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CONTRIBUTION OF INNATE RESPONSES TO *Plasmodium falciparum* INFECTIONS

A thesis submitted
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

Ms. CLARA ERMINE SAWIAN
Registration No. 017 of 2008

School of Science and Technology
Department of Molecular Biology and Biotechnology
Tezpur University
Tezpur-784028, Assam, India

2013

Dedicated to my family

(Ieid ia phi baroh)

ABSTRACT

Introduction

Malaria, an infectious disease is a major health problem with *falciparum* malaria being the most virulent kind. Virulence maybe attributed in part to sequestration of the infected red blood cells in the vasculature of the host organs, an evasive maneuver of the parasite preventing splenic clearance. Cell adhesion molecules (ICAM-1 and VCAM-1) leading to cytoadherence are upregulated by elevated levels of proinflammatory cytokines in response to glycosylphosphatidylinositol (GPI) and other malarial toxins. While people living in malaria endemic regions acquire immunity to the disease, it is a slow process and is transient. Interestingly, not all malaria-infected people progress to the complicated form of the disease, suggesting involvement of certain host factors in conferring protection from severe malaria. Essentially, host and parasite are believed to be co-evolving where footprints of selection of protective traits under malaria pressure in different populations can be seen in human genome as in case of sickle cell haemoglobinopathy which affords protection against mortality from *falciparum* malaria in heterozygous individuals in Africa, balancing the severe consequences of the disease in homozygous individuals. Recent studies have suggested malaria as the driving force in maintaining some polymorphisms in immune genes, however these were seen to differ in populations. SNPs of TLR and CR1 have been reported to show unique distributions in populations from Africa, Asia, and Europe and malaria is suggested to influence these patterns.

Toll-Like Receptors (TLRs) are an important component of the innate immunity and are present on the surfaces of macrophages, dendritic cells, natural killers cells etc. They recognize pathogen associated molecular patterns thereby activating signalling pathways that culminate in the induction of several cytokine genes. Recent studies suggest malaria driven selection of TLR genes with differential prevalence of these genes in different populations.

Induction of proinflammatory cytokines have been shown to minimize parasitaemia and containment of the disease. While a strong and early response helps in protection but a delayed and prolong inflammatory response leads to pathogenesis from disease. The immune status of the individuals, parasite load, virulence of the parasite strains, host genetic factors were seen to actively modulate the inflammatory response.

The GPI anchors the merozoite protein of the parasite and this has been shown to activate TLR2 and TLR4. Cleavage of the merorozoite surface protein (MSP) of *P.falciparum* occurs before the parasite invades the RBC, these cleaved proteins act as decoys and the parasite evades the immune system. MSP2 like many other proteins of the parasite is antigenically diverse among different isolates. Genetic structures and population genetics studies of *P.falciparum* may hold the key for effective disease surveillance and control program.

The present study was designed with the following objectives:

1. Assess the role of polymorphisms and expression of Toll like receptors in relation to clinical manifestations of *P. falciparum.*
2. Role of cytokines and chemokines (IL-1β, IL-8, IL-18 and TNF-α) and CR1 (CD35) polymorphism in clinical malaria.
3. Study the blood group proteins and the minor receptors for *P. falciparum* in relation to malaria.
4. To assess the allelic diversity of MSP2 gene and its interactions with complications and frequency of the disease.

Results

Our data indicated TLR9 1486T/C heterozygosity to be protective (Fishers exact p=0.014) where it was negatively associated with complicated malaria while TLR9 (T-1237C) was seen to predispose to complicated malaria in heterozygosity (p=0.001). Similar findings have been reported earlier with respect to association of TLR9 (T-

1237C) mutation with severe malaria however (T-1486C) polymorphism is contrary to earlier reports where it was associated with susceptibility to severe malaria. Enhanced expression of TLR4 ($p=0.05$) but not of TLR9 was associated with complicated malaria. Austro-Asiatics appeared to have accumulated favourable genotypes of TLR9, perhaps because of their longer exposure to malaria.

mRNA levels of IL-18 (2.14 fold increase; $p=0.05$) and TNF-α (a 1.08 fold increase) were higher in complicated group in comparison to uncomplicated group. When cytokine gene expression between symptomatic and asymptomatic malaria were compared, mRNA levels of IL-1β and IL-8 were seen to be lowered in symptomatic malaria. Comparison of cytokine levels between diseased young and diseased old, IL-1β ($p=0.047$), IL-8, IL-18 and TNF-α were seen to be lower in diseased young in comparison to diseased old. Surprisingly, levels of all cytokines were higher in younger participants of control group IL-1β ($p=0.019$; IL-18 ($p<0.001$) and TNF-α ($p=0.019$).

When blood group antigens Fya, Fyb, K, kpa and kpb were analysed, Fyb was found to be more prevalent at 96.09% than Fya at 49.66% of the study population. It was noted that both Fya and Fyb were found to be negatively associated with fever ($p=0.016$ and $p<0.0001$ respectively). There was an association with headache but was not statistically significant. Fya and Fyb were found to be protective where they were positively associated with infrequent malaria episodes (Fishers test $p=0.010$ and $p=0.016$ respectively). Kpa was found to be negatively associated with fever ($p=0.016$). No association of malaria episodes with K, Kpa and Kpb was observed. Although, an association of Kpa and Fya (i.e. Fya*Kpa) was seen to be positively associated with frequency of malaria disease ($p=0.01$).

Higher prevalence of homozygous mutant (H/H) of the SNP of CR1 (Q981H) was associated with in malaria disease (Fishers test $p=0.003$). Among AA, TB and Khasis, it was noted that the alleles of CR1 (Q981H) were seen predominantly in

heterozygosity. The homozygous mutant genotype was seen to be absent in the Khasis (Fishers Test p=0.040).

At both the study sites, Indochina (IC) was found to be more diverse than FC-27 (isolate FC-27Q/PNG). At Kondoli, increased frequency of an allele of 330 base pair of the FC-27 allelic family was seen during 2006 summer and markedly low frequency of IC allelic family was noted (p=0.005). Moreover, this particular fragment of 330 base pair was negligible in the previous year i.e. summer of 2005; this thus encourages us to speculate on the possibility of its emergence to be associated with malaria outbreak that occurred in 2006. FC-27 allelic composition was seen to be comparable between the two summers at Guabari except for increased frequency of 550 base pair in 2006. Consistent absence of the 330 base pair size fragment in the two summers which was otherwise present in winters, and the presence of a 400 base pair fragment only in the summers suggested these alleles to be season specific.

Conclusion

From our study, we have seen that heterozygous genotypes of TLR9 (T-1486C) and CR1 (Q981H) were seen to provide protection from disease, in contrast to TLR9 (T-1237), which was implicated in disease pathogenesis. Ethnicity was seen to be an important factor in association of frequency of SNPs with malaria pathogenesis. The Austro Asiatics have appeared to have accumulated favourable genotypes of TLR9, have lower mean parasitaemia and multiplicity of infection and this is perhaps because of their longer exposure to malaria as compared to the Tibeto-Burmans of our study population. Interestingly, we also have found positive association of Kell and Fy antigens with *falciparum* malaria infections contributing to the role of host genetic factors in modulation of immune response.

DECLARATION

I hereby declare that the thesis entitled "Contribution of innate responses to *Plasmodium falciparum* infections" is an authentic work carried out by me under the supervision of Dr. Shashi Baruah, Department of Molecular Biology and Biotechnology, Tezpur University, Assam- 784028. No part of this work had been presented for any other degree or diploma earlier. Due to the unavailability of proper facility at Tezpur University, following experiments were carried at other institutes.

1. RT-PCR (Proinflammatory cytokine genes expression) work was carried out at Lab India, Gurgaon, India.

2. RT-PCR (Toll like receptor genes expression) work was carried out at National Institute of Cholera and Enteric Diseases, Indian Council Medical Research, Kolkata, India.

Place: Tezpur

Date: 31.01.13

(Clara Ermine Sawian)

DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY
TEZPUR UNIVERSITY
NAPAAM, TEZPUR-784 028 ASSAM, INDIA

S.Baruah, Ph.D
Assoc Prof. & Head

Tel: +91-3712-267007/8/9 Ext. 5408 (O)
Fax: +91-3712-267005/6
Email: sbaruah@tezu.ernet.in

CERTIFICATE OF THE SUPERVISOR

This is to certify that the thesis entitled "Contribution of innate responses to *Plasmodium falciparum* infections" submitted to the School of Science and Technology, Tezpur University in partial fulfilment for the award of the degree of Doctor of Philosophy in Science is a record of bonafide research work carried out by Ms. Clara Ermine Sawian, Research Scholar of Department of Molecular Biology and Biotechnology, Tezpur University, Assam, under my supervision and guidance at Department of Molecular Biology and Biotechnology, Tezpur University, Assam-784028.

All help received by her from various sources have been duly acknowledged.

The results embodied in the thesis have not been submitted to any other University or, Institution for award of any degree or diploma.

Place: Tezpur

(Dr. Shashi Baruah)

Date: 31.01.2013

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CERTIFICATE OF THE EXTERNAL EXAMINAR AND ODEC

This is to certify that the thesis entitled "Contribution of innate responses to *Plasmodium falciparum* infections" submitted by Ms. Clara Ermine Sawian to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us and found to be satisfactory.

The Oral Defence Evaluation Committee (ODEC) recommends for the award of the degree of Doctor of Philosophy.

Signature of:

Principal Supervisor
Date: 13.12.2013

External examiner
Dr. D N Rao
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(Clara Ermine Sawian)

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LIST OF ABBREVIATIONS

P.falciparum /(Pf)- Plasmodium falciparum

P. vivax- Plasmodium vivax

P. ovale- Plasmodium ovale

P. malariae- Plasmodium malariae

P. knowlesi- Plasmodium knowlesi

WHO-World Health Organisation

SEA- South East Asia

NMCP- National Malaria Control Program

PAMPs-Pathogen-associated molecular patterns

PRRs-Pattern recognition receptors

G6PD-glucose-6-phosphate dehydrogenase

TLRs- Toll-like receptors

NOD-

GPI-glycosylphosphatidylinositol

HZ-haemozoin

IFN- interferon

kDa-Kilo Dalton

*Pf*EMP1-*P. falciparum* erythrocyte membrane protein 1

IgG-Immuno globulin

AMA1-apical membrane antigen 1

GLURP- glutamate-rich protein

SERA5- serine repeat antigen 5

PG-peptidoglycans

LTA-lipoteichoic acid

LPS-lipopolysaccharide

RSV-respiratory syncytial virus

CpG-cytidine–phosphate–guanosine

MyD88-Myeloid differentiation primary response gene 88

TRIF-TIR-domain containing adaptor protein inducing interferon-β

MAL/TIRAP-MyD88-adapter-like protein/ TIR domain-containing adapter protein

DARC-Duffy antigen receptor for chemokines

CR1-Complement receptor type 1

ICAM-1-inter-cellular adhesion molecule 1

PNG-Papua New Guinea

Com M-complicated malaria

DY-Diseased young

COX-cyclooxygenase

TNF-Tumour Necrosis Factor

IL-interleukin

DCs-dendritic cells

NK-natural killer

MCP-monocyte chemotactic protein

VSA-variant surface antigens

MAbs-monoclonal antibodies

MSP- Merozoite surface protein

IC- Indochina

FC-27- Isolate FC-27Q/PNG

PCR - Polymerase chain reaction

MOI- Multiplicity of infection

SNPs-Single nucleotide polymorphisms

AA-Austro-Asiatics

TB-Tibeto-Burmans

IE-Indo Eurppeans

RBC-Red blood cells

MIF-macrophage migration inhibitory factor

RT-PCR –real time PCR

RQ-Relative quantification

HSPs- heat shock proteins

LBP-LPS-binding protein

MCMV-mouse cytomegalovirus

IL-1β-Interleukin-1β

ICs-immune complexes

E-erythrocytes

KO-knockout

EBA-175-erythrocyte binding antigen–175

TRAM-TRIF-related adaptor molecule

SARM-Sterile α- and armadillo-motif-containing protein

cDNA-complimentary DNA

Ct-Cycle threshold

HWE-Hardy-Weinberg equilibrium

Hb-Haemoglobin

Un Com-uncomplicated malaria.

DO-Diseased old

HbF- foetal hemoglobin

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CHAPTER 1: INTRODUCTION

1. *Introduction*

Malaria is a life threatening disease caused by *Plasmodium* species that are transmitted to people through the bites of infected *Anopheles* mosquitoes. Primarily four species of malaria parasites infect humans: *P. falciparum, P. ovale, P. malariae and P. vivax*[1]. In addition, studies in Southeast Asia have shown that *P. knowlesi*, a malaria parasite that typically involves monkeys as the natural reservoir, can also infect humans, and in some cases, result in fatal disease[2].The most virulent of the human malaria parasites is *P. falciparum* which is responsible for the bulk of the malaria related morbidity and mortality[3].

According to World malaria report 2011, there were about 216 million malaria cases and an estimated 655,000 deaths in 2010[1]. *P. falciparum* accounts for 91% of malaria cases worldwide of which the majority (i.e., 86%) occurs in the African region[4].Malaria mortality rates have fallen by more than 25% globally since 2000 and by 35% in the World Health Organization (WHO) African region. It has also been estimated that about 74% of all malaria cases in the South East Asia (SEA) region occur in India (SEARO-WHO)[1, 5]. In 2009, it has been reported by National Malaria Control Program (NMCP) that there were 1.6 millionmalaria cases and 1100 (approximately) deaths. Further, it has been suggested that the malaria incidence is between 9 and 50 times greater than reported[6]with a ~13-fold under-estimation of malaria-related mortality[7]. Such claims reinforce the need for robust and comprehensive epidemiological surveillance studies across the country[8]to determine the actual burden[9].

Northeast India constitutes about 3.8% of the country's total population but accounts for 12.4% of the total malaria cases, 17.4% of the total *P. falciparum* cases and 34.4% of reported malaria deathsin the country. This region is endemic for malaria and has been declared as a 'high risk zone' by National Anti Malaria Program. Assam contributes to 64.7% of the malaria positive cases in the north-eastern region and

74.8% of these cases are due to *P. falciparum*[10]. The spatial distribution of malaria in Northeast is not homogenous and its transmission dynamics and intensity is governed by distinct epidemiological paradigms attributable to eco-geographical, ethnic and socio-cultural diversity[11]. Malaria cases in India are reported through-out the year, since a perfect combination of average temperature (15-30°C), rainfall and precipitation-inducing conditions persist across the different parts of the country over all seasons. With increasing ecological and man-made environmental change(e.g. urbanization, construction of dams, agricultural intensification, deforestation) malaria in India is exhibiting general trends from rural to urban malaria, from forest to plain malaria, and from industrial to travel malaria[12].

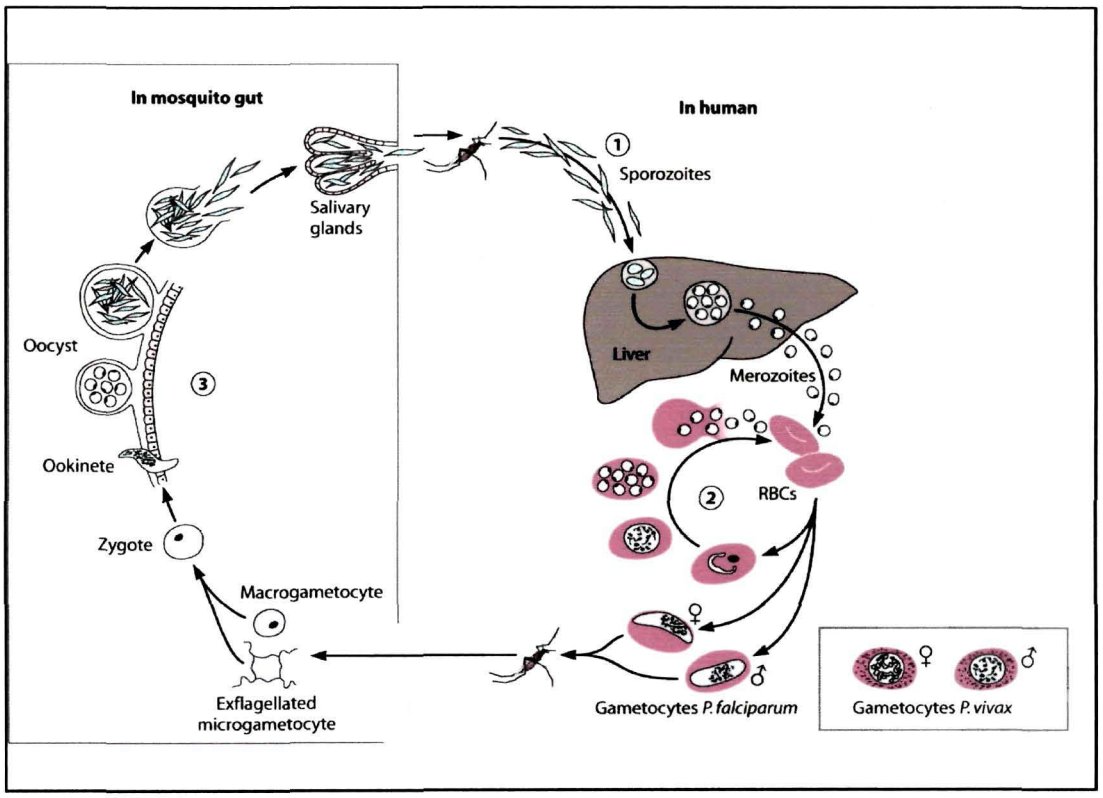

Figure 1.1: Life cycle of *Plasmodium falciparum*.

Adapted from:Bousema and Drakeley. Clin. Microbiol. Rev. April 2011 vol. 24 no. 2 377-41[13]

Malaria parasites enter the human bloodstream in the form of sporozoites that are injected by infected female *Anopheles* mosquitoes taking a blood meal[14] (Figure 1.1). The malaria parasite faces a succession of challenges within the host; it has to attach to, enter and thrive in, first, hepatocytes and then erythrocytes initiating the asexual multiplication cycle[15, 16]. A fraction of merozoites that are released from infected red blood cells form gametocytes, the transmissible parasite form. After having overcome these hurdles,the gametocytes circulating in the peripheral blood are taken up by mosquitoes and the fusion of gametes results in the formation of a zygote that develops into a motile ookinete that can penetrate the midgut wall to form oocysts. The oocysts develop over time and burst to release sporozoites that migrate to the mosquito salivary gland, rendering the mosquito infectious to human beings[13].

While people living in malaria endemic regions acquire immunity to the disease[14], it is a slow process and is transient. Young children with naïve immune systems[17]and pregnant women with potentially compromised immune systems are particularly vulnerable to this disease and so are considered to be the highest risk populations for malaria-related deaths. *P.falciparum* disease severity ranges from severe and complicated, to mild and uncomplicated, to asymptomatic[18, 19]. Interestingly, not all malaria-infected people progress to the complicated form of the disease, suggesting involvement of certain host factors in conferring protection from severe malaria.

Development of optimal immune defenses depends on a critical interaction between the two components of the immune system: innate immunity and acquired immunity. Both these components of immunity recognize invading microorganisms as non-self, which triggers immune responses to eliminate them. In acquired immunity, B and T lymphocytes utilize antigen receptors such as immunoglobulins and T cell receptors to recognize non-self[20]. Specific immune responses are longer in developing but are specific and more durable. The innate immune system constitutes the first line of host defense during infection and relies on recognition of evolutionarily conserved

structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), through a limited number of germ line-encoded pattern recognition receptors (PRRs). Innate defenses are recognised by rapid assimilation, which is particularly valuable in an emergency situation, and nonspecific response, which is of limited duration [21, 22].

A number of human genetic polymorphisms of the red cell membrane for example sicklecell trait,thalassemias, glucose-6-phosphate dehydrogenase (G6PD) deficiency were found to be protective against *P. falciparum* malaria[23]. Receptor polymorphisms of immune cells like macrophages, TLRs, NOD etc. recognises conserved sequences of the parasite and their responses modulated for production of inflammatory cytokines and thus prevented the development of severe pathology.The parasite, *Plasmodium vivax,* utilizes Duffy determinants (Fy^a and Fy^b) during RBC invasion[24]. The two exons of *Fy* gene are encoded by co-dominant alleles *FYA* and *FYB*, located on chromosome 1[25]. It was observed that those individuals that are duffy negative are completely resistant to *Plasmodium vivax* infections[26]. Haldane in 1949 was the first to hypothesize that certain red cell mutations reached unexpectedly high prevalence in malaria endemic areas because these mutations protected against malaria and hence confer survival advantage over non-carriers[27, 28, 29]. Studies have reported over 80% protection against severe malaria among sickle cell heterozygotes[30, 31] and haemoglobin C homozygotes[32]and between 40-60% protection among α+ thalassaemia heterozygotes[31, 33, 34]. The susceptibility of G6PD deficient and thalassaemic cells to oxidative damage which in turn kills the parasite inside has been cited as a possible explanation for their protection against malaria [35, 36, 37].

Toll-like receptors (TLRs) are innate immune receptors that bind to conserved structural motifs, known as PAMPs,expressed by microbial pathogens. TLR2, TLR4, TLR9, and downstream signaling pathways of these proteins have recently been implicated in human malaria pathogenesis [38, 39, 40]. In malaria, *Plasmodium falciparum* (*P.falciparum*)glycosylphosphatidylinositol (GPI) has been shown to induce the

expression of proinflammatory cytokines and immune mediators*in vitro*[41]. Furthermore, GPIwas reported to induce signalling via both TLR2 and TLR4 whereas haemozoin (HZ) in combination with plasmodial DNA activated dendritic cells by engaging TLR9[42]. Changes in TLR responses also modulated the production of inflammatory cytokines and thus prevented the development of severe pathology, emphasising the role of these responses in malaria pathogenesis[43].Increased expression of TLR genes was also seen in *P.falciparum* infected subjects and it was associated with enhanced IFNγ, TNFα and IL10 production[44].

The proinflammatory cytokines produced in defense against parasite infection can be harmful and cause pathological conditions if they are overproduced and not regulated appropriately[45]. During malaria infection, the host produces high levels of many proinflammatory cytokines, including tumor necrosis factor Tumour Necrosis Factor(TNF) -α, interleukin (IL) -1, IL-6, IL-12 and interferon (IFN) -γ, that have important roles in controlling parasite growth[46-54]. TNF-α, IL-12 and IFN-γ activate macrophages to produce reactive oxygen and nitrogen radicals, which kill parasites [45].IFN-γ primes macrophages and dendritic cells (DCs) for the efficient production of cytokines, chemokines and other inflammatory mediators[48]. IL-18 has been recognized as an important regulator of innate and acquired immune responses[55]. It induces IFN-γ production from Th1 cells, and NK cells, particularly in the presence of IL-12 and plays a key role in inducing severe malaria [56].Thoughin another study, IL-18 has been shown to play a protective role in host defense by enhancing IFN-γ production during blood-stage infection by murine malaria[45]. Increased TNF-α level stimulated phagocytosis and thereby enhanced clearance of parasitized erythrocytes [48, 49]. In children, TNF-α plasma levels were higher in cases of fatal malaria compared with non fatal malaria and cerebral malaria in comparison with non complicated malaria[57, 58]. Other cytokines such as IL-1, IL-6, IL-8, IL-10 and IL-12 have been implicated in the pathogenesis of severe malaria cases compared to uncomplicated and matched healthy controls[59, 60].

HZ is a haeme polymer that is produced as a byproduct of the haeme detoxification system in malaria[61]. It is implicated as having an important role in pathophysiology during malaria infection because HZ activates macrophages and dendritic cells to produce a variety of proinflammatory cytokines and chemokines including interleukin IL-6, TNF-a, IL-12, monocyte chemotactic protein (MCP)-1 and IL-8, and certain anti-inflammatory cytokines and chemokines such as IL-10 and macrophage migration inhibitory factor (MIF) [62-67]. The immune status of the individuals, parasite load, virulence of the parasite strains, host genetic factors were seen to actively modulate the inflammatory response[68, 69, 70].

The presence of high levels of circulating proinflammatory cytokines in *P.falciparum* malaria raises the possibility that anticytokine therapy in the form of antibodies, soluble receptors, or counter-regulatory mediators might benefit patients in the period before the parasite burden can be reduced significantly by antimalarial therapy. However, the measurable effects of the intravenous administration of anti-TNF-a monoclonal antibodies (MAbs) in children with cerebral malaria have been confined to fever reduction[71, 72]. One explanation for the disappointing results with anti-TNF-a antibodies is that by the time the patients present to hospital, the cytokine cascade has already been activated. It may therefore be necessary to target multiple steps in the complex sequence of cytokine activation or to use an agent which switches off the whole chain of events[73].

In malaria, *P.falciparum* GPI has been shown to induce the expression of proinflammatory cytokines and immune mediators*in vitro*[41] Furthermore, GPIanchors were reported to induce signalling via both TLR2 and TLR4. Surface proteins (MSPs) are anchored by GPI and are cleaved during the process of RBC invasion of the merozoite. These cleaved proteins act as decoys and facilitates immune evasion. Merozoite surface protein 2 (MSP2) is one of the well-characterized surface proteins of *P.falciparum.*MSP2 is an integral membrane protein (GPI-anchored) and contains

repeat arrays flanked by unique variable domains and conserved N- and C-terminal domains. This protein is encoded by highly divergent alleles grouped into dimorphic families or lineages the FC-27 type (isolate FC-27Q/PNG) and IC/3D7 type (Indochina)[74]. The merozoite is the principal target of current asexual stage vaccine development, the stage of the malarial parasite that is initially released from the infected hepatocyte which then infects other circulating red blood cells [75]. The vaccine strategies aim to elicit antibodies that target merozoites and /or malarial antigens expressed on the RBC surface, thus inducing antibody-dependent cellular cytotoxicity and complement-mediated lysis[76]. These also are meant to elicit T-cell responses that will inhibit the development of the parasite in RBCs and induce memory [76, 77].

Recent studies have suggested malaria as the driving force in maintaining some polymorphisms in innate immune genes, however these were seen to differ in populations. SNPs have been reported to show unique distributions in populations from Africa, Asia, and Europe and malaria is suggested to influence these patterns[78, 79]. Genetic structures and population genetics studies of *P.falciparum* may hold the key for effective disease surveillance and control program especially in Northeast India because there was so limited information available on the genetic structures of *P.falciparum.*In this context, we have examined the association of somered blood cell polymorphisms (CR1, Fya, Fyb, K, Kpa, Kpb), immune receptor polymorphisms (TLR2,4 and 9), proinflammatory cytokines (IL-1β,IL-8, IL-18, TNF-α) of the innate system with risk of malaria in two ethnic groups, the Austro-Asiatics and Tibeto-Burmans, from malaria endemic districts of Assam, and have tried to understand the influence of malaria in selection of polymorphisms and the proinflammatory cytokines/chemokines in these genetically distinct populations.

