Plasmodium knowlesi LIGAND-RECEPTOR MECHANISM IN THE PLACENTA OF OLIVE BABOONS (*Papio anubis*) WITH MALARIA IN PREGNANCY

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Plasmodium knowlesi Ligand-Receptor Mechanism in the Placenta of Olive Baboons (*Papio anubis*) with Malaria in Pregnancy

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my beloved parents; Peter and Esther Ogembo, for their unrelenting support and push to see me through this work. To my fiancée Felisturs, brother Bonafida and sisters Judy, Joan and Sheila, for their constant encouragement. Above all to God, the creator of all things, for strength, health and favour.

"For every failure, there's an alternative course of action. You just have to find it. When you come to a roadblock, take a detour."
(Mary Kay Ash)

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LIST OF ABBREVIATIONS AND ACRONYMS

BLAST	Basic Local Alignment Search Tool
CAM	Cellular Adhesion Molecule
CDD	Conserved Domain Database
CIDR1	Cysteine-rich Inter Domain Region 1
CM	Cerebral Malaria
CS	Caesarean Section
CSA	Chondroitin Sulphate A
CSPG	Chondriotin sulphate proteoglycan
EBP	Erythrocyte binding protein
DB	Database
DBL	Duffy binding like domain
EBA	Erythrocyte binding antigen
GAG	Glycosaminoglycan
HA	Hyaluronic Acid
HAPLN	Hyaluronan and proteoglycan link protein
IACUC	Institutional Animal Care and Use Committee
ICAM 1	Intercellular Adhesion Molecule 1
IEs	Infected Erythrocytes
IPR	Institute of Primate Research
ISERC	Institutional Scientific and Ethical Review Committee
KEMRI	Kenya Medical Research Institute
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
NHP	Non-human primate
PAM	Pregnancy Associated Malaria
PCV	Packed Cell Volume
PECAM	Platelet Endothelial Cell Adhesion Molecule
Pfam	Protein families database
P*f*EMP 1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1

SICA	Schizont-infected cell agglutination
SOPs	Standard Operating Procedures
TNF	Tumour Necrosis Factor
TSP	Thrombospondin
VCAM1	Vascular Cell Adhesion Molecule
WHO	World Health Organization

ABSTRACT

Pregnancy associated malaria (PAM) poses many risks to both women and their infants. It is characterized by accumulation of infected erythrocytes in placental intervillous spaces leading to adverse reactions. Studies using the *P. knowlesi*-Olive baboon model of pregnancy malaria have demonstrated this phenomenon though mechanisms and molecules involved are not known. This study sought to determine the ligands and receptor molecules that permit accumulation of infected erythrocytes in the placenta of *P. knowlesi* infected Olive baboons and to further test placental isolates for adhesion to purified receptors. Sequences of known *Plasmodium* erythrocyte binding antigens and human placental receptors were used in BLAST analysis against the genomes of *P. knowlesi* and *P. anubis* respectively. Hits generated were analysed and characterized to determine the prospective ligands and receptors in *P. knowlesi* and *P. anubis* respectively. Also, four adult female baboons (*P. anubis*) were infected with *P. knowlesi* parasites and their placentas sampled. Infected erythrocytes isolated from these placentas were tested for binding against purified receptors. The results revealed that Predicted chondroitin sulphate proteoglycan 4 (CSPG 4) partial and Predicted hyaluronan and proteoglycan link protein 1 (HAPLN 1) are the putative receptor molecules in the Olive baboon. Phylogenetic analyses clustered them together with sequences from macaques which are he natural hosts of *P. knowlesi.* They also had similar domain organization to known human receptors (CSA and HA). Further, the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha,* EBP-*beta* and EBP-*gamma*) matched closely to the placental *P. falciparum* ligand *Var2csa* and showed similar domains. However, static binding assays with *P. knowlesi* infected erythrocytes did not show any binding to purified receptors. This study has identified and proposed receptors and ligands involved in the adherence mechanism in *P. knowlesi* infected Olive baboons during pregnancy. This presents the Olive baboon as a suitable model for studying PAM at the pre-clinical level and will be important in studies to develop PAM vaccines and therapeutic agents.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

Malaria is the most important parasitic infection in humans; it is endemic throughout the tropical and sub-tropical regions of the world. The World Health Organization (WHO) estimates that there were 216 million cases of malaria worldwide with 655,000 deaths in 2010 (WHO, 2012). Most of these cases were in the African Region (81%) where a child dies every minute of malaria and the disease accounts for 22% of all childhood deaths (WHO, 2012). Other populations and groups of people at risk from malaria are; pregnant women, those infected with HIV, travellers from non-endemic regions and immigrants from endemic regions. Particularly, malaria in pregnancy increases the risk of maternal anaemia, stillbirths, spontaneous abortions, low birth weight and neonatal death (WHO, 2013).

Human malaria is primarily caused by five species of *Plasmodium* parasites; *P. falciparum, P. vivax, P. ovale, P. malariae* and the monkey malaria parasite *P. knowlesi* (Lagerberg, 2008). They are transmitted by the female *Anopheline* mosquito, which injects sporozoites from its salivary glands into the blood stream of the human host during a blood meal. In rare cases, malaria may be transmitted through exposure to infected blood products and congenitally through the placenta (Lagerberg, 2008; WHO, 2013).

Malaria is often categorized as either uncomplicated or severe. Common symptoms in uncomplicated cases are; recurrent fever, nausea, headache and muscle pain. During the erythrocytic stage, infected erythrocytes (IEs) rupture releasing parasite proteins and haemozoin into the bloodstream that cause inflammation. Moreover, increased serum

levels of TNF-α have been associated with the characteristic fever peaks of malaria (Miller *et al.,* 2002). In some cases, when left untreated *P. falciparum* malaria can become severe characterized by acute anaemia, respiratory distress, cerebral malaria (CM) or pregnancy-associated malaria (PAM). Parasite infected erythrocytes sequester to cellular adhesion molecules (CAM) on the surface of endothelial cells and the subsequent immune responses elicited cause pathology in cerebral malaria (CM) and pregnancy-associated malaria (PAM) (Maubert *et al.,* 2000).

During pregnancy, *P. falciparum* infected erythrocytes adhere to the intervillous spaces of the placenta, a phenomenon called sequestration. The parasites in the placenta are at the trophozoite stage which are normally absent in peripheral circulation (Maubert *et al.,* 2000; Mens *et al.,* 2010). The sequestration of IEs is mediated by receptor-ligand interactions between parasite-induced ligands on the erythrocyte membrane and cellular adhesion molecules (CAM) on the surface of vascular endothelial cells. Several studies have characterized these CAMs involved in IE adhesion in the placenta; chondroitin sulphate A (CSA) (Rogerson *et al.,* 1995), hyaluronic acid (HA) (Beeson *et al.,* 2000), CD36 (Barnwell *et al.,* 1983; Febbraio *et al.,* 2001). In *P. falciparum*, the best characterized ligand is *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP 1) encoded by the highly variable *var* gene family (Kraemer & Smith, 2003; Smith *et al.,* 2000). The P*f*EMP 1 proteins are distantly related to the schizont-infected cell agglutination antigens (SICA), that are encoded by the SICAvar genes in *P. knowlesi*, and share binding signature motifs (Korir & Galinski, 2006).

Recently, *Plasmodium knowlesi* (monkey malaria parasite), has become an important public health concern as it can infect humans (Cox-Singh *et al.,* 2008). Many studies have used the parasite to induce infection in non-human primates to study host-parasite interactions. These studies have led to understanding of its biology and pathogenesis. This has demonstrated that it's a suitable model for the human malaria parasite *P. falciparum* (Anderios *et al.,* 2010; Ozwara *et al.,* 2003). The Olive baboon

(*P. anubis*) also displays similar clinical and pathological symptoms when infected with *P. knowlesi* malaria during pregnancy (Mustafa *et al.,* 2010). However, despite evidence showing the accumulation of parasites in the intervillous spaces of the placenta, the mechanism used by *P. knowlesi* IEs to sequester is not known and the molecules involved have not been determined.

The use of *in silico* methods offers an opportunity to discover genetic information. It minimizes time and resource requirements while enhancing scientific inquiry since results obtained are robust and can be further corroborated with wet laboratory experiments. Bioinformatics tools have been developed by researchers to solve different challenges and besides, the database of genomes sequenced and published has been growing. The availability of the published genomes of *P. anubis* and *P. knowlesi* (Pain *et al.,* 2008; Zinner *et al.,* 2013) provides an opportunity to probe them using tools like the Basic Local Alignment Search Tool (BLAST) to determine homologous molecules. Starting with known receptors from humans and ligands from *Plasmodium* species, their homologues can be determined (Altschul *et al.,* 1990).

1.2 Immunity to malaria

1.2.1 Naturally acquired immunity

Immunity to malaria is gradually built after exposure to several episodes of *P. falciparum* infections. Complete immunity is however never achieved and therefore a lower number of parasites are still able to infect the adult but without causing symptoms. Young children on the other hand are very susceptible to malaria infections which lead to high mortality. From human studies it has been demonstrated that although protective immunity to malaria is achieved slowly and following regular exposure to *P. falciparum* parasites, immunity to acute malaria is acquired faster and may be significant after a single episode (Gupta *et al.,* 1999).

On entry the parasite is immediately exposed to the immune system that would potentially fight any foreign organism, but studies show that natural immune responses to pre-erythrocytic stages are of lower importance to prevent disease (Owusu-Agyei *et al.,* 2001). In erythrocytic stage, humoral response plays a crucial role through IgG since infected erythrocytes (IE) lack antigen presenting cell surface proteins. They use three different mechanisms: blocking the IE invasion (Blackman *et al.,* 1990); inducing opsonization and phagocytosis (Bouharoun-Tayoun *et al.,* 1995) and binding to IE surface antigens contributing to parasite clearance (Bull *et al.,* 1998).

1.2.2 Innate immunity

Malaria infection gives rise to elevated blood concentrations of non-specific immunoglobulins. It has been shown that exposure of $CD4^+$ T cells from malaria-naïve donors to malaria antigens leads to *in vitro* proliferation and cytokine production (Currier *et al.,* 1992). Neutrophils, mononuclear phagocytes and natural killer (NK) cells appear to play a role in innate immunity early in malaria infections. In particular, NK cells increase in numbers and lyse *P. falciparum*-infected erythrocytes *in vitro* (Orago and Facer, 1991). They are potent producers of cytokines like interferon gamma (IFN-γ) that activates the parasiticidal activity of macrophages (Mohan *et al.,* 1997).

1.2.3 Humoral immune responses

Antibodies which target the asexual blood-stage parasites are of central importance for protective immunity (Sabchareon *et al.,* 1991). Since RBCs lack an antigen presenting machinery, immunity to blood-stage parasites is mainly conferred by humoral immune responses, although cell-mediated mechanisms (Pombo *et al.,* 2002) and host in-borne factors (Maier *et al.,* 2003) do play a role. However, the efficacy of humoral response is limited by the parasite's antigenic switching. Nevertheless, the immune response is believed to be strengthened by a variety of non-specific effector mechanisms and the

presence of type-1 cytokines, including IFN-γ, IL-2, IL-12, and TNF-α have been reported in infected individuals (Dodoo *et al.,* 2002; Winkler *et al.,* 1999).

IgG antibodies are important for naturally acquired immunity to *P. falciparum* malaria, because of the effect of passively transferred IgG from clinically immune adults to children acutely ill with malaria (Sabchareon *et al.,* 1991). Furthermore, infants younger than six months are thought to be protected against *P. falciparum* infections through the transfer of maternal malarial antibodies to the foetus during pregnancy (Beeson & Duffy, 2005).

1.2.4 Cell mediated immune responses

Cell-mediated immune responses involving T cells induced by malaria infection protect against both pre-erythrocytic and erythrocytic parasite stages.

1.2.4.1 T-helper ($CD4^+$) and T-cytolytic ($CD8^+$) T Cells

The $CD4^+$ T cells are essential for immune protection against asexual blood stages in both murine and human malaria systems. The role of $CD8^+$ T cells is still not clear but they have important effector functions in pre-erythrocytic immunity (Beeson & Duffy, 2005) and contribute to protection against severe malaria (Aidoo & Udhayakumar, 2000). It has been proposed that $CD8^+$ T cells may regulate immunosuppression in acute malaria and down modulate inflammatory responses (Troye-Blomberg *et al.,* 1999a). Since human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by $CD8^+$ cytotoxic T lymphocytes has no role in the defence against blood-stage parasites.

For *P. falciparum* malaria in humans, it has been established that there exist functionally different $CD4^+$ T cells in naturally exposed donors. These cells respond to malaria antigen by *in vitro* proliferation and/or secretion of cytokines such as IFN-γ or IL-4. In general, these *in vitro* responses are poorly correlated with protection (Mohan *et al.,* 1997; Troye-

Blomberg *et al.*, 1999a). Nevertheless, *in vitro* stimulation of $CD4^+$ T cells from malaria-exposed donors may result in the production of IL-4 in concordance with the serum concentrations of antibodies specific for the antigens used for lymphocyte stimulation (Perlmann & Troye-Blomberg, 2002). Furthermore, enhanced IFN-γ production and proliferation have been reported for T cells from donors recovering from a malaria attack (Perlmann & Troye-Blomberg, 2002).

1.2.4.2 Gamma-delta (γδ) T Cells

Cells expressing the γδ TCR normally represent less than 5% of all T cells in the peripheral blood of healthy adults. Activated γδ T cells but not αβT cells from malaria-naïve donors inhibit parasite replication in erythrocytes *in vitro*, supporting their protective function and, in particular, their role in innate defence against the malaria parasites (Elloso *et al.*, 1994; Troye-Blomberg *et al.*, 1999b). The γδ T-cell activation is associated with IL-2 receptor (IL-2R) signalling, initiated by cytokines; IL-2, IL-4 and IL-15 (Pichyangkul *et al.*, 1997). Malaria antigen-activated γδ T cells produce primarily pro-inflammatory cytokines (Troye-Blomberg *et al.*, 1999b) suggesting that protection against the parasites by these cells involves both regulatory and cytotoxic functions. Antigens from plasmodial schizonts stimulate γδ T cells (Elloso *et al.*, 1998; Pichyangkul *et al.*, 1997). These cells recognize antigens in association with MHC class-I or II molecules (Goodier *et al.*, 1992; Waterfall *et al.*, 1998). However, γδ T cells also recognize non-peptide antigens, with no need of MHC presentation (Morita *et al.*, 1995).

1.2.4.3 The Cytokine Network in Malaria

Protective anti-malarial immunity is exerted by cellular activities of lymphocytes, neutrophils and mononuclear phagocytes. However, some of these activities may also cause tissue damage. The course of a malaria infection is dependent on the balance between the cytokines secreted by the various cells when activated (Troye-Blomberg *et*

al., 1999a). In any event, pro-inflammatory cytokines such as IFN-γ, IL-1, IL-6, IL-12 and TNF-α may be protective by inducing parasite killing by monocytes/macrophages and neutrophils (Perlmann and Troye-Blomberg, 2002). The IL-12 produced by mononuclear phagocytes and other cells, contributes to protection against pre-erythrocytic and blood infection by initiating a TH1 anti-malaria response in mice as well as in monkeys (Hoffman *et al.*, 1997; Stevenson *et al.*, 1995).

In contrast, anti-inflammatory cytokines such as IL-10 counteract the production and possible cytopathic effects of pro-inflammatory cytokines (Deloron *et al.*, 1994). Recent studies of human *falciparum* malaria emphasize the importance of the balance between pro- and anti-inflammatory cytokines. Thus, elevated IL-6/IL-10 ratios in plasma due to relative IL-10 deficiencies predict a fatal outcome of severe malaria (Perlmann & Troye-Blomberg, 2002).

Tumour necrosis factor (TNF) has a central role for both protection and malaria pathogenesis. It does not kill parasites directly but exerts protection by activating the anti-parasitic effects of the various effector cells (Deloron *et al.*, 1994; McGuire *et al.*, 1994; Perlmann & Troye-Blomberg, 2002). With regard to pathogenesis, TNF levels are positively correlated with disease severity as well as with malaria fever (Brown *et al.*, 1999). The primary source of this TNF is monocytes/macrophages activated by various parasite products. However, IgE containing immune complexes also contribute to local overproduction of TNF in severe malaria (Perlmann *et al.*, 1997). The amount of TNF produced varies genetically due to altered gene transcription from changes in transcription factor binding to the corresponding TNF promoter region (McGuire *et al.*, 1994).

1.3 *Plasmodium knowlesi*

1.3.1 Biology and epidemiology of *P. knowlesi*

Plasmodium knowlesi is an apicomplexan parasite belonging to the class *Aconoidasida* and order *Haemosporidia*. It has been described as the fifth major human malaria parasite as it can infect humans either naturally or artificially (Cox-Singh *et al.,* 2008; Sabbatani *et al.,* 2010; White, 2008). It was first reported in humans in 1965 and is commonly found in Southeast Asia where it accounts for up to 70% (McCutchan, 2008) of malaria cases. It may cause severe malaria as indicated by its asexual erythrocytic cycle of about 24 hours (Cox-Singh *et al.,* 2008; Jongwutiwes *et al.,* 2004).

P. knowlesi is considered to be a parasite of long-tailed (*Macaca fascicularis*) and pig-tailed (*Macaca nemestrina*) macaques (Ng *et al.,* 2008; Vythilingam *et al.,* 2008). However, in South East Asia humans who enter and work at the borders of rain forests are at risk of infection. Since macaques are getting closer and in direct contact with humans (Vythilingam *et al.,* 2008), and due to increased deforestation and development efforts in South East Asia, people who live in the semi-urban areas are becoming infected with *P. knowlesi* malaria.

The parasite mostly occurs in regions that are reportedly free of the other four types of human malaria. One fifth of the cases of malaria diagnosed in Sarawak, Malaysian Borneo are due to *P. knowlesi* (Singh *et al.,* 2004). It is however absent in Africa because there are no long-tailed and pig-tailed macaques (the reservoir hosts of *P. knowlesi*) and many West Africans lack the Duffy antigen which the parasite uses to invade erythrocytes (Haynes *et al.,* 1988).

1.3.2 The genome of *P. knowlesi*

The genome of the simian and human malaria parasite *P. knowlesi* has been sequenced and published (Pain *et al.,* 2008). The *P. knowlesi* Pk 1 (A+) clone was produced to eightfold coverage by whole-genome shotgun sequencing (Howard *et al.,* 1983). The 23.5 megabase (Mb) nuclear genome is composed of 14 chromosomes. Its overall G+C base composition is 37.5% with a total of 5,188 protein-encoding genes identified which is slightly lower than the predicted proteomes of *P. falciparum* and *P. vivax* (Carlton *et al.,* 2008; Gardner *et al.,* 2002). Its presumed centromeres are similar to those found in other *Plasmodium* species and are positionally conserved within regions sharing synteny with *P. vivax.* For approximately 80% (4,156 out of 5,185) of predicted genes in *P. knowlesi*, orthologues can be identified in both *P. falciparum* and *P. vivax* (Carlton *et al.,* 2008; Gardner *et al.,* 2002).

The ***P. knowlesi***-specific variant antigen gene families, *SICAvar* genes (al-Khedery *et al.,* 1999) and *kir* genes (Janssen *et al.,* 2004) form the largest groups of *P. knowlesi*-specific expansions. In other *Plasmodium* genomes sequenced so far, variant gene families involved in antigenic variation are typically arranged in the sub-telomeres, and only a few members of these families have been found at intrachromosomal sites. Notably, the *P. knowlesi* genome sequence has revealed that the major variant gene families (*SICAvar* and *kir*) are randomly distributed across all 14 chromosomes and often co-localize with ITS-containing repeats. Although *P. knowlesi* and *P. falciparum* are phylogenetically distant, the SICA and *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP 1) variant antigens share many fundamental biological characteristics (Galinski and Corredor, 2004).

Variant SICA (schizont-infected cell agglutination) antigens on the surface of infected red blood cells are associated with parasite virulence and are encoded by the *SICAvar* gene family (al-Khedery *et al.,* 1999; Barnwell *et al.,* 1983; Howard *et al.,* 1983) the largest

variant antigen gene family in *P. knowlesi*. Switching of variant types underlies the establishment of a chronic infection in the vertebrate host, a process that is essential in all species, to ensure mosquito transmission and the completion of the life cycle. Being the first monkey malaria parasite genome to be described, it provides an opportunity for comparison with other sequenced *Plasmodium* genomes (Carlton *et al.,* 2008; Gardner *et al.,* 2002; Hall *et al.,* 2005).

1.3.3 Life cycle of *P. knowlesi*

The life cycle of *P. knowlesi* (Figure 1) begins when an infected mosquito transmits sporozoites into a susceptible vertebrate host during a blood meal. The sporozoites migrate to liver cells, where, in a process termed exo-erythrocytic schizogony (liver stage development); they undergo many rounds of replication and transform into liver schizonts. The infected hepatocytes rupture releasing merozoites which infect circulating erythrocytes immediately.

In the cyclical pattern of development termed intra-erythrocytic schizogony (blood stage development) that follows, parasites develop to rings, trophozoites and schizonts releasing more merozoites that continue to invade, colonize and replicate within erythrocytes. Schizont infected erythrocytes rupture releasing an average of 10 merozoites that continue the cycle of infection. The asexual cycle in blood is quotidian lasting 24 hours. During the blood stage development, *P. knowlesi* invades both mature erythrocytes and reticulocytes (de Koning-Ward *et al.,* 1998). Ring forms appear in blood and appliqué forms are frequently seen. Band forms are common in the trophozoite stage. Mature schizonts have 10 merozoites on average, but the number can go as high as 16. Regular rings have one or more accessory chromatin dots.

A proportion of the re-invading merozoites differentiate into male and female forms (micro- and macro gametocytes respectively). Mature gametocytes are seen in 3 days post-

inoculation if the infection is heavy. Gametocytes are taken up by a mosquito in a subsequent blood meal. In the mosquito mid-gut, gametocytes differentiate into mature micro- and macro gametes that mate to produce a zygote. The zygote differentiates into a motile ookinete that crosses the mosquito mid-gut and develops into a multi-nuclear oocyst. A mature oocyst, filled with sporozoites, ruptures releasing sporozoites into the haemocoel, from where they migrate to the salivary gland, ready to infect a vertebrate host during the next blood meal (de Koning-Ward *et al.*, 1998; Ozwara Suba & LUMC, 2005).

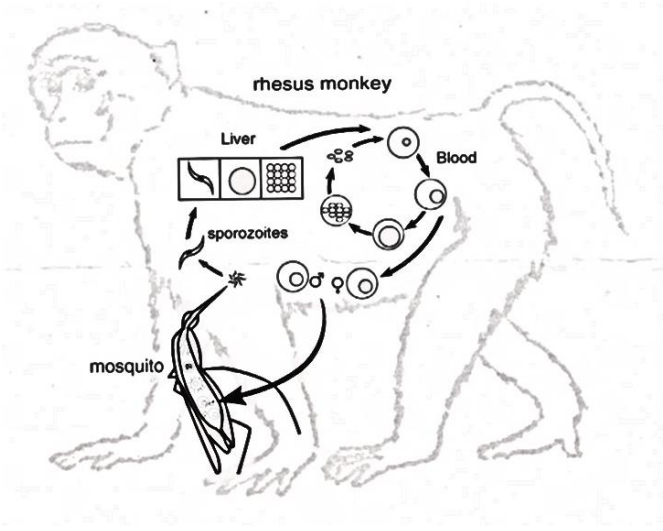


Figure 1: Life cycle of simian malaria parasite, *Plasmodium knowlesi* (Ozwara Suba & LUMC, 2005)

The mosquito hosts for *P. knowlesi* include *Anopheles dirus*, *A. balabacensis* and *A. hacker* all of which are found in Asia. Other possible vectors include *A. freeboni*, *A. maculatus*, and *A. quadrimaculatus* (Coatney, 1971; Collins *et al.*, 1978).Development in the mosquito (sporogony) takes 10-12 days at a temperature of 26° to 28°C while the exo-erythrocytic phase lasts 5.5 days.

1.3.4 Animal models for *P. knowlesi* malaria

The natural vertebrate hosts for *P. knowlesi* are the long-tailed macaques (*Macaca fascicularis*), pig-tailed macaques (*M. nemestrina*) and banded-leaf monkeys (*Presbytis malalophos*) (Eyles and Coatney, 1962; Eyles *et al.,* 1962). Experimental infection has also been described in a number of non-human primates such as *M. mulatta* (Coatney, 1971), *M. radiate* (Dutta *et al.,* 1982), *M. assamensis* (Dutta *et al.,* 1978), *Presbytis entellus* (Dutta *et al.,* 1981), *Callithrix jacchus* (Langhorne & Cohen, 1979), *Macaca fascularis* (Anderios *et al.,* 2010) and *Papio anubis* (Ozwara *et al.,* 2003). The NHPs are the only animal models susceptible to *P. knowlesi* because of their similarity in biological mechanisms to humans.

In baboons, the infection has been induced in *P. cynocepahalus*, *P. doguera*, *P. jubileaus* and *P. papio* (Coatney, 1971), *P. anubis* (Mustafa *et al.,* 2010). In most experimental models *P. knowlesi* infection is acute, whereas in their natural host it generally induces a chronic infection (Butcher, 1996; Coatney, 1971). In humans, *P. knowlesi* causes mild infections seldom exceeding 1% parasitaemia. However, an infection can become virulent upon repeated passages in humans unlike in the natural hosts (White, 2008).

1.4 Pregnancy-associated malaria (PAM)

Millions of women who become pregnant in malaria-endemic areas are at an increased risk of contracting malaria infection that threatens the outcome of pregnancy. It is estimated that 24 million pregnant women in sub Saharan Africa are at risk of suffering from PAM. This prevalence may exceed 50% among primigravidae and secundigravidae in endemic areas (Okoko *et al.,* 2003). Once pregnant and infected there is a tendency towards increased severity of disease (Parise *et al.,* 1998) caused in part by the transient depression of cell-mediated immunity that occurs during pregnancy.

Pregnancy-associated malaria is the sequestration of IEs that accumulate in the intervillous space resulting in pathological alterations. The intervillous space is important for foetal development because it's the main compartment for exchange of nutrients and delivery of oxygen. The accumulation of IEs infiltrates monocytes and macrophages which alter the placental cytokine balance leading to adverse pregnancy outcomes (Mens *et al.,* 2010).

Malarial infection during pregnancy leads to; spontaneous abortion, preterm delivery, low birth weight (LBW), increased risk of stillbirth regardless of parity and congenital infection (Lagerberg, 2008). These effects of PAM differ with various factors, such as the woman's level of immunity, her gravidity status, the trimester of pregnancy, status of malaria in the community and the presence or absence of co-morbidity (Bouyou-Akotet *et al.,* 2003; Coll *et al.,* 2008). In areas of stable, endemic transmission, adult women usually have a protective semi-immunity against *P falciparum* acquired during the first 10 to 15 years of life (Shulman & Dorman, 2003). Immunity is maintained through continued exposure to malarial illness. It has been demonstrated that adult women have no significant level of immunity in areas of unstable, non-endemic transmission. They are more likely to be symptomatic and are at greater risk of developing severe disease leading to death. Lower parity, especially first and second pregnancies, and younger age increase the susceptibility to malaria (Mutabingwa *et al.*, 2005).

1.4.1 Pathogenesis of pregnancy-associated malaria

The WHO considers malaria in pregnancy 'one of the most important preventable causes of low birth weight deliveries worldwide' and 'a major cause of severe maternal anaemia contributing to maternal mortality' (Shulman & Dorman, 2003). In addition, placental malaria contributes to premature delivery, hypertension, infant anaemia and neonatal mortality (Brabin, 1983; Brabin *et al.*, 2004).

The accumulation of IEs in the intervillous spaces of the placenta (where the maternal blood circulates) is the central phenomenon in PAM pathogenesis (Walter *et al.*, 1982). This is referred to as sequestration and it correlates to parasite pathogenesis and antigenic phenotype. The pathological events occur through: obstruction of blood flow, systemic or local production of pro-inflammatory cytokines and blockage of signal transduction (Brabin *et al.*, 2004). This results in altered placental histology due to increased numbers of inflammatory cells and deposition of haemozoin in the intervillous spaces. Chronic infection has been associated with low birth weight due to foetal growth retardation and with maternal anaemia, while acute infection has been related to preterm delivery (Brabin *et al.*, 2004).

Besides reduced birth weight, PAM also causes infant mortality during the first year of life. New-borns of mothers with placental infection have a higher risk of having malaria in early life (Mutabingwa *et al.*, 2005). The mechanisms causing this increased morbidity are not clear but can be linked to decreased transfer of maternal IgG antibodies that confer immunity to the new-born (Brustoski *et al.*, 2005).

Studies have demonstrated that infected erythrocytes (IEs) isolated from placenta do not adhere to the usual endothelial receptors like CD36 and ICAM-1. They instead bind to a placental receptor – chondroitin sulphate A (CSA) (Fried &Duffy, 1996), indicating that placental parasites have distinct antigenic properties from parasites that infect children (Beeson *et al.*, 1999). Women were also found to be immune to *P. falciparum* before being pregnant, having only asymptomatic infections, and that pregnancy associated parasitaemia disappeared after delivery (Nguyen-Dinh *et al.*, 1988).

1.4.2 Immunity to pregnancy-associated malaria

During pregnancy the immune system is altered to accept the foetal allograft while maintaining host defences against foreign antigens. Immunomodulation occurs, which

increases susceptibility of pregnant women to certain infections (Brabin *et al.,* 2004). In high-transmission areas, primigravidae are at greater risk of infection since women develop increasing resistance to PAM over successive pregnancies (Brabin, 1983) while in low transmission areas, women of all gravidities are equally susceptible to PAM (Nosten *et al.,* 1991).

The acquisition of immunity to PAM is associated with development of a broad protective antibody response to placental IEs. These antibodies are generated to recognize specifically variant surface antigens from the PAM type (VSA_{PAM}) and inhibit the binding of placental parasites to chondroitin sulphate A (CSA). Women that have acquired adhesion-blocking antibodies and also have antibodies that react with the surface of PAM parasites are more likely to be protected from malaria in subsequent pregnancies (Fried *et al.,* 1998; Staalsoe *et al.,* 2001). Nevertheless, some women have specific IgG that agglutinate CSA binding parasites but do not avoid their binding to CSA (Beeson *et al.,* 2004), indicating that adhesion-inhibition is dependent on the quality, and not just the quantity of antibodies. Most primigravidae develop anti-adhesive antibodies from around 20 weeks of pregnancy whereas multigravidae develop those antibodies faster (12 weeks) due to the memory of a previous infection (O'Neil-Dunne *et al.,* 2001). However, primigravidae with no evidence of current or past infection lack the crucial adhesion blocking antibodies (Beeson *et al.,* 2004).

Without anti-adhesion antibodies an extensive cell mediated immune response occurs leading to chronic inflammatory placental malaria. Pro-inflammatory cytokines that induce a TH1 response dominate in primigravidae in the absence of an adequate anti-inflammatory TH2 cytokine response (Brabin *et al.,* 2004). Placental malaria up-regulates the cytokines IL-1 and IFN-γ leading to increased levels of placental cytokines IFN-γ, TNF-α and IL-2. Dense accumulation of intervillous inflammatory cells takes place in active placental malaria. Macrophages are the predominant cells and are considered to be the principal arm of protective immunity to placental malaria (Ordi *et al.,* 2001).

Macrophages present in infected placenta express tissue factor that initiates clot formation and the subsequent perivillous clot leads to narrowing and plugging of the intervillous space and disturbance of blood supply (Imamura *et al.,* 2002).

Increased susceptibility of pregnant women to malaria has been attributed to either localized uterine cell-mediated immune (CMI) suppression, or to a systemic reduction in CMI (Brabin *et al.,* 2004). The human placenta is a major anti-inflammatory organ favouring the type 2 helper T-cell (TH2) pathway, through interleukin 10 (IL-10), tissue growth factor-β (TGF-β) and progesterone production (Brabin *et al.,* 2004). The placental anti-inflammatory cytokines are likely to suppress CMI. Expression of type 1 helper T-cell (TH1) pathway cytokines is associated with spontaneous abortion while TH2 pathway cytokines are mandatory for pregnancy maintenance (Wegmann *et al.,* 1993). Gamma interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α) have direct cytotoxic effects towards both intracellular organisms and the villous trophoblast. Malaria stimulates the production of pro-inflammatory mediators, shifting the cytokine balance away from the TH2 response (Fried *et al.,* 1998). Both the type of inflammatory activity and the chronicity of the inflammatory response, rather than the parasite density or peak time of the inflammatory activity, have been associated with poor pregnancy outcomes.

