Immunological characteristics of recombinant fragments of the *Plasmodium falciparum* blood-stage antigen Pf332

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Stockholm 2011
About the cover

These pictures were taken during inhibition assay with rabbit antibodies; they are infected and non-infected red cells, stained with acridine orange. The picture above in collaboration with another infected red cell, formed the “HAPPY” 😊 parasites probably because they escaped the antibody pressure (the parasites were exposed to antibodies for 22 hours). The other pictures below are from parasites that were exposed to antibodies for 42 hours, well they were not so lucky, and they ended up being the “SAD” 😞 parasites.

Well, more mysteries lie ahead in order to combat malaria.

The possession of knowledge does not kill the sense of wonder and mystery. There is always more mystery.

- Anais Nin

Cover image captured & designed by Halima A. Balogun.
I give all praise to Almighty Allah for giving me the strength, when I thought this was not going to be possible.

To my precious Mum (Yeye ni Wura)

...........LAB (R.I.P)
The first step towards knowledge is to know that we are ignorant.

- Richard Cecil
Abstract

An effective malaria vaccine might help in improving control strategies against malaria, but the development of an effective vaccine faces challenges, such as the complexity of interactions between the parasite and its hosts. The asexual blood stage antigen Pf332, a megadalton protein, is transported across the parasitophorous membrane to the iRBC membrane skeleton during schizogony, and has potentials as one of the proteins in understanding the complex host-parasite interactions. The functional role of Pf332 in the parasite's life cycle is still not well defined, as different studies have assigned different roles to this antigen. The interest in Pf332 as a possible target for parasite neutralizing antibodies, evolved from previous studies demonstrating that Pf332-reactive antibodies inhibits parasite growth in vitro. The presence of such significant antibodies during natural P. falciparum infection also indicated that Pf332 has the ability to induce protective antibodies.

In our first study we identified and characterized the immunogenicity of a C-terminal region of Pf332. Immunological analyses carried out with this fragment revealed that rabbit anti-C231 antibodies possess parasite in vitro inhibitory capabilities. In another study, the functional activity of C231 specific antibodies was further confirmed with human-affinity purified antibodies, where the antibodies inhibited late stage parasite development, as evidenced by the presence of abnormal parasites as well as disintegrated red cell membranes.

Using epidemiological data from a malaria endemic area of Senegal, we examined the pattern of antibodies reactive to two different regions of Pf332 (C231 and DBL) with regard to Ig classes and IgG subclasses. With both recombinant antigens, we observed positive correlations between the IgG (R=0.706, p=<0.0001) and IgM (R= 0.711, p= <0.0001) antibody levels against C231 and DBL. The distribution of the anti-C231 antibodies in the IgG subclasses, gave similar levels of IgG2 and IgG3. Correlation studies showed that the levels of anti-C231 antibodies were associated with protection from clinical malaria, which only reached significance with IgE. In contrast, the group with high anti-Pf332-DBL-IgG3 was found to be protected from clinical malaria attack.

We hereby conclude that antigen Pf332 contains immunogenic epitopes, and is a potential target for parasite neutralizing antibodies. The Pf332 protein should thus be considered as a candidate antigen for inclusion in a subunit P. falciparum malaria vaccine.
Content

Abstract............................................................................................................................................... i
Content.............................................................................................................................................. ii
List of included papers ....................................................................................................................... iii
Abbreviations ....................................................................................................................................... iv
1 Introduction.......................................................................................................................................... 1
  1.1 Overview of malaria ....................................................................................................................... 1
  1.2 Control measures against malaria ................................................................................................. 3
  1.3 The malaria parasite ...................................................................................................................... 5
  1.4 Clinical disease and manifestations ............................................................................................... 8
    1.4.1 Disease pathogenesis ........................................................................................................... 9
2 Malaria and the human immune system .......................................................................................... 12
  2.1 Innate immune response to malaria ............................................................................................. 12
  2.2 Pre-erythrocytic stage immunity in malaria ..................................................................................... 15
  2.3 Erythrocytic stage immunity in malaria ......................................................................................... 16
    2.3.1 Mode of action of antibodies ................................................................................................ 18
    2.3.2 Immunoglobulins and immunity to malaria ......................................................................... 19
  2.4 Immune evasion by malaria parasite ............................................................................................. 21
  2.5 Malaria vaccines .......................................................................................................................... 23
    2.5.1 Asexual blood stage vaccines ............................................................................................... 25
3 The infected red cell and its modifications ...................................................................................... 27
  3.1 Antigens of the infected red cell .................................................................................................... 30
  3.2 Antigen 332. .................................................................................................................................. 33
    3.2.1 Related background ............................................................................................................ 33
    3.2.2 Immune responses to antigen Pf332 ................................................................................... 36
4 The Present Investigation .................................................................................................................. 38
  4.1 Preface.......................................................................................................................................... 38
  4.2 Objectives ..................................................................................................................................... 39
  4.3 Experimental approach ................................................................................................................ 40
    4.3.1 Study population ................................................................................................................. 40
    4.3.2 The Malaria Situation in Senegal ......................................................................................... 40
  4.4 Results and discussion .................................................................................................................. 42
    4.4.1 Paper I ............................................................................................................................... 42
    4.4.2 Paper II ............................................................................................................................. 43
    4.4.3 Paper III & IV ................................................................................................................... 45
  4.5 Concluding remarks ..................................................................................................................... 49
  4.6 Future perspectives ....................................................................................................................... 51
  4.7 Other Publications ....................................................................................................................... 52
5 Acknowledgements .......................................................................................................................... 53
6 References ........................................................................................................................................... 56
List of included papers

This doctoral thesis is based on the following original papers, which are referred to in the text by their roman numerals:


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABRA</td>
<td>Acid-basic repeat antigen</td>
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<tr>
<td>ADCI</td>
<td>Antibody-dependent cellular inhibition</td>
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<td>AMA</td>
<td>Apical-membrane antigen</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>CM</td>
<td>Cerebral malaria</td>
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<tr>
<td>DBL</td>
<td>Duffy binding-like domain</td>
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<tr>
<td>GLURP</td>
<td>Glutamate rich protein</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidyl inositol</td>
</tr>
<tr>
<td>His-tag</td>
<td>Histidine tag</td>
</tr>
<tr>
<td>HZ</td>
<td>Haemozoin</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular-adhesion molecule-1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iRBC</td>
<td>Infected red-blood cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite-surface protein</td>
</tr>
<tr>
<td>PAM</td>
<td>Pregnancy associated malaria</td>
</tr>
<tr>
<td>PDC</td>
<td>Plasmacytoid-dendritic cell</td>
</tr>
<tr>
<td>P. f</td>
<td>Plasmodium falciparum</td>
</tr>
<tr>
<td>PV</td>
<td>Parasitophorous vacoule</td>
</tr>
<tr>
<td>RBC</td>
<td>Red-blood cell</td>
</tr>
<tr>
<td>RESA</td>
<td>Ring-infected erythrocyte surface antigen</td>
</tr>
<tr>
<td>SERA</td>
<td>Serine-repeat antigen</td>
</tr>
<tr>
<td>SMA</td>
<td>Severe malaria anaemia</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRAP</td>
<td>Thrombospondin-related anonymous protein</td>
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<tr>
<td>Treg</td>
<td>Regulatory-T cell</td>
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1 Introduction

1.1 Overview of malaria

Despite the fact that humans have evolved to co-exist and survive with the malaria parasite *Plasmodium* for a long period of time, malaria still remains one of the main three infectious diseases in Africa (Fig. 1b). Malaria creates a global health concern with about 225 million clinical cases per year worldwide, with the vast majority of cases (about 85\%) in the African region, and approximately 1 million deaths annually (Alonso *et al.*, 2011). The heaviest burden of malaria lies mainly on non-immune individuals, pregnant women, and children below the age of five years, residing in Sub Saharan Africa. Disease manifestations in malaria, such as severe malaria anemia (SMA), pose socioeconomic challenges and can be life threatening in the case of cerebral malaria (CM).

The malaria situation has aggravated since time immemorial, due to insecticide resistance of the vector, the anopheline mosquito which transmits the parasite, as well as the emergence of drug resistance in the parasite. Eradication of malaria has also been hampered by other factors such as inappropriate public health facilities and bottlenecks in vaccine development. The human host genetic factors have also been shown to play a vital role in clearance of infection (Allison *et al.*, 2009; Pasvol 2010; Giha *et al.*, 2010). The malaria parasite has a complex life cycle during which they infect humans and are transmitted by anopheline mosquitoes. This has also hampered effective control strategies and vaccine development. In malaria endemic countries, progress to reduce the burden of malaria has been achieved to an extent by means of improved health care systems (WHO, 2010). However, malaria still needs great attention in areas of vector control, diagnostics and treatment as impediments still lie in these fields, not exempting basic research on the biology of the parasite and especially vaccine development, which may sooner or later be a long-term solution to this acquainted enemy of man.
Figure 1a: The global distribution of malaria, www.cdc.gov.

Figure 1b: Distribution of malaria in Africa, www.mara.org.za.
1.2 Control measures against malaria

Malaria control has been a great challenge to malariologists, it requires an integrated approach of the various preventive/control measures, which targets reduction of disease incidence, prevalence, morbidity or mortality to an appreciable level. With the current tools and state of knowledge at hand, strategies against obliteration of malaria may not be achievable. One of the reasons is due to the fact that malaria is caused by five *Plasmodium* species, which are being transmitted by more than 30 Anopheline mosquito species (Alonso *et al.*, 2011). These complexities result in diverse disease spectra in different epidemiological settings. Till date, malaria eradication has utilized various interventions, including control of the mosquito vector, use of therapeutic drugs and chemoprophylaxis agents and prompt appropriate case management. Strategies that have proven to have impact on reducing morbidity and mortality/burden of malaria are the home management system (Pagnoni, 2009) and improved health care systems (RBM partnership, 2008). However, eradication of malaria still faces enormous setbacks in most malaria endemic countries.

In most endemic areas, the disease is treated using anti-malarial drugs, which are important in early control of the attack, and the administration of these drugs has either a prophylactic or curative effect. Increasing resistance of *P. falciparum* malaria to antimalarial drugs poses a major threat to the global effort to roll back malaria, but now emphasis is being laid on the use of drugs with a short half-life, which minimizes the risk of development of resistance (WHO, 2010a). Resistance can be prevented, or its onset slowed considerably, by combining antimalarials with different mechanisms of action and ensuring very high cure rates through full adherence to correct dose regimens (WHO, 2010a). Mosquito vector control is an important control approach that should be integrated with other malaria control measures. Significant effect has been observed with the use of indoor residual spraying (IRS) and long lasting insecticide treated nets (LLINs) (Enayati & Hemingway, 2010), with these most malaria endemic areas have been able to reduce vectorial capacity. Notwithstanding, challenges are still encountered, as regions with very high transmission areas will not benefit from this control measure, due to
increased mosquito resistance to insecticides. The global malaria control strategy adopted by governments and W.H.O. emphasized the need for prompt and effective diagnosis, which will aid appropriate treatment. However an effective health care system is very important in this context, and such systems are required to sustain malaria intervention programmes in malaria endemic countries.

Vaccines have been projected to be part of the control measures in malaria eradication, but so far no effective vaccine against this disease has been developed. The reason for this is entangled among various issues, such as breach in knowledge in the area of parasite biology, whereby the molecular interactions between parasite, its human hosts and vector host, has not been properly elucidated (Langhorne et al., 2008; The malERA Consultative Group on Basic Science and Enabling Technologies, 2011). The complexity of the parasite’s life cycle (Gardner et al., 2002; Florens et al., 2002) and extensive antigenic variation (Sherf et al., 2008) also poses setbacks to malaria vaccine development. Vaccines based on asexual blood-stage antigens (will be discussed in section 2.5) may be effective at reducing parasite densities and provide protection against clinical disease. In addition, understanding of the clinically silent stages preceding the blood stage (sporozoite and hepatocyte stages), may help to improve the induction of protective immune responses in vaccine development. Both approaches are being integrated in vaccine development, but Plasmodium species are quite complex. While working on vaccine development, emphasis on adjuvant development should not be forgotten, as the unavailability of a wide range of potent adjuvants has been a bottleneck in development of recombinant protein-based vaccines for malaria.

