Distribution of Knop blood group antigens and association of Triggering receptor expressed on myeloid cells 1 (TREM-1) gene variants in Ghanaian children with malaria

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SUMMARY

The human immune system is continuously evolving to pathogenic challenges and the innate immune system is the first line of defense that tackles any given pathogen. In this dissertation, I have utilized samples from Ghana, a malaria endemic country. I investigated the host innate immune factors that could potentially alter the malaria susceptibility. My investigations are structured as two independent chapters that are published as scientific articles. In the first chapter of my thesis, I have investigated the distribution of Knops blood group antigens located on complement receptor 1 (CR1, CD35) in Ghanaian population and compared these distribution to other world populations that are not endemic to malaria. The distribution of the CR1 alleles, genotypes and haplotypes differed significantly among geographical settings and certain CR1 genetic variants were more specific in African populations, suggesting a potential selective pressure imposed by the malaria parasite. In the second chapter of my thesis, my investigation focuses on the association of innate immune gene variants and cytokine gene variants in a clinically classified malaria cohort. In particular, the investigations were carried out to understand the role of triggering receptor expressed on myeloid cells (TREM)-1, TREM-like 1 (TLT-1), endothelial protein C receptor, interleukin (IL)-8 and IL-18 variants to malaria outcome. Higher sTREM-1 levels were observed among children suffering from severe malaria compared to those with uncomplicated malaria. Low TREM-1 to TREML-1 ratios were associated with uncomplicated malaria. The TREM1 rs2234237T variant causing the amino acid exchange Thr25Ser, which has been associated with higher TREM-1 plasma levels, was significantly more frequent among patients with severe malaria than in those with uncomplicated malaria. Carriage of the TREM1 rs2234237T allele appears to be a risk factor for the development of severe malaria. Taken together this dissertation contributes to an increased understanding of host genetic factors and malaria susceptibility.
ZUSAMMENFASSUNG

1. INTRODUCTION

1.1. Malaria

Malaria is an endemic disease caused by an infective bite of a female *Anopheles* mosquito. Malaria occurs often in tropical and sub-tropical regions, mostly the high burden being reported in sub-Saharan Africa. Young infants, children, and pregnant women living in endemic areas are the most vulnerable group.

Even though the global malaria cases are on the decline, the disease is still a public health problem, particularly in sub-Saharan Africa (Figure 1). An estimated 438,000 individual deaths were reported in 2015. Of those, 90% of deaths were reported in sub-Saharan Africa, especially in children under five years of age [1]. Parasites causing malaria belong to the genus *Plasmodium* and infect a wide range of hosts. Six species, *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are known to infect humans, while the zoonotic *P. knowlesi* and *P. cynomolgi* cause infection both in Macaque monkeys and in humans [1,2]. Among all the different species, *P. falciparum* is the most virulent one and accounts for high morbidity and mortality.

Figure 1: Countries at risk of malaria. Picture courtesy World Malaria Report 2015 [1].
Life cycle of *Plasmodium* spp. and pathogenesis of malaria

*Plasmodium* parasites have a complex and multi-stage life cycle, including sexual and asexual stages, which occurs alternatively within the intermediate human host and in the definitive female *Anopheles* mosquito as depicted graphically in figure 2 below. Infection is initiated when an infected haemophagous female *Anopheles* mosquito inoculates the human host with sporozoites. Once injected, sporozoites quickly infect liver cells, multiply and emerge from this pre-erythrocytic stage in the blood stream as merozoites after 5-7 days to invade erythrocyte and to initiate the erythrocytic stage of infection. In case of *P. ovale* and *P. vivax*, a proportion of the parasites termed hypnozoites may persist in the liver in a dormant condition, and thus leading to emergence of clinical infection later in days, months or years. The erythrocytic stage involves cyclic invasion of erythrocytes by merozoites, maturation of the parasites to schizont stage, rupture of the infected erythrocytes to release more merozoites to repeat the cycle. Few of these parasites develop into the sexual forms (gametocytes) and are taken by the mosquito again and develop as sporozoites and subsequently infecting humans by an infective bite (Figure.2) [1,3].

![Life cycle of P. falciparum](image)

Figure 2: Life cycle of *P. falciparum*. Figure courtesy Miller et al [3].

The asexual stage is associated with clinical manifestation of malaria. The clinical outcome of malaria is influenced by multitude of host and parasite factors (Figure 3). Most often the
underlying factors that determine the clinical outcome in malaria are not completely understood. The clinical episode of malaria ranges from mild (uncomplicated) to severe malaria leading to death (Figure 3).

![Figure 3: Factors influencing the outcome of malaria infection [3].](image)

The asymptomatic *P. falciparum* infection occurs in older children and adults living in endemic areas. It involves carriage of parasites for many days or weeks without any clinical symptoms mainly due to host genetic predisposition and/or a high degree of anti-malaria immunity [3,4]. Infection could result either as uncomplicated malaria, severe malaria, accompanied by other conditions including vital organ dysfunction. Clinical symptoms are rather non-specific, often shared with other febrile conditions and commonly include fever, chills and sweats, headache, musculoskeletal pain, vomiting, anaemia, jaundice, splenomegaly, convulsions, diarrhoea, coughing and rapid shallow breathing [3,4].

Individuals living in regions where malaria is holoendemic are often semi immune to malaria, whereas others not. However, young infants and children in areas where malaria is endemic who have not had repeated exposure to the parasite may be regarded as nonimmune. Such a condition is also extended to travelers and migrants from non-endemic area [5]. *P. falciparum* infection is invariably associated with severe malaria and is responsible for most of the malaria-related deaths. It may manifest as severe malarial anaemia which is characterized by haemoglobin concentration of <5 g/dl or haematocrit <15%. It may also present as cerebral malaria which is
associated with comatoseness. Other signs of severe malaria include deep breathing, respiratory distress, prostration, hypoglycaemia, acidosis amongst others [6]. Uncomplicated malaria is defined as parasitaemia 1000-50,000/μl on admission, no schizontaemia, circulating leukocytes containing malarial pigment <50/μl, not homozygous for haemoglobin S, haemoglobin >80 g/l, platelets >50/μl, leukocytes <12/μl, lactate <3 mmol/l, and blood glucose >50 mg/dl. Severe malaria is defined as severe anaemia (haemoglobin <50 g/l) and/or hyperparasitaemia (>250,000 parasites/μl, corresponding to >5% infected erythrocytes), a Blantyre coma score ≤2 and other facultative signs of severe malaria such as cerebral malaria, convulsions, hypoglycaemia, and respiratory distress. Asymptomatic malaria is defined as individuals harbouring parasites without clinical signs during sample collection.

1.2. Malaria and Immune responses

The various stages of the malaria parasite in the human host elicit both innate and adaptive immune responses, which involve both humoral and cellular arms of immune responses. These responses against the parasite encompasses a complex network of defense mechanisms, that results in the release of molecules capable of limiting the growth and further development of the parasite or causing immunopathology, which may lead to life-threatening conditions such as cerebral malaria.

Adaptive immunity to malaria

Specific immunity to malaria is mediated by lymphocytes and their products especially the cytophilic antibodies. With increasing age, individuals in malaria endemic regions become exposed to a wider spectrum of plasmodial antigens, developing adaptive immunity first against severe malaria and then uncomplicated malaria, thus, achieving a state of premunition [7-10]. Evidence for the involvement of antibodies in the anti-malarial immunity comes from a series passive experiment demonstrating the efficacy of IgG against the clinical manifestation of infection. Artificial transfer of IgG from semi-immune Africans to malaria patients with limited or no anti-malaria immunity drastically improved prognosis [11-13]. Naturally, passive transfer of maternal antibodies to infants is thought to protect them against clinical malaria in the first few months of life [14,15].
Innate immunity to malaria

Innate immunity to malaria has both humoral and cellular components besides the natural resistance to the parasite or the clinical disease. Parasite-specific molecules interact with pattern recognition receptors (PRR) such as toll like receptor (TLR1, TLR2, TLR4, TLR6, and TLR7), RIG-I-like receptors (RLRs) and NOD-like receptors (NLR) to activate phagocytosis [16,17]. Parasite digestive food vacuole containing haemozoin are taken up by leukocytes with the degree of accumulation of haemozoin in the leukocytes reflecting the severity of an infection. Haemozoin also elicits production of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-1β, and certain chemokines from phagocytes [18,19]. *Plasmodium falciparum* derived GPI anchors are recognized by TLR1-TLR2 or TLR2-TLR6 hetero- and TLR4 homodimers, and during the process, innate immune cells equipped with these receptors are activated, producing pro-inflammatory cytokines including TNF [20-22]. Higher expression of TLR-2 and TLR-4 significantly is associated with protection from severe malaria [23]. The complement system is also activated, which in conjunction with antibodies, inhibit merozoites invasion of erythrocytes [24]. Parasites are readily phagocytosed by the phagocytic cells. This phagocytosis is greatly enhanced when parasites are opsonized by complement [25]. Monocytes have also been documented to release soluble factors following the uptake of the parasite to inhibit parasite growth *in vitro* in antibody-dependent cellular inhibition assays [26]. Similarly, ingestion of opsonized parasites by neutrophils triggers respiratory burst in antibody-dependent respiratory burst assay [25]. Effective antibody-dependent cellular inhibition and antibody-dependent respiratory burst have been shown to associate positively with protection from clinical malaria [27,28]. Natural killer (NK) cells participate in this arm of immune responses by producing interferon gamma (IFN-γ) in response to malaria parasite [29]. *In vitro* experimentations have demonstrated an adverse effect of IFN-γ on multiplication of the parasites in a dose-dependent manner [30] and higher levels are associated with reduced risk [31].

Cytokine responses during malaria

In response to *P. falciparum* infection, various cells are activated and subsequently releasing several pro- and anti-inflammatory cytokines [18,19]. Plasma levels of TNF and IL-18 were
positively correlated with parasitaemia and pigment-containing phagocytes [32,33]. In synergy with IL-1β, IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), TNF promotes phagocytosis of merozoites by phagocytic cells [34]. Increasing levels of TNF and IL-8 were associated with rapid parasite clearance and recovery [35,36]. IFN-γ and IL-12 stimulate macrophages to produce free oxygen radicals, which are toxic to \textit{P. falciparum} [37]. Excessively high IL-8, IL-12, IL-18, TNF and IFN-γ levels were associated with severe malaria rather than uncomplicated malaria [32,36,38]. These pro-inflammatory cytokines are not entirely beneficial to the host. A relatively higher level of TNF in comparison with IL-10, a cytokine that limits the detrimental effect of TNF, is mostly associated with severe malaria and severe malarial anaemia in particular [39,40]. In the same vein, lower levels of transforming growth factor β (TGF-β), an anti-inflammatory cytokine, have been documented in children suffering from severe malaria with a small IL-12/TGF-β ratio being a parameter for poor prognosis [41].

1.3. Malaria and the human genome

Epidemiological evidence shows that certain individuals have developed some inherent resistance to malaria, especially due to selection pressure that has been exerted on the human genome over the years by the parasite. Asexual development of the parasite occurs in erythrocytes, as a consequence, a range of erythrocyte variants exist. These include, haemoglobin gene (\textit{HBB}) polymorphisms HbC, HbE and HbS; regulatory defects of HBA and HBB that give rise to α and β thalassaemia, mutations in the \textit{CD233} (\textit{SLC4A1}), which causes ovalocytosis; polymorphism in Duffy-antigen, which encodes DARC/Fy chemokine receptor; and mutation in erythrocyte enzyme gene \textit{G6PD}, that causes glucose-6-phosphate dehydrogenase deficiency [42].