1.4.3 Pregnancy-associated malaria in non-human primates

In the baboon model, placental parasitaemia has been shown to rise to 20 fold higher than peripheral parasitaemia. Furthermore, pregnant baboons were highly susceptible to infection than non-pregnant ones since they developed parasitaemia two days earlier (Mustafa *et al.,* 2010). There was evidence of accumulation of *P. knowlesi* infected erythrocytes in the baboon placentas. The observed accumulation of IEs was suggestive of involvement of molecules in the baboon placenta that mediate parasite adherence to placental tissue. The ring stages were predominantly observed in placental blood smears (Mustafa *et al.,* 2010). This therefore suggests that it's mainly trophozoites and schizonts

that adhered to placental tissue explaining their exclusion in blood smears prepared from placenta.

1.5 Adhesion ligands and receptors in pregnancy-associated malaria

1.5.1 Host adhesion receptors

The placenta and deep microvasculature is the preferential site for sequestration of IEs and can experience high parasitisation while the peripheral circulation is free of trophozoite and schizont stage parasites (Ibhanesebhor and Okolo, 1992). A number of studies have demonstrated that integral proteins of the vascular system support the binding of IEs. Some of the identified receptors include intracellular cell adhesion molecule-1 (ICAM-1), CD36, vascular cell adhesion molecule (VCAM-1), E-selectin, P-selectin, chondroitin sulphate A (CSA) and thrombospondin (TSP) (Fried & Duffy, 1996).

The affinity of binding to a particular endothelial receptor may be related to the pattern of disease. Some receptors such as CD36 and TSP are used by all parasite isolates but others may select some sub-populations of parasites (Newbold *et al.*, 1997). In 1995, independent studies showed that selected populations of IEs could recognize and bind CSA chain in the placenta but not others (Rogerson *et al.*, 1995). It has been shown that CSA (Fried and Duffy, 1996) and hyaluronic acid (HA) (Beeson *et al.*, 2000) are the receptors for adherence of IEs to the human placenta. While the role of HA remains unclear, CSA remains the only uncontroversial receptor for the malaria-IEs in human placenta (Beeson *et al.*, 1999; Fried *et al.*, 2000).

As a glycosaminoglycan (GAG), CSA is composed of repeating disaccharide units of D-glucuronic acid (GlcA) linked to N-acetyl-D-galactosamine (GalNac) with a sulphate group at position C4 of GalNac. Chains of GAGs are usually covalently attached to core proteins (proteoglycans), which are a major component of the extracellular matrix and

play a key role in several biological functions, such as the normal physiology of cartilage tissues, regulation of cell migration and proliferation, cell adhesion and differentiation.

Chondroitin Sulphate A (CSA) is not exclusively localised in the placenta but rather commonly expressed, although at lower levels in the extracellular matrix of various endothelial tissues. However, an unusually low-sulphated form of CSA can be attributed to the syncytiotrophoblasts and the placental intervillous space (Achur *et al.,* 2000). This unique sulphation pattern is thought to be an important factor supporting the selective accumulation of parasites in placental tissue, nevertheless other factors such as the density of CSA and decreased blood flow through the placenta could also play an important role. Further studies have identified the minimum CSA structural motif that supports this IEs adhesion and found that a dodecasaccharide of CSA with ~30% 4-sulfated disaccharide moieties is needed for optimal binding (Achur *et al.,* 2003; Alkhalil *et al.,* 2000).

1.5.2 Erythrocyte binding ligands

Invasion of erythrocytes by malaria parasites requires specific interaction between receptors and ligands. A family of erythrocyte binding proteins have been identified as the parasite proteins that mediate these interactions (Adams *et al.,* 1992). In *P. vivax* and *knowlesi* their binding is dependent on the Duffy blood group antigen while in *P. falciparum* they bind sialic acid residues on glycophorin A and also sialic acid-independent receptors. These proteins are: *P. vivax* Duffy binding protein (Singh *et al.,* 2003), *P. knowlesi* α, β and γ erythrocyte binding proteins (Adams *et al.,* 1992; Chitnis and Miller, 1994), *P. falciparum* erythrocyte binding antigen 175 (EBA-175), EBA-181 (JESEBL), EBA-140 (BAEBL) and EBA-165 that is a pseudogene (Adams *et al.,* 1992; Lopaticki *et al.,* 2011; Orlandi *et al.,* 1990) . The EBLs consist of the F region (region II) which is made up of two related domains (domains F1 and F2) that are involved in receptor binding.

1.5.3 The *P. falciparum* adhesion receptor, P*f*EMP 1 and *Var* genes

In humans, sequestration involves a complex interaction (Figure 2) between parasite-derived neo-antigens expressed on the surface of IEs as proteins, and either one or more receptors expressed on the surface of vascular endothelium or placental syncytiotrophoblasts (Cooke *et al.,* 2000)

Apart from relying on these surface exposed proteins, electron microscopy has shown that parasites also develop 85 to 110 kDa knob-associated histidine rich protein (KAHRP) like protrusions on the membrane of IEs (Figure 2) which serve as points of attachment between infected erythrocytes and endothelial cells (Sherman *et al.,* 2003). It has been demonstrated that maintenance of knobby cultures *in vitro* for a long time will result in knobless variants in which P*f*EMP 1 distribution is altered (Sherman *et al.,* 2003).

Other potential adhesion molecules expressed by IEs but whose function *in vivo* remains unclear include repetitive interspersed family of genes (*rifins*), sequestrin, cytoadherence linked asexual gene (Clag 9), Pf 332 and modified RBC band 3 (Figure 2). Rifins formerly known as rosettins, are a group of 30 to 40 kDa proteins that were earlier described in sera from people in endemic regions due to the ability of a parasite laboratory isolate binding to erythrocytes, thus forming rosettes (Sherman *et al.,* 2003). The *rif* family, subtelomeric variable open reading frames (STEVOR) and Pf60 may also undergo antigenic variation. Clag 9 is a 220kDa protein that is transcribed in mature stage parasites, and is able to bind to the endothelial receptor CD36 while sequestrin, a 270kDa protein has been identified in a knobless cytoadherent parasite line that binds to CD36 (Ockenhouse *et al.,* 1991). Unlike the other parasite derived proteins, band 3, a 95 kDa protein, has been isolated from erythrocyte membranes and was shown to be involved in cytoadherence from studies of *P. falciparum* in *Aotus* or *Saimiri* monkeys (Crandall *et al.,* 1993).

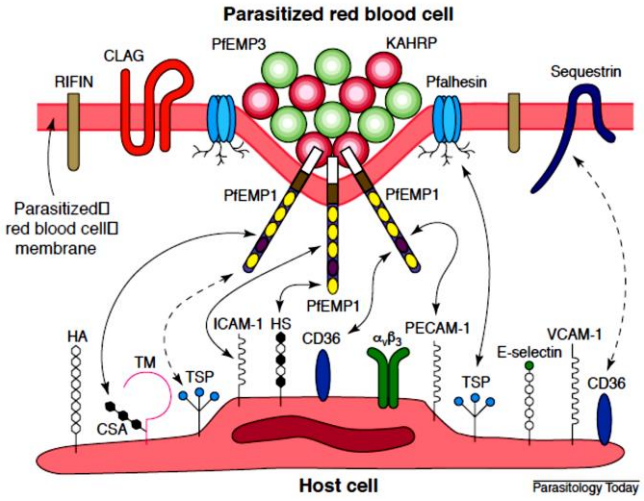


Figure 2: **Schematic diagram of adhesion molecules (infected erythrocyte ligands and host endothelial receptors) involved in sequestration (Cooke *et al.*, 2000)**

The best characterized parasite ligand involved in cytoadherence is the 240-260 kDa *var* gene encoded family of proteins - *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP1). There exist 40-50 *var* genes per haploid genome. P*f*EMP 1 has a highly variable extracellular region consisting of four basic building blocks: N-terminal segment (NTS), Duffy-binding like domains (DBL), Cysteine-rich Inter Domain Region (CIDR) domains and C2 domains as shown in Figure 3 (Smith *et al.,* 2001). It is detected on the surface of IEs 16 to 20 hour's post–invasion and corresponds with parasites disappearance from circulation (Costa *et al.,* 2006). Due to its variable domains and extensive sequence polymorphism, P*f*EMP 1 has been implicated in antigenic variation as well as adhesion (Smith *et al.*, 2000). The P*f*EMP 1 *var* gene has been described as highly polymorphic and undergoes constant changes as a result of frequent recombination that generate a vast repertoire of *var* genes, which alters the cytoadherence and antigenic phenotypes of IEs (Smith *et al.*, 2000).

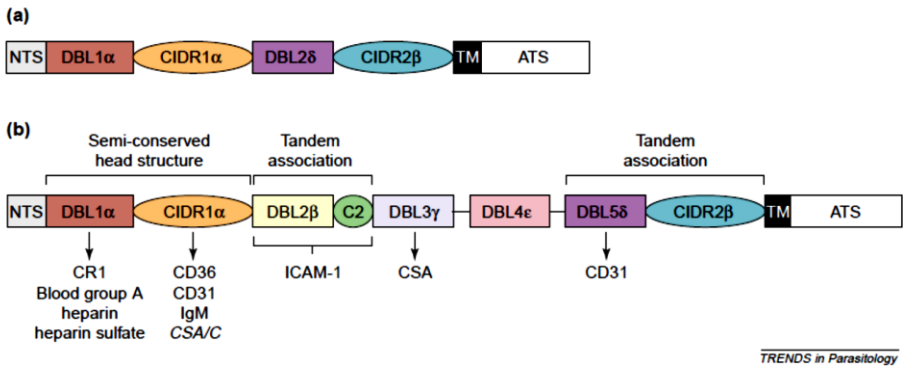


Figure 3: P*f*EMP 1 molecule's protein architecture and binding domains (a,b).

Key: NTS, N-terminal segment; DBL, duffy binding like; CIDR, cysteine rich interdomain region; ATS, acidic terminal segment; CR1, complement receptor 1; CSA, chondroitin sulphate A; ICAM-1, intracellular adhesion molecule 1 (Smith *et al.*, 2000)

1.5.4 Pregnancy-associated malaria *Var* genes

P*f*EMP1 adheres to various host receptors and it is likely that the VSA_{PAM} that causes placental sequestration also belongs to the P*f*EMP1 family (Costa *et al.*, 2006; Su *et al.*, 1995). Several *var* genes have been implicated in PAM, but today's research focuses on the VAR2CSA protein as a vaccine candidate against PAM whereas the other P*f*EMP1 variants have been abandoned. The previous vaccine candidates, *CS2var* (Reeder *et al.*, 1999) and *FCR3varCSA* (Buffet *et al.*, 1999), were predominantly transcribed *var* genes with a DBLγ type domain that mediated binding to CSA *in vitro* (Buffet *et al.*, 1999; Reeder *et al.*, 2000).

In spite of promising results, it has since been shown by many groups that neither of these genes seem to be involved in placental adhesion. The *CS2var* gene was not well-conserved among different isolates (Rowe *et al.*, 2002) and its transcription could not be correlated to the CSA-binding phenotype in selected parasite lines (Beeson & Duffy, 2005). On the other hand, *FCR3varCSA* (or *var1csa*) is well-conserved in genetically distinct parasite isolates (Rowe *et al.*, 2002; Salanti *et al.*, 2002; Winter *et al.*, 2003). However, many studies have presented data that speaks against it as the CSA-ligand mediating adhesion

in the placenta. These findings argue that: the transcription of *var1csa* is equally high in placental parasites and parasites isolated from children (Duffy & Fried, 2003; Winter *et al.*, 2003), the transcription of this gene does not correlate with CSA-binding (Salanti *et al.*, 2003), the P*f*EMP1 product of *var1csa* has never been shown to be expressed on the surface of IEs, and the recombinant VAR1CSA proteins are not recognized in a gender-specific manner (Jensen *et al.*, 2003), which is an important criteria for VSAPAM (Ricke *et al.*, 2000; Staalsoe *et al.*, 2001). All these findings speak against *var1csa's* involvement in placental malaria infections.

More recently, when the 3D7 genome sequencing project was completed (Gardner *et al.*, 2002), a second well-conserved *var* gene subfamily was identified, known as *var2csa* (Salanti *et al.*, 2003). The degenerate *var* primers that had been used previously were designed to target the DBL1α domain of all *var* genes (Taylor *et al.*, 2000). The *var2csa* gene lacks a DBL1α domain and was consequently never detected in these studies.

1.5.5 The principal PAM ligand, Var2csa

Salanti and others (2003) found that the *var2csa* gene was the only *var* gene markedly up-regulated in *P. falciparum* parasites selected *in vitro* for CSA-binding. This was later on also shown for parasites isolated from the placenta (Duffy *et al.*, 2006; Ndam *et al.*, 2005). VAR2CSA belongs to the P*f*EMP1 family and consists of three DBLX domains followed by three DBLε domains. In contrast to other PfEMP1 molecules, it lacks a DBL1α and a CIDR domain. Furthermore, antibodies to the surface expressed VAR2CSA protein are acquired by women exposed to malaria during pregnancy (Ndam *et al.*, 2006; Salanti *et al.*, 2004) and high levels of anti-VAR2CSA antibodies at delivery are associated with protection from LBW babies. In addition, it has been demonstrated that targeted disruption of the *var2csa* gene results in the loss of or marked reduction in the ability of parasites to adhere to CSA (Duffy *et al.*, 2006; Viebig *et al.*, 2006). All these findings support VAR2CSA as the leading PAM ligand.

VAR2CSA is a large protein with an estimated molecular weight of 350 kDa, and even though it belongs to a highly conserved subfamily of PfEMP1 sequence polymorphisms do exist and sequence similarity ranges from 54-94% between parasite isolates (Salanti *et al.*, 2003; Trimnell *et al.*, 2006).

1.6 Statement of the problem

It is estimated that 24 million pregnant women in sub-Saharan Africa are at risk of suffering from pregnancy associated malaria (PAM). Malaria during pregnancy leads to many complications that threaten the livelihood of women and their infants. These complications include; abortion, stillbirth, pre-term birth, low birth weight, reduction in gestational age, anaemia and high fever. Similar pregnancy malaria complications have been demonstrated in the Olive baboon model of PAM. The Olive baboon being susceptible to *P. knowlesi* with similar host-pathogen interactions and reproductive physiology as humans provides a suitable system for studying mechanisms underlying PAM. *Plasmodium falciparum* infected erythrocytes (IEs) sequester in the intervillous space of the placenta in humans by binding to proteins expressed on its endothelium and this prevents splenic clearance. The IEs express parasite-derived ligands that bind to placental receptors like CSA and HA expressed by the syncytiotrophoblast. In the Olive baboon/*P. knowlesi* model of PAM, studies have demonstrated that IEs accumulate in the intervillous spaces of the placenta. However, it is still not yet known whether this accumulation of IEs is mediated by a ligand to receptor mechanism and which molecules are involved.

1.7 Justification

The availability of natural and experimental hosts for *P. knowlesi* offers the possibility to study the biology of PAM in greater detail in a host-parasite combination that is predictive of the human situation. Pregnancy associated malaria has been associated with immune evasion mechanisms like the parasite's antigenic variation and sequestration in deep vascular tissues leading to severe disease. The Olive baboon (*P. anubis*) is ranked highly in primate phylogeny compared to other non-human primates (NHPs) used in biomedical research. Being fully susceptible to experimental infection with *P. knowlesi*, it will provide a good system to overcome shortcomings in human studies such as the mother's health status, inaccurate estimation of gestational age, inadequate tissue for analysis, patient compliance problems, socio-economic conditions, moral, ethical and financial limitations. As a result many questions can be satisfactorily addressed by drawing parallels between NHPs and humans.

The proposed study determined that the accumulation of IEs in the intervillous spaces of the placenta in the *P. knowlesi*/Olive baboon model of PAM is mediated by a ligand to receptor mechanism. The findings suggest that such a mechanism exists in the experimental model and further identified the molecules involved. This confirms that the Olive baboon is a suitable model for studying PAM at the pre-clinical level and thus will be important in studies to develop PAM vaccines and therapeutic agents.

1.8 Research questions

i. Does a ligand-receptor mechanism mediate the placental accumulation of *P. knowlesi* infected erythrocytes in Olive baboons during PAM?
ii. What are the likely molecules mediating this mechanism in *P. knowlesi* and Olive baboons?

iii. What are the binding phenotypes of *P. knowlesi* infected erythrocytes isolated from the placenta of pregnant Olive baboons?

1.9 Hypothesis

Accumulation of *P. knowlesi* infected erythrocytes in the placenta of Olive baboon (*P. anubis)* during pregnancy is not mediated by a ligand-receptor mechanism.

1.10 Objectives

1.10.1 General objective

To determine the ligand-receptor mechanism mediating adhesion of *Plasmodium knowlesi* in placenta of Olive baboons (*P. anubis*) with malaria in pregnancy (PAM)

1.10.2 Specific objectives

1. To determine the molecules that mediate binding to the placenta from the genomes of *P. knowlesi* and Olive baboons (*P. anubis*) using *in silico* methods
2. To characterize the molecules that mediate *P. knowlesi* binding to the placenta in Olive baboons (*P. anubis*)
3. To determine the binding phenotype of *P. knowlesi* infected erythrocytes from Olive baboons to recombinant human Chondroitin sulphate A (CSA), Hyaluronic Acid (HA) and CD36

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study site

The study was carried out at the Institute of Primate Research (IPR): a WHO collaborating centre in Human Reproduction and Tropical Disease Research under the National Museums of Kenya. The institute undertakes basic and applied biomedical research using non-human primates (NHPs) as experimental models for human diseases and infections.

2.2 Research design

This study involved: a descriptive *in silico* study to identify putative ligands and receptor molecules used for adhesion by *P. knowlesi* and the Olive baboon respectively, an after-only with control *in vivo* study to investigate the effects of *P. knowlesi* malaria in Olive baboons during pregnancy, before-and-after with control *in vitro* study to determine the binding phenotype of *P. knowlesi* infected erythrocytes isolated from pregnant baboons (Figure 4).

2.3 Experimental protocol

Four adult female Olive baboons were screened and maintained in the company of an adult male Olive baboon for mating to occur. Pregnancy status and gestation periods of the baboons were confirmed by ultrasound. The baboons were infected together with four non-pregnant baboons (control) on the 150^{th} day of gestation with 2×10^5 *P. knowlesi* blood stage parasites cultured overnight (Ozwara *et al.,* 2003). Following infection, thin blood smear preparations from finger pricks were done daily from day 3 post infection to determine level of parasitaemia. When patent parasitaemia was established in the female Olive baboons (day 7-9 post infection), caesarean sections (CS) were performed by an attending veterinarian to obtain intact sterile placental tissue (Mustafa *et al.,* 2010). The peripheral blood was also collected and processed to acquire infected erythrocytes. These

isolated erythrocytes were then tested to determine their binding phenotypes in a static binding assay against purified receptors.

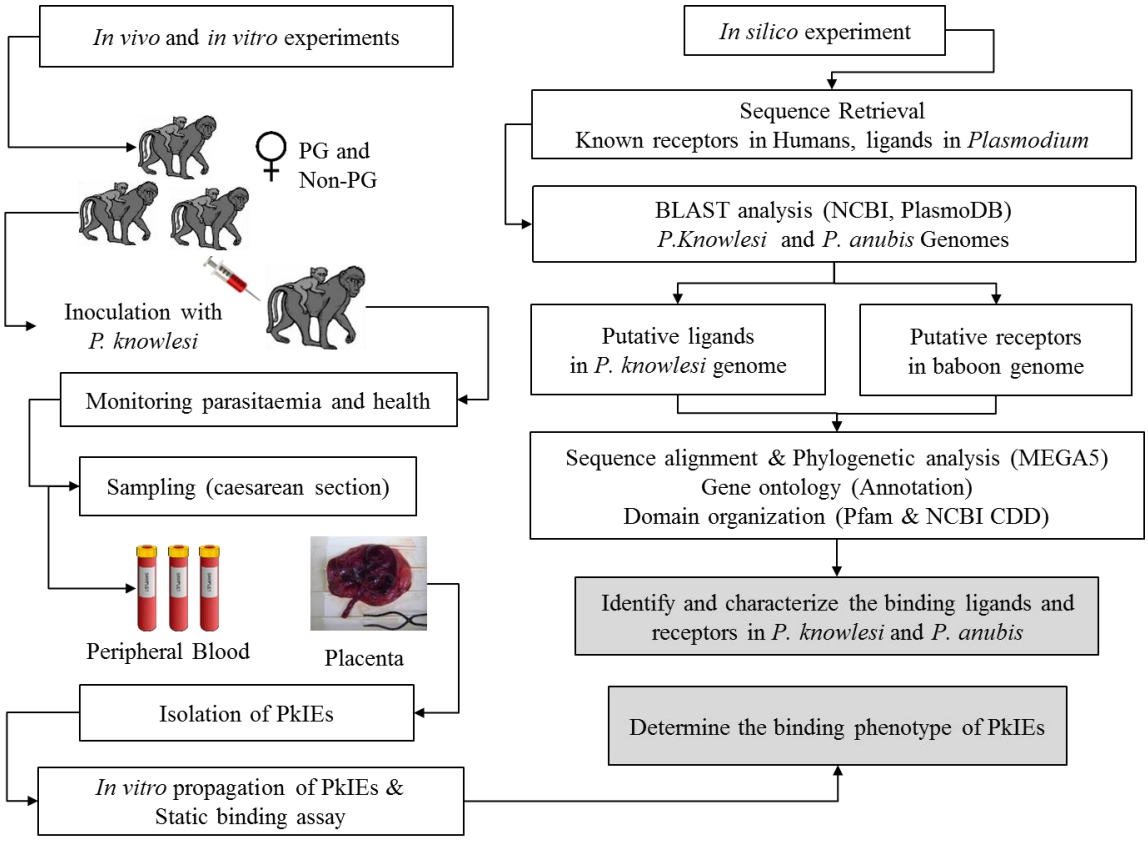


Figure 4: Schematic diagram of the study's experimental design

Key: PG (pregnant), PkIEs (*P. knowlesi* infected erythrocytes)

2.4 Experimental animals

Female Olive baboons (*P. anubis*, weighing between 12–23kg) were used. They were screened by Giemsa stained thick/thin blood smears to ensure they were not infected with *P. knowlesi* prior to the experiment. During quarantine they were also tested to ensure that they were free from hemoprotozoan, gastrointestinal parasites and Simian Immunodeficiency Virus (SIV) before inclusion in the study. Each baboon was housed in an individual squeeze back cage of dimensions 0.6 × 0.6 × 0.8 meters at the bio-

containment facility (according to IPR SOPs and guidelines for holding of animals). They were fed on commercial non-human primate diet (Unga Millers Limited, Nakuru, Kenya), supplemented with fruits, vegetables, minerals, and water *ad libitum* (Olobo & Black, 1990).

2.5 *Plasmodium* parasites

Blood stage parasites of *P. knowlesi* strain H, Pk1 (A+) clone (Barnwell *et al.,* 1983) whose genome has been fully sequenced (Pain *et al.,* 2008) were used to induce malaria infection in the experimental animals. The *P. falciparum* lines CS2 and 3D7 were used to monitor the performance of the static binding assay (obtained through MR4 as part of the BEI Resources Repository, NIAID, NIH, *P. falciparum* CS2, MRA-96, and *P. falciparum* 3D7 KAHRP knockout, MRA-554, deposited by SJ Rogerson and AF Cowman respectively). Clone CS2 binds to CSA and HA with low binding to CD36 and no binding to ICAM-1 while clone 3D7 is applied in cytoadherence and malaria biology studies (Cowman, 1995; Rogerson *et al.,* 1995).

2.6 Sample size determination

Purposive sampling technique was used to determine sample size whereby the researcher deliberately determined the number of animals to be used (Kothari, 2004). The minimum number per experimental group was 3 baboons (n=3) (Ozwara *et al.*, 2003). In accordance with ethical guidelines regarding use of Non-Human Primates (NHPs) in biomedical research, only the minimal number of baboons required to obtain valid and reproducible data was included in this study. This is in compliance with the concept of '3Rs' (Reduce, Replace and Refine) used to minimize the use of the animals in research and any associated suffering without affecting the quality of scientific work (Vitale *et al.,* 2009).

2.7 Ethical and biosafety considerations

Ethical clearance was sought from the Institutional Animal Care and Use Committee (IACUC) and the Institution's Scientific Ethical Review Committee (ISERC) who approved the protocols for this study. Standard operating procedures (SOPs) available at the Malaria Laboratory and the bio-containment facility were followed in all experiments. These included: the anaesthetizing of baboons with Ketamine hydrochloride-xylazine mixture (10mg/Kg) for all invasive procedures, having a qualified veterinarian and animal attendant to assist with C-section procedures. Further precautionary measures included putting on protective gear and carrying out the experiments on samples obtained from animals in a laminar flow hood (Nuair Biological Cabinets).

2.8 *In silico* homology studies

2.8.1 Receptor sequence retrieval from databases

Using publicly available databases (Ensembl release 70, GenBank release 194) the complete coding sequence (CDS) of the human receptor molecules chondroitin sulphate A (CSA), hyaluronic acid (HA), and CD36 (thrombospondin receptor) were retrieved. A text search was done using the name of the receptor against GenBank database at NCBI. Sequences matching the text search and which had complete sequences were selected (Table 1). Their transcript and protein features were obtained from Ensembl database as documented and their accession numbers obtained from NCBI (Ensembl release 70, GenBank release 194). These were used as queries for subsequent investigations using the Basic Local Alignment Search Tool (BLAST) against GenBank Database (Altschul *et al.*, 1990).

Table 1: Characteristics of human receptor molecules HA, CSA and CD36

Characteristic / Feature	Receptor Molecule		
	HA	**CSA**	**CD36**
Full name	Hyaluronan and proteoglycan link protein 1 (HAPLN 1)	Chondroitin sulfate proteoglycan 4 (CSPG 4)	Thrombospondin receptor
Gene ID	ENSG00000145681	ENSG00000173546	ENSG00000135218
Accession No.	ref|NM_001884.3	ref|NM_001897.4	ref|NM_001001548.2
Molecular Weight ($gmol^{-1}$)	40,165.55	250,536.07	53,053.36
Charge	5.0	-48.5	5.0
Residues (aa)	354	2,322	472
Isoelectric Point (pI)	7.44	5.15	8.06
Mean Residue Weight ($gmol^{-1}$)	113.462	107.897	112.401
Location	Chr5: 82,933,624-83,017,432	Chr15: 75,966,663-76,005,189	Chr7: 79,998,891-80,303,725
Strand	Reverse	Reverse	Forward

Source (Ensembl genome browser 70: *Homo sapiens*, GenBank release 194). Transcript and protein features (including molecular weight, length in amino acids, isoelectric point and location) of human receptor molecules obtained from Ensembl DB as documented and their accession numbers obtained from GenBank.

2.8.2 Sequence homology of human receptor molecules

The three sequences (HA, CSA and CD36) of human receptor molecules (Table 1) retrieved were aligned to NCBI's non-redundant protein (Nr) and nucleotide (Nt) databases (GenBank release 194). Briefly, each sequence in FASTA format was input in the query input window and searched against NCBI (Nr and Nt) databases using BLASTn or BLASTx with default parameters (Altschul *et al.,* 1997). Matching hits with highest percentage identities and lowest expectation values (E-values) were documented as the homologues of human receptor molecules in non-human primates.

2.8.3 Sequence similarity search of *P. anubis* genome

Sequences of the non-human primate (NHP) homologues identified (Table 3and 4) were retrieved from GenBank in FASTA format. These were individually searched against the genome of *P. anubis* at NCBI (GenBank release 194, Zinner *et al.,* 2013) using BLASTn

and BLASTx algorithms with default parameters. Hits generated were inspected and those with highest percentage identities and lowest expectation values (E-values) selected as the putative receptor molecules mediating erythrocyte binding in the Olive baboon.

2.8.4 Characterization of putative receptor molecules in *P. anubis* genome

Putative receptor molecules identified in *P. anubis* genome were characterized by comparing their domain organization to those of known human placental receptor molecules CSA, HA and CD36. The protein sequences of the molecules in FASTA format were retrieved from GenBank (GenBank release 194) and used as queries in domain search against Pfam and NCBI's conserved domain database (CDD) (Finn *et al.,* 2010; Marchler-Bauer *et al.,* 2011) with default parameters.

2.8.5 Retrieval of *Plasmodium* ligands from databases

To investigate the putative binding molecules in *P. knowlesi*, known erythrocyte binding ligands in *Plasmodium* (Table 2) were used as queries to BLAST the genome of *P. knowlesi*. These binding molecules were retrieved from PlasmoDB and GenBank by doing a text search and selecting the matching hits that had complete sequences (GenBank release 194, PlasmoDB ver. 9.3, Mar 2013). Their protein features (including molecular weight, isoelectric point and length in amino acids) were obtained from ExPASY-ProParam (Gasteiger *et al.,* 2005) by doing a search using the FASTA sequences of the ligand molecules.

2.8.6 Sequence similarity search of *P. knowlesi* genome

Sequences of erythrocyte binding ligands (Table 2) were retrieved from PlasmoDB in FASTA format (Aurrecoechea *et al.,* 2009). These were used as queries in a BLAST search against the *P. knowlesi* genome hosted at PlasmoDB (Pain *et al.,* 2008; PlasmoDB

ver. 9.3, Mar 2013) using BLASTx algorithm with default parameters. Hits generated were inspected, those with highest percentage identities and expectation value (E-value) of < 1e-04 were designated as the putative erythrocyte binding ligands in *P. knowlesi* genome.

2.8.7 Characterization of putative binding ligands in *P. knowlesi* genome

To predict biological process, molecular function and cellular location of putative erythrocyte binding molecules identified in *P. knowlesi* genome, they were searched against gene ontology (GO) database (Ashburner *et al.,* 2000). They were further characterised for their molecular weights (Mol. Wt.) and isoelectric points (pI) using ExPASYProtParam (Gasteiger *et al.*, 2005). Their domains were determined using InterProScan tool at the InterPro protein families database (Jones *et al.*, 2014, Mitchell *et al.*, 2014 and compared to that *P. falciparum* erythrocyte binding ligand (Var2csa).

Table 2: Summary of features of erythrocyte binding ligands in selected parasites of the genus *Plasmodium*

Ligand	Organism	GI	Source	Location	Length (aa)	Mol. Wt (Da)	pI
Erythrocyte membrane protein 1, (VAR2CSA)	*P. falciparum*	PF3D7_1200600	PlasmoDB	Chr12:46,788-56,805	3056	355247	7.29
Erythrocyte binding antigen-175 (EBA-175)	*P. falciparum*	PF3D7_0731500	PlasmoDB	Chr7: 1,358,055-1,362,929	1502	174589	5.47
Erythrocyte binding antigen-181 (EBA-181)	*P. falciparum*	PF3D7_0102500	PlasmoDB	Chr1: 110,750-115,799	1567	181151	4.71
Erythrocyte binding antigen-165 (EBA-165)	*P. falciparum*	PF3D7_0424300	PlasmoDB	Chr4:1,093,138-1,097,835	1430	165234	6.07
Erythrocyte binding antigen-140 (EBA-140)	*P. falciparum*	PF3D7_1301600	PlasmoDB	Chr13: 89,319-93,352	1210	140597	6.44
Erythrocyte membrane protein 1 (PfEMP1)	*P. falciparum*	NP_700477	GenBank	Chr10: 28,490-36,164	2241	252331.4	5.29
Erythrocyte membrane protein 1	*P. falciparum*	AAQ73923	GenBank	Chr13: 33,959-44,742	3666	422848.3	6.61
FCR3 CSA ligand	*P. falciparum*	CAB59840	GenBank	Chr5: 1,333,470-1,342,964	3542	413096	8.23
PLAFA Variant-specific surface protein	*P. falciparum*	Q26031	GenBank	Chr6: 1,353,946-1,366,430	3078	349301	5.58
Erythrocyte membrane protein 1	*P. falciparum*	AAO67411	GenBank	Chr6: 663,547 to 672,143	2480	286743	6.59
Erythrocyte binding protein (beta)	*P. knowlesi*	PKH_000490	PlasmoDB	1,863-5,920	1154	130481	4.79
Erythrocyte binding protein (gamma)	*P. knowlesi*	PKH_134580	PlasmoDB	Chr13:2,177,465-2,181,275	1072	121212	4.9
Erythrocyte binding protein (alpha)	*P. knowlesi*	PKH_062300	PlasmoDB	Chr6: 1,032,863-1,036,679	1073	120703	5.29
205kDa Pk1(B+)1+ SICAvar antigen	*P. knowlesi*	AF078128	GenBank	NA	2038	227070	7.04
Duffy receptor precursor	*P. vivax*	PVX_110810	PlasmoDB	Chr6: 976,329 - 980,090	1070	119684	5.94

Source (GenBank release 194, Feb 2013, PlasmoDB Ver. 9.3, Mar 2013) **Key:** Gene identifier (GI), molecular weight (Mol. Wt), isoelectric point (pI). Protein features (including molecular weight, isoelectric point and length in amino acids) of known erythrocyte binding obtained from ExPASY-ProParam by doing a search using the FASTA sequences of the ligand molecules.

