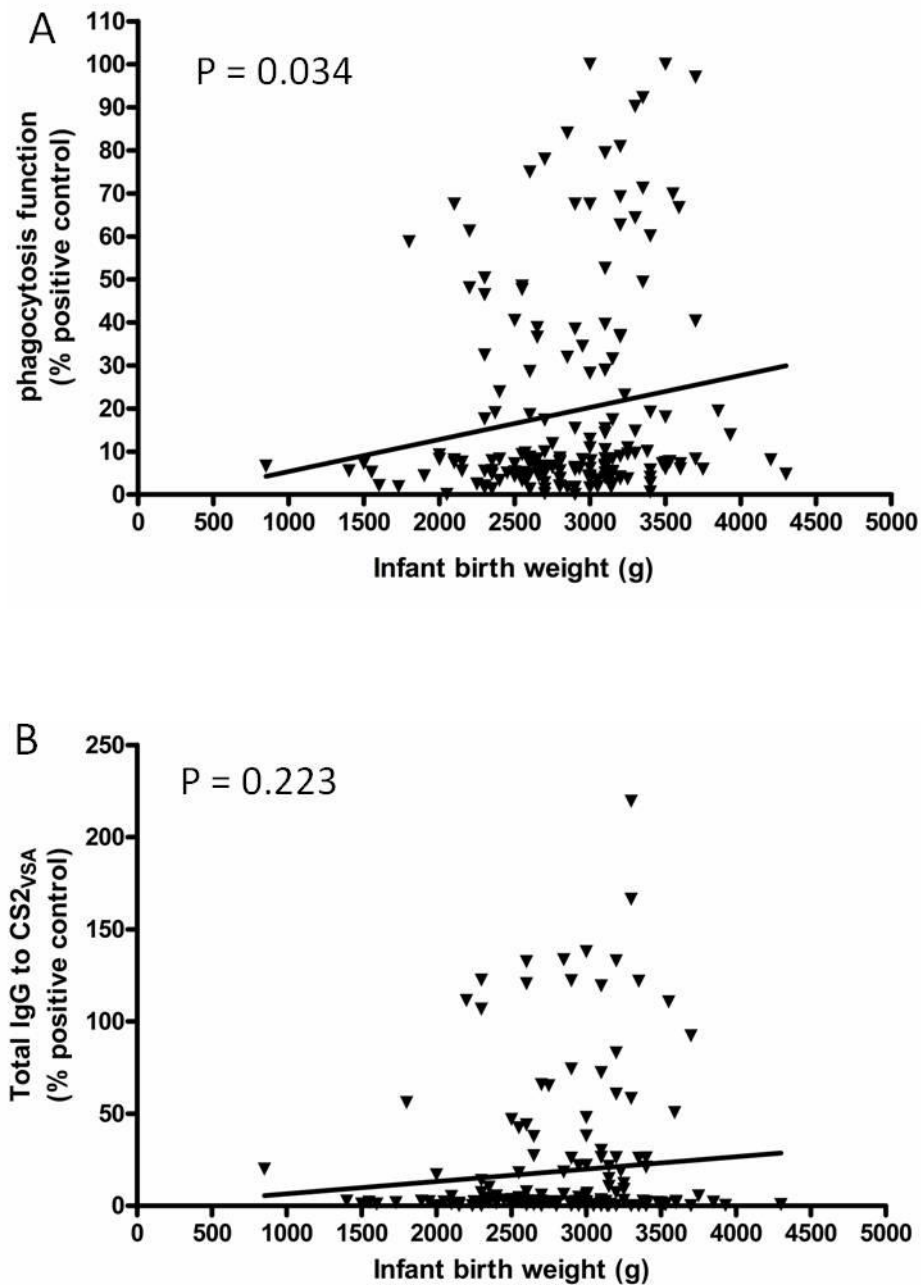


Figure 4-9 – Univariate association between infant birth weight and the anti- CS2_{VSA} antibodies measured in Secundigravidae

Univariate linear regression of phagocytic antibodies (A) and total IgG to CS2_{VSA} (B) with infant birth weight. Both P-values are plotted. Only phagocytic antibodies correlated with infant birth weight ($r^2=0.025$, $p=0.034$).

HIV modulates phagocytic antibodies in the 'No Malaria' histopathology group in Secundigravidae

Although the numbers of SG samples available to perform this study ($n=187$) did not allow for a conclusive analysis of HIV effect according to PM histopathology groups it was possible to verify that the levels of total IgG to CS2_{VSA} and of phagocytic antibodies did not vary according to findings on placental histology, (data not shown), being similar in women with acute, chronic and past infection (Anova across groups, Total IgG to CS2_{VSA}: $p=0.266$; phago: $p=0.429$).

Of notice, in the histopathology group 'No Malaria', in which there was sufficient numbers available for analysis ($n=109$), HIV significantly affected the function of phagocytic antibodies ($z = -2.17$, $P = 0.03$, Figure 4-10A) but not the total IgG to CS2_{VSA} ($Z = -1.35$, $P = 0.179$, Figure 4-10B).

Table 4-5 – Multivariate analysis of infant birth weight in Secundigravidae according to Placental malaria status

Variables	PM negative		PM positive	
	Multivariate analysis ¹		Multivariate analysis ¹	
	Coeff (95% CI)	<i>P</i>	Coeff (95% CI)	<i>P</i>
Infant birth weight (g)	NA		NA	
Phagocytic function	0.42 (-6.28, 7.11)	0.902	3.57 (0.461, 6.68)	0.025
HIV	-257.46 (-21.33, -0.61)	0.043	-272.46 (-462.57, -82.35)	0.006
Maternal weight (Kg)	31.42 (17.83, 45.01)	<0.001	13.37 (1.23, 25.51)	0.031
Maternal age (years)	-2.38 (-33.07, 28.30)	0.878	-1.28 (-39.69, 37.13)	0.947

¹ Multilinear regression analysis of infant birth weight (in grams) against phagocytic antibodies function, HIV status, maternal weight (in kilograms) and maternal age in Placental malaria (PM) positive and negative women. Multi linear model correlation coefficients and respective 95% confidence intervals and *P*-values are shown. (NA) non-applicable, (Coeff) regression coefficient (95% CI) 95% confidence intervals. A negative coefficient implies a decrease of infant birth weight. A positive coefficient implies an increase in antibody levels.

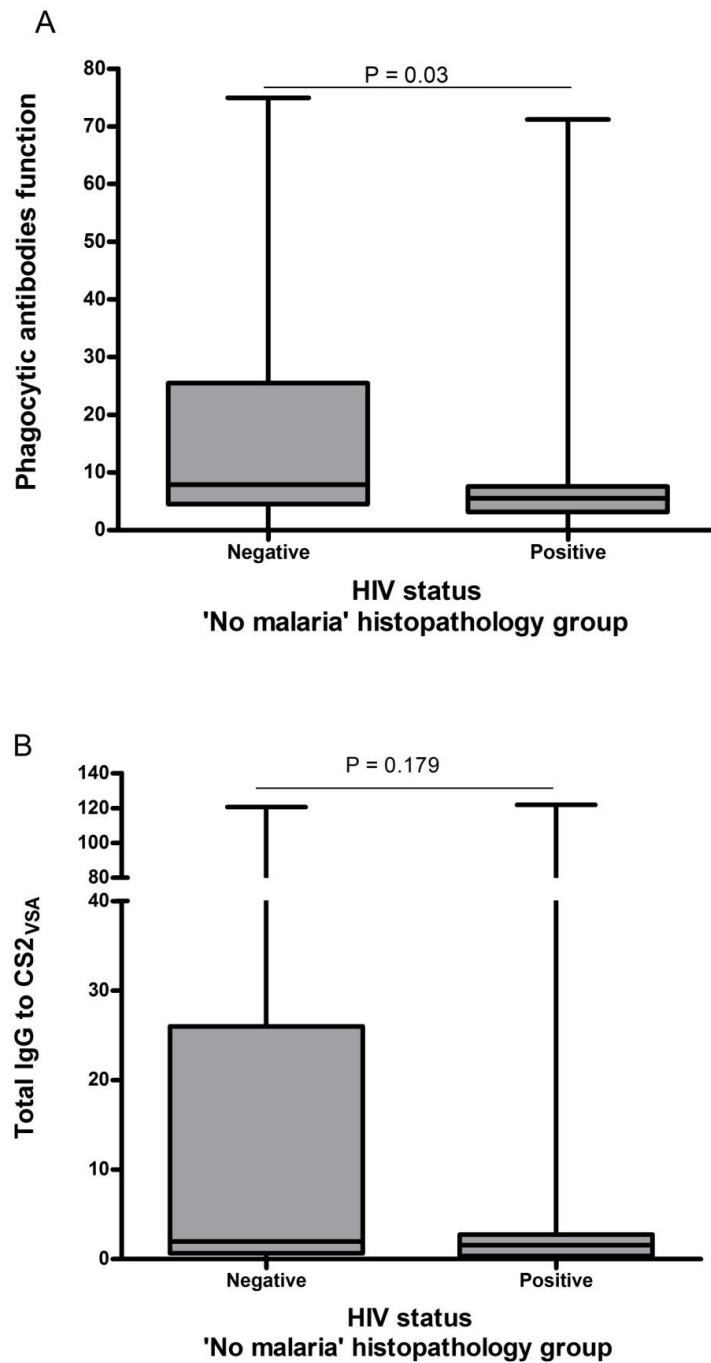


Figure 4-10 – Effect of HIV on the anti-CS2_{VSA} antibody levels in the SG 'No Malaria' histopathology group.

In a univariate analysis of women that showed no evidence of placental malaria infection, using Mann-Whitney ranksum tests, phagocytic antibodies (A) are shown to be significantly modulated by HIV infection, contrary to total IgG to CS2_{VSA} (B). Box, median and interquartile range; whiskers, lowest and highest values. P values are shown.

4.5. Discussion

It is important to start this discussion by pointing out that due to technical constraints (essentially the use of different positive controls for both PG and SG) it was not possible to directly compare the results of the two groups of women, which would have been ideal. Nevertheless, we can compare the results that were obtained within the individual groups and although a quantitative approach cannot be obtained, it is possible to make a qualitative comparison of the results of both groups.

4.5.1. Primigravidae

4.5.1.1. Both HIV and Placental malaria have a negative impact on the clinical outcomes of pregnancy

Both HIV and malaria were strongly and independently associated with the occurrence of low maternal haemoglobin and lower birth weight babies, and as was expected, co-infected women and their babies had the worst outcomes (Table 4-1) [10, 66]. This occurs independently of maternal age as revealed by using multilinear regression analysis. It is interesting to point out that HIV seemed to have a bigger impact on the birth weight of the infants than did PM: HIV associated with a loss of 238.7g when compared to the mean birth weight while PM represented a loss of 193.7g. This revealed to be independent of maternal age and maternal weight by multilinear regression analysis (data not shown).

This is revealing of the possible impact on the maternal and infant health that interventions that target either HIV or malaria infections in this particular group of women might have. The clinical outcomes maternal anaemia and LBW were also more prevalent in women that presented with these infections (Table 4-2).

4.5.1.2. Antibodies to CS2_{VSA}: Function, Titre and Total IgG

Levels of total IgG to CS2_{VSA} as well as function elicited by phagocytic antibodies had a larger spread than the actual titre of the phagocytic antibodies observed (Figure 4-1A), and we postulate that this is due to the limited exposure of PG in this study to pregnancy-specific parasites. In cohorts of MG, with longer histories of exposure and a more developed pregnancy-specific immunity, titres of phagocytic antibodies may have a larger spread, and may thus be more informative. The choice of function or titre for analysis is dependent on the research question, on the characteristics of the study population, etc., but both parameters (function and titre) can provide valuable information.