CHAPTER 2:
REVIEW OF LITERATURE

2. *Review of literature*

2.1 Genetic Diversity of *Plasmodium falciparum* parasites

P. falciparum is the most virulent of thefour parasites which cause malaria in humans[1]. Natural exposure to *P. falciparum* gradually elicits, inhuman hosts, short-lived strain-specific malariaimmunity: first to severe disease and death, and then tomild disease[80].Repeated infections are required tomaintain clinical immunity, which is both antibody and T-cellbased, although evidence is most clear for antibody-mediated immunity to blood-stage malaria[8-84].The malaria parasite *P.falciparum* exhibits extensive antigenic diversity, due to its complex life cycle [85]. The extensive diversity of malarial surface antigens is one ofthe main reasons why clinical immunity develops only afterrepeated infections with the same species over several years[86]. The inherent variability of *P. falciparum* is particularly prevalent in merozoite surface antigens being targeted for malaria vaccines.Merozoite Surface Proteins (MSPs) are extensively studied to assess the genetic diversity of *P. falciparum* in differenttransmission zones of *Pf* malaria as genetic diversity is an indicative of malaria transmission dynamics, complexity of infection and host immune response. This variability provides multiple effective evasion and drugresistance mechanisms for the parasite. It also represents a major challenge for development of an effective malaria vaccine. Thus, the study of geneticdiversity in malaria parasites is expected to providenew insights for the deployment of control measures[87-91].

2.2 Immune evasion strategy

2.2.1 Antigenic diversity

Plasmodium parasite evades host immunity by varying the antigenic character of infected erythrocytes. There is a large and extremely diverse family of *P. falciparum*genes, known as the var genes [92-94]. These genes encode an antigenically diverse

parasite-derivedprotein of 200 to 350 kDa, *P. falciparum* erythrocyte membraneprotein 1 (*Pf*EMP1), on the surface of parasitized erythrocyteswith the expected properties of antigenically variant adhesionmolecules. *Pf*EMP1 has been implicated as the key target antigen involved in naturally acquired immunity to malaria [95-97]. More recently, two other larger families of clonally variant surfacemolecules called rifins [98, 99] and STEVOR [100] havebeen described, although their role in acquired immunity remains unknown. Collectively, these proteins, expressed at theinfected red blood cell membrane, are referred to as variantsurface antigens (VSA), and the immunity directed againstthese antigens is termed variant-specific immunity.

2.2.2 Repetitive sequences

A further strategy for immune evasion may be based in the nature of the antigenic sequence itself.The malaria parasite may use repetitive, immunodominant epitopes as a mechanism to evade theimmune response of the human host [101] and many of malaria antigens contain tandem arrays of relatively short sequences. Tandem repeats may stimulate T-cell-independent B-cell responses [102] that fail to generate memory B cells orsomatic hypermutation, leading to antibody affinity maturation[103]. The existence of cross-reacting antibodies hasoften complicated the problem of identifying specific *Plasmodium* gene products and protectiveimmune responses [105]. Moreover, cross-reactive epitopes on otherwise differentrepetitive antigens may prevent the affinity maturation of antibodies by causing an abnormally high proportion of mutatedB cells to be preserved during clonal expansion [106].

2.3 Asexual blood-stage vaccines

These vaccines, also referred to as erythrocytic stage vaccines, are aimed to primarily protect against severe malariadisease, and not against infection, on the assumption thatinhibition of parasite invasion cycles will lead to reduced parasite burden and decreased morbidity and mortality [106, 107]. The first asexual blood-stage malaria

vaccine to be developed was the SPf66 vaccine candidate that was developed inColombia as a synthetic, multiepitope, multi-stage peptidevaccine mixed with alum as an adjuvant [108]. The vaccinewas tested in several Phase III field trials involving thousandsof volunteers, but its reported efficacy was too low to warrant further development [109-112] although one may suspectthat the vaccine would have fared better with more potent adjuvants [113].

2.3.1 Merozoite Surface Protein (MSP)

Merozoite surface proteins play a role in RBC invasion and hence are important targets of immunity[114]. Antibodies directed against merozoite proteins function by blocking RBC invasion, initiate parasite clearance by opsonisation making the merozoite susceptible to phagocytic cells or complement mediated damage[115, 116]. Merozoite surface proteins (MSPs) are likely to mediate the relatively weak and reversible initial interactions between the parasite and RBC[117]. Antibodies to MSP-1, MSP-2, and MSP-3 have been associated with protection from clinical malaria. Protection from clinical malaria was associated particularly with antibodies of the IgG3 isotype for both MSP-2 and MSP-3[118].

The leading blood-stage vaccine candidates are primarily merozoite proteins either located on the merozoite surface or contained within the apical organelle. These include apical membrane antigen 1 (AMA1), erythrocyte-binding antigen–175 (EBA-175), glutamate-rich protein (GLURP), merozoite surface protein 1 (MSP-1), MSP-2, MSP-3, MSP-4, MSP-5 and serine repeat antigen 5 (SERA5) all of which are highly expressed on the surface of the merozoite [119] (Figure 2.1).

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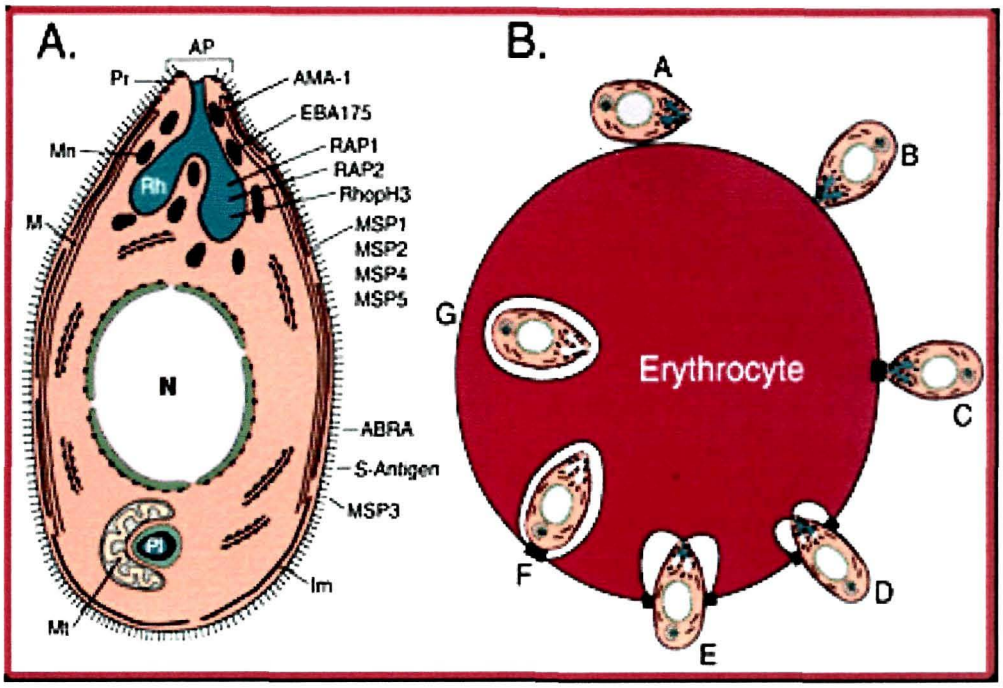


A. B.

Figure 2.1: A) Schematic of a *Plasmodium falciparum* merozoite highlighting merozoite proteins that are potential vaccine candidates and B) Stepwise schematic of the invasion of a *P. falciparum* merozoite into an uninfected erythrocyte (*Cowman A.F. et al. FEBS Letters. 2000;476:84-8*[120]

2.3.2 MSP2- genetic structure

Polymorphic antigens have been described in several parasite life cycle stages but are particularlya notable feature of the antigens associated with the surface of the asexual blood-stage merozoites[121, 122]. Themerozoite surface protein 2 (MSP2) is one of the well-characterized surface proteins of *P.falciparum*. MSP2 is an integral membrane (GPI anchored) protein with highly conservedcarboxy- and amino-

terminal regions flanking a central variable region that is composed of non-repetitive semi-conserved sequences surrounding repetitive highly variable repetitive sequences[123, 124]and identifies two allelic families of MSP2, the FC-27 type (isolate FC-27Q/PNG) and IC/3D7 type (Indochina) [125].

Most studies examining the distribution and frequency of different allelic forms of MSP2 have enumerated the presence of the allelic families[125, 126, 128]and was seen to vary with transmission intensity [86, 87] and geographical regions.A skewed distribution of predominantly 3D7 family alleles exists among laboratory-adapted strains, in the field a more even distribution of FC27 and 3D7 alleles occurs. Often FC27 family alleles are more prevalent than 3D7 alleles, and novel FC27 and 3D7 family alleles have been found in field malaria strains[127, 128]. Detailed genetic structure of MSP2 is shown in Figure 2.2.

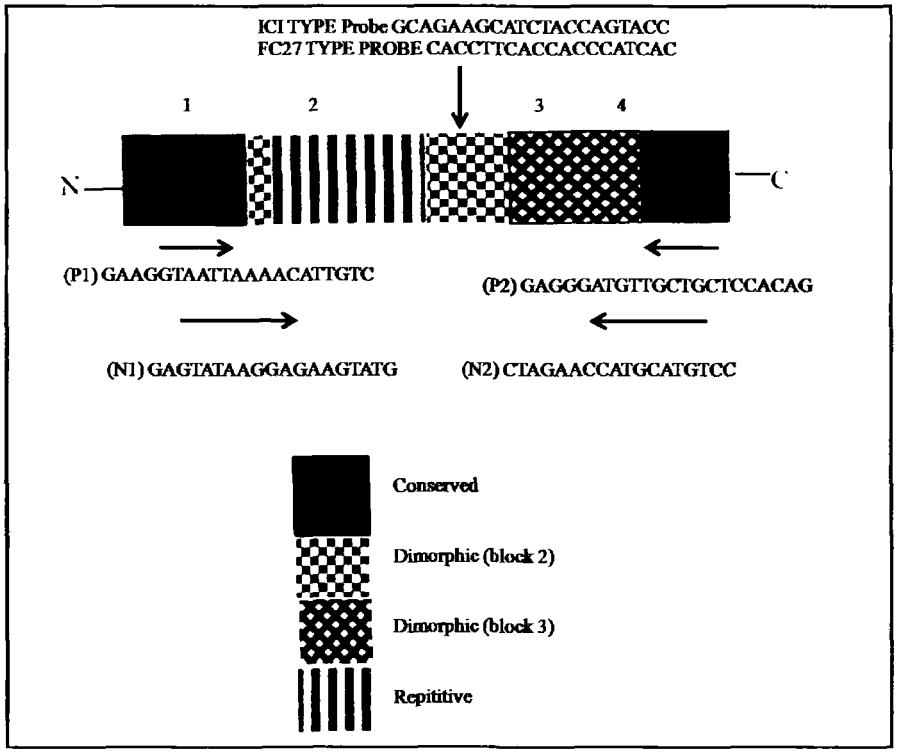


Figure 2.2: Schematic diagram of the merozoite surface protein-2 gene

Adapted from: Ranford-Cartwright and others.[23] *(i) The gene can be conveniently divided into blocks (1–4) according to the conserved and repetitive nature of the sequence. The region of amplification surrounds block 2, which contains tandem repeats and is flanked by a dimorphic sequence against which DNA probes willhybridize. Two pairs of primers are used in consecutive rounds of polymerase chain reaction amplification. The N and C terminal ends of the gene are indicated. (ii) The conserved and repetitive nature of the sequence depicted in (i).*

The MSP2locus is not only a useful marker gene for molecular epidemiology purposes, but the expressedprotein is also a promising vaccine candidate [124].It is a well-known target of naturally acquired clinical immunity to malaria [122] and antibodies to these molecules are reported to block merozoite invasion of erythrocytes [123].Further, vaccines containing only one allelic type (3D7 allele) of a polymorphic antigen induced immune responses that selected for parasites expressing alternative alleles (FC-27 type alleles) [129]. resulting in a higher incidence of morbid episodes. It was also observed at Mali. that the parasites with MSP-1_{19} haplotypes were different from that of the leading vaccine strain, emphasising the importance of determining the genetics of pathogen populations prior to vaccine trials [130]. Inclusion of combinations of large number of conserved antigens and/or multiple components covering all important allelic types in vaccine formulations was recommended [131].Due to the location on the surface of the parasite and the immunological features of MSP2,the protein was suggested as part of a three component vaccine, called Combination B, recently tested in Papua New Guinea [130] [132]. There exists extensive polymorphism of key parasite antigens and this is likely to hamper the effectiveness of subunit vaccines against *Plasmodium falciparum* infection. So little is known about the extent of the antigenic repertoire of naturally circulating strains in different areas where malaria is endemic.Therefore, genetic structures and population genetics studies of *P.falciparum* may hold the key for effective disease surveillance and control program.

2.4 Toll-like receptors (TLRs)

Toll-like receptors are a family of innate immune receptors known as Pattern recognition receptors (PRRs) whose critical role involves the recognition of pathogen-associated molecular patterns (PAMPs) of invading pathogens[133]. They are evolutionarily conserved, their homologs found in mammals, plants and insects. They were first described for their involvement in innate immunity in *Drosophila melanogaster*, where fruitflies with a mutant Toll receptor demonstrated high susceptibility to fungal infection [133 134]. TLRs are broadly distributed on the cells of the immune system such as macrophages, dendritic cells (DC), neutrophils, B cells, as well as mucosal epithelial and endothelial cells [135].

The family of mammalian TLRs are type I trans-membrane receptors characterised by an ectodomain composed of multiple copies of leucine-rich motifs and a Toll/interleukin-1 receptor (TIR) motif in the cytoplasmic domain. The TIR domain, found in other members of the interleukin (IL)-1 receptor family, mediates homophilic and heterophilic interactions between TLRs and TIR-containing adaptors [136]. So far, at least thirteen members of the TLR family have been identified and characterized in the mammalian system. TLR1 to TLR9 are conserved in both humans and mice. TLR10 is expressed in human, while TLR11 to TLR13 are present in mice [133]. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are expressed on the cell surface; TLR3, TLR7, TLR8, and TLR9 are expressed in intracellular vesicles such as endosomes, lysosomes, and the endoplasmic reticulum [137] (Figure 2.3).

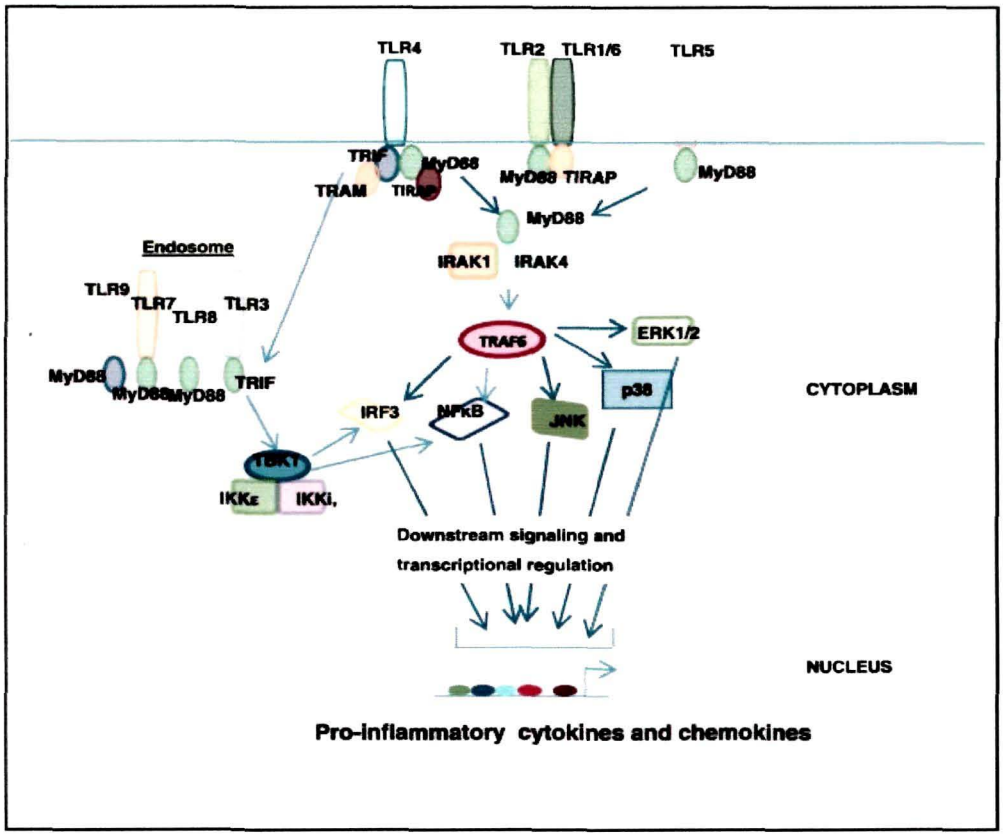


Figure 2.3: Toll-like Receptors (TLRs) recognize specialized structural motifs on the microbes known as pathogen-associated molecular patterns (PAMPs).

The cellular distribution of TLRs represents the site of their encounter with the pathogens - TLRs which recognize cell wall moieties (TLR-2,-4, -5) are present on the cell-membrane whereas TLRs which recognize nucleic-acids (TLR3, -7, -8, -9) are localized in the endosomal compartment. Adapted from: Tarang et al. Cancer Letters Volume 321, Issue 2, 28 2012, Pages 110–11[138].

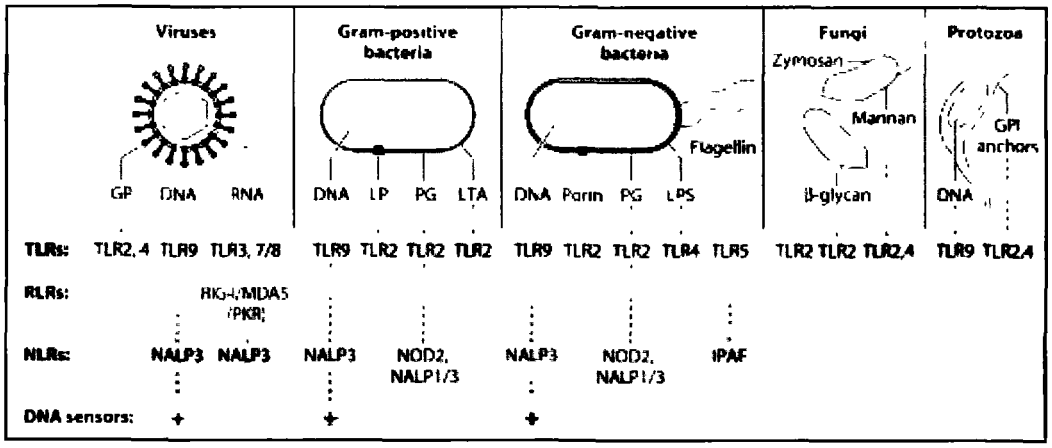


Figure 2.4: Recognition of PAMPs from different classes of microbial pathogens.

Viruses, bacteria, fungi, and protozoa display several different PAMPs, some of which are shared between different classes of pathogens. Major PAMPs are nucleic acids, including DNA, dsRNA, ssRNA, and 5'-triphosphate RNA, as well as surface glycoproteins (GP), lipoproteins (LP), and membrane components (peptidoglycans [PG], lipoteichoic acid [LTA], LPS, and GPI anchors). These PAMPs are recognized by different families of PRRs. Adapted From:Clin Microbiol Rev. 2009 April; 22(2): 240–273 [139].

TLRs can be divided into subfamilies primarily recognizing related PAMPs; TLR1, TLR2, TLR4, and TLR6 recognize lipids, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids [133] (Figure 2.4). Moreover, it appears that TLRs can recognize PAMPs either through direct interaction or via an intermediate PAMP-binding molecule. Thus, TLR1/2, TLR3, and TLR9 directly bind to triacetylated lipopeptides, double-stranded RNA (dsRNA), and CpG DNA, respectively [140-142], whereas TLR4 recognizes lipopolysaccharide (LPS) through the accessory molecule

MD2[142]. Intriguingly, some TLRs are endowed with the capacity to recognize structurally and biochemically unrelated ligands, as exemplified by the ability of TLR4 to recognize such divergent structures as LPS, the fusion protein of respiratory syncytial virus (RSV), and cellular heat shock proteins (HSPs) [133]. The molecular basis of this phenomenon may be the ability of different regions of the extracellular portion of TLRs to bind their cognate ligands or the involvement of different PAMP-binding molecules, such as MD2 [142, 143].

2.4.1 Toll-like receptors 2, 4 and 9

2.4.1.1 Toll-like receptor 2 (TLR2)

TLR2 recognizes a wide range of PAMPs derived from various pathogens, ranging from bacteria, fungi, parasites and viruses [133]. These ligands include di and tri-acyl lipopeptides, peptidoglycan, lipoteichoic acid, porin, lipoarabinomannan, zymosan Trypanosoma GPI-mucin and hemagglutinin protein[155, 156, 157]. It has been suggested that TLR2 recognizes a wide spectrum of microbial components because it forms heterodimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2. TLR2 also forms heterodimers with non-TLR molecules such as CD36, CD14 and dectin-1 [158].

2.4.1.2Toll-like receptor 4 (TLR4)

Of the thirteen TLRs, TLR4 was characterized first [159]. TLR4 is involved in recognition of bacterial lipopolysaccharide (LPS) [160, 161] In addition, TLR4 is implicated in the recognition of taxol, a diterpene purified from the bark of the western yew (*Taxus brevifolia*) [162, 163]. Furthermore, TLR4 has been shown to be involved in the recognition of endogenous ligands, such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen[164]. TLR4 forms a complex with another LRR protein known as MD-2, and this is mediated by ionic and hydrogen bonds in two oppositely

charged patches. There is no direct interaction between TLR4 and LPS, but MD-2 functions as the LPS-binding component in the TLR4–MD-2 complex. LPS-binding protein (LBP) and CD14 deliver and load the LPS to the TLR4-bound MD-2 [165].

2.4.1.3 Toll-like receptor 9 (TLR9)

TLR9 was originally found to detect bacterial DNA. Its specificity was initially thought to be directed toward cytidine–phosphate–guanosine (CpG) motifs,which are four times less abundant (and mostly methylated) inmammalian genomic DNA than in bacterial or viral DNA. TLR9 recognizes dsDNA viruses such as mouse cytomegalovirus (MCMV) and herpes simplex viruses 1 and 2 [166, 167, 168] and the genomes of protozoa,including Trypanosoma cruzi [168]. Although malarial DNA is by itself only weakly stimulatory to TLR9, it inducesstrong TLR9 responses when delivered to the endosomalcompartment with a transfection reagent or when bound to malarial hemozoin [169 170]

2.4.2 TLR downstream signalling

The ability of a TLR to tailor an inflammatory response, specific for individual ligands, has recently focused attention onto the cytosolic subfamily of adapter molecules that orchestrate and fractionate these downstream signalling events. There are currently five cytosolic TIR-containing proteins (MyD88, Mal, TRIF, TRAM, SARM) that are thought to play a crucial role in specificity of individual TLR-mediated signalling pathways, where most TLR members differentially utilise many of these signalling components[144].

2.4.2.1 Myeloid differentiation primary response gene 88 (MyD88)

MyD88 is one of the representative adaptor molecules in TLR signaling[145]. MyD88 is a protein that is induced by terminal differentiation of M1D$^+$ myeloid precursors and responses to IL-6(66). MyD88 is located in the cytosol near the cytosolic part of

TLRs and delivers an activation signal that is initiated by receptor activation. MyD88 is used by all TLR family members, except TLR3, to activate NF-κB[146, 147].

2.4.2.2 TIR-domain containing adaptor protein inducing interferon-β (TRIF)

TRIF is another adaptor molecule associated with TLR signaling TRIF was found by database screening during the search for a TIR domain containing protein. TRIF interacts with TLRs through TIR-TIR interaction. In contrast with MyD88, which is broadly used as an adaptor molecule in TLR signaling, TRIF is only involved in the signaling pathways of TLR3 and TLR4. TRIF is considered to be closely related to anti-viral signaling, since signals mediated by TRIF are linked to IRF activation and production of IFN [148]. While TLR3 only uses TRIF as its adaptor molecule, TLR4 uses TRIF under limited conditions in a MyD88-independent manner[149].

2.4.2.3 MyD88-adapter-like protein/TIR domain-containing adapter protein (MAL/TIRAP)

MAL/TIRAP is an adaptor molecule essential to the TLR2 and TLR4 signaling pathways. MAL/TIRAP acts as a bridge between MyD88 and TLR. MAL/TIRAP has an N-terminus binding domain that binds to phosphati-dylinositol-4,5-bisphosphate; this process mediates the recruitment of MAL/TIRAP to the plasma membrane and, in particular, to the microdomains that contain TLR4. MyD88 does not bind directly to TLR4, but instead interacts with MAL/TIRAP in association with TLR4 [146, 150].

2.4.2.4 TRIF-related adaptor molecule(TRAM)

TRAM also known as TICAM2 plays an essential role in the MyD88-independent signaling pathway of TLR4. TRAM has a TIR domain, and acts as a bridge connecting TLR and TRIF, which allows for the activation of the TRIF dependent pathway in response to LPS [151]. The activation of TRAM affects IRF3 and NF-κB activation as well. TRAM is regulated by myristoylation, which is required for the adaptor molecule to be localized within plasma membrane[152].

2.4.2.5 Sterile α- and armadillo-motif-containing protein (SARM)

SARM consists of a sterile α motif (SAM) and a TIR domain[153, 154]. The SAM domain is known to mediate protein-protein interactions whereas the Armadillo repeat mediates the interaction of β-catenin with its ligands as well as forming structural complexes with other proteins. SARM has been shown to be a negative regulator of NF-κB and IRF in TLR signaling[154].

2.4.3 Toll-like receptors associated with disease

Activation of TLRs leads not only to the robust production of proinflammatory mediators but also to the production of unique effectors, which provide pathogen-tailored immune responses.A positive correlation between TLR2 and TLR4 expression stimulated the innate immune response against bacterial pathogens, including *H. pylori*-LPS[172]. Recent studies suggest that tumor cells bear TLRs and that TLR signaling promotes tumor growth and immune evasion [172, 173].Kelly *et al.* (2006) find that activation of TLR4 signaling promotes the growth and chemoresistance of epithelial ovarian cancer cells.

Gene mutations and polymorphisms in TLRs have revealed the importance of TLRs in human defense against diseases[174]. Studies of the TLR4 gene have shown that two co-segregating SNPs, gene-Asp299Gly and Thr399Ile, have been found to positively correlate with several infectious diseases such as increased incidence of systemic inflammatory response syndrome [175], reduced risk for carotid artery atherosclerosis [176] and decreased rheumatoid arthritis disease susceptibility [177]. Polymorphisms have also been identified in TLR2, where gene-Arg753Gln, was associated with a decreased response to bacterial peptides derived from *B. burgdorferi* and*T. pallidum* in septic shock patients [178]. In addition, this polymorphism may also predispose persons to staphylococcal infections [179], or tuberculosis [180].

In a study of 870 children in Ghana, a polymorphism in TLR4 but not TLR9 was found to influence malaria susceptibility [181]. Specifically, TLR4 Asp299Gly and Thr399Ile SNPs were associated with the increased risks of severe malaria. Polymorphisms in the promoter regions of TLR9 had no influence on malaria severity[181, 182].