Amidst the decrease in malaria prevalence in some endemic areas, due to various expanded control programmes, such as preventive mosquito control with ITN and indoor insecticide spraying (Kappe et al., 2010). Attempts to eradicate or at least ameliorate the disease are still hampered, due to resistance of parasites to anti-malarial drugs and of mosquitoes to insecticides, plus socio-economic turmoil in many countries. For these reasons, there is still need for additional studies in order to understand the interactions between the malaria parasite and its host, leading to discovery of potential target antigens for vaccine development.
1.3 The malaria parasite

Malaria is caused by a unicellular eukaryotic protozoan parasite belonging to the kingdom *Protista*, phylum *Apicomplexa*, class *Sporozoa*, order *Eucoccidia* and genus *Plasmodium*. There are 172 species of *Plasmodium* that infect birds, reptiles, and mammals, but only five cause malaria in humans, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* and *P. knowlesi*. *Anopheles* mosquitoes are the vectors for transmission of malaria. There are 435 known species of *Anopheles* mosquitoes, out of which 30-40 species can transmit malaria. In sub-Saharan Africa, the three major species that transmit malaria are: *Anopheles gambiae*, *A. funestus*, and *A. arabiensis*. As a means of survival the malaria parasite inhabits two natural hosts, the female *Anopheles* mosquitoes and, during the other half of its life cycle, the mammalian host (Fig. 2). Described below is the life cycle of *P. falciparum*, the most lethal and virulent of the five species mentioned above, and this will be main species referred to in this thesis.

**Transmission of *P. falciparum* into the host cell:** During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host, which then circulate in the blood for minutes, before they attach and invade hepatocytes (Florens *et al.*, 2002). Within the liver the parasite undergoes the pre-erythrocytic stage in which it multiplies asexually. Since the parasite is in the liver for at least five days, this is a good site for attack by the immune cells, such as the T-cells and cytokines. Of note, in *P. vivax* and *P. ovale* a dormant stage hypnozoites can persist in the liver and cause relapses by invading the bloodstream, weeks or even years later. After this initial replication in the liver (pre-erythrocytic stage), a single sporozoite gives rise to tens of thousands of asexual parasites called merozoites (Prudêncio *et al.*, 2006). Around one week after the initial liver infection, merozoites are released from the infected hepatocytes into the bloodstream, infecting red blood cells (RBCs). In the RBCs the parasites undergo asexual multiplication (erythrocytic stages) (Fig. 2), were the ring stage parasite develops in a parasitophorous vacuole (PV) into trophozoites, the feeding stage of the parasite, where it digests haemoglobin from the RBC (Florens *et al.*, 2002).
The schizont stage is the final stage of asexual development in the human host. At this stage the asexual daughter cells, called the merozoites, develop, and when mature the infected RBC (iRBC) ruptures and the newly formed merozoites may infect new RBCs. Each merozoite can multiply up to 20-fold every 48 h in cycle from erythrocyte invasion, to erythrocyte rupture, and this cycle is responsible for the clinical manifestations of the disease.

**Transmission of *P. falciparum* from host cell to mosquito:** Some merozoites differentiate into sexual erythrocytic stages (gametocytes). The transmission of Plasmodium from the human to mosquitoes requires infectious gametocytes that reside in the capillaries of the inner organs of the infected human. Gametocytes will only appear 3 to 10 days after the merozoites have entered into the bloodstream. However the length of time depends on the strain of the Plasmodium (WHO: FAQ about malaria, 2009). Once the female mosquito bites and sucks the blood from the infected host, the formation of male (microgametes) and female (macrogametes) gametes are released and fusion takes place to produce zygotes. The zygotes in turn become motile and elongated (ookinetes), which invade the midgut wall of the mosquito, where they develop into oocysts. The oocysts grow, rupture, and release thousands of sporozoites, which become infectious once they migrate to the mosquito’s salivary gland. The infected mosquito then finds a host to feed on to get blood, and in the process injects the infectious sporozoites into its victim (Mikolajczak et al., 2008), and the vicious cycle of infection starts all over again.
Figure 2: Life cycle of *P. falciparum*, showing the development of the parasite in the mosquito and human host. Potential immunological mechanisms at the different stages of the parasite’s life cycle are also indicated, A-D. (Diagram is adapted and modified from, Crompton *et al.*, 2010).
1.4 Clinical disease and manifestations

The malaria parasite’s life cycle initially involves a clinically silent liver stage (Prudêncio et al., 2006, Shofield & Hacket 1993), and thereafter the asexual blood stage, where multiple molecular processes contribute to the remodelling of infected as well as the uninfected red blood cells, (Maier et al., 2009; Layez et al., 2005). These processes lead to various clinical outcomes, such as severe malaria (SM), including severe malarial anaemia (SMA) and cerebral malaria (CM), as well as pregnancy-associated malaria (PAM). In humans, severe cases of malaria are multifaceted and most likely cannot be represented by one single pathogenic scheme, rather by compounding effects of multiple disorders. Such effects include, destruction of uninfected red blood cells (uRBCs), microvascular obstruction, immunopathological and inflammatory processes (Mecheri, 2011), and not exempting the age and previous immunological experience of the host (Schofield & Grau, 2005). In malaria endemic areas, the burden of the disease is mainly borne by infants and young children leading to a severe outcome of the disease, including SMA and CM. In areas of lower transmission, primary infections might occur in adulthood, in which severe disease is manifested. In most cases, transmission dynamics, host age, host genetics and immunological responses are important determinants of disease severity (Schofield & Grau 2005).

The traditional presentation of malaria, observed in about 50-70% of malarial cases consists of paroxysms of fever alternating with periods of fatigue (Zaki & Shanbag, 2011). Signs associated with febrile outbursts include high fever, rigors, sweats and headaches, myalgia, nausea, diarrhoea, pallor and jaundice (Krause et al., 2007). Other obvious clinical symptoms include fatigue, respiratory distress, pulmonary edema, convulsions, circulatory collapse, hemoglobinuria, severe anaemia and impaired consciousness (Ferreira et al., 2008). However, in different endemic regions malaria can present itself with unusual manifestations due to development of immunity and increasing resistance to antimalarial drugs (Zaki & Shanbag, 2011). Severe manifestations of *P. falciparum* malaria in hyperendemic areas are defined by the occurrence of one or more of the symptoms mentioned above. SMA is the earliest most
severe and frequent dangerous complication of malaria, and it might be the leading cause of deaths from malaria worldwide in most hyperendemic areas, resulting from a massive RBC loss and/or impaired erythropoiesis (Lamikanra et al., 2007). CM, a syndrome of unarguable coma is often associated with fits and other neurological abnormalities, such as seizures, increased intramuscular muscle tone, and elevated intracranial pressure (Kwiatkowski 1994; Mishra & Newton 2009). It is one of the most common and potentially life-threatening complications of P. falciparum malaria commonly found in low endemic areas.

1.4.1 Disease pathogenesis

The pathogenesis of severe cases of malaria occurring during the erythrocytic cycle is multifactorial and intertwined. It involves processes from the early parasite stage (rings) to the mature stages (trophozoites & schizonts) (Fig 2), interactions with the host, as well as immunological feedbacks from the host immune system. During P. falciparum infection, the asexual cycle, where the release of daughter merozoites occurs every 48 hrs from iRBCs, is a major contributor to the fever chills observed during manifestation of the disease.

Glycosyl-phosphatidyl inositol (GPI), an anchor molecule of some plasmodial membrane proteins, is thought to function as a critical toxin, which contributes to severe malarial pathogenesis by eliciting the production of pro-inflammatory responses by the innate immune system of mammalian hosts (see section 2.1) (Schofield et al., 1993; Schofield & Hackett 1993; Tachado et al., 1997; Naik et al., 2000). In cases of SMA, which can be a chronic complication of malaria, it does not only arise from destruction of iRBCs, but also from the significant loss of uninfected RBCs and decreased production of RBCs. Indeed infection by the P. falciparum parasite modifies the uninfected RBCs (Maier et al., 2009; Layez et al., 2005), as a proportion of uninfected RBCs are “decorated” with parasite molecules (such as RSP2/RAP 2) released during invasion (Layez et al., 2005; Pouvelle B, 2000; Awah et al., 2009), and these RBCs are susceptible to splenic retention, phagocytosis or complement mediated lysis (Layez et al., 2005; Awah et al., 2009). These immunological reactions likely aggravate RBC loss in malaria patients,
leading to severe cases of malaria.

In addition there is induction of an array of bioactive molecules (pro-inflammatory and anti-inflammatory cytokines) which either upregulate or downregulate pathogenic processes. Sequestration of iRBCs in the microvasculature of vital organs is one of the most important factors in pathogenesis of \textit{P.f.} malaria (Turner \textit{et al.}, 1994), caused mainly by one of the parasite proteins, \textit{Plasmodium falciparum} erythrocyte membrane protein 1 (PfEMP1) (for details see section 3.1). Sequestration is characterized as the removal of iRBCs from the circulation by binding to the vascular endothelium of the host cells, mostly in the post capillary venules of the deep tissues. Impaired oxygen delivery due to occlusion of the blood flow in vessels may result in organ dysfunction.

\textit{P. falciparum} infection during pregnancy can bring about immense adverse effects such as maternal anaemia. Low birth weight and infant anaemia, have been a hallmark of pregnancy-associated malaria and these are believed to be the consequence of sequestration of the parasites in the intervillous spaces of the placenta using the placental receptor Chondroitin sulphate A (CSA) (Hviid \& Salanti, 2007; Rogerson \textit{et al.}, 2007).

Immense sequestration in the brain is alleged to be the cause of coma in CM (Turner \textit{et al.}, 1994). In patients with CM, the total parasite biomass is greater than in patients with uncomplicated or other severe attacks (Buffet \textit{et al.}, 2011). In addition to sequestration of mature forms of the parasites in the brain leading to organ and tissue dysfunction, there is recruitment and sequestration of host cells, (such as leukocytes or platelets) which contribute to the pathogenesis of CM (Wassmer \textit{et al.}, 2003), either through local effects in brain microvessels or through distant effects mediated by the production of potentially deleterious mediators, such as pro-inflammatory cytokines and induction of nitric oxide (NO) (Clark \textit{et al.}, 1992).

Another phenomenon with immense pathological effect is rosetting, described as when matured trophozoite iRBCs attach to two or more uninfected RBCs to form rosettes, or the adhesion of several iRBCs and RBCs to each other to form giant rosettes (Wahlgren
et al., 1990, Udomsangpetch et al., 1989). Rosetting parasites are associated with severe clinical disease including CM in the human host (Heddini et al., 2001, Rowe et al., 1995, Rowe J.A et al., 2002, Treutiger et al., 1992, Carlson et al., 1990). In addition rosetting may also aid in camouflaging of iRBCs from immune cells, and increase the efficiency of merozoite invasion, imparted by the proximitity of newly ruptured schizonts to uninfected red blood cells, this ability has been speculated for antigen Pf332 (Moll et al., 2007).
2 Malaria and the human immune system

As mentioned earlier, *P. falciparum* infection is the major cause of morbidity and mortality in malaria endemic areas. The erythrocytic stage of the parasite is the main culprit in malaria pathogenesis, and the main target of immune responses to malaria. Immunity to malaria is complex, and has been shown to be based on factors such as age, transmission intensity and continuity of exposure to parasite. As it has been emphasized, immunity to malaria is partial, as older children and adults living in malaria hyper-endemic regions can develop malarial disease (Marsh & Kinyanjui, 2006). Newborns do not contract the disease during the first months of life, owing to the maternal antibodies that crossed the placenta. However, the role of maternal antibodies in this context has been questioned (Riley *et al.*, 2000). As the level of the passively acquired antibodies wane around 6-9 months of age, infants in malaria endemic areas may experience their first clinical episode at this age (Bruce-Chwatt, 1952). This leaves the young children (about 1-5 years of age), non-immune individuals and pregnant women with the burden of the disease. In other words, the acquisition of malaria specific immunity is important in preventing morbidity and mortality from malaria (Sanni *et al.*, 2003).

Immunity to malaria is based on the different stages (pre-erythrocytic and erythrocytic) of the parasites life cycle, as each stage induces different mechanisms of immune response (Fig. 2A-D). In this thesis, we will be addressing one of the mechanisms employed by the host against the erythrocytic stage. Above all, there is no single measure to predict a protective immunity to malaria, as it involves an interplay of various arms of the immune system, including the innate as well as the humoral and cellular arms of the adaptive system.