Not all antibodies that are able to target the surface of the pRBC are immediately associated with a specific function. According to the IgG sub-class to which the antibodies belong there are a number of functions that can be attributed to them, as was reviewed in Chapter 3 [14, 67-71]. Although in this study there was no attempt at associating the sub-class of IgG present in the serum samples with the amount of phagocytosis obtained (which has already been the focus of different studies [68, 72]), I was nonetheless interested in the correlation between the total IgG to CS2_{VSA} and the phagocytosis levels obtained. In this study group of PG the correlation between those two antibody measurements was about 48% by Spearman's rank and about 56% by linear regression (see section 4.4.2.2 and Figure 4-1B). These values give us an idea of how the values of one measurement co-vary (Spearman's rank) and how strong is one of the measurements in predicting the other (linear regression).

4.5.1.3. Phagocytic antibodies to CS2_{VSA} are decreased in HIV-infected women and are not associated with protection

Antibodies towards CS2_{VSA} (in this case measured by phagocytosis assays or total IgG assays) were found to be elevated in women who presented with evidence of current or past *Plasmodium falciparum* infection in their placentas. It seems well established that these anti-VSA_{CSA} antibodies represent a good marker of infection in PG [7, 31, 47, 48] (Figure 4-2A, Figure 4-3A) at delivery.

Levels of IgG to CS2_{VSA} and phagocytic antibodies were lower in HIV positive women when compared to HIV negative women (Figure 4-2B, Figure 4-3B), as had been suggested by other studies [55].

In our cohort of PG, antibodies to CS2_{VSA} were not found to be associated with protection from LBW or low maternal haemoglobin levels (known to be important outcomes associated with placental malaria (reviewed in [73, 74]) (Figure 4-4 and Figure 4-5), instead antibodies reflect mainly exposure to antigen. Similar results, with a lack of association between adverse outcomes and presence of antibodies to MiP related antigens, have recently been published [51]. It may be possible that a certain titre and "quality" of such antibodies has to be present in circulation at very early stages during the pregnancy for a protective effect (or an association with one) to be observed, something already suggested by other studies [8, 51, 75]. Only studies spanning from pre-pregnancy until delivery looking at these antibodies would be able to answer this question convincingly.

In a multivariate analysis including HIV, maternal haemoglobin level and infant birth weight, using the malaria infected women, HIV was found to be associated with a de-

crease of phagocytic antibodies function but not of levels of IgG to CS2_{VSA} (Table 4-3). A similar result with HIV affecting phagocytic antibodies function (opsonic-activity) but not other types of antibody was obtained by Jaworowski et al, using MG women from this same study cohort [12]. This raises two distinct possibilities: either HIV impacts differently on the production of functionally distinct antibodies (as suggested also by [14]) and/or the new phagocytosis assay is more sensitive for the detection of small differences between groups.

4.5.1.4. HIV impacts greatly on the acquisition of antibodies to VSA_{CSA}

When the women were grouped according to their PM status, measures of the function of phagocytic antibodies and the total IgG to CS2_{VSA} tended to vary between current, chronic and past placental infection. Antibody levels were highest in the women with chronic infection (as observed by others [7]), supporting the view that constant exposure to MiP-type parasites increases production of anti-VSA_{CSA} antibodies. Unlike the previously mentioned study, which included women of all gravidities, this study focussed solely on PG, indicating that a single pregnancy where chronic infection is present is sufficient to generate strong anti-VSA antibody responses. In the absence of HIV infection, the levels of antibody in acute infection were very similar to those in chronic infection, suggesting that robust antibody responses to these antigens can develop rapidly, as observed also in other studies [75] (Figure 4-6). Further investigation of the relationship between placental histopathology, duration of infection and levels of antibodies in HIV positive versus HIV negative women would be of significant interest in confirming the differences observed.

Grouping according to PM status provides also a putative timeline of exposure to pregnancy specific antigens and of antibody acquisition: Women with 'No Malaria' may have had little or no exposure to antigen. In 'Acute Infection', antibody responses are developing, following recent exposure to antigen. 'Chronic Infection' represents repeated exposure to antigen, and antibody maturation and/or recall, and 'Past Infection' is associated with antibody maintenance, following past exposure to antigen. This is in keeping with established paradigms of the time course of placental infection [76].

When analysed under this classification it seems that HIV infection reduced the generation of antibodies and possibly affected the maintenance of antibodies after clearance of malaria infection, when comparing HIV-positive with HIV-negative women. Although the time-frame of one pregnancy is probably not ample enough to fully assess the impact that HIV can have on the maintenance of antibody repertoires, it is well established that HIV negatively influences memory B cells, impairing the long-term serological memory of the

infected individuals [77, 78]. Future studies looking at the levels of these same antibodies six months after delivery or in subsequent pregnancies in HIV negative and HIV positive women and comparing numbers and activity of memory B-cells may prove to be more informative. Even, so, and since phagocytic antibodies seem to be more affected by HIV than total IgG to CS2_{VSA} I hypothesised that this difference would be of seminal importance when a second pregnancy starts since HIV positive women would have, theoretically, less phagocytic antibodies than HIV negative women.

In the chronic infection group, HIV did not seem to impact on the level of IgG to CS2 VSA or function of these antibodies, suggesting that a longer exposure period may overcome the impairment in antibody production elicited by HIV infection.

4.5.2. Secundigravidae

4.5.2.1. PM does not associate with clinical outcomes. HIV associates with lower infant birth weight.

The mean haemoglobin level of the women participating in this study was low (mean= 10.6 g/dL), but the exact cause of this high prevalence of anaemia is unknown, and likely to be multifactorial in this population [79]. Probably as a consequence of those low levels, haemoglobin levels did not differ between HIV negative and HIV positive women, nor between women with and without PM. HIV infection, but not PM, significantly influenced infant birth weight (Table 4-4).

4.5.2.2. Antibody levels: spread and correlations between antibodies measured

The correlation between total IgG to CS2_{VSA} and phagocytic antibodies observed was similar in strength in both the SG and PG from the same cohort (Figure 4-7A). It is interesting to note, though, that the values for both the Spearman's rank correlation and the linear regression are slightly higher in the SG group than in the PG group (see sections 4.4.2.2 and 4.4.3.2), something that I will discuss below. The spread of the antibodies measured revealed a higher spread for total IgG to CS2_{VSA} in this SG group (Figure 4-7B), reflecting, I hypothesise, the recall of immunity from previous exposures.

4.5.2.3. Associations of antibodies measured with HIV and PM

Both phagocytic antibodies and total IgG to CS2_{VSA} were higher in PM+ women than in PM- women. HIV appeared to have significantly greater impact on phagocytic antibodies than on total IgG to CS2_{VSA} (Figure 4-8). This impact of HIV supports the view that either,

in cohorts of women infected with HIV, IgG sub-classes that contribute to phagocytosis are more impaired than those that do not or that the phagocytosis assay developed is a more sensitive way of detecting these differences. Several studies have shown that different functional antibodies and even levels of total antibodies do not always correlate well with each other when controlling for gravidity or HIV [12, 14, 80].

4.5.2.4. Association of infant birth weight with phagocytic antibodies

When a univariate analysis of infant birth weight and maternal haemoglobin levels was performed against the antibodies measured, infant birth weight revealed a significant association with phagocytic antibodies but not total IgG to CS2_{VSA} (Figure 4-9). I tried to dissect this association by including in the analysis parameters that are associated with changes in low birth weight. When the women were divided according to presence or absence of PM, levels of total IgG to CS2_{VSA} were still not associated with birth weight or haemoglobin levels in either group. On the other hand, phagocytic antibodies to CS2_{VSA} within PM+ women remained associated with infant birth weight even after controlling for HIV, maternal age and maternal weight. Infant birth weight increased 3.57g per unit of antibody measured (coef= 3.57, $p=0.025$) (Table 4-5). These observations have similarities to those of Duffy et al, who measured anti-adhesion antibodies and identified SG as a particular important group in the search for associations between antibodies and outcomes [8].

4.5.3. Antibodies towards VSA_{CSA} in Primigravidae and Secundigravidae: an attempt at a final discussion

As I specified earlier, it is not possible to directly compare the results of the two groups of women. Nevertheless, I believe that by comparing some of the results obtained with one and the other it is possible to begin to paint the picture of how in the context of HIV infection, immunity against MiP is being developed. Although an attempt for a rational and factual discussion is going to be made, it is unavoidable to enter the realm of speculation at times, and I hope that this discussion can foster the development of new ideas.

I would like to start by stating that I believe that in cases when longitudinal studies are not able to be performed, cross-sectional studies of antibody levels against MiP-type antigens should take placental histopathology results in consideration and not depend on the presence or absence of parasites in the peripheral circulation, or the placental circulation only. Taking placental histopathology results in consideration allows us not to (or at least to minimise it considerably) overlook cases of past infections that were resolved, and that

might have significantly contributed for the levels of antibodies that are being detected at the time of collection of samples.

In this study, the effect that HIV had on the levels of antibodies measured in PG was quite dramatic and we were able to give a tentative explanation of how this occurred by making use of the histopathology grading of the placentas of the women involved. This revealed that it is during the acquisition of immunity that HIV seems to impact on the immunity to new antigens, which in this case were MiP-type parasites. Besides this, phagocytic antibodies in HIV positive women that had evidence of past infections also seemed to have lower levels than in women that were HIV negative. This led me to hypothesise that, if these women were to have a second pregnancy, those that had higher levels of antibodies, from their first pregnancy, would be able to fight a second PM infection better. So, and despite revealing to be only a marker of infection in the PG group, these phagocytic antibodies could actually be protective in SG. It was with this in mind that I decided to investigate a group of SG for which the placental histopathology results were also available.

Although the numbers of women were not enough to completely dissect the role of these antibodies according to the different PM groups, the fact that in the 'No malaria' group HIV positive women had lower levels of phagocytic antibodies than did HIV negative women (with no significant differences for total IgG to CS2_{VSA}) seems to confirm the results obtained in the PG group for women with past infection. The significant effect that having phagocytic antibodies had on the infant birth weight in the SG women, may reflect a very important parameter that I was not able to assess during this study but which I (together with others) believe to be important to determine and to which I alluded before: probably a certain titre and "quality" of antibodies has to be present in circulation at very early stages during the pregnancy for a protective effect (or an association with one) to be observed [8, 51, 75]. As such, being in a position of transition between susceptible PG and immune MG, SG can be an important group for the detection of associations between antibodies and clinical outcomes and thus present a very interesting focus of research.

4.5.4. Final remarks and future directions

This study was primarily directed at looking at the effects of HIV on a PG group of women who are the group more likely to suffer from MiP. The study on SG women that followed, was the result of observations made in the first study and revealed and consolidated the idea that these two groups of pregnant women sufficiently different from each other (and probably from MG, though I did not analyse MG in this study), to be analysed separately and even be the subject of independently designed studies.

It is not possible to conclude with absolute certainty that the results obtained in this study will be applicable to all populations of African pregnant women since it is well established that the dynamics of infection (both HIV and malaria) highly influence the way in which these two interact [11, 53]. Nevertheless, there are several points that can be taken and tested in different settings. Although the effects of anti-malarial treatment were not evaluated, it is clear that HIV should be fought from the onset of pregnancy in PG so as to allow for the generation of immunity against MiP. This immunity is likely to be of importance for the outcomes of subsequent pregnancies. Together with this, interventions that target maternal mal-nutrition should be used in order to increase maternal well-being and as revealed in the SG study, minimise the adverse outcomes of maternal HIV infection on the infants.

Longitudinal studies that are conducted specifically to look at the development of immunity during pregnancy in HIV positive versus HIV negative women from PG to SG would, no doubt, be the ideal way of confirming some of the results that I have obtained. Future studies looking at the numbers and activity of memory B-cells may also prove to be more informative.