An unusual Leu658Pro mutation in TLR2 was present in this population, however, its association with malaria remains to be studied [181].TLR4 and TLR9 play a role in the manifestation of malaria during pregnancy. In 304 Ghanian primigravid women studied, TLR4 Asp299Gly and TLR9 T1486C mutations showed increased risks of delivering low-birth-weight babies. The TLR4 Asp299Gly mutation also exhibited higher risks of maternal anemia. More recently, a large population study demonstrated that functional impairment of Mal/TIRAP caused by a single SNP corresponding to Ser180Leu, results in significant protection against malaria[182].

2.5 Proinflammatory cytokines

Cytokines are low molecular weight regulatory proteins or glycoproteins secreted by various immune effector cells in the body in response to a number of stimuli. These proteins assist in regulating the development of immune effector cells, and somecytokines possess direct effector functions of their own[183]. Some cytokines clearly promote inflammationand are called proinflammatory cytokines, whereasother cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines[184].A successful type 1 response to malaria requires a well-timed and proportional release of proinflammatory cytokines such as IL-12, IL-1β, IFN-γ and TNF-α to minimize infection [185].Cytokines such as IL-4, IL-10, IL-13, and TGF-β suppress the production ofIL-1, TNF, chemokines such as IL-8, and vascularadhesion molecules. The ratio between the pro- and anti- inflammatory cytokine responses should be also considered to evaluate the outcome of the disease.

High ratios of TNF-α, IFN-γ and IL-12 to TGF-β were found to be associated with increased risk of fever and complications.Therefore, a "balance" betweenthe effects of proinflammatory and anti-inflammatory cytokines is thought to determine the outcomeof disease[186].

2.5.1 Proinflammatory cytokines: IL-1β, IL-8, IL-18 and TNF- α

2.5.1.1 Interleukin-1β (IL-1β)

IL-1β is a proinflamatory cytokine and It belongs to the IL-1 family cluster that includes the IL-1α, and IL1-RN genes It is expressed by many cells including macrophage, NK cells, monocytes, and neutrophils as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [187]. Inflammatory hypersensitivity has been found to be the result of IL-1β activation of cyclooxygenase-2 (PTGS2/COX2). IL-1beta has also been associated with septic shock, and wound healing [188].

2.5.1.2 Interleukin-8 (IL-8)

The protein encoded by this gene is a member of the CXC chemokine family. This chemokine is one of the major mediators of the inflammatory response. This chemokine is secreted by several cell types. It functions as a chemoattractant, and is also a potent angiogenic factor. This gene is believed to play a role in the pathogenesis of bronchiolitis, a common respiratory tract disease caused by viral infection. This gene and other ten members of the CXC chemokine gene family form a chemokine gene cluster in a region mapped to chromosome 4q[189].

2.5.1.3 Interleukin-18 (IL-18)

IL-18 is expressed in macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortexcells, intestinal epithelial cells, microglial cells, and synovialfibroblasts. IL-18, like IL-1β, with which it shares structural homology,is produced as a 24-kD inactive precursor lacking a signal peptide (proIL-18). Pro IL-18 is cleaved after Asp35 by theendoprotease IL-1β -converting enzyme (ICE; caspase-1) togenerate a biologically active, mature 18-kD moiety (). IL-18 has pleiotropic effects, which include production of IFN-γ and GM-CSF in PBMCs and in T cells and enhancement of Fas-ligand expression on Th1 cells[190]. Itwas also found that addition of IL-18 together with IL-12 syner-gistically induced IFN-γ production and that IL-12 was involvedin up-regulation of IL-18R[191].

2.5.1.4 Tumour Necrosis Factor-α (TNF- α)

The human TNF-α protein contains 233 amino acids with a predicated molecular weight of 25.6 kDa. TNF-α is the first prototypic member identified in the TNF superfamily. The most abundant cellular sources of TNF-α are macrophage and monocyte. In response to inflammatory stimulation, macrophage or monocyte secretes TNF-α that can induce apoptotic or necrotic cell death of certain tumor cell lines[192]. In addition, TNF-α is also capable of inducing cell proliferation and differentiation in many types of cells under certain circumstances. TNF-α can be a pyrogen that causes fever by its direct action or by stimulation of interleukin 1 secretion. Sustained generation of TNF-α in a variety of human diseases, especially cancer and severe infection, can cause cachexia-like syndrome. The increased expression of TNF-α in adipose tissue was considered to be responsible for the development of obesity or diabetes due to the induction of insulin resistance by TNF-α[192].

2.5.2 Cytokines and malaria

Cytokines seem to be involved both in protection and pathology in malaria infection. Early and effective inflammatory response, mediated by IFN-γ in the IL-12 and IL-18 dependent manner, seems to be crucial for the control of parasitaemia and resolution of malaria infection through the mechanisms of the

TNF-α induction and enhanced release of the antiparasitic reactive nitrogen and oxygen radicals [193]. On the other hand, severe malaria has long been associatedwith high circulating levels of proinflammatory cytokines such as TNF-α, IFN-γ, IL-1 and IL-6 [194-198]. Their excessive production may affect the disease outcome through their direct systemic effect and by increasing cytoadherence of parasitized erythrocytes to the endothelium via up regulation of adhesion molecules in *P. falciparum* infections[199]. The expression of cytokines in general as well as the balance of pro- and antiinflammatory response are supposed to be involved in malaria pathogenesis, but their relationship with the pattern and extent of vital organ dysfunction in malaria infection has not been well defined yet [199]. Severe malarial anaemia has been associated with low serum levels of IL-12 and low IL-10 to TNF-α serum concentrations ratio in a few studies of childhood malaria in holoendemic areas[195].

However, the manifestations of severe malaria vary with geographic location and malaria transmission intensity as well as with the age of the patient [200-202]. In non-immune adults severe malaria often presents as a [105] multiorgan disorder with renal failure, hepatic dysfunction with jaundice and shock while in African children cerebral malaria and severe anaemia predominate [201, 203]. Recent in vitro experiments have shown that peripheral blood mononuclear cellsfrom clinically immune individuals from areas of high endemicity produce lower amounts of IFN-γ in

response to *P. falciparum* schizont antigens than those from previously unexposed donors, indicating that the control of clinical symptoms may depend on the host ability to regulate strictly the inflammatory response [204]. Studies in mice undergoing primary malaria infection have suggested that the profile of cytokines, including IFN-γ, released early in the course of the infection, may predict the final outcome of the disease [205, 206]. Data concerning involvement of cytokines in naturally acquired malaria infection in non-immune adults, particularly with severe manifestations of the disease, are scarce.

2.6 Red blood cell polymorphisms

BLOOD GROUP ANTIGENS are inherited, polymorphicamino acid or carbohydrate motifs on the surface of redblood cells (RBCs) and because they are easy to detect, amultitude of naturally occurring variants have been identified[207].Knowledge of the molecular basis of these genes enabled the development ofmolecular biology typing methods, the identification of new mutations, an understandingof polymorphisms and the discovery of new alleles. Genes of red blood cell groups arehighly polymorphic and the distribution of allelic frequencies in these systems variesbetween different regions of the world[208, 209]. The red blood cell groups are composed of numerous antigens, of which the mainones are: ABO, Rh, Kell, Duffy, Kidd and MNS. ABO and Rh are the most important systems.

2.6.1 Duffy antigens: Fya and Fyb

The major Duffy group antigens are composed of two principal antigens Fya and Fyb that are encoded by the co-dominant alleles *FYA* and *FYB*, located on chromosome 1 [210, 211]. The corresponding anti-Fya and anti-Fyb antibodies define four different phenotypes; Fy(a+b+), Fy(a+b-), Fy(a-b+) and Fy(ab-) [212]. The *FYA* and *FYB* alleles differ by a point mutation in the major cDNA transcript, encoding glycine in Fya or aspartic acid in Fyb at residue 42 of the most important form of the protein, encoded

by the exon 2. The Fy(a–b–) phenotype is the major phenotype in Blacks, but is very rarely found in Caucasians. The phenotype found in Blacks is characterized by the presence of Fyb antigen on nonerythroid cells, but an absence of the Fyb antigen on RBCs [213, 214, 215]. A mutation in the erythroid promoter GATA-1 binding motif explains why Fy(a–b–) individuals do not make anti-Fyb. The FY (a–b–) phenotype found in Caucasians is characterized by a lack of Duffy antigen expression in both erythroid and nonerythroid tissues. Different mutations are present in either the *FYA* or *FYB* gene, which prevent the Duffy protein from being formed. These individuals, interestingly, tend to form anti-Fy3 [214, 215].

Chemokines are proteins secreted by cells, such as immune cells, which are used as communication signals to guide their interactions. Chemokine messages secreted from one cell are received and decoded by another cell via specific receptors, leading to various responses such as leukocyte chemotaxis and adhesion [216]. Similar to the Duffy glycoprotein, many chemokine receptors have seven transmembrane domains [217]. However, whereas other chemokines receptors specifically bind chemokines of a single class, the Duffy glycoprotein was found to bind a variety of chemokines and is known as the Duffy antigen receptor for chemokines (DARC) [218].

The function of DARC is yet to be clearly defined. It has been suggested that DARC may permit the erythrocyte to serve as a chemokine "sink" or scavenger, thus limiting activation of leukocytes in the systemic circulation[217, 218]. However, it is unclear how long chemokines remain bound to the cell surface or what happens to the chemokines at the end of the erythrocyte lifespan. In addition, it is unclear as to the importance of this function in inflammatory or infectious disease as Fy(a–b–) erythrocytes do not bind chemokines, although Fy(a–b+w) erythrocytes bind reduced amounts compared with Fy(a–b+) cells[214].

2.6.2 Kell antigens: K, Kpa and Kpb

The Kell blood group system is one of the most polymorphicantigenic systems in human RBCs with at least 23 Kell antigensincluding sets of antithetical antigens K (K1) and k (K2); Kpa(K3), Kpb (K4), and Kpc (K21); Jsa (K6) and Jsb (K7)[219]. Kellsystem antigens are highly immunogenic and the resultingantibodies can cause severe reactions to transfusion of incompatible blood as well as causing fetal anemia and hemolytic disease in newborns [220].It was recently shown that anemia in the foetus/newborn is exacerbated by suppression of erythropoiesis[221].With the exception of the K antigen [219], it has not beenpossible to determine whether single point mutations are solelyresponsible for each of the Kell antigens, probably due to lackof strongly reactive monoclonal antibodies (MoAbs) as detection tools. In addition, despite a deliberate search, a KEL allelethat encodes two low incidence antigens has not yet beendescribed[222].

The Kell system consists of 31 antigens, including six pairs of antithetical antigens.The Kell protein is associated with other proteins on the red blood cell membrane[219].The gene encoding the antigens of this system (KEL) is located on chromosome 7q33 andits expression occurs in erythroid cells and in some tissues, such as the brain, lymphoidorgans, the heart and muscles[223].Kell antigens reside on a 93-kD type II glycoprotein, with a665-amino acid carboxy terminal extracellular domain, a singletransmembrane domain, and a 47–amino acid N-terminal cytoplasmic domain [224]. The Kell protein has a striking sequencehomology with neutral zinc endo peptidases [224], which activate orinactivate bioactive peptidesand was recently shown to have proteolytic activity [225]. A weakening of all inherited, highincidence Kell antigens has been reported with RBCs of the Kp(a1) phenotype, [220, 226, 227]but the mechanism by which thisoccurs is not understood. An amino acid substitution ofArg281Trp is associated with the Kpa antigen. Because Kell isa highly folded protein, this amino acid change may cause aconformational change throughout the protein, thereby

affectingthe accessibility of the antigens to Kell antibodies. Alternatively, the amino acid substitution may affect the stability of theprotein or its ability to reach the cell membrane.Understanding theunderlying mechanism for the difference in these RBC phenotypes can potentially yield useful information on the structure ofKell and its related family members and/or help in elucidatingthe intracellular trafficking of Kell and its requirements forsurface expression[229].

2.6.3Complement receptor 1 (CR1/CD35)

Complement receptor type 1 (CR1) is a large (~200-kDa), single-chain, immunoregulatory membrane glycoprotein[230] expressedon various cell types, including erythrocytes (red bloodcells), granulocytes, monocytes, all B cells andsome T cells, glomerular podocytes, and follicular dendritic cells [231, 232]. In humans, the functions of CR1 principally include opsonization, control of complement activation, and removal of immune complexes (ICs) [232].CR1 plays a major role in IC clearance because of its highaffinity for C3b and C4b [233]. Erythrocyte CR1 (E-CR1),through a process known as "immune adherence," bindsthe ICs (C1q, C4b, C3b, and C3bi) in the peripheralblood and transports them to the phagocytes in the liverand spleen, to remove them from the circulation [234].The rate of clearance of immune complexes (ICs) from the circulation is directly related to the number of CR1 molecules expressed on the erythrocytes [235].

2.6.3.1 CR1 and malaria

Human CR1binds to a major malarial adhesin, the *P.falciparum*erythrocyte membrane protein-one (PfEMP-1) [236]. *Pf*EMP-1is responsible for the vast array of binding activities ofparasitized erythrocytes (E), including the phenomenoncalled resetting inwhichmalariaparasitizedEbindto non parasitized ones [237-240]. The *Pf*EMP-1 protein can interactwith several types of surface molecules, including inter-cellular adhesion molecule 1 (ICAM-1), type A and B bloodgroups, thrombospondin,

E-selectin, chondroitin sulfate,CD36 and CR1, as well as with soluble ligands. Moreover,the identification of CR1 polymorphisms in association with malaria in Africa,[241]raises concerns regarding the actual roleof CR1 or that of the binding fragments (C3b and C4b) inimmune complex clearance and malaria pathogenesis.

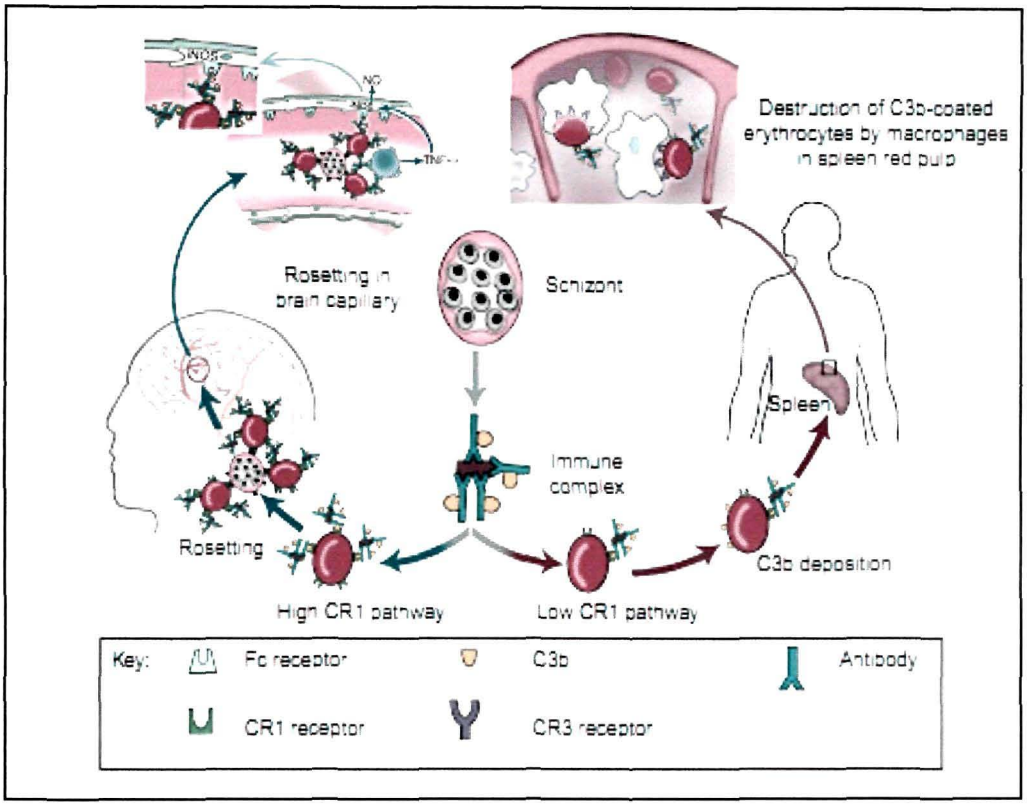


Figure 2.5: Susceptibility to severe malaria in relation to the level of erythrocyte CR1 expression.

Adapted from: Stoute, J.A. Complement-regulatory proteins in severe malaria: too little or too much of a good thing? TRENDS in Parasitology 21,(5) 218-223 (2005).

ICsare produced during malaria infection because of the interaction of anti-malaria antibodies with antigens released during schizont rupture. Erythrocytes of individuals with low-level CR1 are more susceptible to complement-mediated damage and removal of erythrocytes in the spleen, leading to severe anaemia. This susceptibility could be compounded by decreases in levels of erythrocyte complement-regulatory proteins due to repeated malaria attacks. However, individuals with high levels of erythrocyte-CR1 (E-CR1) carry more ICs and are more likely to form rosettes that contribute to sequestration in brain capillaries [242](Figure 2.5)

E-CR1 has been considered to play an important role in "rosetteformation," a phenomenon resulting from adhesion of *Plasmodium falciparum* erythrocyte membrane protein 1 (PFEMP-1) onthe surface of infected RBCs to a variety of uninfected RBC membrane receptors, such as CR1[243-246]. This aggregation causesconsiderable obstruction of the cerebral microvasculature, thuscontributing to the pathologic changes in cerebral malaria [247, 248], severe malaria-associated anemia [249, 250] or both [251, 252]. Erythrocytes having low CR1 expression have beenshown to form reduced number of rosettes with *Pf*-infected cells.Since E having low CR1copy numbers form fewer rosettes, it has been postulatedthat low E-CR1 might protect from severe malaria. In astudy by Cockburn' et al,[253] found that low CR1 expression protected from severe malaria.

In Africa, however,E-CR1 deficiency seems to be less important since otherCR1 polymorphisms, that is, Knops blood groupantigens,[254]appear to confer a protective advantageagainst malaria infection in this continent. These observations support the notion thatthe selective pressure of malaria has acted differently onthe CR1 gene in different populations. The highincidence of these CR1 mutations in Asia depicts yetanother example of the evolutionary pressure exerted by*Plasmodium* on human population, with these mutationsa response to adaptive evolution in these locations as aresult of human migration from Africa to Asia [255–257].

Genomic studies of the CR1 gene reveals three types ofgenetic polymorphisms: firstly, a size variation createdby LHR duplications and deletions; secondly, a HindIIIrestriction fragment length polymorphism [258], which, inCaucasians but not in Africans, correlates with E-CR1copy number and the third represented by the Knopsblood group system. Recently, a number of other CR1polymorphisms have been identified in Caucasianslinked to constitutive E-CR1 expression levels [259–261]. Twoof these newly identified single-nucleotide polymorphisms (SNPs) occur in regions of known ligand-bindingdomains, at amino acid I643T (T2078C) at the 30 end ofSCR 10, and at Q981H (G3093T) in SCR 16. The finding ofthese polymorphisms resulted in the hypothesis thatthey constituted a low expression (L) SNP haplotype thatcould have ligand-binding activity different from CR1encoded by the high expression allele (H) [261].

In Papua New Guinea (PNG), a high rate of the heterozygous(Q981H) gene state was noted, indicating that this mutation may bestill evolving in this location resulting in a significantshift from the wild (QQ) to the mutant types (HH).Interestingly, in PNG,[262]there is greater protection fromsevere malaria among individuals heterozygous for theHindIII H/L alleles. The low E-CR1 density found in LLpatients may protect from cerebral complications ofmalaria but predispose to severe malarial anemia.

CHAPTER 3: Polymorphisms and expression of TLR2, 4 and 9 with malaria in two ethnic groups of Assam, Northeast India

3. *Polymorphisms and expression of TLR2, 4 and 9 with malaria in two ethnic groups of Assam, Northeast India*

3.1 Abstract

Infectious diseases have been postulated to play an important role in exerting pressure and in selection of TLR polymorphisms. SNPs of TLR4 have been reported to show unique distributions in populations from Africa, Asia, and Europe and malaria is suggested to influence these patterns. In this context, we have examined association of TLR polymorphisms with risk of malaria in two ethnic groups the Austro-Asiatics and Tibeto-Burmans, from malaria endemic districts of Assam, to understand the influence of malaria in selection of TLRs in these genetically distinct populations.

TLR9 (T-1237C) mutation was positively associated with complicated (p=0.001) and frequent (p=0.035) malaria in Austro-Asiatics (Relative risk =0.595 95% CI: 0.479 to 0.836), but not in Tibeto-Burmans. Nonetheless, these alleles were not in Hardy-Weinberg Equilibrium in Tibeto-Burmans (p<0.001). In contrast, TLR9 1486T/C genotype was favourable where it was negatively associated with complicated malaria (Fishers exact p=0.014). Sequencing data revealed the two populations differed in nucleotide diversity of TLR9 promoter region. No association of TLR2 (Arg753Gln) with either severity or frequency of disease was noted.

Enhanced expression of TLR4 (p=0.05) and TLR2 (p=0.083) but not of TLR9 was associated with complicated malaria. Austro-Asiatics appeared to have accumulated favourable genotypes of TLR9, perhaps because of their longer exposure to malaria.

3.2 Introduction

Innate immunity is gaining importance for understanding the immune responses to infectious organisms. Cells of the immune system armed with germ-line encoded receptors (Pathogen Recognition Receptors) of limited repertoires respond to the molecular motifs on exogenous antigens by secreting cytokines and chemokines that significantly influence adaptive immunity and disease outcome.[213]

Toll like receptors (TLRs) are an important component of the innate immune system and their role in bacterial and viral infections is well documented and they are becoming important in immune responses to protozoan infections.[214-216] Activation of TLRs leads not only to the robust production of proinflammatory mediators but also to the production of unique effectors, which provide pathogen-tailored immune responses. In malaria, *Plasmodium falciparum* (*P.falciparum*)glycosylphosphatidylinositol (GPI) has been shown to induce the expression of proinflammatory cytokines and immune mediators*in vitro* [41]. Furthermore, GPIwas reported to induce signalling via both TLR2 and TLR4 whereas haemozoin (HZ) in combination with plasmodial DNA activated dendritic cells by engaging TLR9.[42] Changes in TLR responses also modulated the production of inflammatory cytokines and thus prevented the development of severe pathology, emphasising the role of these responses in malaria pathogenesis.[43] Increased expression of TLR genes was also seen in *P.falciparum* infected subjects and it was associated with enhanced IFNγ, TNFα and IL10 production.[44]

Genetic variations of TLRs can change the ability of the organism to respond to different stimuli and hence the interest in the possible associations of TLR polymorphisms with altered susceptibility to infections and inflammatory diseases is growing.[214] Studies have linked TLR2 (Arg753Gln) with rheumatic fever in children and TLR4 polymorphisms with increased susceptibility to bacterial infections.[217-219]

TLR9 promoter polymorphisms (T-1237C and T-1486C) have been assumed to influence transcription regulation and they have been associated with asthma, Crohns disease, atopic eczema and lymphoma. [220-224] Many studies have also implicated polymorphisms of TLR2, 4 and 9 to be associated with disease manifestation of malaria.SNPs ofTLR4 (Asp299Gly) and TLR9 (T-1486C) but not TLR9 (T-1237C) were associated with low birth rate, maternal anaemia and with high parasitaemia suggesting that these SNPs play a role in the manifestation of malaria during pregnancy.[223-224] SNPs of TLR4 Asp299Gly and Thr399Ile were seen to increase the risk of severe malaria in Ghanian children. [38, 223]

A number of SNPs associated with inflammatory and immunologic diseases also show large frequency differences among ethnically distinct populations.[214, 225] A study in India, showed that high frequency of FcγR2A exon4 AA genotype protected the Tharus, a population living in the foothills of the Himalayas, from *falciparum* malaria.[226]

Distribution of TLR polymorphisms have been shown to differ among the ethnically different populations in Asia, Africa and Europe and malaria was suggested to be the driving force. [227] In this context, we have examined the association of SNPs in TLR2, 4 and 9 and the expression of these genes in relation to malaria and ethnicity in Assam State of Northeast India which is endemic for malaria.[228] We report the differential association of polymorphisms and expression of TLR9 with malaria in two ethnic groups. Increased expression of TLR 2 and 4 genes was seen in complicated malaria. The Austro-Asiatics (plains) had the favourable genotype conferring protection from complicated malaria.

3.3 Materials and methods

3.3.1. Study sites

The study was conducted at two study sites: Guabari, a village of Baksa district which lies at the foothills of Bhutan, and at Kondoli in Karbi Anglong foothills of Nagaon district of Assam (Figure 3.1). The two study sites may be classified as mesoendemic for malaria. Guabari has poor access to health care and has a mixed population of Bodos, Assamese, Bengalis, Nepalese and a few Adivasis. Kondoli has fairly homogenous population comprised of tea tribes, is an organic garden and has negligible *P. falciparum* malaria in winter. Malaria in Guabari [228] is perennial but highly seasonal with two peaks, the main peak in the summer from May to July and a smaller peak from October to November Slide positivity rate varies from ≤ 15% in the dry winter months, when transmission is low, to 30–55% in the high transmission period spanning mid March to mid July, varying with temperature and rainfall [229]. According to annual slide positivity rate and spleenomagaly index, these areas maybe classified as 'mesoendemic'. However, sporadic malaria outbreaks occur at these sites.