2.1 Innate immune response to malaria

Innate immunity to malaria is an immediate inhibitory response to the inception of the infection, not dependent on any previous encounter with the parasite (Doolan *et al.*, 2009). This type of response may be based on inherent properties of the host which may
help to reduce the parasite multiplication rate and in the end prevents disease severity (Mohan & Stevenson 1998; Doolan et al., 2009). Pattern recognition receptors (PRR) are molecules on host cells which the innate system use to detect pathogens, they comprise a family of type I transmembrane receptors which are characterized by an extracellular leucine-rich repeat (LRR) domain and intracellular Toll/IL-1 receptor (TIR) domain (Medzhitov, 2001; Janeway & Medzhitov, 2002; Beutler, 2004). One family of pattern recognition molecules present on or in host cells is the Toll-like receptors (TLRs).

The infected RBC, or parasite products, can interact with several Toll-like receptors (TLRs) on host cells, especially monocytes/macrophages and dendritic cells, which are used to recognize and respond to a plasmodial infection. The interaction between the iRBC and most TLRs can orchestrate an early defense, and in most cases dependent on a crucial adaptor molecule MyD88 (myeloid differentiation primary gene 88) and activation of nuclear factor kappa β (NF-κβ), which often leads to the production of pro-inflammatory cytokines (Ropert et al., 2008; Gowda, 2007; Arama et al., 2011).

Cells of the innate immune system contribute to innate immunity against malaria, these cells include NK, gamma delta (γδ) and NK T cells, macrophages and dendritic cells (DCs). Polymorphonuclear cells such as neutrophils and eosinophils, soluble factors, such as interferons and complement factors, are also involved in the innate immune responses against malaria (Perlmann & Troye-Blomberg 2002). NK cells have been shown to be the first cells to respond to P. falciparum infection by increasing in number and the ability to lyse infected RBC in vitro (Orago & Facer 1991). In P. falciparum infection, direct contact between parasitized RBCs and NK cells leads to IL-12 and 18 production, which further leads to IFN-γ production (Artavanis-Tsakonas et al., 2003). Thus, the IFN-γ produced by the NK cells activates macrophages to eliminate parasitized RBCs. During malaria infection, γδ T cells are more expanded in the circulation than other T cell subsets, and they have been shown to directly inhibit the growth of blood-stage parasites (Farouk et al., 2004). The role of γδ T cells expressing NK receptors are dominant source of IFN-γ in response to P. f. infection (D’Ombrian et al., 2007).
Evidence for the role of macrophages and DCs in innate immunity is their ability to phagocytose infected erythrocytes in the absence of cytophilic or opsonizing malaria-specific antibodies (Gyan et al., 1994; Serghides et al., 2003), and thereby contributing to the reduction of initial parasitemia. The GPI molecule interacts with macrophages and DCs to induce pro-inflammatory cytokines such as TNF-α, IL 1 and IL-12 (Schofield & Hackett, 1993; Naik et al., 2000, Gowda, 2007). It activates DCs, through TLR2 and TLR4 (Krishnegowda et al., 2005), studies on TLRs and malaria in humans, indicated that common TLR-4 mutations in African children may increase the risk of severe malaria (Mockenhaupt et al., 2006). Parasite material such as haemozoin (HZ) has effects on the immune system as demonstrated by HZ purified from P. falciparum being a ligand for TLR-9, which may further modulate the function of DCs (Coban et al., 2005; Pichyangkul et al., 2004). However it has also been demonstrated that the parasite DNA attached to HZ is responsible for the observations with DCs (Parroche et al., 2007). The divergencies observed with the specific ligand for TLR-9, may be due to factors such as heterogeneity of the DCs, different parasites strains and type of HZ used (Urban & Todryk, 2006; Bousheri & Cao 2008).

The influence of the genetic make-up of the host on susceptibility to malaria infection in endemic areas has been established, especially in the genetic red cell disorders, including sickle cell trait, thalassemia, enzyme deficiencies, ovalocytosis and ABO blood groups (Miller et al., 1994; Modiano et al., 2001, Aidoo et al., 2002; Williams et al., 2005; Weatherall et al., 2002; Ayi et al., 2004; Mockenhaupt et al., 2003; Wambua et al., 2006; Durand & Coetz 2008). These host factors may confer natural protection against malaria infection. Other innate factors, such as polymorphisms in ICAM-1, a putative receptor for erythrocyte binding to the brain endothelium, and a polymorphism in the promoter region of TNF-α, appear related to the frequency of severe disease (Fernandez-Reyes et al., 1997, McGuire et al., 1994; Keusap et al., 2010). Innate differences in host’s control of immune responses and differences in parasite virulence each have their part in determining the response to infection. Polymorphisms in host genes such as those encoding IFN-γ, TNF, IL-10 and IL-4 (Bayley et al., 2004, Carpenter et al., 2007, Henri et al., 2002, Jüliger et al 2002) have been associated with susceptibility to the disease.
However, proper balance between the regulation of pro- and anti-inflammatory cytokines vis-à-vis disease endemicity may be critical in determining the extent of pathology (Kurtzhals et al., 1999, Sinha et al., 2010, Hafalla et al., 2011). Proper understanding of the innate immune responses to pathogens is very crucial, because it directs the development of an efficient adaptive immune system (Janeway & Medzhitov 2002).

2.2 Pre-erythrocytic stage immunity in malaria

Natural immune responses to the pre-erythrocytic stages (sporozoite and liver) of malaria have been suggested to have limited involvement in malaria immunity (Hoffman 1987, Hoffman 1996, Owusu Agyei et al., 2001, Marsh & Kinyanjui, 2006, Langhorne et al., 2008; Shwenk & Richie 2011). This immunity precedes the pathogenic stage and if very strong and sterilizing may be very important in malaria immunity, one of the challenges encountered in vaccine development against the liver stage. However, our understanding on immune effectors underlying an effective and sterilizing pre-erythrocytic immunity is limited. Antibodies to sporozoites may act through neutralizing opsonization of sporozoites and/or blocking of the invasion of hepatocytes (Fig 2B), yet they are thought to have negligible function, probably due to inadequate titers of high affinity antibodies (Nardin et al., 1999).

The infected hepatocyte is an important target of protective immunity, because the presence of parasites in this MHC-I-expressing cell may render parasite antigens more accessible to a cell-mediated immune response (Speake & Duffy 2009). During this liver stage, effector functions of the CD4+ and CD8+ T cells are important (Tsuji & Zafala, 2003; Wykes & Good, 2009). There are evidences that IFNγ-secreting T cells against liver stage antigens (Fig 2C), are associated with reduced malaria incidence (Todryk & Bejon 2009; Perlaza et al., 2008). However, the definitive roles of CD4+ and CD8+ T cells in malaria immunity are yet to be determined (Tsuji, 2010). In addition, studies have demonstrated that antibodies also contribute to protection against the pre-erythrocytic stages during malaria infection (Hollingdale et al., 1984; Kebaier et al., 2009; Schwenk &Richie 2011).
2.3 Erythrocytic stage immunity in malaria

Red-cell invasion is a crucial activity in malaria pathogenesis and is an important target for protective immune responses. Blood-stage immunity can be acquired in individuals that are repeatedly exposed to the pathogen, and it is substantially mediated by antibodies, but, protective mechanisms such as, innate immune responses, secretion of IFN-γ by CD4⁺ T cells are also utilized (Fig 2D), (Plebanski & Hill 2000). During the asexual blood stage, CD4⁺ T cells are pivotal in malaria immunity (Pombo et al., 2002) as well as in the development and regulation of humoral immune response, as they form interplay with the B cells. This leads to effective class switching based on the cytokine milieu and efficient antibody production. It has been shown that CD4⁺ T cells from malaria exposed individuals naturally exposed to malaria, respond to blood stage antigens of *P. falciparum* by proliferation, production of IFNγ and / or IL-4 secretion *in vitro* (Troye-Blomberg et al., 1990). γδT cells, whose activation is initiated by IL-2, IL-4 and IL-15, have been shown to expand both in mice and humans during malaria infection (Rzepczyk et al., 1997). These cells also recognize schizont derived–phosphorylated molecules (Pichyangkul et al., 1997) and produce proinflammatory cytokines.

Pathology of severe disease has been linked to an excessive inflammatory response, and the ability to regulate these responses is important in immunity against severe malaria (Artavanis-Tsakonas et al., 2003). The regulatory T cells (T-regs) comprise 5-10% of CD4 T-cells of the immune system (Riley et al., 2006), these IL-10 and TGF-B producing cells are involved in immunosuppression (Hansen & Schofield, 2010). It has been shown that these cells achieve this function due to their abilities to regulate the magnitude and timing of the cellular immune response, and allowing sequential induction of appropriate levels of inflammatory- and anti-inflammatory cytokines at key stages of the infection (Li et al, 1999, 2003, Walther et al., 2005, 2009).

A number of evidence has shown that antibodies are very important in the clearance of parasite loads in both animal and human blood stage infections (Berzins et al., 1991; Bolad & Berzins, 2000). In most malaria endemic countries, malaria infection induces
humoral immune responses, involving production of predominantly IgM and IgG but also of other immunoglobulin isotypes. While a majority of this immunoglobulin is a result of polyclonal B-cell activation, up to 5% or more represent species as well as stage-specific antibodies, reacting with a wide variety of parasite antigens. The level of total antimalarial antibodies in most cases increases with age, and is usually taken as a measure of the length and intensity of exposure, and sometimes may indicate protection against malaria. The biological properties of antibodies produced in response to malaria are of particular importance, due to their efficiency in antibody-mediated actions and ability to confer protection based on the presence of one or combination of isotypes and subisotypes.

Figure 3: Possible mechanisms for parasite neutralization of *P. f.* asexual blood stages.
2.3.1 Mode of action of antibodies

Antibodies specific for malaria can carry out their actions through various mechanisms directed towards different stages of the parasite in the erythrocytic cycle. Antibodies against erythrocyte surface-associated proteins may block RBC invasion by blocking merozoite release from ruptured schizonts, a phenomenon referred to as agglutination (Green et al., 1981; Wåhlin et al., 1984). Antibodies may also enter the infected RBC through the leaky membrane at the time close to rupture (Green et al., 1981), or through the parasitophorous duct, which has been suggested to form a connection for direct access of serum macromolecules to the parasite (Fig 3) (Pouvelle et al., 1991; Taraschi 1999). These actions may interfere with intracellular development of the parasite and essentially lead to growth inhibition (Ahlborg et al., 1996; Bergmann-Leitner et al., 2006). In addition, antibodies against antigens expressed during the trophozoite- schizont stages can block sequestration of iRBC to endothelial cells in internal organs thus enhancing clearance of parasites by the spleen (David et al., 1983), as well inhibiting rosetting of RBC to iRBC (Carlson et al., 1990). These actions may help prevent severe malaria complications. Antibodies to the GPI molecule may neutralize parasite toxins and prevent the induction of excessive inflammation.

Antibodies may carry out their effector functions in alliance with ancillary cells. They opsonize iRBC or free merozoites to enhance the phagocytic activity of monocytes (Bouharoun-Tayoun et al, 1995). The mechanism underlying this effective killing of parasites is the capture of antibodies on the surface of monocytes through receptors that bind the Fc part of the antibody, while the Fab part of the antibody molecule is bound to antigen/s on the surface of merozoites (Bouharoun-Tayoun et al., 1990) or late stage iRBCs (Gysin et al., 1993). This co-operation between malaria specific antibodies (see 2.3.2) and monocytes via Fcγ receptors could induce cellular functions such as phagocytosis, antibody dependent cellular inhibition (ADCI), mediated by monocyte-derived factors (Tebo et al., 2001; Jafarshad et al., 2007).
Antibodies against well studied target antigens such as merozoite-surface associated antigens (MSP1, MSP2), and antigens present in the apical complex organelles of merozoites (EBA-175 in micronemes, Rhop1-3, RAP 1-3 and AMA 1 in rhoptries) or in dense granules (Pf155/ RESA) (Wåhlin et al., 1984), may block RBC invasion either by neutralization of free merozoites and further interfering with the merozoite invasion process (Berzins & Anders, 1999). Other antibodies to antigens expressed during trophozoite development (SERA, ABRA, GLURP, PfEMP1, RIFINS, STEVORS and Pf332) have been demonstrated as efficient inhibitors of *P. falciparum* growth *in vitro* and/ or merozoite invasion (reviewed in Bolad & Berzins, 2000).