It cannot be forgotten, also, that this is a study that has looked only at 'one side of the coin'. Evaluating the levels of antibodies in these women has given us an idea of how immunity is being developed, but it fails to show us how the immune cells (particularly monocytes and macrophages) of these women are responding to these antibody levels. It is possible that women that have shown high phagocytic antibodies levels but no protection from PM may have impaired monocytic function. It would be extremely interesting to measure the function of these cells in pregnant women and correlate this with their antibody levels and PM status.

In conclusion, this work indicates that the measurement of functional antibody is superior to the common measurement of total IgG to CS2_{VSA}, (which has been the "gold-standard" assay for immunity acquisition in the field of malaria in pregnancy).

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5. Early studies into the potential role for IgE in the induction of phagocytosis in primigravidae

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5.1. Summary

It was clear from the studies conducted on PG and SG (refer to chapter 4) that there was a significant number of serum samples that were able to induce phagocytosis in association with extremely low levels of total IgG to VARCSA. This, together with the knowledge that Thp-1 cells also express the FcR for IgE, prompted me to investigate whether PG, particularly the ones who had very low levels of Total IgG to VARCSA could have levels of IgE that would correlate with the observed phagocytosis measured.

5.2. Literature Review

5.2.1. Finding and describing IgE

Of all the immunoglobulin (Ig) classes known to exist in humans, IgE was the last to be discovered with its existence, and acceptance as a distinct Ig, coming in 1966-69 [1]. Before this, experiments by Prausnitz and Kustner in 1921 had proved the existence of a serum factor that could passively be transferred from an allergic patient to a non-allergic patient and cause, in the latter, an allergic reaction once the appropriate antigen was administered, thus providing us with the first demonstration of the biological activity of IgE [1].

IgE is the least abundant of all the Ig classes. This Ig exists as a monomer and has a short half-life, of about 3 days, in the serum, compared to the 21 days attributed to IgG [2]. Most IgE is sequestered in the tissues associated with immune cells like mast-cells [3]. Despite the sequestration of IgE in the tissues (e.g. skin, lung or bowel), and its low concentration in serum (approximately 150 µg/mL compared to 10mg/mL for IgG), it can be extremely elevated in reaction to parasitic infections or allergens [4] and the increased level of IgE in the serum is accompanied by an increase in the number of circulating plasma cells that produce it [5], suggesting IgE may have an important role in immunity. However, the role of IgE as an immune mediator is mostly recognised in allergies (including asthma [6] and food allergies [7], for example) and in response to helminthic infections like Schistosomiasis [8]. Its role during a malaria infection, if any, is still not completely resolved (see **IgE and Malaria** below).

5.2.2. The IgE receptors

As was briefly reviewed in Chapter 3 of this thesis, there are three known receptors for IgE: FcεRI, FcεRII (CD23) [2] and the recently identified Galactin-3 [9, 10].

5.2.2.1. FcεRI

The Fc ϵ RI high-affinity receptor is found on mast cells and basophils in which it induces their degranulation [2]. It can also be found on Langerhans cells and monocytes/macrophages [11-14]. This receptor is composed of four chains in both mast cells and basophils, but only of three chains in monocytes/macrophages [15].

5.2.2.2. Fc ϵ RII (CD23)

Fc ϵ RII (CD23) was identified as a receptor for IgE in the early 1990's [16, 17]. This receptor is not a member of the immunoglobulin family, unlike all the Fc γ receptors and also Fc ϵ RI, rather belonging to the C-type lectin receptors [18]. This receptor, although considered the low-affinity receptor for IgE, is still capable of binding IgE with as much strength as CD64, the high-affinity receptor for IgG, binds to its ligand [2]. Found on several types of cells including B-cells, monocytes/macrophages, eosinophils and follicular dendritic cells, CD23 has two-forms: CD23a and CD23b which mediate either endocytosis and B-cell stimulation (CD23a) or phagocytosis (CD23b) [19].

5.2.2.3. Galectin-3

The last IgE receptor to be identified, galectin-3 is a beta-galactoside-binding lectin [9, 10] found to be expressed on neutrophils (where it plays a role in their activation) [20] and in placental trophoblasts [21].

5.2.3. IgE and phagocytosis

After the discovery of IgE receptors on the surface of human macrophages/monocytes [12, 13] the capacity that IgE and its associated receptors could have to induce the phagocytic function of mononuclear phagocytes was shown by Boltz-Niculescu et al [22]. Using IgE-sensitised sheep RBC, they were able to show that human-donor derived monocytes were able to ingest these particles and thus establish the presence and function of IgE-receptors on the surface of those cells. The receptors responsible for the phagocytic activity were still not identified at this stage. This was done a few years later after CD23 was identified as one of the receptors for IgE [16, 17]. It was shown, by Yokota et al [19] that CD23 (Fc ϵ RII) had two variants: Fc ϵ RIIa and Fc ϵ RIIb. By using stable transfectants expressing the wild-type or mutated forms of these receptors, they were able to demonstrate that Fc ϵ RIIb was the variant of CD23 responsible for the phagocytosis activity of IgE while Fc ϵ RIIa had a role in endocytic processes. The role of IgE as a mediator of phagocytosis has been since elegantly shown in a series of papers from Hannah J. Gould's lab, where after designing IgE antibodies against Folate Binding Protein (a marker of ovarian cancer), they were able to reveal the roles that both IgE and CD23 play in the

induction of phagocytosis of cancerous cells by monocytes. This induction of phagocytosis is enhanced when in the presence of the cytokine IL-4 [23-25].

5.2.3.1. IgE receptors and Thp-1 cells

The presence of receptors for IgE on the surface of Thp-1 cells was first demonstrated in a couple of papers where the effect of exposure of various monocytic cell lines to either IL-4 or IL-6 and the subsequent increase in expression of CD23 was investigated [26, 27]. These studies, together with later ones, established the presence of CD23 on the surface of Thp-1 cells. It is important to notice that these studies were conducted with uThp-1 cells.

Agents such as Tumour Necrosis Factor-alpha (TNF- α), Interferon-gamma (IFN- γ) and 1 alpha,25-dihydroxyvitamin D3 (Vitamin D3) were shown to either increase (TNF- α , IFN- γ) or decrease (Vitamin D3) the expression of this receptor on the surface of uThp-1 cells, in a pattern that closely resembled the one obtained with blood-derived monocytes [28, 29].

5.2.4. IgE and Malaria

The search for the significance of IgE during a malaria infection, if any, has been marked by contradictory results and more doubts and questions than answers, and remains, even after 40 years of studies, largely an unknown.

5.2.4.1. IgE levels

The first studies that looked at IgE and malaria, relied on the observation that Nigerian patients seemed to have much higher levels of IgE in their serum than did Swiss nationals, which prompted the question: could malaria be responsible for this increase? The results of their studies seemed to point to malaria not being associated with an increase of the total levels of IgE in the serum of those patients [30]. Desowitz was the first to investigate the presence of Plasmodium-specific IgE in serum samples from residents of malaria endemic areas finding that 33% of adults tested revealed IgE to *Plasmodium falciparum* antigens by ELISA [31]. After these initial studies, several others have reported on the levels of IgE in malaria patients. The levels of total IgE were reported to be elevated in the sera of patients from malaria endemic regions of Africa and Asia and specific IgE to Plasmodium antigens was verified using both ELISA and protein immunoblotting (Western Blots) [32] and these correlated with malaria parasitaemia or malaria attacks [33-37]. There are some reports, though, of increased levels of IgE (specific for *P. falciparum*) associated with a decrease in parasitaemia [38].

5.2.4.2. IgE: protective or detrimental?

The question of whether IgE levels (total or parasite-specific) are protective or detrimental may be a difficult one to answer.

The first reports on the effect that IgE might have on the outcome of a malarial infection presented evidence of an increase in IgE levels in children that presented with cerebral malaria when compared with patients that had uncomplicated disease [32]. A higher level of IgE was also observed in Thai patients with severe malaria when compared with those that presented uncomplicated malaria [33]. In a study conducted in Burkina Faso, children with severe malaria had a significant increase of IgE levels in the serum than those with non-severe disease and, in the Ivory Coast, children that presented severe malaria also had higher levels of IgE [35].

On the other hand, in a holoendemic region of Tanzania, and when looking at a population of asymptomatic individuals followed longitudinally, high levels of *P. falciparum* specific IgE were associated with a reduced risk of acute malaria, independently of the age group [39]. Farouk et al, when studying two ethnic groups in Mali exposed to the same environment, showed that protection from parasitaemia was associated with higher levels of anti-malarial IgG and IgE [40].

5.2.4.3. Beyond IgE: IL-4 and TNF- α

Perlmann et al's observation that IgG4 seemed to be present in most serum samples where IgE against malaria antigens was evident, led them to hypothesise that this might be an IL-4 mediated mechanism [32]. Studies conducted using animal models of malaria seemed to show that IL-4 had a role in the induction of IgE, but was not absolutely necessary [41]. In human populations living in *P. falciparum* endemic areas individuals with an IL-4/IFN- γ ratio higher than 1 (which would presumably reflect a more Th2 oriented immune response) had higher levels of anti-malarial IgE than did individuals where that ratio was lower than 1. This association, although a weak one, was further pursued and it was shown that an allelic variant of the IL-4 promoter region (IL4-589T) associated with higher levels of IgE in children with severe malaria [42] and, together with another polymorphism in the IL-4 promoter region IL4+33, associated with higher IgE levels in cerebral malaria victims [43]. The associations were not always towards IL-4 and IgE being detrimental, as was shown by the study of Farouk et al that linked IL-4 producing cells and IgE to protection from malaria [40] and that observed, once again, the IL4-589T could be linked to this [44].

TNF- α was found to be secreted by monocytes exposed to IgE-complexes, through the action of CD23, and since its release in the capillaries has been associated with tissue lesions, it is believed to form part of the process by which IgE might mediate the severity of the disease [45].

5.2.4.4. IgE, malaria and helminth infections

Helminths are eukaryotic worm-like parasites that live inside their host [46]. The study of the possible interactions between helminthic parasites and *Plasmodium sp* is a vast field and extremely interesting. The main points of these possible interactions are presented, but by no means exhaustively. Extremely thorough and informative reviews on the subject have been published both on helminths alone [46, 47] as well as on malaria and helminth interactions [48, 49].

Murray et al showed in the late 1970's that, when African populations were treated against *Ascaris lumbricoides*, there was an increase in the risk of recrudescant malaria attacks which led investigators to conclude that the immunological milieu created by the worms was protecting the host from clinical malaria [50, 51]. This was also the case in a series of studies conducted in Thailand where the burden of infection with that same worm, *Ascaris lumbricoides*, was associated with protection from cerebral malaria, independently of the nutritional status of the host [52, 53]. More recent studies though have not provided such results, rather showing that worm infections might worsen the malaria outcome of those that have co-infections [54, 55]. All the results obtained in these studies may be dependent on factors like the host age (the studies in Thailand were performed in adults, the studies in Africa are mainly done in children), setting (hospital based or community based), helminth species present, malaria species present and even the study type (interventional, observational, cross-sectional, longitudinal, etc).

During pregnancy it is believed that worm and plasmodium co-infections lead to exacerbated anaemia and even to low birth weight babies especially in PG where co-infection is highly prevalent [56, 57]. It is well established that helminthic infections induce a strong Th-2 oriented immune response, with an emphasis on the production of IL-4, IL-5 and IL-13 that drives B-cells to the Ig class switching necessary for the production of IgE [46, 47]. CD23, the IgE low-affinity receptor, has been implicated in the interaction between helminths and malaria in cerebral malaria [58] nevertheless, in terms of MiP there are, at present, no studies that have addressed the presence of IgE and co-infection with helminth parasites and *Plasmodium spp*.