\textit{P. vivax} uses duffy gene antigen as a receptor for attachment to erythrocytes and the lack of DARC/Fy chemokine receptor on erythrocyte of the Duffy negative blood individuals living in \textit{P. vivax} endemic regions makes such people refractory to \textit{P. vivax} infection. Haemoglobin (Hb) C protects against clinical malaria with HbS, though having little effect on infection per se protects against severe malaria. Other genetic factors such as α and β- Thalassaemias and Glucose-6-Phosphate Dehydrogenase deficiency are protective against severe malaria. South-
east Asian ovalocytosis/Melanesian form of ovalocytosis offers almost complete refractoriness against the development of cerebral malaria, even though; an individual carrying these genes is not immune to infection. In spite of their protective effects, the mechanisms of the protection accorded by these genetic factors against malaria are still not clear. However, it is evident that the parasite has exerted enough selective pressure leading to a positive selection of many human genes [42].

1.4. Complement system

The complement system is made up of both membrane and plasma proteins, of which there are about 30 plasma proteins, which amount to approximately 3 g/L and 15% of the globular fraction of plasma [43]. The system was first identified in 1896 by Bordet as a heat-sensitive factor in serum and named “complement” system because of its ability to complement the antibacterial properties of antibodies in the heat labile portion of serum [44]. Complement proteins influence inflammation and play a pivotal role in host defense against pathogens. Complement protein connect the innate and adaptive immune systems together, in particular through the classical pathway of complement system activation. Complement system has three distinctive pathways; classical, mannan-binding lectin (MBL) and alternative pathways [45].

The classical pathway of complement system activation is triggered when complement factor C1q in complex with C1r and C1s serine proteases bind to Fc region of complement fixing immunoglobulin (Ig) G1 and IgM attached to the surface of pathogens. C1r and C1s then undergo autocatalytic activation and cleave C4 and C2 into C2a, C2b, C4a and C4b fragments. C2a and C4b then form C4bC2a complex, also called classical pathway C3 convertase, on the surface of pathogens. The C3 convertase cleaves C3 to a smaller C3a fragment which leaves the surface of the pathogen as an inflammatory mediator while the larger C3b is retained on the surface of the pathogen as an opsonin, marking the pathogen out as a non-self molecule for phagocytic uptake by phagocytes equipped with a C3b receptor. Additionally, a molecule of C3b may complex with C4bC2a to form C4bC2aC3b, the so-called C5 convertase of the classical pathway, which then produces C5a and C5b from C5. Complement system activation through the lectin pathway begins when mannose groups on microbes are recognized and bound by MBL or ficolin to activate the esterase activity of MBL-associated serine proteins (MASPs), which cleave
C4 and C2 to C2a, C2b, C4a and C4b. The succeeding activation and complex formation of the next complement zymogens through cleavage results in the formation of C3 and C5 convertases just like the classical pathway.

The alternative pathway is initiated through the spontaneous hydrolysis of C3 to iC3. The later binds complement factor B, which is then cleaved by complement factor D to Ba and Bb, producing the so-called fluid phase C3 convertase (iC3Bb). The fluid phase C3 convertase cleaves C3 to form C3a and C3b, creating more C3b to associate with Bb to form the C3 convertase (C3bBb) of the alternative pathway. The C3bBb as formed may be stabilized on microbes by properdin, which may associate with additional C3b to give rise to C5 convertase (C3bBbC3b) on microbes [45].

The C5 convertase from any of the pathways then acts on C5 to produce C5a and C5b. The later then initiates the ‘late’ phase of complement action, a sequence of polymerization reactions during which the terminal complement components (C6, C7, C8 and C9) interact to form a membrane attack complex. Subsequently, membrane attack complex inserts into the membrane of the pathogen, creating pores within and disrupting the ionic and osmotic balance of the pathogen to kill it (Figure 4). Other effector mechanisms associated with complement system activation include chemotaxis (attraction of leukocytes to the site of a pathogen) and opsonophagocytosis. Complement system activation is potentially detrimental to the host unless it is tightly regulated. One of the molecules that regulate all the three complement pathways is complement receptor 1 (CR1) [45].
Figure 4: A schematic presentation of pathways of complement system activation. Diagram courtesy Taylor et al [45].

1.4.1. Complement receptor 1
Human CR1 is a single chain; type 1 membrane-bound glycoprotein, encoded by CR1 gene located on the extended arm of Chromosome 1 at 1q32 locus [46]. CR1 belongs to the family of regulators of complement activation family of proteins. Its extracellular region is organized into 30 short consensus repeats, also known as short complement regulators (SCR) domains, sushi domains or complement control protein repeats, each having 59-75 amino acids [47-49]. SCR are further organized, based upon the degree of internal homology, into four larger units termed long homologous repeats (LHR A, B, C and D). Each of the LHR A, B and C contains seven SCR domains while D has at least nine [47,48,50,51]. The structure of CR1 is formed by 3-6 long homologous repeats. The variation in the number of long homologous repeats is responsible for
the different CR1 isoforms: *CR1*1, *CR1*2, *CR1*3 and *CR1*4 which correspond to, under non-reducing conditions, molecular sizes of 190 kDa, 220 kDa, 250 kDa and 280 kDa, respectively [52]. The four allotypes probably arose from unequal chromosomal crossover event [52]. The most frequently observed of the four is *CR1*1 (*CR1 A or F*), followed by *CR1*2 (*CR1 B or S*) and *CR1*3 (*CR1 F*') with *CR1*3 (*CR1 D*) being the rarest. *CR1*1 is made up of 38 exons, spanning 133kb, and code for a protein of 2,039 amino acids [52].

The polymorphisms that give rise to all known Knops blood group system antigens have been described in either exon 26 or exon 29 of the *CR1*. These polymorphisms and antigens include, York antigen (Yk<sup>a+</sup>/Yk<sup>b</sup>, rs3737002C/T) located on exon 26 while Knops a and b (Kn<sup>a</sup>/Kn<sup>b</sup>, rs41274768G/A, p.V1561M), McCoy a and b (McC<sup>a</sup>/McC<sup>b</sup>, rs17047660A/G, p.K1590E), Swain-Langley/Vilien 1 and 2 (Sl1/Sl2, rs17047661A/G, p. R1601G) [53-55], and KCAM + and – (KCAM+/-, rs6691117A/G, p. I1615V) are situated on exon 29 [53]. Besides the molecular weight, and Knop blood group system antigen polymorphisms is the density-expression polymorphism which defines the quantitative expression of the CR1 molecule on erythrocytes with some individuals being low (LL) expressors while others are either high (HH) or intermediate (HL) expressors [54].

Functionally, CR1 regulates all three pathways of complement activation by binding to C1q, C3b, C4b, and mannan-binding protein. CR1 dampens the intensification of the complement cascade by disrupting C3 convertase that cleave C3 to activate C3 and also by providing cofactor function to Factor I that cleaves C3b to an inactive form, iC3b, which is incapable of assembling a functional C3 convertase [56]. It serves as a receptor for complement fixed immune complexes and enhances phagocytic uptake of complement opsonized pathogens and particles by phagocytes and promotes antigen presentation to T lymphocytes [57].

Several pathogens have subverted the functions of CR1 to their advantage. Epstein-Barr virus, human immuno deficient virus, *Mycobacterium leprae*, *M. tuberculosis*, *Legionella pneumophila*, *Leshmania spp*, and *P. falciparum* interact with and use CR1 to gain access to targeted host cells that include monocytes, macrophages, lymphocytes and erythrocytes [58-62]. Clinical presentations of several infectious diseases have been linked to CR1 expression density. A
decreased CR1 expression is associated with pathogenesis of severe acute respiratory syndrome [63] and the chronic phase of Chagas disease [64].

The pathology of these infections, in some cases, is subjective to variants of CR1 that result from the various mutations in CR1 [65-69]. In vitro experimentation associated Sl1 (rs17047661A) allele with more rosette formation by P. falciparum-infected erythrocytes in relation to Sl2 (rs17047661G) allele [70]. Erythrocytes of individuals carrying Sl1 (rs17047661A) bind more efficiently to recombinant COS7 cells expressing Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP-1) compared to Sl1 (rs17047661A) bearing erythrocytes [70]. Field isolates of P. falciparum from malaria patients also behave similarly [71] suggesting that these CR1 variants could be vital in the pathophysiology of malaria and might have co-evolved with P. falciparum.

1.5. TREM-1 and TLT-1 immune receptors
Triggering receptor expressed on myeloid cells-1 (TREM-1) is a 30-kDa immune-regulatory protein, a member of the immunoglobulin superfamily, and whose expression is up-regulated on cells of the myeloid lineage in response to inflammatory insult [72-76]. Its activation stimulates the development of those cells [77], including polarization of classically activated macrophages [75]. It promotes inflammation in general, including the release of IL-8, monocyte chemoattractant protein 1, Monocyte inhibitory protein 1, TNF and Granulocyte-macrophage colony-stimulating factor [73,77,78], and makes dendritic cells better antigen presenting cells [77].

TREM-1 is expressed on polymorphonuclear neutrophils, monocytes and macrophages (M/M) in response to an inflammatory insult from inflammatory cytokines, some substances from inflammation-damage-associated molecular, bacterial and fungal origins [73,79,80]. TREM-1 regulates innate immunity by enhancing inflammatory signals which are initiated by pathogen-associated molecular pattern (PAMP) receptors, for example, Toll-like receptors (TLR) [78,80-82]. Its engagement stimulates proliferation and maturation of cells of myeloid lineage, expression of costimulatory molecules on macrophages, better antigen presentation by dendritic cells to T lymphocytes for their proliferation, and increased synthesis and secretion of proinflammatory cytokines [78]. Activation of neutrophils through TREM-1, in particular, enhances their respiratory burst activity, degranulation, phagocytosis, the release of
myeloperoxidase, and IL-8 [80]. By promoting inflammatory responses, TREM-1, therefore, accelerates activation of immune cells, but with a down side of enhancing tissue destruction too [77,82,83], which in certain disease conditions, for instance, malaria may be highly pathological. Anti-inflammatory cytokines however individually or synergistically inhibit its expression and function [84]. TREM-1 lacks signaling molecule, and therefore signals upon association with the immunoreceptor tyrosine-based activation motif-containing adapter protein, DAP12 to activate the cell species it affects [72,85].

A soluble isoform of TREM-1 (sTREM-1) exists and can be quantified in biological fluids [86-88]. It is provided through proteolytic cleavage of the membrane-bound form [89] or through alternative splicing [90]. Increased levels of sTREM-1 in plasma are associated with systemic inflammation and severe pathology [86-88]. The plasma level of sTREM-1 is linked to intronic SNP- rs2234237 [91]. The mutant allele, rs2234237T is associated with higher sTREM-1 level, though other factors of unknown origin account for about 30% of its interindividual variability [91]. The SNP- rs2234237 has been linked with severe pathologies in a number of inflammation associated illnesses [88,91,92] but not by others [93,94].