2.8.8 Sequence alignment and phylogenetic analysis

Putative protein sequences from *P. anubis* genomes were aligned with known ligands and receptors respectively using MUSCLE program. Evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987) incorporated in MEGA5 (Tamura *et al.,* 2011). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test were shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Tamura *et al.,* 2011) and were in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated.

2.9 *In vitro* culture of *Plasmodium* parasites

2.9.1 Retrieval of parasites from liquid nitrogen

Plasmodium falciparum and *P. knowlesi* parasite vials were separately retrieved from liquid nitrogen, immediately thawed at 37°C in a water bath and transferred into 50ml centrifuge tubes. To every 1ml of parasite suspension: 200µl of 12% NaCl was added drop wise while shaking and allowed to stand for 5 minutes, followed by 10ml of 1.6% NaCl before finally adding 10ml of 0.9% NaCl (Moll *et al.,* 2008). The mixture was allowed to stand for 5 minutes and centrifuged at 500G for 10 minutes at 24°C (Hettich Zentriffugen Rotanta 460R, Germany). The supernatant was aspirated and the resulting pellet washed twice in incomplete culture medium by centrifuging, before the parasite pellet was transferred into culture (Diggs *et al.,* 1975).

2.9.2 Erythrocyte processing from baboon blood

Baboon whole blood collected in Alsever's solution was transferred into a sterile 50ml tube and centrifuged at 500G for 10 minutes at 24°C (Hettich Zentriffugen Rotanta 460R, Germany). The supernatant was aspirated to remove the plasma and leukocytes. The resulting erythrocyte pellet was then washed three times in incomplete media (200mM L-Glutamine, 20% Glucose, 1M NaOH, 1M HEPES, Gentamycin and RPMI 1640) by centrifugation as before. After the last wash, the supernatant was aspirated and the pellet mixed with equal volume of RPMI 1640 (Sigma) resulting into a 50% packed cell volume (PCV). This was stored at 4°C and used within two weeks for *in vitro* propagation of *P. knowlesi* parasites (Ozwara *et al.,* 2003).

2.9.3 Serum preparation from baboon blood

Approximately 10ml of baboon blood was collected in a plain tube and placed on bench for 4 hours to allow clotting to occur. An applicator stick was used to dislodge the clot formed and centrifuged at 885G for 10 minutes at 24°C (Hettich Zentriffugen Rotanta 460R, Germany). The supernatant (serum) was aspirated and transferred into a well labelled sterile 50ml tube. The serum was heat inactivated (HI) in a water bath at 56°C for 30 minutes. The heat inactivated serum was allowed to cool down and stored in aliquots at -20°C until use in culturing of *P. knowlesi* parasites (IPR SOPs).

2.9.4 Erythrocyte processing from human blood

Donated human blood with anticoagulant (EDTA) obtained from KEMRI was transferred under sterile conditions to 50ml centrifuge tubes. It was centrifuged at 500G for 5 minutes at 24°C (Hettich Zentriffugen Rotanta 460R, Germany). The plasma and buffy coat were aspirated off and the resulting erythrocyte pellet washed three times with twice volume of incomplete RPMI 1640 by centrifugation. After the last wash, the supernatant was aspirated and the pellet mixed with equal volume of RPMI 1640 (Sigma) resulting in 50%

PCV. This was stored at 4°C and used within two weeks for *in vitro* propagation of *P. falciparum* parasites (Moll *et al.*, 2008).

2.9.5 Serum processing from human blood

Human blood (O+ve) was obtained from KEMRI in blood collection bags without anticoagulant and left overnight at 4°C. The serum was transferred into well labelled sterile 50ml centrifuge tubes in a laminar flow cabinet followed by heat inactivation (HI) in a water bath at 56°C for 30 minutes. The heat inactivated serum was allowed to cool down and then stored in aliquots at -20°C until use for *in vitro* culture of *P. falciparum* (IPR SOPs).

2.9.6 *In vitro* culture of *P. knowlesi*

In vitro cultures were initiated using cryopreserved *P. knowlesi* parasites previously isolated from infected baboons and kept frozen in liquid nitrogen. The parasite pellet obtained on retrieval was transferred into culture to a starting erythrocyte PCV of 2.5% in complete culture medium [RPMI 1640 (Sigma) supplemented with 2.5% baboon PCV, 20% baboon heat inactivated serum and 15µg/ml gentamycin solution (Sigma)].

Thin smear preparations were made using 100µl of culture to determine the starting and daily parasitaemia. Cultures were mixed by gently pipetting and transferred into sterile labelled T-25cm^3 culture flask (Corning, UK). The culture flasks were then gassed with a mixture of gases (5% CO_2, 5% O_2, and 90% N_2) for 30 seconds, tightly capped and transferred to an incubator at 37°C (Ozwara Suba & LUMC, 2005).

2.9.7 *In vitro* culture of *P. falciparum*

In vitro cultures were initiated using cryopreserved laboratory parasite lines (*P. falciparum* 3D7 and CS2). The resulting parasite pellet was mixed with 5ml of

complete RPMI 1640 medium [supplemented with 37.5mM HEPES, 7mM D-glucose, 6mM NaOH, 25µg/ml of gentamicin sulphate, 2mM L-glutamine, and 10% human serum] at pH7.2 and transferred to a T-25cm^3 culture flask (Corning, UK). A drop of the culture mixture was used to make a thin smear to determine the starting parasitaemia. The parasitaemia was adjusted to 2% using human PCV (O+ve erythrocytes). The culture mixture was then gassed with a mixture of 95% nitrogen, 5% carbon dioxide, and 5% oxygen and transferred to an incubator at 37°C (Trager & Jensen, 1976).

2.9.8 Refreshing of *P. falciparum and P. knowlesi* cultures

The parasite cultures were refreshed every two days to measure their parasitaemia level and maintained at a parasitaemia of 1–2% to ensure better growth. Briefly, the culture medium was pre-heated at 37°C. The culture flasks were transferred into the biosafety cabinet. Excess media was aspirated from the flasks and 10ml of fresh complete media added while gently pipetting to mix. An aliquot of 100µl was obtained from each of the flasks and used to prepare thin smears that were later stained and assessed microscopically to determine level of parasitaemia. Depending on level of parasitaemia, appropriate amount of erythrocytes (PCV) were added to each flask respectively. The flasks were then thoroughly gassed for 45 seconds and quickly resealed before being incubated at 37°C (Trager & Jensen, 1976).

2.9.9 Preparation of thin blood smears

A 100µl aliquot of the *Plasmodium* cultures was taken from each of the flasks and transferred to a labelled eppendorf tube. These were spun in a microfuge, the supernatant aspirated and the remaining erythrocyte pellet used to make a thin smear. A small amount of the pellet was placed on a microscope slide. A second slide was placed on the first and moved back into the drop, spreading it. The second slide was then moved forward, smearing a film of blood across the first slide. The smear was allowed to dry and fixed with methanol. The methanol was discarded and the slide covered with 10% Giemsa

solution (freshly prepared) for 15 minutes to stain. The staining solution was rinsed with water and allowed to dry, where after it was read under a microscope at ×100 oil immersion objective (Moll *et al.*, 2008).

2.9.10 Determination of parasitaemia

Parasitaemia was evaluated every 24 hours for *P. knowlesi* cultures and 48 hours for *P. falciparum* (3D7 and CS2) cultures by making thin smears. To determine parasitaemia, at least 2000 erythrocytes were enumerated at ×100 objective. The number of infected erythrocytes (IEs) out of the 2000 was counted and the percentage parasitaemia calculated using the formula:

$$\%\ Parasitemia = \left(\frac{Number\ of\ Infected\ Erythrocytes\ (IEs)\ counted}{2000\ Erythrocytes} \times 100\right)$$

(Adapted with modifications from (Moll *et al.*, 2008)

Once established in the culture, the parasites were maintained under similar conditions except for medium changes every 48 hours and sub culturing done when parasitaemia exceeded 5% (Ozwara Suba & LUMC, 2005).

2.9.11 Synchronization of cultures

Synchronization of *in vitro* cultures was done using 5% sorbitol to obtain homogenous staged parasites (Lambros & Vanderberg, 1979). This was done when culture parasites were at ring stage. The parasite culture was centrifuged at 500G for 5 minutes at room temperature (Hettich Zentriffugen Rotanta 460R, Germany). To the resulting pellet equal amount of sorbitol [5% sorbitol (Sigma) in distilled water] was added and incubated at 37°C for 10 minutes at room temperature. The parasite-sorbitol mixture was then centrifuged at 500G for 5 minutes at room temperature (Hettich Zentriffugen Rotanta 460R, Germany), washed 3 times in incomplete culture medium and diluted to 5%

haematocrit. Parasitaemia was counted by making a thin smear and infected erythrocytes sub-cultured as usual (Lambros & Vanderberg, 1979).

2.9.12 Freezing of *Plasmodium* parasites

In vitro culture to be frozen was transferred into a 50ml centrifuge tube and centrifuged at 500G for 5 minutes at room temperature (Hettich Zentriffugen Rotanta 460R, Germany). The supernatant was aspirated and for every 200µl of erythrocyte pellet, 300µl of foetal bovine serum (FBS) was added. Lastly, 500µl of freezing medium was added drop-by-drop while gently shaking the vial. Approximately 1 ml of the mixture was then aliquoted into well labelled cryovials. These were put in a freezing chamber and frozen overnight at -80°C. The vials were transferred into liquid N_2 the next day for long term storage (Moll *et al.,* 2008).

2.10 Infection of pregnant baboons (*P. anubis*) with *P. knowlesi*

Four adult female baboons together with four non pregnant control baboons were infected with 2×10^5 *P. knowlesi* blood stage parasites, cultured overnight (Ozwara *et al.,* 2003) on the gestation day 150. These animals were either in their first or second pregnancies (primigravidae or secundigravidae).

Cryopreserved stocks of *P. knowlesi* H strain were used to initiate blood stage infection in the baboons. The parasites stocks were retrieved from liquid nitrogen and used to initiate *in vitro* cultures as previously described for about 18 hours. After incubation, thin blood smears of the cultures were made to check for bacterial contaminations, parasite viability and level of parasitaemia. The culture suspension was then centrifuged at 500G, 20^{o}C for 10 minutes. The supernatant was discarded and pellet volume determined. Parasite concentration was then adjusted to a population of 2.0×10^5 parasites/ml in incomplete RPMI 1640. Baboons were first sedated with Ketamine hydrochloride-xylazine mixture before inoculation via the sephaseous vein with the 1ml parasite-RPMI mixture (Ozwara Suba & LUMC, 2005).

2.10.1 Monitoring parasitaemia in baboons

Following infection, thin blood smears from finger pricks were prepared daily from day 3 post infection to determine level of parasitaemia (Ozwara *et al.,* 2003). The baboon's finger to be pricked was first cleaned by alcohol swabbing before pricking with a sterile needle. A drop of blood was transferred to a slide using capillary tubes and used to prepare thin smears. These were stained and read under a microscope at ×100 oil immersion objective to determine level of parasitaemia.

2.10.2 Caesarean section procedure in baboons

Caesarean sections (CS) were performed by an attending veterinarian according to IPR SOPs in order to obtain intact sterile placental tissue (Mustafa *et al.,* 2010). These procedures were undertaken when patent parasitaemia was established in baboons after inoculation with *P. knowlesi* (day 7-9 post infection).

2.10.3 Collection and processing of peripheral and placental samples

Peripheral blood was collected from baboons (both experimental and control) during CS in tubes containing lithium heparin anticoagulant. It was centrifuged to remove plasma and buffy coat, washed three times in phosphate buffered saline (PBS, pH 7.4) to obtain infected erythrocytes prior to culture (Beeson *et al.,* 1999).

To harvest infected erythrocytes from freshly delivered placentas, several biopsies of placental tissue (5–10cm^3) were cut from the maternal side and put immediately into 50ml tubes containing PBS with 50mM EDTA. These were then placed on a tube roller for 1 hour. The medium containing infected erythrocytes and other blood cells was separated from the placental tissue and centrifuged. The pellet was washed three times in PBS before use in assays or cultures. Placental parasites predominantly consisted of mature stages and could be tested for static binding without prior culture (Beeson *et al.,* 1999).

2.11 Static adhesion assay

2.11.1 Culture and preparation of samples and assay performance controls

Samples obtained were cultured for up to 24 hours until mature trophozoite forms predominated (Beeson *et al.,* 2000). Laboratory parasite clones (*P. falciparum* 3D7 and CS2) were also cultured using standard culturing techniques (Trager & Jensen, 1976). The parasitaemia was adjusted to 3% and 1% haematocrit for all adhesion assays.

2.11.2 Recombinant proteins for static adhesion

Purified receptors used in this study were: Recombinant Human CD36/SR-B3 Fc Chimera (R & D Systems, UK), Chondroitin 4 sulphate sodium salt from bovine trachea, CSA (Sigma) and Hyaluronic acid from bovine vitreous humour, HA (Sigma).

They were reconstituted into stock solutions with concentrations of 1mg/ml for CSA, 100µg/ml for HA and 50µg/ml for CD36 in PBS. Prior to the static binding assay, the receptors were first prepared by reconstituting them into the coating concentrations used in the assay. To make a 100µg/ml solution of CSA, 10µl of 1mg/ml stock was dissolved in 90µl PBS which was then dissolved in 100µl of PBS to get a 50µg/ml solution. The stock solution prepared for HA had a concentration of 100µg/ml and was used as is but to make a 50µg/ml solution the stock solution was dissolved in equal volume of PBS. The stock solution prepared for CD36 had a concentration of 50µg/ml and was used as is but to make a 25µg/ml solution the stock solution was dissolved in equal volume of PBS. The static binding experiment was done under two pH conditions of pH6.8 and pH7.2.

2.11.3 Static protein binding assay

Three identical areas of each Petri dish (60×15mm diameter, Falcon 1007, Becton Dickenson, Oxford, UK) were treated with 2µl aliquots of purified CSA, HA each at 100µg/ml and 50µg/ml and CD36 at 50µg/ml and 25µg/ml. Control areas were treated

with phosphate buffered saline (PBS) and three marked areas were left untreated. The dishes were incubated in a humid chamber at 37°C for two hours before aspirating off excess protein and blocking all areas with 1% w/v bovine serum albumin in PBS for 2 hours at 37°C. The blocking solution was removed by gentle pipetting. *Ex vivo* matured infected erythrocyte cultures were added to 3ml warmed binding buffer (RPMI 1640 media supplemented with 2% D-glucose) to a final haematocrit of 3%.

Each receptor protein and PBS control were spotted in triplicate per dish and duplicate dishes were seeded per isolate. Dishes were seeded with 1.5ml of the prepared *ex vivo* cultured cell suspension per isolate and additional assay dishes with *P. falciparum* clone CS2 and 3D7 as assay performance controls. The dishes were incubated at 37°C for 1 hour, with gentle mixing at 10 minutes intervals. Unbound cells were removed by gentle washing seven times with binding buffer. Bound cells were fixed with 1% v/v glutaraldehyde (Sigma) for 1 hour and stained with 10% Giemsa (BDH, VWR international Ltd, England) for 20 minutes (Fatih *et al.*, 2012).

Using an inverted light microscope at × 300 magnification the number of bound infected erythrocytes was enumerated from non-overlapping fields for each protein and PBS treated area. The area of the field of view was equivalent to 0.19635mm^2. The results were expressed as the number of infected cells (IE) bound/ mm^2 to each of the proteins or control [IE/mm^2 = (1/0.19635) × mean number of bound IE per field] (Fatih *et al.*, 2012).

2.12 Data analysis

Data obtained was entered, formatted, and stored in Excel Spreadsheets (Microsoft Corp.). Results of the static protein binding assays were expressed as Medians and interquartile ranges (IQR). Statistical analyses were performed using R 2.15.2 (R Core Team, 2012). The Mann-Whitney U-test and the Kruskal-Wallis test were used to evaluate statistical significance of the data since distributions were skewed from normal (Fatih *et al.*, 2012; Heddini *et al.*, 2001). Probability values of $P < 0.05$ were considered significant.

CHAPTER THREE

RESULTS

3.1 Putative receptor molecules in Olive baboon (*P. anubis*)

3.1.1 Homologues of human CSA, HA and CD36 in non-human primates

The human receptor molecules CSA (CSPG 4), HA (HAPLN 1) and CD36 all aligned to their homologues in non-human primates with high identities (PID=88–99%) and low expectation values (E-value < 0.0) (Table 3 and 4). Human CSA aligned to predicted chondroitin sulphate proteoglycan (CSPG 4 and CSPG 4-like) nucleotides and proteins, human HA aligned to predicted hyaluronan and protein link protein 1(HAPLN 1 and transcript variants 2 and 3), while CD36 molecule aligned to CD36 molecules (platelet glycoprotein isoforms and transcript variants 2, 3 and 4).

The matching non-human primates identified were: Gorilla, Bonobo–Pigmy chimpanzee, Olive baboon, Chimpanzee, Rhesus macaque, Sumatran orang-utan, White-cheeked gibbon, White-tufted ear marmoset, Crab-eating macaque, Black-capped squirrel monkey, Bush baby (Table 3 and 4). The high identity of putative molecules identified in non-human primates to the human receptor molecules suggests they are homologous.

Table 3: Nucleotide sequences producing high-scoring segment pairs (HSP) in non-human primates' genomes

Query	Predicted Sequences producing HSP	Organism	Accession No.	E-value	PID
Homo sapiens CSPG 4, mRNA (8305bp)	CSPG4	*Gorilla gorilla gorilla*	XM_004056554.1	0.00	99
	CSPG4	*Pan paniscus*	XM_003846064.2	0.00	99
	CSPG4	*Pan troglodytes*	XM_009429673.1	0.00	98
	CSPG4	*Nomascus leucogenys*	XM_004088380.1	0.00	98
	CSPG 4-like	*Pongo abelii*	XM_009250046.1	0.00	97
	CSPG4, partial	*Papio anubis*	XM_009210666.1	0.00	96
	CSPG4	*Macaca mulatta*	XM_002804882.1	0.00	96
	CSPG 4	*Callithrix jacchus*	XM_009005291.1	0.00	94
	CSPG4	*Saimiri boliviensis boliviensis*	XM_003942991.2	0.00	92
Homo sapiens HAPLN 1 (4678bp)	HAPLN 1	*Gorilla gorilla gorilla*	XM_004042246.1	0.00	99
	HAPLN 1	*Pan paniscus*	XM_003822242.1	0.00	99
	HAPLN 1, transcript variant 3	*Pan troglodytes*	XM_001147083.2	0.00	99
	HAPLN 1	*Pongo abelii*	XM_003899897.1	0.00	98
	HAPLN 1	*Nomascus leucogenys*	XM_003261564.1	0.00	97
	HAPLN 1	*Papio anubis*	XM_003899897.1	0.00	97
	HAPLN 1, transcript variant 1	*Macaca mulatta*	XM_001112341.2	0.00	97
	HAPLN 1	*Macaca fascicularis*	XM_005557322.1	0.00	97
	HAPLN 1, transcript variant 2	*Callithrix jacchus*	XM_002744793.1	0.00	96
	HAPLN 1	*Saimiri boliviensis boliviensis*	XM_003920783.1	0.00	94
Homo sapiens CD36 molecule (thrombospondin receptor) (CD36), transcript variant 1, mRNA (4727bp)	CD36 molecule	*Gorilla gorilla gorilla*	XM_004045656.1	0.00	99
	CD36 molecule, transcript variant 1, 2, 3 and 4	*Pan troglodytes*	XM_003318554.1	0.00	98
	CD36 molecule	*Pan paniscus*	XM_003822746.1	0.00	98
	CD36 molecule, transcript variant 1, 3, 5 and 6	*Nomascus leucogenys*	XM_003252173.1	0.00	97
	CD36 molecule	*Pongo abelii*	XM_002818297.2	0.00	97
	Testis cDNA, clone, similar to human CD36 antigen	*Macaca fascicularis*	AB169273.1	0.00	96
	CD36 molecule, transcript variant 1, 2	*Papio anubis*	XM_003896397.1	0.00	96
	CD36 molecule	*Macaca mulatta*	NM_001032913.1	0.00	96
	CD36 molecule	*Callithrix jacchus*	XM_002751659.2	0.00	94

Source (GenBank release 194) **Key**: CSPG (chondroitin sulphate proteoglycan), HAPLN (hyaluronan and protein link protein), PID (percentage identity), HSP (high scoring segment pair), *Macaca mulata* (Rhesus macaque), *Pongo abelii* (Sumatran orangutan), *Gorilla gorilla*, *Nomascus leucogenys* (White-cheeked gibbon), *Pan troglodytes* (Chimpanzee), *Papio anubis* (Olive baboon), *Callithrix jacchus* (White-tufted ear marmoset), *Macaca fascularis* (Crab-eating macaque), *Homo sapiens* (man), *Pan paniscus* (Bonobo – Pigmy chimpanzee), *Saimir boliviensis boliviensis* (Black-capped squirrel monkey), *Otolemur garnetii* (Bushbaby)

Table 4: Protein sequences producing high-scoring segment pairs (HSP) in non-human primates' genomes

Query	Predicted Sequences producing HSP	Organism	Accession No.	E-value	PID
H. sapiens CSPG4 (8305bp)	CSPG 4	*Gorilla gorilla gorilla*	XM_004056554.1	0.00	99
	LQP: CSPG 4	*Pan paniscus*	XM_003846064.1	0.00	99
	CSPG 4	*Pan troglodytes*	XM_001144835.3	0.00	99
	CSPG 4, partial	*Papio anubis*	XP_003901280.1	0.00	97
	CSPG 4	*Saimiri boliviensis boliviensis*	XM_003942991.1	0.00	95
	CSPG 4-like, partial	*Macaca mulatta*	XM_002804882.1	0.00	94
	CSPG 4	*Otolemur garnettii*	XM_003784677.1	0.00	89
	CSPG 4	*Nomascus leucogenys*	XM_004088380.1	0.00	92
	LQP: CSPG 4	*Callithrix jacchus*	XM_002807180.2	0.00	95
Homo sapiens HAPLN 1 (4678bp)	HAPLN 1 isoform 3	*Pan troglodytes*	XM_001147083.2	0.00	99
	HAPLN 1	*Gorilla gorilla gorilla*	XM_004042246.1	0.00	99
	HAPLN 1	*Saimiri boliviensis boliviensis*	XM_003920783.1	0.00	97
	LQP: HAPLN 1	*Papio anubis*	XP_009206994.1	0.00	98
	Proteoglycan link protein	*Macaca fascicularis*	XP_005557379.1	0.00	98
	HAPLN 1	*Nomascus leucogenys*	XM_003261564.1	0.00	99
	HAPLN 1 isoform 2	*Pongo abelii*	XM_003776628.1	0.00	98
	HAPLN isoform 1	*Macaca mulatta*	XM_001112341.2	0.00	98
	HAPLN 1	*Otolemur garnettii*	XM_003785961.1	0.00	97
	unnamed protein product	*Macaca fascicularis*	BAE87564.1	0.00	98
	HAPLN 1 isoform 2	*Callithrix jacchus*	XP_002744839.1	0.00	97
Homo sapiens CD36 molecule (thrombospondin receptor) (CD36), transcript variant 1 (4727bp)	PG 4 isoform 4	*Pan troglodytes*	XM_519573.3	0.00	99
	PG 4	*Pongo abelii*	XM_002818297.2	0.00	98
	PG 4	*Gorilla gorilla gorilla*	XM_004045652.1	0.00	98
	PG 4 isoform 1	*Nomascus leucogenys*	XM_003252175.1	0.00	96
	PG 4	*Macaca mulatta*	NM_001032913.1	0.00	95
	PG 4 isoform 1	*Saimiri boliviensis boliviensis*	XM_003921144.1	0.00	92
	PG 4	*Callithrix jacchus*	XM_002751659.2	0.00	92
	PG 4-like	*Otolemur garnettii*	XP_009000809.1	0.00	88

Source (GenBank release 194) **Key**: CSPG (chondroitin sulphate glycoprotein), HAPLN (hyaluronan and link protein), LQP (low quality protein), PG (platelet glycoprotein)

3.1.2 Sequence similarity search of Olive baboon (*P. anubis*) genome

The homologues of human receptor molecules identified in non-human primate genomes (Table 3 and 4) were used as queries to BLAST the *P. anubis* genome. All the individual sequences aligned in similar manner to the non-redundant nucleotide and protein databases of *P. anubis* (Table 5 and 6). The top hits from the BLAST results had maximum identities ranging from 83 to 99% with low expectation values (E-value=0.0) for both the nucleotide and protein sequences. The predicted CSPG 4 homologues aligned to CSPG 4 and CSPG 4-like partial sequences in *P. anubis* genome (PID=>90%, E=0.0), while the predicted HAPLN 1 homologues aligned to HAPLN 1 (PID=>93%, E=0.0).

The BLAST also identified other CSPGs namely neurocan, versican, aggrecan and brevican. In particular, the CSPG core protein aggregan with two link modules interacts with hyaluronan (HA) forming aggregates that stabilize cartilage linkages contributing to a tissue's load bearing properties. Aggregates having other CSPGs (neurocan, versican, and brevican) contribute to structural integrity of different tissues.

Table 5: Summary of BLAST hits in *P. anubis* genome when aligned with homologous nucleotide sequences from non-human primates

Query	Predicted high scoring segment pairs (HSPs) in *P. anubis*			
	Nucleotide	**Accession No.**	**PID**	**E-value**
Predicted CSPG 4 homologs				
CSPG 3 mRNA, partial (*Macaca fascicularis)*	Neurocan (NCAN)	XM_003915238.1	99	0.00
CSPG 4 (*H. sapiens ,Callithrix jacchus, Gorilla gorilla gorilla, Macaca mulatta, Nomascus leucogenys, Otolemur garnettii, Pan paniscus, Pan troglodytes, Saimiri boliviensis boliviensis*), CSPG 4-like (*Pongo abelii*)	CSPG4, partial,	XM_003901231.1	96	0.00
	CSPG 4-like, partial,	XR_163612.1	93	0.00
	PUP (FLJ43447)	XM_003901225.1	93	0.00
Predicted HAPLN 1 homologs				
HAPLN 1 (*Homo sapiens, Gorilla gorilla gorilla, Nomascus leucogenys, Otolemur garnettii, Pan paniscus, Pongo abelii, Saimiri boliviensis boliviensis*), HAPLN 1, Transc Var 2 (*Callithrix jacchus, Pongo abelii*), HAPLN 1 Transc Var 1 (*Macaca mulatta,*), HAPLN 1, Transc Var 3 (*Macaca mulatta, Pan troglodytes*)	HAPLN 1	XM_003899897.1	93	0.00
Predicted CD36 molecule homologs				0.00
CD36 molecule Transc Var 1 (*Homo sapien, Nomascus leucogenys, Pan troglodytes*), Testis cDNA, clone similar to human CD36 antigen (*Macaca fascicularis*), CD36 molecule (*Macaca mulatta, Callithrix jacchus, Gorilla gorilla gorilla, Pan paniscus, Pongo abelii, Saimiri boliviensis boliviensis*) PG 4-like (*Otolemur garnettii*)	CD36 molecule, Transc Var 1,	XM_003896397.1	92	0.00
	CD36 molecule, Transc Var 2	XM_003896398.1	96	0.00

Source (GenBank release 194**) Key**: HAPLN 1 (hyaluronan and proteoglycan link protein 1), CSPG (chondroitin sulphate proteoglycan 4), CD36 (CD36 molecule, thrombospondin receptor), EMP (extracellular matrix protein), SRCBM (scavenger receptor class B member), Transc Var (transcript variant), PG (platelet glycoprotein), PUP (putative uncharacterized protein)

Table 6: Summary of BLAST hits in *P. anubis* genome when aligned with homologous protein sequences from non-human primates

Query	Predicted high scoring segment pairs (HSPs) in *P. anubis*			
	Protein	**Accession No.**	**PID**	**E-value**
Predicted CSPG 4 homologs				
CSPG 3 mRNA, partial (*Macaca fascicularis)*	Neurocan core protein	XP_00391 5287.1	98	0.0
	Aggrecan core protein	XP_003901 403.1	56	2e-92
	Versican core protein	XP_003899948.1	55	6e-92
	Brevican core protein	XP_003892883.1	66	2e-87
CSPG 4 (*H. sapiens ,Callithrix jacchus, Gorilla gorilla gorilla,* M*acaca mulatta, Nomascus leucogenys, Otolemur garnettii, Pan paniscus, Pan troglodytes, Saimiri boliviensis boliviensis*), CSPG 4-like (*Pongo abelii*)	CSPG 4, partial	XP_003901 280.1	94	0.0
	PUP (FLJ43447)	XP_003901 274.1	63	4e-24
	FRAS1-related EMP 3	XP_003899274.1	35	3e-49
	FRAS1-related EMP 2	XP_00391 3840.1	30	3e-41
Predicted HAPLN 1 homologs				
HAPLN 1 (*Homo sapiens, Gorilla gorilla gorilla, Nomascus leucogenys, Otolemur garnettii, Pan paniscus, Pongo abelii, Saimiri boliviensis boliviensis*), HAPLN 1, Transc Var 2 (*Callithrix jacchus, Pongo abelii*), HAPLN 1 Transc Var 1 (*Macaca mulatta,*), HAPLN 1, Transc Var 3 (*Macaca mulatta, Pan troglodytes*)	HAPLN 1	XP_003899946.1	98	0.0
	HAPLN 3	XP_003901 408.1	56	3e-116
	HAPLN 4	XP_00391 5288.1	51	9e-100
	HAPLN 2	XP_003892880.1	46	2e-95
Predicted CD36 molecule homologs				
CD36 molecule Transc Var 1 (*Homo sapien, Nomascus leucogenys, Pan troglodytes*), Testis cDNA, clone similar to human CD36 antigen (*Macaca fascicularis*), CD36 molecule (*Macaca mulatta, Callithrix jacchus, Gorilla gorilla gorilla, Pan paniscus, Pongo abelii, Saimiri boliviensis boliviensis*) PG 4-like (*Otolemur garnettii*)	PG 4 isoform 1	XP_003896446.1	95	0.0
	Lysosome membrane protein 2	XP_003898782.1	35	3e-79
	SRCBM 1 isoform 2,	XP_003907409.1	34	4e-73
	SRCBM 1 isoform 1	XP_003907408.1	34	7e-73

Source (GenBank release 194**) Key**: HAPLN 1 (hyaluronan and proteoglycan link protein 1), CSPG (chondroitin sulphate proteoglycan 4), CD36 (CD36 molecule, thrombospondin receptor), EMP (extracellular matrix protein), SRCBM (scavenger receptor class B member), Transc Var (transcript variant), PG (platelet glycoprotein), PUP (putative uncharacterized protein)

The *P. anubis* sequences with the highest percentage identities and least E-values from BLAST analysis against GenBank Database (GenBank release 194) were designated as the likely molecules that mediate placental erythrocyte binding (Table 7 and Appendix 1). The top hits for CSPG receptor were CSPG 4 and CSPG 4-like partial sequences. Also, a group of aggregate proteins associated with CSPGs namely; neurocan, aggregan, versican and brevican core proteins were identified. Equally, the top hit for the HAPLN 1 receptor was HAPLN 1 in the *P. anubis*. Other hits that were closely associated were; HAPLN 2, 3 and 4 which are variants of HAPLN 1. Lastly, CD36 molecule transcript variants 1 and 2 and other scavenger receptor class B member proteins were identified using the human CD36 receptor.