Importantly, adequate knowledge of blood stage immunity as well as antigen-specific responses (an issue addressed in this thesis) is very essential in malaria immunology, as this will give appropriate information about antigens responsible for protection, and to further scrutinize these antigens for vaccine development.

2.3.2 *Immunoglobulins and immunity to malaria*

As it has been emphasized, immunoglobulins (Ig) are crucial in immunity to malaria especially during the pathogenic blood stage. Antibodies produced against malaria parasites are of different isotypes, with different functional capabilities regarding being protective or otherwise. **IgM** is the primary antibody produced on the first encounter with an antigen (Leoratti *et al.*, 2008), and most puzzling of all isotypes. It has been speculated that these antibodies are involved in anti-parasite immunity (Baird, 1995), also in anti-toxic immunity which helps to prevent the clinical appearance of a malaria attack (Bate *et al.*, 1990; Boudin *et al.*, 1993). Elevated levels of IgM, as compared to controls have been observed in several studies performed in relation to malaria specific antigens, and has been suggested to be important in agglutination (Doolan *et al.*, 2009) as well as in neutralizing pathogens, owing to its polymeric structure (Czajkowsky *et al.*, 2010; Ehrenstein & Notley, 2010 ). In addition it is a potent complement activator (Czajkowsky & Shao, 2009). In a study it
was shown that immune IgM is protective against *P. chaubadi* infection (Couper *et al.*, 2005). In contrast, other studies have shown that IgM may not play a significant role in *P. f.* infection (Branch *et al.*, 1998), which still leaves us with the impression that there is no clear role of IgM during malaria infection.

The **IgG** isotype has been shown to be the mostly produced in humans in response to pathogens, this isotype consists of four subclasses (IgG 1, 2, 3, 4). IgG and its subclasses have been demonstrated to be significant towards immunity to malaria. The subclasses differ in their structure and mediate different immune effector functions (Nimmerjahn& Ravetch, 2008). The cytophilic ones, **IgG1** and **IgG3**, have been shown to predominate regarding protective humoral responses to *P. f.* malaria (Bouharoun –Tayoun & Druihlhe 1992, Sarthou *et al.*, 1997, Shi *et al.*, 1999). By acting through various mechanisms mentioned above (Bouharoun Tayoun *et al.*, 1995; Jafarshad *et al.*, 2007; Tebo *et al.*, 2001), these IgG subclasses of antibodies have been demonstrated to be very important in immunity to malaria. **IgG3**, the most short-lived subclass out of all IgG subclasses, has been the prevailing isotype in terms of responses associated with protection in malaria (Garraud *et al.*, 2003a), a finding which we also observed in one of our studies.

The other subclasses, **IgG2** and IgG4 are non-cytophilic, and have been speculated to be non-protective. Although in some malaria endemic areas, elevated levels of IgG2 have been related to decreased risk of infection, especially in individuals carrying a specific allelic variant of FcγRIIA, which binds IgG2 (Aucan *et al.*, 2000, Garraud *et al.*, 2003b, Nasr *et al.*, 2009). Meanwhile, **IgG4** is thought to compete with the IgG1 and 3 binding epitopes, thereby inhibiting the parasite neutralizing effects of cytophilic antibodies (Nutman, 2001, Hagan, 1991, Garraud *et al.*, 2003b). In malaria immunology, the dynamics of subclass responses and association with protection is important, and at the same time vary, especially with specific antigens and this area should be explored properly in order to understand immunity to malaria, as well guiding vaccine development (Garraud *et al.*, 2003a; Stanisic *et al.*, 2009).

The role of **IgE** antibodies in malaria is still vague, as it on one hand has been shown that malaria specific IgE may be associated with protection (Bereczky *et al.*, 2004, Farouk *et
al., 2005, Duarte et al., 2007). On the other hand, elevated levels of IgE appear to be associated with pathogenesis, as indicated in patients with severe and cerebral malaria (Perlmann et al., 1994; Perlmann et al., 2000; Perlmann et al., 1997; Troye-Blomberg et al., 1999; Seka-Seka et al., 2004, Leoratti et al., 2008).

Regarding IgA, there is no evidence of specific function regarding malaria. Some studies have shown high titres of naturally occurring plasmodium-specific IgA in sera (Biswas et al., 1995) and breast milk (Kassim et al., 2000), however some showed that the frequency of positivity of IgA antibodies does not have a correlation with the number of previous malaria episodes (Leoratti et al., 2008).

In malaria immunity, different antigens of P. f. induce different classes and subclasses of antibodies, and production of some of these immunoglobulins in individuals in relation to protection exists. The different patterns of antimalarial antibody responses observed have been suspected to be associated to various factors such as antigen properties, host age, cumulative exposure (Scopel et al., 2006; Nimmerjahn & Ravetch 2008; Garraud et al., 2002; Stanisic et al., 2009) and genetics (Duah et al., 2009; Nasr et al., 2009; Ntoumi et al., 2002; Ntoumi et al., 2005). In addition, the quality of antibodies and proper balance/proportion is a very crucial factor in protective immunity towards malaria.

2.4 Immune evasion by malaria parasite

The human immune system has evolved in order to eliminate infectious diseases, but the P. f. parasites, which are obligate parasites of human RBCs, have evolved to a complex life cycle and have been able to maintain an invasive blood-borne infection in its host. A complex co-existence has been established between the parasite and its host by evading human immune responses and occasionally not killing their hosts, making P. falciparum a cunning evader. The ability to evade the immune system is the bases of the lethality of P. f. infection when compared to other Plasmodium species, and has made the design of an effective vaccine amazingly difficult as evasion can occur at the various stages of the parasites’ life cycle.
Suppression of the host’s immunity during the pre-erythrocytic stage of malaria infection takes place. The sporozoite-infected hepatocytes are targets for CD8$^+$ T cells, thus leading to immune activation upon recognition of parasite derived peptides (Schofield et al., 1987, Klotz et al., 1995). To avoid this, the parasite suppresses the activation of the T-cell immune response due to the presence of multiple tandem repeats (Gilbert et al., 1998, Plebanski et al., 1999).

The pathogenic erythrocytic stage has an armory of evasive machineries, one of them being the inability of iRBC to process and present antigens, thereby providing protection from the host’s immune system. Some P. f.-proteins mimic the immune system by producing antibodies to non-protective repetitive regions of the antigens, thereby preventing the normal affinity and isotype maturation of an effective immune response (Anders, 1986; Ramasamy, 1998). The hemozoin from the late trophozoite/schizont stages have also been demonstrated to be involved in evasion, as HZ modulates the maturation of DC (Urban et al., 1999; Guisti et al., 2011), and monocytes (Skorokhod et al., 2004), which may downregulate the immune response.

In most cases, antigenic variation is employed by P. f. as an avenue to evade the immune system, based on the presence of immunodominant surface antigens. PfEMP1 has achieved a form of variability (see section 3.1 below), which in turn has allowed the parasite to cope with the host immune system. Furthermore, P. falciparum parasites show a remarkably high degree of polymorphism at the various stages of their life cycle (Lockyer et al., 1989; Miller, 1993; Fenton et al., 1991; Konate et al., 1999), which has important implications for the efficacy of parasite neutralizing immune responses. Antigenic diversity may thus delay acquisition of protective immunity to malaria, the development of which may thus require repeated exposure to different antigenic types or strains present in the epidemiological area. The antigenic diversity observed in most malaria antigens is a reflection of allelic gene polymorphisms, which is caused by variations in the sequence of short tandem repeats and which frequently constitute immunodominant regions. The best studied are msp1 and 2 genes regarding allelic
polymorphisms (Snounou et al., 1999, Aubouy et al., 2003, Kang et al., 2010).

The spleen is an immunologically active organ, where resident macrophages recognize and remove RBCs with compromised deformability or altered antigenicity (a form of innate immunity), which is the case with the *P. f.* infected red blood cells. Mature stage parasites express proteins which alter the properties of the host RBC membrane (section 3) and thus promotes adhesion of the iRBCs to ICAM-1 of vascular endothelium using the antigen PfEMP1 (section 3.1), resulting in sequestration and primarily evasion from clearance by the spleen.

PfEMP1 and RSP2/RAP2 proteins are not the only culprit antigens involved in pathogenesis and evasive mechanisms observed in *P. f.* malaria. A number of proteins involved in modifications of erythrocyte cytoskeleton and/or iRBC membrane might be involved (Maier et al., 2009), which will be discussed later in section 3. It is believed that most of the iRBC proteins act alone or in combination with other proteins to perform their molecular functions (Tilley et al., 2011). Understanding the roles of these antigens is important in the field of parasite biology, as is the way they elicit strong immunological responses, which might be protective or used to artifice the human immune system. These questions are relevant and represent a key point addressed in this thesis.

2.5 Malaria vaccines

The aim of vaccination is to generate immune responses with capacity to protect the recipient against the natural infection. The immune response induced by vaccination should be of long duration and should have the capacity to be effectively reactivated by a natural infection. The rationale behind the development of a malaria vaccine is supported by previous studies, which have shown that anti-sporozoite vaccines based on irradiated sporozoites could elicit sterile immunity in humans (Schofield et al., 2002), and also that passive transfer of IgG from immune individuals can provide some protection against malaria (Cohen et al., 1961, Sabchareon et al., 1991). Attempts to develop a malaria
A vaccine began in the early twentieth century (Desowitz, 1991), and in spite of advances in biomedical technology and periodic bouts of unsubstantiated optimism in the field, no effective vaccine is available for widespread use till date.

Ideally, a vaccine should be able to follow the central dogma of immune responses. After antigen encounter, the CD4+ T cells develop into T helper (Th) 1 or Th 2 effector cells, depending on the cytokine milieu. The Th1 effector cells produce proinflammatory cytokines, such as IFN-γ and TNF-α, and these lead to further activation of the CD8+ T cells and their cytotoxic effector function. The Th2 cells, on the other hand, produce interleukins, such as IL-4, 5, 10 and 13, which further stimulate B cells to undergo activation into effector B cells (plasma cells), which secrete antibodies (IgD, IgM, IgE, IgA or IgG). Memory cells, which are only about 5-10% of the lymphocyte population, are phenotypically different from the naïve cells, and they proliferate and produce cytokines faster upon activation. Whether the persistence of antigen on follicular dendritic cells is needed or not for maintenance of immunological memory, is not clear. Several studies have indicated that memory B and T cells can survive without antigen, while others have suggested otherwise (Haberman & Selomchick 2003). However, cytokines such as IL-15 are crucial for the longevity of memory T cells.

The advent of recombinant DNA technology and protein engineering enabled the expression of immunogenic proteins in bacterial, (an approach employed in our study) yeast or mammalian cells. After being cloned in a suitable expression vector, such expressed antigens are used for studies in vaccine development (Dertzbaugh, 1998; Liu 1998; Babiuk, 1999; Liljeqvist & Ståhl 1999). The basics of this technology is to transfer a gene encoding an antigen, responsible for inducing good humoral responses sufficient for protection, to a non-pathogenic expression vehicles (Liljeqvist & Ståhl 1999), thereby making the production of the antigen safer and generally more efficient. There are several limitations with recombinant proteins; they are generally poor immunogens when administered alone and are unable to induce effector T–cell responses, such as CD8+ CTLs, that are necessary for elimination of the intracellular pathogens.
As mentioned above (section 2.4), Plasmodium species have evolved multiple mechanisms of immune evasion at the individual and population levels, including stage specific antigen expression, allelic diversity, variability within T cell epitope sequences and antigenic variation. During the course of its complex life cycle, the Plasmodium parasite expresses different, complex mixtures of antigens. Therefore, a vaccine against a single stage in the parasite life cycle may need to be 100% effective, because parasites which progress to the next stage may express a new set of antigens, that may be unaffected by the vaccine induced response. Most malaria antigens are stage-specific and therefore there are distinct immune mechanisms operating against the different stages of the complex life cycle. Vaccines against all stages of the parasite life cycle are important, however for conciseness of this thesis, we will address only issues around vaccines against the blood stage cycle.