5.2.5. IgE during MiP

Not many studies have addressed questions related to the role of IgE during MiP and, as of the time of writing of this thesis, the role of IgE during a normal pregnancy is also an unknown [59].

The first studies that investigated IgE (Total and Plasmodium-specific) during MiP were conducted by Desowitz et al in Papua New Guinea and were mainly concerned with the role that plasmodium-specific foetally generated IgE (stimulated by antigens transferred from the mother's circulation) could play [60, 61]. As well as cord blood responses, some pregnant women were also found to have Plasmodium-specific IgE, and this was more common with increasing parity. It is curious that in these studies, plasmodium-specific IgE (as measured by ELISA) was not able to be detected in PG. Deposition of IgE in the placentas of infected women (from both Malawi and Papua New Guinea) was demonstrated by Maeno et al and a possible negative association with the degree of parasitaemia was found [62]. Deloron et al also were able to detect plasmodium-specific IgE in maternal serum from Cameroonian pregnant women but failed to detect any possible association or role for these IgE with malaria infection [63]. The last study that has focused on maternal IgE and MiP was conducted by Rindsjo et al, where the presence of IgE associated with cells in the placenta was evaluated immunohistochemically. IgE was found to be mainly associated with Hofbauer-like cells, macrophage-like cells resident in the placenta, and no differences in the pattern of expression of IgE, between placentas of Ghanaian women with or without evidence of malaria parasites were reported. There was also no correlation between the levels of IgE in the maternal serum and the presence of these IgE+ cells [64].

5.2.6. IgE and PG: why investigate?

The few studies that have focused on MiP and IgE [60-64] have failed to give us a clear idea of the role that IgE might play in the development or outcome of the disease. Besides being mainly focused at detecting the presence or absence of IgE in pregnant women and trying to relate that presence/absence with the presence of malaria parasites, not much more has been answered.

What are the levels of IgE at the beginning and end of pregnancy? How do they relate to IgG? Are IgE levels associated with helminth infections during pregnancy? How do the different helminth infections affect the levels of malaria-specific IgE and how are they related to the outcomes of MiP? Does IPTp treatment influence the levels of IgE in pregnant women?

There are a number of questions left to answer and especially important may be the questions that concern PG. Do PG, with no IgG to the MiP-like malaria parasites have, at the

beginning of pregnancy, IgE that recognises the parasite? If so, where does that IgE come from? Is it cross-reacting? Are PG that exhibit higher levels of these IgE more or less protected from MiP? What is the pathway by which these IgE work? Do they induce ADCC responses in the placenta that might correlate with increased levels of inflammatory cytokines? Do they work as anti-adhesion antibodies, or are they a first line of antibodies that might contribute to the clearance of parasites by phagocytosis? Since it seems clear that placental trophoblasts express galectin-3, what could the presence of IgE-covered *Plasmodium* pRBC mean in terms of pregnancy outcome?

There are too many questions unanswered, and this thesis does not aim to answer them all, but my results, as described in Chapter 4, led me to hypothesize that, in PG that exhibit low levels of total IgG to CS2_{VSA} but still elicited good levels of phagocytosis, parasite-specific IgE could be playing the role of opsonising antibodies.

5.3. Methods

The results pertaining to the function of IgG towards CS2_{VSA} and total IgG to CS2_{VSA} phagocytosis assay were obtained during the work described in Chapter 4 and the methods are described in detail in Chapter 4, section 3.

I would like to thank Sara Prickett for providing me with some anti-IgE antibodies used in the preliminary stages of this study and Michael Duffy and Michaela Peter for providing me with the anti-ATS antibodies.

I would like to thank Elizabeth Aitken for providing me with her unpublished protocols for the detection of parasite-specific IgE by ELISA.

5.3.1. Ethics statement

The work presented in this chapter used samples from the MHP study cohort conducted in Blantyre, Malawi, which got its ethical clearance from the College of Medical Research and Ethics Committee, University of Malawi and the Melbourne Health Human Research Ethics Committee.

5.3.2. Western Blotting

This protocol is an adaptation of methods previously published [65, 66]. Twenty samples of serum that had low total IgG to CS2_{VSA} were used.

5.3.2.1. Solutions used

Protease inhibitors: 1 tablet Cocktail tablet Complete-Mini (Roche) in 10 mL PBS

1% Tx-100: 1mL Triton-X 100 (Sigma-Aldrich, St Louis, Missouri) in 99mL PBS

2%-SDS: 2g Sodium Dodecyl Sulphate (SDS) in 90 mL PBS. This was heated to 68°C and stirred. The pH was then adjusted to 7.2 with HCL 1M and the volume adjusted to 100mL.

1x Tris-Acetate (TA) Running Buffer: 100 mL of Tris-Acetate 10x in 900 mL of MilliQ water

1x Tris-Glycine Transfer Buffer: 100 mL Tris-Glycine 10x (with 0.1% SDS), 200 mL Methanol in 700 mL MilliQ water., kept ice-cold until use.

10x Tris Buffer Saline – Tween (TBS-T): 30g Tris, 45g NaCl, 5 mL neat Tween20, 495 mL H₂O.

Blocking Solution: 50 mL TBS-T 10x. Add 300 mL H₂O and 50 g Skinned Milk (powder), stirred for 10 min, adjusted to a final volume to 500 mL and filtered.

TBS-T with Milk (TTM): 1/2 dilution of Blocking Solution in TBS-T

TTM-High salt: TTM + 2g per 100 mL of NaCl

5.3.2.2. Preparation of protein extract

A CS2-GFP parasite culture, of > 5% trophozoites was collected and spun for 5 min at 400 rpm. The pellet was resuspended in 2mL of RPMI-HEPES and the trophozoites were purified by Percoll and counted. Briefly, parasite cultures were overlaid on a gradient of 80%, 60% and 40% Percoll (Amersham) in RPMI-HEPES. After centrifugation at 2,000g x 15 min, cells resting on the 60% layer were collected and washed thrice at RT with RPMI-HEPES (400g for 5 min). After counting, a final spin was performed and the supernatant was discarded. From here on after, all the procedures were done on ice. The parasite pellet was lysed with the help of 1% Triton-X 100 in PBS containing protease inhibitors (TX-100PI) and spun at high speed at 4°C for 10 min. The supernatant was discarded and the pellet washed in 1 mL of TX-100PI and re-spun at high speed at 4°C for 10 min. The pellet was then resuspended vigorously in 2% SDS in PBS at a ratio of 10⁸ parasites/mL (800 µL). The solution was then spun at high speed (just long enough to bring the pellet down), and the pellet resuspended again until no more pellet was able to be dissolved. The solution was then spun at high speed for 10 min and the supernatant collected.

5.3.2.3. Running of the protein extract on a gel

A 3-8% Tris-Acetate gel with only one sample well and a control well (NuPAGE, pre-cast gels, Invitrogen) was mounted on a small gel apparatus filled with TA-running buffer, making sure that the well of the gel was filled with the buffer. One-hundred microliters of the protein extract preparation (containing 490µg/mL of protein) was added to 100 µL of sample buffer (4% SDS, 10% ME, 20% Glycerol, 0.004% bromophenol blue, 0.125M Tris-HCl pH 6.8) and this mix was vortexed and boiled for 3 min in a heating block. This was done to reduce and denature the proteins. The 200 µL of sample were then applied to the sample well of the gel and 5 µL of pre-stained protein molecular weight marker (max of 210 kD) (Invitrogen) was used in the control well. The sample was then run for 10 min at 90 volts and then for 45 min (or until the migration front exited the gel) at 180 volts. A protein ladder (SeeBlue®Plus2 pre-stained standard, Invitrogen) was used to determine the molecular weight of the protein bands.

5.3.2.4. Transfer of the protein bands to nitrocellulose membranes

Fifteen minutes before beginning the transfer, an ice block and the transfer buffer were put at -80°C. The gel was cast off its support and 'sandwiched' between one sponge pad, two pieces of Whatman blotting paper, and one nitrocellulose membrane, two pieces of Whatman blotting paper and a sponge pad. The transfer of the protein bands to the nitrocellulose membrane was achieved by electrophoresis in transfer buffer with agitation and in the presence of a frozen ice block for one hour at 100v.

5.3.2.5. Blocking, probing and developing the membrane

The membrane was marked with a line on top and bottom and then cut into fine strips approximately five millimetres wide. Each strip was numbered. The strips were then incubated with blocking solution with agitation for 2.30 hours.

Probing of the strips was done for one hour with one mL final volume of serum or antibodies diluted in TTM. The dilutions used were 1:2000 dilutions of serum for IgG and 1:25 dilutions of serum for IgE. Two pools of serum samples that had low total IgG to CS2_{VSA} and either high (HP) or low (LP) phagocytosis values towards CS2_{VSA} were used. Control antibodies used were a 1:200 dilution of an anti-ATS (Acidic Terminal Sequence) 2747/affinity purified antibody [65] (recognises in CS2 parasites a single band corresponding to the localization of PfEMP-1), 1:200 dilution of anti-ATS 3031/rabbit serum (recognises 2 bands, at least one of which should be PfEMP-1 and the other one may be a RBC membrane component), a 1:10,000 dilution of RaH (the same antibody used as a control during the phagocytosis assays). After 1h incubation, the strips were washed 1x5min with TBS-T, 1x5min with TTM-high-salt and 1x5min with TTM (Wash). A 1h incubation followed with

rabbit anti-human IgE antibody (DAKO, ref: A0094) used at a dilution of 1:1,000 for the detection of human IgE, or with a 1:500 dilution of rabbit antihuman IgG antibody (Dako-Cytomation, Denmark) for the detection of IgG. A new wash was performed and a final 1h incubation, this time with a 1:2,000 dilution of (WHAT?) anti-rabbit coupled with horse-radish peroxidase (HRP) (Invitrogen). A new wash was performed. Finally, equal volumes of reagent A and reagent B from ECL kit (GE Healthcare) were mixed and added to the strips for 1 min. The strips were then gently taken out of the ECL and aligned on transparent sheets, before being put on Hypercassette (Amersham Biosciences) and revealed in an Agfa 1000 developer.

5.3.3. IgE measurement by ELISA

For the measurement of IgE by ELISA, the original serum samples (not heat-inactivated) of the PG cohort were used. A total of 115 samples were analysed corresponding to all the PG samples that had a total IgG to CS2_{vSA} level of less than 3% of positive control.

5.3.3.1. Total IgE

Total IgE was measured using a sandwich ELISA. In brief a mouse anti-human IgE monoclonal Ab 107 (MABTECH) 2 μ g/mL in PBS 100 μ L/well, was left to adhere to Nunc MaxiSorp™ flat bottom 96 well plates (Nunc, Thermo Fisher Scientific, Roskilde, US) overnight at 4°C. The plates were then washed thrice with wash buffer (PBS 0.1% Tween20) and blocked (100 μ L/well blocking solution-PBS 0.1% Tween20 with 10% Skim milk) for 1 hour at room temperature (RT) followed by three washes with wash buffer and incubated with 100 μ L/well samples diluted 1/1000 in reagent diluent (PBS 0.1% Tween20 with 5% Skim milk) or purified IgE standards (MABTECH) 0-40ng/mL (8 points) for two hours at RT. The plates were then washed thrice and incubated with 100 μ L/well of biotinylated Mouse anti-human IgE monoclonal antibody 182 0.5 μ g/mL in reagent diluent for 1 hour at RT, after which plates were washed thrice and 100 μ L/well streptavidin-HRP conjugated antibody (P0397 from DAKO, Denmark) diluted 1/300 in reagent diluent and incubated on the plate for 1 hour at RT. The plate was washed thrice with wash buffer and 100 μ L/well of HRP substrate made as described by manufacturer from ECL substrate kit (Amersham) for 5 minutes until colour developed, reaction was stopped with the addition of 50 μ L/well 1N HCl solution. The plate was read at 490 nm.