Table 7: List of putative nucleotide and protein sequences in *P. anubis* genome used as receptor molecules

Nucleotide	Accession no	Protein	Accession no
CSPG			
CSPG4, partial	XM_003901231.1	CSPG 4, partial	XP_003901280.1
CSPG 4-like, partial	XR_163612.1	neurocan core protein	XP_003915287.1
PUP (FLJ43447)	XM_003901225.1	FRAS1-related EMP 3	XP_003899274.1
neurocan (NCAN)	XM_003915238.1	PUP (FLJ43447)	XP_003901274.1
		aggrecan core protein	XP_003901403.1
		versican core protein	XP_003899948.1
		brevican core protein	XP_003892883.1
		FRAS1-related EMP 2	XP_003913840.1
HAPLN 1			
HAPLN 1	XM_003899897.1	HAPLN 1	XP_003899946.1
		HAPLN 3	XP_003901408.1
		HAPLN 4	XP_003915288.1
		HAPLN 2	XP_003892880.1
CD36 molecule			
CD36 molecule, transcript variant 1	XM_003896397.1	PG 4 isoform 1	XP_003896446.1
CD36 molecule, transcript variant 2	XM_003896398.1	LMP 2	XP_003898782.1
		SRCBM 1 isoform 2	XP_003907409.1
		SRCBM 1 isoform 1	XP_003907408.1

Key: EMP (extracellular matrix protein), PG (platelet glycoprotein), PUP (putative uncharacterized protein), LMP (lysosome membrane protein), SRCBM (scavenger receptor class B member)

3.2 Characterization of putative *P. anubis* receptors

3.2.1 Phylogenetic analysis of Olive baboon (*P. anubis*) receptor molecules

Phylogenetic relationship was inferred from multiple sequence alignment of putative protein sequences, known receptor sequences and homologous non-human primate sequences with the *P. falciparum* sequence Pf3D7_1220600 EMP 1 VAR2CSA used as an outgroup. Overall, the putative sequences from *P. anubis* clustered to homologous sequences in macaque monkeys *M. mulatta* and *M. fascularis* (Figure 5, 6 and 7). These are the natural hosts of *P. knowlesi* thereby suggesting that the sequences could be for proteins used as adhesive receptors for *P. knowlesi* ligands. The human receptor sequences (CSPG, HAPLN 1 and CD36 antigen) clustered with homologous sequences from higher non-human primates like gorilla and chimpanzees. However, it was noted that all the homologous sequences share a common ancestral origin as demonstrated by branching of the phylogenetic tree generated and bootstrap values.

The CSPG core protein sequences (neurocan, aggregan, versican and brevican) from *P. anubis* all clustered together forming a clade that diverged from other CSPG homologues in *P. anubis* and other non-human primates (Figure 5). The *P. anubis* CSPG 4 sequence clustered to CSPG 4 from *M. mulatta* while human CSPG 4 clustered to homologues from higher non-human primates like chimpanzee and gorilla. The relationship suggests that the FRAS 1-related emp 2 and 3 from *P. anubis* are closely related to the CSPG 4 homologues than the CSPG core proteins since they diverged later from their common ancestry.

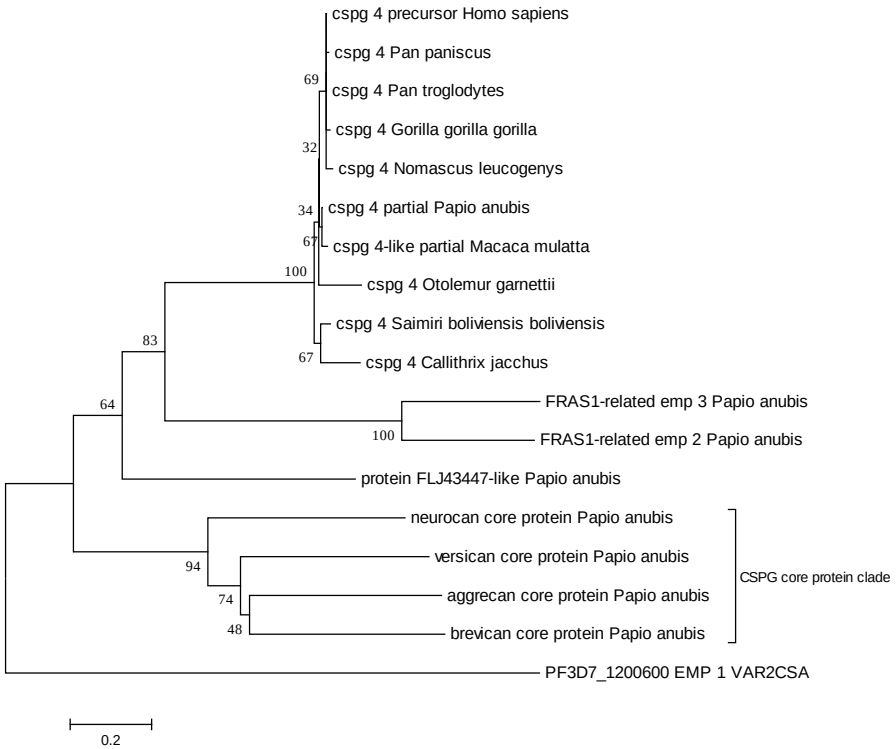


Figure 5: Phylogenetic relationship of putative CSPG proteins in *P. anubis* and other non-human primate homologues

The Neighbor-Joining tree was constructed using the Poisson correction method for 18 CSPG sequences. The numbers at the juncture of two branches are the percent of 1000 bootstrap trees with the same branch length. *P. falciparum* sequence Pf3D7_1220600 EMP 1 VAR2CSA was included as an out-group. **Key**: CSPG (chondroitin sulphate proteoglycan), EMP (erythrocyte membrane protein), emp (extracellular matrix protein)

The variants HAPLN 2, 3 and 4 from *P. anubis* all clustered together forming a clade (bootstrap 73%) that diverged from the HAPLN 1 sequence in *P. anubis* and other non-human primates homologues (Figure 6). The *P. anubis* HAPLN 1 sequence clustered with homologous sequences from macaque monkeys *M. mulatta* and *M. fascularis*. The *P. anubis* sequences were closer to the lower monkeys (marmoset, bushbaby) than higher monkeys (gorilla and chimpanzee) that clustered to human HAPLN 1 sequence forming a clade. Although distant from the human HAPLN 1, the *P. anubis* HAPLN 1 sequence must have diverged earlier since they both share a common ancestry.

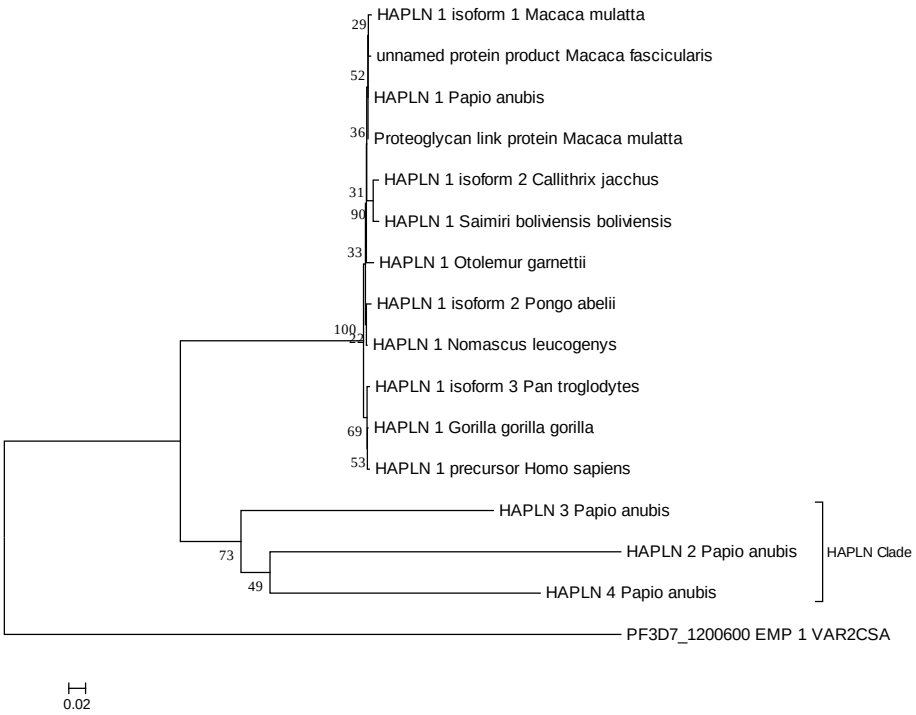


Figure 6: Phylogenetic relationship of putative HAPLN proteins in *P. anubis* and other non-human primate homologues

The Neighbor-Joining tree was constructed using the Poisson correction method for 16 HAPLN sequences. The numbers at the juncture of two branches are the percent of 1000 bootstrap trees with the same branch length. *P. falciparum* sequence Pf3D7_1220600 EMP 1 VAR2CSA was included as an out-group. **Key**: HAPLN (hyaluronan and proteoglycan link protein), EMP (erythrocyte membrane protein)

The scavenger receptor class B member (SRCBM) proteins 1 and 2 diverged from similar ancestry to platelet glycoprotein 4 (PG 4) from *P. anubis* and other non-human primate homologues (Figure 7). They both belong to a diverse group of scavenger receptor proteins. The PG 4 sequence from *P. anubis* clustered to PG 4 sequence from *M. mulatta* while human CD36 antigen clustered with higher non-human primate homologues though they both share common ancestry.

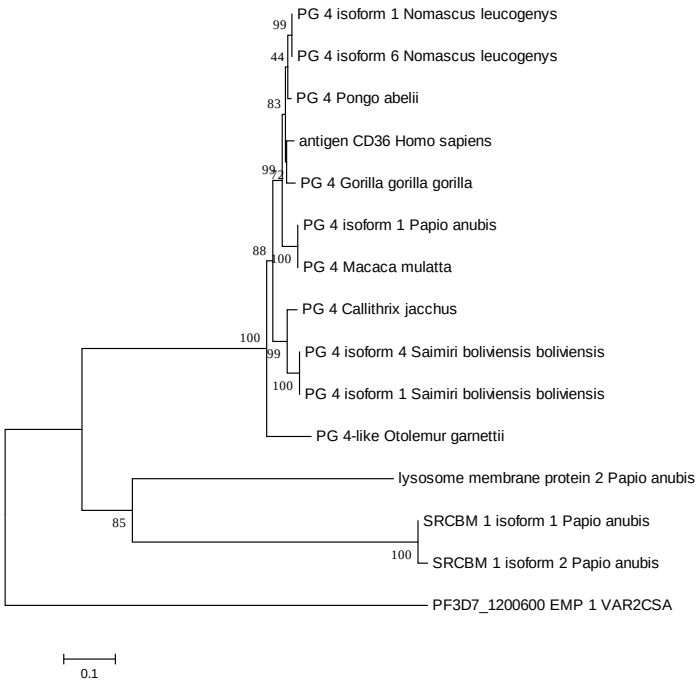


Figure 7: Phylogenetic relationship of putative CD36 proteins in *P. anubis* and other non-human primate homologues

The Neighbor-Joining tree was constructed using the Poisson correction method for 15 CD36 sequences. The numbers at the juncture of two branches are the percent of 1000 bootstrap trees with the same branch length. *P. falciparum* sequence Pf3D7_1220600 EMP 1 VAR2CSA was included as an out-group. **Key**: PG (platelet glycoprotein), EMP (erythrocyte membrane protein), SRCBM (scavenger receptor class B member protein)

3.2.2 Domain organization of the putative *P. anubis* receptors

The domain structure of the identified receptor molecules was determined by aligning them to Pfam and NCBI CDD databases and comparing them to domains of the human receptor molecules to determine probable amino acid residues involved in binding. The structure of the domains was determined from InterPro domain database. The identified Olive baboon and human receptor sequences showed similar domain organization (Figure 8 and Table 8). The predicted CSPG 4, partial sequence of *P. anubis* and CSA (CSPG) of human origin had the domain Laminin-G. Likewise, the predicted HAPLN 1 sequence of *P. anubis* and HA (HAPLN 1) from humans had the domains immunoglobulin V-set and

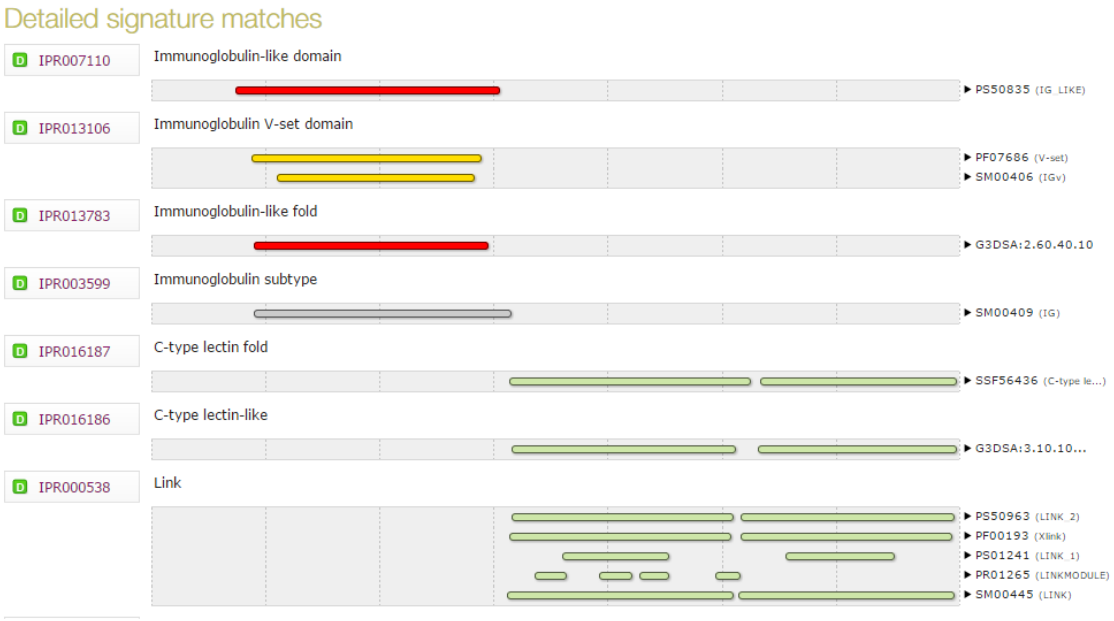
extracellular link protein. The human receptor CD36 molecule (thrombospondin receptor) also had similar domain organization as the predicted *P. anubis* receptors.

Table 8: Comparison of domain organization of identified baboon receptor molecules and human receptors

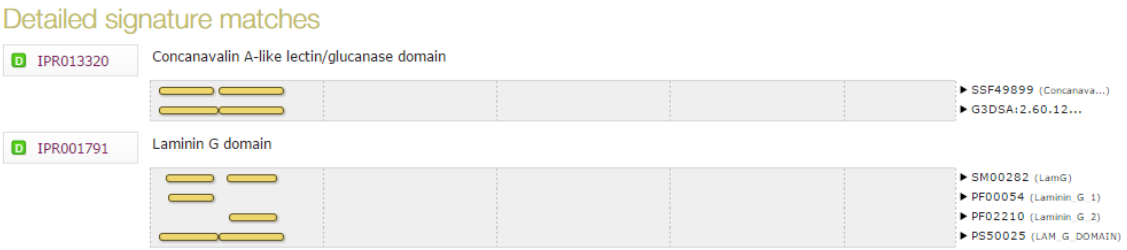
Sequence	Organism	Pfam and NCBI CDD Domains
CSPG		
CSA (CSPG)	*H. sapiens*	Laminin Domain (G1 and G2)
Predicted: CSPG 4, partial	*P. anubis*	Laminin Domain (G1 and G2)
PREDICTED: neurocan core protein; PREDICTED: aggrecan core protein; PREDICTED: LQP versican core protein; PREDICTED: brevican core protein	*P. anubis*	Immunoglobin V-set domain, Xlink (Extracellular link domain), Xlink (Extracellular link domain), EGF-like domain, Lectin C (Lectin C-type domain), Sushi (Sushi domain – SCR repeat)
PREDICTED: FRAS1-related EMP 3; PREDICTED: LQP FRAS1-related EMP 2	*P. anubis*	Calx-beta domain
PREDICTED: LQP: PUP FLJ43447-like	*P. anubis*	None
HAPLN 1		
HA (HAPLN 1)	*H. sapiens*	Immunoglobin V-set domain ; Xlink (Extracellular link domain), Xlink (Extracellular link domain)
Predicted: HAPLN 1; PREDICTED: LQP: HAPLN 3; PREDICTED: HAPLN 4; PREDICTED: HAPLN 2	*P. anubis*	Immunoglobin V-set domain ; Xlink (Extracellular link domain), Xlink (Extracellular link domain)
CD36 molecule		
CD36 (Thrombospondin receptor)	*H. sapiens*	CD36 (CD36 family)
Predicted: PG 4 isoform 1; PREDICTED: LMP 2; PREDICTED: SRCBM 1 isoform 2; PREDICTED: SRCBM 1 isoform 1	*P. anubis*	CD36 (CD36 family)

Source (Pfam and NCBI CDD website) **Key**: CSPG (chondroitin sulphate proteoglycan), HAPLN (hyaluronan and protein link protein), LQP (low quality protein), PUP (putative uncharacterized protein), PG (platelate glycoprotein), LMP (lysosome membrane protein), SRCBM (scavenger receptor class B member (SRCBM), SCR (short consensus repeats)

A



B



C

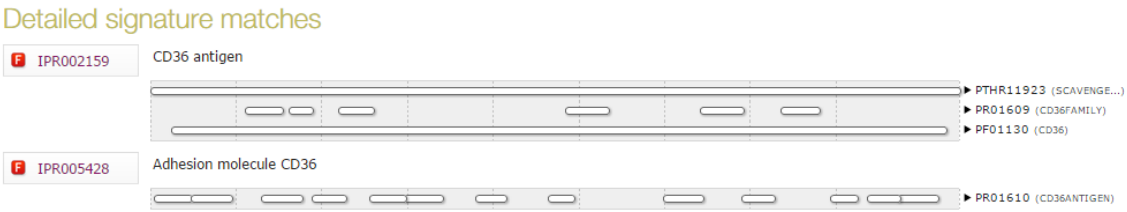


Figure 8: Domain organization of receptor molecules.

Source (InterPro 2015) **Key**: Hyaluronan and protein link protein 1 (HAPLN 1) domains (A), Chondroitin sulphate proteoglycan (CSPG) domains (B), and CD36 domains (C)

3.3 Putative ligand molecules in *P. knowlesi*

3.3.1 Sequence similarity search of *P. knowlesi* genome

The sequences of known erythrocyte binding ligands from *Plasmodium* (Table 2) were aligned against the *P. knowlesi* genome hosted at PlasmoDB (ver. 9.3, Mar 13) using BLASTp. The top BLAST hits with highest identities and least expectation value (E-value cut off <1e-04) were selected for further analysis (Table 9). The top hits were against the *P. knowlesi* erythrocyte binding proteins; EBP-*alpha*, EBP-*beta* and EBP-*gamma* which on alignment had low expectation values (4.3e-09 to 5.3e-13, cut off <1e-04 against *P. falciparum* Var2csa). Most of the other matches were conserved *Plasmodium* proteins of unknown function while some have been annotated for functions other than adhesion.

The SICAvar antigen (SICAvar-HB205) of *P. knowlesi* produced hits of low expectation values (E-value=0.00, cut off <1e-04) with high identity to its variants (SICAvar antigen and SICAvar antigen (fragment)) (Table 9). Even though *P. knowlesi* and *P. falciparum* are phylogenetically distantly related, no significant level of sequence identity has been observed in alignments of SICAvar and var gene families or their encoded proteins. The SICAvar antigens are orthologous in relation to var antigens and only share significant common sequences. These data affirm that the SICAVar antigen molecules found in *P. knowlesi* do not align to the *P. falciparum* binding ligands but align to its variants in *P. knowlesi* genome.

Table 9: Summary of BLAST hits from *P. knowlesi* genome when aligned with known erythrocyte binding ligands

Query sequence	Sequences producing HSP (proteins)	Accession No	PID	E-value
PKH_000490 (erythrocyte binding protein (beta))	Erythrocyte binding protein (beta)	PKH_000490	94	0.0
	Erythrocyte binding protein (gamma)	PKH_134580	56	1.3e-274
	Erythrocyte binding protein (alpha)	PKH_062300	57	1.2e-273
	Merozoite adhesive erythrocytic binding protein	PKH_094500	37	3.6e-15
	Merozoite surface protein	PKH_145630	23	1.4e-05
	Hypothetical protein, conserved in *Plasmodium*	PKH_130670	22	5.5e-05
	Ada2-like protein, putative	PKH_081460	23	1.9e-04
	Dynein heavy chain, putative	PKH_130100	25	2.1e-04
	Hypothetical protein, conserved in *Plasmodium*	PKH_140190	19	5.1e-04
	Ribonuclease, putative	PKH_113360	21	7.9e-04
PKH_062300 (erythrocyte binding protein (alpha))	Erythrocyte binding protein (alpha)	PKH_062300	94	0.0
	Erythrocyte binding protein (gamma)	PKH_134580	81	0.0
	Erythrocyte binding protein (beta)	PKH_000490	60	0.0
	Merozoite adhesive erythrocytic binding protein	PKH_094500	37	1.1e-17
	Protein phosphatase, putative	PKH_093290	22	3.6e-05
	Dynein heavy chain, putative	PKH_130100	25	4.0e-05
	Conserved *Plasmodium* protein, unknown function	PKH_060900	25	2.2e-04
	Conserved *Plasmodium* protein, unknown function	PKH_140190	20	6.8e-04
	Conserved *Plasmodium* protein, unknown function	PKH_061100	21	8.1e-04
PKH_134580 (erythrocyte binding protein (gamma))	Erythrocyte binding protein (gamma)	PKH_134580	93	0.0
	Erythrocyte binding protein (alpha)	PKH_062300	81	0.0
	Erythrocyte binding protein (beta)	PKH_000490	62	2.0e-262
	Merozoite adhesive erythrocytic binding protein	PKH_094500	37	1.8e-18
	Dynein heavy chain, putative	PKH_130100	23	8.3e-06
	Conserved *Plasmodium* protein, unknown function	PKH_103220	20	8.3e-04
PF3D7_0731500 (erythrocyte binding antigen-175 (EBA175))	Erythrocyte binding protein (gamma)	PKH_134580	34	3.6e-62
	Erythrocyte binding protein (alpha)	PKH_062300	32	1.6e-56
	Erythrocyte binding protein (beta)	PKH_000490	21	2.8e-52
	Conserved *Plasmodium* protein, unknown function	PKH_143120	20	1.1e-15
	Merozoite adhesive erythrocytic binding protein	PKH_094500	19	4.3e-15
	Conserved *Plasmodium* protein, unknown function	PKH_103220	25	2.1e-12
	Conserved *Plasmodium* protein, unknown function	PKH_121410	22	2.7e-11
	Conserved *Plasmodium* protein, unknown function	PKH_094540	20	1.7e-10
	Conserved *Plasmodium* protein, unknown function	PKH_092240	22	3.7e-10
	Conserved *Plasmodium* protein, unknown function	PKH_041690	21	4.7e-10
	Conserved *Plasmodium* protein, unknown function	PKH_031040	22	1.2e-09
	Tryptophan-rich antigen	PKH_146990	22	2.1e-08
PF3D7_0102500 (erythrocyte binding antigen-181 (EBA181))	Erythrocyte binding protein (beta)	PKH_000490	30	3.8e-53
	Erythrocyte binding protein (gamma)	PKH_134580	28	5.1e-50
	Erythrocyte binding protein (alpha)	PKH_062300	31	3.2e-48
	Merozoite adhesive erythrocytic binding protein	PKH_094500	25	2.1e-18
	Conserved *Plasmodium* protein, unknown function	PKH_126570	41	2.1e-06
	Conserved *Plasmodium* protein, unknown function	PKH_092240	22	5.8e-06
	Conserved *Plasmodium* protein, unknown function	PKH_131300	23	8.5e-06
	Conserved *Plasmodium* protein, unknown function	PKH_052170	24	1.1e-05
	Dynein heavy chain, putative	PKH_092040	21	1.2e-04

Query sequence	Sequences producing HSP (proteins)	Accession No	PID	E-value
	Conserved *Plasmodium* protein, unknown function	PKH_121410	19	3.0e-04
	Conserved *Plasmodium* protein, unknown function	PKH_101030	23	3.7e-04
	Rhomboid protease, putative	PKH_133640	24	4.4e-04
	Conserved *Plasmodium* protein, unknown function	PKH_143120	19	4.4e-04
	Conserved *Plasmodium* protein, unknown function	PKH_103220	20	4.6e-04
PF3D7_0424300 (erythrocyte binding antigen-165, pseudogene (EBA165))	Erythrocyte binding protein (beta)	PKH_000490	39	1.5e-58
	Erythrocyte binding protein (gamma)	PKH_134580	23	1.1e-42
	Erythrocyte binding protein (alpha)	PKH_062300	23	1.9e-39
	Merozoite adhesive erythrocytic binding protein	PKH_094500	21	1.2e-16
	Conserved *Plasmodium* protein, unknown function	PKH_130670	23	4.6e-05
	Conserved *Plasmodium* protein, unknown function	PKH_081490	21	3.6e-04
	Zinc finger protein, putative	PKH_100710	22	5.7e-04
	Conserved *Plasmodium* protein, unknown function	PKH_062110	21	7.4e-04
PF3D7_1301600 (erythrocyte binding antigen-140 (EBA140))	Erythrocyte binding protein (gamma)	PKH_134580	30	2.3e-47
	Erythrocyte binding protein (alpha)	PKH_062300	32	1.6e-38
	Erythrocyte binding protein (beta)	PKH_000490	25	5.7e-37
	Merozoite adhesive erythrocytic binding protein	PKH_094500	22	1.0e-23
	Hypothetical protein, conserved in Apicomplexans	PKH_130600	28	1.8e-10
	Conserved *Plasmodium* protein, unknown function	PKH_041690	21	4.2e-08
	Conserved *Plasmodium* protein, unknown function	PKH_100260	22	4.3e-06
	Conserved *Plasmodium* protein, unknown function	PKH_125560	21	6.0e-06
	Conserved *Plasmodium* protein, unknown function	PKH_121410	21	1.2e-05
	Conserved *Plasmodium* protein, unknown function	PKH_126220	27	1.5e-05
	Conserved *Plasmodium* protein, unknown function	PKH_101030	24	2.2e-05
	Conserved *Plasmodium* protein, unknown function	PKH_133810	28	2.6e-05
	Plasmodium exported protein, unknown function	PKH_120020	23	3.4e-05
	Atypical protein kinase, ABC-1 family, putative	PKH_133270	30	4.8e-05
	Conserved *Plasmodium* protein, unknown function	PKH_030800	26	5.7e-05
	Conserved *Plasmodium* protein, unknown function	PKH_050220	26	8.9e-05
PF3D7_1200600 (erythrocyte membrane protein 1, PfEMP1 (VAR2CSA))	Erythrocyte binding protein (beta)	PKH_000490	27	5.3e-13
	Erythrocyte binding protein (gamma)	PKH_134580	25	2.2e-12
	Erythrocyte binding protein (alpha)	PKH_062300	23	4.3e-09
	Reticulocyte binding protein, putative	PKH_070003	24	4.0e-08
	Conserved *Plasmodium* protein, unknown function	PKH_081890	21	6.1e-07
	Conserved *Plasmodium* protein, unknown function	PKH_145780	20	2.5e-05
	Conserved *Plasmodium* protein, unknown function	PKH_090490	21	6.0e-05
	Conserved *Plasmodium* protein, unknown function	PKH_100260	23	2.5e-04
	RNA binding protein, putative	PKH_142610	24	5.6e-04
	Chromosome associated protein, putative	PKH_051770	20	5.8e-04
	Conserved *Plasmodium* protein, unknown function	PKH_112280	22	7.1e-04
	Conserved *Plasmodium* protein, unknown function	PKH_133180	23	7.5e-04
GI:23507807 (Erythrocyte membrane protein 1 (PfEMP1))	Erythrocyte binding protein (beta)	PKH_000490	23	3.1e-06
	Erythrocyte binding protein (alpha)	PKH_062300	21	2.7e-04
	Erythrocyte binding protein (gamma)	PKH_134580	19	7.4e-04
GI:34525754 (Erythrocyte membrane protein 1)	Erythrocyte binding protein (alpha)	PKH_062300	26	6.2e-10
	Erythrocyte binding protein (gamma)	PKH_134580	25	1.3e-09
	Erythrocyte binding protein (beta)	PKH_000490	22	4.2e-08
	Ubiquitin transferase, putative	PKH_112120	29	8.4e-04
	Erythrocyte binding protein (gamma)	PKH_134580	30	4.2e-14

Query sequence	Sequences producing HSP (proteins)	Accession No	PID	E-value
GI:6165411 (FCR3 CSA ligand)	Erythrocyte binding protein (beta)	PKH_000490	32	2.0e-13
	Erythrocyte binding protein (alpha)	PKH_062300	29	2.4e-11
	Hypothetical protein, conserved in Apicomplexans	PKH_130600	27	2.1e-07
	Conserved *Plasmodium* protein, unknown function	PKH_145780	23	1.9e-06
	Conserved *Plasmodium* protein, unknown function	PKH_062110	22	5.0e-04
GI:74766456 (PLAFA Variant-specific surface protein)	Erythrocyte binding protein (beta)	PKH_000490	23	2.6e-11
	Erythrocyte binding protein (gamma)	PKH_134580	22	4.0e-11
	Erythrocyte binding protein (alpha)	PKH_062300	25	6.0e-06
	Rhoptry associated membrane antigen, putative	PKH_010540	21	2.2e-05
	CCAAT-box DNA binding protein	PKH_094400	19	2.1e-04
GI: 29293851 (Erythrocyte membrane protein 1)	Erythrocyte binding protein (beta)	PKH_000490	28	2.4e-08
	Erythrocyte binding protein (gamma)	PKH_134580	21	2.8e-08
	Erythrocyte binding protein (alpha)	PKH_062300	26	2.1e-07
	Hypothetical protein, conserved in Apicomplexans	PKH_130600	22	7.0e-05
	Conserved *Plasmodium* protein, unknown function	PKH_133810	19	1.0e-04
PVX_110810 (Duffy receptor precursor)	Erythrocyte binding protein (alpha)	PKH_062300	52	7.1e-252
	Erythrocyte binding protein (gamma)	PKH_134580	50	1.0e-243
	Erythrocyte binding protein (beta)	PKH_000490	51	5.3e-199
	Merozoite adhesive erythrocytic binding protein	PKH_094500	34	2.8e-29
	Conserved *Plasmodium* protein, unknown function	PKH_083470	32	2.2e-07
	Conserved *Plasmodium* protein, unknown function	PKH_146600	30	2.7e-07
	Conserved *Plasmodium* protein, unknown function	PKH_140190	31	1.8e-06
	Pre-mRNA splicing factor, putative	PKH_060650	29	2.7e-06
	Eukaryotic translation initiation factor 4 gamma	PKH_141200	26	2.2e-05
	Conserved *Plasmodium* protein, unknown function	PKH_121410	22	6.8e-05
	Splicing factor, putative	PKH_123140	19	3.1e-04
	Conserved *Plasmodisum* protein, unknown function	PKH_081330	20	6.6e-04
	Conserved *Plasmodium* protein, unknown function	PKH_031040	17	6.9e-04
(SICAvar-HB205)	SICAvar antigen (fragment)	PKH_052830	97	0.00
	SICAvar antigen	PKH_112900	49	0.00
	SICAvar antigen (fragment)	PKH_144880	36	0.00
	SICAvar antigen	PKH_120300	36	0.00

Source (PlasmoDB Ver 9.3, Mar 13) **Key**: E-value (Expectation value), PID (Percentage identity), SICA (schizont-infected cell agglutination antigen). PKH_XXX (*P. knowlesi* accession number). An E-value cut-off of <1e-04 was used to select homologous *P. knowlesi* sequences

Fifty seven (57) protein sequences were selected following analysis of BLAST hits as the putative ligands in *P. knowlesi* genome (Table 10). Three of these sequences (5%) are erythrocyte binding proteins – *alpha*, *beta* and *gamma* (Appendix 2). Thirty four of the sequences (60%) are conserved *Plasmodium* proteins of unknown function, some are merozoite surface proteins and others are annotated for varying functions (for example Ada2-like protein, Dynein heavy chain, , Rhomboid protease, Zinc finger protein, Reticulocyte binding protein) (Table 10). Therefore, since the *P. knowlesi* erythrocyte

binding ligands (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) demonstrated high identities and low expectation values (E-value cut off <1e-04) against the known erythrocyte binding ligands they were supposed to be the putative ligands used for adhesion.