2.5.1 Asexual blood stage vaccines

Vaccines targeted against antigens expressed by the asexual blood stages of the parasite’s life cycle are aimed at preventing the complications of the disease, such as cerebral malaria or anemia. Since these are the stages responsible for pathology of the disease, antibodies to the target antigens should be able to inhibit parasite sequestration, induce neutralizing antibodies against parasite derived antigens, or to eliminate/reduce the parasite load, which might further reduce mortality. Despite encouraging progress, the lack of immune correlates of protection, and insufficient predictive animal models, as well as antigenic polymorphisms and strain variability of most asexual blood-stage antigens, constitute major challenges to the development effort of asexual stage vaccines. Inhibition of parasite invasion, as often measured in in vitro assays, may not be predictive of immune status in endemic areas. This assay is termed as one of the prerequisite assays important for evaluating blood-stage vaccine candidates and for identifying targets of protective antibodies against malaria (Persson et al., 2006).
Till date, a small number of blood-stage antigens expressed on the surface of merozoites are in clinical development as vaccines (Crompton et al., 2010). These include the apical membrane antigen 1, AMA1 (Sagara et al., 2009), erythrocyte-binding antigen-175, EBA-175 (El Sahly et al., 2010), glutamate rich protein, GLURP (Esen et al., 2009, Hermsen et al., 2007), merozoite surface protein 1, MSP1 (Ogutu et al., 2009), MSP2 (Genton et al., 2002), MSP3 (Esen et al., 2009, Audran et al., 2005, Sirima et al., 2009, Druilhe et al., 2005), and the serine repeat antigen 5, SERA5 (Horii et al., 2010). However, in a phase II trial some of these vaccine candidates, AMA1 and MSP1, with novel adjuvants have failed to demonstrate efficacy in African children (Sagara et al., 2009, Ogutu et al., 2009).

An important setback in the development of vaccines is that the *P. falciparum* genome encodes about 5,300 proteins (Gardner et al., 2002), and identification of potential blood-stage vaccine candidates capable of inducing effective immune response is quite challenging. Steps such as high-throughput protein expression systems to construct microarrays of large numbers of *P. f.*-proteins are being currently employed to circumvent this setback (Tsuboi et al., 2008, Doolan et al., 2008, Crompton et al., 2010). Another major factor preventing the development of an effective malaria vaccine, is extensive parasite genetic diversity due to selective pressure exerted by the human immune response, which leads to extremely polymorphic antigens and effective measures to overcome antigenic polymorphism are important to control this setback (Weedall & Conway 2010; Takala et al., 2009).

To ensure a high degree of protection from malaria disease, a malaria vaccine may encompass antigens from different stages of the parasite’s life cycle. If found effective, such a vaccine will interfere with parasite development within the mosquito and further reduce parasite transmission (transmission-blocking vaccine), as well as prevent blood stage infection (pre-erythrocytic vaccine) and target pathogenesis (erythrocytic vaccine).
3 The infected red cell and its modifications

Simply, the uninfected erythrocyte is a sack of haemoglobin, inherently adapted to perform specialized tasks of O₂ and CO₂ transport. This cell is anucleated and therefore unable to synthesize new proteins and its inability to perform neither intracellular trafficking nor antigen presentation. The RBC (diameter ~8 µm) is highly deformable without undergoing fragmentation, demonstrated by its ability to transit through 1-2 µm interendothelial slits (An & Mohandas, 2008). The capability of the RBCs membrane to undergo this deformability is due to the composition of the membrane skeleton, which is made up of a network of proteins, actin and spectrin (Mohandas & Chasis, 1993; Luna & Hitt 1992), as well as to their low internal viscosity (Maier et al., 2008).

The *P. falciparum* parasite finds refuge in the uninfected erythrocyte, a perfect cellular niche that will accommodate its havoc. As the parasite grows within the erythrocyte it loses its deformability and becomes spherocytic and more rigid (Cooke et al., 2004). These properties are thought to contribute to the pathogenesis of malaria, in addition to vascular adhesion of parasitized erythrocytes. The altered deformability is manifested by export of proteins into erythrocytes that interact with the host cell cytoskeleton and inserted into the membrane. Upon initial invasion the parasite is enclosed in a parasitophorous vacuole (PV) and its own parasite plasma membrane, which separate the parasite from the RBC (Hanssen et al., 2010). It develops into a ring form, where it starts to take up small amounts of haemoglobin and nutrients, using its cystostome (Bannister & Mitchell, 2003), as well as synthesizing molecules specific to this stage (Spielmann & Beck, 2000).

The ring develops into the trophozoite (~20-38 h post infection), the period where the parasite is metabolically active, growth increases, and major modifications occur (Bannister, 2003; Hanssen et al., 2010). Most of the cell structures are visible, and parasite organelles such as the Maurer’s clefts (MC) are observed in the iRBC’s cytoplasm (Aikawa et al., 1986, Atkinson & Aikawa, 1990). **Maurer’s clefts** are flattened cisternae that arise from the PV membrane, bud into the RBC cytoplasm, and
become attached to the RBC membrane by specialized tubular structures (Hanssen et al., 2010). It is a secretory organelle that concentrates proteins for delivery to the host iRBC membrane (Lanzer et al., 2006; Sam-Yellowe, 2009). This organelle harbours a number of P. f. proteins and is an intermediate compartment in the trafficking some of the parasite proteins associated with parasite virulence. Observations have shown that defects in Maurer’s cleft proteins can cause altered morphology of the exo-membrane system (Tilley et al., 2011).

As the parasite matures it initiates alterations to its host RBC, which facilitates entry of nutrients and exit of its byproducts, such as digested haemoglobin and synthesized proteins. The properties of the RBCs are modified by some of these proteins, which are further transported across the PV membrane via the MC to different sites in the RBC cytoplasm and the iRBC membrane (Lanzer et al., 2006; Maier et al., 2009).

The concluding phase of the cycle is the schizont stage (38-48 h p.i.), where the parasite occupies most of the host cell volume. At this stage the parasite consumes and digests approximately 80% of the host cell haemoglobin (Loria et al., 1999). The parasite undergoes a sequence of nuclear divisions, generating 16-20 daughter merozoites (about 1.2 µm long) ready for re-invasion as well as intense synthesis and assembly of molecules needed for RBC invasion (Bannister & Mitchell, 2003). The individual merozoites, armed with their own invasion machinery, remain within PV until the iRBC and the PVM ruptures by a protease-dependent process (Salmon et al., 2001).
Figure 4: (a) diagrammatic representation of a *P. f.*-infected erythrocyte, showing the organelles, Maier et al., 2008, (b) an expanded view of the membrane skeleton of *P. f.*-infected red blood cell, showing the location of Pf332 and other membrane related proteins (modified from Maier et al., 2009).
3.1 Antigens of the infected red cell

The *P. falciparum* parasite, might be a small haploid organism, but genomically complicated, with about 5300 proteins expressed by the organism at the different stages of its life cycle. The parasite exports an estimated 400 proteins, including kinases, lipases, adhesions, proteases and chaperone-like proteins, to the host cell during the blood stage. These proteins mediate a profound remodelling of the iRBC, performing different functions, from structural and functional changes of the iRBC to parasite growth (Tilley *et al.*, 2011; Maier *et al.*, 2008; Maier *et al.*, 2009). They are also involved in the modification of the host’s RBCs, such as increasing the iRBCs rigidity, capability to adhere to the walls of vascular endothelial cells and ability to vary the antigenic coat of the iRBC to avoid protective antibodies (Cooke *et al.*, 2004; Rowe *et al.*, 2009), which eventually promote disease severity due to its pathogenic mechanisms. The mechanism of export of some *P. f.* proteins from the parasite into the RBC cytoplasm depends on the possession of an NH$_2$-terminal motif, termed plasmodium export element (PEXEL) or vacuolar transport signal (VTS), which is conserved across the *Plasmodium* genus (Hiller *et al.*, 2004; Marti *et al.*, 2004), some but not all of the *P. f.* proteins possess this motif. Very few of these proteins have been highly studied in relation to understanding the biology of the parasite, therein, some of the proteins transported to iRBC cytoplasm and membrane as well as those involved in modification of the iRBC are described briefly.

As earlier mentioned, golgi-like structures such as Maurer’s clefts appear in the cytoplasm of the iRBC as the parasite matures. Significant resident proteins of the MCs and PEXEL-negative include, SBP1, MAHRP1, REX1, REX 2. Antigen Pf332 (the antigen of interest in this thesis) is also involved with the cleft but the exact association with the MC is still elusive. Although there is a recent data, that demonstrated that Pf332 is associated with the cytoplasmic face of the cleft (Nilsson *et al.*, unpublished).

**SBP1**: the skeleton–binding protein-1, is one of the first MC proteins to be described. It is a 48 kDa integral membrane protein that spans the Maurer’s cleft membrane (Blisnick *et al.*, 2000). The NH$_2$-terminal domain is found within the cleft, whereas the COOH-
terminal domain is exposed within the iRBC cytoplasm and interacts with a RBC membrane skeleton protein, possibly participating in the anchoring of the clefts to the RBC membrane skeleton (Blisnick et al., 2000). SBP1 has been suggested to be involved in the translocation of the virulence associated protein PfEMP1 to the surface of infected red blood cells (Cooke et al., 2006).

**MAHRP1:** the membrane-associated histidine-rich protein-1 is a 28.9 kDa protein, which consists of a highly conserved N-terminal region, a putative transmembrane (TM) domain in its middle region, and a variable histidine-rich domain at its C-terminal end (Spycher et al., 2003). The N-terminal and the TM domain have been suggested to be important for proper translocation of this protein towards MCs (Spycher et al., 2006). This protein is also being speculated to be involved in haemozoin production (Lynn et al., 1999, Sullivan et al., 1996, Garcia et al., 2009). It is important for the intraerythrocytic development of the parasite (Garcia et al., 2009) as well as the morphology of the MCs, as disruption of the MAHRP1 leads to increased fragility of MCs, accumulation of PfEMP1 in the parasite and PV, and in the end no surface expression of PfEMP1 (Spycher et al., 2008).

**REX1 & 2:** the ring-exported proteins 1 and 2. REX1 is the largest member of a cluster of the exported proteins (REX1-4) expressed in the ring-stage parasite (Dixon et al., 2008). It persists at the Maurer’s cleft throughout the erythrocyte infection. REX1 is peripherally associated with the cytoplasmic surface of the cleft, this protein plays a role in controlling the overall architecture of the MCs, as disruption of REX1 causes stacking of the MC lamellae and reduction in their numbers (Spielmann et al., 2006, Hanssen et al., 2008). REX2 is a 96 amino acid long protein that contains a N-terminal region and a TM domain, which are suggested to be involved in the transporting the protein to the MCs (Spielmann et al., 2006, Haase et al., 2009).

Additional proteins such as, KAHRP and PfEMP3 are PEXEL positive MC proteins associated with the cytoplasmic face of the Maurer’s clefts. About 16 hours post-invasion of merozoites, the surface of iRBC expresses knob-like protusions (Gruenberg et al., 1983), these structures contain the knob-associated histidine-rich protein, **KAHRP** (Taylor et al., 1987). KAHRP interacts with spectrin and actin (Kilejian et al., 1991, Oh
et al., 2000, Chishti et al., 1992), and is essential for knob formation (Crabb et al., 1997; Wickham et al., 2001; Rug et al., 2006). PfEMP3, P. f erythrocyte membrane protein 3 (Pasloske et al., 1993, Glenister et al., 2002), is important for trafficking of PfEMP1 to the erythrocyte surface and, hence, for cytoadherence. The truncation of PfEMP3 affects the MC morphology (Waterkeyn et al., 2000).

Several proteins synthesized by the intracellular parasite have been shown to be transported to the erythrocyte surface (Cooke et al., 2004). The most characterized protein on the surface of iRBC is P. f erythrocyte membrane protein-1, PfEMP 1, an adhesive protein encoded by the hypervariable var gene family, which consists of about 60 genes encoding proteins ranging in size from 220-350 kDa (Baruch et al., 1995; Su 1995; Smith et al., 1995). During the ring stage several var genes are expressed but during the trophozoite only one PfEMP1 is dominant. The expression of PfEMP1 on the iRBC surface leads to sequestration of matured parasites in the vascular endothelium.