TLT-1 is expressed in megakaryocytes and platelets, and co-localizes with P-Selectin, also called platelet alpha-granule Membrane protein or CD62 in alpha-granules of non-activated platelets [95]. An alternative form of this recently described as TLT-1s with shorter cytoplasmic strand is, however, expressed on macrophages and osteoclast [96]. Unlike the other members of the TREM family, TLT-1 contains an immunoreceptor tyrosine-based inhibitory motif and is thus capable of delivering inhibitory signals when activated [97]. However, the intact TLT-1 promotes mobilization of calcium ions when activated and thus enhances cell activation rather than inhibition [98]. When activated, TLT-1 becomes exposed on the membrane [95] and is afterwards cleaved and released as soluble fragments [99-101]. The soluble isoforms of TLT (sTLT) may compete with the cell surface molecules as well as surface bound TREM-1 for a common ligand and interfere with the productive interaction between these cell surface receptors and their ligands [99,100]. Experimentally, binding and trapping of TREM-1 ligand by sTLT-1 leads to reduced inflammatory response, which limits collateral organ damage, and improves
upon the outcome of microbial sepsis in mice [99] and protect against inflammation-associated haemorrhage [99].

1.6. EPCR and inflammation-associated diseases

Endothelial protein C receptor (EPCR), also called activated protein C receptor or CD201 is a type 1 transmembrane glycoprotein and a member of the major histocompatibility complex (MHC)-class 1/CD1 family of proteins [102]. EPCR is encoded in humans by Protein C receptor gene (PROCR) and serves as a receptor for protein C [102]. It enhances protein C activation, which leads to inhibition of Th1 cytokine secretion and prevention of inflammation-associated damage of vascular endothelium [102,103]. In experimental models, increased expression of EPCR correlates with heightened downregulation of inflammatory responses [104].

A recent report has however implicated EPCR in the pathophysiology of malaria. It facilitates parasite sequestration via binding to PfEMP-1 [105], and serological analyses have so far indicated loss of expression of ECPR during malaria [88,106,107], and in some cases, a lower level in uncomplicated malaria compared to severe malaria patients or healthy controls [108]. About 60% of interindividual variability in levels of EPCR are influenced by rs867186AG [109], a locus linked to the outcome of *P. falciparum* infection in a recent report [108,109] but not in several others [88,106,110].

2. STUDY AIMS

Two distinct investigations were carried out. The first investigation with samples collected from healthy individuals from different world populations, the second investigation on samples from Ghanaian children who suffered from uncomplicated and severe malaria.

The specific objectives addressed are:

1. Diversity of Knop blood group antigens in malaria and non-malaria endemic populations (Paper I) were investigated.

2. The circulating levels of soluble TREM-1, TLT-1 and EPCR were investigated for correlation with clinical malaria (Paper II),
3. TREM-1, PROCR, IL-8 and IL-18 genetic variants were investigated for possible associations with clinical malaria (Paper II).

3. RESULTS

3.1. Manuscript one


*Shared first authorship (equal contribution).

The distribution of the various CR1 genotypes in all the populations investigated was in Hardy-Weinberg equilibrium (HWE) ($p>0.05$). The genotype and allele frequencies of CR1 rs17259045 ($p=0.002$), rs17047660 ($p<0.0001$), rs17047661 ($p=0.01$) and KCAM+/− (rs6691117) ($p<0.006$) SNPs differed significantly amongst the five populations while those of CR1 rs41274768 and rs4844609 SNPs were comparable ($p>0.05$). The distribution of variant allele of each of McCa/b (rs17047660A/G), SI1/2 (rs17047661A/G) and KCAM+/− (rs6691117A/G) SNPs was similar in Ghanaian and Congolese populations ($p>0.05$). However, each of the derived allele of McCa/b (rs17047660A/G), SI1/2 (rs17047661A/G) and KCAM+/− (rs6691117A/G) SNPs was significantly over-represented in the Ghanaian and Congolese populations compared to other populations ($p<0.01$). All the investigated SNPs were polymorphic in the Brazilian population. The minor alleles of CR1 rs17259045A/G, Knab (rs41274768G/A) and rs4844609T/A SNPs were identified in the Brazilian population only. Except CR1 KCAM+/− (rs6691117), all the investigated loci were monomorphic in the Vietnamese population. The heterozygote CR1 rs17259045AG genotype and the CR1 rs17259045G allele were more frequent in the Brazilian population than the other ones. In the Indian population, three SNPs, rs17259045, Knab (rs41274768G/A) and KCAM+/− (rs6691117A/G) were observed.

Haplotypes reconstruction with the six CR1 SNPs under investigation revealed a total of nine haplotypes. The commonest haplotype observed in Ghanaian and Congolese populations was
CR1*AGAGTG while CR1*AGAATA haplotype occurred more frequently in the Brazilian and Vietnamese populations. The CR1*AGAATG haplotype was detected mostly in the Indian population than any other. Linkage disequilibrium (LD) analyses indicated a medium level LD for CR1 SNPs SI1/2 (rs17047661) and KCAM+/− (rs6691117), and for McC\textsuperscript{ab} (rs17047660) and SI1/2 (rs17047661) in both the Congolese and Ghanaian study groups.

3.2. Manuscript two


Acute levels of sTLT-1, sTREM-1 and sEPCR were differentially associated with severity of the infection. A significantly high level of sTREM-1 was found in severe malaria group compared to uncomplicated malaria group (p=0.049). Absolute plasma levels of sTLT-1 in both groups were similar (p=0.115) but a greater sTLT-1 to sTREM-1 ratio observed in severe malaria compared to uncomplicated malaria (p=0.033). Acute-state plasma sEPCR levels were similar in severe malaria and uncomplicated (p=0.095). By day 14 following the initiation of antimalarial treatment, plasma levels of EPCR had increased significantly in both uncomplicated malaria (p=0.0007) and severe malaria (p=0.0002) groups.

The distribution of TREM-1 rs2234237A/T (p=0.451) and TREM-1 rs34727391A/C (p=0.858) SNPs was in HWE. The distribution of the various TREM-1 rs2234237A/T genotypes in uncomplicated malaria was significantly different from that of severe malaria children in a codominant model (AA vs. AT vs. TT, p=0.036). The AA genotype, which is known to be associated with low plasma sTREM-1 level, was observed less frequently in uncomplicated malaria (AA vs. AT + TT, OR=2.4; 95% CI: 1.2-4.5, p=0.01). Comparing allele frequencies also revealed over-representation of the rs2234237A in children who suffered from uncomplicated malaria (p=0.018). Irrespective of the genetic model employed in the analyses, none of the genotypes of TREM-1 rs34727391A/C associated with uncomplicated or severe malaria (p=0.7).
The distributions of the two SNPs, rs867186 \((p=0.567)\) and rs1051021 \((p=0.822)\) detected in the \textit{PROCR} were in HWE; their genotype frequencies were similar between the children who suffered from uncomplicated and severe malaria \((p=0.789\) and \(p=0.625\), respectively).

The distribution of \textit{IL-8} SNPs, rs4073A/T \((p=0.874)\) and rs2227538C/T \((p=0.533)\) was in HWE. The frequencies of the various genotypes of \textit{IL-8} rs4073A/T were similar in the two paediatric malaria groups \((p=0.466)\) irrespective of the genetic model used in the analyses. Allele distribution analysis revealed no association between any of the allele and either uncomplicated malaria or severe malaria \((\text{OR}=1.5, \text{95\% CI:} 0.9-1.7, p=0.18)\). Likewise, the distribution of \textit{IL-8} rs2227538C/T SNPs was comparable in the two groups \((p=0.592)\). None of the allele showed an association with either uncomplicated malaria or severe malaria \((\text{OR}=1.5, \text{95\% CI:} 0.9-2.5, p=0.123)\).

The rs1946518C/A and rs187238G/C SNPs of \textit{IL-18} were polymorphic enough for evaluation and were assessed about the malaria disease phenotype expression. The distribution of all \textit{IL-18} SNPs, rs1946518C/A \((p=0.973)\) and rs187238G/C \((p=0.883)\), was in HWE. The frequencies of various genotypes of rs1946518C/A were similar in both uncomplicated malaria and severe malaria groups \((\text{AA vs. AC vs. CC}, p=0.167)\). Analysis of allele frequencies of the rs1946518C/A SNP revealed no association between any allele and uncomplicated or severe malaria \((\text{OR}=0.7, \text{95\% CI:} 0.66 – 1.0, p=0.065)\). The distribution of genotypes of \textit{IL-18} rs187238G/C SNP did not differ between uncomplicated or severe malaria \((\text{GG vs. GC vs. CC}, p=0.237)\). Similarly, the distribution of the alleles of the rs187238G/C SNP was comparable between the children who suffered from uncomplicated malaria and severe malaria \((\text{OR}=1.5, \text{95\% CI:} 0.9-2.0, p=0.123)\).

\textbf{4. GENERAL DISCUSSION AND CONCLUSION}

Every organism defends against pathogens by evolving certain immune defense mechanisms involving both innate and adaptive immunity \cite{111}. In order to effectively thwart an infection, the pathogens should be killed either by lysis or opsonization by specific immune cells. Such processes are mediated by PRRs, which present the pathogen to specific immune cells of the innate or adaptive immune system. Among many such receptors that act as immune mediators or as PRRs, TREM-1 and CR1 are involved in tackling the pathogen and containing them. Genes encoding these receptor molecules are subjected to constant pressure imposed by the parasite.
The pressure imposed on such immune gene loci may be selective. For instance, human genes undergo selection in regions where malaria is holoendemic. Such observations were reported. In this context, my investigations were aimed to investigate certain immune genetic variants and their role in malaria and non-malaria endemic population.

**CRI variants in malaria and non-malaria endemic population**

*CRI* genetic variants in exon 29 are associated with *CRI* expression levels, C1q or C3b binding activity and increased susceptibility to various infectious diseases. This study investigated the entire exon 29 of *CRI* in five diverse populations to assess the distribution of Knops blood group antigens and the distinct functional *CRI* SNPs. Such studies on geographically diverse populations can provide insights on how these *CRI* alleles have spread in populations and contribute to the understanding of natural selection. Allele and genotype frequencies of *CRI* variants in exon 29 [rs17259045, rs41274768 (Kna/b), rs17047660 (McCa/b), rs17047661 (Sl1/Sl2), rs4844609 (Sl4/Sl5), rs6691117 (KCAM+/-)] as well as their haplotype frequencies were differently distributed among the Brazilian, Vietnamese, Indian, Congolese and Ghanaian study groups. So far, the frequencies of these variants and especially, the distribution of blood group antigens have not been described explicitly for central African populations yet. *CRI* variants rs17047660A/G (McCa/b) and rs17047661A/G (Sl1/Sl2) were observed to be polymorphic only in the African groups compared to those from Asia and Brazil, indicating that the frequencies of these two SNPs result from a strong selective bias exerted by exposure to distinct pathogens, especially by *P. falciparum*. This is substantiated by a high linkage disequilibrium between the two variants. This locus also determines the Knops blood group antigen McCa/b.