3.3.2 Study participants

Patients were enrolled into the study after obtaining a written informed consent. Active as well as passive case detection was followed which was carried out by local health workers in Guabari while at Kondoli it was done by hospital staff. Based on linguistic group affinities, the study population was stratified into two groups namely the speakers of Tibeto-Burman (TB) that included the Bodo-Kachari and Nepalis, theAustro-Asiatic (AA) that comprised the tea tribes who are Mundari speakers.[226] Sampling was done such that the samples were drawn independently and randomly from each group particularly at Guabari that has ethnically mixed population. Though

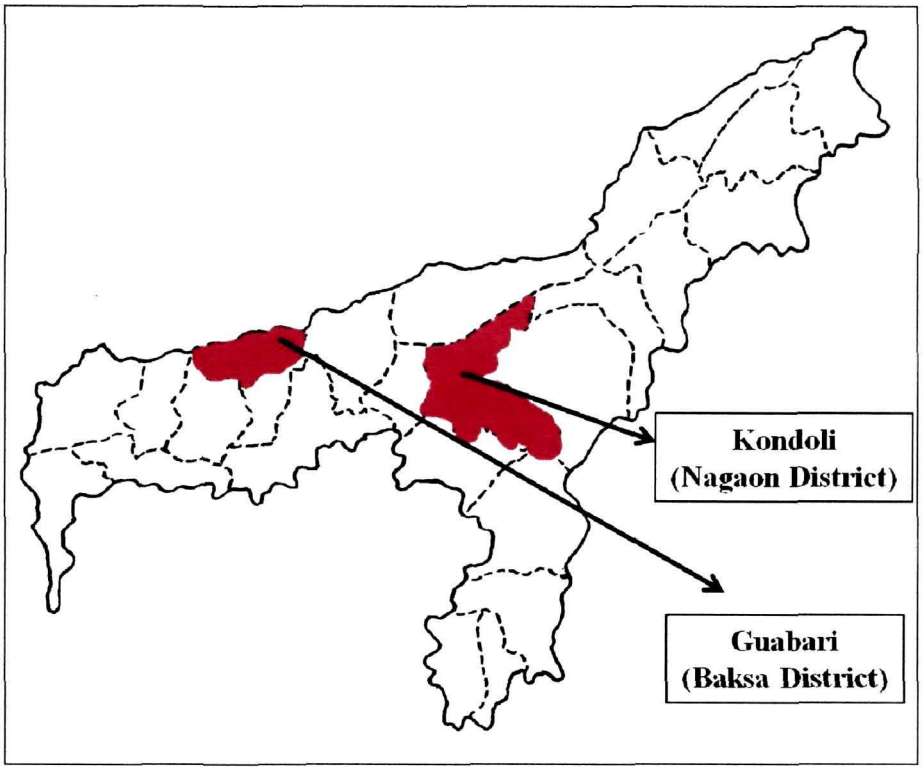


Figure 3.1: Map of Assam (India) showing the two study sites: Guabari and Kondoli

the people belonging to AA and TB groups are found in Assam at present, but their origin is very different. The AA group is believed to be the earliest inhabitants of Indian plains whereas the Tibeto-Burmans are believed to have migrated from South China and Tibet approximately 5000-10,000 years ago.[230-232] Moreover based on mitochondrial DNA and NRY haplotypes the AA was found to be genetically distinct from TB.[232] Khasis are a group of people residing in the state of Meghalaya and they are as AA because their language belongs to the Khasi-Khmic subfamily of the Austro-Asiatics.[233] In the present study, AA group from Assam was denoted as AA (plains) and the Khasis as AA (hills). Khasi participants (n=30) were from hilly

regions (>4900 ft above sea-level) where exposure to malaria is unlikely and so served as controls for AA (plains) in SNP-malaria association studies.

3.3.3 Sample collection

Blood samples were obtained from individuals irrespective of their *P.falciparum* positive (*Pf*+) status at the time of collection. Inclusion criteria were individuals with *Pf*+ or with history of *falciparum* malaria. Exclusion criteria were children with age less than 1 year, pregnant women and individuals suffering from any other diseases. *P. falciparum* positivity was checked using Rapid Diagnostic Kits and confirmed by microscopy of thin and thick blood smears. Individuals were categorized according to their disease symptoms and frequency of malaria incidence which were simultaneously recorded. Complicated malaria was defined following WHO guidelines (2000).[234]Uncomplicated malaria included fever, headache, body ache and other mild symptoms or asymptomatic cases with parasitaemia of ≤5000/µl of blood. On the basis of number of clinical episodes of malaria, the participants were classified as infrequent (≤ 2 episodes) or frequent malaria (> 2 episodes) groups as detailed in our earlier work.[235]The study was approved by the Tezpur University Ethical Committee (Resolution number 3 dated 13/06/06).

3.3.4 Single Nucleotide Polymorphism typing of Toll like receptors 2, 4 and 9

200ul of blood was collected in EDTA-tubes and genomic DNA extraction was carried out using Qiagen blood extraction kit (Qiagen, Germany). The TLR2, 4 and 9 genes were amplified by polymerase chain reaction and then digested using restriction enzymes [218, 236-238] as described below:

3.3.4.1 For TLR2 (Arg753Gln) polymorphism: PCR was performed in a total volume of 25 ml containing 100ngms of DNA, 25 picomoles of both forward and reverse primers, 2mM of PCR buffer, 0.1µl of 50µM of dNTPs each and 1U of the Taq polymerase (Sigma Aldrich, India). A sequence of 340 base pair was amplified by

PCR. The PCR consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of 30seconds at 95 °C, 30seconds at 58°C and 25 seconds at 75°C, and a final extension of 5 minutes at 72°C. The PCR products were digested for 2 hours at 37°C with Aci1 restriction enzyme (New England Biolabs).

3.3.4.2 For TLR4 (Asp299Gly) polymorphism:PCR was performed in a total volume of 25 ml containing 100ngms of DNA, 20 picomoles of both forward and reverse primers, 2.5mM of PCR buffer 2.166μM of dNTPs each and 2U of the Taq polymerase (Sigma Aldrich, India) . A sequence of 263 base pair was amplified by PCR. The PCR consisted of an initial denaturation at 95 °C for 4 min, followed by 30 cycles of 30 s at 95 °C, 30 seconds at 55 °C and 30 seconds at 72 °C, and a final extension of 5 min at 72 °C. The PCR products were digested overnight with Nco1 restriction enzyme (New England Biolabs).

3.3.4.3 TLR9 (T-1237C) polymorphism: PCR was performed in a total volume of 25 ml containing 100ngms of DNA), 0.8μM o f both forward and reverse primers, 2.5mM of PCR buffer 2.166μM of dNTPs each and 2U of the Taq polymerase (Sigma Aldrich, India) . The PCR parameters were as follows: 4 min denaturation at 95°C, 40 cycles of amplification: 30seconds denaturation at 95°C, 20seconds annealing at 61°C, 18seconds elongation at 72°C, and one cycle of elongation for 5 min at 72°C. We incubated 12 μl of the PCR product with 7.5 U of BstNI (Ne w England Biolabs) in a volume of 20ml at 60°C for 3 hours.

3.3.4.4 TLR9 (T-1486C) polymorphism: PCR was performed in a total volume of 25 ml containing 100ngm of DNA), 0.8μM of both forward and reverse primers, 2.5mM of PCR buffer 2.166μM of dNTPs each and 2U of the Taq polymerase (Sigma Aldrich, India) . The PCR parameters were as follows: 4 minutes denaturation at 95°C, 35 cycles of amplification: 30seconds denaturation at 95°C, 20seconds annealing at 60°C, 20 seconds elongation at 72°C, and one cycle of elongation for 5 minutes at 72°C. We incubated 12 μl of the PCR product with 20 U of AflII (New England

Biolabs) and the respective restriction buffer in a total in a volume of 15ml at 37°C for 3 hours.

After restriction digestion, the fragments were then analysed on 3% agarose gels pre-stained with Ethidium Bromide.

3.3.5 Sequence analysis

TLR9 promoter regions were sequenced (Applied Biosystems, Sequencer) and aligned with the sequence available in the GenBank (Accession number: NT_022517.18) using CLUSTAL X and the matrix selected for the purpose is Clustal W 1.6. The alignment file created was used for further analysis using DNASp V 5.0. TLR9 promoter sequences have been submitted to GenBank with the following accession numbers: HM231310, HM231311, HM231312 and HM231313.

3.3.6 Toll like receptors gene expression study

The cohort used for TLR2, 4 and 9 genotyping study was also used for expression study. 46 samples were randomly drawn from each of the three diseased groups (comprising of patients having complicated malaria, uncomplicated malaria and non malaria fever) and from the healthy control group.Total RNA was then extracted from these blood samples and was stored in RNAlater as per the manufacturer's instructions (Ribopure, Ambion). Reverse transcription (RT) reactions were set up by using cDNA Archive kit (Applied Biosystems). Real time PCR was performed in 7300 Real time PCR system (Applied Biosystems). TLR genes expression was calculated in relation to the expression of RnaseP which was used as an endogenous control.[239]

3.3.7 Data Analysis

The data was analysed using Excel Stat Software, 2010 version. Variation in the frequency of the TLR2,4 and 9 genes with respect to disease status, frequency of disease, ethnicity was analysed by Fishers exact test. The allele frequencies were

calculated (estimation of p values by Pearson test) to test if they were in agreement with Hardy-Weinberg equilibrium (HWE). For TLR gene expression data analysis, we used the comparative CT method (ΔΔCT method). Expression of TLR genes was analysed by t-test used for comparing the means in the different groups. Expression levels of TLR genes were compared between the younger age group (≤ 10 years) and older age group (> 10 years) who had malaria at that point of time.

3.4 Results

3.4.1 Overall frequency of Single Nucleotide Polymorphisms of Toll like receptor 2, 4 and 9 genes

A total of 191 samples were studied, where we found median age of the participants was 26.174 (Range: 2-58years). The younger age group (2-10 years) was seen to have a higher mean parasitaemia of 2526.09±1764.92 trophozoites/μl of blood than the older age group (11-58 years) which was 1816.833±700.9 trophozoites/μl of blood. These samples were examined for the frequency of SNPs in TLR2 (Arg753Gln), TLR4 (Asp299Gly), TLR9 (T-1237C) and (T-1486C) (Figure 3.2).

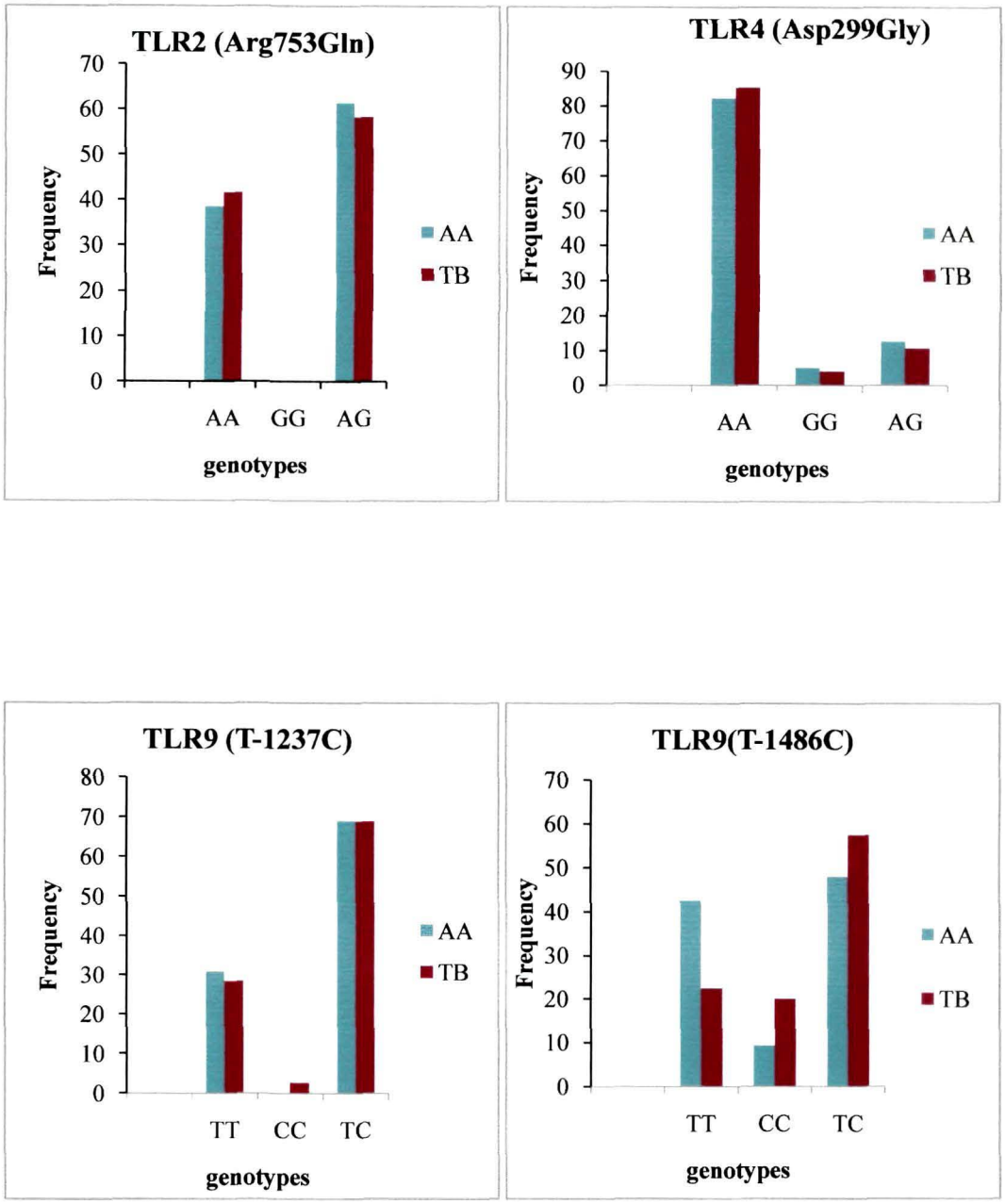


Figure 3.2: Comparison of the genotype frequencies of the TLR2, 4 and 9 loci in the study population

Analysis was performed to check the interactions in the three genotypes of each TLR gene by Fishers exact test. AA-Austro-Asiatic; TB- Tibeto-Burman

The mutant alleles of TLR2 and TLR9were seen predominantly in heterozygosity in the three ethnic groups. In case of TLR4, the wild genotype (A/A) was seen to dominate. The two alleles of TLR4 were not in HWE, neither in the overall population nor in each of the two ethnic groups ($p<0.001$) (Table 3.1). The frequency of the homozygous mutant genotype of the studied TLR genes was low except for TLR9 (T-1486C) where it was seen to be present at higher frequency in the TB (Fishers exact $p=0.014$; Figure 3.2). The genotype frequencies of TLR4 and 9 were compared between AA (hills) and AA (plains) were comparable (Table 3.2), suggesting that malaria may not be a factor for the variation. TLR2 was not compared, since AA (hills) samples did not get amplified.

	Overall population n=191		AA n=112		TB n-80	
SNP	W-allele	M-allele	W-allele	M-allele	W-allele	M-allele
TLR2 Arg753Gln	[a]0.703	[b]0.297	0.69	0.31	[c]0.708	[d]0.292
TLR4 Asp299Gly	[q]0.895	[q]0.105	[m]0.89	[m]0.11	[r]0.9	[r]0.1
TLR9 (T-1237C)	[s]0.64	[s]0.36	[t]0.65	[t]0.35	[n]0.628	[n]0.372
TLR9 (T-1486C)	0.587	0.413	0.66	0.34	0.512	0.488

Table 3.1: Comparison of the allele frequencies of the SNPs of TLR2, 4 and 9 loci in the study population

a.b.c.d.q.m.n.r.t and s allele frequencies were not in Hardy-Weinberg Equilibrium (Pearsons $p<0.05$). Allele frequencies of TLR4 suggest that there is a low prevalence of the mutation in the study population. AA- Austro-Asiatic; TB- Tibeto-Burman; W-wild; M-mutant.

	TLR4 Asp299Gly				
Genotype	n	AA	GG	AG	p-value
AA (plains)	79	65	4	10	0.685
AA (hills)	30	26	0	4	
	TLR9 (T-1237C)				
Genotype	n	TT	CC	TC	p-value
AA (plains)	81	25	0	56	0.123
AA (hills)	30	5	0	25	
	TLR9 (T-1486C)				
Genotype	n	TT	CC	TC	p-value
AA (plains)	75	32	7	36	0.20
AA (hills)	30	7	4	18	

Table 3.2: Comparison of the genotype frequencies of the TLR4 and 9 between AA (plains) and AA (hills)

Analysis was performed to check the interactions in the three genotypes of each TLR gene by Fishers exact test between the Mundari speakers, AA (plains) and the Khasi-

Khmic AA (hills) subfamilies of the Austro-Asiatics. TLR2 was not compared, since AA (hills) samples did not get amplified.

3.4.2 Sequence Analysis

Differences in SNP frequencies in the two ethnic groups were further analysed by sequencing the PCR products of TLR9 promoter region. The sequences were compared with the sequence data present in Gen Bank (GenBank accession number: NT_022517.18). Analysis of the two subject populations with the sequence present in Gen Bank showed that the TB population had a maximum variation in the nucleotide sequence in the range of nucleotide from 162-340 over 178 base pair (bp) length (Figure 3.3a). In contrast, in the AA population, variation was seen in the later part of the sequence over a region of 180 bp, lying in range of nucleotide number 420-600 (Figure 3.3b). Analysis between the two populations showed a distinct pattern of the number of nucleotide variation as shown in Figure 3.3c where the TB population showed maximum variation in the first 200bp region while the AA population was seen to show maximum variations in the 400-600bp region.

3.4.3 Association of Single Nucleotide Polymorphisms of TLR2, 4 and 9 genes with malaria

We analysed the frequency of SNPs in relation to disease severity: complicated malaria and uncomplicated malaria and with respect to frequency of episodes.

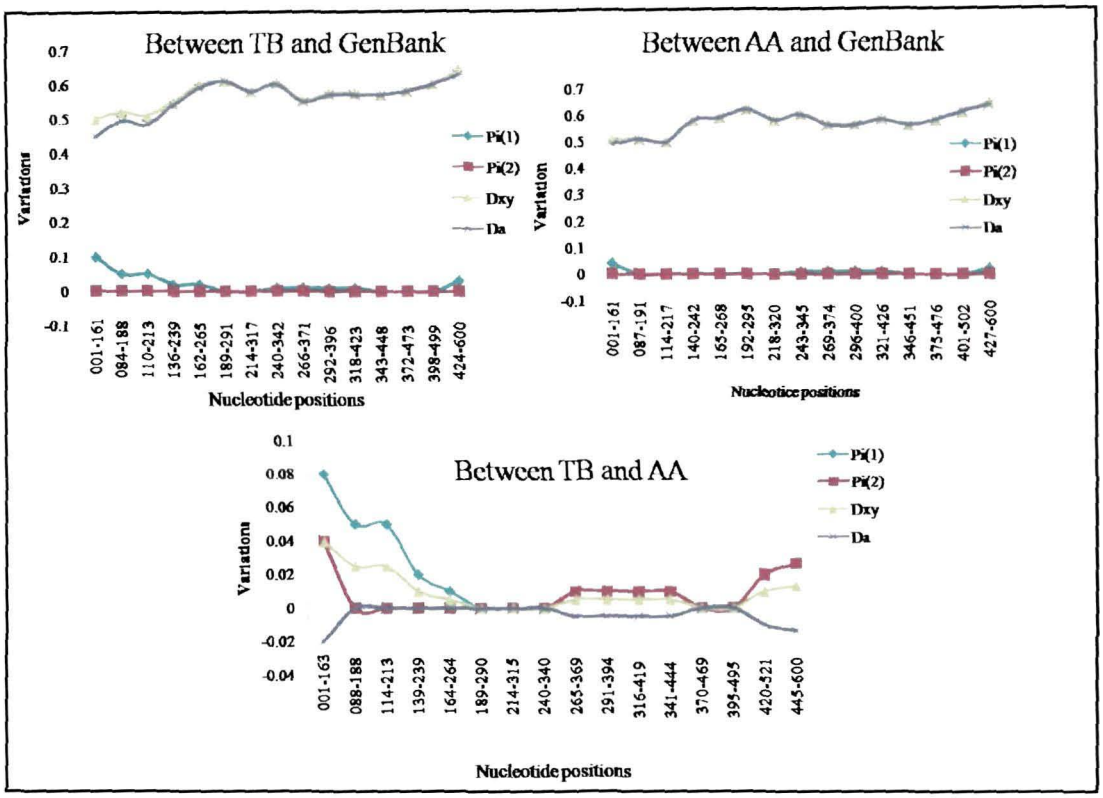


Figure 3.3: Comparison of nucleotide divergence.

The figure compares nucleotide divergence (a) between TB and GenBank (NT_022517.18) (b) between AA and GenBank (NT_022517.18) and (c) between AA and TB. Nucleotide diversity (Pi), the average number of nucleotide differences per site between two sequences, Average Number of nucleotide substitution per site between populations (Dxy),Number of net nucleotide substitutions per site between populations (Da). Alignment of the sequences was done using CLUSTAL X and further analysis using DNASp V 5.0. AA-Austro-Asiatic; TB-Tibeto-Burman.

3.4.3.1 Complicated and uncomplicated malaria

Frequency of TLR4 (Asp299Gly) was comparable irrespective of disease status and ethnicity (Table 3.3). Overall frequency of TLR9 1237T/T genotype was higher in the uncomplicated group (Fishers exact p=0.009), whereas T/C genotype was seen at higher frequency in the complicated group. Stratifying data on the basis of ethnicity, T/T genotype was seen at higher frequency in AA (plains) in the uncomplicated group suggesting an association of TLR9 (T-1237C) mutation with complicated malaria (Relative risk =0.595 95% CI: 0.479 to 0.836; Fishers exact p=0.001; Table 3.3). In TB too, the frequency of T/T genotype was higher in uncomplicated malaria group but did not reach statistical significance (p>0.05). Interestingly, this mutation was not in HWE in both AA (plains) and TB (p<0.001) suggesting this could be under selection in these population (Table 3.1). Intriguingly for TLR9 1486T/C genotype was found to be protective in TB where it was negatively associated with complicated malaria (Fishers exact p=0.01; Table 3.3).

3.4.3.2 Frequency of the disease (number of episodes)

The frequencies of SNPs in TLR2, 4 and 9 genes were unrelated to frequency of the disease. When the data was stratified according to ethnicity, we found that malaria occurred less frequently in absence of (T-1237C) mutation of TLR9 in AA (plains) (p=0.035; Table 3.4), indicating the mutation was not favourable to malaria outcome.

			AA				TB		
SNP	Genotype	n	Com M	Un Com	p-values	n	Com M	Un Com	p-values
TLR2	AA		3	7			8	17	
Arg753Gln	GG	26	0	0	1	60	0	0	0.59
	AG		5	11			14	21	
TLR4	AA		24	40			26	38	
Asp299Gly	GG	78	2	2	0.57	75	1	2	0.87
	AG		5	5			4	4	
TLR9	TT		4	21			7	14	
(T-1237C)	CC	81	0	0	[a]**0.001**	74	1	1	0.8
	TC		30	26			21	30	
TLR9	TT		14	18			9	9	
(T-1486C)	CC	75	1	6	0.28	77	10	6	[b]**0.01**
	TC		13	23			11	32	

Table 3.3: Comparison of the genotype frequencies of the SNPs of TLR2, 4 and 9 in the two ethnicities in complicated and uncomplicated malaria groups.

Analysis was performed to check the interactions in the three genotypes of each TLR gene. [a] *and* [b] *p-values are significant (p<0.05). AA- Austro-Asiatic; TB- Tibeto-Burman; n-number of samples; Com M-complicated malaria; Un Com-uncomplicated malaria.*

		AA				TB			
SNP	Genotype	n	F	IF	p-values	n	F	IF	p-values
TLR2	AA		7	1			7	13	
Arg753Gln	GG	20	0	0	0.55	46	0	0	0.55
	AG		10	2			12	14	
TLR4	AA		30	34			28	36	
Asp299Gly	GG	78	2	2	0.3	75	1	2	0.5
	AG		7	3			5	3	
TLR9	TT		11	14			10	11	
(T-1237C)	CC	81	0	0	**[w]0.035**	74	1	1	0.3
	TC		40	16			16	35	
TLR9	TT		19	13			8	10	
(T-1486C)	CC	75	3	4	0.6	77	8	8	0.9
	TC		18	18			20	23	

Table 3.4: Comparison of the genotype frequencies of the SNPs of TLR2, 4 and 9 in the two ethnicities (AA and TB) in frequent and infrequent malaria episodes

Comparison of the frequency of the given SNPs between frequent and infrequent malaria episodes in the two ethnic groups. Analysis was done using χ^2 test to check whether the frequencies of SNPs in TLR2, 4 and 9 genes were related to frequency ofthe disease. Significant difference was seen only for TLR9 (T-1237C) ([w]) between frequent and infrequent malaria episodes in AA and TB. Malaria occurred less frequently in absence of (T-1237C) mutation of TLR9 in AA (plains) indicating the mutation was not favourable to malaria outcome. F-Frequent; IF-Infrequent; AA-Austro-Asiatic; TB- Tibeto-Burman; n-number of samples.

3.4.4 SNP-grouping of TLR9

Based on the combination of the presence or absence of the wild and mutant alleles for the two SNPs of TLR9 (T-1237C and T-1486C) a total of eight groups could be identified (Table 3.5). Group 1 with co-occurrence of the two mutations in heterozygosity was seen to be the dominant group and occurred in 67 individuals. Interestingly, 77.77% of the individuals of this group belonged to the uncomplicated group (Kruskal-Wallis test p=0.05) suggesting its association with protection from having complicated malaria. Group 4 which contained T/T genotype of both mutations was more frequent than group 7 containing C/C genotype for both the polymorphisms. In group 4, nine individuals were of AA (plains) and 76.9% of them suffered from complicated malaria (Table 3.5).

3.4.5 TLR2, 4 and 9 genes expression

46 samples were analysed for the mRNA levels of TLR2, 4 and 9 genes and data analysed in relation to disease status. TLR2, 4 and 9 mRNA levels showed no difference between malaria and no malaria groups (p>0.05). When the data were analysed in the context of complicated and uncomplicated malaria (Figure 3.4), TLR4 gene expression was higher in the complicated group (p=0.05) whereas in uncomplicated group it was lower in comparison to no malaria group. Increased gene expression of TLR2 was also seen in complicated malaria though the association was weak (p=0.083).

Group	T1237C	T1237C	T1486C	T1486C	Genotype	Total (Pf+ and control)	In relation to malaria severity	
	W	M	W	M			CM	UCM
1	■	■	■	■	TCTC	67	10	36
2	■	■	■	□	TCTT	39	14	14
3	■	■	□	■	TCCC	24	8	9
4	■	□	■	□	TTTT	17	1	13
5	■	□	□	■	TTCC	4	1	2
6	■	□	■	■	TTTC	36	2	24
7	□	■	□	■	CCCC	1	1	-
8	□	■	■	■	CCTC	1	-	1

Table 3.5: SNP-grouping of TLR9 (T-1237C and T-1486C) in the study population.

The participants (n=189) positive for both the TLR9 (T-1237C and T-1486C) mutations were SNP-grouped. Groups 4, 5, 7 and 8 was absent in the AA (hills). Negligible prevalence of C/C genotype for both the mutations may be noted. The proportions of complicated malaria were higher if TLR9 (T-1237C) mutation was present but if it was present along with the (T-1486C) a larger proportion of uncomplicated malaria was observed. CM-complicated malaria; UCM-uncomplicated malaria; Pf+ - P.falciparum positive.Black and white fields denote the presence and absence of wild or mutant alleles respectively.