Binding specificities of PfEMP1 variants have been mapped to different adhesion domains of the proteins and have revealed that DBL1α binds blood group A, CR1 and HS on both endothelial cells and erythrocytes (Barragan et al., 2000a, 2000b; Rowe et al., 1997; Chen et al., 2000; Vogt et al., 2003). CIDR1α domain has been shown to bind CD36 and IgM (Chen et al., 2000, Baruch et al., 1997, Robinson et al., 2003), and the DBLβ-C2 region binds to CD36 and ICAM-1 (Baruch et al., 1996, Chattopadhyay et al., 2004). Different patterns in disease severity have been observed with different PfEMP1 expressed at the time of infection. The variant associated with severe disease is Var2CSA, which mediates maternal malaria through sequestration to CSA in the placenta (Salanti et al., 2004; Dahlbäck et al., 2006).

Other variant protein families trafficked via the Maurer’s clefts include the STEVOR (subtelomeric variable open reading frame) proteins a family encoded by 27-39 gene copies (Przyborski et al., 2005). The RIFIN (repetitive interspersed family) proteins are encoded by the largest multicopy gene family in P. falciparum, comprising about 150-200 gene copies (Helmby et al., 1993; Kyes et al., 1999; Sam-Yellowe, 2009). The
**SURFIN** proteins are encoded by *surf* genes, consisting of a family of 10 genes. It is exported along with RIFIN and PfEMP1 to the MCs (Winter *et al.*, 2005).

Additional proteins of interest include: the ring-infected erythrocyte surface antigen, **Pf155/RESA**, which is released from the merozoite dense granules during or shortly after invasion, and it ends up in the RBC cytoplasm destined to the surface of the infected red cell (Aikawa *et al.*, 1990; Culvenor *et al.*, 1991). RESA is one of the first proteins exported across the PV membrane to iRBC membrane, where it interacts with the spectrin network and appears to suppress further invasion by other merozoites (Foley *et al.*, 1991; Ruangjirachuporn *et al.*, 1991; Pei *et al.*, 2007). This antigen has been suggested to be involved in modulating the mechanical properties of ring-stage iRBC, thereby reducing deformability of early ring infected- host red blood cells (Mills *et al.*, 2007). The mature-parasite-infected erythrocyte surface antigen, **MESA**, is a high molecular-mass protein expressed in trophozoites and schizonts this protein is also associated with the RBC skeleton (Coppel *et al.*, 1988, Lustigman *et al.*, 1990; Przyborski *et al.*, 2005).

### 3.2 Antigen 332

**3.2.1 Related background**

The *P. falciparum* antigen 332 (Pf332) identified by Mattei *et al.* (1989), is so far the largest of all *P. f.* proteins (~ 1 megadalton) exported to the infected red blood cell membrane. The Pf332 gene has a two exon structure and is located in the subtelomeric region on chromosome 11, and it has been detected in all clinical parasite isolates examined so far. Subtelomeric genes are prone to frequent gene recombination event, the Pf332 gene displays a marked sequence variation as demonstrated by restriction fragment length polymorphism (RFLP) (Mattei & Scherf 1992). Pf332 consists of a DBL (Duffy binding like) domain at the N terminus, followed by a predicted transmembrane domain and a large number of highly degenerate Glu-rich repeats (Fig.5) (plasmodb; Moll *et al.*, 2007; Scherf & Mattei 1992). With an overrepresentation of glutamic acids (30%) and
valine (13%) (Hinterberg et al., 1994), Pf332 shares related amino acid repeat sequences with other Glutamic acid rich antigens such as RESA (Cappai et al., 1989) and Pf11.1 (Scherf et al., 1992), and consequently these proteins show antigenic cross-reactivity (Ahlborg et al., 1991; Mattei et al., 1992).

During the later stages of the intraerythrocytic parasite development, this protein appears in vesicle-like structures (MCs) in the iRBCs cytoplasm and later becomes associated with the iRBC membrane skeleton (Hinterberg et al., 1994; Waller et al., 2010). The functional role of Pf332 in the parasite’s life cycle has not yet been fully elucidated. Nevertheless, studies have shown that the antigen could be involved in the trafficking of the major virulence adhesion protein PfEMP1 to the surface of the iRBC, as seen in a co-localization study, where Pf332 was transported in vesicles together with RIFIN and PfEMP-1 (Haeggström et al., 2004). Other findings showed that the N-terminal DBL region of Pf332 might be involved in re-invasion of red blood cells by facilitating uRBC adhesion (Moll et al., 2007), and as well as modulating iRBC membrane rigidity and assisting trafficking of PfEMP1 (Glenister et al., 2009, Hodder et al., 2009). Another region of Pf332, located in the C- terminus binds to actin, suggesting that Pf332 might be important in modulating the RBC membrane skeleton (Waller et al., 2010).

Studies on another region located C-terminally has showed that anti-Pf332 antibodies were involved in growth inhibition of parasites and the iRBC integrity (Paper I, Paper II). As well, it has been demonstrated that a centrally located region might be a target for opsonizing antibodies (Gysin et al., 1993; Perraut et al., 1995). The different observations in predicted functional role of antigen Pf332 is probably due to the large size of the protein and the fact that the different groups have focused on the different regions of the protein. These has led to the proposition that different regions of Pf332 may be involved in different functions during the erythrocytic cycle of the parasites life cycle (Hodder et al., 2009; Glenister et al., 2009; Waller et al., 2010; Paper II). Studies on Pf332 have been carried out using recombinant fragments of the antigen, and some of the fragments used in our studies are described below:
**EB200** is a fragment of antigen Pf332 (Mattei & Scherf 1992). It is about 157 aa long, and centrally located. It consists of mainly glutamic acid (E) repeats often occurring in pairs. This fragment has been defined as a target of anti-EB200 opsonizing antibodies in *P. falciparum*-exposed *Saimiri* monkeys (Gysin *et al.*, 1993; Perraut *et al.*, 1995). A study on sera from Senegalese adults displayed EB200-reactive antibodies of mainly the IgG3 subclass (Perraut *et al.*, 2000) and in another study the levels of anti-EB200 antibodies were found to be associated with fewer future malaria attacks (Ahlborg *et al.*, 2002).

**Pf332-C231** is recombinant fragment located at the C-terminal region of the antigen, which was characterized in papers I-III in this thesis, and it will be dealt with below in Section 4.

**Pf332-DBL** is a region located in the N-terminal region of the antigen. This region has a Duffy binding like (DBL) domain, which is related to the DBL domain of erythrocyte-binding-like (EBL) family of proteins (Moll *et al.*, 2007). Studies on this region by Moll *et al.*, 2007, disclosed that antigen Pf332 may be important for merozoite re-invasion, as infected RBCs were shown to bind to uninfected RBCs. (Moll *et al.*, 2007). Immunization studies with animal model revealed that recombinant Pf332-DBL was able to induce a strong IgG response in the presence of an adjuvant approved for human use (Du *et al.*, 2010). Recently, reactivity with plasma from individuals living in malaria endemic regions (Uganda, Burkina Faso and Mali) has been reported (Nilsson *et al.*, 2011, (In press)). This fragment was used in the Paper IV of this thesis.

![Figure 5: A schematic representation of antigen Pf332.](image-url)
As mentioned previously, an effective protective immunity against *P. f.* malaria in humans develop gradually over time after repeated exposure, protecting against progression to symptomatic and severe illness. The importance and quality of the response in relation to protective immunity is associated with different classes and subclasses of antibodies induced by the antigen in naturally exposed individuals. Several data have indicated that Pf332 might be a target of protective immune response as it has been demonstrated to be a target for ADCI and growth inhibitory antibodies.

The Pf332-reactive human monoclonal antibody (mAb) 33G2 has been demonstrated to inhibit both *P. falciparum* growth and cytoadherence *in vitro* (Udomsangpetetch *et al.*, 1989). Furthermore, various rabbit polyclonal antibodies specific for Pf332 (Ahlborg *et al.*, 1996; Ahlborg *et al.*, 1995; paper I), and human polyclonal IgG antibodies, affinity-purified on Pf332 fragments, also display similar parasite growth inhibitory capacity (Ahlborg *et al.*, 1993; paper II). In addition, Pf332-reactive antibodies in humans are associated with decreased number of malaria incidents (Ahlborg *et al.*, 2002; paper III). Experimental animal vaccinations with Pf332 have mainly been conducted with EB200 fragment (Mattei & Scherf, 1992). In this primate model, EB200 was identified as a target of opsonizing antibodies present in hyperimmune sera from *P. f.*-exposed *Saimiri* monkeys (Gysin *et al.*, 1993), and the presence of these antibodies correlated with protection against disease (Perraut *et al.*, 1995).

Evidence from various sero-epidemiological studies with the C231 fragment of Pf332 (Sudan- Nasr *et al.*, 2007; Giha *et al.*, 2009, 2010a, & 2010b; Nasr *et al.*, 2009; Senegal-paper I, paper III; Nigeria- Iriemenam *et al.*, 2009, BurkinaFaso, Liberia, Mali- paper I) have demonstrated that antibody responses to this antigen are present during natural infections. Recently, immunization studies with an experimental model, showed that Pf332-DBL generated a strong and significant IgG response (Du *et al.*, 2010), and in
addition reactivity with several plasma from different malaria endemic regions have been demonstrated (Nilsson et al., 2011, In press). From a prospective study using microarray, Malian sera used were highly reactive with antigen Pf332 (Crompton et al., 2010), further strengthening the fact that antigen Pf332 might be a target for protective antibody responses during natural *P. f.* infection to malaria.
4 The Present Investigation

4.1 Preface

Despite efforts made towards vaccine development, malaria still remains uncontrolled due to the meager knowledge of the natural protective immunity against this disease. Induction of an effective immune response to malaria is multifaceted and mechanisms behind the induction, regulation and maintenance, should be considered on antigen-specific level. As mentioned earlier, the asexual erythrocytic stages of the malaria parasite are the major cause of morbidity and mortality, and major focus is based on identifying and characterizing antigens from the blood stage cycle. As the importance of antibodies in immunity against malaria has been emphasized, antigens from the blood stage cycle are expected to induce significant antibodies, which may be functional in protective immunity. These antigens might in the end be effective for vaccine development.

This present study is based on Pf332, an antigen derived from the late stage of the erythrocytic cycle, with focus on the two recombinant fragments Pf332-C231 and Pf332-DBL, derived from two different regions of this megadalton protein. Being an uncommonly large protein it will be feasible to work with fragments rather than the large protein as a whole. The rationale behind Pf332-C231 is based on earlier studies which focused on the central part of Pf332, EB200. This fragment is quite overrepresented with glutamic acid repeats and there have been difficulties in defining the actual target for parasite growth inhibitory antibodies due to its antigenic cross-reactivity with other malaria antigens. In order to address this problem, it was proposed that a less repetitive fragment Pf332-C231 located in the C-terminal region of the antigen be investigated. This C-terminal fragment is well represented with various amino acids and most importantly has a cysteine, which might be involved in the folding of the protein. Studies on the second fragment, Pf332-DBL, located at the N-terminus are based on the findings that antibodies targeted against this region might be effective against parasite re-invasion (Moll et al., 2007) as well as immunization studies carried out in experimental models (Du et al., 2010).
Within the context of this thesis, we addressed issues such as cloning of recombinant fragments (Paper I), a molecular technology that has facilitated numerous studies such as characterization of the antigen, generation of antigen specific antibodies, and the ability of specific antibodies to be effective in \textit{in vitro} killing of parasites and inhibiting growth of parasites (Paper I & II). Understanding the humoral response to malarial antigens has been a major objective in malaria research, initially this centered on the identification of protective immune responses and the classical serum replacement of Cohen and McGregor (Cohen \textit{et al.}, 1961; McGregor, 1964). In this thesis we looked at the humoral response of naturally exposed Senegalese against two different fragments (Pf332-C231 – Paper III, Pf332-DBL – Paper IV) of antigen Pf332, using the conventional ELISA.

4.2 Objectives

The specific aims of the research work carried out in this thesis were:

- To study the immunological capabilities of the fragment Pf332-C231 using an experimental model (Paper I).

- To investigate the immunological capacities and possible mode of action of affinity purified human antibodies reactive with the Pf332-C231 fragment of antigen Pf332 (Paper II).

- To study the profile of the antibody responses to Pf332-C231 in naturally exposed individuals living in Senegal (Paper III).

- To determine the pattern of immunoglobulin responses to Pf332-DBL fragment of Pf332 in exposed individuals living in Senegal (Paper IV).
4.3 Experimental approach

The materials and methods used, as well as ethical approvals are described in detail in the respective studies (paper I-IV). A brief description of the study area used in the sero-epidemiological studies (paper III & IV) is given below.