5.3.3.2. Antigen-specific IgE

P. falciparum IgE was measured after using an indirect ELISA to overcome the problem of IgG competing out IgE binding of antigen (unpublished protocol provided by Elizabeth Ait-

ken). In brief a mouse anti-human IgE monoclonal (Ab 107, MABTECH), 2 µg/mL in PBS 100 µL/well, was incubated overnight at 4°C in Nunc MaxiSorp™ flat bottom 96 well plates (Nunc, Thermo Fisher Scientific, Roskilde, US). The plates were then washed thrice with wash buffer and blocked for 1 hour at RT, followed by three washes with wash buffer and incubated with 100 µL/well of samples or controls, diluted 1/50 in reagent diluent for 2 hours at RT. The plates were then washed thrice and incubated with schizont extract (1/300) at RT for 2h. The plates were then washed thrice and 100 µL/well of hyperimmune sera (pps sera, also used in the phagocytosis assays as positive control) diluted 1/1000 was added to each well and left for 2 hours at RT, following which the plates were washed thrice and 100 µL/well Goat anti-human IgG–HRP (GAH, Silenius Labs) 1/5,000 in reagent diluent for 1 hour. The plates were then washed thrice and 100 µL/well HRP substrate made as described by manufacturer from ECL substrate kit (Amersham) for 10 minutes until colour developed. The reaction was stopped with the addition of 50 µL/well 1N HCl solution. The plate was read at 490 nm.

5.3.3.3. Analysis of results

Total IgE was calculated as µg/mL using the standard curve.

The values for IgE to the CS2 *Plasmodium falciparum* extract were standardised and expressed relative to controls (mean of non-exposed controls 0, mean of pps, 100) as relative absorbance in arbitrary units.

5.4. Results

5.4.1. Choosing the samples for IgE measurement.

As was seen for the PG and SG cohorts on chapter 4 (section 4.2.2 and section 4.3.2), a percentage of women in whom the levels of total IgG to CS2_{VSA} were very low had high or relatively high values for phagocytic function. Adding to this, PG had a lower degree of correlation than SG between the two antibodies' parameters measured (Figure 5-1). Focusing on the PG samples, where IgG had the worst correlation between total and functional levels, I decided to study the group of samples that had less than 3% total IgG to CS2_{VSA} (median value for all the samples of PG excluding all the samples for which total IgG was zero) (Figure 5-2).

5.4.2. Western blot analysis: IgE associates with higher values of phagocytosis

Samples chosen for the detection of IgE were derived from the same aliquots used in Chapter 4. These were pooled into two groups according to the levels of phagocytosis obtained. Samples were grouped as having high phagocytosis (HP, n=20) values if phagocytic function was $> 10\%$ and as having low phagocytosis if phagocytic function was $<10\%$ (LP, n=21). A pool of male serum, taken from fathers of children admitted to the same hospital (Queen Elizabeth Hospital, Blantyre, Malawi) where the PG samples were taken, and a pool of serum from Melbourne donors were used as controls.

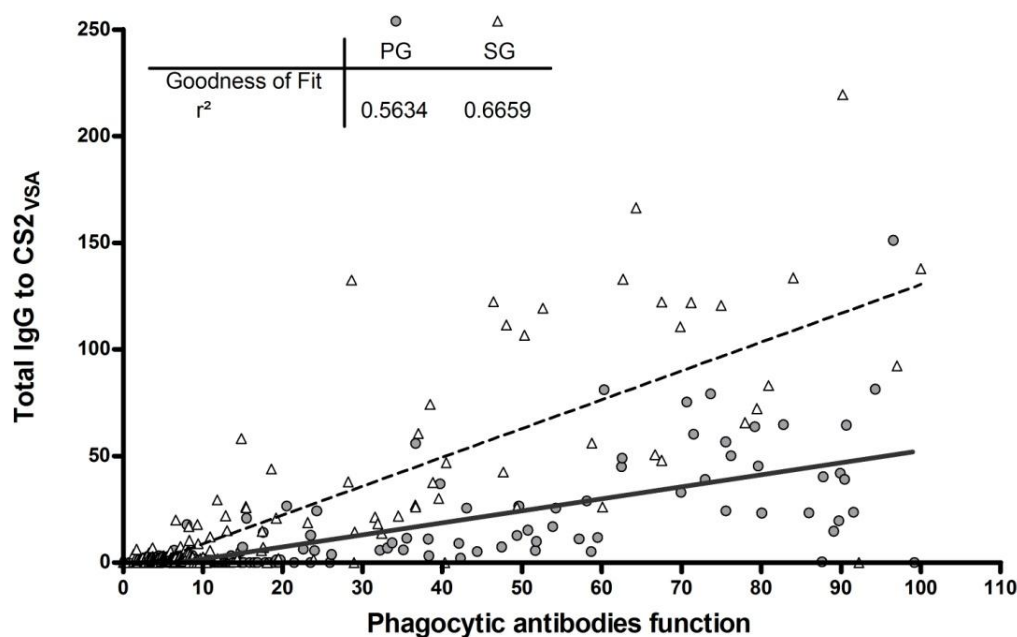


Figure 5-1 – Correlation between total IgG to CS2_{VSA} and phagocytic function for PG and SG

Both PG (circles) and SG (triangles) values for total IgG to CS2_{VSA} and phagocytic function are plotted. Linear regression lines (full line – PG, broken line – SG) and r^2 values are shown.

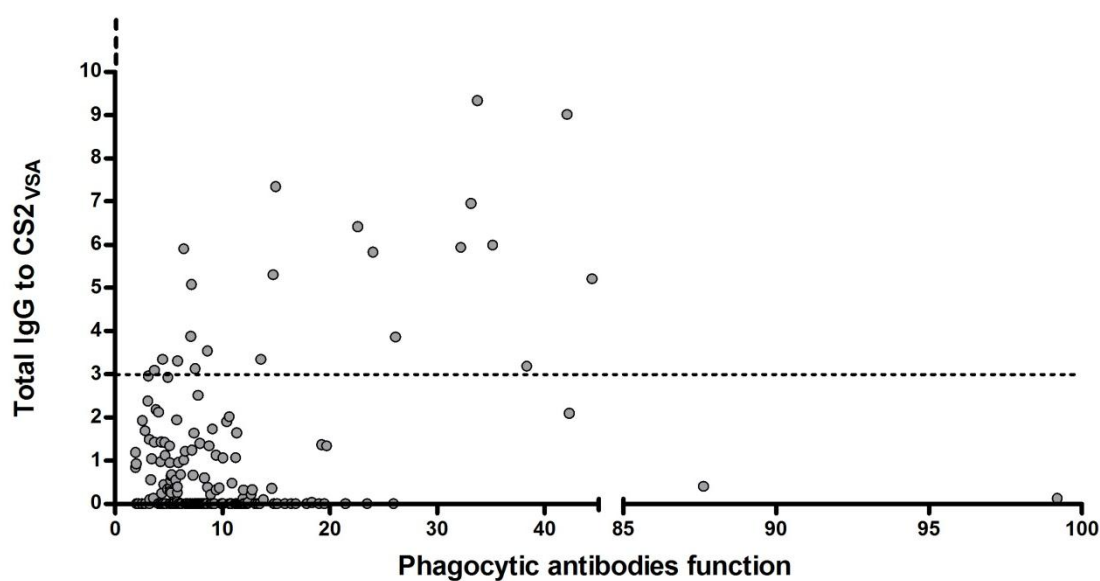


Figure 5-2 – Plot of total IgG to CS2_{VSA} and phagocytic function in primigravidae with cut-off for IgE studies.

Plot of the cut-off line used for the selection of samples to be used on IgE studies. Dotted line at 3% of total IgG to CS2_{VSA} was determined by calculating the median of the values for total IgG to CS2_{VSA} for all PG samples with the exception of those to which the value was zero.

5.4.2.1. Western blots reveal protein bands to which IgE associates in the group with increased levels of phagocytic function

Protein extracts from the parasite line *Plasmodium falciparum* CS2-GFP were separated on a 3-8% Tris-Acetate gel and probed with serum samples that demonstrated to have low total IgG to CS2_{VSA} (<3%). These were divided into two pools, a pool with low phagocytosis function (<10%, LP) and a pool showing high phagocytosis function (>10%). Pools of male serum, Melbourne donor control serum and CS2 positive serum were used as controls. An affinity purified anti-ATS antibody was used to detect PfEMP-1 as well as rabbit-sera against the ATS of PfEMP-1.

As can be seen in Figure 5-3, the anti-ATS control antibody recognised a single high-molecular weight (~300kD) protein band corresponding to the molecular weight of PfEMP-1 of *Plasmodium falciparum* CS2 (strip 10), while the rabbit-sera against the ATS of PfEMP-1 recognised not only a similar band corresponding to PfEMP-1 but also a band corresponding to a probable RBC derived protein (strip 9) that is also recognised by the rabbit anti-human RBC antibody used in phagocytosis assays (strip 1), as well as by all the strips probed with human serum (2-8). At the dilutions and exposure time used, no protein bands exclusive to the strips probed with human were found. After exposing the films to the strips for one minute (Figure 5-4) it was possible to visualise a number of protein bands that are equally recognised by all strips as well as 2 possible protein bands (strips 6 and 7, white asterisks) of molecular weight between 111 and 71 kD.

To confirm the presence of the protein bands associated with the HP pool of sera, new membranes were prepared and probed with HP serum, LP serum and affinity purified anti-ATS antibody. As can be seen in Figure 5-5, at a dilution of 1:25, and after 20s exposure there are clearly two protein bands of molecular weight between 111 and 71 kD that are recognised by IgE in the HP group but that are absent in the LP group. These protein bands do not correspond to PfEMP-1.

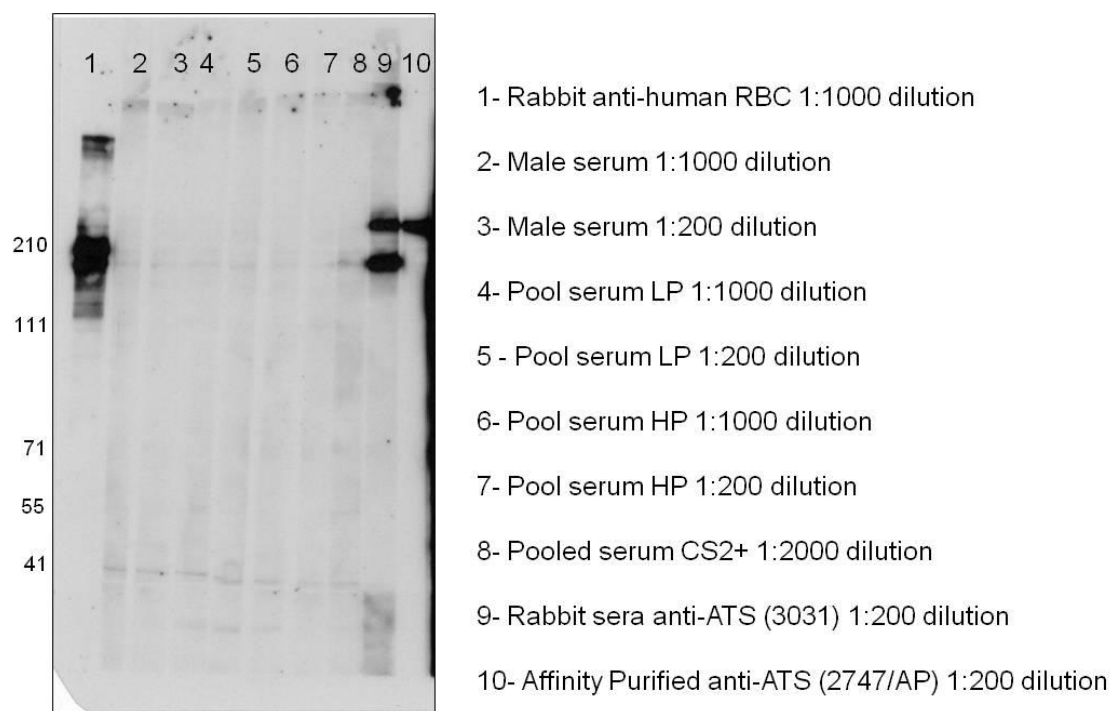


Figure 5-3 – Western blot of CS2-GFP membrane proteins probed with Primigravidae serum, 20 seconds exposure.