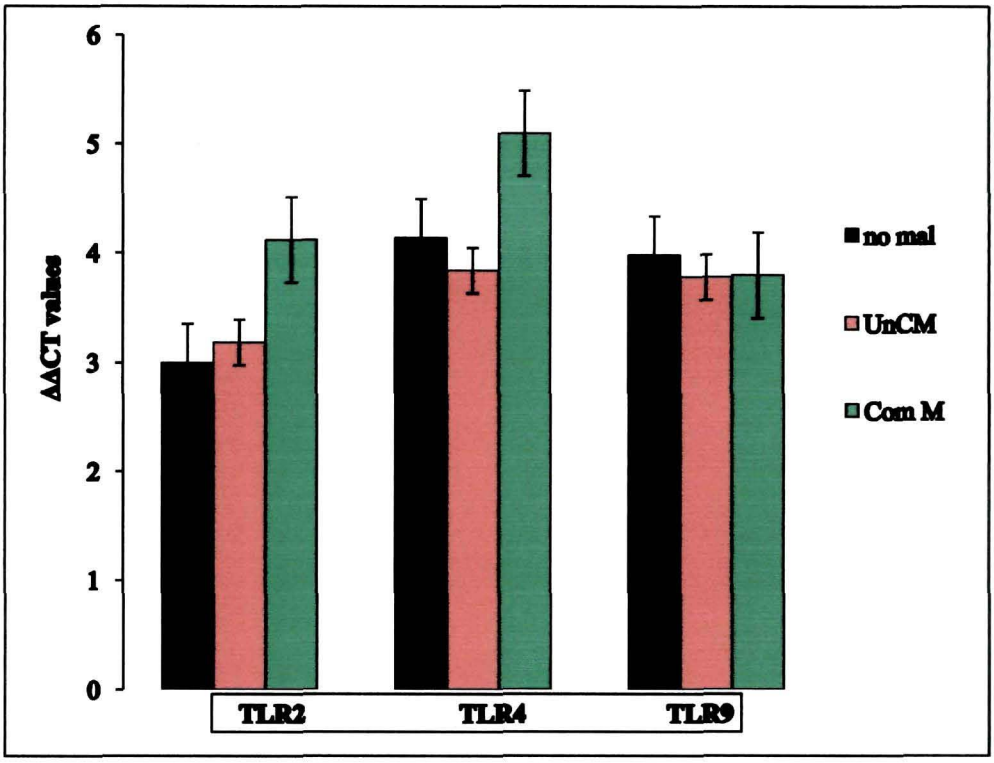


Figure 3.4: Expressions of TLR2, 4 and 9 genes in relation to malaria severity

Mean expression of TLR2, 4 and 9 genes in complicated and uncomplicated malaria was compared by t-test. TLR4 expression was higher in the complicated group (p=0.05). No mal-no malaria; UnCM-uncomplicated malaria; Com M-complicated malaria.

As age is an important factor in immunity to malaria, the data was analysed in context of age. TLR9 expression was higher in the younger age group with clinical malaria (p=0.002; Figure 3.5). Expression of TLR2 and 4 genes were comparable between younger and older age groups with malaria (Figure 3.5).

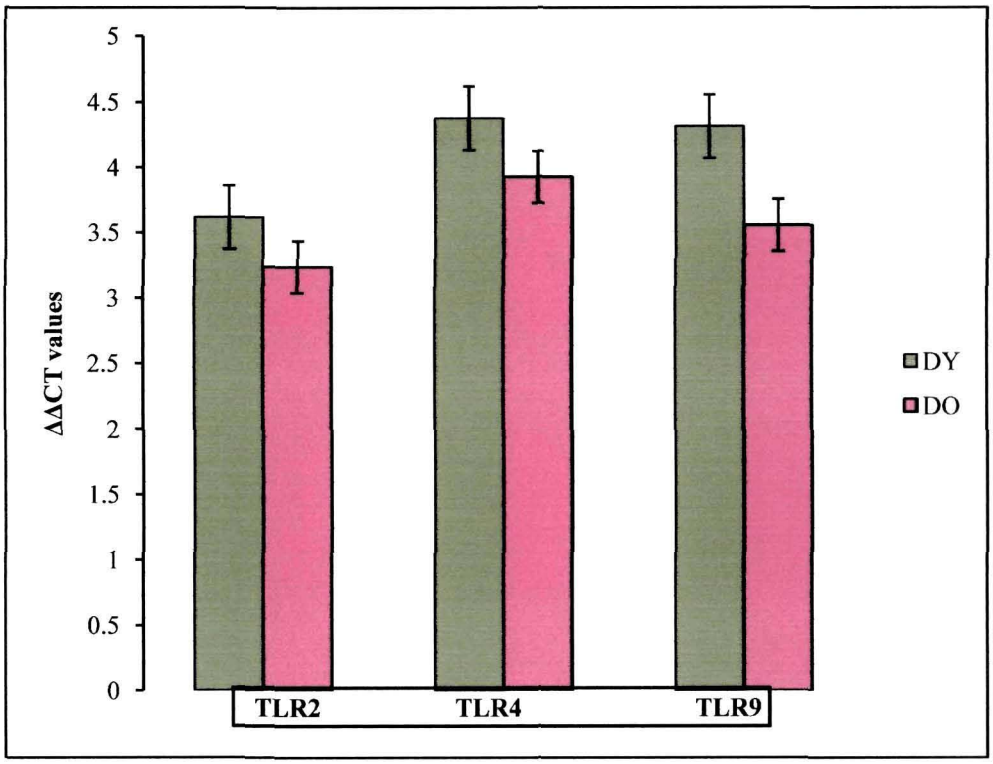


Figure 3.5: Expressions of TLR2, 4 and 9 genes in relation to age

Expression of TLR2, 4 and 9 genes in between the younger age group and the older age group was compared by t-test. It was seen that the expression of TLR2, 4 and 9 genes was higher in the younger age group in comparison to the older group, but did not reach statistical significance except for TLR9 (p=0.002). DY= Diseased young and DO= Diseased old.

3.5 Discussion

Vertebrate TLRs play a crucial role in immune protection and have undergone selection in response to immune challenge as noted in studies that have associated TLRs and their SNPs with diseases.[215] Selection favouring homozygosity or heterozygosity is one of the mechanisms that maintain polymorphism and genetic variability. Our data indicated TLR9 1486T/C heterozygosity to be protective, while it was negatively associated with complicated malaria, whereas TLR9 (T-1237C) was seen to predispose to complicated malaria in heterozygosity. Similar findings have been reported earlier with respect to association of TLR9 (T-1237C) mutation with severe malaria.[240] However, our observations in context of (T-1486C) polymorphism are in contrast with earlier reports where it was associated with susceptibility to severe malaria in children and also with the adverse foetal outcome in women with placental malaria.[223, 38] Although TLR9 (T-1237C) mutation has been correlated with complicated malaria, but in the presence of (T-1486C) mutation which was associated with protection from complicated malaria, a larger proportion of uncomplicated malaria was seen in our study, suggesting that (T-1486C) was the determining mutation. Advantages conferred by heterozygosity are well documented as in case of sickle cell haemoglobinopathy which affords protection against mortality from *falciparum* malaria in heterozygous individuals, balancing the severe consequences of the disease in homozygous individuals.[241] Similarly, it was also found that those individuals with heterozygous class I and class II HLA alleles progressed from HIV infection to an AIDS-defining illness more slowly and had the ability to clear hepatitis B infection respectively.[242, 243]

We did not observe association of SNPs of TLR4 either with malaria severity or frequency of clinical episodes. In contrast, Mockenhaupt et al.(2006) reported higher frequency of TLR4 polymorphisms Asp299Gly and Thr399Ile in malaria cases that increased the risk of severe malaria. [38]Although the proportion of A/A genotype of

TLR4 was seen to be higher in both the ethnicities in the present study, it was however not associated with either protection or susceptibility to malaria. TLR4 alleles were not in HWE in the overall population including each of the two ethnic groups. Natural selection acting on human TLR4 ascribed to the presence of excess rare non-synonymous polymorphisms has been reported by Nakajima et al. (2008) postulating that the selection was linked to pathogen pressure. [244]

Expression of TLR4 genes was enhanced in patients having complicated malaria seen in the present study which could possibly be an outcome of binding to GPI anchors of parasite surface proteins leading to increased levels of proinflammatory cytokines and also result in up regulation of cell adhesion molecules (ICAM-1 and VCAM-1) leading to cytoadherence.[41, 245] An earlier study too reported increased expression of TLR4 in adults with severe and mild malaria but a decreased TLR9 expression in people with no history of malaria.[246] Also, Coban et al. (2006) found that innate immune responses via TLR2 and 9 and, adaptor molecule, MyD88 dependent pathways are critically involved in the pathogenesis of cerebral malaria.[247] On the contrary, Lepenies et al. (2007) demonstrated that induction of cerebral malaria was independent of TLR2, TLR4 and TLR9 gene expression. [248] TLR9 gene expression was seen to be age related with higher expression in younger malaria patients. Interestingly, proportion of uncomplicated malaria was higher in this group and may be seen as an outcome of increased TLR9 gene expression which induced early pro-inflammatory response resulting in containment of the disease. In a parallel study we have analysed the gene expression of a panel of cytokines and we found that early pro-inflammatory cytokines were up regulated in diseased cases in comparison to healthy controls (Unpublished data). Increased TLR9 expression could be induced by HZ, a malarial pigment formed after digestion of the haemoglobin by malarial parasites.[249]It is well documented that the younger age group generally have higher parasitaemia and suffer more severe consequences of malaria. [250, 251]Increased infection would

result in increased concentration of HZ, in the reticulo-endothelial system, which has been demonstrated to activate the immune system by binding to TLR9 and activating the downstream signaling pathway via MyD88, thereby resulting in increased TLR9 expression as one of the outcomes[42]. Parroche et al. (2007) also reported that HZ activates the immune system similar to Coban et al.(2006), though the mechanism of activation is different.[249, 42] Notably, higher parasitaemia was seen in the younger age group of our study population. However, no difference in mRNA levels was seen between control and disease which was surprising as we had observed association between the SNPs in promoter region of TLR9 with disease. This was however, not a unique finding, as earlier Fuse et al. (2010) had also found correlation of TLR9 SNPs with Japanese ulcerative colitis but no correlation between TLR9 mRNA expression and disease. [252]Modulation of TLR9 gene expression through cis-regulatory variants offers an explanation for the lack of correlation between SNPs in promoter region and levels of mRNA expression.[253]

Genetic variations in TLRs have been linked to susceptibility to infectious diseases and these variations could be the result of selective pressure on the TLR genes in those populations living in malaria endemic areas. Ethnicity was seen to be an important factor in association of frequency of SNPs of TLR9 with malaria pathogenesis in the present study. The promoter region of TLR9 was seen to vary between the Austro-Asiatics and Tibeto-Burmans as confirmed by sequencing. This was not surprising as AA (plains) of our study group belonged to tea tribes of Mundari language affinity and are genetically distinct from Tibeto-Burmans based on mitochondrial DNA and NRY haplotypes.[230, 231] Our data suggested that AA (plains) had the favourable genotype that protected them from complicated malaria as: (1) TLR9 1237T/T was seen to confer protection from complicated malaria and from frequent episodes of malaria (2) negligible TLR9 1486C/C genotype in AA which was implicated in complicated malaria in TB. We propose that their favourable genotype could be a reflection of their

longer exposure to malaria as compared to the TB of our study group, in which the AA are believed to be the earliest inhabitants of Indian plains.[230] We also compared the genotype frequencies of TLR9 between the two different AA groups and we found that they did not differ, emphasising the role of ethnicity. Thus, it may be postulated that TLR9 gene may have been under selective pressure exerted by malaria. Our observation that TLR9 (T-1237C) alleles were not in HWE in Tibeto-Burmans perhaps reflects an ongoing selection in this group, who are believed to have migrated from South China and Tibet approximately 5000-10,000 years ago.[231-232]

In conclusion, TLR9 (T-1237C) mutation was seen to be implicated in malaria pathogenesis. These two ethnic groups, namely Austro-Asiatics having genetic affinity with the earliest inhabitants of Indian plains and Tibeto-Burmans whose origin leans more towards the Southeast Asians, have accumulated different SNPs in the TLR9 promoter region as indicated from our sequencing results. Our study demonstrated that AA (plains) had the favourable genotype with respect to TLR9 polymorphisms which protected them from malaria pathogenesis and this may be related to their longer exposure to malaria. No association of TLR2 (Arg753Gln) with either severity or frequency of disease was noted. While TLR9 over expression was favourable, that of TLR4 was a risk factor for complicated malaria. These findings thus open the possibilities of application of TLR9 SNPs as markers of population at risk of severe malaria.

CHAPTER 4: Proinflammatory cytokines: IL-1β, IL-8, IL-18 and TNF-α with falciparum malaria in Assam, India

4. *Proinflammatory cytokines: IL-1β, IL-8, IL-18 and TNF- α with falciparum malaria in Assam, India*

4.1 Abstract

Successful immune response to malaria requires a well-timed and proportional release of proinflammatory cytokines to contain infection. A strong and early response mediates protection by inhibiting the growth and replication of the malarial parasites and thereby limiting infection, whereas a delayed and prolonged inflammatory response results in pathogenesis from disease. In this context, we have examined proinflammatory cytokines gene expression (IL-1β, IL-8, IL-18 and TNF-α) by RT-PCR technique in individuals with different symptomology of malaria.

The mRNA levels of IL-18 (2.14 fold increase; p=0.05) and TNF-α (a 1.08 fold increase) were higher in complicated in comparison to uncomplicated malaria. By logistic regression, it was seen that the combination of cytokines IL-1β*IL18*TNF-α were negatively associated with complicated malaria (p<0.0001). The combination of IL-1β*IL18 was positively associated with complicated malaria for Tibeto-Burmans (p=0.062). When cytokine gene expression between symptomatic and asymptomatic malaria were compared, mRNA levels of IL-1β and IL-8 were seen to be lowered in symptomatic malaria. Comparison of cytokine levels between diseased young and diseased old, IL-1β (p=0.047), IL-8, IL-18 and TNF-α were seen to be lower in diseased young in comparison to diseased old. Surprisingly, levels of all cytokines were higher in younger participants of control group IL-1β (p=0.019); IL-18 (p<0.001) and TNF-α (p=0.019).

In conclusion, elevated level of IL-18 was implicated in complicated malaria and therefore, IL-18 may be used as a marker for complicated malaria. Also, increased levels of IL-18 along with TNF-α was seen to discriminate between healthy and non malaria fever controls and also between symptomatic and asymptomatic malaria. Cytokine profiles were also seen to change with age and with malaria disease.

4.2. Introduction

Proinflammatory cytokines are regulators of the immune responses [254] and they provide the first line of defense against invading pathogen [139]. Their actions are like a double-edged sword, on one hand, a strong and early response mediates protection by limiting infection by inhibiting the growth and replication of the malarial parasites [255]. On the other hand, a delayed and prolonged inflammatory response results in pathogenesis from disease. The outcome of infection depends on a delicate balance between appropriate and inappropriate induction of these mediators [45].

Age is considered to be one of the most important factors that correlate with protective immunity in malaria endemic areas where young children are most susceptible to malaria infections and disease onset [60]. Severe *falciparum* malaria in children living in holoendemic endemic regions is characterized by high-density parasitaemia and severe malarial anaemia but less frequently by cerebral malaria [256].Although the exact mechanisms underlying the pathogenesis of severe malaria are not well known, available evidence implies an exaggerated and prolonged proinflammatory response[257].

IL-18 has been recognized as an important regulator of innate and acquired immune responses [258] that induces IFN-γ production from Th1 cells and NK cells particularly in the presence of IL-12 and plays a key role in inducing severe malaria [56]. Other studies have shown IL-18 to play a protective role in host defense by enhancing IFN-γ production during blood-stage infection by murine malaria [259]. Another important proinflammatory cytokine, TNF-α, is produced and released by immune host cells following exposure to various malarial antigens [260, 261]. Increased TNF-α level stimulated phagocytosis and thereby enhanced clearance of parasitized erythrocytes [262 263]. In children, TNF-α plasma levels were higher in cases of fatal malaria compared with non fatal malaria and in cerebral malaria compared with non complicated malaria [57,58]. Other cytokines such as IL-1, IL-6, IL-8, IL-10 and IL-12

have been implicated in the pathogenesis of severe malaria cases compared to uncomplicated and matched healthy controls [194-198].

Panels of cytokines have been demonstrated to be associated with different clinical manifestations of the disease. In a study in endemic region of central India, levels of IL-12, IL-5, and IL-6 discriminated severe forms of malaria from mild malaria while levels of IL-1β, IL-12, and IFN- γ was relevant for the discrimination of cerebral malaria [264]. Cytokine profiles have also been observed to change with age [265]. A study by Perkin et al. (2011) revealed that children with severe malaria anaemia had significantly lower levels of IL-1β than parasitized children without severe malaria anaemia [266]. A study in Gabonese children revealed low IL-12 levels with severe malaria [267], on the contrary, a study for central India found no correlation between age with malaria severity[264].

The immune status of the individuals, parasite load, virulence of the parasite strains, host genetic factors were seen to actively modulate the inflammatory response [268. 269. 270]. The cause of such differential responses in the degree of severity, as well as the underlying physiopathologic process by which severe *P.falciparum* malaria progresses to cerebral complications, are not well understood. In this context, we have examined the association of expression of the proinflammatory cytokines (IL-1β, IL-8, IL-18 and TNF-α) in relation to different status of *falciparum* malaria in Assam State of Northeast India which is endemic for malaria.

4.3Material and Methods

4.3.1 Study Sites

The study was conducted at two study sites: Guabari, a village of Baksa district which lies at the foothills of Bhutan, and at Kondoli in Karbi Anglong foothills of Nagaon

district of Assam. The two study sites may be classified as mesoendemic for malaria. The characteristics of the two study sites have been described in chapter 3.

4.3.2 Study participants Patients were enrolled into the study after obtaining a written informed consent. Active as well as passive case detection was followed which was carried out by local health workers in Guabari while at Kondoli it was done by hospital staff. Based on linguistic group affinities, the study population was stratified into two groups namely the speakers of Tibeto-Burman (TB) that included the Bodo-Kachari and Nepalis and the Austro-Asiatic (AA) that comprised the tea tribes who are Mundari speakers and others consist mostly of Indo European (IE) that included the Bengalis and non tribal Assamese [232, 233]. Sampling was done such that the samples were drawn independently and randomly from each group particularly at Guabari that has ethnically mixed population as described in our earlier study.

4.3.3 Study design

A total of 58 *Plasmodium falciparum* positive individuals and 30 *Plasmodium falciparum*negative individuals were included in the study. *Pf* negative individuals were further classified into groups of individuals having non malaria specific fever and healthy controls. Individuals were included in the study irrespective of age, sex and their ethnic backgrounds. Blood samples were obtained from individuals irrespective of their *P.falciparum* positive (*Pf*+) status at the time of collection. Inclusion criteria were individuals with *Pf*+ or with history of *falciparum* malaria. Exclusion criteria were children with age less than 1 year, pregnant women and individuals suffering from any other diseases. *P. falciparum* positivity was checked using Rapid Diagnostic Kits and confirmed by microscopy of thin and thick blood smears. Individuals were categorized according to their disease symptoms and frequency of malaria incidence which were simultaneously recorded. Complicated

malaria was defined following WHO guidelines (2000) [234].Uncomplicated malaria included fever, headache, body ache and other mild symptoms or asymptomatic cases with parasitaemia of ≤5000/μl of blood. The study was approved by the Tezpur University Ethical Committee (Resolution number 3 dated 13/06/06).

4.3.4 Blood collection and RNA isolation

1 ml of intravenous blood was collected in tubes containing EDTA, of which 500 μl was mixed thoroughly with 1.3 ml of RNA later solution (Ambion) and stored at -20°C for further use. Total RNA was extracted from whole blood using Ribopure blood Kit as per manufacturer protocol (Ambion) and the eluted RNA was converted to cDNA and stored at –20°C.

4.3.5. Quantitative real time RT-PCR

Total RNA (1μg) was reverse-transcribed into cDNA using high capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random hexamers. The reactions were carried out at 25°C for 10 minutes, 37°C for 120 minutes followed by 85°C for 5 seconds. Cytokine gene expression was analyzed by quantitative real time PCR on an Applied Biosystems 7900 Real Time PCR System (Applied Biosystems, Foster City, CA). cDNA was amplified in duplicate for the following cytokines IL-1β, IL-8, IL-18 and TNF-α using Assay on Demand (AOD), Applied Biosystems and Taqman Master mix, Applied Biosystems. The conditions for the PCR were as follows: 1 cycle at 50°C for 2 minutes followed by 40 cycles at 95°C for 10 minutes, melting at 95°C for 15seconds and final annealing at 60°C for 1 minute. The endogenous control gene 18s (Applied Biosystems) was used as a reference gene to normalize the expression levels of target genes by correcting differences in the amount of cDNA loaded between samples. Relative amounts of the target genes were calculated by using the comparative CT method.

4.3.6 Statistical Analysis

The Statistical analysis of the data was done using Spotfire software 2010. Cytokine transcriptional expression was measured in duplicate and presented as mean ± standard error of the mean. Comparison of the cytokines genes expression between groups was performed using student's t-test. Correlation of cytokines expression was checked using Pearson'scorrelation coefficient test. Interaction of age and cytokines genes expression was determined by ANOVA. Relationships between cytokine genes expression levels and variables like age, sex and ethnicity was predicted using logistic regression models.

4.4 Results

A total of 88 individuals were included in the study and examined for cytokines gene expression and Table 4.1 showed the characteristics of the study group. 89% of them had *P.falciparum* malaria related fever and headache as the most common symptoms among others and 67% of the control participants had non malaria specific fever (>98.6°F). 10% of the *P.falciparum* cases were asymptomatic for the disease whose *P. falciparum* positivity was confirmed by microscopy. The cytokine genes expression was also compared among the three ethnic groups: AA, TB and IE. The expression of the cytokines- IL-1β, IL-8, IL-18 and TNF-α could be detected and were valid in the samples studied as can be seen in the heat map (Figure 4.1).Aggregation method for imputation was done for missing values. Missing values have been filled out from averaged Ct (Cycle threshold) values (Figure 4.2).

Age (average)	21.5 years (range:2-70years)
Sex ratio	0.8 (M/F)
Control group	30
Malaria diseased group	58
Complicated cases	12
Uncomplicated cases	46
Febrile cases and headaches	89%
Non malaria fever (control group)	67%
Ethnicity (%)	
TT	42.3
TB	46.8
Others	10.9

Table 4.1: Patient characteristics

Tibeto-Burman (TB); Austro-Asiatic (AA); Others consist mostly of Indo European (IE) that included the Bengalis and non tribal Assamese.

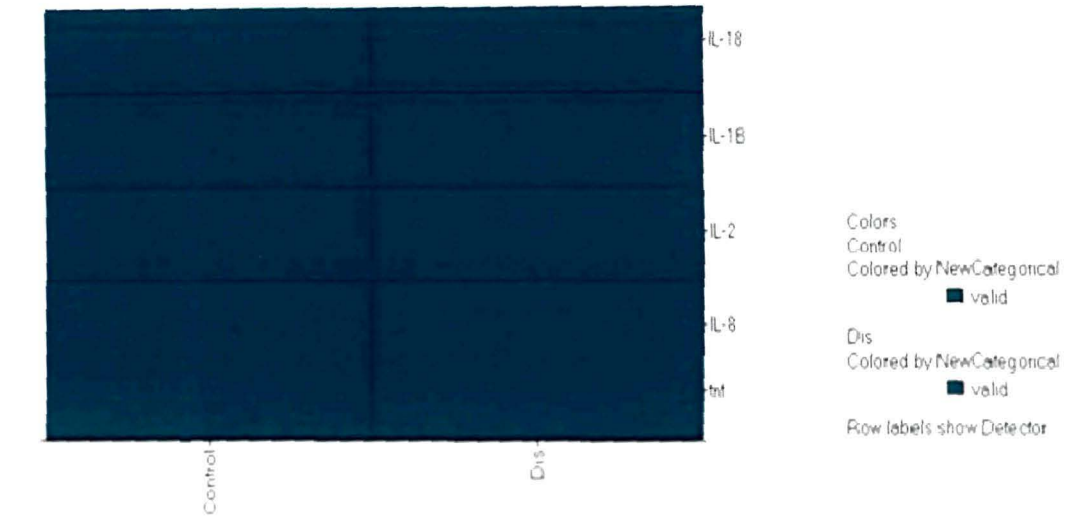


Figure 4.1: This heatmap shows detectors (rows) that were flagged as detected (blue) across different experimental factor values (columns).

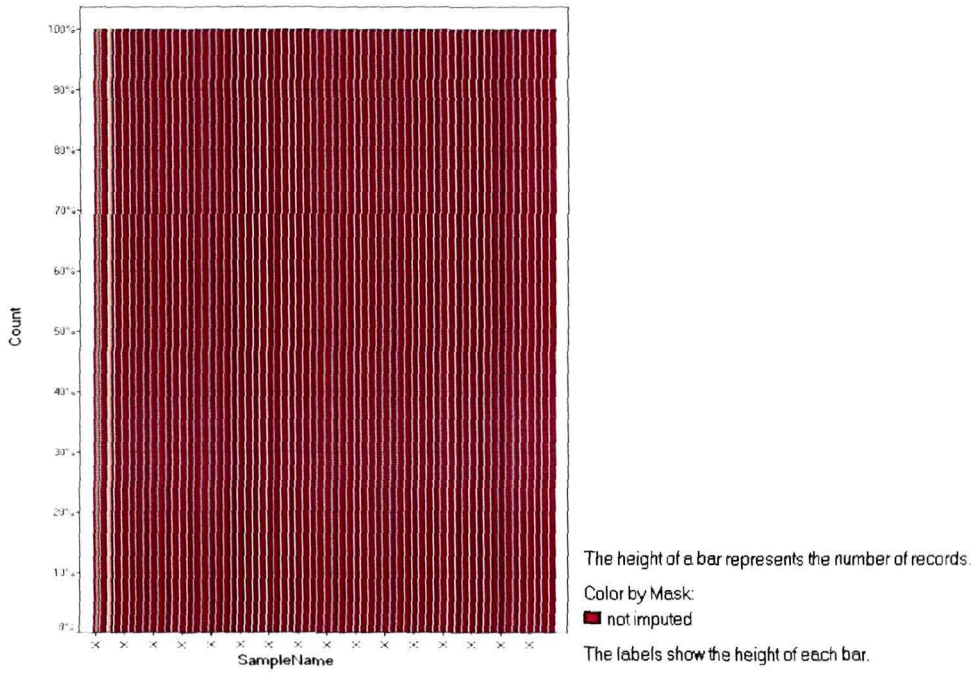


Figure 4.2: Imputation graph

Imputation is the process used to determine and assign replacement values for missing, invalid or inconsistent data that have failed edits. This is done by assigning values from averaged Ct (Cycle threshold) values when they are missing on the record being edited to ensure that estimates are of high quality and that a plausible, internally consistent record is created.

4.4.1 Disease and control

The proinflammatory cytokines (IL-1β, IL-8, IL-18 and TNF-α) were downregulated in diseased group in comparison to control group with IL-18 ($p<0.0001$) and TNF-α ($p=0.012$) being significantly lowered in the diseased group (Figure 4.3). When the control samples were stratified into healthy controls and non malaria fever controls, an increased expression of IL-18 and TNF-α in the non malaria fever control samples was noted. Comparison of mRNA levels between *Pf*+ and non malaria fever control samples revealed a highly significant increase in IL-18 ($p<0.001$) and TNF-α ($p=0.004$) in non malaria fever controls, whereas only a small increase in IL-18 ($p=0.068$) in *Pf*+ in comparison to healthy controls.