Table 10: List of identified putative ligand molecules in *P. knowlesi* genome

Sequences producing HSP (proteins)	Accession number
Erythrocyte binding protein (beta)	PKH_000490
Erythrocyte binding protein (gamma)	PKH_134580
Erythrocyte binding protein (alpha)	PKH_062300
Reticulocyte binding protein, putative	PKH_070003
Chromosome associated protein, putative	PKH_051770
Conserved *Plasmodium* protein, unknown function	PKH_060900, PKH_061100, PKH_103220, PKH_143120, PKH_121410, PKH_094540, PKH_092240, PKH_041690, PKH_031040, PKH_126570, PKH_131300, PKH_052170, PKH_101030, PKH_081490, PKH_062110, PKH_100260, PKH_125560, PKH_126220, PKH_133810, PKH_030800, PKH_050220, PKH_081890, PKH_145780, PKH_090490, PKH_112280, PKH_133180, PKH_062110, PKH_083470, PKH_146600, PKH_081330
RNA binding protein, putative	PKH_142610
Ada2-like protein, putative	PKH_081460
Atypical protein kinase, ABC-1 family, putative	PKH_133270
CCAAT-box DNA binding protein subunit B	PKH_094400
Dynein heavy chain, putative	PKH_130100, PKH_092040
Eukaryotic translation initiation factor 4 gamma	PKH_141200
Hypothetical protein, conserved in Apicomplexans	PKH_130600
Hypothetical protein, conserved in *Plasmodium*	PKH_130670, PKH_140190
Merozoite adhesive erythrocytic binding protein	PKH_094500
Merozoite surface protein	PKH_145630
Plasmodium exported protein, unknown function	PKH_120020
Pre-mRNA splicing factor, putative	PKH_060650
Protein phosphatase, putative	PKH_093290
Rhomboid protease, putative	PKH_133640
Rhoptry associated membrane antigen, (RAMA)	PKH_010540
Ribonuclease, putative	PKH_113360
Splicing factor, putative	PKH_123140
Tryptophan-rich antigen	PKH_146990
Ubiquitin transferase, putative	PKH_112120
Zinc finger protein, putative	PKH_100710

Source (PlasmoDB Ver 9.3, Mar 13) **Key**: E-value (Expectation value), PID (Percentage identity). PKH_XXX (*P. knowlesi* accession number). An E-value cut-off of <1e-04 was used to select homologous *P. knowlesi* sequences

3.4 Characterization of putative *P. knowlesi* ligands

3.4.1 Analysis of putative *P. knowlesi* ligands

To functionally annotate the putative *P. knowlesi* ligand molecules they were searched against gene ontology (GO) database. Gene ontology annotates molecules in terms of their biological process, cellular component and molecular function. Further, the molecules' protein parameters were determined and documented from ExPASY-ProtParam. Some of the molecules (47%) are not annotated for any function in their ontology. The rest are annotated for various functions (Table 11). Notably, some are annotated for the biological process of pathogenesis, are integral to the membrane in location and function in either binding or receptor activity (PKH_062300, PKH_134580, PKH_000490, PKH_094500, PKH_140190 and PKH_083470).

The putative ligand molecules are located on different chromosomes in the genome of *P. knowlesi*. Majority of them are found along chromosomes 12 to 14 (42%), few along chromosomes 1 to 7 (23%) and the rest (35%) are found along chromosomes 8 to 11 (Table 12). The numbers of amino acids for the putative ligand molecules ranged from 175 to 9173. Predicted molecular weight also varied with range of 20.58 to 1054.77 kDa while the isoelectric points reported were mostly alkaline with an average of 7.06 (range 3.86-10.48). The molecules (PKH_062300, PKH_134580, PKH_000490, PKH_094500, PKH_140190 and PKH_083470) are involved in binding in *P. knowlesi* since they show similar gene ontology to the *P. falciparum* binding ligand var2csa.

Table 11: Functional annotation of putative ligand molecules from *P. knowlesi* genome producing best hits to gene ontology (GO) database

Sequence	Match to GO DB					
	BP	**GO No.**	**CC**	**GO No.**	**MF**	**GO No.**
PKH_000490	Pathogenesis	0009405	integral to membrane	0016021	receptor activity	0004872
PKH_134580	Pathogenesis	0009405	integral to membrane	0016021	receptor activity	0004872
PKH_062300	Pathogenesis	0009405	integral to membrane	0016021	receptor activity	0004872
PKH_070003	-	-	-	-	calcium ion binding	0005509
PKH_051770	chromosome organization	0051276	chromosome	0005694	protein and ATP binding	0005515 and 0005524
PKH_060900	-	-	apicoplast	0020011	-	-
PKH_061100	-	-	apicoplast	0020011	-	-
PKH_103220	-	-	cell surface	0009986		
PKH_143120	-	-	-	-	RNA, Protein binding, Transferase activity	0003723, 0005515 and 0016740
PKH_121410	carbohydrate metabolic process	0005975	extracellular region	0005576	carbohydrate binding	0030246
PKH_094540	-	-	-	-	RNA binding	0003723
PKH_092240	-	-	apicoplast	0020011	-	-
PKH_041690	-	-				
PKH_031040	intracellular transport	0046907	-	-	-	-
PKH_126570	-	-	Cellular component	0005575	Molecular function	0003674
PKH_131300	-	-	apicoplast	0020011	-	-
PKH_052170	-	-	apicoplast	0020011	-	-
;PKH_101030	-	-	Cellular component, nucleus	0005575 and 0005634	Molecular function	0003674
PKH_081490	-	-	apicoplast	0020011	-	-
PKH_062110	-	-	Host cell cytoplasm	0030430	Kinase activity	0016301
PKH_100260	-	-	Cytoplasm, membrane	0005737 and 0016020	Molecular function	0003674
PKH_125560	Entry to host	0044409	Rhoptry neck	1990225	-	-
PKH_126220	-	-	membrane	0016020	-	-
PKH_133810			apicoplast	0020011		
PKH_030800	Entry to host cell	0030260	Apical complex, rhoptry	00200007 and 00200008	Host cell surface binding	0046812
PKH_050220	-	-	Cellular component, cytoplasm	0005575 and 0005737	Molecular function	0003674

Sequence	Match to GO DB					
	BP	**GO No.**	**CC**	**GO No.**	**MF**	**GO No.**
PKH_081890	-	-	Cytoplasm, membrane	0005737 and 0016020	Molecular function	0003674
PKH_145780	-	-	-	-	nucleotide binding	0000166
PKH_090490	-	-	Cytoplasm, membrane	0005737 and 0016020	Molecular function	0003674
PKH_112280	-	-	Cytoplasm, membrane	0005737 and 0016020	Molecular function	0003674
PKH_133180	-	-	Host cell cytoplasm	0030430	Kinase activity	0016301
PKH_083470	protein targeting to Golgi	0000042			protein binding	0005515
PKH_146600	-	-	Cellular component	0005575	Molecular function, protein domain specific binding	0003674, 0019904
PKH_081330	-	-	-	-	Molecular function	0003674
PKH_142610	-	-	-	-	nucleic acid binding	0003676
PKH_081460	-	-	-	-	DNA, chromatin and Zinc ion binding	0003677, 0003682 and 0008270
PKH_133270	-	-	-	-	-	-
PKH_094400			intracellular	0005622	Sequence specific DNA binding	0043565
PKH_130100	microtubule-based movement	0007018	dynein complex	0030286	microtubule motor activity	0003777
PKH_092040	microtubule-based movement	0007018	dynein complex	0030286	microtubule motor activity, ATP binding, ATPase and nucleoside triphosphate activity	0003777, 0005524, 0016887 and 0017111
PKH_141200	RNA metabolic process	0016070	-	-	DNA, RNA and protein binding	0003677, 0003723 and 0005515
PKH_130600	-	-	Cellular component	0005575	Molecular function, protein domain specific binding	0003674, 0019904
PKH_130690	translation	0006412	ribosome	0005840	structural constituent of ribosome	0003735
PKH_140190	-	-	-	-	protein binding	0005515
PKH_094500	pathogenesis	0009405	Membrane and apical complex	0016020 and 0020007	binding	0005488
PKH_145630	Entry into host cell	0030260	Cell surface, plasma membrane	0009986 and 0005886	Heme binding	0020037
PKH_120020	-	-	chromosome, telomeric region	0000781	telomeric DNA binding	0042162
PKH_060650	-	-	-	-	nucleic acid binding	0003676
PKH_093290	-	-	-	-	catalytic activity	0003824
PKH_133640	-	-	integral to membrane	0016021	serine-type endopeptidase activity	0004252
PKH_010540	-	-	apicoplast	0020011	-	-
PKH_113360					RNA binding and ribonuclease activity	0003723 and 0004540

Sequence	Match to GO DB					
	BP	**GO No.**	**CC**	**GO No.**	**MF**	**GO No.**
PKH_123140	mRNA processing	0006397	nucleus	0005634	Nucleic acid and RNA binding	0003676 and 0003723
PKH_146990	-	-	Cell surface	0009986	-	-
PKH_112120	cellular protein modification process	0006464	intracellular	0005622	Protein binding and acid-amino acid ligase activity	0005515 and 0016881
PKH_100710					Nucleic acid and zinc ion binding	0003676 and 0008270

Source (Gene ontology database release 2015-04-18). **Key**: biological process (BP), cellular component (CC), molecular function (MF), gene ontology (GO).

Table 12: Predicted Molecular weight and Isoelectric points for putative *P. knowlesi* ligand molecules

Accession No.	Location	Length (Aa)	Mol. Weight (Da)	pI
PKH_000490	1,863 to 5,920	1154	130481	4.79
PKH_134580	Chr13: 2,177,465 to 2,181,275	1072	121212	4.9
PKH_062300	Chr6: 1,032,863 to 1,036,679	1073	120703	5.29
PKH_070003	Chr7: 8,828 to 18,024	2844	344108	6.69
PKH_051770	Chr5: 793,561 to 800,609	1193	140531	7.7
PKH_060900	Chr6: 426,927 to 430,622	1231	141313	4.29
PKH_061100	Chr6: 502,113 to 507,287	1724	192739	10.02
PKH_103220	Chr10: 1,455,731 to 1,466,680	1568	159469	5.15
PKH_143120	Chr14: 1,429,011 to 1,438,213	1652	194311	4.17
PKH_121410	Chr12: 630,081 to 634,832	1583	176959	5.08
PKH_094540	Chr9: 2,106,607 to 2,111,130	1130	135372	4.27
PKH_092240	Chr9: 986,223 to 991,010	1595	181982	4.37
PKH_041690	Chr4: 734,902 to 740,466	1854	217077	5
PKH_031040	Chr3: 523,717 to 527,129	607	68156	4.06
PKH_126570	Chr12: 2,845,961 to 2,850,019	481	57705	9.6
PKH_131300	Chr13: 615,512 to 619,429	1306	149710	4.13
PKH_052170	Chr5: 965,222 to 968,315	842	97857	4.29
PKH_101030	Chr10: 514,632 to 519,997	1486	173506	8.82
PKH_081490	Chr8: 680,039 to 686,932	2297	255988	7.67
PKH_062110	Chr6: 940,976 to 946,909	1861	205736	9.79
PKH_100260	Chr10: 170,011 to 175,479	1822	209315	6.59
PKH_125560	Chr12: 2,399,748 to 2,402,267	839	97360	10.4
PKH_126220	Chr12: 2,675,025 to 2,697,654	6806	792939	8.57
PKH_133810	Chr13: 1,830,784 to 1,835,121	1445	167741	4.74
PKH_030800	Chr3: 399,430 to 403,702	851	100445	8.61
PKH_050220	Chr5: 148,645 to 151,737	1030	121772	6.65
PKH_081890	Chr8: 866,815 to 872,536	1691	195316	5.08
PKH_145780	Chr14: 2,586,597 to 2,602,757	5386	639217	5.28
PKH_090490	Chr9: 210,532 to 213,774	1080	127731	9.59
PKH_112280	Chr11: 1,140,808 to 1,143,552	914	106513	9.19
PKH_133180	Chr13: 1,534,695 to 1,539,509	1604	182749	9.14
PKH_083470	Chr8: 1,606,360 to 1,609,299	979	112853	4.48
PKH_146600	Chr14: 2,950,491 to 2,956,205	1904	208322	9.01
PKH_081330	Chr8: 602,756 to 604,958	451	53905	6.17
PKH_142610	Chr14: 1,158,270 to 1,161,173	967	114841	9.47
PKH_081460	Chr8: 660,328 to 667,950	2540	286150	8.51
PKH_133270	Chr13: 1,575,039 to 1,582,623	2274	261069	9.75
PKH_094400	Chr9: 1,982,157 to 1,986,309	1183	133258	4.36
PKH_130100	Chr13: 41,232 to 63,914	5607	666771	9.03
PKH_092040	Chr9: 882,319 to 898,091	5220	596531	4.9
PKH_141200	Chr14: 547,280 to 551,049	1132	127263	7.57
PKH_130600	Chr13: 275,798 to 283,680	2528	292616	8.25
PKH_130690	Chr13: 310,111 to 310,835	175	20581	9.87
PKH_140190	Chr14: 74,144 to 77,074	976	108375	5.78
PKH_094500	Chr9: 2,060,514 to 2,067,075	1971	226916	7.74
PKH_145630	Chr14: 2,529,932 to 2,532,694	920	101555	4.43
PKH_120020	Chr12: 3,896 to 6,013	491	57166	9.57

Accession No.	Location	Length (Aa)	Mol. Weight (Da)	pI
PKH_060650	Chr6: 292,097 to 294,727	495	56603	10.48
PKH_093290	Chr9: 1,460,126 to 1,463,059	977	104757	4.88
PKH_133640	Chr13: 1,747,160 to 1,750,002	791	92186	9.67
PKH_010540	Chr1: 297,692 to 300,683	711	80580	3.86
PKH_113360	Chr11: 1,607,800 to 1,617,144	2637	302798	9.76
PKH_123140	Chr12: 1,337,880 to 1,340,975	970	110720	9.57
PKH_146990	Chr14: 3,132,750 to 3,135,092	732	88299	7.44
PKH_112120	Chr11: 1,058,771 to 1,086,292	9173	1054765	7.92
PKH_100710	Chr10: 360,688 to 363,330	880	97291	9.51

Source: ExPASY-ProtParam 2015. **Key**: molecular weight (Mol. Weight), isoelectric point (pI). PKH_XXX (Accession number)

3.4.2 Domain organization of the putative *P. knowlesi* ligands

The putative ligand sequences were characterized by determining their domains from the InterPro domain databases. The reference *P. falciparum* ligand (Var2csa) was organized into 7 duffy binding domains. Following analysis more than half of the sequences (61%) had different domain organizations while 22 sequences (39%) were not annotated (Table 13). Among the annotated sequences, the erythrocyte binding proteins (PKH_000490, PKH_062300, and PKH_134580) are members of the protein families; duffy-antigen binding and erythrocyte binding antigen 175 (EBA-175 VI) which are similar to the reference *P. falciparum* ligand (Var2csa). The other sequences were annotated for different protein families like; structural maintenance of chromosomes protein, histidine phosphatase superfamily and dynein heavy chain (Table 13).

The analysis also revealed that the erythrocyte binding proteins (PKH_000490, PKH_062300, and PKH_134580) had the domains; duffy-antigen binding, *Plasmodium falciparum* erythrocyte membrane protein-duffy binding like (PfEMP-DBL) domain and erythrocyte binding antigen 175 (EBA-175 VI) (Figure 9). Similarly, the *P. falciparum* binding ligand (Var2csa) was organized into duffy-antigen binding domain, PfEMP-DBL domain and PfEMP 1 acidic terminal segment domain. Notably, one of the sequences (PKH_094500) was annotated for the protein family apical membrane antigen (AMA-1) and the domains AMA-1 and erythrocyte binding antigen 175 (EBA-175 VI) which

closely match those of the erythrocyte binding proteins. The other sequences were annotated to various domains for example zinc finger, dynein heavy chain, transcription adaptor 2, tryptophan/threonine-rich *Plasmodium* antigen and nucleic acid binding domains (Table 13). These data show that the *P. knowlesi* erythrocyte binding proteins (PKH_000490, PKH_062300, and PKH_134580) share domains with the *P. falciparum* binding ligand (Var2csa) indicating that they may be performing similar functions.

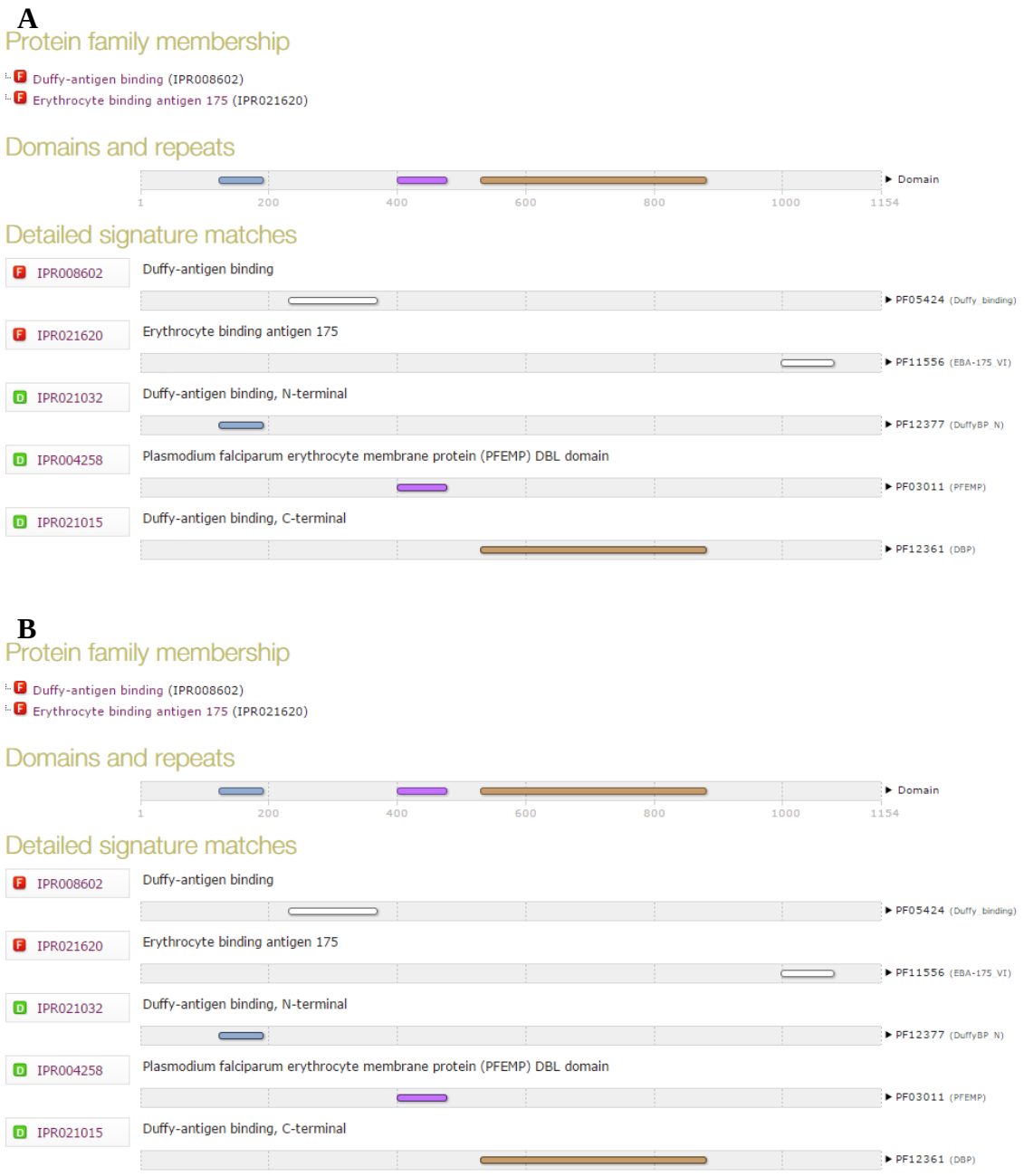


Figure 9: Domain organization of ligand molecules

Source (InterPro 2015) **Key**: PF3D7_1200600 (*P. falciparum* ligand Var2csa) domains (A); PKH_000490 (*P. knowlesi* erythrocyte binding protein-*beta*) domains (B)

Table 13: Summary of the domains of putative *P. knowlesi* ligands

Accession No.	InterPro Domains		Source
	Protein family membership	Domains and repeats	
PF3D7_1200600	Duffy-antigen binding	Duffy-antigen binding, PfEMP-DBL domain, PfEMP 1 acidic terminal segment	Pfam
PKH_000490	Duffy-antigen binding, EBA-175 VI	Duffy-antigen binding, EBA-175, duffy-antigen binding, N and C terminal, PfEMP DBL domain	Pfam
PKH_134580	Duffy-antigen binding, EBA-175 VI	Duffy-antigen binding, EBA-175, duffy-antigen binding, N and C terminal	Pfam
PKH_062300	Duffy-antigen binding, EBA-175 VI	Duffy-antigen binding, EBA-175, duffy-antigen binding, N and C terminal	Pfam
PKH_070003	NP	EF-hand domain	PS
PKH_051770	Structural maintenance of chromosomes protein	SMC-protein, SMC- protein 3, P-loop containing nucleoside triphosphate hydrolase, SMC-N terminal, SMC-hinge	PIR, PTHR, SSF, Pfam, SM
PKH_060900	NP	NP	-
PKH_061100	NP	NP	-
PKH_103220	NP	Tryptophan/threonine-rich plasmodium antigen, C-terminal	Pfam
PKH_143120	NP	Nucleotide-diphospho-sugar transferases, Trimeric LpxA-like, Armadillo-type fold, W2 domain, Pumilio RNA-binding repeat	SSFS, SSFS, SSF, PS, PS
PKH_121410	NP	Carbohydrate-binding module family 5/12	Pfam
PKH_094540	NP	RNA-binding, CRM domain, Tryptophan/threonine-rich plasmodium antigen, C-terminal, Pumilio RNA-binding repeat	PS, Pfam, PS
PKH_092240	NP	NP	-
PKH_041690	NP	NP	-
PKH_031040	NP	Pleckstrin homology-like domain, Ran binding domain	G3D, Pfam/SM
PKH_126570	NP	NP	-
PKH_131300	NP	NP	-
PKH_052170	NP	NP	-
PKH_101030	NP	NP	-
PKH_081490	NP	NP	-
PKH_062110	NP	NP	-
PKH_100260	NP	NP	-
PKH_125560	NP	mRNA splicing factor, Cwf21	Pfam
PKH_126220	NP	NP	-
PKH_133810	Histidine phosphatase superfamily	Histidine phosphatase superfamily	SSF
PKH_030800	NP	NP	-
PKH_050220	NP	NP	-
PKH_081890	NP	NP	-

Accession No.	InterPro Domains		Source
	Protein family membership	Domains and repeats	
PKH_145780	NP	NP	-
PKH_090490	NP	NP	-
PKH_112280	NP	NP	-
PKH_133180	NP	Btz domain	SM
PKH_083470	NP	GRIP	PS
PKH_146600	NP	NP	-
PKH_081330	NP	NP	-
PKH_142610	Cyclophilin-type peptidyl-prolyl cis-trans isomerase	Cyclophilin-type peptidyl-prolyl cis-trans isomerase, Nucleotide-binding alpha-beta plait domain, RNA recognition motif domain	PTHR, G3DS, Pfam/SM/ PS
PKH_081460	Transcriptional adaptor 2	Transcriptional adaptor 2, Zinc finger, ZZ-type, Homeodomain-like, SANT/Myb domain, SANT domain	PTHR, SM, SSF, Pfam, PS
PKH_133270	NP	Protein kinase-like domain, UbiB domain, Beta-lactamase-related	SSF, Pfam,
PKH_094400	NP	Histone-fold, Transcription factor CBF/NF-Y/archaeal histone	SSF, Pfam
PKH_130100	Dynein heavy chain	Dynein heavy chain, Dynein heavy chain domain	PTHR, Pfam
PKH_092040	Dynein heavy chain	Dynein heavy chain, P-loop containing D4 domain, AAA+ ATPase domain, Filamin/ABP280 repeat-like	Pfam, SM, PS
PKH_141200	NP	Armadillo-type fold, MIF4-like, type 3	SSF, Pfam/SM
PKH_130600	NP	NP	-
PKH_130690	Ribosomal protein L29	Ribosomal protein L29	SSF
PKH_140190	NP	LIS1 homology motif	Pfam/SM
PKH_094500	AMA-1, EBA-175	AMA-1, EBA-175	Pfam
PKH_145630	NP	NP	-
PKH_120020	NP	NP	-
PKH_060650	NP	Nucleotide-binding alpha-beta plait domain, RNA recognition motif domain	G3D, PS/SM
PKH_093290	NP	Protein phosphatase 2C (PP2C)-like domain	SM/Pfam
PKH_133640	Peptidase S54, rhomboid	Peptidase S54, rhomboid, Peptidase S54, rhomboid domain	PTHR, Pfam
PKH_010540	NP	NP	-
PKH_113360	NP	Nucleic acid-binding, OB-fold, Ribonuclease B, N-terminal OB domain, Ribonuclease II/R, conserved site	SSF, Pfam, PS
PKH_123140	Splicing factor, RBM39-like	Splicing factor, RBM39-like, Nucleotide-binding alpha-beta plait domain, RNA recognition motif domain, RNA recognition motif domain, eukaryote	TIGR, G3D, Pfam, SM
PKH_146990	NP	Tryptophan/threonine-rich plasmodium antigen, C-terminal	Pfam

Accession No.	InterPro Domains		Source
	Protein family membership	Domains and repeats	
PKH_112120	NP	Ankyrin repeat-containing domain, HECT, Ankyrin repeat	PS, Pfam, SM
PKH_100710	NP	Zinc finger, CCCH-type	SM

Source (InterPro 2015). **Key**: None predicted (NP), structural maintenance of chromosomes (SMC), erythrocyte binding antigen (EBA), Apical membrane antigen 1 (AMA-1), Domain databases: PTHR (PANTHER), G3D (CATH-Gene3D), Pfam (Protein families), SM (SMART- a Simple Modular Architecture Research Tool), PS (Prosite), TIGR (TIGRFAM), SSF (SUPERFAMILY),

3.5 Binding phenotype of *P. knowlesi* infected erythrocytes from *P. anubis*

3.5.1 Binding of parasite clones *P. falciparum* CS2 and 3D7

Binding of *P. falciparum* CS2 infected erythrocytes (IEs) to the receptors CSA and HA was significantly different (p=0.00) unlike in Pf3D7 where this was not significant (p=0.80) (Figure 10). The level of binding of the receptors CSA and HA when compared between the two parasite clones was significantly different (p=0.00 and p=0.00 respectively). Therefore, PfCS2 binds at high levels to CSA and HA while Pf3D7 binds at high levels to CD36.

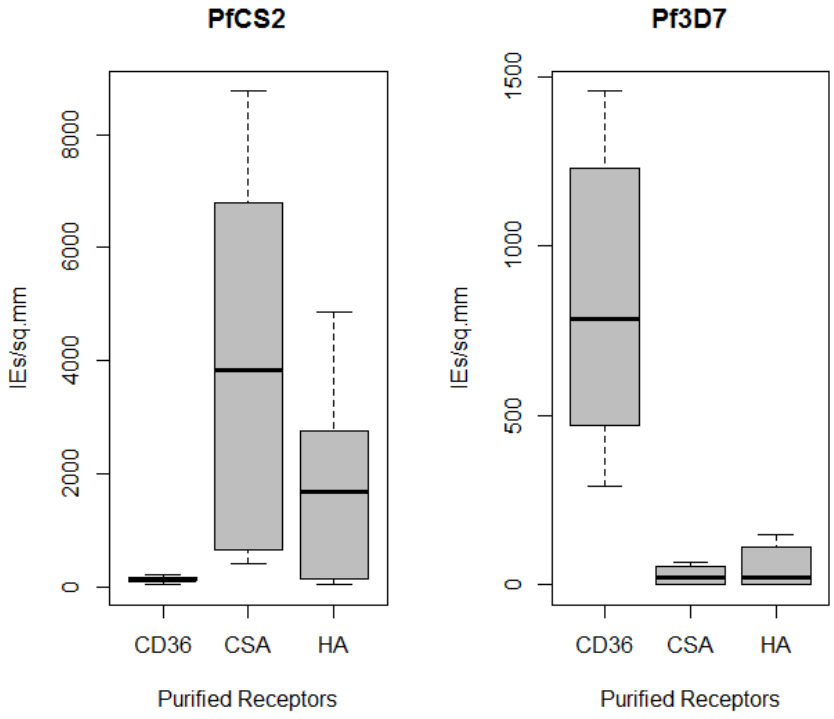


Figure 10: ***P. falciparum*** **CS2 and 3D7 infected erythrocytes binding to purified receptors CD36, CSA and HA.**

Data represents the median and interquartile ranges of adherent infected erythrocytes per mm^2 for the purified receptors at 50µg/ml concentration.

3.5.2 Effect of pH and receptor concentration on binding of infected erythrocytes

Binding of PfCS2 to CSA receptor at pH6.8 and 100µg/ml concentration was not significantly different from its binding at pH7.2 and 50µg/ml concentration (p=0.61 and p=0.53 respectively) (Figure 11A). The clone bound at low levels to CD36 receptor at pH7.2 and 25µg/ml concentration (107 IEs/mm^2). There was a significant difference in the binding of the clone at the two test concentrations of 25µg/ml and 50µg/ml (p=0.00) but no difference in binding between pH6.8 and pH7.2 (p=0.66) (Figure 11B).

Binding of Pf3D7 to CD36 receptor at pH6.8 and 50µg/ml concentration was not significantly different from its binding at pH7.2 and 25µg/ml concentration (p=0.18 and p=0.24 respectively). The clone did not bind to CSA at pH7.2 and to HA at pH6.8 (Figure 12B). The level of binding of the clone was lowest to CSA at pH6.8 and 50µg/ml concentration (66 IEs/mm^2) compared to the other receptors. There was no significant difference when binding was compared at 100µg/ml concentration for CSA and HA (p=0.67 and p=0.73 respectively) (Figure 12A). Therefore, the level of binding of Pf3D7 to CSA and HA was pH dependent but independent on concentration of receptors.