4.3.1 Study population

Senegal is a country south of the Sénégal River in western Africa. The country is externally bounded by the Atlantic Ocean to the west, Mauritania to the north, Mali to the east, and Guinea and Guinea-Bissau to the south; internally it almost completely surrounds The Gambia (Fig. 6). Senegal covers a land area of almost 197,000 square kilometres (76,000 sq mi), and has an estimated population of about 14 million. The climate is tropical with two seasons: the dry season and the rainy season. Dakar, the capital city of Senegal, is located at the westernmost tip of the country on the Cap-Vert peninsula. The specific study area in this thesis for Paper III & IV is the village of Dielmo (Fig. 6). Dielmo is in an area of the Sudan-type, savanna region of Senegal, located 280 km southeast of Dakar (about 4 hr drive from Dakar) and about 15 km north of the Gambian border (Trape et al., 1994). Rainfall occurs during a four-month period (from end of June-mid October). The village is situated on the marshy bank of a small permanent stream (the Nema) that permits the persistence of anopheline breeding sites year round (Trape et al., 1994). The inhabitants of Dielmo are settled agricultural workers (Trape et al., 1994).

4.3.2 The Malaria Situation in Senegal

Malaria in Senegal is defined as endemic/stable with seasonal transmission occurring from June to November, with almost all cases caused by *P. falciparum*. There are significant epidemiological variations from north (hypoendemic) to south
(hyperendemic). Malaria has been a venerable public health problem in Senegal. At the national level, 2 million cases (> 2000 deaths) of malaria were recorded. In Dielmo, malaria transmission is intense and permanent. The annual entomological inoculation rate (EIR) recorded in 1996 in Dielmo was 305 \textit{P. falciparum} infective bites per individual. The longitudinal epidemiological follow-up of this study area has been described in detail in (Trape \textit{et al.}, 1994). However, in Senegal, the malaria cases and deaths has declined markedly between 2007 and 2009, due to the effective malaria control programme established since 2005(WHO, 2010).


Figure 6: Map of Senegal, showing the study area Dielmo. (Modified from http://en.wikipedia.org/wiki/Senegal).
4.4 Results and discussion

4.4.1 Paper I

The majority of all functional studies on malarial proteins have been initiated by introducing genes downstream of promoters ported by plasmids, and transfection into *E. coli* for recombinant expression. There are however other expression systems such as baculovirus, yeast, mammalian and insect cells, but the approach which we have used in our study poses some advantages, such as simplicity and low cost, availability of compatible molecular tools, absence of post translational modifications, higher yield per unit biomass. In this paper, we were able to clone the recombinant protein of interest, using the plasmid vector and expression was carried out in *E. coli*. In molecular biology, expression in *E. coli* has been debarred by the formation of insoluble aggregates of the recombinant protein being expressed, and to circumvent this problem, we enhanced the purification of our protein, using a solubility enhancing fusion tag. The 6xHis tag is a small, poorly immunogenic tag, which does not interfere with the structure or function of the purified protein.

This study on C231, possesses theoretical and practical advantages over those on EB200 regarding functional and immunological studies, as we observed that the primary sequence of C231 is more complex as the contribution of each type of amino acid is more equal, and the C231 sequence also consists of one cysteine residue, which theoretically may contribute to sequence conservation and formation of a disulphide bond. The characterization of this recombinant Pf332-C231 fragment is one of the steps that have facilitated the unravelling of the entangled Pf332. Additional bioinformatic analysis also revealed that C231 consists of conserved B-cell epitopes.

Regarding the immunogenicity of this protein, Pf332-C231 induced high antibody titers in rabbits, and these antibodies also recognized the native protein expressed in infected erythrocytes, as revealed by immunoflourescence and immunoblotting. The functionality of antibodies induced against the asexual blood stages of *P. falciparum* are evaluated on
the basis of their ability to induce anti-parasite activity. Such antibodies may have different effector functions, e.g. inhibition of invasion or inhibition of parasite growth/development, depending on the target antigen. In \textit{in vitro} experiments carried out with anti-C231 antibodies we observed inhibition of merozoite invasion, as reflected by inhibition of parasite development of infected RBCs. Reactivities by ELISA with recombinant C231 of plasma samples of malaria-immune individuals from Senegal, Liberia, Burkina Faso and Mali, revealed that the Pf332-C231 region is immunogenic during natural malaria infections, as revealed by its recognition by antibodies at high frequency. Thus, the B-cell epitopes of Pf332 are not only limited to the glutamic acid rich repeats.

As mentioned earlier, antibodies to EB200 displayed cross-reactivity with other malaria antigens, however in this study we could not totally circumvent this effect, as some degree of cross-reactivity of rabbit anti-C231 antibodies was observed with RESA.

\textbf{4.4.2 Paper II}

Humans repeatedly exposed to malaria have been shown to be reactive with antigen Pf332 (Ahlborg \textit{et al.}, 2002; Paper I; Paper III), and Pf332 reactive antibodies have been demonstrated to inhibit the growth of F32 parasites \textit{in vitro} (Paper I), although the mechanisms by which such antibodies inhibit invasion/parasite growth is not explicit. In this paper, using an \textit{in vitro} model, we investigated the ability of Pf332-C231 affinity purified human antibodies to inhibit the growth of F32 parasites, and the likely mode of action of these specific antibodies was studied.

Using seven sera from malaria exposed Liberian adults, Pf332-C231 reactive-antibodies were affinity purified using CNBr activated Sepharose. All affinity-purified antibodies were reactive by ELISA, and positive in immunofluorescence, giving the typical Pf332 dot-like staining pattern. To analyse the functional activities of the C231-specific antibodies, a parasite growth inhibition assay was performed, and it was found that all
C231-specific antibody preparations except one inhibited (> 50%) the growth of parasites, leading to subsequent reduction in parasitaemia. The specificity of this inhibition was further established by an antigen reversal assay, where the inhibitory effect seen was reduced/blocking by the recombinant C231. In this study affinity purified antibodies were of the IgM and IgG isotypes, but the IgG isotype seemed to be responsible for the inhibitory effect observed.

In addition to the ability of Pf332-specific antibodies to inhibit parasite growth, external matured parasites were observed in the form of pyknotic parasites as well as parasites with anomalous morphologies. The presence of these external late stage parasites may indicate that Pf332-C231 targets the cytoskeleton of the infected RBC. This observation is highly important, as Pf332 has been affirmed to be involved in modulating the rigidity of the cytoskeleton of iRBCs (Glenister et al., 2009, Hodder et al., 2009), specifically by binding to F-actin through a region in the C-terminal domain (Waller et al., 2010). Thus, the presence of Pf332-specific antibodies may lead to interference with the cytoskeletal network, eventually leading to destruction of the iRBCs and exposure of the mature parasites, which may imply a minimal or no reinvasion.

The effect of anti-C231 antibodies was further examined, when antibody pressure on F32 parasites was removed after 42 h and parasites growth monitored. Initially, the growth of the antibody treated parasites was retarded, as compared to that of the control parasites, but the parasite growth was recuperated after 5 invasive cycles. However, there may be a different situation in vivo, as other immune mechanisms (Bolad & Berzins, 2000) may aid parasite clearance. Indeed, immunity to malaria is not sterile, and asymptomatic infections are very common in malaria endemic countries, and it has been mentioned that antibodies to some intracellular malaria antigens might be important in curbing parasite load (Crompton et al., 2010). Therefore, the implication of antibody pressure is that it may give rise to parasite clones with a differential sensitivity to growth inhibitory antibodies, an observation seen earlier with Pf332-reactive antibodies (Iqbal et al., 1997), and which may be an avenue for immune evasive mechanisms due to antigenic variation.
The ultimate position of Pf332 regarding the infected erythrocyte is still uncertain. Moll et al., (2007) showed that the antigen is expressed on the surface of infected RBCs, but another study failed to detect this (Hodder et al., 2009). We performed immunofluorescence on live infected RBCs, but could not reach any conclusion regarding the surface exposure of the C-terminal part of Pf332, as we only detected a very weak staining. Although different fragments were used in the above mentioned studies, we may speculate that C-terminal region may be more involved in the interaction with the iRBC membrane skeleton, as suggested by Waller et al., (2010), but may also be partially on the surface of iRBCs. In addition, recent unpublished data showed that the Pf332 protein is present on the cytosolic face of the cleft via protein-protein interaction (Nilsson et al., 2011 (in PhD thesis by S. Nilsson). Obviously, the actual location of Pf332 regarding surface or internal membrane of the iRBC needs intensive structural studies. If the final location of Pf332 is not on the iRBC membrane, the mode of entry of antibodies into the iRBC to effect growth inhibition or parasite killing is still unrequited. Most likely, antibodies may gain access to intraerythrocytic epitopes by passing through the leaky membrane during the late parasite stages, close to the time of rupture or via the parasitophorous duct (Pouvelle et al 1991, Taraschi 1999).

4.4.3 Paper III & IV

To understand the dynamics of the host-parasite balance, its relationship to transmission, and to identify those factors that contribute to the development of disease, it is essential to carry out studies of well defined cohorts, in which malaria parameters are recorded longitudinally. As is well established, antibodies are thought to be the primary immune effectors in the defense against erythrocytic stage *P. falciparum*. The level of total anti-malarial antibodies increases with age, and is usually taken as a measure of the length and intensity of exposure, and sometimes may indicate protection against malaria.

In Paper III, we performed analyses with recombinant **Pf332-C231** as antigen in comparison to crude malaria extract, and looked at the profile of isotypes and IgG
subclasses of antibodies in malaria exposed individuals from Senegal. In general, anti-
C231 reactive antibodies were detected in all sera tested regarding all isotypes (IgG, IgM,
IgE) investigated, and the levels increased significantly with age.

The polarization of antibody responses towards the IgG1 and IgG3 subclasses, which
possess the ability to bind to Fcγ receptors on the surface of monocytes, macrophages, and
neutrophils, is believed to play a key-role in immunity to blood-stage *P. falciparum*
infection. Such cytophilic antibodies (IgG1 and IgG3) have been demonstrated to mediate
parasite-killing responses, such as opsonization and phagocytosis of extracellular
parasites or parasitized red blood cells, and antibody-dependent cellular inhibition of
intracellular parasites. However, there are studies suggesting a protective role of non-
cytophilic IgG2 *in vivo* (Aucan *et al.*, 2000; Ntoumi *et al.*, 2005), which may to some
extent be explained by a 131 R/H polymorphism in the Fcγ receptor IIa. As a result, IgG2
is cytophilic in individuals carrying the H131 allele positive FcγIIa gene (Warmerdam *et
al.*, 1991). When analyzing the subclasses of C231 specific IgG antibodies in sera from
individuals naturally primed to *P. falciparum* (Paper III), we observed a bias towards
IgG2 and IgG3 relative to IgG1. We conclude that the IgG subclass distribution of
naturally acquired antibodies to Pf332-C231 in malaria exposed individuals in Senegal
may be epitope driven. Since polarization towards IgG2 was more evident for anti-C231,
this may be affected by cumulative or current exposure to malaria by the age and FcγRIIa
genotype of the subjects. These findings have clear implications for the rational design
and evaluation of antimalarial vaccines that induce antibody-mediated protection.
However, further studies and analysis should be carried out for possible correlation of
IgG2 and H131 allele of FcγRIIa.

Comparison of serological and clinical data in a previous study with the same Senegalese
donors showed that high levels of IgG antibodies to crude malaria antigen and to EB200
were predictive of fewer future clinical attacks of malaria (Ahlborg *et al.*, 2002). With
C231, antibodies of all isotypes tended to be higher in individuals not experiencing any
malaria attack after 12 months of follow up, which however did not reach statistical
significance in a multivariate analysis.
In Paper IV, using the same samples and epidemiological data employed in paper III, we investigated the antibody reactivity pattern to the N-terminal Duffy binding like domain of Pf332 (Pf332-DBL). In this study we detected antibodies of all isotypes and IgG subclasses measured in all age groups, with IgG as the main antibody detected. The effect of age, as seen with most malaria antigens, was observed mainly with IgG, IgG1 and IgG3.

Most malaria antigens have been shown to induce antibodies of the cytophilic IgG1 and IgG3 subclasses, which is based on many factors, such as antigenic composition and age of donors (Tongren et al., 2006; Matondo Maya et al., 2006). In our study with Pf332-DBL, IgG1 and IgG3 subclasses predominated the non-cytophilic IgG2 and IgG4 antibodies. When investigating if there were correlations among the levels of antibodies of the different subclasses detected, we found all had a positive correlation, except for IgG2, which displayed consistent negative associations.