4.4.2 Complicated and Uncomplicated malaria

Levels of IL-18 ($p=0.05$) and TNF-α were higher in complicated malaria whereas IL-1β and IL-8 were lowered in comparison to uncomplicated malaria. IL-18 was seen to have 2.14 fold increase and TNF-α 1.08 fold increase in complicated malaria (Figure 4.4). IL-18 levels were also seen to be higher in complicated malaria when compared to healthy controls but were lowered when compared to non malaria fever. IL-1β levels in both the control groups, healthy ($p=0.022$) and non malaria fever ($p=0.020$), were higher in comparison to complicated malaria. By logistic regression, it was seen that the combination of cytokines IL-1β*IL18*TNF-α were negatively associated with complicated malaria ($p<0.0001$).

No association of cytokine gene expression was observed with any of the ethnic groups. Interestingly, in Tibeto-Burmans the combination of IL-1β*IL18 was positively associated with complicated malaria (p=0.062).

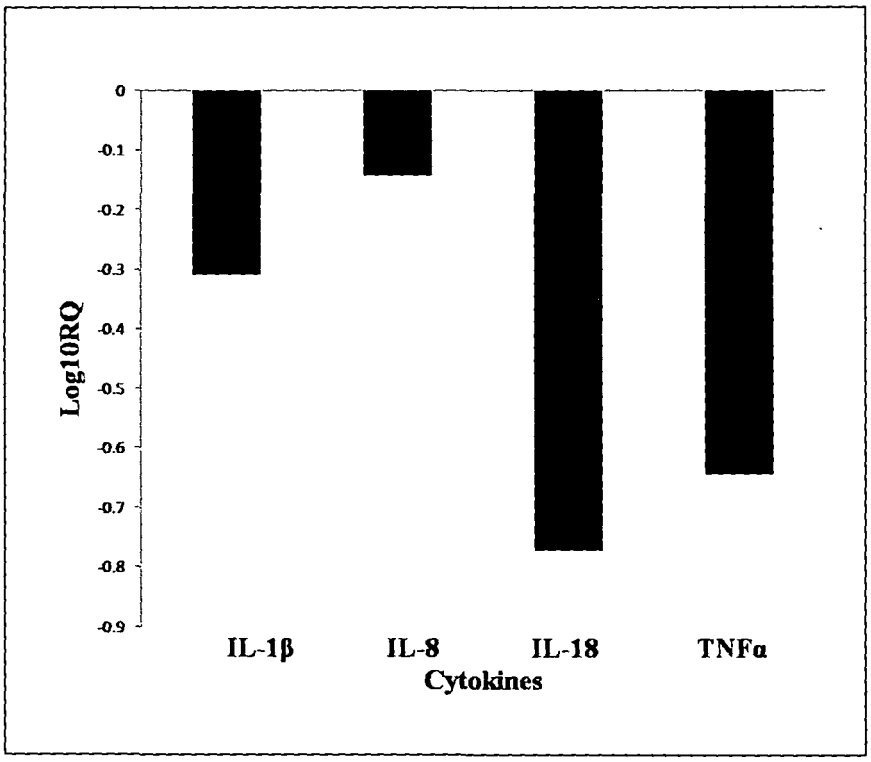


Figure 4.3: Relative quantification (RQ) of cytokines expression of diseased group compared to control.

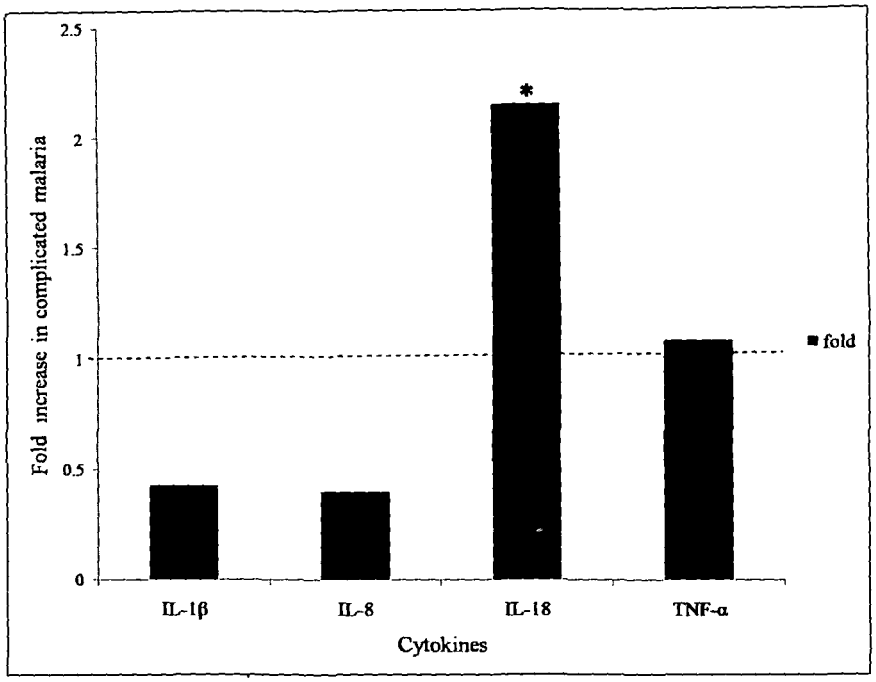


Figure 4.4: Fold increase in the various proinflammatory cytokines in complicated malaria as compared to uncomplicated malaria

**Upregulation of IL-18 was observed in complicated malaria (p=0.05).*

4.4.3 Symptomatic and asymptomatic malaria

When cytokine gene expression between symptomatic and asymptomatic malaria were compared, a similar trend as complicated malaria was observed where we see that levels of IL-18 and TNF-α were higher and levels of IL-1β and IL-8 were lowered in symptomatic malaria (Figure 4.5) though it was not statistically significant.

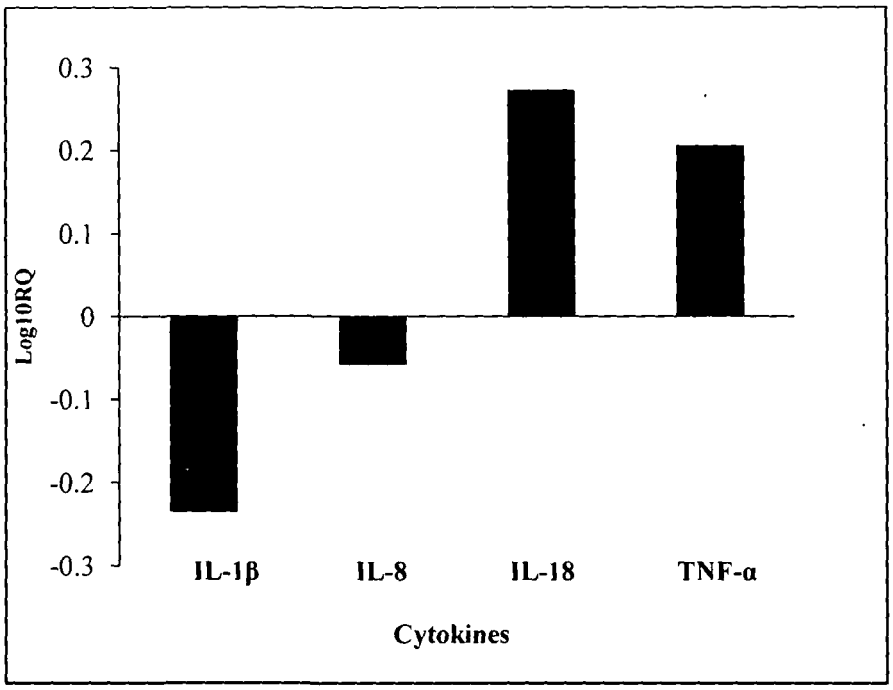


Figure 4.5: Relative quantification (RQ) of cytokines expression of symptomatic malaria compared to asymptomatic malaria.

Expression levels of cytokines were compared between the younger age group (≤ 10 years) and older age group (> 10 years). Further, data was stratified into control young control old, diseased young and diseased old. When control young and old were compared it was seen that the cytokine gene expression were markedly higher in the younger group IL-1β ($p=0.019$; IL-18 ($p<0.001$) and TNF-α ($p=0.019$). But, when diseased young and diseased old were compared we saw a different pattern where the cytokines IL-1β ($p=0.047$), IL-8, IL-18 and TNF-α were seen to be lower in diseased young (Figure 4.6).

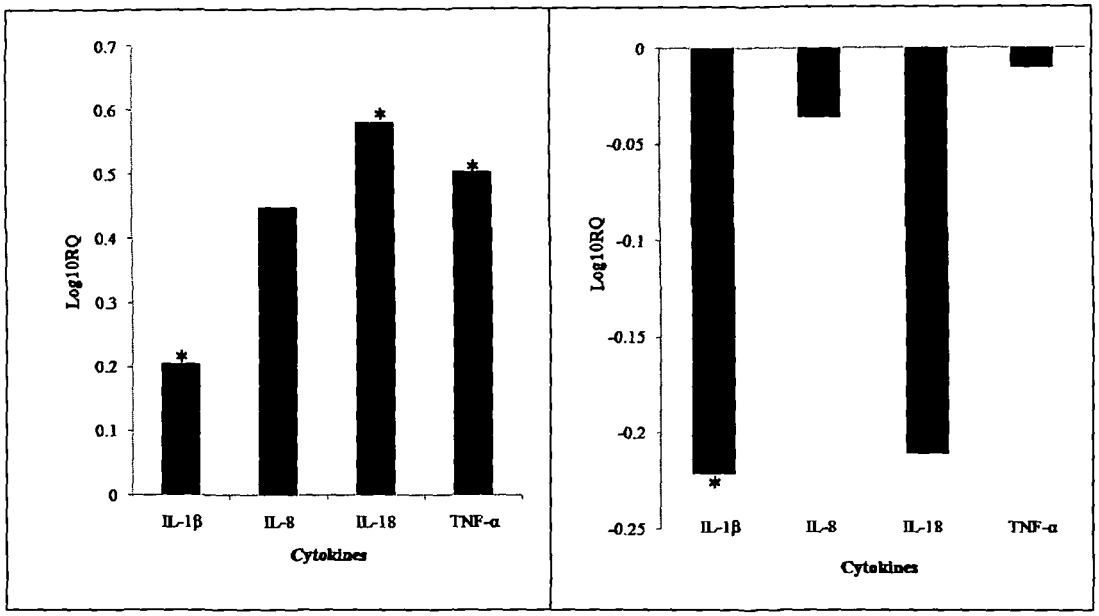


Figure 4.6: Relative quantification (RQ) of cytokines expression between (A) Younger and older age control groups and (B) Younger and older age groups who have *falciparum* malaria

**Significant levels of (A) IL-1β, IL-18 and TNF-α were observed between younger and older control groups (B) IL-1β between younger and older diseased groups.*

4.5 Discussion

Cytokine profiles were seen to differ with malaria positivity, severity of the disease and also between the two age groups in the present study. Levels of IL-18 were significantly higher in complicated group than in uncomplicated group which is consistent with studies by Kojima et al. (2004) [56] and Nagamine et al. (2003) [271]. Although, contrasting results were noted in African children where IL-18 levels were lower in children with severe malaria [45]. In our study, we also observed small increase in the levels of TNF-α in complicated malaria along with IL-18 and this was in agreement with a study by Wroczyńska et al. (2005) [199]. In the present study, levels of IL-1β and IL-8 were downregulated in complicated malaria in comparison to uncomplicated malaria. IL-18 is a potent proinflammatory cytokine [272] and has been recently shown to induce the production of TNF-α by human natural killer and T cells [273]. Furthermore, it is also known that IL-18 induces IFN-γ production from Th1 cells and NK cells, particularly in the presence of IL-12 [56]. It wasnoted that increased IL-18 levels during acute and recovery phases of uncomplicated *P.falciparum* malaria may be due to an early and effective immune response regulated by proinflammatory Th1 cytokines and thus limit the progression from uncomplicated malaria to severe and life-threatening complications [274]. This is in contrast to our findings, where we observed increased levels of IL-18 and TNF-α in complicated but not in uncomplicated malaria. In addition, we also observed increased levels of IFN-γ (results not shown here) in complicated malaria and this persistent heightened response perhaps explains the transition in the manifestation of disease from uncomplicated to complicated malaria.

Increased TNF-α levels stimulated phagocytosis and thereby enhanced clearance of parasitized erythrocytes [262, 263] but prolonged response was seen to contribute to adverse disease and thus was associated with severe disease syndromes in both human and experimental animals [275, 276, 45]. In our study, we also observed higher

levels of TNF-α, but these levels were not significant. A decreased level of IL-1β was observed in complicated malaria in our study which was unusual as it is an important inflammatory cytokine. This could be due to the delayed production of IL-10 which counter-balances the initial proinflammatory response elicited by the parasite [262]. In a study from Western Kenya by Ouma et al. (2008), also found reduced circulating IL-1β levels in severe malarial anaemia, and this could be attributed to promoter variations in the IL-1β promoter haplotype -31C/-511A (CA) [277].

Though it was noted, in the present study, that IL-1β was down regulated in complicated malaria, but when data was modeled for complicated malaria as outcome in TB, the combination of IL-18*IL-1β was found to be associated with complicated malaria.

IL-8 is a chemoattractant cytokine known to be involved in recruiting neutrophils to inflammatory sites and its role in malaria whether it provides protection or pathogenesis is not known clearly [278]. Levels of IL-8 has been shown to be elevated in a small group of severe malaria cases in adults and also been demonstrated in placental malaria[279]. Boström et al. (2012), observed lower levels IL-8 in *Plasmodium falciparum* infected individuals of Fulani tribe in comparison with uninfected individuals [280]. In the present study, we observed low levels of IL-8 in infected individuals and also in complicated malaria, the reason for this is presently unknown and needs to be further investigated.

When parasitaemia crosses a critical threshold and also the production of endogenous pyrogens (like IL-1β, IL-6 and TNF-α) induces symptoms associated with malarial infection [257]. In our study we observed that IL-18 and TNF-α were higher in symptomatic malaria as also seen in earlier studies [56, 57]. TNF-α has been shown to increase the severity of inflammation by inducing cyclooxygenase (COX)-2 and subsequently generated effector molecules, such as prostaglandins [282]. Many of the signs and symptoms associated with malaria, such as fever, headache, nausea,

vomiting, among others can be linked to TNF-α [283]. This tends to explain the higher levels of TNF-α in symptomology malaria. Surprisingly, IL-1β was upregulated in asymptomatic malaria in our study. Besides, increased expression of IL-1β in some studies has been associated with clinical pathology to malaria [264, 281].

Age is considered one of the most important factors that correlate with protective immunity in malaria endemic areas [60]. When younger and older age groups were compared, it was seen that in the younger age group the levels of all the proinflammatory cytokines were higher than the older group. But, when diseased young and old were compared we saw a shift in the cytokine profile where it was seen that the cytokines were all downregulated in younger diseased age group. Studies have showed that levels of proinflammatory cytokines (IFN-γ, IL-2, IL-8) were higher in the younger age group in malaria as well as complicated malaria [255. 284], suggesting a possible age related pattern in the secretion of proinflammatory cytokines with a peaking at an age when the individuals are susceptible to severe malaria infection and then decline in older ages. In contrast, we observed that the older diseased group to have increased levels of proinflammatory cytokines. In some other studies, severe malarial anaemia has been associated with low serum levels of IL-12 [268,285] and low IL-10 to TNF-α serum ratio concentrations in a childhood malaria in holoendemic areas [262]. Although many studies have associated severe malaria with elevated levels of IL-1β [264, 286], others have found no significant changes in the levels in children with severe malaria anaemia [262]. Winkler et al. (2006) [287] investigated the dichotomy of type1/type2 of T-cells in patients of different age groups to examine the immunoregulatory mechanisms that would result in different levels of anti malarial protection. They found increased frequency of type 1 cytokine–expressing cells in adults compared with children. Adults displayed a 2-fold higher frequency of IFN-γ–expressing $CD4^+$ cells, most of which were also IL-2 producers, than did infants. These differences were not restricted to $CD4^+$ T cells but were also evident for the

$CD8^+$ subset. The increased frequency of both type 1 and type 2 cytokine–producing T cells in adults is likely to be of significance in the protection against *P. falciparum* malaria [287].

In conclusion, we found that an elevated level of IL-18 was implicated in complicated malaria and therefore, IL-18 may be used as a marker of complicated malaria. Also, increased levels of IL-18 along with TNF-α was seen to discriminate between healthy and non malaria fever controls and also between symptomatic and asymptomatic malaria. Age was seen to be an important factor in proinflammatory cytokine response in malaria.Increased levels of IL-18 together with IL-1β increased the risk of complicated malaria for Tibeto-Burmans. This was not evident for Austro Asiatics group, thus the result suggests that the increased levels of these cytokines seen in Tibeto-Burmans may play a critical role in the difference in susceptibility to complicated malaria in these two ethnic groups.

CHAPTER 5:
A study of erythrocyte receptor polymorphisms in relation to falciparum malaria in the ethnic populations of Assam, North east India

5. A study of erythrocyte receptor polymorphisms in relation to falciparum malaria in the ethnic populations of Assam, North east India

5.1 Abstract

Several Red Blood Cell polymorphisms, for example thalassemias, glucose-6-phosphate dehydrogenase (G6PD) deficiencypyruvate kinase,complement receptor-1 and haemoglobinopathies, blood group antigens etc. have a role in the clinical outcome of malaria and they have shown to vary with geographical areas and malaria is documented to be a factor in their selection.

Fyb was found to be more prevalent at 96.09% than Fya at 49.66% of the study population. It was noted that both Fya and Fyb were found to be negatively associated with fever ($p=0.016$ and $p<0.0001$ respectively). There was an association with headache but was not statistically significant. Fya and Fyb were found to be protective where they were positively associated with infrequent malaria episodes (Fishers test $p=0.010$and $p=0.016$ respectively).Kpa was found to be negatively associated with fever ($p=0.016$). No association of malaria episodes with K, Kpa and Kpb was observed. Although, an association of Kpa and Fya (i.e. Fya*Kpa) was seen to be positively associated with frequency of malaria disease ($p=0.01$). Higher prevalence of homozygous mutant (H/H) of the SNP of CR1 (Q981H) was associated with in malaria disease (Fishers test $p=0.003$). Among AA, TB and Khasis, it was noted that the alleles of CR1 (Q981H) were seen predominantly in heterozygosity. The homozygous mutant genotype was seen to be absent in the Khasis (Fishers Test $p=0.040$).

In conclusion, it was seen that there was an association of Fya and Fyb antigens with malaria symptomology. An interaction between Fya*Kpa was observed where they are positively associated with frequent malaria episodes. Our data indicated that CR1 981H/Q genotype to be protective from having *falciparum* malaria. Homozygous

mutant (H/H) was completely absent in the Khasis. The shift from the wild (QQ) to the heterozygous type (QH) in our study population indicates that this mutation of the CR1 gene may be still evolving in theseareas.

5.2 Introduction

There is a dynamic interaction between the merozoite, the asexual blood stage of the malaria parasite, with the red blood cell since it has to gain entry into the cell, grow and divide within it.A number of human genetic polymorphisms affecting the structure, function and physiology of the red cell membrane are known to influence the parasites ability to invade and survive in it. Haemoglobinopathies (Haemoglobin (Hb) C and Hb S), thalassemias, glucose-6-phosphate dehydrogenase (G6PD) deficiency were found to be protective against *P. falciparum* malaria and were selected under malaria pressure in different populations[27, 30, 35-37].

The parasite, *Plasmodium vivax,* utilizes Duffy antigens (Fya and Fyb) present on the surface of red blood cell (RBC) and utilizes them during RBC invasion[288] and individuals negative for these antigens were observed to be resistant to *Plasmodium vivax* infections. Fya and Fyb, are codominantly expressed, and the alleles, FyA and FyB, are encoded on chromosome 1[289, 290]. The distribution of these antigens varies in different ethnic groups because the null phenotype, Fy (a-b-), is more common in African populations than in Caucasians[291]. Unlike *P.vivax, P.falciparum* can invade Duffy negative and positive erythrocytes equally well and is known to utilize a number of distinct receptors [292].

During erythrocyte invasion by merozoite, proteases are known to act at two levels, one at the host cell surface and at the surface or within the apical organelles of the invading parasite [293]. Some genes of *P.falciparum* encoding subtisilin-like serine and cysteine proteases have been identified (pfsub-1, pfsub-2, pfsub-3, falcipain 1 etc.) [294, 295].

The Duffy glycoprotein was found to bind a variety of chemokines and is known as the Duffy Antigen Receptor for Chemokines (DARC)[296]. The function of DARC is yet to be clearly defined. It has been suggested that DARC may permit the erythrocyte to serve as a chemokine "sink" or scavenger, thus limiting activation of leukocytes in the systemic circulation[217, 218, 296]. However, it is unclear how long chemokines remain bound to the cell surface or what happens to the chemokines at the end of the erythrocyte lifespan[214]. In addition, it is unclear as to the importance of this function in inflammatory or infectious disease as Fy (a–b–) erythrocytes do not bind chemokines, although Fy(a–b+weak) erythrocytes bind reduced amounts compared with Fy (a–b+) cells[214].

There are some antigens present on the erythrocyte that have proteolytic activity [225] and these proteases may aid in the invasion process of the merozoite. The Kell blood group system is one of the most polymorphic antigenic systems in human red blood cells and is composed of 23 Kell antigens including K (K1), k (K2), Kpa (K3), kpb (K4), Kpc (K21), Jsa (K6) and Jsb (K7)[220]. Kell protein has a striking sequencehomology with metalloendopeptidases which activate orinactivate bioactive peptides and was recently shown to haveproteolytic activity[225,297-299]. Kell antigens are highly immunogenic and the resulting antibodies can cause severe reactions to transfusion of incompatible as well as causing foetal anaemia and haemolytic disease in new borns[220]. Depression of the Kell system antigens have been reported in individuals with the rare Kpa (a+b-) phenotype[300]. An amino acid substitution of Arg281Trp is associated with Kpa antigen causing conformational changes throughout the glycoprotein and may affect its stability or its expression [301].

Another red blood cell polymorphism, complement receptor1 (CR1) polymorphism has been note to influenced severity of malaria disease. Human CR1 binds to a major malarial adhesin, the *P.falciparum*erythrocyte membrane protein-one (PfEMP-1) [236]. In malaria, CR1 has been reported to play an important role of both rosette formation phenomenoncalled resetting inwhichmalariaparasitized erythrocytes

bindtononparasitized ones and immune complex clearance during malaria infection leading to the pathogenesis of cerebral malaria and severe malaria anaemia respectively[237-240].

A number of polymorphisms in CR1 gene have been implicated in the pathogenesis and protection of various diseases. However, conflicting results have been obtained from studies in different malaria-endemic regions regarding association of CR1 genetic variants that influences the level of CR1 expression with disease severity[203,255-257].

Study of polymorphisms in ligand binding domains at amino acid I643T (nucleotide T2078C) at the end ofshort consensus repeats(SCR) 10 at long homologous repeats (LHR) B and at Q981H (nucleotide G3093T) in SCR16 in LHRC showed that the low expression allele encodes a CR1 with greater binding activity due to increase in the positive charge in the ligand binding domain [211]. Another study showed correlation of the Q981H polymorphism with the Hind III RFLP suggesting it to be part of a low CR1 expression haplotype, the gene frequency for this haplotype being highest in the malaria-endemic areas of Asia [212].

Erythrocyte-associated antigenic polymorphisms or their absence have evolved in the human population to protect against malarial infection [302]. The blood group variants are characteristic of population groups,they have shown to vary with geographical areas [303], and malaria is documented to be a factor in their selection. In this study we have examined the association of some rare red blood cell antigens like Fya, Fyb, K, Kpa, Kpb and also SNP (Q981H) of CR1 in relation to malaria and ethnicity in Assam State of Northeast India which is endemic for malaria.

5.3 Materials and methods

5.3.1 Study sites

The study was conducted at two study sites: Guabari, a village of Baksa district which lies at the foothills of Bhutan, and at Kondoli in Karbi Anglong foothills of Nagaon district of Assam. The two study sites may be classified as mesoendemic for malaria. The characteristics of the two study sites have been in chapter 3.

5.3.2 Study participants

Patients were enrolled into the study after they or their accompanying relatives gave full consent. Active as well as passive case detection was followed which was carried out by local health workers in Guabari while at Kondoli it was done by hospital staff. Based on linguistic group affinities, the study population was stratified into two groups namely the speakers of Tibeto-Burman (TB) that included the Bodo-Kachari and Nepalis, the Austro-Asiatic (AA) that comprised the tea tribes who are Mundari speakers and the Indo Europeans (IE) comprised of nontribal Assamese, Bengalis and few Biharis who are the speakers of Indo Aryan languages[232, 233]. Sampling was done such that the samples were drawn independently and randomly from each group particularly at Guabari that has ethnically mixed population. The characteristics of the study population have been described in detail in Chapter 3.

5.3.3 Sample collection

Blood samples were obtained from individuals irrespective of their *P.falciparum* positive (*Pf*+) status at the time of collection. Inclusion criteria were individuals with *Pf* positivity or with history of *falciparum* malaria. Exclusion criteria were children with age less than 1 year, pregnant women and individuals suffering from any other diseases. *P. falciparum* positivity was checked using Rapid Diagnostic Kits and confirmed by microscopy of thin and thick blood smears. 200ul of blood was collected in EDTA-tubes from both *Pf*+ positive patients as well as from healthy controls. Individuals were categorized according to their disease symptoms and frequency of malaria incidence which were simultaneously recorded. Complicated malaria was defined following WHO guidelines (2000).Uncomplicated malaria included fever,

headache, body ache and other mild symptoms or asymptomatic cases with parasitaemia of ≤5000/µl of blood. On the basis of number of clinical episodes of malaria, the participants were classified as infrequent (≤ 2 episodes) or frequent malaria (> 2 episodes) groups as detailed in our earlier work.The study was approved by the Tezpur University Ethical Committee (Resolution number 3 dated 13/06/06).

5.3.4 Serological tests for the presence of RBC antigens Fya, Fyb, K, Kpa and Kpb

Fresh drawn blood samples were collected in anticoagulated tubes (n=304). The red blood cells were washed in PBS. The washed RBCs were then incubated with respective antibody (anti Fya, anti Fyb, anti K, anti Kpa and anti Kpb; DiaMed, Switzerland). Agglutination was viewed microscopically.

5.3.5 Validation of the micro agglutination assays of Fya and Fyb by molecular genotyping

200ul of blood was collected in EDTA-tubes and genomic DNA extraction was carried out using Qiagen blood extraction kit (Qiagen, Germany). The Fy gene was then amplified by polymerase chain reaction and then digested using BstNI restriction enzyme as described[304]. After digestion the fragments were then analysed on 3% agarose gels pre-stained with Ethidium Bromide.