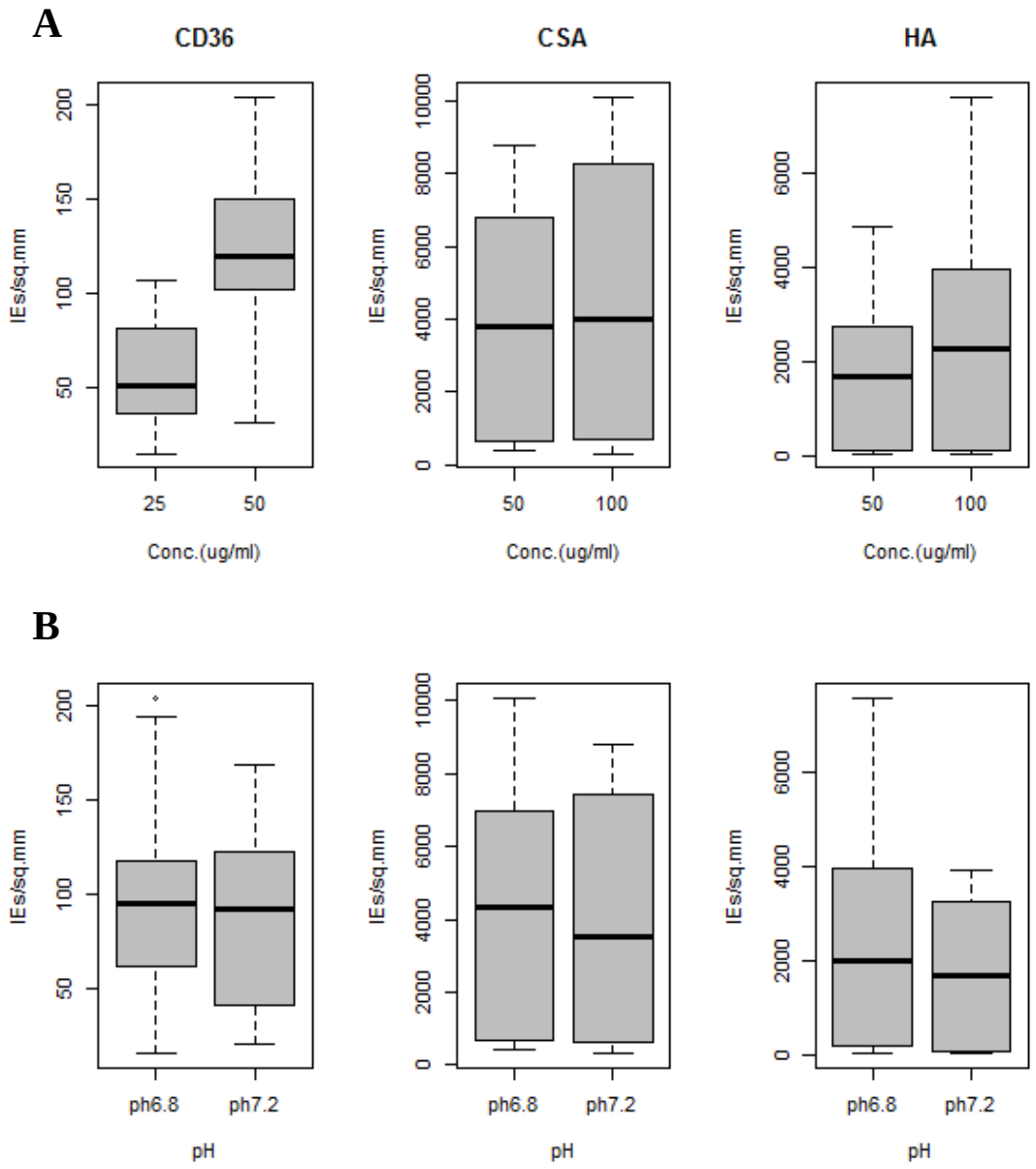


Figure 11: ***P. falciparum*** **CS2 infected erythrocytes binding to purified receptors CD36, CSA and HA.**

Data represents the median and interquartile ranges of adherent infected erythrocytes per mm^2 for the purified receptors

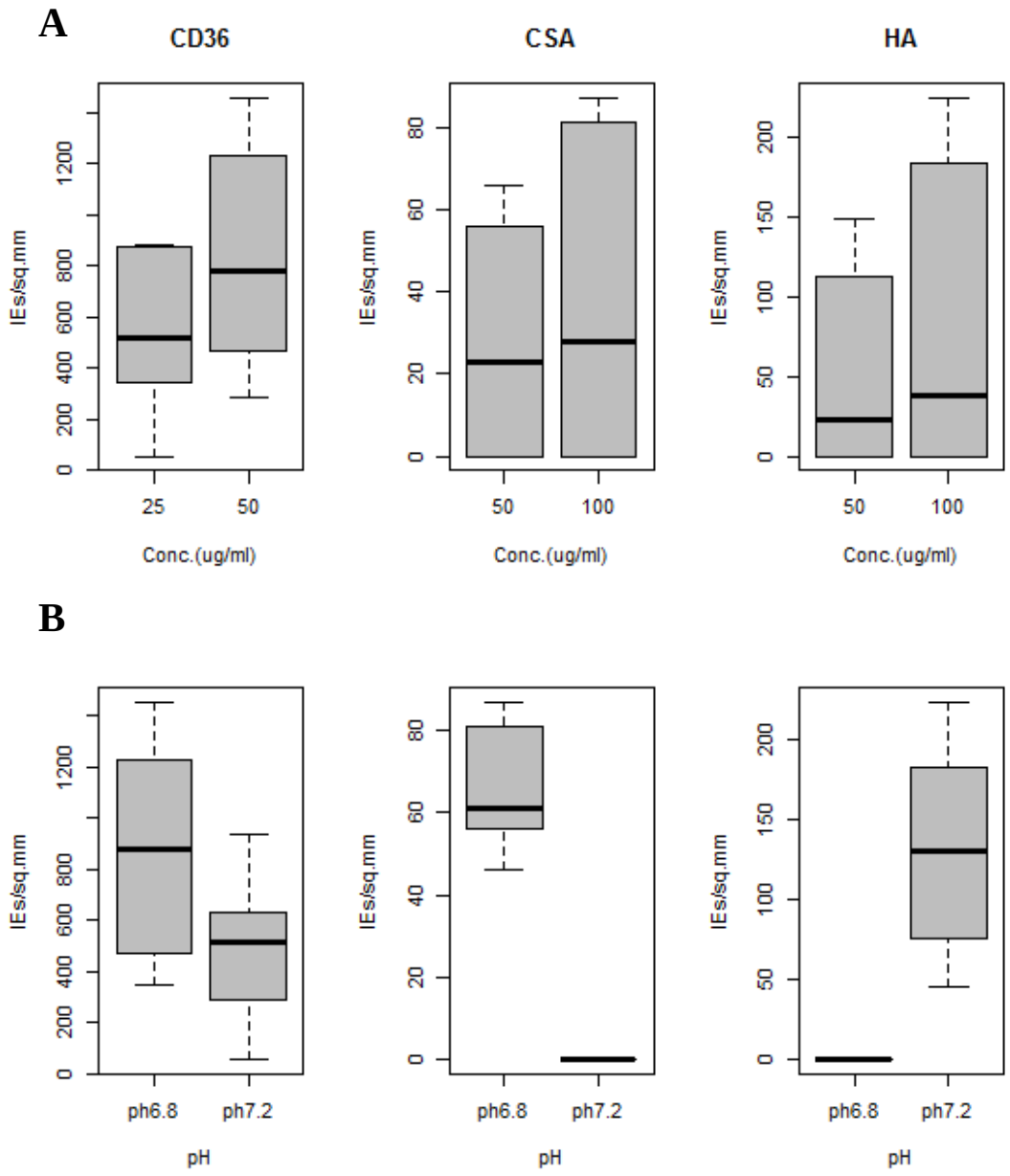


Figure 12: ***P. falciparum*** **3D7 infected erythrocytes binding to purified receptors CD36, CSA and HA.**

Data represents the median and interquartile ranges of adherent infected erythrocytes per mm^2 for the purified receptors

3.5.3 Binding of *P. knowlesi* infected erythrocytes isolated from baboons

Plasmodium knowlesi infected erythrocytes were isolated from the placenta and peripheral blood of Olive baboons (*P. anubis*) and used in binding assays to the purified receptors CD36, CSA and HA. The binding of the isolated parasites was then compared to those of the assay performance *P. falciparum* clones CS2 and 3D7.

None of the *P. knowlesi* parasites (placental-Pan3443, peripheral-Pan3614 and Pan3443) isolated from baboon placenta or peripheral blood bound to the purified receptors CD36, CSA or HA (Figure 13, 14 and 15). The performance of the binding assay was monitored by the *P. falciparum* clones CS2 and 3D7. They both bound to the receptors at varying levels with PfCS2 binding highly to CSA and HA (Median, 3820 and 1681 IEs/mm^2 respectively) while Pf3D7 bound highly to CD36 (Median, 785 IEs/mm^2)

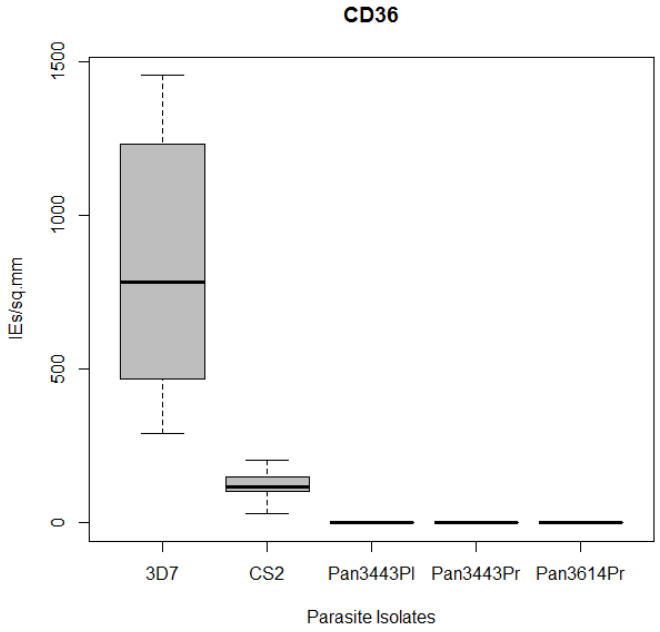


Figure 13: Binding of *P. knowlesi* infected erythrocytes from baboons to CD36 receptor compared to *Plasmodium* clones (CS2 and 3D7).

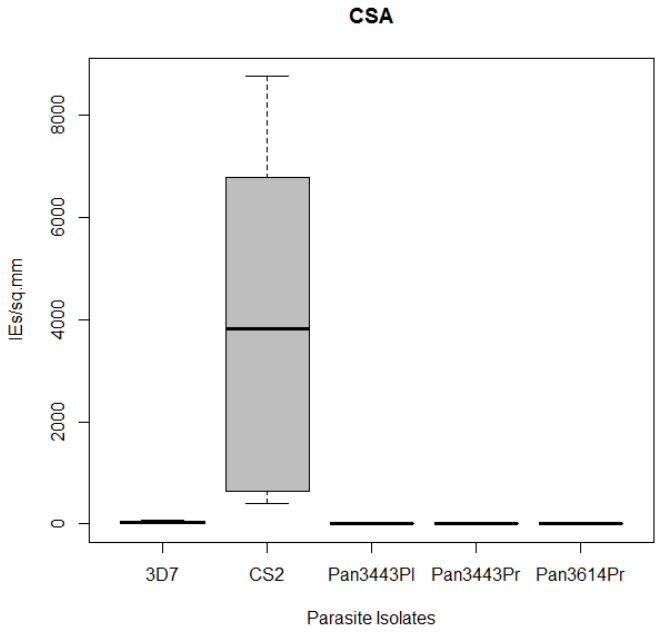


Figure 14: **Binding of *P. knowlesi* infected erythrocytes from baboons to Chondroitin sulphate A (CSA) receptor compared to *Plasmodium* clones (CS2 and 3D7).**

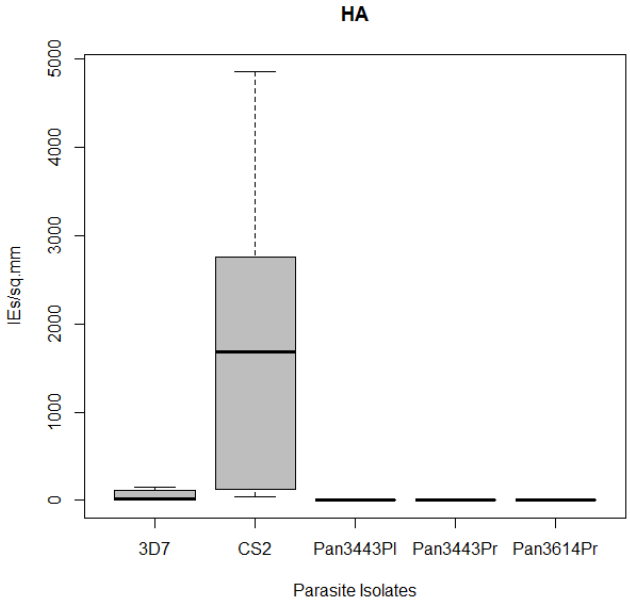


Figure 15: **Binding of *P. knowlesi* infected erythrocytes from baboons to Hyaluronic Acid (HA) receptor compared to *Plasmodium* clones (CS2 and 3D7).**

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

Until recently *Plasmodium knowlesi* was a malaria parasite of macaques but it is now considered the fifth malaria parasite affecting humans in terms of severity (White, 2008). It is an attractive experimental system for malaria research: it's a natural parasite of monkeys with similar immune and metabolic systems to humans (King *et al.,* 1988); is phylogenetically closer to *P. vivax* sharing many vaccine candidate molecules, and it can infect humans leading to fatalities (Cox-Singh *et al.,* 2008; White, 2008). Infection with the parasite in the Olive baboon during pregnancy has shown similar outcomes to that of *P. falciparum* in humans (Mustafa *et al.,* 2010; Ozwara *et al.,* 2003).

The present study determined the factors that mediate binding in *P. knowlesi* and the Olive baboon using two approaches: (1) comparing putative receptor and ligand sequences in baboon with human and *P. knowlesi* with *P. falciparum* respectively and (2) static adhesion assays against purified receptors using *P. knowlesi* infected erythrocytes (*Pk*IEs) isolated from Olive baboons.

4.1.1 Putative receptors and ligands in *P. anubis* and *P. knowlesi*

In *P. anubis* the putative receptors identified are: predicted CSPG 4, predicted HAPLN 1 and CD36 molecule transcript variants 1, 2 for the corresponding human receptors CSA (CSPG), HA (HAPLN) and CD36 respectively. These were the top BLAST hits with the highest percentage identities and the lowest e-values. Previous studies had shown that non-human primates are phylogenetically closer to human beings (Galinski & Barnwell, 2012) and this would explain why the data obtained showed high identities and low e-values for the top BLAST hits. Sequences from higher monkeys (Gorilla, Chimpanzee)

were homologous to human receptors followed by Olive baboon sequences while the least homologous were lower monkey sequences (Marmosets, Bush babies).

Phylogenetic analyses showed that the putative receptors identified in *P. anubis* all cluster together with sequences from macaques particularly *M. mulatta* and *M. fascularis* which are known natural hosts of *P. knowlesi* (Cox-Singh *et al.,* 2010, 2008). Since the Olive baboon is susceptible to *P. knowlesi* with similar disease outcomes to humans (Mustafa *et al.,* 2010; Ozwara *et al.,* 2003) these data suggest that the putative molecules identified could be used for adhesion. On the other hand, receptor sequences from human receptors clustered with sequences from the great apes; gorilla, bonobo and chimpanzee which are phylogenetically closer to humans (Galinski & Barnwell, 2012; Sibal & Samson, 2001). Despite this clustering, the putative receptors have common ancestry to the human receptors since they diverged from base and their evolutionary distance is close.

In *P. knowlesi*, the top hits identified were the *P. knowlesi* erythrocyte binding proteins; EBP-*alpha* (PKH_062300), EBP-*beta* (PKH_000490), and EBP-*gamma* (PKH_134580). They had the highest identities and least e-values to queries. These were initially also used as queries on the premise that they are erythrocyte invasion ligands (Chitnis & Miller, 1994) and could be homologous to molecules that aid in binding of *P. knowlesi* infected erythrocytes to the placenta in Olive baboons. They were among fifty seven (57) protein sequences identified from BLAST results as the putative molecules homologous to *Plasmodium* erythrocyte binding ligands. An e-value cut-off of <1-e4 was used to select the best aligning sequences. Majority of these protein sequences were conserved *Plasmodium* proteins of unknown function while some have been annotated for functions other than adhesion like Ada2-like protein, Dynein heavy chain or Ribonuclease.

Sequence similarity searches showed that SICAVar antigen molecule in *P. knowlesi* does not align to *P. falciparum* binding ligands but aligns to its variants in *P. knowlesi*. The SICAvar and KIR genes are the largest variant gene families in *P. knowlesi* genome (Pain *et al.,* 2008). These antigens found on the surface of infected erythrocytes are associated

with parasite virulence and have been shown to be essential in antigenic variation (Korir & Galinski, 2006). Previous studies had shown that despite the *P. knowlesi* SICAvar and *P. falciparum* var antigen families encoding proteins that enable antigenic variation in the respective organisms, they do not share significant level of sequence identity when aligned. Although distantly related, some *P. knowlesi* SICA peptides show identity with particular *P. falciparum* EMP1, mapping throughout all characterized domains, including the externally exposed cysteine-rich domains characteristic of both proteins (Korir & Galinski, 2006).

4.1.2 Characterization of ligands and receptors in *P. knowlesi* and *P. Anubis*

The putative receptors identified in *P. anubis* had similar domain organization to the known human receptors. The Laminin-G (LamG) domains common in human and *P. anubis* CSPG sequences are Ca^{2+} mediated receptors with binding sites for steroids, beta1 integrins, heparin, sulfatides, fibulin-1, and alpha-dystroglycans. They play a role in signal transduction via cell-surface steroid receptors, adhesion, migration and differentiation through mediation of cell adhesion molecules (Marchler-Bauer *et al.,* 2011).

Likewise, HAPLN 1 sequences had the domains immunoglobulin V-set and extracellular link protein. The link proteins are involved in interaction with HA and contribute to the structural integrity of different tissues (Marchler-Bauer *et al.,* 2011). Lastly, the CD36 family domain identified in the CD36 molecule of both *P. anubis* and human genome is thought to be a novel class of scavenger receptors with a role in signal transduction and cell adhesion (Marchler-Bauer *et al.,* 2011). CD36 is ubiquitous in system, mostly found in vasculature, not in placental tissue (Febbraio *et al.,* 2001; McCormick *et al.,* 1997). These data suggest that the identified molecules in *P. anubis* are likely used for adhesion due to their similar domain structure to the human receptors. They could belong to a family of receptors conserved in the baboon, human beings and other non-human primates.

The putative ligand molecules in *P. knowlesi* are located on different chromosomes in the genome of *P. knowlesi* with majority of them found along chromosomes 12 to 14. The erythrocyte binding proteins identified in *P. knowlesi* belong to duffy-antigen binding and erythrocyte binding antigen 175 (EBA-175 VI) protein families. They have the domains: duffy-antigen binding, *P. falciparum* erythrocyte membrane protein duffy binding like (PfEMP-DBL) and EBA-175 VI that are similar to the duffy-antigen binding and PfEMP-DBL domains of the principal *P. falciparum* ligand (Var2csa) (Smith *et al.,* 2001). Similarly, in gene ontology (GO) they are annotated for the biological process of pathogenesis, are integral to the membrane in location and function in either binding or receptor activity. This suggests that they could be performing similar functions to the DBL domains of Var2csa and are likely the *P. knowesi* ligands responsible for binding to the placenta of Olive baboons.

4.1.3 Binding phenotype of *P. knowlesi* infected erythrocytes

Plasmodium knowlesi infected erythrocytes (*Pk*IEs) isolated from placenta and peripheral blood of baboons were tested for their ability to bind purified receptors in a static binding assay. The study found that none of the *P. knowlesi* infected erythrocytes isolated adhered to either CSA, HA or CD36, the receptors responsible for *P. falciparum* sequestration in the placenta and microvasculature in humans (Beeson *et al.,* 2000; Fried & Duffy, 1996). This finding contradicts the results by Mustafa and others who characterized *P. knowlesi* malaria in the Olive baboon during pregnancy. They showed that the infection led to the accumulation of parasites in the intervillous spaces of the placenta leading to pathology (Mustafa *et al.,* 2010).

A cerebral malaria study by Fatih and co-workers (2012), found that *Pk*IEs from human subjects bound in a variable manner to the endothelial receptors ICAM-1 and VCAM but not to CD36. In retrospect, *P. knowlesi* malaria in the Olive baboon leads to accumulation of parasites in the intervillous spaces of the placenta (Mustafa *et al.,* 2010). In our study, we used frozen samples instead of fresh isolates in binding assays which might have

interfered with the binding capacity of the infected erythrocytes. A study by Ochola and others (2011) obtained different patterns of association between binding and clinical phenotypes by using freshly grown parasite isolates than frozen ones (Newbold *et al.*, 1997). Placental isolated parasites are usually in the trophozoite stage and can be used for binding assays directly unlike peripheral parasites that need to be cultured to the trophozoite stage before being used in assays (Beeson *et al.*, 2002, 2000). The *P. knowlesi* isolates may have failed to bind to purified receptors because these receptors could be unique to *P. falciparum* isolates. Since the data presented here suggests that there are receptor molecules in the baboon that mediate adhesion, they may have a modified structure making them different from human receptors.

The *P. falciparum* clones CS2 and 3D7 (PfCS2 and Pf3D7) both bound to the receptors being tested at different levels of adhesion. In general, the data collected showed that PfCS2 bound at high levels to the receptors CSA and HA while Pf3D7 bound at high levels to CD36. This is consistent with previous studies by Beeson and others (2000) which demonstrated that PfCS2 binds the receptors CSA and HA but at low levels to CD36 while Pf3D7 binds at low levels to HA.

In summary, this study has demonstrated that there exist molecules that aid binding of *Pk*IEs to the placenta of Olive baboons. These molecules have been identified and characterized using *in silico* methods. This builds the paradigm that a ligand-receptor process in the placental interface of Olive baboons is what directs accumulation of parasites leading to fatal outcomes of low birth weight in infants, abortion and still births and severe malaria.

The findings provide a platform for further investigations to ascertain these molecules identified are involved in placental accumulation of *Pk*IEs in the Olive baboon. They further affirm the close likeness of the *P. knowlesi* infection in baboons to the *P. falciparum* infection in humans. They show that the baboon can be a good model for *in vivo* placental malaria studies at the pre-clinical level. This will be useful in evaluating

placental malaria vaccines and other therapeutic agents that can prevent malaria infection during pregnancy leading to better outcomes for mothers and their unborn.

4.2 Conclusions

The results of this study show that accumulation of *P. knowlesi* infected erythrocytes in the placenta of Olive baboon (*P. anubis)* during pregnancy is mediated by a ligand-receptor process. In summary, the study:

i. Has identified: CSPG 4, CSPG 4-like and HAPLN 1 sequences as the putative receptors molecules in *P. anubis* and demonstrated they share similar domains to the human receptors. They are homologous to the known human placental binding receptors (CSA, HA) and are proposed to be the molecules used by the Olive baboon in binding of infected erythrocytes in the placenta during malaria in pregnancy.

ii. Has shown that the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) could be playing a role in infected erythrocyte adhesion apart from their traditional role of invasion.

iii. Has shown that static binding using *P. knowlesi* infected erythrocytes isolated from the Olive baboon do not bind to purified receptors (CSA, HA and CD36).

4.3 Limitations

There was difficulty in obtaining placental samples from pregnant Olive baboons due to the adverse outcomes of malaria in pregnancy for example stillbirths, abortions, and loss of conceptus before undertaking a caesarian section.

4.4 Recommendations

Based on this study, it is recommended that:

i. It would be good to investigate further whether the *Plasmodium knowlesi* uses its erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) to bind to placental receptors in Olive baboons (*P. anubis*).

ii. Further studies are also required to establish whether the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) bind to any or all of the *P. anubis* receptor molecules found.

iii. Studies should be conducted on interventions targeting receptors in placenta or ligands on infected erythrocytes which may prevent adverse outcomes in malaria during pregnancy.

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APPENDICES

Appendix 1: Sequences of putative receptor molecules in *P. anubis*

>gi|402874939|ref|XP_003901280.1| PREDICTED: chondroitin sulfate proteoglycan 4, partial [Papio anubis]

PRVGTCGREPSVRPLSASVDQAQLLPPHLPLLPLFFQTGYLNWKCSPMSRSPALTLNYGPRHSPPAILPS
SFRLPDFLPYKVRQQQTPRNATAPMTQPGCGAAPRQPGMWAYKSPTLPMAEAAFSGRWQLAQAGQGPPLP
APVFSLSLRETPFLGAVTTKPGDQASDGSLCGGRGPRPGRKTPLGPRGGKHLSLDHSSLSHTASFFGENH
LEVPVATALTDIDLQLQFSTSQPEALLLLAAGPADHLLLQLYSGRLQVRLVLGQEELRLQTPAETLLSDS
VPHTTVLTVVEGWATLSVDGFLNTSSAVPGAPLEVPYGLFVGGTGSLGLPYLRGTSRPLRGCLHAATLNG
RSLLRPLTPDVHEGCAEEFSAGDDVALGFSGPHSLAAFPAWGTQDEGTLEFTLTTQSRQAPLAFQAGGRR
GDFIYVDIFEGHLRAVVEKGQGTVLLHNSVPVADGQPHEVSVHINAHQLEVSVDQYPTRTSNRGVLSYLE
PRGSLLLGGLDAEASRHLQEHRLGLTPEATNASLLGCMEDLSVNGQRRGLREALLTRNMVAGCRLEEEEY
EDDAYGHYEAFSTLAPEAWPAMELPEPCVPEPGLPPVFANFTQLLTISPLVVAEGGTAWLEWRHVQPTLD
LMEAELRKSQVLFSVTQGARHGELELDIPGAQARKMFTLLDVVNRKARFIHDGSEDTSDQLVLEVSVTAR
VPMPSCLRRGQTYLLPIQVNPVNDPPRIIFPHGSLMVILEHTQKLLGPEVFQAYDPDSACEGLTFQFLGT
PSGLPVQRRDQPGEPATEFSCQELEAGSLVYVHRGGPAQDLTFRVSDGLQASPPATLKVVAVRPAIQIHR
STGLHLAQGSAMPILPANLSVETNAVGQDVSVLFRVTGALQFGELQKQGAGGVEGAEWWATQAFHQRDVE
QGRVRYLSTDPQHRTEDTVENLALEVQVGQEILSNLSFPVTIQRATVWMLRLEPLHTQNTQQETLTTAHL
EATLEEAGPSPPTFHYEVVQAPRKGNLQLQGTRLSDGQGFTQDDIQAGRVTYGATARASEAVEDTFRFRV
TAPPYFSPLYTFPIHIGGDPDAPVLTNVLLVVPEGGEGVLSADHLFVKSLNSASYLYEVMERPRHGRLAW
RGTQDKTTMVTSFTNEDLLHGRLVYQHDDSETTEDDIPFVATRQGESSGDMAWEEVRGVFRVAIQPVNDH
APVQTISRVFHVARGGRRLLTTDDVAFSDADSGFADSQLVLTRKDLLFGSIVAVDEPTRPIYRFTQEDLR
KRRVLFVHSGADRGWIQLQVSDGQHQATALLEVQASEPYLRVANGSRLVVPQGGQGTIDTAVFHLDTNLD
IRSGDEVHYHVTAGPRWGQVLRAGQPATAFSQQDLLDGAVLYSHNGSLSPRDTLAFSVEAGPVHTDATLQ
VTVALEGPLAPLKLVRHKKIYVFQGEAAEIRRDQLEAAQEAVPPADIVFSVKSPPSAGYLVMVSRGILAD
EPPSLDPVQSFSQEAVDAGRVLYLHSRPEAWSDAFSLDVASGLGAPLEGVRVELEVLPAAIPLDAQNFSV
PEGGSLTLAPPLLRVTGPYFPTLPGLGLQVLEPPRHGALQKEDGPQARTLSAFSWREVEEQLIRYVHDGS
ETLTDSFVLMANASEMDRQSHPVAFTVTILPVNDQPPILTTNTGLQMWEGATAPIPAEVLRSTDGDSGSE
DLVYTIEQPSNGRVVLRAAPGTEVRSFTQAQLDGGLVLFSHRGTLDGGFRFGLSDGEHTSSGHFFRVTAQ
KQVLLSLEGSRTLTVCPGSVQPLSSQTLRASSSAGTDPQLLLYHVVRGPQLGRLFHAQQDSTGETLVNFT
QAEVYAGNILYEHEMPTEPFWEAHDILELQLSSPPARDVAATLAVAVSFEAACPQRPSHLWKNKGLWVPE
GQRAKITMAALDASNLLASVPSSQRLEHDVLFQVTQFPSRGQLLVSEEPLHAGQPHFLQSQLAAGQLVYA
HGGGGTQQDGFHFHAHLQGPAGATVAGPQTSEAFAITVRDVNERPPQPQASVPLRITRGSRAPISRAQLS
VVDPDSAPGEIEYEVQRAPHNGFLSLVGGGPGPVTRFTQADVDSGRLAFVANGSSVAGVFQLSMSDGASP
PLPMSLAVDILPSTIEVQLRAPLEVPQALGRSSLSQQQLRVVSDREEPEAAYRLIQGPKYGHLLVGGRPA
SAFSQLQIDQGEVVFAFTNFSSSHDHFRVLALARGVNASAVVNITVRALLHVWAGGPWPQGATLRLDPTI
LDAGELANRTGSVPRFRLLEGPRHGRVVRVPRARTEPGGSQLVEQFTQQDLEDGRLGLEVGRPEGRAPSP
TGDSLTLELWAQGVPPAVASLDFATEPYNAARPYSVALLSVPEATRTEAGKPESSTPTGEPGPMASSPVP
AVAKGGFLGFLEANMFSVIIPVCLVLLLLALILPLLFYLRKRNKTGKHDVQVLTAKPRNGLAGDTETFRK
VEPGQAIPLTAVPGQGPPPGGQPDPELLQFCRTPNPALKNGQYWV

>gi|402872030|ref|XP_003899946.1| PREDICTED: LOW QUALITY PROTEIN: hyaluronan and proteoglycan link protein 1 [Papio anubis]

MKKILWAIKMKSLLLLVLISICWADHLSDNYTLDHDRVIHLQAENGPRLLVEAEQAKVFSHRGGNVTLPC
KFYRDPTAFGSGIHKIRIKWTKLTSDYLKEVDXFVSMGYHKKTYGGYQGRVFLKGGSDNDASLVITDLTL
EDYGRYKCEVIEGLEDDTAVVALDLQGVVFPYFPRLGRYNLNFHEAQQACLDQDAVIASFDQLYDAWRGG
LDWCNAGWLSDGSVQYPITKPREPCGGQNTVPGVRNYGFWDKDKSRYDVFCFTSNFNGRFYYLIHPTKLT
YDEAVQACLNDGAQIAKVGQIFAAWKLLGYDRCDAGWLADGSVRYPISRPRRRCSPTEAAVRFVGFPDKK
HKLYGVYCFRAYN

Appendix 2: Sequences of putative ligand molecules in *P. knowlesi*

>PKH_000490 | Plasmodium knowlesi strain H | erythrocyte binding protein (beta) | protein | length=1154

MEGKKKRPLFFLLVLLLSHKANNVLFERMKGILLLECENEYVKNENGYKLATGHHYMDND
QIERWLQGTDRSRRVKIEENVKYKYNVEELNTKYEQTKGKRINRILKESTYEAQNVADNN
YIDDKANGEYKTDNKTNKGEGARNMVMLDYDISGSGHPDGIIDNVVELGTEDEGNFLENS
SKGGDHPYRMNRKERMCSGVINQTFLQKNVMRRCNNKRKRGARDWDCPTKKDVCIPDRRY
QLCMKELTNLVNNTKTHSHNDITFLKLNLKEKLTYDAAVEGDLLLKKYNNVYSEDLCKDI
KWSLEDFGDIIMGTDMEGIGYSQVVENNLRSIFGTGTSAQLDRKKWWNDHKKYIWEATIL
SVKKKLNGYSAWNCKEDVQINVEPQIYRWIREWGMDYMSELPKEQRKIKEKCDRKLYYTN
LRICTMSPCNDSCKLYDQWITRKKKQWDVLSTKFSSVKKGQIIETENITTAYDILKQELN
GFNEVMFENEINKRDNVYIDICLCAADEPNKNTQEHLKKLKSAPKLETQRSHSTIQPMSS
SGAEKVQGDLAHGNINDAAYKSTTDEAAKGDGQNGNQTVAESNIKGTDNIENEAAKNVDT
YKFVTERSADTRGATDITETGEEKLNTSYSGSSEITVKENIPGDGIVKDVSAAVENSENP
LETKHKIFEPSKDNSDNSENSGSMEFKATSSNPITEAVESSSAEGQVQEDSAHRSVNTGR
DNSTISAATSDDGLSSGDKRVESLTSIENADDGGDPVQGSLWNLLNDPSVGAGGGKSHIK
TEENEGSQAEIDGKNVDIAEQRTATITEVQPERPDLSDTDNGNVPRSGNKQNEGATALSG
AESLESNESVHKTIDNTTHGLENKNGGNEKDFQKHDFMNNDMLNDQTSSDQTSSDQTSSN
QTSSDQTSSNQTSSDQTSSDQISSDQTSSDQTSSNQTSSDQTIDTEEHHRDNVRNPEIKS
SEDMSKGDFMRNSNSNELYSHNNLNNRKLNIDQYEHRDVKATREKIILMSEVNKCNNRAS
LKYCNTIEDRMLSSTCSRERSKNLCCSISDFCLNYFELYSYEFYNCMKKEFEDSSYECFT
KGSSTGIVYFATGGAFLIILLLFVSKNVASNDSYSYEEEATFDEFVEYSDDIHRTPLMPD
DIEHMLQLSPLDYS