Membrane associated proteins were extracted from *Plasmodium falciparum* CS2-GFP parasites and separated on a 3-8% Tris-Acetate gel. These were then transferred to a nitrocellulose membrane and probed with human serum (strips number 2-8) or control rabbit antibodies (strips number 1, 9 and 10). A Rabbit anti-human IgE antibody was used to detect any IgE associated with the proteins on membrane strips 2-8. Film was exposed to the strips for 20 second (after incubation with a horse-radish peroxidase coupled antibody and ECL substrate). Numbers to the left represent the position and molecular weight (kD) of the standard (See-Blue®Plus2 pre-stained standard, Invitrogen).

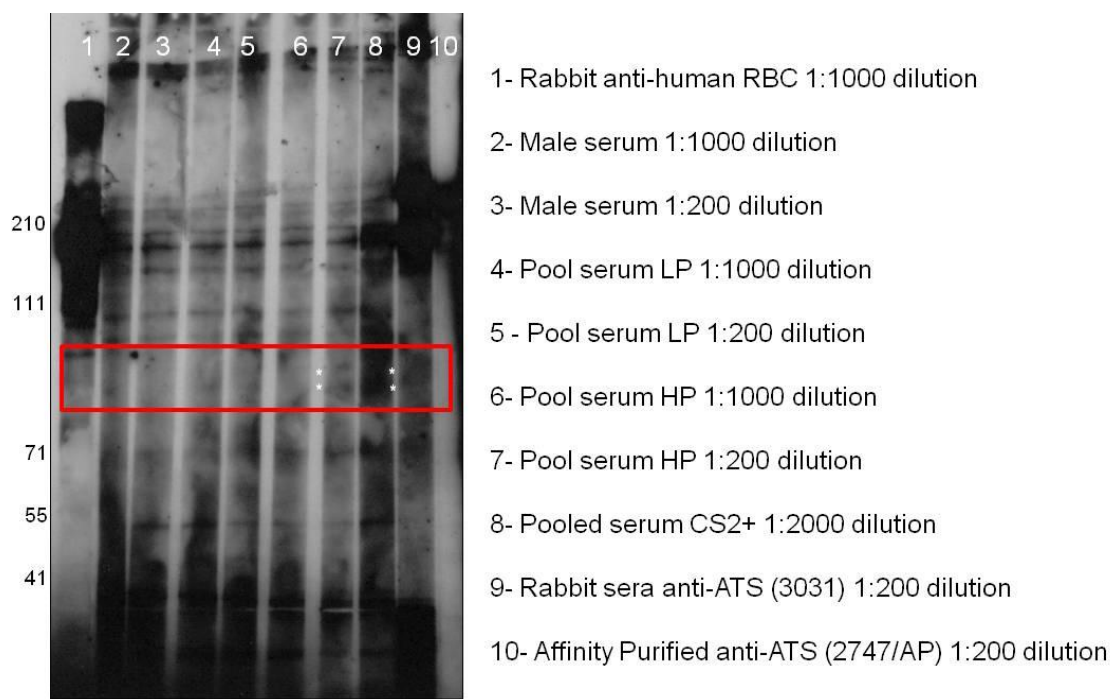


Figure 5-4 - Western blot of CS2-GFP membrane proteins probed with Primigravidae serum, 60 seconds exposure.

Membrane associated proteins were extracted from *Plasmodium falciparum* CS2-GFP parasites and separated on a 3-8% Tris-Acetate gel. These were then transferred to a nitrocellulose membrane and probed with human serum (strips number 2-8) or control rabbit antibodies (strips number 1, 9 and 10). A Rabbit anti-human IgE antibody was used to detect any IgE associated with the proteins on membrane strips 2-8. Film was exposed to the strips for 20 second (after incubation with a horse-radish peroxidase coupled antibody and ECL substrate). White asterisks represent 2 putative protein bands to which IgE seems to be associated in the HP group of pooled serum. Numbers to the left represent the position and molecular weight (kD) of the standard (SeeBlue®Plus2 pre-stained standard, Invitrogen).

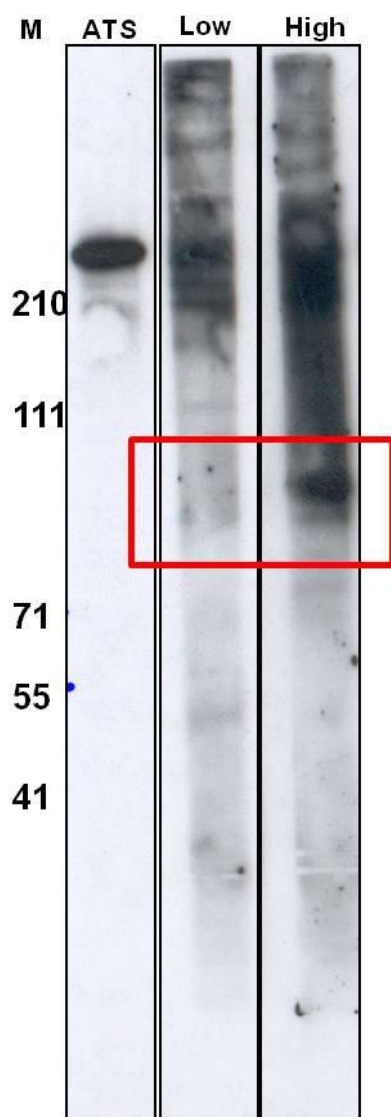


Figure 5-5 – Confirmation western blot of CS2-GFP membrane proteins probed with Primigravidae serum, 20 seconds exposure.

Membrane associated proteins were extracted from *Plasmodium falciparum* CS2-GFP parasites and separated on a 3-8% Tris-Acetate gel. These were then transferred to a nitrocellulose membrane and probed with two pools of human serum (**Low**: Low phagocytosis pool; **High**: High phagocytosis pool) or with a control affinity purified rabbit anti-ATS antibody (**ATS**). A Rabbit anti-human IgE antibody was used to detect any IgE associated with the proteins in the HP and LP strips. Film was exposed to the strips for 20 second (after incubation with a horse-radish peroxidase coupled antibody and ECL substrate). Red box represents the location of the two putative protein bands recognised by IgE in the high group but not in the low group. (**M**) Molecular marker, represents the position and molecular weight (kD) of the standard (SeeBlue®Plus2 pre-stained standard, Invitrogen).

5.4.2.2. Levels of IgE detected on western blots are not dependent on maternal age or on levels of total IgG to CS2_{VSA}

Due to the dependence of IgE on age, the maternal age of both the HP and LP groups were compared using student's t Tests. No significant differences were detected between both groups ($P=0.299$), excluding age as an influencing factor (Figure 5-6A). The levels of total IgG to CS2_{VSA} were also assessed between both the LP and HP groups, using Mann-Whitney ranksum tests. The LP group revealed to have higher values of total IgG to CS2_{VSA} than the HP group ($P=0.03$) (Figure 5-6B).

5.4.3. Evaluation of IgE levels by ELISA

The levels of both total and antigen specific IgE present in the sera of PG that had less than 3% of total IgG to CS2_{VSA} were evaluated by ELISA ($n=115$). The results were then introduced in the database used for the analysis of the results shown in Chapter 4 and analysed for association of IgE with IgG parameters measured and HIV status of the women.

5.4.3.1. Distribution and correlation of total IgE and antigen specific IgE in the primigravidae cohort.

The median level of total IgE present in this group of PG was 763, 285-5358 $\mu\text{g/mL}$ (median, 95% CI) while the distribution of antigen-specific IgE was (in relative, arbitrary units) 53.01, 18.2-140.6 (Figure 5-7A).

There was no correlation between the levels of total IgE and antigen-specific IgE in this group of PG (Figure 5-7B). Spearman's rank correlation yielded a P-value of 0.835, with a rho of -0.02 while linear regression failed also to show any association ($r^2=0.0009$, $P=0.756$).

5.4.3.2. Associations between levels of IgE measured and IgG, maternal age and HIV.

The levels of both total IgE and antigen-specific IgE were analysed with regards to the possible associations with total IgG to CS2_{VSA}, phagocytic antibodies' function, maternal age and HIV status (Table 5-1). Univariate linear regression methods, revealed a lack of association between the levels of total IgE and any of the other antibodies' measurements obtained for this group of PG.

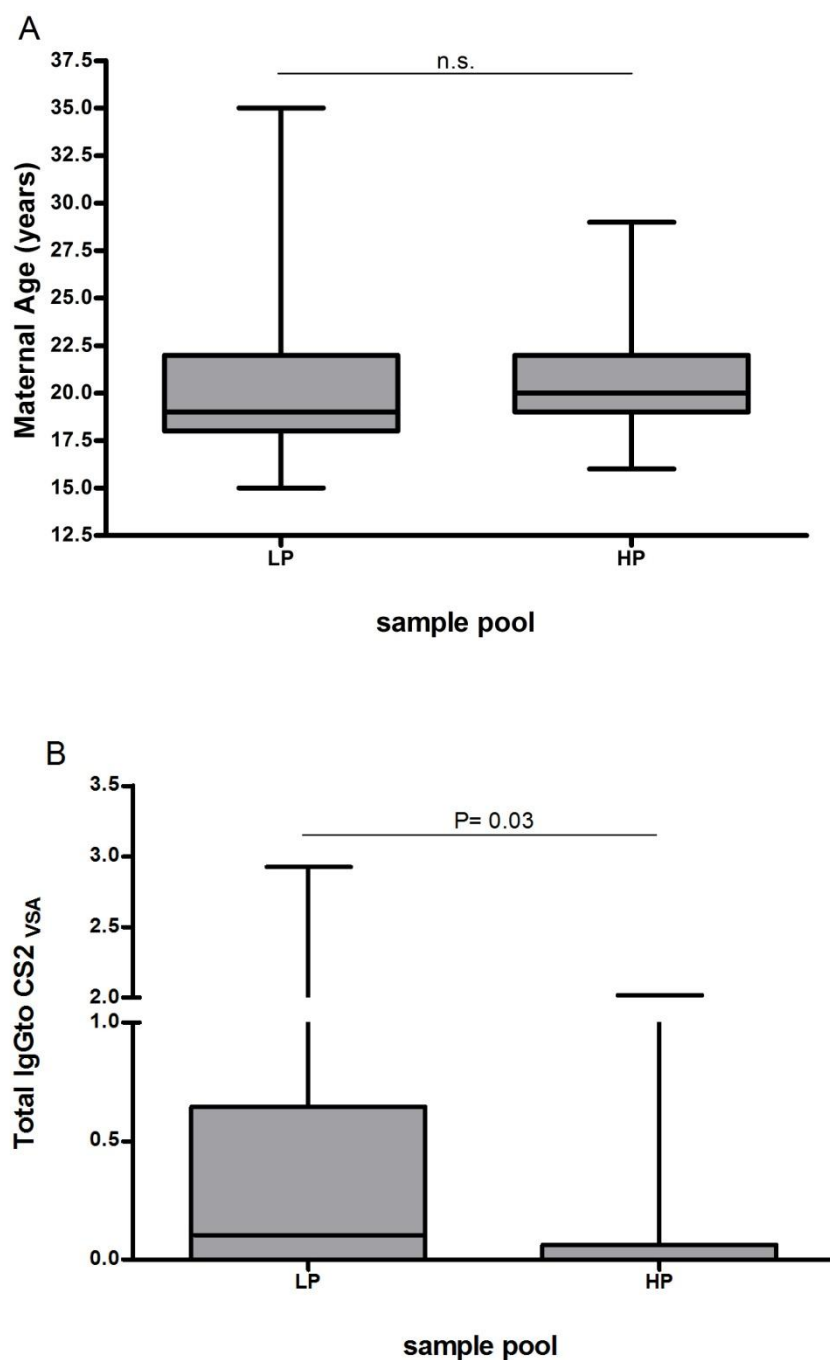


Figure 5-6 – Maternal age and Total IgG to CS2_{VSA} distribution among the two pools of primigravidae serum used in Western Blot

A) The two groups of women (LP-low phagocytosis, HP- high phagocytosis) included in the pools of PG serum used to probe nitrocellulose membranes containing membrane antigens of *Plasmodium falciparum* CS2 parasites were found to have no difference in maternal age (Student's t-test: $P=0.296$). **B)** The levels of total IgG to CS2_{VSA} were found to be higher in the LP group than in the HP group, reaching statistical significance (Mann-whitney ranksum: $P=0.03$). n.s. (non-significant). Total IgG to CS2_{VSA} is given as a percentage of positive control.