The knowledge of IgG subclass responses associated with protection against malaria with specific antigens varies (Stanisic et al., 2009). Thus, we investigated the association of anti-Pf332-DBL IgG subclasses in relation to number of malaria attacks experienced by the donors between 6 months and 3 years of follow up. The IgG3 response was found negatively associated with the number of malaria attacks observed after 2 years of follow up. This indicates that anti-Pf332-DBL IgG3, amidst other factors, might play a role in the asymptomatic malaria observed in the Dielmo village during the study period. To further elucidate the role of IgG3, the donors were divided into two groups based on the level of IgG3 antibodies induced. It was found that the group with higher levels Pf332-DBL IgG3 was associated with fewer malaria attacks still after 2 years of follow up, and this remained significant when all other variables were controlled. In a recent preliminary microarray analysis, it was observed that the antibody reactivity to Pf332 was significantly higher in protected individuals (Crompton et al., 2010). The observation with antibodies to Pf332 might be a reflection of anti-disease immunity whereby anti-Pf332 antibodies (Pf332-DBL IgG3) might prevent pathological symptoms.
Even though we observed positive correlations between the IgG (R=0.706, p=<0.0001) and IgM (R= 0.711, p= <0.0001) antibody responses against Pf332- C231 (paper III) and Pf332-DBL (paper IV), the pattern of antibody responses differed, even with the previously studied EB200 fragment (Ahlborg et al., 2002). These results are indicative of antigen Pf332 being capable of inducing specific polyclonal antibodies. This is not unexpected, as these fragments, even if from the same antigen, possess different antigenic properties, owing to the differences in their amino acid composition. In addition, the size of Pf332 permits this, as it has been suggested that such a megadalton protein may engage its different regions in various functions (Hodder et al., 2009; Glenister et al., 2009; Waller et al., 2010; Paper I).
4.5 Concluding remarks

The natural immunity to malaria consists of a complex mixture of diverse immune responses, some non-protective and some protective or inhibitory. Within the capacity of our studies, we have shown that the C-terminal C231 fragment of antigen Pf332, an asexual blood stage antigen is immunogenic. Furthermore we have been able to ascertain the functional activity of Pf332-specific (Pf332-C231), human antibodies using the *in vitro* inhibition assay, a prerequisite for evaluation of blood-stage vaccine candidates and for identifying targets of protective antibodies against malaria. In addition, the presence of Pf332 (C231 & DBL) reactive antibodies associated with protection, was found in all age groups of asymptomatic individuals naturally exposed to *P. falciparum* infection.

The development of an effective malaria vaccine encompasses a whole array of setbacks. The complexity of the parasite cycle offers various candidates, and there might be doubts whether these candidates will induce the right type of immunological response, even if it does in experimental models and some clinical trials. Furthermore, it might not be able to induce the long term immunological memory essential for long term protection. Antibodies to intracellular proteins, such as Pf332, are typically viewed as markers of past infection and not as evidence of vaccine potential, based on the location of such antigens. However, it is possible that higher antibody levels against intracellular proteins among protected individuals, as seen in our studies, could be a predictor of anti-disease immunity and/or a marker of enhanced parasite killing (Crompton *et al.*, 2010). On this note, it is assumed that it might be too early to dismiss intracellularly located immunogenic proteins, such as antigen Pf332, as potential vaccine targets based solely on their subcellular location, as little is known about the molecular host-malaria parasite interactions. Based on our findings (Paper I-IV), and other recent studies on Pf332 (Glenister *et al.*, 2009; Moll *et al.*, 2007; Hodder *et al.*, 2009; Du *et al.*, 2010, Crompton *et al.*, 2010), we propose that Pf332 is a target for parasite neutralizing antibodies, which emphasizes that Pf332 might be a potential vaccine candidate.
Figure 7a-c: Schematic representation of the possible mechanism of antibody mediated inhibition of intraerythrocytic development of parasites by anti-Pf332 antibodies. a) by antibodies binding to antigens on the surface of iRBC, b) by antibodies entering through the leaky membrane, c) antibodies entering through the parasitophorous duct, and d) the morphological effects of (C231-Pf332) affinity purified human antibodies on F32 parasites, in vitro.
4.6 Future perspectives

Amidst all inferences mentioned above, further studies should be carried out addressing different aspects of antibodies to Pf332.

Antibodies induced by proteins are commonly directed against surface exposed regions of the protein, and certain epitopes may be immunodominant. One strategy for the selection of antigenic sequences to be included in a subunit vaccine against *P. falciparum* malaria, is to define epitopes seen by antibodies which have the ability to interfere with parasite development. A Pf332 reactive monoclonal antibody (mAb 33G2), which was shown to have the capacity to inhibit parasite growth/merozoite invasion *in vitro*, harbours an epitope of five amino acids long sequence, VTEEI (Ahlborg *et al*., 1991). In line with this, the elucidation of specific B-cell epitopes by analyzing the ability of peptides to induce antibodies may reveal different epitope specificities regarding Pf332-C231 and other fragments. Furthermore, production of new human monoclonal antibodies against epitopes in different regions of Pf332 would be important tools for dissecting the antigen in specific functional assays.

Further studies on the parasite inhibitory capacity of affinity purified human anti-C231 antibodies from human sera from other malaria endemic areas should be carried out. It might also be interesting to see the inhibitory effect of affinity purified antibodies of other Pf332 fragments, using the same samples. Also of importance is to carry out inhibition assays with other parasite strains as we only used the F32 strain. The parasite neutralizing capacity of these human antibodies can also be tested with human monocytes, as this mimics the immunological situation *in vivo*.

To further elucidate the mechanism of the antibody mediated inhibition of parasite growth, it may be functional to perform inhibition experiments with Pf332 reactive antibodies on parasites lacking expression of Pf332, (Hodder *et al*. 2009; Glenister *et al*., 2009), as well as inhibition experiments with mixtures of antibodies to different regions of Pf332 to see if there will be synergistic effects. Such experiments would also give information about whether Pf332 is the true target for the inhibitory antibodies.
4.7 Other Publications

The following articles are of relative importance but not included within the framework of the thesis:


5 Acknowledgements

These studies were supported by grants from Swedish International Development Cooperation agency (SIDA, SAREC), the Swedish Medical Research Council (VR) and grants from the Biomalpar European Network of Excellence (LSMP-CT-2004-503578).

I sincerely, express my profound gratitude to all who have in one way or the other contributed to my studies.

My supervisor, Prof. Klaus Berzins, thank you for your kind heartedness, and patience. I will not forget the adventures of chasing the rabbits, all the regular journals, dealing with statistics and remaining supportive until I got to the finish line. You are such a great teacher, because you instill the knowledge and always made sure your students do not scale through “short cuts” but learn from the basics. It was a pleasure being your student.

The seniors at the department of immunology, Marita Troye-Blomberg (for your charismatic leadership), Carmen Fernandez (for your constructive criticism), Eva Sverremark-Ekström (for your concern always and those “re-assuring smiles”, they do mean a lot to me, you know), and Eva Severinson (for the nice discussions during the JCs and Seminars), thank you all for making the department a nice and lovely environment.

Ulrika, thank you for the motivation. Yohannes, thank you for all the encouraging words, advice and articles.

Ann Sjölund, I enjoyed the times we spent during our work both day and night, and the cheerful Margretha Hagstedt, for the nice assistances rendered always both in the lab and also outside lab issues, you are such a wonderful person. Hedvig Perlmann, thanks for the lovely smiles always.

Gelana Yadeta, for having your doors open always, and ready to answer all question regarding administrative work. Anna-Leena Jarva, you have been such a kind and cheerful person, I still have all the nice things you gave me, and thanks for always helping with “blanket” issues.

The present Students at the department: Charles (thanks for helping me with statistics), Ever smiling, Yeneneh, Maiga (for the nice & encouraging words), Samad (for your gentle
personality). And the Allergy girls: Ebba, Maria, Shanie, for being nice and wonderful colleagues. And Olga Flores, you are such a cheerful person, thanks for the invitations to have dinner, and nice chats during lunch, and also company during the weekends. Natalja G, Christine Z, Marine D, Dagbjort P, for the nice smiles and being cheerfulness always.

My roommates. Stéphanie Boström (thanks for the cookies always), Ioana Boujila (thanks for putting up with the junks in the malaria lab), and Dr. Pablo, “the almighty” (you are such a kind person & your presence alone radiates humor), I am going to miss you guys, especially the dude and his kompis ☺, I had always enjoyed your company and all your gists……

All former students at the department of immunology: and most especially Ben Gyan and Alice Nyakeriga, for being so accommodating. Nancy Awah, for being a nice contemporary at different times and thanks for the lovely desserts, Nina-Maria Vasconcelos, thanks for the lovely gestures and all assistance rendered right from the beginning. Ahmed Bolad, Salah Farouk (I enjoyed working with you), Jacob, Amre, Nnaemeka, Lisa (I miss the Swedish and Italian lessons), Jubayer, Petra, Yvonne, Ylva, Nora, Manijeh (the lucky charm worked finally), Camilla, Muktı, and Anna T. I am so happy I met you all one time or the other, ’cos you have all been wonderful in different ways.

The malaria group at MTC, KI, you guys have been so helpful and welcoming, especially my collaborators Qujin Chen, Kirsten Moll and Malin Haegström, Sandra Nilsson and Mats Wahlgren.

My mentor, Asst. Prof. Bolaji Thomas, finally this is it! Thank you so much for everything.

All NIDOE members & AEC members, thanks for the wonderful moments we had together, maybe I have time for more activities now.

Close family friends in Sverige. Sister Rahma, Prof. Atuma, Dr Raheem, The Garbas, Iyke Grandma Princess, Yetty, Aunty Maggie, Owode’s, Zainab & Sis. Lilian You guys have been more than friends and very supportive and encouraging. To people like, Mummy Ene (you are such a kind hearted person), Afa Tj (you have been a great company and I wish you all the best), Lanre & Hanifa, Matthew, you have all been wonderful people.
My first two teachers in Life: *My dear Mum* (you initiated this as my biology teacher, right from your Kitchen, I am so grateful for showing me this direction and all those other things you taught me and for helping with no.9, you will always remain close to my heart), *My gentle & easygoing Dad*; those days with English lessons at home, did not go down the drains. I really do not know how to express my immeasurable gratitude. 

And to my brothers, *BrosB*, I can’t stop being your kid sister, thank you so much for all the support from the beginning of my studies and I wish you all the best in life & goodluck with your studies as well. *Bros Femo* (my dearest egbon), all those late night lessons did pay off I guess, although I was too young for that at that time, thank you for everything. My dearest Sister *Tosin (aka- Toscarni)* thanks for watching my back, and being a good company, growing up with you was really fun (*especially with aroma styles and funny acting skills*) and I wish you all the best in life. 

To my buddies & the best girl club ever, *Hi 5 girls* (Shola, Lawunmi, Tosin, Bukky & moi....). You guys have been the senior sisters I never had.

My great –in– laws, *The Oboirien’s*– thanks for all your support and understanding through these years.

And to the only best friend “*Salle*”, thanks for being more than a better half, for being a solid rock through the hard times, and for the help rendered through the odd hours of the night as well as in the lab (filling the pipette tip boxes,.....), I guess I can have the “Ph” while you have the “D” & to my wonderful gift from God “Ebunoluwa”, (Farid bobo©), I know the uniqueness in you will bring added achievements.

※ For everyone that succeeds, it’s because there’s someone there to show you the way out; for me it was my parents, my teachers right from kindergarten, through the university and up to this level, and finally God Almighty.

*And to all those whom I have met along this extensive journey of mine, one way or the other.*

*I am very grateful to everyone & TACK SÁ MYCKET*
6 References


Kappe S.H., Vaughan A.M., Boddey J.A. & Cowman A.F. 2010. That was then but this is now: malaria research in the time of an eradication agenda. Science 328: 862-866.


**Perlmann H., Helmby H., Hagstedt M., Carlson J., Larsson P.H., Troye-Blomberg M. & Perlmann P.** 1994. IgE elevation and IgE anti-malarial antibodies in


World Health Organization FAQ about malaria 2009.


Science is a wonderful thing if one does not have to earn one's living at it.

- Albert Einstein