>PKH_134580 | Plasmodium knowlesi strain H | erythrocyte binding protein (gamma) | protein | length=1072

MEGKKKRPLFFLLVLLLSHKANNVLFERMNGILLLECENEYVKNENGYKLATGHHYMDND
QIERWLQGTDRSRRVKIEENVKYKYNVEELNTKYEQMKGKRINRILKESTYEAQNVADNN
YIDDKANGEYKTDNKTNKGEGARNMVMLDYDIFGSGHPDGIIDNVVEFVTEDEGNFLKNS
SKGDDHPYRMKRKEKMSSGAINQIFLQNNVMDKCNDKRKRGERDWDCPTEKDVCIPDRRY
QLCMMEITNLVDNTNTHFHSDIIFRKSYFERRLIYDVGAEGDLLLKKYNNVYSEDLCKDI
KWSLQDFGDIIMGTDMEGIGYSLVVENNLRSIFGTGTSAELDRKKWWNDHKKDIWKAMIL
SVKEKNRYSAWNCKEDVQINVEPQIYRWIREWGRDYMSEFREQRRKLNEKCEDKLYYSTM
LICTLPPCNNACKSYDEWITGKKKQWDVLSTKFSSVKKAQKIETENIARAYDILKQELNG
FNEVTFENEINKRDKLYNYFCVCIVQEARKNTQENVKNVGSGVESKAPSSNPINEAVKSS
SGEGKVQEDSAHRSVNEGEGKSSTNEADPGSQPGGPASRSVDEKAGVPALSAGQGHDKVP
PAEAAATESAVPHSADKTPITATEENKQRTQVDGVAGGDGKAPGPTVSSDVPSVGGKDSG
PSTPASHALAGENGEVHNGTDTEPKEDGEKADPQKNIEVKGKQDTDDRSQGSLGPHTDER
ASLGETHMEKDTETTGGSTLTPEQNVSVASDNGNVPGSGNKQNEGATALSGAESLESSES
VHKTIDNTTHGLENKNGGNEKDFQKHDFMNNDMLNDQTSSDHTSSDQTSSDQTSSDQTSS
DQTSSDQTSSDQTSSDQTSSDQTIDTEGHHRDNVRNPEIKSSEDMSKGDFMRNSNSNELY
SHNNLNNRKLNRDQYEHRDVKATREKIILMSEVNKCNNRTSLKYCNTIEDRMLSSTCSRE
RSKNLCCSISDFCLNYFELYSYEFYNCMKKEFEDPSYECFTKGSSTGIVYFATGGAFLII
LLLFASWNAASNDYEEEATFDEFEEYCYNIHRTPQMPNDIEHMQQFTPLDYS

>PKH_062300 | Plasmodium knowlesi strain H | erythrocyte binding protein (alpha) | protein | length=1073

MEGKKKRPLFFLLVLLLSHKANNVLFERMNGILLLECENEYVKNENGYKLATGHHYMDND
QIEQWLQGTDRSRRVKIEENVKYKYNVEELNTKYEQMKGKRINRILKESTYEAQNVADNN
YIDDKANGEHKTDNKTNKGEGARNMVMLDYDISGSGHPDGIIDNVVELGTEDEGNFLENS
SKGGDHPYRMNRKERMSNGVINQTFLQNNVMDKCNDKRKRGERDWDCPAEKDICISDRRY
QLCMKELTNLVNNTRTHSHNDITFLKLNLKRKLMYDAAVEGDLLLKKNNYQYNKEFCKDI
RWGLGDFGDIIMGTNMEGIGYSQVVENNLRSIFGTDEKAKQDRKQWWNESKEHIWRAMMF
SIRSRLKEKFVWICKKDVTLKVEPQIYRWIREWGRDYMSELPKEQGKLNEKCASKLYYNN
MAICMLPLCHDACKSYDQWITRKKKQWDVLSTKFSSVKKTQKIGTENIATAYDILKQELN
GFKEATFENEINKRDNLYNHLCPCVVEEARKNTQENVKNVGSGVESKAASSNPITEAVKS
SSGEGKVQEDSAHKSVNKGEGKSSTNEADPGSQSGAPASRSVDEKAGVPALSAGQGHDKV
PPAEAAATESAVLHSADKTPNTVTEENKEGTQMDGAAGGDGKAPGPTVSSDVPSVGGKDS
GPSTSASHALAGENGEVHNGTDTEPKEDGEKADPQKDIEVKGKQDTDDRSQGSLGPHTDE
RATLGETHMEKDTETAGGSTLTPEQNVSVASDNGNVPGSGNKQNEGATALSGAESLKSNE
SVHKTIDNTTHGLENKNGGNEKDFQKHDFMNNDMLNDQASSDHTSSDQTSSDHTSSDQTS
SDHTSSDHTSSDQTSSDQTSSDQTIDTEGHHRDNVRNPEIKSSEDMSKGDFMRNSNSNEL
YSHNNLNNRKLNRDQYEHRDVKATREKIILMSEVNKCNNRASVKYCNTIEDRMLSSTCSR
ERSKNLCCSISDFCLNYFELYSYEFYNCMKKEFEDPSYECFTKGSSTGIVYFATGGAFLI
ILLLFASWNAASNDYEEEATFDEFVEYSDDIHRTPLMPNDIEHMQQFTPLDYS

Appendix VIII

INSTITUTE OF PRIMATE RESEARCH
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INSTITUTIONAL SCIENTIFIC AND ETHICAL REVIEW COMMITTEE (ISERC)

PROPOSAL ETHICAL APPROVAL FORM

Dear Dr. Hastings Ozwara,

It is my pleasure to inform you that your proposal entitled" **Characterisation Of Placental Malaria In Baboons Infected With Wild Type And Transfected *Plasmodium Knowlesi*** in collaboration with Dr. John Ayisi of Kenya Medical Research Institute, Dr. Julie Moore of University of Georgia Athens and Dr. Alan Thomas of Biomedical Primate Research Centre in The Netherlands has been reviewed on the scientific merit and ethical considerations on the use of baboons (*Papio anubis*) for research purposes.
The committee is guided by the Institutional guidelines (e.g., S.O.Ps) as well as international regulations, including those of WHO, NIH, PVEN and Helsinki convention on humane treatment of animals for scientific purposes and GLP.
The proposal has been approved unconditionally on 2nd June 2005 by the above named committee. You may therefore proceed and seek funding for the proportion of work to be done at this institution.

Signed _____ Dr. Peter G. Mwethera (Chairman ISERC)

Date: 3/06/05

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Plasmodium knowlesi Ligand-receptor Process in Baboon (*Papio anubis*) Placenta

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Abstract

Pregnancy associated malaria poses many risks to both women and their infants. It is characterized by the accumulation of infected erythrocytes in the intervillous spaces of the placenta leading to adverse reactions. Studies using the *P. knowlesi*-Olive baboon model of pregnancy malaria have demonstrated this phenomenon though the mechanisms and molecules involved are not known. This study sought to identify the ligands and receptor molecules that permit accumulation of infected erythrocytes in the placenta of *P. knowlesi* infected Olive baboons and to further test placental isolates for adhesion to purified receptors. Sequences of known *Plasmodium* erythrocyte binding antigens and human placental receptors were BLASTed against the genome of *P. knowlesi* and *P. anubis* respectively. Hits generated were analysed and characterized to determine the prospective ligands and receptors in *P. knowlesi* and *P. anubis* respectively. Also, four adult female baboons (*P. anubis*) were infected with *P. knowlesi* parasites and their placentas sampled. Infected erythrocytes isolated from these placentas were tested for binding against purified receptors. We identified Predicted CSPG 4 partial and Predicted HAPLN 1 as the putative receptor molecules in the Olive baboon. Further, the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) matched closely to the placental *P. falciparum* ligand *Var2csa*. However, static binding assays with *P. knowlesi* infected erythrocytes did not show any binding to purified receptors. This study has identified and proposed receptors and ligands involved in the adherence process in *P. knowlesi* infected Olive baboons during pregnancy.

Keywords: *Plasmodium knowlesi*, Olive baboon, receptor, ligand, malaria, pregnancy

1. Introduction

Malaria is a public health problem that mainly affects pregnant women and children under 5 years old. During pregnancy, *Plasmodium falciparum* infected erythrocytes adhere to the intervillous spaces of the placenta, a phenomenon called sequestration (Costa *et al.,* 2006; Sherman *et al.,* 2003). Mature forms of the parasite which are commonly absent in peripheral circulation tend to dominate the placenta (Maubert *et al.,* 2000). Sequestration is mediated by receptor-ligand interactions between parasite-induced ligands on the erythrocyte membrane and cellular adhesion molecules (CAM) on the surface of vascular endothelial cells. Several studies have characterized these CAMs involved in adhesion of infected erythrocytes (IEs) in the placenta: chondroitin sulphate A (CSA) (Reeder *et al.,* 1999), hyaluronic acid (HA) (Beeson *et al.,* 2000), CD36 (Febbraio *et al.,* 2001). The best characterized ligand in *P. falciparum* is *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP 1) encoded by the highly variable *var* gene family (Smith *et al.,* 2001). The P*f*EMP 1 proteins are distantly related to the schizont-infected cell agglutination antigens (SICA), that are encoded by the SICAvar genes in *P. knowlesi,* and share binding signature motifs (Korir and Galinski, 2006).

Recently, the monkey malaria parasite *Plasmodium knowlesi* has become an important public health concern as it can infect humans (Cox-Singh *et al.,* 2010, 2008; Sabbatani *et al.*, 2010; White, 2008). Studies have demonstrated that its infection of non-human primates parallels that of *P. falciparum* thereby making it a suitable model for study of human malaria (Anderios *et al.,* 2010; Dutta *et al.,* 1982, 1981, 1978; Langhorne and Cohen, 1979; Ozwara *et al.,* 2003). The *P. knowlesi*-Olive baboon model has been shown to display clinical and pathological symptoms during pregnancy similar to *P. falciparum* in humans (Mustafa *et al.,* 2010). However, despite evidence showing the accumulation of parasites in the intervillous spaces of the placenta, the mechanism used by *P. knowlesi* IEs to sequester is not known and the molecules involved have not been determined.

In this study, we used bioinformatics analyses and *in vitro* static based adhesion assays to identify and characterize the ligand and receptor molecules involved in the accumulation of *P. knowlesi* infected erythrocytes in the placenta of Olive baboons.

2. Materials and Methods

2.1 Ethics statement

The proposal for this study was reviewed and passed as ethically acceptable by the Institutional Animal Care and Use Committee (IACUC) and the Institution's Scientific Ethical Review Committee (ISERC) of the Institute of Primate Research-Kenya where this work was done.

2.2 Study design

The study involved: a descriptive *in silico* study to identify the putative ligands and receptor molecules used by *P. knowlesi* and the Olive baboon to adhere to the placenta, *in vivo* study to investigate the effects of *P. knowlesi* malaria in Olive baboons during pregnancy and an *in vitro* study to determine the binding phenotype of *P. knowlesi* infected erythrocytes isolated from baboons during pregnancy (Figure 1).

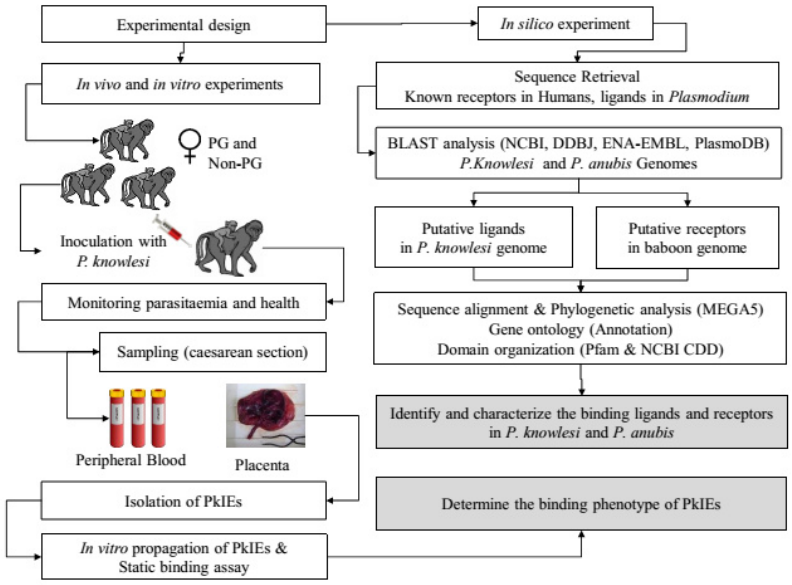


Figure 1. Schematic diagram of the study's experimental design

Four adult female baboons were screened and maintained in the company of an adult male for mating to occur. Pregnancy status and gestation periods of the baboons were confirmed by ultrasound. The baboons were infected together with four non-pregnant baboons (control) on gestation day 150 with 2×10^5 *P. knowlesi* blood stage parasites cultured overnight (Ozwara *et al.,* 2003). Following infection, level of parasitaemia was determined daily from day 3 post infection. When patent parasitaemia was established in baboons (day 7-9 post infection), caesarean sections (CS) were performed by an attending veterinarian to obtain intact sterile placental tissue (Mustafa *et al.,* 2010). Peripheral blood was also collected and processed together with the placentae to acquire infected erythrocytes. These isolated infected erythrocytes were then tested to determine their binding phenotypes in a static binding assay.

2.3 In vivo and in vitro experiments

2.3.1 Parasites

Baboons were infected using *P. knowlesi* H strain parasites originally from Pk1(A+) clone (Barnwell *et al.,* 1983) and whose genome has been fully sequenced (Pain *et al.,* 2008). *P. falciparum* lines CS2 and 3D7 were used to monitor the performance of static binding assay (obtained through MR4 as part of the BEI Resources Repository, NIAID, NIH, *P. falciparum* CS2, MRA-96, and *P. falciparum* 3D7 KAHRP knockout, MRA-554, deposited by SJ Rogerson and AF Cowman respectively) (Cowman, 1995; Rogerson *et al.*, 1995).

2.3.2 Animals

Female baboons (*P. anubis,* weighing between 12–23kg) were used. They were screened to ensure they were not

infected with *P. knowlesi*, hemoprotozoans, gastrointestinal parasites and Simian Immunodeficiency Virus (SIV) before inclusion in the study. Each baboon was housed in an individual squeeze back cage of dimensions 0.6 × 0.6 × 0.8 meters at the bio-containment facility (according to Institute of Primate Research Standard Operating Procedures and guidelines for holding of animals) and fed on commercial non-human primate diet (Olobo and Black, 1990).

2.3.3 Parasite preparation and culture

To obtain infected erythrocytes from placenta and peripheral blood a method by Beeson and co-workers was used (Beeson *et al.,* 2000).

Laboratory *P. falciparum* lines CS2 and 3D7 were used as controls for testing binding to CSA, HA and CD36 (Cowman, 1995; Rogerson *et al.,* 1995). *In vitro* cultures were initiated with cryopreserved parasites using standard culturing techniques (Trager and Jensen, 1976). The parasite pellet was mixed with 5ml of complete RPMI 1640 medium [supplemented with 37.5mM HEPES, 7mM D-glucose, 6mM NaOH, 25µg/ml of gentamicin sulphate, 2mM L-glutamine, and 10% human serum] at pH7.2 and transferred to a T-25cm^3 culture flask (Corning, UK). The parasitaemia was adjusted to 2% using human PCV (O+ve erythrocytes), gassed with a mixture of 90% nitrogen, 5% carbon dioxide, and 5% oxygen and incubated at 37°C (Trager and Jensen, 1976).

In vitro culture of *P. knowlesi* parasites was initiated with cryopreserved parasites previously isolated from infected baboon placenta and peripheral blood. The pellet obtained was transferred into culture to a starting erythrocyte PCV of 2.5% in complete culture medium [RPMI 1640 (Sigma) supplemented with 2.5% baboon PCV, 20% baboon heat inactivated serum and 15µg/ml gentamycin solution (Sigma)]. Cultures were transferred into sterile labelled T-25cm^3 culture flasks, gently mixed, gassed with 5% CO_2, 5% O_2, and 90% N_2 for 30 seconds, and incubated at 36.5°C (Ozwara Suba and LUMC, 2005). Synchronization of *in vitro* cultures was done using 5% sorbitol to obtain homogenous staged parasites (Lambros and Vanderberg, 1979) and in cases of excess yields from propagation, they were cryopreserved using standard techniques (Moll *et al.,* 2008).

2.3.4 Static adherence assay

Samples obtained were cultured as described for up to 24 hours until mature trophozoite forms predominated (Beeson *et al.,* 2000). Laboratory parasite clones (*P. falciparum* 3D7 and CS2) were cultured using standard culturing techniques (Trager and Jensen, 1976). The parasitaemia was adjusted to 3% and 1% haematocrit for all adhesion assays.

Purified receptors used in this study were: Recombinant Human CD36/SR-B3 Fc Chimera (R & D Systems, UK), Chondroitin 4 sulphate sodium salt from bovine trachea, CSA (Sigma) and Hyaluronic acid from bovine vitreous humour, HA (Sigma).

A method by Fatih and others was used for binding experiments with modifications (Fatih *et al.,* 2012). Briefly, three identical areas of each Petri dish (60×15mm diameter, Falcon 1007, Becton Dickenson, Oxford, UK) were treated with 2µl aliquots of purified CSA, HA each at 100µg/ml and 50µg/ml and CD36 at 50µg/ml and 25µg/ml. Control areas were treated with phosphate buffered saline (PBS) and three marked areas were left untreated. The dishes were incubated in a humid chamber at 37°C for two hours before aspirating excess protein and blocking all areas with 1% w/v bovine serum albumin in PBS for 2 hours at 37°C. The blocking solution was removed and *in vitro* matured infected erythrocyte cultures were added to 3 ml warmed binding buffer (RPMI 1640 media supplemented with 2% D-glucose) to a final haematocrit of 3%. Each protein and the PBS control were represented in triplicate per dish and duplicate dishes were seeded per isolate. Dishes were seeded with 1.5ml of the prepared *in vitro* cultured cell suspension per isolate and additional assay dishes with *P. falciparum* clone CS2 and 3D7 as assay performance controls. The dishes were incubated at 37°C for 1 hour, with gentle mixing at 10 minutes intervals. Unbound cells were removed by gentle washing seven times with binding buffer. Bound cells were fixed with 1% v/v glutaraldehyde (Sigma) for 1 hour and stained with 10% Giemsa (BDH, VWR international Ltd, England) for 20 minutes. Using an inverted light microscope at × 300 magnification the number of bound infected erythrocytes was enumerated from non-overlapping fields for each protein and PBS treated area. The area of the field of view was equivalent to 0.19635mm^2. The results were expressed as the number of infected cells (IE) bound/ mm^2 to each of the proteins or control [IE/mm^2 = (1/0.19635) × mean number of bound IE per field] (Fatih *et al.,* 2012).

2.4 Bioinformatics

2.4.1 Sequence retrieval and homology search of *P. anubis* genome

Coding sequences (CDS) of the human receptor molecules chondroitin sulphate A (CSA) hyaluronic acid (HA) and CD36 (thrombospondin receptor) [GenBank: 126091140, 194018435 and 188536058 respectively] were

retrieved from NCBI (NCBI, http://www.ncbi.nlm.nih.gov). These were aligned to NCBI's non-redundant protein and nucleotide databases, the DNA Database of Japan and the European Molecular Biology Laboratory's European Bioinformatics Institute (DDBJ, http://www.ddbj.nig.ac.jp/; ENA-EMBL, http://www.ebi.ac.uk/ena/; NCBI, http://www.ncbi.nlm.nih.gov) databases to identify their homologues in non-human primates. The BLASTn or BLASTx algorithms were used while maintaining the default parameters (Altschul *et al.,* 1990). The non-human primate (NHP) homologues identified were retrieved from NCBI's GenBank and individually aligned to the entire genome of *P. anubis* at NCBI (Zinner *et al.,* 2013) using BLASTn and BLASTx. The hits were manually inspected and those with highest percentage identities (PID) and lowest expectation values (E-values) were designated as the putative receptor molecules in *P. anubis*.

2.4.2 Characterization of putative receptor molecules in *P. anubis* genome

Putative protein sequences from *P. anubis* were aligned with sequences of known human receptors using MUSCLE and evolutionary history inferred using the Neighbor-Joining method in MEGA5 with bootstrap consensus tree inferred from 1000 replicates (Tamura *et al.,* 2011). To determine the probable function and location of the putative molecules, they were aligned to NCBI's non-redundant database to determine closest matching homologues. They were further characterized by comparing their domain organization to those of the known human placental receptor molecules in Pfam and NCBI CDD domain databases (Finn *et al.,* 2010; Marchler-Bauer *et al.,* 2011).

2.4.3 Sequence similarity search of *P. knowlesi* genome

Protein sequences of known erythrocyte binding ligands in *Plasmodium* were retrieved from PlasmoDB (Aurrecoechea *et al.,* 2009) and NCBI [PlasmoDB: PF3D7_1200600, PF3D7_0731500, PF3D7_0102500, PF3D7_0424300, PF3D7_1301600, PKH_000490, PKH_134580, PKH_062300, PVX_110810; GenBank: 23507807, 34525754, 6165411, 74766456, 29293851, 226438086]. These sequences were used in a BLAST search against the entire *P. knowlesi* genome hosted at PlasmoDB (Pain *et al.,* 2008). Further, the SICAvar antigen (SICAvar HB205, *P. knowlesi* from Malaysia 205kDa Pk1 (B+) 1+) [GenBank: AF078128.2] was also used as a query for BLAST against *P. falciparum* and *P. knowlesi* genomes hosted at PlasmoDB to determine its homologues. The hits generated were inspected, those with highest percentage identities and an expectation value (E-value) of < 1e-04 were designated as the putative erythrocyte binding ligands in the *P. knowlesi* genome.

2.4.4 Characterization of putative binding ligands in *P. knowlesi* genome

To predict the molecular function and cellular location of the putative erythrocyte binding molecules identified, they were aligned against the NCBI non-redundant database to determine closest matching homologues. Their protein parameters and inferred gene ontology (GO) terms were obtained from PlasmoDB and SIB's ProtParam tool (ExPASy - ProtParam tool, http://web.expasy.org/protparam/; PlasmoDB, http://www.plasmodb.org). Furthermore, their domain organization was obtained in a domain search against the Pfam and NCBI CDD domain databases and compared to the known *P. falciparum* erythrocyte binding ligand (Var2csa) (Finn *et al.,* 2010; Marchler-Bauer *et al.,* 2011).

2.5 Statistical analysis

Statistical analyses were performed using R 2.15.2 (R Core Team, 2012). The Mann-Whitney U-test and the Kruskal-Wallis test were used to evaluate statistical significance of the data since distributions were skewed from normal (Fatih *et al.,* 2012; Heddini *et al.,* 2001). Probability values of $P < 0.05$ were considered significant.

3. Results

3.1 Receptor molecules (CSPG 4 and HAPLN 1) discovered in P. anubis are similar to human receptors CSA and HA

To determine the putative receptors in *P. anubis*, sequences of known human placental receptors were BLASTed against the genome of *P. anubis* from NCBI server and the hits generated analysed. The human receptor molecules CSA (CSPG 4), HA (HAPLN 1) and CD36 all aligned to their homologues in non-human primates with high identities and low expectation values (PID 80–99%, E-value <0.0) (Table 1). Human CSA aligned to predicted chondroitin sulphate proteoglycan (CSPG 4 and CSPG 4-like) nucleotides and proteins, human HA aligned to predicted hyaluronan and protein link protein 1(HAPLN 1 and variants 2 and 3), while CD36 molecule aligned to CD36 molecules (platelet glycoprotein isoforms and transcript variants 2, 3 and 4).

The non-human primate homologues were used as queries to BLAST the *P. anubis* genome. The BLAST hits showed that the individual sequences aligned in similar manner to the non-redundant nucleotide and protein

databases of *P. anubis*. The top BLAST hits had identities ranging from 83 to 99% with low expectation values (E-value=0.0). The predicted CSPG 4 homologues aligned to CSPG 4 and CSPG 4-like partial sequences in *P. anubis* genome (PID=>90%, E=0.0), while the predicted HAPLN 1 homologues aligned to HAPLN 1 (PID=>93%, E=0.0). The BLAST also identified other CSPGs namely neurocan, versican, aggrecan and brevican. This suggests that the baboon has the receptor molecules CSPG 4 and HAPLN 1 that are similar to the human receptors CSA and HA.

Table 1. BLAST hits of known human receptors to non-human primates

Query	Predicted Sequences producing HSPs (proteins)	Organism	% ID
H. sapiens CSPG4 (8305bp)	CSPG 4	*Gorilla gorilla gorilla*	99
	LQP: CSPG 4	*Pan paniscus*	99
	CSPG 4	*Pan troglodytes*	99
	CSPG 4, partial	*Papio anubis*	97
	CSPG 4	*Saimiri boliviensis boliviensis*	95
	CSPG 4-like, partial	*Macaca mulatta*	94
	CSPG 4	*Otolemur garnettii*	89
	CSPG 4	*Nomascus leucogenys*	92
	LQP: CSPG 4	*Callithrix jacchus*	95
	CSPG 4-like, partial	*Pan troglodytes*	94
Homo sapiens HAPLN 1 (4678bp)	HAPLN 1 isoform 3	*Pan troglodytes*	99
	HAPLN 1	*Gorilla gorilla gorilla*	99
	HAPLN 1	*Saimiri boliviensis boliviensis*	97
	LQP: HAPLN 1	*Papio anubis*	98
	Proteoglycan link protein	*Macaca fascicularis*	98
	HAPLN 1	*Nomascus leucogenys*	99
	HAPLN 1 isoform 2	*Pongo abelii*	98
	HAPLN isoform 1	*Macaca mulatta*	98
	HAPLN 1	*Otolemur garnettii*	97
	unnamed protein product	*Macaca fascicularis*	98
	HAPLN 1 isoform 2	*Callithrix jacchus*	97
Homo sapiens CD36 molecule (thrombospondin receptor) (CD36), transcript variant 1, (4727bp)	PG 4 isoform 4	*Pan troglodytes*	99
	PG 4	*Pongo abelii*	98
	PG 4	*Gorilla gorilla gorilla*	98
	PG 4 isoform 1	*Nomascus leucogenys*	96
	PG 4	*Macaca mulatta*	95
	PG 4 isoform 1	*Saimiri boliviensis boliviensis*	92
	PG 4	*Callithrix jacchus*	92
	PG 4-like	*Otolemur garnettii*	88
	PG 4 isoform 6	*Nomascus leucogenys*	97
	PG 4 isoform 4	*Saimiri boliviensis boliviensis*	91
	PG 4-like	*Otolemur garnettii*	75

Key: *Macaca mulata* (Rhesus macaque), *Pongo abelii* (Sumatran orangutan), *Gorilla gorilla* (Gorilla), *Nomascus leucogenys* (White-cheeked gibbon), *Pan troglodytes* (Chimpanzee), *Papio anubis* (Olive baboon),

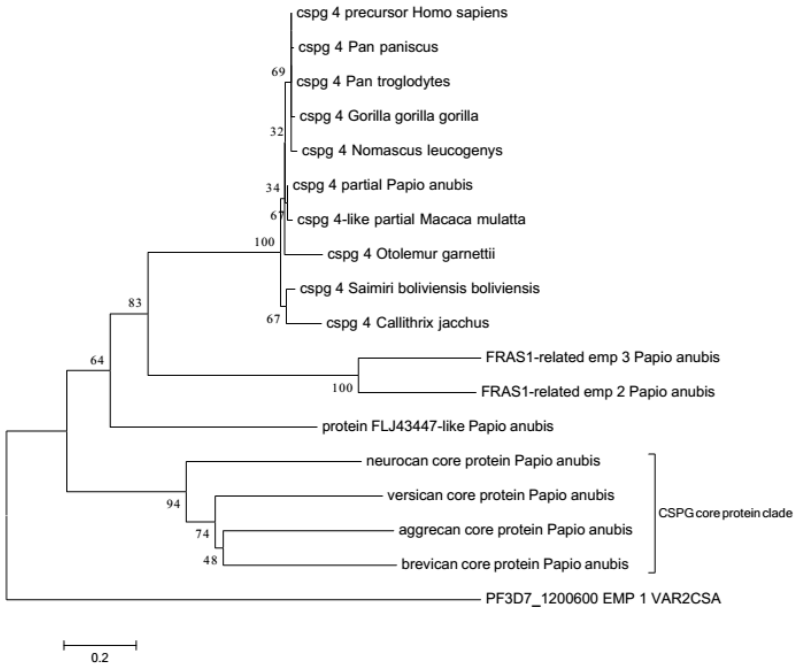
Callithrix jacchus (White-tufted ear marmoset), *Macaca fascularis* (Crab-eating macaque), *Homo sapiens* (man), *Pan paniscus* (Bonobo – Pigmy chimpanzee), *Saimir boliviensis boliviensis* (Black-capped squirrel monkey), *Otolemur garnetii* (Bushbaby).

CSPG (chondroitin sulphate proteoglycan), HAPLN (hyaluronan and protein link protein), LQP (low quality protein), PG (platelet glycoprotein)

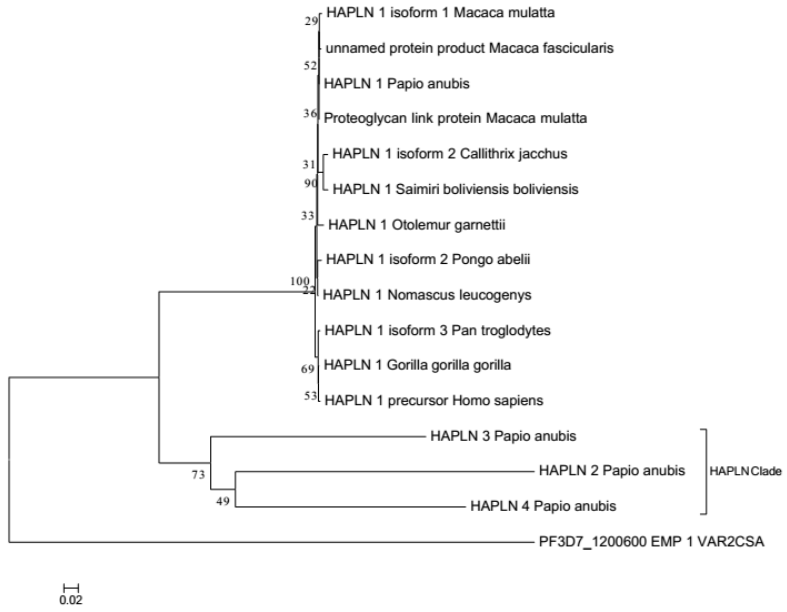
3.2 Putative P. anubis receptor sequences cluster with Macaque monkeys' sequences

Phylogenetic relationship was inferred using the putative *P. anubis* receptor sequences and sequences from human and non-human primate receptors. Putative sequences from *P. anubis* clustered to homologous sequences in macaque monkeys *M. mulatta* and *M. fascularis*, the natural hosts of *P. knowlesi* (Figure 2 A, B and C). In general, the human receptor sequences (CSPG, HAPLN 1 and CD36 antigen) clustered with homologous sequences from higher non-human primates like gorilla and chimpanzees. However, it was noted that all the homologous sequences share a common ancestral origin as demonstrated by branching of the phylogenetic tree and bootstrap values. The CSPG core protein sequences (neurocan, aggregan, versican and brevican) from *P. anubis* all clustered together forming a clade that diverged from other CSPG homologues in *P. anubis* and other non-human primates (Figure 2A).