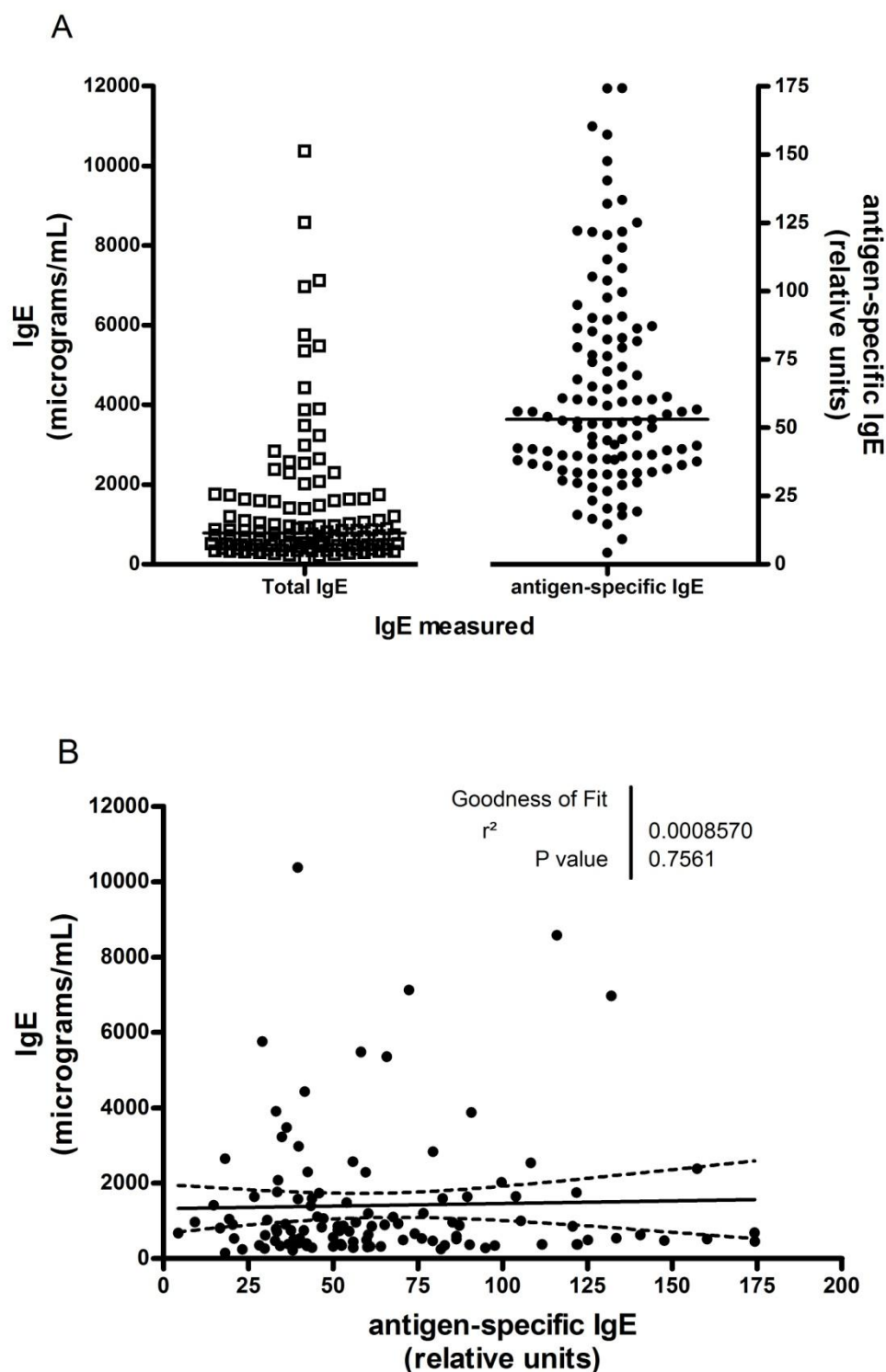


Figure 5-7 – Distribution and correlation of total and antigen specific IgE in the cohort of primigravidae with low total IgG to CS2_{VSA} levels

(A) Dot plots representing the distribution of total IgE (open squares) and antigen –specific IgE (filled circles). The median lines are plotted. **(B)** Correlation between total IgE and antigen-specific IgE. The linear regression curve and 95% confidence intervals are plotted. Values for both the P-value and the r^2 -value are also shown.

Levels of antigen-specific IgE failed to show any association with the total amount of IgG to CS2_{VSA} but showed a significant association with the function of phagocytic antibodies (coeff: 0.874, P=0.002). Maternal age showed an association with the levels of total IgE (coeff: -113.90, P=0.017) but not with the levels of antigen-specific IgE.

Total IgE levels did not seem to be influenced by the presence of an HIV infection (Mann-Whitney ranksum: z=1.26, P=0.209, Figure 5-8A) but antigen-specific IgE was significantly increased in HIV positive PG compared to HIV negative PG (Mann-Whitney ranksum: z=4.48, P<0.001, Figure 5-8B). In contrast, evidence of placental malaria (PM) was associated with lower levels of antigen-specific IgE (Mann-Whitney ranksum: z=2.06, P=0.04, Figure 5-8D). No association was found between total levels of IgE and evidence of PM (Mann-Whitney ranksum: z= 0.19, P=0.848, Figure 5-8C).

5.4.3.3. After multilinear regression analysis, antigen-specific IgE is associated with phagocytic antibodies and HIV in primigravidae with low total IgG to CS2_{VSA}.

In order to confirm the strength of the associations obtained with the univariate analysis conducted, both the total levels of IgE and the levels of antigen-specific IgE were analysed by multilinear regression analysis against maternal age, phagocytic function, total IgG to CS2_{VSA} and HIV status. Since both phagocytic antibodies and total IgG to CS2_{VSA} are highly associated to PM status (Chapter 4) multivariate analysis was performed after dividing the women into PM positive and PM negative.

Total IgG to CS2_{VSA} failed to associate with any of the parameters of IgE measured (data not shown). Multivariate analysis revealed that antigen-specific IgE retained its association to phagocytic antibodies (coeff: 0.74, P=0.005) and HIV status (coeff: 31.45, P=0.001) only in PM negative women. Total levels of IgE also remained associated with maternal age (Coeff: -136.71, P=0.042) after adjusting for HIV infection and phagocytic antibodies (Table 5-2) (results for women with PM yielded no significant results and are not shown).

Table 5-1 – Univariate associations between IgE levels, IgG parameters and maternal age

	Total IgE ¹ [Coef, (P-value), CI] ³	Antigen-specific IgE ² [Coef, P-value, CI]
Phagocytic antibodies	-8.64 (0.516) -34.95, 17.65	0.874 (0.002) 0.33, 1.42
Total IgG to CS2 _{VSA}	184.33 (0.391) -239.65, 608.31	-4.19 (0.371) -13.41, 5.04
Maternal age (years)	-113.90 (0.017), -206.97, -20.82	1.60 (0.129) -0.47, 3.69

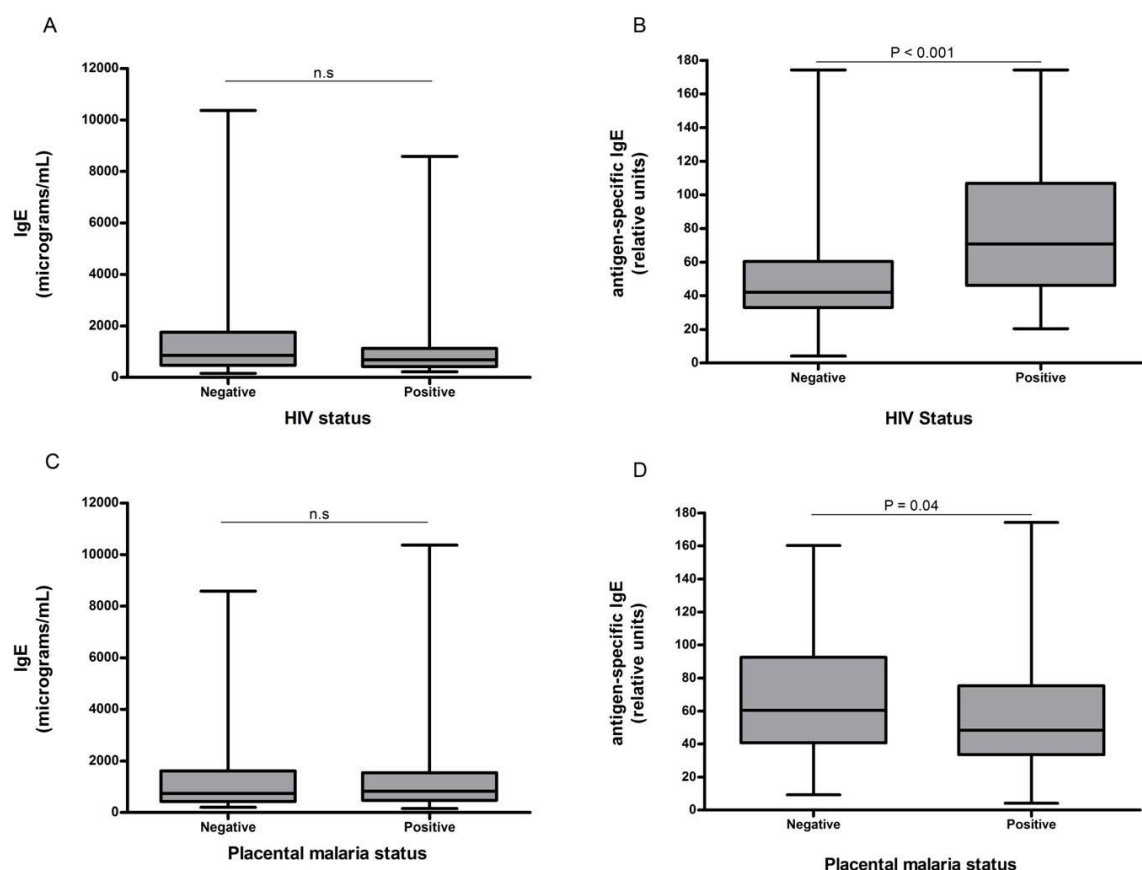
¹Total IgE in ng/mL²Antigen-specific IgE is in relative, arbitrary units³Linear regression coefficients (coeff), P-values and 95% confidence intervals (CI)

Figure 5-8 – Univariate associations between IgE level, HIV infection and placental malaria status

The associations between the levels of total IgE (A and C) and antigen-specific IgE (B and D) with HIV and Placental malaria were evaluated. Only the antigen-specific IgE associated with HIV status (B) and the placental malaria status (D). Mann-Whitney ranksum test was performed to evaluate the significance of the associations. P-values are shown. (n.s.) non significant.