5.3.6 CR1 Q981H genotype determination

Genomic DNA was prepared from 200µl of EDTA blood using Qiagen blood extraction kit according to the manufacturer's instructions (Qiagen, Germany). The frequencies of the CR1 Q981H polymorphism in the study population was determined by following the protocol of Thomas et al. (2005) [212]. PCR amplification for the Q981H SNP was carried out using allele specific primers. Sequences for primer pair used for amplification are 5'-GCTACATGCACGTTGAGACCT-TAC-3' (forward

primer) and 5'-AGCAAGCATACA-GATTTTCCCC-3' (reverse primer). Amplification using these primers yielded a 366 base pair product which was analyzed

by electrophoresis in a 2% agarose gel stained with ethidium bromide and observed by ultraviolet transillumination. These PCR products then were further restricted digested. For restriction fraction length polymorphism determination, 18 ml PCR product, 2.5 ml buffer, 2.5 ml bovine serum albumin and 2.0 ml of BstNI were digested at 60°C for 2 hours and analyzed on a 3% ethidium bromide gel. Using this protocol, the wild-type 3093G residue yielded fragments of 54, 91 and 221 base pairs, while the 3093T mutant yielded fragments of 54 and 312 base pairs.

5.3.7 Data analysis

The data was analysed using Excel Stat Software, 2012 version. Statistical analysis for the differences in distribution of blood group antigens and genotypes between malaria cases and controls was carried out using Fisher's Exact test. Statistical significance was denoted by $P < 0.05$. The chi-square test was performed to evaluate whether the allele frequencies of the populations are in Hardy-Weinberg equilibrium.

5.4 Results

Total of 304 samples were analysed for microagglutination assays for the presence or absence of Fya, Fyb, K, Kpa, Kpb (Figure 5.1). The average age of the study population was 24.1 years (range: 2-70 years). The characteristics of the study population have been given in Table 5.1.In the samples typed for ABO blood groups (n=434), it was seen that blood group B was the most prevalent (33.87%),group A being the next prevalent blood group (27.88%; Table 5.1).

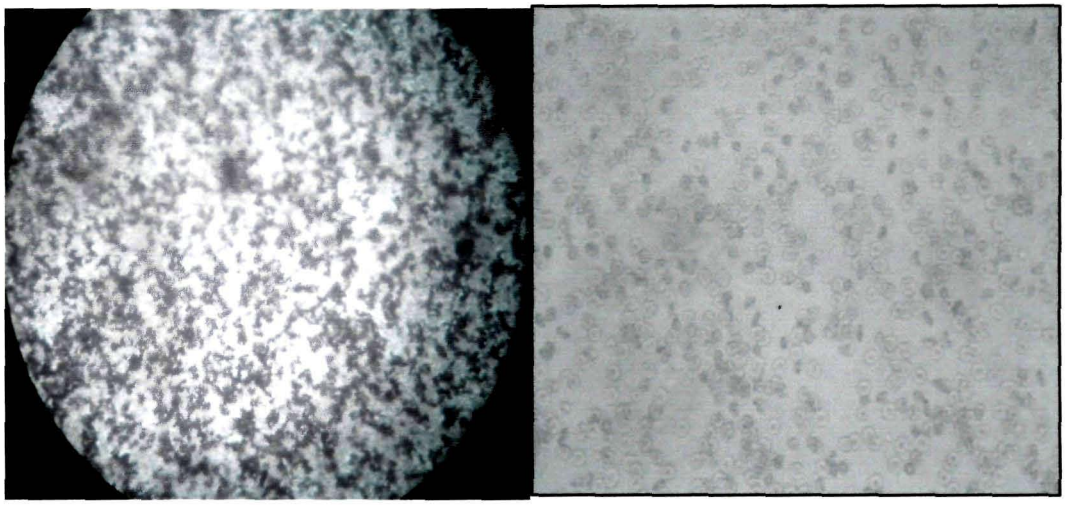
When the different blood groups were analysed it was observed that none of them were found to be associated with *Pf* status. However, regression analysis done for the

different blood groups along with *Pf* status and sex showed that *Pf* negative females were positively associated with blood group B (p=0.05).

Age	24.1 (Range 1.5-70 years)			
Frequent malaria episodes	55			
Infrequent malaria episodes	207			
Ethnic groups	N			
AA	140			
TB	111			
IE	57			
Blood group (n=434)	A+	B+	AB+	O+
Overall population	121	147	55	111
AA	49	63	21	48
TB	56	51	26	41
IE	16	33	8	22

Table 5.1: Characteristics of the study population

Blood group B was the most prevalent (33.87%) and group A the next prevalent blood group (27.88%) in the study population. AA-Austro-Asiatic; TB- Tibeto-Burman; IE-Indo Europeans



A B

Figure 5.1: Microagglutination of RBCs (A) Agglutination occurred with anti Kpa (B) No agglutination occurred with anti Kpa

5.4.1 Fya and Fyb with malaria

Fyb was found to be more prevalent at 96.09% than Fya at 49.66% of the study population (Figure 5.2). One third of the samples were validated by genotyping studies and 91.04% corresponded with the microagglutination assays. From genotyping data, Fyb was also found to be more prevalent (96.26%) than Fya (54.20%) in the studied samples.

From microagglutination data, the two phenotypes Fy(a+b+) and Fy(a-b+) were observed to occur more frequently (45.4% and 44.6% respectively) than Fy(a+b+) and

Fy(a-b-) phenotypes.The frequency of Fy antigens was analysed in relation to malaria symptomology: fever and headache, and with frequency of malaria episodes. It was noted that both Fya and Fyb were found to be negatively associated with fever ($p=0.016$ and $p<0.0001$ respectively). There was an association with headache but was not statistically significant. Fya and Fyb were found to be protective where they were positively associated with infrequent malaria episodes (Fishers test $p=0.010$and $p=0.016$ respectively).

Fy(a+b-) was the least prevalent phenotype. It was observed that 9.7% of the study population had the Fy(a-b-) phenotype. When data was further stratified into different ethnic groups: AA, TB and IE, an interaction among the three ethnic groups and the different phenotypes was observed (Fishers test $p=0.008$). Fy(a+b+) and Fy(a-b+) were observed to occur more frequently in all the three ethnic groups. Fy(a+b-) phenotype was present marginally for AA, but was absent for both TB and IE (Figure 5.3).

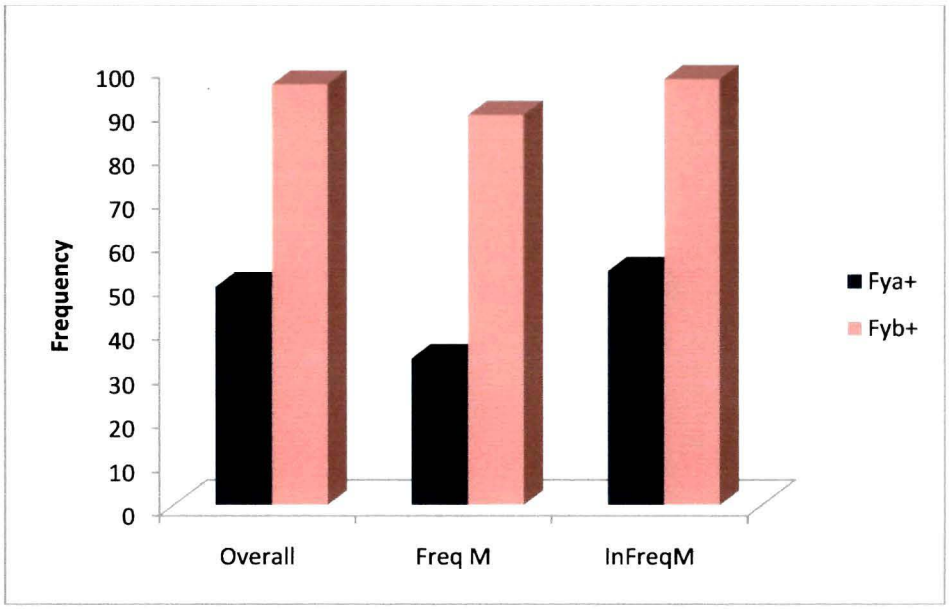


Figure 5.2: Comparison of the frequency of Fya and Fyb antigens with malaria episodes.

Fya and Fyb were found to be protective where they were positively associated with infrequent malaria episodes (Fishers test p=0.010and p=0.016 respectively).FreqM-Frequent malaria episodes; InFreqM-Infrequent malaria episodes.

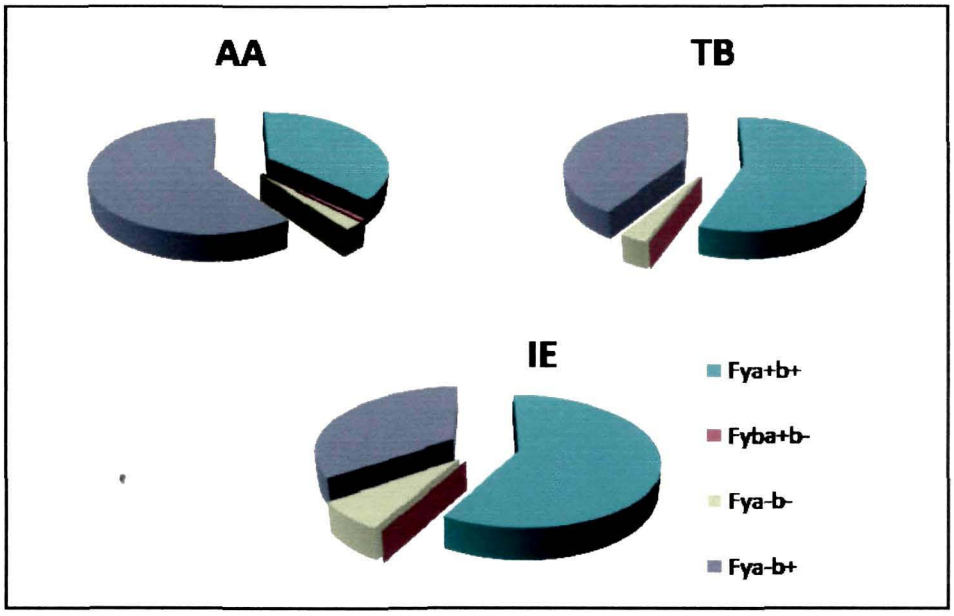


Figure 5.3: Frequency of the four phenotypes [Fy (a+b+), Fy (a+b-), Fy(a-b-) and Fy(a-b+)] in the three ethnic groups: AA, TB and IE

The phenotypes Fy(a+b+) and Fy(a-b+) were the most prevalent in the three ethnic groups.Fy(a+b-) phenotype was present marginally for AA, but was absent for both TB and IE. AA-Austro-Asiatic; TB- Tibeto-Burman; IE- Indo Europeans;

5.4.2 K, Kpa and Kpb with malaria

K+ was seen in most of the samples (87.66%), also when stratified into frequent and infrequent malaria episodes (Figure 5.4). Kpa+(24.66%) was more prevalent than

Kpb+ (13.37%) in the study samples. Kpa+ prevalence did not change with frequency of malaria episodes but a lowered percentage for Kpb+ in the infrequent malaria groupwas noted. K antigen was seen to be negatively associated with symptoms of malaria, fever ($p=0.0220$ and headache ($p=0.033$). Kpa was found to be negatively associated with fever ($p=0.016$). No association of malaria episodes with K, Kpa and Kpb was observed. An interaction of Kpa with the three ethnic groups was noted, Kpa being the least in AA group (Fishers test $p<0.001$). Kp(a-b-) phenotype was the most prevalent, whereas Kp(a+b+) was the least observed in the study population (Figure 5.5). Kp(a-b-) phenotype was also observed the maximum in the three ethnic groups, with AA having the maximum prevalence (83.07%).

When the different antigens (Fya, Fyb, K, Kpa and Kpb) were analysed with frequency of malaria episodes, an interaction between Fya and Kpa was observed ($p=0.01$). By itself, Fya was seen to be negatively associated with frequent malaria episodes, but together with Kpa (i.e. Fya*Kpa) was seen to be positively associated with frequency of malaria disease.

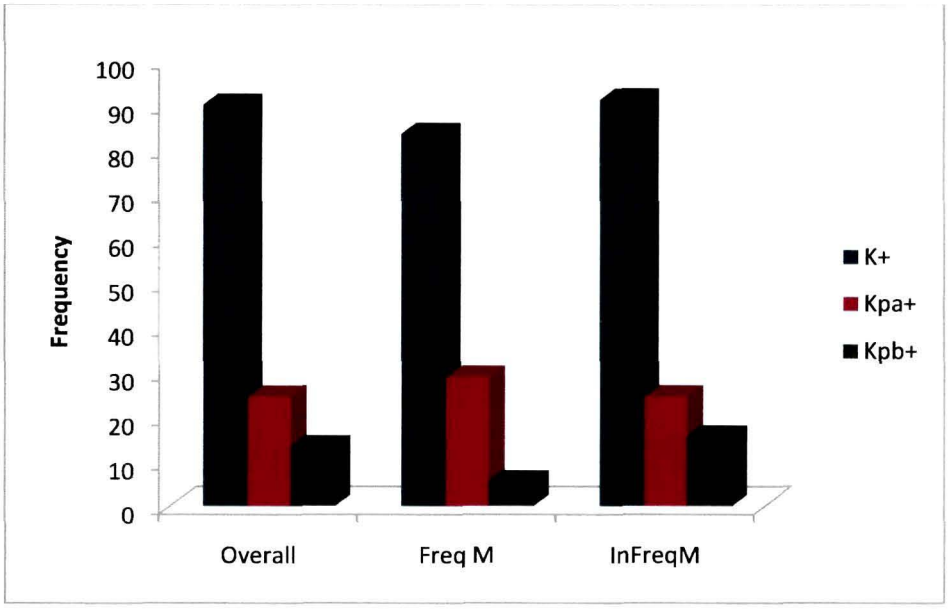


Figure 5.4: Frequency of K, Kpa and Kpb antigens in the different malaria groups.

K was seen in 87.66% samples. Kpa (24.66%) was more prevalent than Kpb (13.37%) No association of malaria episodes with K, Kpa and Kpb was observed. Freq M-Frequent malaria episodes; InFreqM-Infrequent malaria episodes

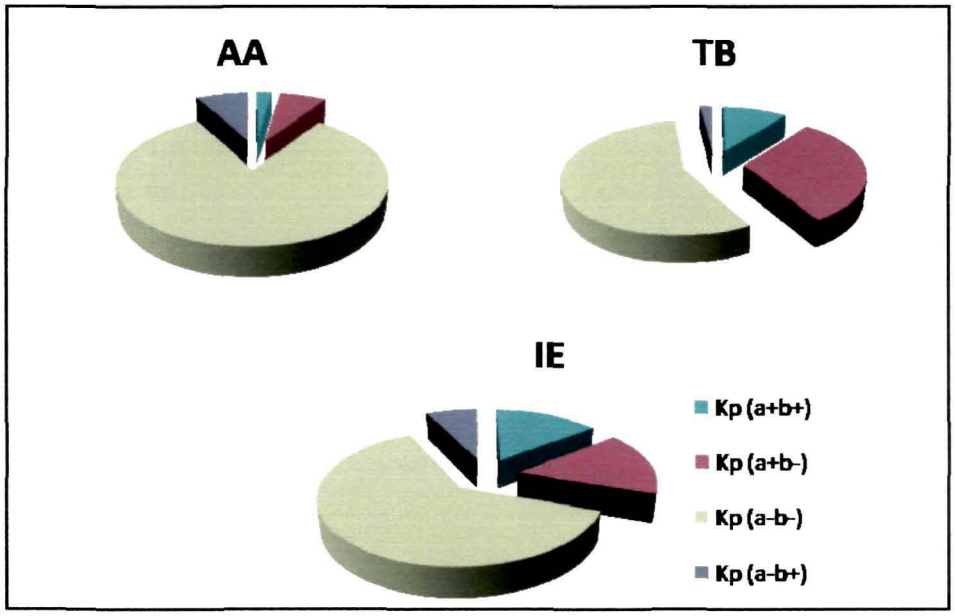


Figure 5.5: Frequency of the four phenotypes [Kp(a+b+), Kp(a+b-), Kp(a-b-) and Kp(a-b+)] in the three ethnic groups

Kp(a-b-) phenotype was also observed the maximum in all the ethnic groups, with AA having the maximum prevalence (83.07%).AA-Austro-Asiatic; TB- Tibeto-Burman; IE- Indo Europeans;

5.4.3 CR1 polymorphism

5.4.3.1 Overall frequency of Single Nucleotide Polymorphism of CR1 (Q981H) gene

A total of 282 samples were studied, where we found median age of the participants was 27.6 (Range: 2-70 years). These samples were examined for the frequency of SNP in CR1 (Q981H) (Table 5.2). When the genotype frequencies of the CR1 locus were compared among AA, TB and Khasis, it was noted that the alleles of CR1 (Q981H) were seen predominantly in heterozygosity. The homozygous mutant genotype was seen to be absent in the Khasis, though the frequency of the wildgenotype (Q/Q) was comparable with that of the other two populations (Fishers Test p=0.040; Table 5.2).

	Genotypes				
Ethnicity	n	Q/Q	H/H	Q/H	p-value
AA(plains)	63	11	12	40	
TB	63	6	12	35	**0.040**
Khasis	30	9	0	21	

Table 5.2: Comparison of the genotype frequencies of the CR1 (Q981H) polymorphism in the three ethnic groups: AA, TB and Khasis

Three ethnicities were considered (AA, TB and Khasis) and the others which consist of mixed ethnicities were not taken. Analysis was performed to check the interactions in the three genotypes of the CR1 gene by Fishers exact test. AA-Austro-Asiatic; TB-Tibeto-Burmans; n-number of samples

		Genotypes			
	n	Q/Q	H/H	Q/H	p-values
Disease	165	36	31	98	**0.003**
Control	117	14	11	92	
Severe malaria	59	16	10	33	Ns
Non severe malaria	106	20	21	65	
Frequent malaria	81	15	15	51	Ns
Infrequent malaria	48	13	3	32	

Table 5.3: Comparison of the genotypes of CR1 (Q981H) between the different malaria groups

Comparison of the frequency genotypes of the SNP of CR1 (Q981H) between disease and control between by Fisher'stest, it noted as that homozygous mutant (H/H) was found to be significantly higher and Q/H genotype lower in disease group than control (Fishers test p=0.003). ns- Not significant

5.4.3.2 Association of SNP of CR1 (Q981H) with malaria

We analysed the frequency of SNP in patients having malaria against healthy controls, disease severity: complicated malaria and uncomplicated malaria and also with respect to frequency of malaria episodes.

Comparing the genotype frequencies of the SNP of CR1 (Q981H) between disease and control, it was seen that homozygous mutant (H/H) was found to be significantly higher and Q/H genotype lower in disease group than control (Fishers test p=0.003; Table). Stratifying the data on the basis of disease severity, it was seen that the genotypes were comparable in the two groups with the mutant allele being in the heterozygous condition was more predominant (Table 5.3). H/H genotype was found to be higher in participants having frequent malaria episodes (18.51%) than those that are infrequent (6%) but was not found to be significant (Fishers test p=0.154). It was also noted that when data was stratified on the basis of ethnicity (AA and TB) with respect to frequency of malaria episodes, H/H genotype was significantly lower (Fishers test p=0.05) in infrequent malaria group than in frequent malaria group in both the ethnic groups.

The frequency of mutant allele (H) was noted to increase as one moves away from Africa towards Asia, and we also found that the frequency of mutant allele in Assam is also high (H=0.75). Many alleles were not in Hardy-Weinberg Equilibrium this implies that they are still in the process of evolving so as to reach equilibrium.

5.5 Discussion

Several RBC polymorphisms, including those linked toglucose-6-phosphate dehydrogenase, pyruvate kinase,complement receptor-1 and haemoglobinopathies, have a role in the clinical outcome of malaria[302]. It has been suggested that the presence of erythrocyte-associated antigenic polymorphisms or their absence have evolved in the human population to protect against malarial infection [27, 30, 35-37]. The blood group variants are characteristic of population groups, and can show conspicuous geographic patterns[303].

The spatial distribution of the Fy antigens was found to differ in different geographical areas. Frequencies of FY*A prevalence was shown to increase with distance from Africa and Europe, becoming dominant across south-east Asia, including those areas where *P.vivax* endemicity is highest[305]. In our study we observed that Fyb was more prevalent of the two antigens in our population. The global maps in a study by Howes et al. (2011) [306], showed that the distribution and frequency of the FY*B allele is highly restricted , with highest prevalence found in Europe and parts of the Americas, with further patches of increased prevalence in areas buffering the region of FY*BES (erythroid silent), predominance in sub-Saharan Africa[306].

Fy (a+b+) phenotype was found to be the most prevalent in 45.4% of the cases in the present study and a similar finding by Mohanty et al.(2011) [307] also found that this phenotype wasthe most prevalent but the frequency was much higher in tribal (Bhil group; 70.4%) than in non tribal group (54.8%) of India. In Europe, this phenotype was seen to be the most prevalent but in Asia Fy (a+b-) was the most prevalent. Erythrocytes expressing Fya had 41-50% lower binding of *P. vivax* compared with Fyb cells. Individuals with the Fy(a+b-) phenotype have a 30-80% reduced risk of clinical *vivax* but not *falciparum* malaria[24].

We have found an association of Fya and Fyb with fever, one of the symptoms of malaria but it was a negative association. It has been suggested that DARC may permit the erythrocyte to serve as a chemokine "sink" or scavenger, reducing the levels of circulating inflammatory chemokines, thus dampening systemic leucocyte activation [217.218]. In DARC-transfected cells, DARC is internalized following ligand binding and this led to the hypothesis that expression of DARC on the surface of erythrocytes, endothelial, neuronal cells andepithelial cells may act as a sponge and provide a mechanism by which inflammatory chemokines may be removed from circulation as well as their concentration modified in the local environment[308]. So, with the levels of cytokines being reduced, assuming pyrogens IL-1β and IL-6 are those cytokines getting reduced, accordingly fever also gets reduced. Although, activation of TLRs by GPI anchors lead to proinflammatory cytokines [41] being produced including IL-1β and IL-6, and because of these pyrogens, fever occurs. Therefore, probability of having fever is dependent on various factors.

There are two conflicting observations in DARC-deficient miceexposed to various inflammatory stimuli [309-313]. In one study DARC knockout (KO) animals received systemic LPS and responded by a marked increasein neutrophil infiltrate in the lungs and livers as compared to the wild type controls [309]. Lee et al. (2003), showedthat DARC KO mice have significantly less leucocyte infiltratein the bronchoalveolar lavage in response to chemokine instilled into pulmonary airspace [314]. In the present study,Fya and Fyb were found to be protective from having frequent *falciparum* malaria episodes.

Though many studies have found no association of duffy antigens with *P.falciparum* infections [24], however in a study by Beiguelmanet al. (2003) [315] observed lesser mean number of malarial episodes in Fy(a-b-) individuals as compared to other positive Duffy phenotypes. Very less is known about K, Kpa and Kpb antigens and their association with *P.falciparum* malaria. K antigen was prevalent in most of the study

participants though other studies have shown that a small percentage of the population have this antigen [316]. Fya was seen to be negatively associated with frequent malaria, however, it was seen that there is an interaction between Fya*Kpa where they are positively associated with frequent malaria episodes indicating that Kpa is the deciding antigen. We proposed that kell antigens may facilitate in merozoite invasion process by providing the proteolytic activity required for cleavage. When Kpa allele is not transported to the surface of the erythrocyte, it does not get expressed and so therefore no enzymatic activity in aiding the cleavage occurs and hence it is important in invasion process. The role of the kell antigen in erythrocyte invasion by *falciparum* malaria parasite needs to be further investigated.

Selection favouring heterozygosity conferred advantages that have been documented as in case of sickle cell haemoglobinopathy, which affords protection against mortality from *falciparum* malaria in heterozygous individuals, balancing the severe consequences of the disease in homozygous individuals. Our data indicated that CR1 981H/Q genotype protected from *falciparum* malaria. It was also observed in our earlier work on TLR which indicated TLR9 1486T/C heterozygosity to be protective, where it was negatively associated with complicated malaria [79]. Increased frequency of the CR1 homozygous mutant genotype (H/H) may also increase the probability of having *falciparum* malaria and also frequent malaria episodes, suggesting balancing selection on CR1 (Q981H) allele.

In the present study, the heterozygous genotype (Q981H) was seen to be the most prevalent. Similar finding was observed where a high rate of the heterozygous(Q981H) gene state in PNG [262]. Also, when our data was stratified into different ethnic groups, it was seen here that the heterozygous genotype was the most prevalent, but what was unusual was that homozygous mutant (H/H) was completely absent in the Khasis. This shift from the wild (QQ) to the heterozygous type (QH) indicates that this mutation of the CR1 gene may be still evolving in these locations.

CR1 Q981H has been identified in Caucasians linking to constitutive Erythrocyte-CR1 (E-CR1) expression levels.Erythrocytes having low CR1 expression have beenshown to form reduced number of rosettes with *Pf*-infected cells [242]. In Africa, rosetting has been shown tocorrelate with disease severity. Since erythrocytes having low CR1copy numbers form fewer rosettes, it has been postulatedthat low E-CR1 might protect from severe malaria[23]. Though in the present study, we did not observe any association of the CR1 SNP with severe malaria.

Frequency of blood group B is the highest in our study population, though studies from different parts of India have reported that frequency of blood group O is the highest. It has been hypothesized that blood group O offered a survival advantage during infection with *P.falciparum* because it has been demonstrated that there is an association between O blood group and lower rosetting capacity [317]. Group O has been observed to be more prevalent in sub Saharan Africa, Turkey, Persia etc. where *falciparum* malaria is endemic[318, 319].Many studies on patients from Zimbabwe, Gabon and Ethiopia [320-322]showed a significant association of group A with severe malaria, in contrast a study by Panda et al (2012)[302] in Odisha India, showed that the frequency of blood group B was associated with severe malaria. We did not find any association of A, B or O groups with *falciparum* malaria though Assam is endemic for *P.falciparum* malaria, this variation may be a population specific phenomenon. Though, we did observe that females who were *Pf* negative are positively associated with group B.

In conclusion, it was seen that there was an association of Fya and Fyb antigens with malaria symptomology. An interaction between Fya*Kpa was observed where they are positively associated with frequent malaria episodes. The frequency of the various RBC antigens varied with different ethnicities of our study population.Our data indicated that CR1 981H/Q genotype to be protective from having *falciparum* malaria. It was also observed that increased frequency of the CR1 homozygous mutant

genotype (H/H) may also increase the probability of having *falciparum* malaria and also frequent malaria episodes. Homozygous mutant (H/H) was completely absent in the Khasis. The shift from the wild (QQ) to the heterozygous type (QH) in our study population indicates that this mutation of the CR1 gene may be still evolving in theseareas.

CHAPTER 6:

MSP2 allelic composition and its variation with transition in transmission season of *Plasmodium falciparum* -A study from Assam state North East India.