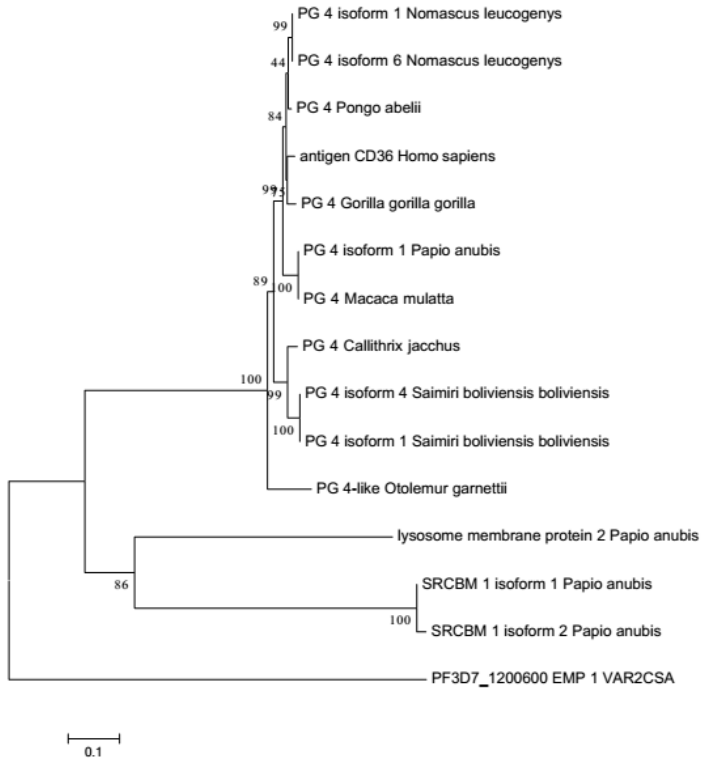
The variants HAPLN 2, 3 and 4 from *P. anubis* all clustered together forming a clade divergent from the HAPLN 1 sequence in *P. anubis* and other non-human primates homologues (Figure 2B). It was noted that the *P. anubis* sequences were closer to the lower monkeys (marmoset, bushbaby) than higher monkeys (gorilla and chimpanzee) that clustered to human HAPLN 1 sequence forming a clade. Likewise, the scavenger receptor class B member (SRCBM) proteins 1 and 2 diverged from similar ancestry to platelet glycoprotein 4 (PG 4) from *P. anubis* and other non-human primate homologues (Figure 2C).



A



B



C

Figure 2. Phylogenetic relationships of sequences from *P. anubis* and other non-human primates.

The Neighbor-Joining trees were constructed using the Poisson correction method for 18 CSPG sequences (A), 16 HAPLN sequences (B) and 15 CD36 sequences (C). The numbers at the juncture of two branches are the percent of 1000 bootstrap trees with the same branch length. *P. falciparum* sequence Pf3D7_1220600 EMP 1 VAR2CSA was included as an out-group.

The *P. anubis* sequences with the highest identities and least E-values from BLAST analysis were designated as the likely molecules that mediate placental erythrocyte binding (Table 2). The top hits for the CSPG receptor

were CSPG 4 and CSPG 4-like partial sequences. However, other hits were discovered which are a group of aggregate proteins associated with CSPGs (neurocan, aggregan, versican and brevican core proteins). Equally, the top hit for the HAPLN 1 receptor was HAPLN 1 in the *P. anubis* genome. Other hits that were closely associated were; HAPLN 2, 3 and 4 which are variants of HAPLN 1. The human CD36 molecule receptor identified similar CD36 molecule transcript variants 1 and 2 along with other scavenger receptor class B member proteins in the *P. anubis* genome.

Table 2. Putative sequences in the Olive baboon (*P. anubis*) genome used as receptor molecules

Nucleotide	Accession no	Protein	Accession no
CSPG			
CSPG4, partial	XM_003901231.1	CSPG 4, partial	XP_003901280.1
CSPG 4-like, partial	XR_163612.1	neurocan core protein	XP_003915287.1
PUP (FLJ43447)	XM_003901225.1	FRAS1-related EMP 3	XP_003899274.1
neurocan (NCAN)	XM_003915238.1	PUP (FLJ43447)	XP_003901274.1
		aggrecan core protein	XP_003901403.1
		versican core protein	XP_003899948.1
		brevican core protein	XP_003892883.1
		FRAS1-related EMP 2	XP_003913840.1
HAPLN 1			
HAPLN 1	XM_003899897.1	HAPLN 1	XP_003899946.1
		HAPLN 3	XP_003901408.1
		HAPLN 4	XP_003915288.1
		HAPLN 2	XP_003892880.1
CD36 molecule			
CD36 molecule, transcript variant 1	XM_003896397.1	PG 4 isoform 1	XP_003896446.1
CD36 molecule, transcript variant 2	XM_003896398.1	LMP 2	XP_003898782.1
		SRCBM 1 isoform 2	XP_003907409.1
		SRCBM 1 isoform 1	XP_003907408.1

Key: HAPLN 1 (hyaluronan and proteoglycan link protein 1), CSPG (chondroitin sulphate proteoglycan 4), CD36 (CD36 molecule, thrombospondin receptor), EMP (extracellular matrix protein), PG (platelet glycoprotein), PUP (putative uncharacterized protein), LMP (lysosome membrane protein), SRCBM (scavenger receptor class B member)

3.3 P. knowlesi EBP-alpha, beta and gamma as putative ligands for placental adhesion

To determine prospective ligands in *P. knowlesi*, sequences of known *Plasmodium* erythrocyte binding antigens were BLASTed against the genome of *P. knowlesi* and hits generated were analysed. The *P. knowlesi* SICAvar antigen (SICAvar-HB205) sequence aligned to various molecules in *P. falciparum* genome but the hits were of high expectation values (E-value > 0.062, cut off <1e-04). However, hits against *P. knowlesi* were of low expectation values (E-value 0.0, cut off <1e-04) with high identity to its variants (SICAvar antigen and SICAvar antigen (fragment)).

Fifty seven (57) protein sequences were selected following BLAST analysis as the putative ligands in *P. knowlesi* genome (E-value cut off <1e-04) (Table 3). The top hits are the erythrocyte binding proteins – *alpha*, *beta* and *gamma*. They aligned to *P. falciparum* Var2csa, a known binding ligand, with low expectation values (4.3e-09 to 5.3e-13, cut off <1e-04). Thirty four of the sequences (60%) are conserved *Plasmodium* proteins of unknown function, some are merozoite surface proteins and others are annotated for different functions. Since the *P. knowlesi* erythrocyte binding ligands (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) demonstrated high identities and low expectation values against the known erythrocyte binding ligands, we designated them as the putative ligands used for adhesion.

Table 3. Putative sequences in the *P. knowlesi* genome used as ligand molecules

Sequences producing HSP (proteins)	**Accession number**
Erythrocyte binding protein (beta)	PKH_000490
Erythrocyte binding protein (gamma)	PKH_134580
Erythrocyte binding protein (alpha)	PKH_062300
Reticulocyte binding protein, putative	PKH_070003
Chromosome associated protein, putative	PKH_051770
Conserved *Plasmodium* protein, unknown function	PKH_060900, PKH_061100, PKH_103220, PKH_143120, PKH_121410, PKH_094540, PKH_092240, PKH_041690, PKH_031040, PKH_126570, PKH_131300, PKH_052170, PKH_101030, PKH_081490, PKH_062110, PKH_100260, PKH_125560, PKH_126220, PKH_133810, PKH_030800, PKH_050220, PKH_081890, PKH_145780, PKH_090490, PKH_112280, PKH_133180, PKH_062110, PKH_083470, PKH_146600, PKH_081330
RNA binding protein, putative	PKH_142610
Ada2-like protein, putative	PKH_081460
Atypical protein kinase, ABC-1 family, putative	PKH_133270
CCAAT-box DNA binding protein subunit B	PKH_094400
Dynein heavy chain, putative	PKH_130100, PKH_092040
Eukaryotic translation initiation factor 4 gamma	PKH_141200
Hypothetical protein, conserved in Apicomplexans	PKH_130600
Hypothetical protein, conserved in *Plasmodium*	PKH_130670, PKH_140190
Merozoite adhesive erythrocytic binding protein	PKH_094500
Merozoite surface protein	PKH_145630
Plasmodium exported protein, unknown function	PKH_120020
Pre-mRNA splicing factor, putative	PKH_060650
Protein phosphatase, putative	PKH_093290
Rhomboid protease, putative	PKH_133640
Rhoptry associated membrane antigen, putative	PKH_010540
Ribonuclease, putative	PKH_113360
Splicing factor, putative	PKH_123140
Tryptophan-rich antigen	PKH_146990
Ubiquitin transferase, putative	PKH_112120
Zinc finger protein, putative	PKH_100710

(Source PlasmoDB ver 9: March 2013)

3.4 Analogous domain organization of putative ligands in P. knowlesi and receptors in P. anubis to P. falciparum and humans respectively

To characterize the putative receptor molecules in *P. anubis* their domain organization was compared to known human receptors using Pfam and NCBI CDD databases. The putative baboon sequences showed similar domain organization to the human receptor sequences (Table 4). The predicted CSPG 4, partial sequence of *P. anubis* and CSA (CSPG) of human origin had the domain Laminin-G. Likewise, the predicted HAPLN 1 sequence of *P.*

anubis and HA (HAPLN 1) of human origin had the domains immunoglobulin V-set and extracellular link protein. The human receptor CD36 molecule (thrombospondin receptor) also had similar domain organization as the predicted *P. anubis* receptors. Similar domains suggest that they are conserved in the Olive baboon and could have evolved to perform a similar function like in humans.

Table 4. Domain organization of putative baboon and known human receptor molecules' sequences

Sequence	Organism	Pfam and NCBI CDD Domains
CSPG		
CSA (CSPG)	*H. sapiens*	Laminin Domain (G1 and G2)
Predicted: CSPG 4, partial	*P. anubis*	Laminin Domain (G1 and G2)
PREDICTED: neurocan core protein; PREDICTED: aggrecan core protein; PREDICTED: LQP versican core protein; PREDICTED: brevican core protein	*P. anubis*	Immunoglobin V-set domain, Xlink (Extracellular link domain), Xlink (Extracellular link domain), EGF-like domain, Lectin C (Lectin C-type domain), Sushi (Sushi domain – SCR repeat)
PREDICTED: FRAS1-related EMP 3; PREDICTED: LQP FRAS1-related EMP 2	*P. anubis*	Calx-beta domain
PREDICTED: LQP: PUP FLJ43447-like	*P. anubis*	None
HAPLN 1		
HA (HAPLN 1)	*H. sapiens*	Immunoglobin V-set domain ; Xlink (Extracellular link domain), Xlink (Extracellular link domain)
Predicted: HAPLN 1; PREDICTED: LQP: HAPLN 3; PREDICTED: HAPLN 4; PREDICTED: HAPLN 2	*P. anubis*	Immunoglobin V-set domain ; Xlink (Extracellular link domain), Xlink (Extracellular link domain)
CD36 molecule		
CD36 (Thrombospondin receptor)	*H. sapiens*	CD36 (CD36 family)
Predicted: PG 4 isoform 1; PREDICTED: LMP 2; PREDICTED: SRCBM 1 isoform 2; PREDICTED: SRCBM 1 isoform 1	*P. anubis*	CD36 (CD36 family)

(Source Pfam and NCBI CDD website)

Key: PG-4 (platelet glycoprotein 4), HAPLN 1 (hyaluronan and proteoglycan link protein 1), PUP (putative uncharacterized protein), LQP (low quality protein), LMP (lysosome membrane protein), SRCBM (scavenger receptor class B member), CSPG (chondroitin sulphate proteoglycan), SCR (short consensus repeats)

To characterize the putative ligand molecules in *P. knowlesi* their protein parameters and gene ontology (GO) terms were determined from PlasmoDB and ProtParam. The GO terms denote the inferred biological process involved in, cellular component and molecular function of the proteins. The putative ligand molecules are located on different chromosomes in the *P. knowlesi* genome. Majority of them are found along chromosomes 12 to 14 (44%), few along chromosomes 1 to 7 (24%) and the rest (29%) along chromosomes 8 to 11 (Table 5). Most of the molecules (71%) are not annotated for any function in their GO terms. The rest are annotated for various functions not related to adhesion. Notably, some are annotated for the biological process of pathogenesis, are integral to the membrane in location and function in either binding or receptor activity (PKH_062300, PKH_134580, PKH_000490, PKH_094500, PKH_140190 and PKH_083470). The molecules have an average molecular weight of 182,449Da (range 53,905-792,939Da) and average isoelectric point of 6.67 (range 3.84-10.4). The molecules (PKH_062300, PKH_134580, PKH_000490, PKH_094500, PKH_140190 and PKH_083470) could be involved in binding in *P. knowlesi* since they show similar gene ontology to the *P.*

falciparum binding ligand var2csa.

Table 5. Protein parameters and gene ontology of the putative ligand molecules in *P. knowlesi*

Accession No.	Location	GO Term Name	Mol.Wt (Da)	pI
PKH_121410	Chr12: 630,081 to 634,832	Carbohydrate metabolic process (BP), extracellular region (CC), carbohydrate binding (MF)	176959	5.08
PKH_120020	Chr12: 3,896 to 6,013	Chromosome telomeric region (CC), telomeric DNA binding (BP)	57166	9.57
PKH_031040	Chr3: 523,717 to 527,129	Intracellular transport (BP)	68156	4.06
PKH_060900	Chr06: 426,927 to 430,622	None	141313	4.29
PKH_061100	Chr06: 502,113 to 507,287	None	192739	10.02
PKH_092240	Chr09: 986,223 to 991,010	None	181982	4.37
PKH_041690	Chr04: 734,902 to 740,466	None	217077	5
PKH_131300	Chr13: 615,512 to 619,429	None	149710	4.13
PKH_052170	Chr05: 965,222 to 968,315	None	97857	4.29
PKH_101030	Chr10: 514,632 to 519,997	None	173506	8.82
PKH_081490	Chr08: 680,039 to 686,932	None	255988	7.67
PKH_062110	Chr06: 940,976 to 946,909	None	205736	9.79
PKH_130670	Chr13: 304,702 to 307,452	None	102905	3.84
PKH_126570	Chr12: 2,845,961 to 2,850,019	None	57705	9.6
PKH_130600	Chr13: 275,798 to 283,680	None	292616	8.25
PKH_100260	Chr10: 170,011 to 175,479	None	209315	6.59
PKH_125560	Chr12: 2,399,748 to 2,402,267	None	97360	10.4
PKH_126220	Chr12: 2,675,025 to 2,697,654	None	792939	8.57
PKH_133810	Chr13: 1,830,784 to 1,835,121	None	167741	4.74
PKH_030800	Chr03: 399,430 to 403,702	None	100445	8.61
PKH_050220	Chr05: 148,645 to 151,737	None	121772	6.65
PKH_081890	Chr08: 866,815 to 872,536	None	195316	5.08
PKH_090490	Chr09: 210,532 to 213,774	None	127731	9.59
PKH_112280	Chr11: 1,140,808 to 1,143,552	None	106513	9.19
PKH_133180	Chr13: 1,534,695 to 1,539,509	None	182749	9.14
PKH_103220	Chr10: 1,455,731 to 1,466,680	None	159469	5.15
PKH_126570	Chr12: 2,845,961 to 2,850,019	None	57705	9.6
PKH_146600	Chr14: 2,950,491 to 2,956,205	None	208322	9.01
PKH_081330	Chr8: 602,756 to 604,958	None	53905	6.17
PKH_130600	Chr13: 275,798 to 283,680	None	292616	8.25
PKH_070003	Chr7: 8,828 to 18,024	None	344108	6.69
PKH_145630	Chr14: 2,529,932 to 2,532,694	None	101555	4.43
PKH_145780	Chr14: 2,586,597 to 2,602,757	Nucleotide binding (BP)	639217	5.28
PKH_062300	Chr6: 1,032,863 to 1,036,679	Pathogenesis (BP), integral to membrane (CC), receptor activity	120703	5.29

PKH_134580	Chr13: 2,177,465 to 2,181,275	Pathogenesis (BP), integral to membrane (CC), receptor activity	121212	4.9
PKH_000490	1,863 to 5,920	Pathogenesis (BP), integral to membrane (CC), receptor activity	130481	4.79
PKH_094500	Chr9: 2,060,514 to 2,067,075	Pathogenesis (BP), membrane (CC), apical complex (CC), binding (MF)	226916	7.74
PKH_140190	Chr14: 74,144 to 77,074	Protein binding	108375	5.78
PKH_083470	Chr8: 1,606,360 to 1,609,299	Protein binding (MF)	112853	4.48
PKH_094540	Chr09: 2,106,607 to 2,111,130	RNA binding (BP)	135372	4.27
PKH_143120	Chr14: 1,429,011 to 1,438,213	RNA binding (BP), binding (MF), transferase activity (MF)	194311	4.17

(Source PlasmoDB ver 9: March 2013)

Key: Biological process (BP), cellular component (CC), molecular function (MF)

Following Pfam analysis less than half of the sequences (30%) were annotated for various domain organizations while 22 sequences (67%) failed to be annotated. In comparison only 11 sequences (33%) failed to be annotated with the rest having various domain organizations in the NCBI CDD analysis (Table 6). Among the annotated sequences, the erythrocyte binding proteins (PKH_000490, PKH_062300, and PKH_134580) had the domains; duffy binding protein N, duffy binding domain, and EBA-175 VI which are similar to the reference *P. falciparum* ligand (Var2csa) which has 7 duffy binding domains. Seven sequences (21%) had the domain Smc (Chromosome aggregation ATPases), and the remaining sequences (36%) had different domains. The *P. knowlesi* erythrocyte binding proteins (PKH_000490, PKH_062300, and PKH_134580) share some domains with the *P. falciparum* binding ligand var2csa indicating that they may be performing similar functions.

Table 6. Domain organization of the putative ligand molecules in *P. knowlesi* genome

Accession No.	**Pfam Domains**
PF3D7_1200600	Duffy binding domains (7)
PKH_094500	Apical membrane antigen 1 (AMA-1), EBA-175 VI
PKH_060900	CDC45
PKH_125560	cwf21 domain
PKH_070003	DUF2937 (unknown function)
PKH_000490, PKH_062300, PKH_134580	Duffy BP N, Duffy binding domain, DBP, EBA-175 VI
PKH_030800	KLRAQ (predicted coiled-coil domain)
PKH_140190	LisH
PKH_130670, PKH_061100, PKH_092240, PKH_041690, PKH_126570, PKH_131300, PKH_052170, PKH_101030, PKH_081490, PKH_062110, PKH_130600, PKH_100260, PKH_133810, PKH_050220, PKH_081890, PKH_090490, PKH_112280, PKH_133180, PKH_083470, PKH_146600, PKH_081330, PKH_145630	None
PKH_126220	PapD-like

PKH_103220		Tryptophan-Threonine-rich Plasmodium antigen C terminal (TryThrA_C)
		NCBI CDD Domains
PKH_094500		Apical membrane antigen 1 (AMA-1), EBA-175 VI, PTZ0012 (MAEBL provisional)
PKH_060900, PKH_092240, PKH_081490, PKH_133810, PKH_081330, PKH_126220,	PKH_130670, PKH_101030, PKH_062110, PKH_146600, PKH_145630, PKH_133180	None
PKH_125560		cwf21 domain
PKH_070003		PTZ00440
PKH_000490, PKH_134580	PKH_062300,	Duffy BP N, Duffy binding domain, DBP, EBA-175 VI
PKH_030800		SH3_and_anchor
PKH_140190		LisH
PKH_061100		2A1904 K+-dependent Na+/Ca+ exchanger
PKH_126570,	PKH_081890	COG2433Uncharacterized conserved protein
PKH_131300		DNA_pol-phi super family
PKH_100260		Inter-Src homology 2 (iSH2) helical domain of Class IA Phosphoinositide 3-kinase; COG1340Uncharacterized archaeal coiled-coil protein
PKH_050220, PKH_112280, PKH_041690, PKH_130600	PKH_090490, PKH_083470, PKH_052170,	Smc, SMC_prok_B
PKH_103220		Tryptophan-Threonine-rich Plasmodium antigen C terminal (TryThrA_C)

(Source Pfam and NCBI CDD)

Key: Smc (Chromosome segregation ATPases), SMC_prok (structural maintenance of chromosomes, prokaryotes)

3.5 Binding of P. knowlesi infected erythrocytes to purified receptors

P. knowlesi infected erythrocytes isolated from pregnant Olive baboons were tested for binding against immobilized purified receptors. None of the *P. knowlesi* infected erythrocytes isolated from either baboon placenta (Pan3443) or peripheral blood (Pan3614 and Pan3443) bound to the purified receptors CD36, CSA or HA (Figure 3B, C and D). The two parasite clones used to optimize the static binding assay bound variably to the purified receptors being tested (Figure 3A). In general, *P. falciparum* CS2 bound at high levels to the CSA and HA receptors (Median, 3820 and 1681 IEs/mm^2 respectively) while *P. falciparum* 3D7 bound at high levels to CD36 (Median, 785 IEs/mm^2). Binding to the receptors CSA and HA was significantly different in PfCS2 (p=0.00) unlike in Pf3D7 where this was not significant (p=0.80). The level of binding of the receptors CSA and HA when compared between the two parasite clones was significantly different (p=0.00 and p=0.00) respectively/

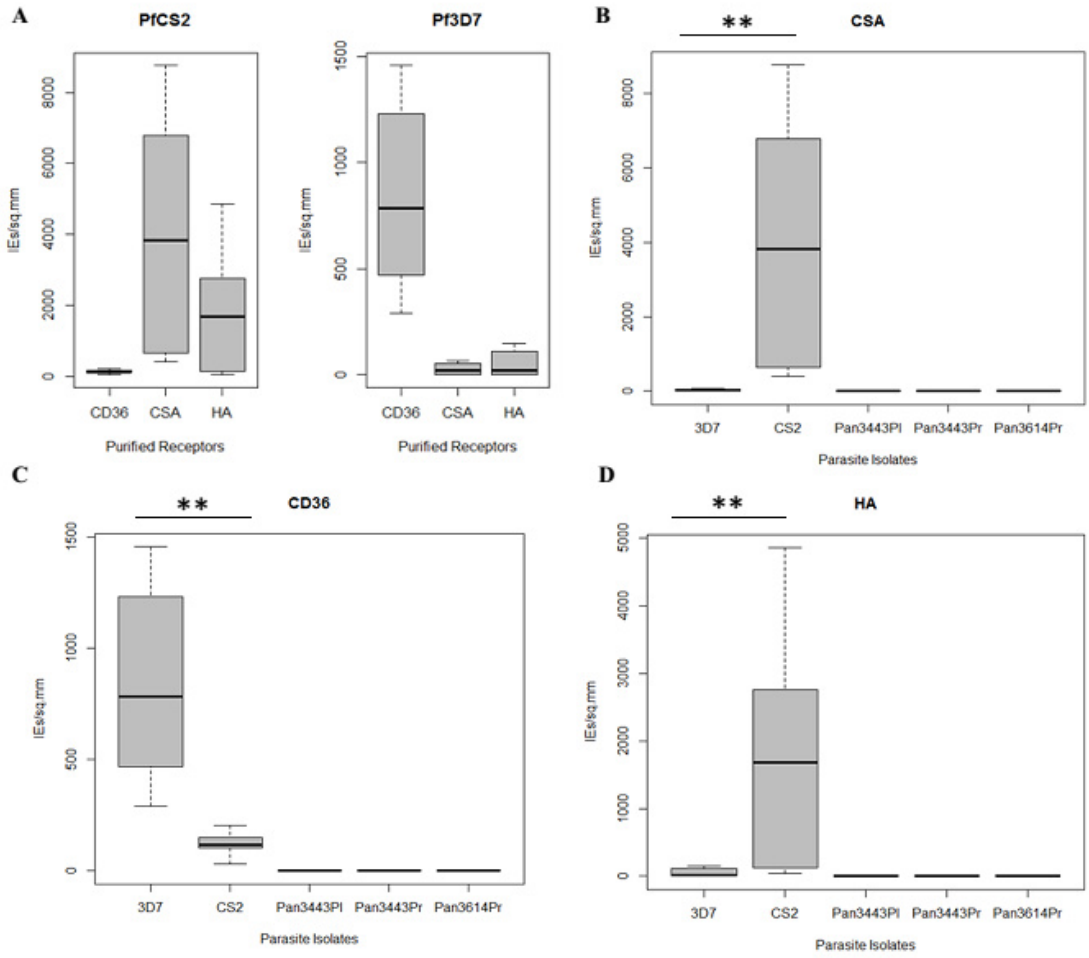


Figure 3. Box and whisker plots of infected erythrocytes binding to purified receptors CSA, HA and CD36

(A) Binding of *P. falciparum* CS2 and 3D7 IEs used as control. (B, C and D) Binding of *P. knowlesi* infected erythrocytes from placenta (PAN3443Pl) and peripheral blood (PAN3614Pr, PAN3443Pr) to purified receptors. Data represents the median and interquartile ranges of adherent infected erythrocytes per mm^2. PfCS2 and Pf3D7 were used as assay performance controls. **p< 0.05

4. Discussion

In the current study, we elucidated the factors that mediate binding in *P. knowlesi* and the Olive baboon using two approaches: (1) comparing putative receptor and ligand sequences in baboon with human and *P. knowlesi* with *P. falciparum* respectively and (2) adhesion assays.

In *P. anubis*, we identified the putative receptors as: predicted CSPG 4, predicted HAPLN 1 and CD36 molecule transcript variants 1, 2 for the corresponding human receptors CSA (CSPG), HA (HAPLN) and CD36 respectively. Previous studies have shown that non-human primates are phylogenetically closer to human beings (Galinski and Barnwell, 2012) and this would explain why the data obtained showed high identities and low e-values for the top BLAST hits. Phylogenetic analyses showed that the putative receptors identified in *P. anubis* all cluster together with sequences from macaques particularly *M. mulatta* and *M. fascularis* which are known natural hosts of *P. knowlesi* (Cox-Singh *et al.,* 2010, 2008). Since the Olive baboon is susceptible to *P. knowlesi* with similar disease outcomes to humans (Mustafa *et al*., 2010; Ozwara *et al.,* 2003) these data suggest that the putative molecules identified could be used for adhesion. On the other hand, receptor sequences from human receptors clustered with sequences from the great apes; gorilla, bonobo and chimpanzee which are phylogenetically closer to humans (Galinski and Barnwell, 2012; Sibal and Samson, 2001). Despite this clustering, the putative receptors have common ancestry to the human receptors since they diverged from base and their evolutionary distance is close.

The putative receptors identified in *P. anubis* had similar domain organization to the known human receptors.

The Laminin-G (LamG) domains common in human and *P. anubis* CSPG sequences are Ca^{2+} mediated receptors with binding sites for steroids, beta1 integrins, heparin, sulfatides, fibulin-1, and alpha-dystroglycans. They play a role in signal transduction via cell-surface steroid receptors, adhesion, migration and differentiation through mediation of cell adhesion molecules (Marchler-Bauer *et al.,* 2011). Likewise, HAPLN 1 sequences had the domains immunoglobulin V-set and extracellular link protein. The link proteins are involved in interaction with HA and contribute to the structural integrity of different tissues (Marchler-Bauer *et al.,* 2011). Lastly, the CD36 family domain identified in the CD36 molecule of both *P. anubis* and human genome is thought to be a novel class of scavenger receptors with a role in signal transduction and cell adhesion (Marchler-Bauer *et al.,* 2011). CD36 is ubiquitous in system, mostly found in vasculature, not in placental tissue (Febbraio *et al.,* 2001; McCormick *et al.,* 1997). These data suggest that the identified molecules in *P. anubis* are likely used for adhesion due to their similar domain structure to the human receptors.

We identified 57 protein sequences from BLAST results as the putative molecules in the *P. knowlesi* homologous to *Plasmodium* binding ligands. Although there was no clear homology observed due to high expectation values, an E-value of <1e-04 was used as cut off to select the best aligning sequences. Majority of these protein sequences were conserved *Plasmodium* proteins of unknown function while some have been annotated for functions other than adhesion like Ada2-like protein, Dynein heavy chain or Ribonuclease. The putative ligand molecules aligned to sequences that are conserved in either *Plasmodium vivax* Sal-1 or *Plasmodium cynomolgi* strain B organisms but which have not been annotated for any function. This suggests close homology between these three species and is consistent with previous studies that have shown *P. knowlesi* and *P. vivax* to be phylogenetically related (Carlton *et al.,* 2008).

Notably, the top hits identified were the *P. knowlesi* erythrocyte binding proteins; EBP-*alpha* (PKH_062300), EBP-*beta* (PKH_000490), and EBP-*gamma* (PKH_134580). These were initially used as queries in this study on the premise that they could be homologous to molecules used in binding of *P. knowlesi* IEs to the placenta of Olive baboons. They are known erythrocyte invasion ligands (Chitnis and Miller, 1994). They have the domains: duffy binding protein N, duffy binding domain, and EBA-175 VI that are similar to the duffy binding domains of the principal *P. falciparum* ligand (Var2csa). Similarly, they are annotated for the biological process of pathogenesis, are integral to the membrane in location and function in either binding or receptor activity (GO terms). This suggests that they could be performing similar functions to the DBL domains of var2csa and are likely the *P. knowesi* ligands responsible for binding to the placenta of Olive baboons.

Sequence similarity searches showed that SICAVar antigen molecule in *P. knowlesi* does not align to *P. falciparum* binding ligands but aligns to its variants in *P. knowlesi*. The SICAvar and KIR genes are the largest variant gene families in the published genome of *P. knowlesi* (Pain *et al.,* 2008). These antigens found on the surface of infected erythrocytes are associated with parasite virulence and have been shown to be essential in antigenic variation (Korir and Galinski, 2006). They have shown that despite the *P. knowlesi* SICAvar and *P. falciparum* var antigen families encoding proteins that enable antigenic variation in the respective organisms, they do not share significant level of sequence identity when aligned. Although distantly related, some *P. knowlesi* SICA peptides show identity with a particular *P. falciparum* EMP1, mapping throughout all characterized domains, including the externally exposed cysteine-rich domains that are characteristic of both proteins (Korir and Galinski, 2006).

We tested *P. knowlesi* infected erythrocytes (*Pk*IEs) isolated from the placenta and the peripheral blood of Olive baboons for their ability to bind to purified receptors in a static binding assay. We found that none of the *Pk*IEs adhered to either CSA, HA or CD36, the receptors responsible for *P. falciparum* sequestration in the placenta and microvasculature in humans (Beeson *et al.,* 2000; Fried and Duffy, 1996). A cerebral malaria study by Fatih and co-workers (2012), found that *Pk*IEs from human subjects bound in a variable manner to the endothelial receptors ICAM-1 and VCAM but not to CD36. In retrospect, *P. knowlesi* malaria in the Olive baboon during pregnancy showed that the infection led to the accumulation of parasites in the intervillous spaces of the placenta leading to pathology (Mustafa *et al.,* 2010). In our study, we used frozen samples instead of fresh isolates in binding assays which might have interfered with the binding capacity of the infected erythrocytes. A study by Ochola and others (2011) reported that they had obtained different patterns of association between binding and clinical phenotypes by using freshly grown parasite isolates than frozen ones (Newbold *et al.,* 1997). Placental isolated parasites are usually in the trophozoite stage and can be used for binding assays directly unlike peripheral parasites that need to be cultured to the trophozoite stage before being used in assays (Beeson *et al.,* 2002). *P. knowlesi* isolates may have failed to bind to purified receptors because these receptors could be unique to *P. falciparum* isolates. Since the data presented here suggests that there are receptor molecules in the baboon that mediate adhesion, they may have a modified structure making them different from human receptors.

Our study has investigated the ligands and receptors that mediate *P. knowlesi* infected erythrocytes' (*Pk*IEs) binding in the Olive baboon. We attest that the molecules discovered are likely the ones mediating adhesion of parasites in the placenta. We propose further work on molecular biology to corroborate our findings. The findings in this study further affirm the close likeness of the *P. knowlesi* infection in baboons to the *P. falciparum* infection in humans. They show that the baboon can be a good model for *in vivo* placental malaria studies at the pre-clinical level. This will be useful in evaluating placental malaria vaccines and other therapeutic agents that can prevent malaria infection during pregnancy leading to better outcomes for mothers and their unborn.

5. Conclusion

The present study has identified and proposed: CSPG 4, CSPG 4-like and HAPLN 1 sequences as the putative receptors molecules in *P. anubis* and shown they share similar domains to the human receptors. We have also shown that the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) could be playing a role in infected erythrocyte adhesion apart from their traditional role of invasion. Static binding using *Pk*IEs however did not show any binding to purified receptors. The findings presented further affirm that the *P. knowlesi*-Olive baboon model can provide a useful pre-clinical model for evaluating placental malaria vaccines and other therapeutic agents.

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