Table 5-2 – Multivariate analysis of levels of IgE in placental malaria negative primigravidae

Variables	Total IgE		Antigen-specific IgE	
	Multivariate analysis ¹ Coeff (95% CI)	P ²	Multivariate analysis ¹ Coeff (95% CI)	P
Phagocytic antibodies	-9.85 (-39.95, 20.26)	0.516	0.74 (0.23, 1.26)	0.005
HIV infection	306.99 (-680.96, 1294.95)	0.537	31.45 (14.21, 48.70)	0.001
Maternal age (years)	-136.71 (-268.35, -5.08)	0.042	1.44 (-0.81, 3.70)	0.206

¹ Multilinear regression analysis coefficient (coeff) and 95% confidence intervals (95% CI). ² P-values. A negative coefficient indicates a negative association and a positive coefficient indicates a positive association

5.5. Discussion

The work presented in this chapter represents a preliminary attempt at answering a question that has not been the focus of many research studies: what is the function of IgE during a *Plasmodium falciparum* infection in pregnant women?

5.5.1. Total IgG to CS2_{VSA} and phagocytic antibodies do not correlate leaving room for another player.

The observation that there are women whose serum can induce a phagocytic response, when used in experimental settings, despite having low levels of the phagocytosis inducing antibody class IgG towards MiP-type pRBC surface antigens, led me to believe that other opsonins might have been present in the serum of those women. If so, these opsonins would surely be of importance especially in women where the probability of having developed IgG towards VSA_{CSA} was low. If this was in fact true, then the correlation between the levels of IgG to VSA_{CSA} in PG would have to have a lower correlation coefficient to phagocytosis function than the one obtained with more exposed women who had more time to develop IgG to VSA_{CSA}. This proved to be correct, with SG having a higher correlation coefficient between both total IgG and phagocytic antibodies than PG (Figure 5-1).

This observation led me to search for the identity of the opsonin responsible. All the serum samples used in the phagocytosis assays conducted in Chapter 4 were heat-inactivated, which excluded a possible role for Complement. The knowledge that IgE receptors were present on the surface of uThp-1 cells [26, 27] and that IgEs (both total IgE and *Plasmodium* specific IgE) were already found associated to MiP [60, 62, 63, 67], made IgE a candidate. Besides, there were no previous studies addressing IgE as a possible phagocytosis enhancer during a *Plasmodium* infection despite the well known role that this Ig can play in phagocytosis [23, 25].

Trying to conduct assays similar to the FACS-based total IgG to CS2_{VSA} assays in order to detect IgE proved to be extremely difficult and may be dependent on the development of a better protocol (results for the FACS based Total IgE to CS2_{VSA} are not shown).

5.5.2. Serum used in phagocytosis assays revealed to have IgE associated with higher levels of phagocytosis by western blot.

One impediment for the role that IgE might have had on the phagocytosis results obtained in Chapter 4 is the fact that heat inactivation seems to partially degrade IgE [68, 69]. Despite this, and since the phagocytosis assay relies on surface antigens of the pRBC, it

was decided to conduct the western blots using the exact same aliquots used in Chapter 4. Protein preparations of membrane associated structures of CS2-GFP *Plasmodium falciparum* were made with the use of extraction by Triton-X and solubilisation in SDS. The presence of a high molecular band recognised by the anti-ATS antibodies in all the preparations used assures us that if PfEMP-1 was present, surely other surface-membrane associated proteins must also have been present and thus available for probing (Figure 5-3).

By probing the CS2-GFP *Plasmodium falciparum* protein preparation with male serum and Melbourne serum, a possible unspecific role of IgE would be eliminated and the absence of specific protein bands associated with these serum pools seems to suggest that (Figure 5-3 and Figure 5-4). If the case can be argued that Melbourne serum donors may have much less IgE when compared to residents of malaria endemic countries, the same cannot be said for the male serum donors (in fact, early results done in collaboration with Sara Prickett, from the Immunology Dept of Monash University, Melbourne, Australia showed no differences between the levels of Total IgE in these male donors and in MG from the same MHP cohort. Data presented in poster format at the International Conference for Parasitology, ICOPA, in August 2010, Melbourne, Australia).

At higher dilutions two protein bands of between 111 and 71 kD seemed visible in the membrane strips probed with HP serum but absent in other strips, namely the LP strips (Figure 5-4) which was then confirmed by probing new strips with a lower dilution of the same serum pools (Figure 5-5). Even if some heat-inactivation may have degraded IgE present in those samples, there was still enough present to show that HP samples have IgE that recognises two protein bands associated with the membrane of CS2-GFP *Plasmodium falciparum* and that the same cannot be said of the LP serum pool. This was not due to a difference in the age of the women whose serum was used in the pools (Figure 5-6A). Some IgG-mediated effect (like the binding of IgG2 or IgG4 to the same epitopes recognised by IgE) could be occurring in the LP samples since the LP pool had significantly higher levels of total IgG to CS2_{VSA} than the HP pool although the extremely low levels of IgG in both groups render this explanation unlikely (Figure 5-6B).

It was not the goal of this part of the study to identify any of the proteins present in the protein bands recognised by IgE. What can be said though is that these are very unlikely to be PfEMP-1 or any of the proteins recognised by the polyclonal rabbit anti-human RBC antibody used as a control in phagocytosis assays. These proteins, identified by western blot as being recognised by IgE should be the focus of future research although it has to be taken into account the fact that a process of reduction and denaturing of the proteins

was conducted in order for them to run in the Tris-Acetate gel, which can expose epitopes that are not naturally exposed or even hide epitopes that are usually present.

5.5.3. Antigen-specific IgE associates with phagocytosis in the context of HIV infection

The decision to search for IgE, both total and antigen specific, by ELISA was taken with the higher throughput of the technique and its higher sensitivity in mind.

Again, only the PG samples that revealed to have less than 3% of total IgG to CS2_{VSA} were used, but this time the original serum samples (not heat-inactivated) were used which provided an increased availability of serum samples allowing for 115 samples to be processed in duplicate for both total IgE and antigen-specific IgE. The choice of antigen to be used (same as on the western blots or a more conformational correct one) fell on the commonly used schizont extract, prepared by cycles of freeze and thaw [70].

Due to the design of the study, with a very limited range of total IgG to CS2_{VSA} on all samples used, it was not expected that associations of this parameter with any of the IgE measured would be found, and that was indeed the case.

The levels of total IgE and antigen-specific IgE detected in this cohort of PG were not correlated with each other. This reduces the possibility that the results obtained for the antigen-specific IgE may be a reflection of a general IgE response by these women.

Total IgE levels were associated with maternal age, being reduced by 113.90 µg/mL with every added year of age (Table 5-1). This was an unexpected result as IgE is usually considered to increase with age in areas where malaria is endemic [34, 71], although one study by Perlman et al seems to show a decrease of IgE occurring after 16 years of age [45]. The negative association of total IgE with age still prevailed after adjusting for HIV infection and levels of phagocytic antibodies, in PM negative women (Table 5-2). Similarly, in PM negative women, antigen-specific IgE levels were independently associated with HIV infection and phagocytic antibodies, after adjusting for maternal age (Table 5-2). This is another clue to the possible involvement of IgE as a phagocytic antibody class in malaria (something that was vaguely suggested by Khusmith [34]), which may be of particular relevance in HIV positive women who possess low levels of IgG to fight a *Plasmodium falciparum* infection during pregnancy.

5.5.4. Final discussion, ideas and future work

This study was intended to be a platform from which other studies are to be conducted. For a complete idea of the role of IgE as a phagocytic antibody class in MiP other, more complete, studies should be performed. It will be important to show that IgE are actually able to recognise and bind to MiP-type pRBC and that this will in turn induce phagocytosis. This can be achieved, for example, by affinity purifying IgE from pools of serum from pregnant women from malaria endemic regions, exposing pRBC to that IgE and conducting phagocytosis assays. It will also be interesting to show if these IgE are, as I hypothesise, already present at the onset of infection and are thus not MiP-specific, but may be cross-reacting IgE. This would open the scene for other players like non-MiP pRBC or even (and more likely) helminths, which have been shown to be able to induce high levels of IgE and even associate with protection from malaria outcomes [48, 49]. Studies looking at the prevalence of helminth infection and levels of IgE at enrolment in PG versus MG and their correspondent levels of phagocytic antibodies' function against VSA_{CSA} expressing *Plasmodium falciparum* parasites would be extremely interesting to perform. Once again, and although very speculative at this point, I believe that IgE would prove to be of some importance in PG and not MG, where the levels of IgG to VSA_{CSA} would no doubt be sufficient to "hide" possible IgE-binding epitopes (Figure 5-9).

The possible effect that IgE complexes, or IgE deposits in the placenta might have is also something that is left unanswered, as are the potential roles that cytokines like IL-4 and TNF- α might be playing in this model. As I said, this study is a first insight into a field that still has a lot to offer. IgE has been extensively studied by malariologists, but it is time to stop being satisfied with stating their presence or absence and understand what possible functions might be attributed to them, and how a better rationale for the design of strategies to combat malaria can be achieved.

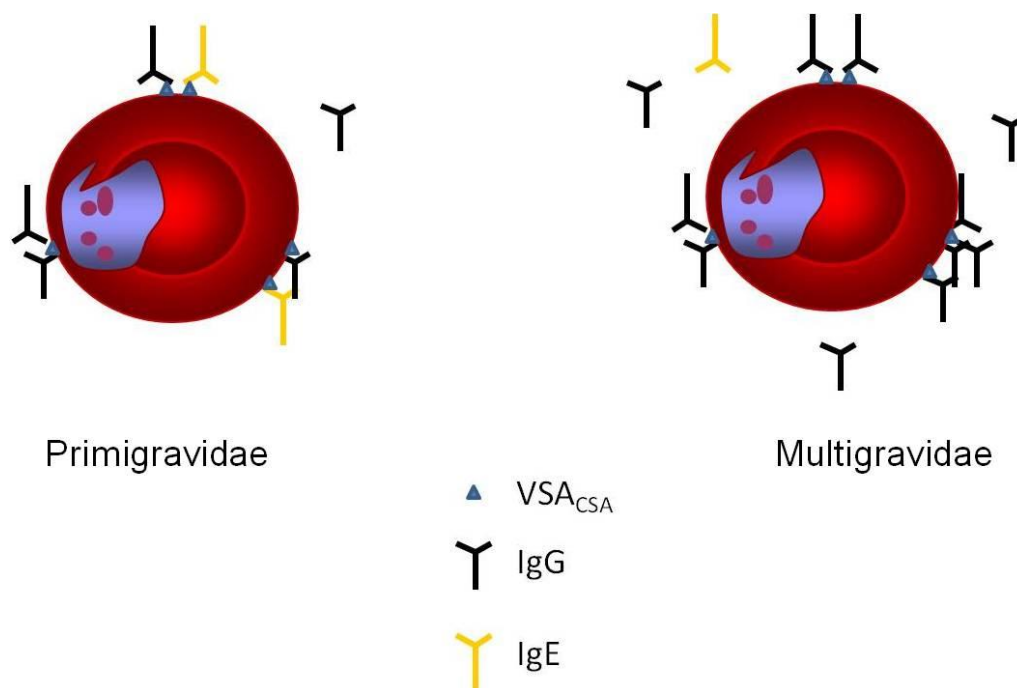


Figure 5-9 – Model of how IgE/IgG to VSA_{CSA} ratios could differently influence the effect of IgE as an opsonin in primigravidae and multigravidae.

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Appendix I - Papers

Appendix II - Curriculum vitae