Studies have demonstrated that this blood group antigen is dominant among many ethnic groups of African ancestry living in malaria endemic regions. Also, the reported frequencies of these two loci, rs17047660A/G (Sl4/Sl5) and rs1704661A/G (Sl1/Sl2), in this study were in accordance with frequencies observed in other East and West African ethnicities as reported in the 1000 Genomes database ([https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes](https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes)). The frequencies in other African populations correspond to the frequencies observed in this study [rs17047660A/G (McCa/b): Gambian 0.67/0.32, Kenyan 0.69/0.31, Sierra Leone 0.71/0.29 and
Yoruba 0.73/0.27; whereas for rs17047661A/G (Sl1/Sl2): Gambian 0.21/0.78, Kenyan 0.30/0.70, Sierra Leone 0.21/0.79 and Yoruba 0.30/0.70]. Higher rates of adaptive evolution are expected to occur especially in genes involved in the immune system, as these gene loci coevolve with pathogens. This is largely contributed by two factors the genetics of the population and natural selection. Immune genes tend to evolve rapidly as selection pressure is changing continuously due to various pathogenic challenges. Therefore, positive selection of rs17047660A/G (McCa/b) and rs1704661A/G (Sl1/Sl2) loci is expected in sub-Saharan African populations exposed to distinct pathogenic challenges (e.g. falciparum malaria). Such a selective advantage occurs mainly in immune genes involved in pathogen recognition and signaling, and the CRI is one of such genes involved in innate immunity. Taken together, this study revealed significant differences in allele, genotype and haplotype frequencies of CRI SNPs in five populations. This study, first to include population from Central Africa, may provide an increased understanding of the contribution of red blood cell phenotypes and the complement regulator protein with regard to possible associations with infectious diseases including malaria.

**TREM-1 and malaria**

TREM-1 regulates inflammatory responses during microbial infections and plays a role in anti-malarial immunity [77,86]. The relationship between clinical malaria and serum levels of TREM-1 were assessed. Also, a possible association between clinical malaria and TREM1 variants was investigated. The data indicated plasma sTREM-1 levels were significantly higher in uncomplicated malaria patients compared to the severe malaria patients, which concurs with an earlier report [86]. As high levels of sTREM-1 are linked to inflammatory pathology, the higher levels of sTREM-1 in severe malaria cases might, therefore suggest a very intense inflammatory response during the acute phase of infection, especially, in children who succumbed to severe malaria. Activation of immune cells through TREM-1 leads to skewed secretion of a larger amount of TNF in comparison to IL-10. This phenomenon is associated with the pathogenesis of severe malaria. Thus, stronger production of sTREM-1 in severe malaria might be pathogenic in nature.

Nearly two-third of the inter-individual variations in serum levels of TREM-1 were accounted for by TREM1 rs2234237A/T; the rs2234237AA and rs2234237TT genotypes are associated with
the lowest and the highest levels respectively [91]. The rs2234237T allele is associated with severe malaria, indicating that one is under increased risk of developing severe malaria compared to uncomplicated malaria as a result of the carriage of this allele. This observation is consistent with the serological data and falls in line with earlier studies [86,91]. Thus, carriage of the T allele seems a risk factor for the development of severe malaria once an individual is infected with *P. falciparum*. Thus, the observations made here suggest that the role of TREM-1 in malaria may parallel its role in other inflammation associated disorders, including sepsis, where its high serum levels and the rs2234237T- allele have been linked with severe pathology [76,87,92].

TLT-1 transcripts share homology with the transmembrane domains of TREM-1 and compete with membrane-bound TREM-1 for the yet unknown TREM-1 ligand. It is, therefore, viewed as a de novo inhibitor of TREM-1-mediated activation of immune cells [99]. Absolute TLT-1 levels could not differentiate between severe and uncomplicated malaria, significantly high ratios of sTREM-1 to sTLT-1 were associated with severe malaria, indicating that inadequate production of TLT-1 relative to TREM-1 levels during malaria infection can contribute to pathology. Evidence from clinical trials and *in vivo* experiments suggests that anti-inflammatory properties of TLT-1 could be harnessed for therapeutic use against inflammation associated diseases [97,99,112]. Inadequate production of TLT-1 may, therefore, lead to severe malaria.

**Endothelial protein C receptor and malaria**

Malaria infections are associated with endothelial cell activation and damage with several biomarkers of endothelial damage relating positively to the severity of malaria [113,114]. High plasma levels of sEPCR indicate extensive endothelial damage [115,116] and/or higher cell membrane bound EPCR (mEPCR) expression. A trend to reduced levels of sEPCR was observed in severe malaria. This is in contrast to the findings of Moussiliou et al. [110]. The difference in that and our report is difficult to comprehend. One reason may be that the patients in our study were slightly older than those described earlier [110]. Our report is, however, consistent with the study by Moxon et al., who clearly demonstrated that unlike malaria, infection with several other pathogens could lead to plasma levels of EPCR above baseline values [107]. The current report thus fits well with the loss of EPCR expression [107] which can aggravate inflammation during malarial infection. Evidence from experimental studies indicates that increasing EPCR
expression translates into increasing anti-inflammatory response [104]. Following the interaction with mEPCR, endothelial protein C becomes activated and inhibits monocyte migration via EPCR, upregulates IL-10 production and thereby dampens inflammation [117-119].

Loss of EPCR expression during *P. falciparum* infection appears to be a general phenomenon as sEPCR levels increased in both uncomplicated and severe malaria patients by day 14 post initiation of anti-malarial therapy. Even though one cannot rule out the presence of residual parasite materials in circulation to activate cells, even on day 14, our participants were aparasitaemic by microscopy and had fully recovered. Thus, the loss of EPCR expression during the acute state can rather be attributed to parasite factors, which could limit the activation of a cytoprotective and anti-inflammatory function of endothelial protein C and disrupt the blood-brain barrier [107,120]. The limitation may precipitate increased inflammatory responses to cause haemorrhage in sites of parasite sequestration [105]. The occurrence of haemorrhages in the brain microvasculature associated with cerebral malaria [121]. This vicious cycle, involving reducing the interaction between mEPCR and endothelial protein C to promote pro-inflammatory cytokine production, may aggravate the severity of malaria. At the genetic level, no association between any of the genotypes and clinical malaria was observed, confirming earlier studies [110,122].

**IL8 and IL18 variants in clinical malaria**

None of the cytokine gene variants (*IL8* rs4073, *IL8* rs2227538, *IL18* rs1946518, *IL18* rs187238) could be linked to the outcome of malaria. The role of *IL-8* rs4073A/T SNP in clinical malaria has contradictory associations [123,124]. The lack of significance could be due to the small sample size and the size of the effect of each of these investigated Interleukin loci.

**CONCLUSION**

Taken together, the first study demonstrates that distinct distribution of *CRI* alleles especially in African populations, may contribute to the fact that, this locus might have a selective advantage conferred to immune genes involved in pathogen recognition and signaling, possibly contributing to disease susceptibility or resistance. The second study demonstrates that higher plasma levels of sTREM-1 alone or relative to sTLT-1 during malaria predisposes to the phenotype of severe
malaria. Carriage of the *TREM1* rs2234237T allele appears to be a risk factor for the development of severe malaria.
5. PERSONAL CONTRIBUTIONS

My contributions to the two papers that form the basis of this thesis include:

Paper I

➢ Collected samples,
➢ Performed laboratory experiments,
➢ Participated in analyses and interpretation of the datasets,
➢ Wrote and approved the manuscript for publication,
➢ Participated in addressing the reviewers’ comments.

Paper II

➢ Conceived the original idea,
➢ Designed the study,
➢ Collected samples,
➢ Performed the laboratory experiments,
➢ Analyzed and interpreted the datasets,
➢ Wrote and approved the manuscript for publication,
➢ Participated in addressing the reviewer’s comments.
6. REFERENCE LIST

(1) WHO. World malaria report 2015. WHO.


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Publications


9. APPENDIX:

PUBLICATIONS I and II
Geographical distribution of complement receptor type 1 variants and their associated disease risk

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Abstract

Background

Pathogens exert selective pressure which may lead to substantial changes in host immune responses. The human complement receptor type 1 (CR1) is an innate immune recognition glycoprotein that regulates the activation of the complement pathway and removes opsonized immune complexes. CR1 genetic variants in exon 29 have been associated with expression levels, C1q or C3b binding and increased susceptibility to several infectious diseases. Five distinct CR1 nucleotide substitutions determine the Knops blood group phenotypes, namely Kn+a/b, McC+a/b, SI1/Si2, Si4/Si5 and KCAM+-/−.

Methods

CR1 variants were genotyped by direct sequencing in a cohort of 441 healthy individuals from Brazil, Vietnam, India, Republic of Congo and Ghana.

Results

The distribution of the CR1 alleles, genotypes and haplotypes differed significantly among geographical settings (p<0.001). CR1 variants rs17047660A/G (McC+a/b) and rs17047661A/G (SI1/Si2) were exclusively observed to be polymorphic in African populations compared to the groups from Asia and South-America, strongly suggesting that these two SNPs may be subjected to selection. This is further substantiated by a high linkage disequilibrium between the two variants in the Congolese and Ghanaian populations. A total of nine CR1 haplotypes...
were observed. The \textit{CR1*AGAATA} haplotype was found more frequently among the Brazilian and Vietnamese study groups; the \textit{CR1*AGAATG} haplotype was frequent in the Indian and Vietnamese populations, while the \textit{CR1*AGAGTG} haplotype was frequent among Congolese and Ghanaian individuals.

**Conclusion**

The African populations included in this study might have a selective advantage conferred to immune genes involved in pathogen recognition and signaling, possibly contributing to disease susceptibility or resistance.

**Introduction**

Complement receptor type 1 (CR1) is widely recognized to play a role in disease pathophysiology, diagnosis, prognosis and in therapy [1]. The gene encoding human CR1 is located on chromosome 1 (1q32.2; OMIM 120620) [2–4]. CR1 belongs to the regulator of complement activation family (RCA) and is a transmembrane glycoprotein (single chain type 1), which occurs either in membrane-bound or soluble forms [2,5]. CR1 is predominantly involved in the transport of circulating immune complexes to the reticuloendothelial system.

CR1 acts as a regulator in the three pathways of the complement system [2], namely the classical, the lectin and the alternative pathway. It enhances phagocytosis of opsonized particles together with the complement components C3b, C4b, C1q, mannose-binding lectin and ficolin-2, thereby facilitating clearance of opsonized immune complexes. In the presence of Factor I, CR1 suppresses the complement cascade by inactivating C3b and C4b [6]. CR1 comprises of 30 short complement regulator (SCR) domains, known as complement control protein repeats (CCPs). Four protein isoforms have been identified based on their molecular weight and the number of \textit{CR1} exons [3]. Groups of seven CCPs are organized into four long homologous repeats (LHRs A to D) [7,8].

CR1 is also expressed on cells involved in both innate and adaptive immune responses [9–11]. The erythrocyte CR1 binds to circulating immune complexes and to complement-coated particles to transport them to the liver or spleen for subsequent phagocytosis [2,3]. CR1 deficient mice showed decreased and delayed IgM and IgG responses to West-Nile virus, thus increasing mortality [12]. Moreover, \textit{in vitro} studies have shown that CR1 has distinct adjuvant properties [13–16], probably due to its involvement in uptake of antigen by antigen-presenting cells [17].

Three types of polymorphisms have been characterized in the \textit{CR1} gene, namely those generating size variants, those resulting in copy number differences on red blood cells and polymorphisms forming the Knops blood group antigens [1,18]. Five distinct \textit{CR1} nucleotide substitutions determine the Knops blood group phenotypes: Knops (rs41274768, Kn\textsubscript{a/b}, p.N1540S), McCoy (rs17047660, McC\textsubscript{a/b}, p.K1590E), Swain-Langley/Villien (rs17047661, Sl1/Sl2, p.R1601G), Swain-Langley (rs4844609, Sl4/Sl5, p.T1610S), and the KCAM antigens (rs6691117, KCAM+/−, p.I1615V) [19–23].

In the process of pathogen evasion from the host’s immune system, pathogens bind to complement receptors and other regulatory proteins to facilitate their uptake by host cells. This may considerably downregulate and impair the function of the complement system [24]. For instance, CR1 has been reported to facilitate entry of intracellular pathogens into host cells and CR1 protein levels are associated with disease susceptibility. Among protozoan parasites,
CR1 mediates immune adherence of intracellular *Leishmania* amastigotes [25] to present them to macrophages, the preferred habitat of *Leishmania* [26,27]. Low CR1 levels were associated with a decreased degree of opsonisation in patients with chronic *Trypanosoma cruzi* infection [28]. Among viral infections, CR1 has been shown to be a secondary receptor for Epstein-Barr virus (EBV) [29] and to expedite the entry of EBV into cells [30,31]. CR1 is associated with the pathogenesis caused by SARS-CoV [32], adenoviruses [33] and other viral infections such as HIV and HCV [30].

The present study utilized samples from five populations originating from Brazil, Ghana, Republic of Congo, India and Vietnam and aimed to assess the distribution of the different Knops blood group antigens and functional CR1 genetic variants [rs17259045, rs41274768 (Kn<sup>ab</sup>), rs17047660 (McC<sup>ab</sup>), rs17047661 (Sl1/Sl2), rs4844609 (Sl4/Sl5), rs6691117 (KCAM+/-)] in exon 29 that were involved in pathogen recognition and signaling, possibly contributing to disease susceptibility or resistance.

**Methods**

**Ethics statement**

The study was approved by the Ethics Committee of the Hospital de Clínicas in Curitiba, Brazil, the institutional Review Board of the Tran Hung Dao Hospital, Hanoi, Vietnam, the Ethics Committee of the CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India, Ethics Committee of the LEPRa-Blue Peter Public Health and Research Centre; the Ethics Committee of the Fondation Congolaise pour la Recherche Médicale, Brazzaville, Republic of Congo and the Ethics Committee of the Noguchi Memorial Institute for Medical Research, Ghana. Informed written consent was received from all studied participants (consent from parents if the participant was under 18 years old).

**Study population**

A total of 441 DNA samples from healthy individuals were utilized. Investigations were carried out in populations from Brazil [n = 102; mean age 51±7; 48% (49/102) were female and 52% (53/102) male], Ghana [n = 77; mean age 5±3; 45% (28/62) were female and 55% (34/62) male], Republic of Congo [n = 77; mean age 3±3; 49% (38/77) were female and 51% (39/77) male], India [n = 86; mean age 32±18; 39% (30/78) were female and 61% (48/78) male] and Vietnam [n = 99; mean age 26±5; 40% (36/89) were female and 60% (53/89) male].

**CR1 genotyping**

In order to assess the distribution of six functional variants [rs17259045, rs41274768 (Kn<sup>ab</sup>), rs17047660 (McC<sup>ab</sup>), rs17047661 (Sl1/Sl2), rs4844609 (Sl4/Sl5), rs6691117 (KCAM+/-)], the complete *CR1* exon 29 including their intron-exon boundaries was screened by direct sequencing in the 441 DNA samples (Table 1). A fragment of 884 bp in exon 29 of the *CR1* gene was amplified by polymerase chain reaction (PCR) using the *CR1* locus specific primer *CR1F* (5′-TCT TCA TAA ATA ATG CCA GAA GTG G-3′) and *CR1R* (5′-TGC CAA TTT CAT AGT CCT TAT ACA C-3′). PCR amplifications were carried out in a 25 μl volume of reaction mixture containing 10X PCR buffer, 3.0 mM MgCl2, 0.2 mM dNTPs, 0.2 μM of each primer, 1 unit of Taq polymerase (Qiagen GmbH, Hilden, Germany) and 20 ng of genomic DNA on a TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany). Cycling parameters were initial denaturation at 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute, and a final elongation step at 72°C for 10 minutes. PCR fragments were stained with SYBR
Safe DNA Gel Stain (Invitrogen, Carlsbad, USA) and visualized on 1.5% agarose gels. PCR products were subsequently purified using Exo-SAP-IT (USB, Affymetrix, Santa Clara, CA, USA) and the purified products were directly used as templates for sequencing using the Big-Dye terminator v. 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130XL DNA sequencer according to the manufacturer’s instructions. DNA polymorphisms were identified by assembling the sequences with the reference sequence of the CR1 (NM_000573) using Geneious v9.1.4 software (Biomatters Ltd, Auckland, New Zealand) and reconfirmed visually from their respective electropherograms.

### Statistical analysis

Statistical analyses were performed using the GraphPad Prism 3.0 software package (GraphPad Software, La Jolla, CA, USA) and Stata 12.0 (StataCorp, College Station, TX, USA). Normal Chi square and two tailed Fisher’s exact tests were calculated to determine the differences of

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<th>Vietnamese n = 99 (%)</th>
<th>Indian n = 86 (%)</th>
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<td>119 (77)</td>
<td>104 (67)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G 2 (1)</td>
<td>0</td>
<td>0</td>
<td>35 (23)</td>
<td>50 (33)</td>
<td></td>
</tr>
<tr>
<td>rs17047661A/G</td>
<td>AA 94 (93)</td>
<td>99 (100)</td>
<td>86 (100)</td>
<td>7 (9)</td>
<td>7 (9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>AG 7 (7)</td>
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<td>0</td>
<td>31 (40)</td>
<td>28 (36)</td>
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<tr>
<td></td>
<td>GG 0</td>
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<td>0</td>
<td>39 (51)</td>
<td>42 (55)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A 195 (96)</td>
<td>198 (100)</td>
<td>172 (100)</td>
<td>45 (29)</td>
<td>42 (26)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G 7 (4)</td>
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<td>0</td>
<td>109 (71)</td>
<td>112 (74)</td>
<td></td>
</tr>
<tr>
<td>rs4844609T/A</td>
<td>TT 99 (98)</td>
<td>99 (100)</td>
<td>86 (100)</td>
<td>77 (100)</td>
<td>77 (100)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TA 2 (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>AA 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T 200 (99)</td>
<td>198 (100)</td>
<td>172 (100)</td>
<td>154 (100)</td>
<td>154 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A 2 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6691117A/G</td>
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<td>37 (37)</td>
<td>21 (24)</td>
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<td>&lt; 0.006</td>
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<td></td>
<td>AG 33 (33)</td>
<td>53 (53)</td>
<td>39 (46)</td>
<td>19 (25)</td>
<td>10 (13)</td>
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</tr>
<tr>
<td></td>
<td>GG 7 (7)</td>
<td>9 (9)</td>
<td>26 (30)</td>
<td>58 (75)</td>
<td>64 (83)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A 155 (77)</td>
<td>127 (64)</td>
<td>81 (47)</td>
<td>19 (12)</td>
<td>16 (9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G 47 (23)</td>
<td>71 (36)</td>
<td>91 (53)</td>
<td>135 (88)</td>
<td>138 (91)</td>
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</table>

NS, not significant; NA, not applicable

https://doi.org/10.1371/journal.pone.0175973.t001
genotype, allele and haplotype frequencies among the different ethnicities. Genotype and allele frequencies were determined by simple gene counting and haplotypes were reconstructed by using the expectation-maximum (EM) algorithm as implemented in the Arlequin v3.5.2.2 software (http://cmpg.unibe.ch/software/arlequin35/Arl35Downloads.html). The significance of deviations from Hardy Weinberg equilibrium was tested using the approach of Guo and Thompson random-permutation procedure implemented in Arlequin v. 3.5.2.2 software. Linkage disequilibrium (LD) analysis was performed using the Haploview v. 3.2 program (https://www.broadinstitute.org/haploview/downloads). The level of significance was set to a p-value of <0.05.

Results

The frequencies of CRI genotypes in the five populations were in Hardy Weinberg equilibrium (p>0.05). The allele and genotype frequencies of the CRI SNPs rs17259045, rs17047660 (McC\(^{ab}\)), rs17047661 (Sl1/Sl2) and rs6691117 (KCAM+/+) differed significantly among the groups (p<0.01) (Table 1). Genotype frequencies of the CRI variants rs41274768 (Kn\(^{ab}\)) and rs4844609 (Sl4/Sl5) did not differ. The rs17259045AG genotype and the rs17259045G allele were more frequent in the Brazilian population. Moreover, the G carriers (AG and GG) and the G allele of variants rs17047660 (McC\(^{ab}\)), rs17047661 (Sl1/Sl2) and rs6691117 (KCAM+/-) were observed more commonly among the two African populations (Republic of Congo, Ghana). Interestingly, among Congolese and Ghanaian individuals the minor allele of SNPs rs17259045A/G, rs41274768G/A (Kn\(^{ab}\)) and rs4844609T/A (Sl4/Sl5) did not occur at all; this allele was observed exclusively in Brazilian individuals. Except for rs6691117 (KCAM+/-), the Vietnamese population was monomorphic. The Indian group was monomorphic for three of the SNPs, but not for rs17259045, rs41274768 (Kn\(^{ab}\)) and rs6691117 (KCAM+/-). Brazilian individuals were polymorphic for all SNPs (Table 1). The Knops blood antigen distribution among the studied populations is summarized in Table 2.

Haplotypes were reconstructed from the six CRI variants. A total of nine haplotypes were observed. The haplotype distributions are summarized in Table 3 and Fig 1. The CRI'AGAATA haplotype was more frequent among the Brazilian and Vietnamese populations; CRI'AGAGTG occurred frequently among the Indian and Vietnamese groups, while CRI'AGAGTG was observed frequently among Congolese and Ghanaian individuals. The

Table 2. Knops blood group antigens distribution among world populations.

<table>
<thead>
<tr>
<th>CRI variants</th>
<th>Amino acid substitution</th>
<th>Knops blood antigens</th>
<th>Brazil n = 202 (%)</th>
<th>Vietnam n = 198 (%)</th>
<th>India n = 172 (%)</th>
<th>Congo n = 154 (%)</th>
<th>Ghana n = 154 (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs41274768</td>
<td>V1561M</td>
<td>Kn(^{a}) 200 (98)</td>
<td>198 (100)</td>
<td>171 (99.4)</td>
<td>154 (100)</td>
<td>154 (100)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kn(^{b}) 4 (2)</td>
<td>0</td>
<td>1 (0.6)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17047660</td>
<td>K1590E</td>
<td>McC(^{a}) 200 (99)</td>
<td>198 (100)</td>
<td>172 (100)</td>
<td>119 (77.3)</td>
<td>104 (67.5)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>McC(^{b}) 2 (1)</td>
<td>0</td>
<td>0</td>
<td>35 (22.7)</td>
<td>50 (32.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17047661</td>
<td>R1601G</td>
<td>Sl1 195 (96.5)</td>
<td>198 (100)</td>
<td>172 (100)</td>
<td>45 (29.2)</td>
<td>42 (27.3)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sl2 7 (3.5)</td>
<td>0</td>
<td>0</td>
<td>109 (70.8)</td>
<td>112 (72.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4844609</td>
<td>T1610S</td>
<td>Sl4 200 (99)</td>
<td>198 (100)</td>
<td>172 (100)</td>
<td>19 (12.3)</td>
<td>16 (10.4)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sl5 2 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6691117</td>
<td>I1615V</td>
<td>KCAM+ 155 (76.7)</td>
<td>127 (64.1)</td>
<td>81 (47.1)</td>
<td>19 (12.3)</td>
<td>16 (10.4)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>KCAM- 47 (23.3)</td>
<td>71 (35.9)</td>
<td>91 (52.9)</td>
<td>135 (87.7)</td>
<td>138 (89.6)</td>
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<td></td>
</tr>
</tbody>
</table>

NS, not significant; NA, not applicable

https://doi.org/10.1371/journal.pone.0175973.t002
CR1 variants in different world populations

Table 3. Reconstructed CR1 haplotype distribution among world populations.

<table>
<thead>
<tr>
<th>CR1 haplotypes (+4659/+4721/+4808/+4841/+4868/+4883)</th>
<th>Brazil n = 202 (%)</th>
<th>Vietnam n = 198 (%)</th>
<th>India n = 172 (%)</th>
<th>Congo n = 154 (%)</th>
<th>Ghana n = 154 (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1*AGAATA</td>
<td>130 (64)</td>
<td>127 (64)</td>
<td>79 (45.9)</td>
<td>19 (12.3)</td>
<td>14 (9)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>CR1*AGAATG</td>
<td>39 (19)</td>
<td>71 (36)</td>
<td>90 (52.3)</td>
<td>26 (17)</td>
<td>26 (16)</td>
<td>&lt;0.0016</td>
</tr>
<tr>
<td>CR1*GGAATA</td>
<td>19 (9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CR1*AGGGTG</td>
<td>1 (0.5)</td>
<td>0</td>
<td>0</td>
<td>35 (22.7)</td>
<td>51 (33)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CR1*AGAGTG</td>
<td>3 (1.5)</td>
<td>0</td>
<td>0</td>
<td>74 (48)</td>
<td>63 (41)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CR1*AAAATG</td>
<td>4 (2)</td>
<td>0</td>
<td>1 (0.6)</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>CR1*AGGATA</td>
<td>1 (0.5)</td>
<td>0</td>
<td>2 (1.2)</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>CR1*AGAAAA</td>
<td>2 (1)</td>
<td>0</td>
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<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>CR1*AGAGTA</td>
<td>3 (1.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

NS, not significant; NA, not applicable

https://doi.org/10.1371/journal.pone.0175973.t003

CR1*AGGGTG and CR1*AGAGTG haplotypes were observed only in Brazil and Africa, being far more frequent among the Congolese and Ghanaian groups. Interestingly, CR1*GGAATA was exclusively observed in the Brazilian population. Linkage disequilibrium (LD) analysis between SNPs revealed medium levels of LD for SNPs rs17047661 (Sl1/Sl2) and rs6691117 (KCAM+/−) and for rs17047660 (McCα/β) and rs17047661 (Sl1/Sl2) in the Congolese and Ghanaian study groups (Fig 2).

![Fig 1. Distribution of CR1 haplotypes in world populations.](https://doi.org/10.1371/journal.pone.0175973.g001)
Discussion

Pathogens exert strong selective pressure on the human host, leading to substantial changes in host immune regulation thereby evading immune responses. This study utilized samples from population exposed to diverse infectious diseases, where a strong selective pressure is exerted by these infectious pathogens on the human immune locus. The samples utilized in this study are from different case-control cohorts investigated for possible associations of CR1 variants with different infectious diseases (unpublished data). Brazilian, Vietnamese and Indian samples utilized in this study are from an endemic area to Chagas disease, viral hepatitis and leprosy respectively. The Republic of Congo and Ghanaian samples are from malaria holoendemic sites.

CR1 genetic variants in exon 29 are associated with CR1 expression levels, C1q or C3b binding activity and increased susceptibility to various infectious diseases. This study investigated the entire exon 29 of CR1 in five diverse populations in order to assess the distribution of Knops blood group antigens and the distinct functional CR1 SNPs. Such studies on geographically diverse populations can provide insights on how these CR1 alleles have spread in populations and contribute to the understanding of natural selection.

Allele and genotype frequencies of CR1 variants in exon 29 [rs17259045, rs41274768 (Kn\textsuperscript{a/b}), rs17047660 (McC\textsuperscript{a/b}), rs17047661 (Sl1/Sl2), rs4844609 (Sl4/Sl5), rs6691117 (KCAM+/-)] as well as their haplotype frequencies were differently distributed among the Brazilian, Vietnamese, Indian, Congolese and Ghanaian study groups. So far, the frequencies of these variants and especially, the distribution of blood group antigens have not been described explicitly for central African populations yet.

CR1 variants rs17047660A/G (McC\textsuperscript{a/b}) and rs17047661A/G (Sl1/Sl2) were observed to be polymorphic only in the African groups compared to those from Asia and Brazil, indicating that the frequencies of these two SNPs result from a strong selective bias exerted by exposure...
to distinct pathogens especially by *Plasmodium falciparum*. This is substantiated by a high linkage disequilibrium between the two variants. Of the reconstructed *CRI* haplotypes, *CRI* ^AGAGTG^ and *CRI* ^AGGGTG^ were observed to be unique among the Congolese and Ghanaian groups. *CRI* ^AGAGTG^ contains the allele of the rs17047660A. This locus also determines the Knops blood group antigen McC^a/b^. Studies have demonstrated that this blood group antigen is dominant among many ethnic groups of African ancestry living in malaria endemic regions [34].

Higher rates of adaptive evolution are expected to occur especially in genes involved in the immune system, as these gene loci coevolve with pathogens. This is largely contributed by two factors the genetics of the population and natural selection. Immune genes tend to evolve rapidly as selection pressure is changing continuously due to various pathogenic challenges. Therefore, positive selection of rs17047660A/G (McC^a/b^) and rs1704661A/G (Sl1/Sl2) loci is expected in sub-Saharan African populations exposed to distinct pathogenic challenges (e.g. falciparum malaria). Such a selective advantage occurs mainly in immune genes involved in pathogen recognition and signaling, and the *CRI* is one of such genes involved in innate immunity.

In addition, the reported frequencies of these two loci, rs17047660A/G (Sl4/Sl5) and rs1704661A/G (Sl1/Sl2), in this study were in accordance with frequencies observed in other East and West African ethnicities as reported in the 1000 Genomes database (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes). The frequencies in other African populations correspond to the frequencies observed in this study [rs17047660A/G (McC^a/b^): Gambian 0.67/0.32, Kenyan 0.69/0.31, Sierra Leone 0.71/0.29 and Yoruba 0.73/0.27; whereas for rs17047661A/G (Sl1/Sl2): Gambian 0.21/0.78, Kenyan 0.30/0.70, Sierra Leone 0.21/0.79 and Yoruba 0.30/0.70]. Also the reported frequencies in other studied Asian and Brazilian populations were in accordance with the frequencies described in the 1000 Genomes database.

There is growing evidence of ethnic differences in susceptibility to some infectious diseases and of genetic adaptation to diverse pathogens [18,35]. This study investigated five antigens of the Knops blood group including the Knops (rs41274768, Kn^ab^, p.N1540S), the McCoy (rs17047660, McC^a/b^, p.K1590E), the Swain-Langley/Villien (rs17047661, Sl1/Sl2, p.R1601G), the Swain-Langley (rs4844609, Sl4/Sl5, p.T1610S), and the KCAM antigens (rs6691117, KCAM^+/-^, p.I1615V) [19–23]. These Knops blood group polymorphisms have been found associated with various infectious diseases (Table 4). In particular, the two Knops blood group variants McC^b^ (rs1704660G, E1590K) and Sl2 (rs1704661G, R1601G) have specific distributions among African populations, which has been related to selective pressure by malaria in Africa [36–42]. The substitution of lysine to glutamic acid at 1590 aa position modulates the epitope conformation and serologic reactivity due to its surface exposed feature, affecting the overall CR1 binding capacity [22]. A high frequency of the rs1704661G (Sl2) allele was observed in the African groups. The high frequency of the rs6691117G (KCAM^-, I1615V) allele in Africa and India indicates that this allele, similar as the rs1704660G (McC^b^) and rs1704661G (Sl2) alleles, might also be subjected to selection. The presence of rs1704661G (McC^b^), which is almost limited to African populations, suggests that rs1704661A (Sl1) may be the ancestral allele [43]. Also a differential distribution of rs6691117A/G (KCAM^+/-^) variants was observed. For instance, in the Vietnamese and Brazilian groups, rs6691117A (KCAM^+) is a major allele, while the variant rs6691117G (KCAM^-) was observed to be the major allele in Africa. A study from India compared exon 29 *CRI* variants in endemic and non-endemic populations and concluded that a differential association with falciparum malaria in regions of varying disease endemicity exists [44]. However, the Indian samples from the present study originate from an area not endemic for malaria.
Taken together, this study revealed significant differences in allele, genotype and haplotype frequencies of CR1 SNPs in five populations. A limitation of this study might be a small sample size. However, this study, first to include population from Central Africa, may provide an increased understanding of the contribution of red blood cell phenotypes and the complement regulator protein with regard to possible associations with infectious diseases. Further studies are warranted with increased sample sizes, to determine the role of CR1 in disease associations and pathogenesis mechanisms.

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Author Contributions
Conceptualization: TPV.
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Formal analysis: TLS FAA HVT.
Funding acquisition: PGK TPV.
Investigation: TLS SA DPG CNN.
Resources: NLT LHS PE KT VLL FN IJMR.
Supervision: TPV.
Writing – original draft: TLS TPV.
Writing – review & editing: CGM TPV HVT.
References


Abstract

BACKGROUND  Malaria elicits inflammatory responses, which, if not well regulated, may exert detrimental effects. When activated, triggering receptor expressed on myeloid cells 1 (TREM-1) enhances inflammatory responses by increasing secretion of IL-8 and other Th1 cytokines. In contrast, TREM-like transcript 1 (TREML-1) promotes anti-inflammatory responses by binding to TREM-1 ligands and competing with TREM-1, thus antagonizing TREM-1 activation to reduce inflammation. Endothelial protein C receptor (EPCR) also mediates anti-inflammatory responses by activating endothelial protein C (PC). Upon microbial stimulation, soluble forms of TREM-1 (sTREM-1) and soluble EPCR (sEPCR) are released. Their plasma levels reflect the degree of inflammation and the severity of infection.

METHODS  In a cross-sectional study comparing patients with severe with uncomplicated malaria, sTREM-1, soluble TREML-1 (sTREML-1) and sEPCR plasma levels as well as plasma levels of sEPCR derived from convalescent patients were quantified. Samples were collected on admittance of paediatric patients infected with Plasmodium falciparum to hospitals in Accra, Ghana. Distinct genetic regions of the genes encoding TREM-1, EPCR, interleukin (IL)-8 and IL-18 encompassing known genetic polymorphisms that influence plasma levels underwent DNA sequencing.

RESULTS  Higher sTREM-1 levels were observed among children suffering from severe malaria compared to those with uncomplicated malaria (P = 0.049). Low TREM-1 to TREML-1 ratios were associated with uncomplicated malaria (P = 0.033). The TREM1 rs2234237T variant causing the amino acid exchange Thr25Ser, which has been associated with higher TREM-1 plasma levels, was significantly more frequent among patients with severe malaria than in those with uncomplicated malaria (P = 0.036). Low levels of sEPCR were observed in severe and uncomplicated malaria, while variant genotypes of IL8, IL18 and EPCR did not show any association.

CONCLUSION  Higher plasma levels of sTREM-1 alone or relative to sTREML-1 during malaria predispose to the phenotype of severe malaria. Carriage of the TREM1 rs2234237T allele appears to be a risk factor for the development of severe malaria.

keywords  Malaria, TREM-1, TREML-1, EPCR, sTREM-1, sTREML-1, sEPCR

Introduction

Malaria-associated fatality results almost exclusively from severe malaria, which manifests mainly as severe malarial anaemia and cerebral malaria. These syndromes predominantly occur in children in malaria-endemic regions, whose adaptive immunity is still maturing, and in pregnant women and malaria-naïve individuals. Severe infection may develop when the initial host immune response required to clear the parasite [1] becomes dysregulated [2], resulting in the production of excessive amounts of pro-inflammatory factors and possibly in death [3].

The innate immune response is the first line of defence against malaria parasites in non-immune individuals. Triggering receptor expressed on myeloid cells 1
(TREM-1) regulates innate immune responses by increasing inflammatory signals initiated by pathogen recognition receptors such as Toll-like receptors (TLRs) and NOD-like receptors (NLRS). TREM-1 is also critical in inducing inflammation [4–7]. Cross-linking of TREM-1 with DAP12, a signalling adapter for recognition receptors, activates distinct cells of the myeloid lineage, in particular neutrophils, monocytes, macrophages and dendritic cells [8, 9] to enhance inflammatory responses. Activation of TREM-1 increases secretion of the Th1 cytokines IL-8, tumour necrosis factor and monocyte chemotactic protein-1 [4–7]. Production of interleukin (IL)-8 is also induced by IL-18 [10] with high plasma levels of both IL-8 and IL-18 linked to the pathogenesis of severe malaria [11–13]. Soluble TREM-1 (sTREM-1) is released after proteolytic cleavage of the membrane-bound form or provided through alternative splicing [14]. Activation and increased levels of TREM-1 in plasma are associated with systemic inflammation [15] and implicated in several infectious disorders [15, 16].

Although early vigorous inflammatory responses are required to curtail infection, a timely and sufficient anti-inflammatory response is crucial to prevent immunopathology [2, 17]. TREM1-1, the only member of the TREM family of receptors with an immunoreceptor tyrosine-based inhibitory motif, can deliver inhibitory signals when activated [18, 19]. Structurally, soluble TREM-1 (sTREM-1) is similar to TREM-1 and may compete with TREM-1 for its ligand [20]. Such competition can minimise TREM-1 and its ligand interactions and reduce TREM-1-mediated activation of immune cells and hence inflammation. TREM1-1 has therefore been suggested to be the natural inhibitor of TREM-1 [21]. It has been experimentally shown in mouse models to protect against inflammation-associated haemorrhage [22].

Cytoprotection is also mediated by the endothelial protein C receptor (EPCR) through activation of the endothelial protein C (PC) following interactions between the two. Activation of the PC upon interaction with EPCR leads to inhibition of Th1 cytokine secretion and prevention of inflammation-associated damage of vascular endothelia [23]. In experimental models, increasing expression of EPCR corresponds with increasing anti-inflammatory responses to endotoxins [24]. Excessive inflammation and endothelial damage have both been suggested to aggravate severity of malaria. Given the clinical significance of TREM-1, TREM1-1 and EPCR pathways in modulating inflammatory processes and integrity of the vascular endothelium, it may be important to elucidate their role in the prognosis of malaria.

We measured and compared levels of sTREM-1, sTREM1-1 and soluble EPCR (sEPCR) between patients with uncomplicated malaria (UM) and severe malaria (SM). About 60% of variability in levels of sTREM-1 and sEPCR are influenced by the TREM1 rs2234237 and EPCR rs867186 variants, respectively [25, 26]. Therefore, we determined the genotype distribution of TREM1 rs2234237 and EPCR rs867186 in different malaria phenotypes in a cross-sectional design involving children who presented at hospitals in Accra, Ghana. IL-8 is positively regulated by both TREM-1 and IL-18. As plasma levels of IL-8 are associated with the IL8 variant rs4073 [27] and IL-18 levels are linked to the IL18 variants rs1946518 and rs187238 [28], the distribution of these variants was also evaluated.

Methods

Malaria patients and sampling

Blood samples from paediatric malaria patients who reported to the Department of Child Health of the Korle-Bu Teaching Hospital during 2003–2014 with a diagnosis of P. falciparum malaria were included in the study. All participants were febrile at enrolment (≥37.5 °C) and P. falciparum positive as assessed by light microscopy, with no other diagnosis. The study participants were of haemoglobin AA genotype only. Plasma samples were available from 37 and 49 children with a diagnosis of UM and SM, respectively. Buffy coat samples for genetic analyses were from 95 SM and 147 UM patients. Malaria treatment was carried out according to the Ghanaian National Guidelines at the time of enrolment. Blood samples were obtained from all participants before initiation of antimalarial treatment and in convalescence (14 days post-initiation of treatment) when they were atraumatic by light microscopy and showed no clinical signs suggestive of malaria. Each blood sample was centrifuged and separated into plasma and cell samples before storing at −80 °C until further use.

Serological assays

TREM-1 ELISA. Microtitre plates (NUNC MAXISORP™, Roskilde, Denmark) were coated with purified monoclonal mouse antibody against human TREM-1 (R&D Systems, Minneapolis, USA) in phosphate-buffered saline (PBS; Gibco, Waltham, MA, USA) at a concentration of 800 ng/ml and incubated overnight at room temperature. Each well was blocked with 300 μl of 1% bovine serum albumin (BSA) for 2 h at ambient temperature. Test sera and serially diluted recombinant human
TREM-1 (6000–46.875 pg/ml with 1% BSA; R&D Systems, USA) were added and incubated for 2 h at room temperature. Thereafter, 400 ng/ml of biotinylated goat anti-human TREM-1 antibody (R&D Systems, Wiesbaden, Germany) was added to each well and incubated for 2 h at room temperature. Streptavidin–horseradish peroxidase (Streptavidin-HRP; R&D Systems) was added at 200 ng/ml, and the reaction mixtures were incubated for 30 min at room temperature. Colour development was carried out in the dark for 20 min with 3,3′,5′-Tetramethylbenzidine substrate (TMB; R&D Systems, Wiesbaden, Germany), and optical densities were read at 450/570 nm. PBS with 0.05% Tween-20 was used as washing buffer. The plates were washed three times after each incubation step preceding addition of the substrate.

**TREML-1 ELISA.** Purified mouse monoclonal antibody against human TREML-1 (R&D Systems, Wiesbaden, Germany) in PBS (GIBCO, Waltham, MA, USA) was added to the plates at a concentration of 800 ng/ml and at 100 μl/well. The plates were incubated overnight at room temperature followed by blocking of each well with 300 μl of 1% BSA for 2 h at ambient temperature. Sera and serially diluted recombinant human TREML-1 (1000–7.8 pg/ml with 1% BSA; R&D Systems) were added and incubated for 2 h at room temperature. Subsequently, 200 ng/ml of biotinylated goat anti-human TREML-1 antibody (R&D Systems) was added to each well and incubated for 2 h (room temperature). After incubation, 200 ng/ml of Streptavidin-HRP (R&D Systems, Wiesbaden, Germany) was added, and the reaction mixtures were incubated for 30 min, again at room temperature. The plates were developed with TMB (R&D Systems) substrate in the dark for 20 min and optical densities read at 450/570 nm. All incubation steps were performed at room temperature. After each incubation step, plates were washed three times using PBS with 0.05% Tween-20 as washing buffer.

**Extraction of DNA, PCR, DNA sequencing**

DNA was purified from buffy coat samples using the Qiagen DNA purification kit (Qiagen, Hilden, Germany). The concentration of the DNA was estimated by measuring absorbance at 260 nm using the NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, NC, USA). A total of 809 base pairs encompassing variant rs2234237 within exon 2 of the *TREM1* gene (OMIM 605085) (primers 5′-GGAGGCTCAAGAACCCTCAT-3′ [forward], 5′-CACAAACGCATCTTGGGA-3′ [reverse]), 619 bp of the promoter region of *IL8* (OMIM 146930) (primers 5′-TTGCTGGTCTATCTTCCACCA-3′ [forward], 5′-AGGAAAACGCTGATAGTTCAGA-3′ [reverse]) and 651 bp of the promoter region of *IL18* (OMIM 600953) (primers AACACTGGAAACTGCAA GTAAAT-3′ [forward], 5′-TCGAGGCAATTG AAGTCCG3′ [reverse]) were amplified. In the case of *EPCR* (OMIM 600646), published primers [26] were used to amplify 668-bp surrounding variant rs867186 A/G in exon 4. The total volume of each reaction mix was 20 μl. The conditions were 2 mM of each primer, 1.25 mM of each dNTP, 1 unit of Taq DNA polymerase (Biomol, Hamburg, Germany) and the corresponding 10× reaction buffer. PCR conditions were denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C (59 °C for EPCR) for 30 s and 72 °C for 1 min with a final extension step at 72 °C for 10 min. PCR products were purified using exonuclease 1 and alkaline phosphatase. Amplicons were sequenced using the sense PCR primer (BigDye® Terminator v3.1; Applied Biosystem, Foster City, CA, USA) and analysed with the ABI 3031 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA).

**Statistical analyses**

Levels of sTREM-1, TREML-1 and sEPCR in the two groups were compared using Mann–Whitney *U*-tests as data were not normally distributed. Genotype frequencies were tested for deviation from Hardy–Weinberg equilibrium by chi-square ($\chi^2$) test [29]. Two-tailed Fisher’s exact test was performed to compare allelic frequencies in UM and SM groups. The relationship between genotypes and disease severity is given as odds ratios (OR)
with 95% confidence intervals (CI). In all comparisons, the level of significance was set at $P < 0.05$. All analyses and graphical presentations were performed using GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA).

**Ethics**

We used plasma and buffy coat samples from previous prospective studies [30–32] conducted at the Department of Child Health of the Korle-Bu Teaching Hospital in Accra, Ghana, and the paediatric units of three other health facilities in the Accra Metropolitan Area in this study. Ethical approval was granted by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research and the Ethics Committee of the University of Ghana Medical and Dental School. Study participants or parents/guardians of children provided written informed consent before inclusion in the study and granted approval for re-use of their samples for research purposes.

**Results**

**Patient characteristics**

The participants were aged between 6 months and 12 years. Results are presented as medians with minimum and maximum values. The median age in the SM group was significantly lower than that of the UM group ($P < 0.0001$). Likewise, the median haemoglobin levels in the SM group were significantly lower than in the UM group ($P < 0.0001$) while parasitaemia levels were higher in the SM than in the UM group ($P = 0.04$) (Table 1).

**Plasma levels of TREM-1 and TREML-1 of patients at admission**

Acute-state plasma levels of sTREM-1 were measured in samples obtained from all patients. Levels were significantly higher in SM than UM children ($P = 0.049$) (Figure 1), indicating increased expression of TREM-1 in SM. In contrast, acute-state plasma levels of sTREML-1 did not differ between UM and SM patients ($P = 0.115$) (Figure 2), but a greater TREM-1 to TREML-1 ratio was associated with SM ($P = 0.033$) (Figure 2b).

**sEPCR levels in the acute and convalescent phase of malaria**

Acute-state sEPCR levels were similar in UM and SM ($P = 0.095$) (Figure 3). By day 14 after initiation of antimalarial treatment, sEPCR levels had increased significantly in both SM ($P = 0.0002$) and UM ($P = 0.0007$) groups (Figure 3).

**TREM1 polymorphisms rs2234237A>T and rs34727391A>C**

The frequencies of TREM1 variants rs2234237 and rs34727391 were in Hardy–Weinberg equilibrium (HWE) ($P = 0.451$ and $P = 0.858$, respectively). The genotypic and allelic distributions of rs2234237A>T and rs34727391A>C SNPs are given as counts and frequencies (Table 2). The distributions of the various genotypes in UM differed significantly from those in SM children in a codominant model (AA vs. AT vs. TT; $P = 0.036$). There was a significantly higher representation of the AT+TT genotypes, which are known to be associated with intermediate-to-high TREM-1 production [25], in SM (AA vs. AT & TT, OR = 2.4; 95% CI: 1.2–4.5, $P = 0.01$). Comparing allele frequencies also revealed a disproportionate representation of the T allele in SM (OR = 2.1, 95% CI: 1.2–3.8, $P = 0.018$) vs. UM (Table 2). Rs34727391 was not associated with any disease phenotype either at the genotype or allele level (Table 2).

**EPCR polymorphisms rs867186A>G and rs1051021G>C**

The distributions of EPCR variants rs867186 and rs1051021 were in HWE ($P = 0.567$ and $P = 0.822$, respectively); their genotype frequencies are shown in

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical, parasitological and demographic characteristics of the uncomplicated (UM) and severe malaria (SM) study population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncomplicated malaria</td>
</tr>
<tr>
<td>Number of patients</td>
<td>147</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>5 (0.5–12)</td>
</tr>
<tr>
<td>Male/Female ratio</td>
<td>74/73</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>9.9 (8–14.5)</td>
</tr>
<tr>
<td>Parasite density (10$^3$/μl blood)</td>
<td>26.0 (0.203–393.6)</td>
</tr>
</tbody>
</table>

NS, not significant. 
Values reported are medians with minimum and maximum values in parentheses. 
Gender composition of each group is reported as male/female ratio. Differences between groups were considered significant at a $P < 0.05$. 

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Table 2. None of the SNPs appeared to influence the outcome of infection, irrespective of the genetic model employed.

**IL8 and IL18 promoter polymorphisms and severe malaria**

The frequencies of *IL8* SNPs rs4073 and rs2227538 were in HWE (*P* = 0.873 and *P* = 0.533, respectively). Likewise, the distribution of *IL18* SNPs rs1946518 and rs187238 (*P* = 0.973 and *P* = 0.883, respectively) was in HWE. None of the SNPs showed any association with UM or SM (Table 2), irrespective of whether codominant, dominant or recessive models were applied in the analyses.

**Discussion**

TREM-1 regulates inflammatory responses during microbial infections [33] and plays a role in antimalarial immunity. In this study, high plasma levels of TREM-1 were associated with the development of SM (Figure 1). As increased levels of sTREM-1 associate positively with inflammatory pathology [34], increased levels of sTREM-1 during severe malaria might, therefore, indicate stronger inflammatory responses during the acute phase of infection [35, 36].

About 60% of interindividual variation in TREM-1 levels are accounted for by rs2234237 with its AA genotype associated with lower levels of sTREM-1 [25]. Under a dominant model, the T allele is associated with SM, with the odds of developing SM as a result of T allele carriage being 2.1 (Table 2), a finding consistent with the serological data and confirming earlier studies [25, 37]. Carriage of either the TT or the AT genotype is, therefore, a risk factor for SM. Thus, our observations
### Table 2 Allele and genotype frequencies in the UM and SM

<table>
<thead>
<tr>
<th>SNPs</th>
<th>UM n (%)</th>
<th>SM n (%)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREM1-rs2234237 A/T</td>
<td></td>
<td></td>
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<td>Codominant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>94 (83)</td>
<td>61 (68)</td>
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<td></td>
</tr>
<tr>
<td>AT</td>
<td>18 (16)</td>
<td>28 (31)</td>
<td>2.4 (1.2–4.7)</td>
<td>0.036</td>
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<tr>
<td>TT</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>0.7 (0.04–10.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>206 (91)</td>
<td>150 (83)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>20 (9)</td>
<td>30 (17)</td>
<td>2.1 (1.2–3.8)</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>94 (83.2)</td>
<td>61 (67.8)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>AT &amp; TT</td>
<td>199 (16.8)</td>
<td>26 (32.2)</td>
<td>2.4 (1.2–4.5)</td>
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</tr>
<tr>
<td>Recessive</td>
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<td></td>
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</tr>
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<td>AA &amp; AT</td>
<td>112 (99)</td>
<td>89 (99)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
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<td>1.2 (0.08–20.4)</td>
<td>NS</td>
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<tr>
<td>TREM1-rs34727391 A/C</td>
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<td>Codominant</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>107 (97)</td>
<td>87 (98)</td>
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<tr>
<td>AC</td>
<td>3 (3)</td>
<td>2 (2)</td>
<td>0.8 (0.1–5.2)</td>
<td>NS</td>
</tr>
<tr>
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<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
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<td>A</td>
<td>217 (99)</td>
<td>176 (99)</td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>3 (1)</td>
<td>2 (1)</td>
<td>0.82 (0.1–4.9)</td>
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<tr>
<td>EPCR-rs867186 A/G</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>AA</td>
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<td>79 (93)</td>
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<tr>
<td>AG</td>
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<td>6 (7)</td>
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</tr>
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<td>0</td>
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<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>191 (95)</td>
<td>164 (96)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>9 (5)</td>
<td>6 (4)</td>
<td>0.78 (0.3–2.2)</td>
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</tr>
<tr>
<td>EPCR-rs1051021 G/C</td>
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<td></td>
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</tr>
<tr>
<td>GG</td>
<td>67 (66)</td>
<td>63 (72)</td>
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<td></td>
</tr>
<tr>
<td>GC</td>
<td>30 (29)</td>
<td>23 (26)</td>
<td>0.8 (0.4–1.6)</td>
<td>NS</td>
</tr>
<tr>
<td>CC</td>
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<td>1 (1)</td>
<td>0.2 (0.002–1.9)</td>
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<td>149 (86)</td>
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<td>C</td>
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<td>25 (14)</td>
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<td>Dominant</td>
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<tr>
<td>Recessive</td>
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<tr>
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<td>86 (99)</td>
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<tr>
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<td>1 (1)</td>
<td>0.2 (0.03–2.0)</td>
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<tr>
<td>IL8-rs4073 A/T</td>
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<td></td>
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<tr>
<td>AA</td>
<td>88 (79)</td>
<td>68 (72)</td>
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<td></td>
</tr>
<tr>
<td>AT</td>
<td>21 (19)</td>
<td>25 (26)</td>
<td>1.5 (0.8–2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>TT</td>
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<td>2 (2)</td>
<td>1.3 (0.2–9.4)</td>
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</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>197 (89)</td>
<td>161 (85)</td>
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<td></td>
</tr>
<tr>
<td>T</td>
<td>23 (11)</td>
<td>29 (15)</td>
<td>1.4 (0.8–2.5)</td>
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<tr>
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<td>68 (72)</td>
<td>Reference</td>
<td></td>
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<tr>
<td>AT &amp; TT</td>
<td>23 (21)</td>
<td>27 (28)</td>
<td>1.5 (0.8–2.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>
suggest that the role of TREM-1 in malaria may parallel its role in other inflammation-associated disorders, where its high levels and the rs2234237T allele have been linked with severe pathology [7, 15, 33, 34, 38].

TREM1 transcripts share homology with the transmembrane domains of TREM-1 and compete with membrane-bound TREM-1 (mTREM-1) for the yet unknown TREM-1 ligand. It is, therefore, viewed as a \textit{de novo}
Malaria infections are associated with endothelial cell activation and damage with several biomarkers of endothelial damage relating positively to the severity of malaria [32, 43]. High plasma levels of sEPCR indicate extensive endothelial damage [44, 45] and/or higher cell membrane-bound EPCR (mEPCR) expression. A trend to reduced levels of sEPCR was observed in SM (Figure 3). This is in contrast to the findings of Moussiliou et al. [46], and difficult to comprehend. One reason may be that the patients in our study were slightly older than those described in Ref. [46]. Our report is, however, consistent with the study by Moxon et al., who clearly demonstrated that unlike malaria, infection with several other pathogens can lead to plasma levels of EPCR above baseline values [47]. The current report thus fits well with the loss of EPCR expression [47] that can aggravate inflammation during malarial infection. Evidence from experimental studies indicates that increasing EPCR expression translates into increasing anti-inflammatory response [24]. After interaction with mEPCR, PC becomes activated and inhibits monocyte migration via EPCR, upregulates immunoregulatory cytokine IL-10 production and thereby dampens inflammation [48–51].

Loss of EPCR expression during P. falciparum infection appears to be a general phenomenon, as sEPCR levels increased in both SM and UM patients by day 14 postinitiation of anti-malarial therapy (Figure 3). Although one cannot rule out the presence of residual parasite materials in circulation to activate cells, even on day 14, our participants were aparasitaemic by microscopy and had fully recovered. Thus, the loss of EPCR expression during the acute state may be attributed to parasite factors that could limit the activation of a cyto-protective and anti-inflammatory function of PC and disrupt the blood brain barrier [47, 52]. The limitation may precipitate increased inflammatory responses to cause haemorrhage in sites of parasite sequestration [53]. Haemorrhages in the brain microvasculature are associated with cerebral malaria [54]. This vicious cycle, involving reducing the interaction between mEPCR and PC to promote pro-inflammatory cytokine production, may aggravate the severity of malaria. At the genetic level, no association between any of the genotypes and disease phenotype was observed (Table 2), confirming earlier studies [46, 55].

None of the cytokine gene variants (IL8 rs4073, IL8 rs2227538, IL18 rs1946518, IL18 rs187238) could be linked to the outcome of malaria. The lack of significance could be due to the small sample size and the size of the effect of each of these loci.

The limitations of this work are a small sample size, not allowing us to measure the convalescent state levels of sTREM-1 and sEPCR. Nevertheless, we have confirmed in our study earlier reports on the involvement of TREM-1 and EPCR in the pathogenesis of malaria. We also provide evidence that links reduced production of TREML-1 relative to TREM-1 to the outcome of malaria.

Acknowledgements

We thank the study participants and their parents/guardians for agreeing to donate samples to us for this study. We also thank the staff in the Immunology Department of Noguchi Memorial Institute For Medical Research, University of Ghana, Legon, and Institute for Tropical Medicine, University of Tübingen, for their technical and laboratory support. We are also indebted to the medical staff of Korle-Bu Teaching and allied hospitals that helped collect the samples.

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