6. *MSP2 allelic composition and its variation with transition in transmission season of Plasmodium falciparum -A study from Assam state North East India.*

6.1 Abstract

Merozoite surface protein (MSP) 2 of *Plasmodium falciparum* like many other proteins of the parasite is antigenically diverse among different isolates. This protein is a promising candidate for inclusion in a malaria subunit vaccine. We have examined here the allelic diversity of the MSP2 and also attempted to understand its variation with transmission season and malaria epidemiology at different sites.

At both the study sites, Indochina (IC) was found to be more diverse than FC-27 (isolate FC-27Q/PNG). At Kondoli, increased frequency of an allele of 330 base pair of the FC-27 allelic family was seen during 2006 summer withmarkedly low frequency of IC allelic family (p=0.005).Moreover, this particular fragment of 330 base pair was negligible in the previous year i.e. summer of 2005; this thus encourages us to speculate on the possibility of its emergence to be associated with malaria outbreak that occurred in 2006. FC-27 allelic composition was seen to be comparable between the two summers at Guabari except for increased frequency of 550 base pair in 2006. Consistent absence of the 330 base pair size fragment in the two summers which was otherwise present in winters, and the presence of a 400 base pair fragment only in the summers suggested these alleles to be season specific.

A positive correlation between number of clones and age was seen in the winter of 2006 only at Guabari (p=0.008, R^2=0.384). The Adivasis tended to have less number of bands/clones as compared to the other ethnicities(p=0.042;Odds ratio=2.509). Study site, ethnicity, age and gender were identified as risk factors in infection complexity. The present study demonstrated clonal composition of *P. falciparum* varied with study site and between periods of high and low transmission as

well as in different years of study and the possible association of a 330 base pair allele of FC-27 allelic family with malaria outbreak.

6.2 Introduction

Antigenic diversity exhibited by *P. falciparum*[323, 324] remains a major challengein vaccine development. World over the genetic structure of *P. falciparum* has been demonstrated to be highly diverse [325, 326]. Studies in acquired immunity in endemic areassuggest that the variable genetic structure of the pathogencontributed significantly to the evasion of immune responseleading to selection of certain clones, arguably those that do notevoke strong immune responses leading to dominance of somegenotypes in certain populations or geographical areas [327]. Apart from immune status of the population,treatment and genetic background of the host have also beenpostulated to have significant bearing on the genetic structure of*P. falciparum*[325, 328].

The merozoite is the principal target of current asexual stage vaccine development, the stage of the malarial parasite that is initially released from the infected hepatocyte which then infects other circulating red blood cells[75]. The vaccine strategies aim to elicit antibodies that target merozoites and /or malarial antigens expressed on the RBC surface, thus inducing antibody-dependent cellular cytotoxicity and complement-mediated lysis[76]. These also are meant to elicit T-cell responses that will inhibit the development of the parasite in hepatocytes and induce memory[76,77].

Among the Merozoite Surface Proteins (MSPs); MSP1, MSP2 and MSP3 are the primary candidate antigens for blood stage malaria [329]. MSP2 of *P. falciparum* is a well-known target of naturally acquired clinical immunity to malaria [122] and antibodies to these molecules are reported to block merozoite invasion of erythrocytes [123]. MSP2 is an integral membrane protein and contains repeat arrays flanked by unique variable domains and conserved N- and C-terminal domains. This protein is encoded by highly

divergent alleles grouped into dimorphic families or lineages the FC-27 type (isolate FC-27Q/PNG) and IC/3D7 type (Indochina)[124].

MSP2, like most antigenic molecules exhibits allelic variation [122] and this was seen to vary with transmission intensity [124] [125] and geographical regions. Association of some allele types or genotypes with disease severity was observed [323] [331] though a lack of association in some other studies this was also noted [322]. Further, vaccines containing only one allelic type (3D7 allele) of a polymorphic antigen induced immune responses that selected for parasites expressing alternative alleles (FC-27 type alleles) [130], resulting in a higher incidence of morbid episodes. It was also observed at Mali, that the parasites with MSP-1_{19} haplotypes were different from that of the leading vaccine strain, emphasising the importance of determining the genetics of pathogen populations prior to vaccine trials [131]. Inclusion of combinations of large number of conserved antigens and/or multiple components covering all important allelic types in vaccine formulations was recommended [132].

Assam, Northeast India experiences frequent epidemic outbreaks of malaria[228]. The transmission dynamics and intensity of malaria in the region is governed by distinct epidemiological paradigms attributable to eco- geographical, ethnic and socio-cultural diversity [333]. Genetic structures and population genetics studies of *P.falciparum* may hold the key for effective disease surveillance and control program especially in Northeast India because there was so limited information available on the genetic structures of *P.falciparum*[334, 229]. In an earlier study of the MSP1 gene, we have observed that there was interaction of allelic families and the clonal composition between the two transmission periods and it varies with site. A 380 base pair allele of RO33 allelic family of MSP1 gene was over represented in high transmission summer season and seen frequently in isolates with high parasitaemia raising the possibility of its association with *P. falciparum* virulence[229]. The present investigation explored the changes in allelic composition of MSP2 with change in transmission season and malaria epidemiology to understand if the increased

P.falciparum incidence seen in summers was related to clonal variation and how it differed in two different malaria epidemiology situations prevalent at Guabari (Baksa district)and Kondoli(Nagaon district) of Assam, India.

6.3 Materials and methods

6.3.1 Study sites

The study was conducted at two study sites: Guabari, a village of Baksa district which lies at the foothills of Bhutan and at Kondoli, located at Karbi Anglong foothills of Nagaon district of Assam. Guabari has a mixed population of Bodos, Assameses, Bengalis, Nepalese and a few Adivasis; whereas Kondoli has a fairly homogenous population comprised of Adivasis. Malaria transmission dynamics differed at the two study sites. At Guabari village, the study period spanned low transmission in the dry winter months to period of peak transmission in summer months for two consecutive years from December 2005- June 2007. At Kondoli, the study was conducted for three consecutive summers from 2005–2007. Slide positivity rate in Guabari varies from ≤15% in the dry winter months, when transmission is low, to 30-55% in the high transmission period spanning Mid March – Mid July. Kondoli has seasonal malaria during the high transmission period and experiences negligible malaria in dry winter months. According to annual slide positivity rate and spleenomagaly index, these areas may be classified as 'mesoendemic'. However, sporadic malaria outbreaks occur at these sites. The study was conducted during themalaria outbreak of 2006 and of 2007.

6.3.2 Sample collection

The study was designed as a longitudinal study in two districtsof Assam.A standardised patient information sheet had been developed and read to the participants and explained to them about the study by the field workers. Patients were enrolled into the study after they or their accompanying relatives were satisfied and gave full

informed consent. Active as well as passive case detection was followed which was carried out by local health workers and research staff in Guabari while at Kondoli it was done by hospital staff. Field visits were carried out by the researchers. *P.falciparum* positivity was the criteria for inclusion in the study. Exclusion criteria were children with age less than 1 year, pregnant women and individuals suffering from any other diseases. Studies have shown that pregnant women have depressed immune functions, exposing them to malaria infections and other stress-causing factors. During pregnancy onset, there appears to be down-regulation or suppression of the T-cell-mediated or adaptive immune response [335]. Resistance has previously been attributed in part to poor parasite growth in foetal hemoglobin (HbF) containing red blood cells and maternal immune IgG [336].

The samples were collected from patients who tested positive for *P.falciparum* infection using Rapid Diagnostic Kits. Those positive for malaria were given medication and those with severe malaria were referred to the health centre doctors for evaluation and proper treatment. *P.falciparum* positivity was confirmed by microscopy of thin and thick blood smears.Individuals were categorized according to their disease symptoms and frequency of malaria incidence which were simultaneously recorded. On the basis of number of clinical episodes of malaria, the participants were classified as infrequent ($\leq$ 2 episodes) or frequent malaria (> 2 episodes) groups. Data were collected by trained field staff and checked by the supervisor of the field staff by regular field visits. Clinical symptoms characteristic of malaria like history of malaria, headache, body ache, fever, age, ethnicity and sex were recorded. Axillary temperature also was takenand recorded. Apart from tests for malaria, haemoglobin determination was also done. The study was approved by the Tezpur University Ethical Committee (Resolution number 3 dated 13/06/06).

6.3.3 Parasite Sampling

Blood samples were collected by finger prick method and spotted on Whatman 3MM and on Whatman FTA cards for parasite DNA extraction and PCR (polymerase chain reaction) analysis. The DNA was extracted from the Whatmann paper using the Chelex extraction method [337] and from the FTA cards as per the manufacturer specified protocol. The extracted DNA of the positive samples was taken and the Block 2 of the MSP2 was amplified by primary PCR followed by nested PCR using allele specific primers. The PCR cycles and the primer sequences used were as described [337]. The amplicons were then run on 1.8% agarose gels pre stained with Ethidium Bromide. 100 base pair and 50 base pair molecular markers (Fermentas) were used to analyse the size of the bands. Blood samples that were negative for *P.falciparum* and bacterial genomic DNA were used as negative controls.

6.3.4 Statistical Analysis

The data was analysed using Excel Stat Software, 2011 version. Variation in the frequency of the MSP2 allelic families FC-27 and IC with respect to study sites and transmission season was analysed by Chi square (χ^2) test with Yates correction as required. Interaction of alleles with sites and also between transmission seasonswas analysed using χ^2test. The proportion of isolates with an allelic family of MSP2 was compared using k-test for multiple proportions. Variance in the alleles in the three sites was compared using two-tailed z-test. The distribution of alleles was compared using Smirnov-Kolmogrov two-tailed test. The association between clones and age was analysed by Pearson Correlation test.

6.4 Results

165 samples were amplified for allelic diversity and size polymorphism and data was analysed with respect to transmission season and study site. The characteristics of the study population are given in Table 6.1. A high degree of MSP2 polymorphism was found in isolates from the two study sites with 22 alleles of MSP2 seen at Guabari village and 19 alleles at Kondoli. Notably, an allele was defined as amplification of a band of distinct size of an allelic family. There was no interaction between the number of alleles of an allelic family with study site, where number of alleles were comparable at the twostudy sites(Table 6.2).

	Guabari	Kondoli
Median age	17.8	24.833
Febrile cases (>98.6 F)	90.08 %	80.56 %
†Parasetaemia		
Summer	10608.88±2541.450	8821±2280.5114
Winter	3130.132±1572.2948	NA
M/F ratio	1	3.16

Table 6.1: Patient characteristics at the two study sites

†mean parasitaemia ±standard error. 47% of the study population in Guabari was seen to be Bodos whereas 96% of the population in Kondoli was the Adivasis. NA-not applicable.

Site	N	FC	IC	Total MSP2 alleles	MOI
Guabari	112	10	12	22	3.25
Kondoli	53	10	9	19	2.5

Table 6.2: Distinct alleles of MSP2 and MOI

N-sample number; MOI-multiplicity of infection. The number of distinct alleles of the two allelic families of MSP2 seen at Guabari and Kondoli. No interaction between the number of distinct alleles of each of the families and study site existed (χ^2 = 0.021, p = 0.885).

6.4.1 Temporal Variation

Changes in allelic composition of FC-27 were gradual, between winter of 2005 and summer of 2007 (p=0.009; Table 6.3; Figure 6.1a). Interestingly, the allelic composition was seen to be comparable between the two summers at Guabari except for increased frequency of 550 base pair in 2006. Between the two winters, a variation in the allelic composition was noted with appearance of fragments in the range of 150-300 base pair in 2006 but not in 2005(Figure 6.1a). Consistent absence of the 330 base pair size fragment in the two summers which was otherwise present in winters, and the presence of a 400 base pair fragment only in the summers suggested these alleles to be

season specific. Proportion of isolates with FC-27 allelic family was also seen to vary, being lowest in winter of 2006 (p=0.025; Table 6.4).

	Guabari			Kondoli	
comparison between transmission seasons	p-values FC	IC	Comparison between transmission years	p-values FC	IC
W2005- S2006	0.362	**0.038**	S2005- S2006	0.154	0.209
S2006- W2006	0.562	0.659	S2006- S2007	**0.005**	0.057
W2006- S2007	0.109	0.720	S2005-S2007	0.143	**0.023**
W2005-W2006	0.358	**0.003**			
S2006- S2007	0.305	0.477			
W2005- S2007	**0.009**	**0.002**			

Table 6.3: Statistical comparison of distribution of alleles in different transmission period.

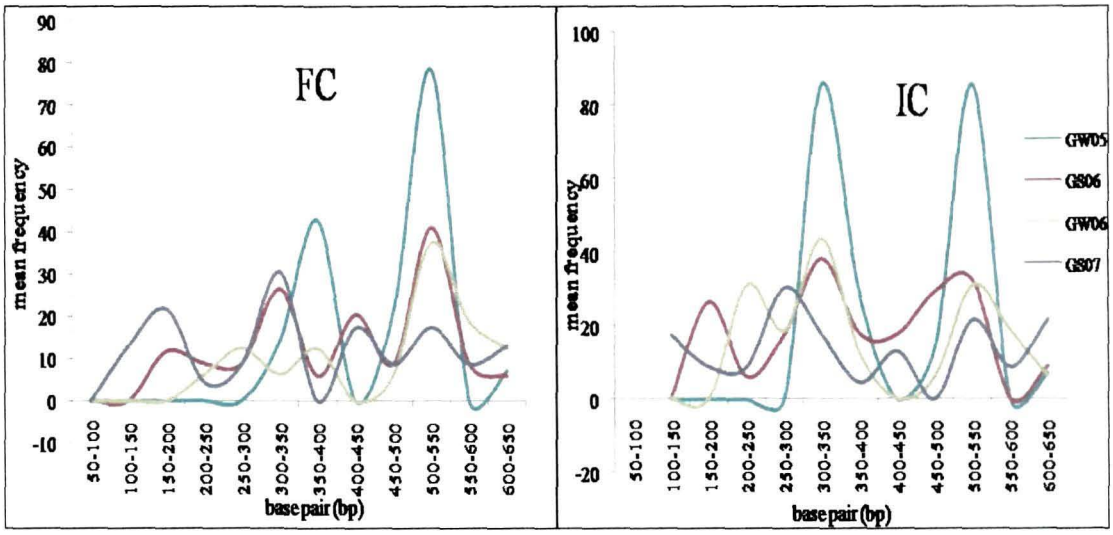
The temporal variation in frequency of allele distribution at Guabari was compared between the different transmission seasons, i.e. between winter (W) and summer (S), between the two winters and between the two summers.At Kondoli, comparison was done between the three transmission years. Analysis was performed using two tailed Smirnov–Kolmogrov test.

Transmission	Guabari		Transmission	Kondoli	
Season and year	FC	IC	Year	FC	IC
W2005	1	1	S2005	0.846	0.769
S2006	0.852	0.911	S2006	0.888	0.555
W2006	0.588	0.88	S2007	0.937	0.875
S2007	0.818	0.863			
	p-values			p-values	
$\chi 2$	**0.025**	0.557	$\chi 2$	0.726	0.196

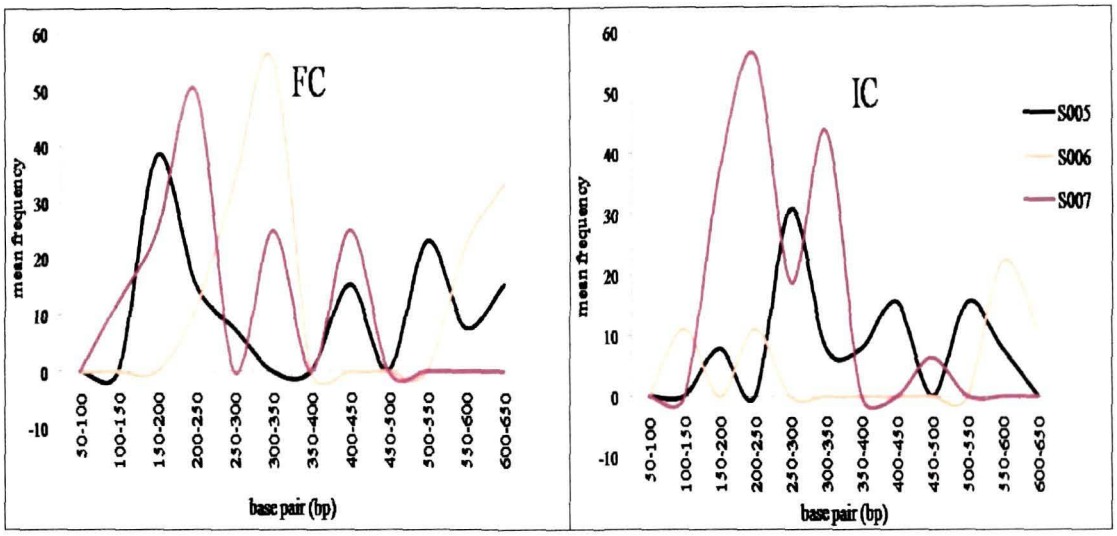
Table 6.4: The proportion of isolates with an allelic family of MSP2.

The proportion of isolates with an allelic family was analysed for temporal variation employing the two-tailed k-test for multiple proportions at Guabari and Kondoli. At Guabari the variation was studied between the high transmission summer and the low transmission winter while at Kondoli between the three study years. Data analysis shows a distinct difference between the two study sites with respect to temporal variation in number of isolates. Winter (W) and Summer (S)

In contrast, a marked difference was noted in allelic composition of IC allelic family at Guabari, where the alleles of winter 2005 were different in size polymorphism and in frequency from the two summers of 2006 and 2007 (p=0.0.38 and p=0.002 respectively) and also from the subsequent winter of 2006 (p=0.003, Figure 6.1 A).



(A)



(B)

Figure 6.1: Temporal variation in size polymorphism of the MSP2 alleles at (A) Guabari and at (B) Kondoli.

Polymorphism of alleles of MSP2 may be noted in both the allelic families at Guabari (A) and at (B) at Kondoli with variation in the transition of transmission season. For FC allelic family at Kondoli, it may be noted that alleles of 300-350 base pair being dominant in 2006 summer. W: Winter and S: Summer

At Kondoli, allelic composition of FC-27 allelic family was seen to differ with each transmission year. In 2006, alleles of FC-27 family consisted of mainly 330 base pair sized fragments , while IC allelic family was nearly absent during this period (Figure6.1B and Table 6.3; p=0.005). Incidentally, there was a major outbreak in the summer of 2006 which was associated with high mortality, suggesting association of FC-27 with virulence. Proportion of isolates with FC-27 allelic family increased with change in transmission years from summer of 2005 to summer of 2007 (Table 6.4). For IC

allelic family, allelic composition differed in the three summers. The presence and absence of alleles were the reasons for the difference in allelic composition between summers of 2005 and 2007 (p=0.023) where alleles seen in 2005 were not seen in 2007 (Figure 6.1B).

The frequency of allele distribution was compared between the two study sites and was observed that though the sites shared many alleles, there were also site restricted alleles and these were differently distributed (p<0.01; Figure 6.2), with 500-550 base pair of FC-27 allelic family was most frequent at Guabariand occurred less frequently at Kondoli, while 150-200 base pair and300-350 base pair were dominant at Kondoli. Proportions of isolates of IC allelic family was seen to be higher at Guabari than at Kondoli (p=0.030). Higher sized fragments were seen to dominate for IC allelic family at Guabari.

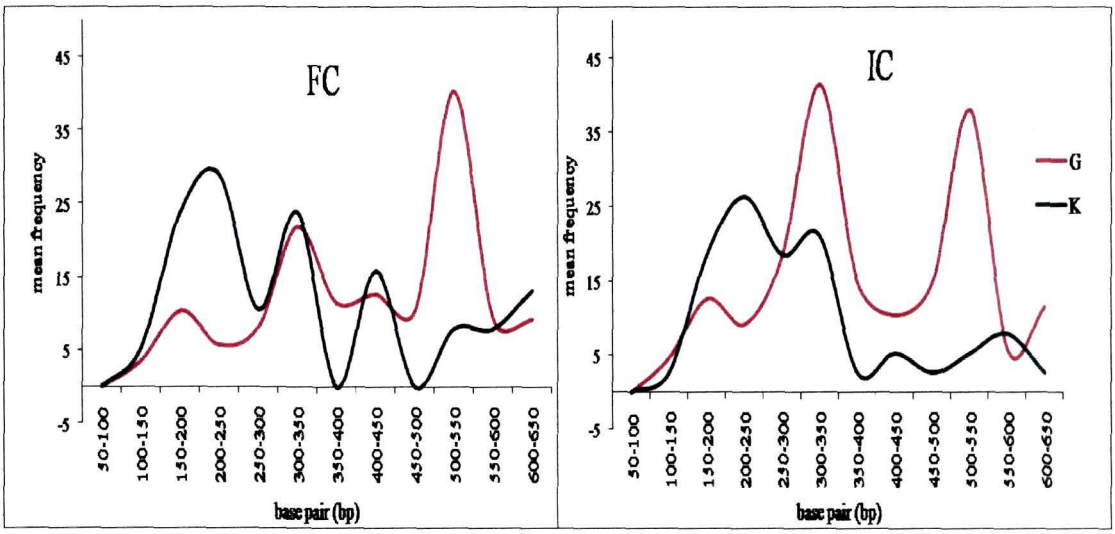


Figure 6.2: Spatial variation in size polymorphism of the MSP2 alleles between the two study sites.

It may be noted that size polymorphism of IC allelic family differed between Guabari (G), and Kondoli (K).

6.4.2 Complexity of Infection

At Guabari and Kondoli, 73.56 and 65.78 percent of infections respectively were found to be multiclonal. The mean number of clones per isolate was 3.25 at Guabari and 2.5 at Kondoli (Table 6.2). The infections tended to be complex both because of presence of more than one allelic family as well as more than one allele of the same family in an isolate (Figure 6.3). Changes in MOI with transmission season were noted being the highest in winter of 2005 (MOI of 3.8 clones/isolate) and lowest in winter of 2006 (MOI of 2.6 clones/isolate) at Guabari village, whereas highest MOI at Kondoli was noted in summer of 2007 (MOI of 3 clones/isolate). Guabari was also identified as a risk factor (p=0.014; Odds ratio=2.49), with the probability of having more than 2 clones per infection by logistic regression analysis. A positive correlation between number of clones and age was seen in the winter of 2006 only at Guabari (p=0.008; Figure 6.4).

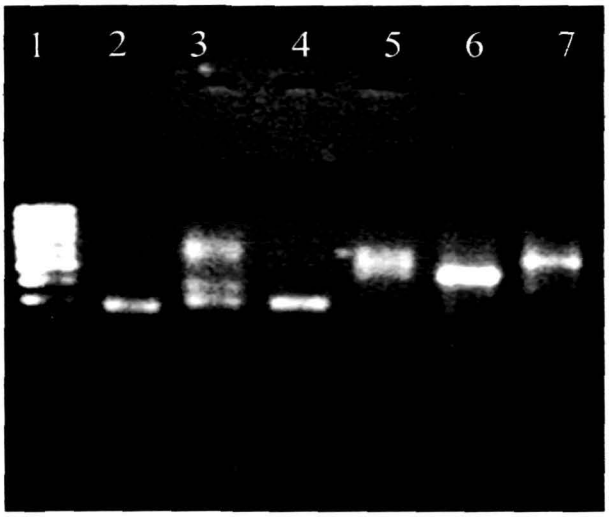


Figure 6.3: Gel picture depicting nested PCR products.

Lane: 2, 3, 4, 5, 6 and 7 shows amplified products of FC-27 allele of MSP2 gene. Lane 1:- 100 bp molecular marker (Fermentas).Multiple bands in lanes 3 and 5 correspond to multiple clones present per isolate.

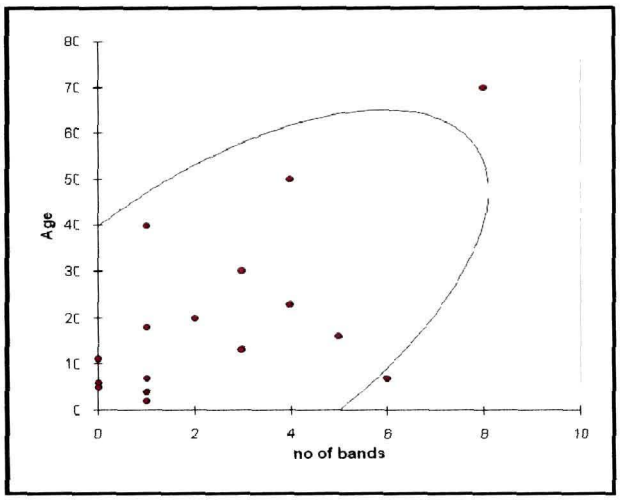


Figure 6.4: Correlation graph between age and number of bands.

Analysis between age and number of bands by Pearson correlation showed that with the increase in age, the number of bands also was seen to increase (p=0.008) at Guabari only.

Ethnicity was also identified as a risk factor in the probability of having >2 bands per infection by logistic regression analysis. The Adivasis (p=0.042; Odds ratio=2.509) were seen to be protected from having more number of bands (>2 bands) as compared to the other ethnicities (Bodos, Bengalis and Nepalese). Gender was also seen as a confounding factor, where males are seen to have more number of bands (>2 bands) than the females at Guabari (p=0.034; Odds ratio=0.364). At Kondoli, no association of multiplicity of infection with age, gender or ethnicity was observed.

6.5 Discussion

The present investigation explored the changes in allelic composition of MSP2 with change in transmission season and malaria epidemiology to understand if the increased *P.falciparum* incidence seen in summers was related to clonal variation and how it differed in two different malaria epidemiology situations prevalent at Guabari and Kondoli.

Diverse and extensive allelic polymorphism was seen for the MSP2 locus at both the study sites which was similar to reports from Gabon [323], Ndiop [338] and Myanmar[339] which are meso to hyperendemic areas. In the present study, a spatial fluctuation was seen in which some genotypes were present in one site but absent in the other site and also their frequencies differed indicating that the genotypes are site dependent. A study in two neighbouring villages, Dielmo and Ndiop, also observed similar observations where allele frequencies differed in both villages, indicating considerable microgeographical heterogeneity of parasite populations [340].Similar findings were observed with the change of transmission seasons. Most studies examining the distribution and frequency of different allelic forms of MSP2 have shown that they vary with transmission intensity and geographical regions[74, 330]. A skewed distribution of predominantly 3D7 family alleles exists among laboratory-adapted strains, in the field a more even distribution of FC27 and 3D7 alleles have been noted. Though in some studies, FC27 family alleles were noted to bemore prevalent than 3D7 alleles [341], also novel FC27 and 3D7 family alleles have been found in field malaria strains [342].However, in the present study, the alleles of the two allelic families of FC27 and IC were comparable at the two study sites. Increased frequency of a particular allele of 330 base pair of the FC-27 allelic family was seen at Kondoli during 2006 summer and also in the next summer though at a lower frequency.

Notably, 2006 and 2007 were the years of outbreak associated with high morbidity in Assam [343].Moreover, this particular fragment of 330 base pair was negligible in the

previous year i.e. summer of 2005; this thus encourages us to speculate on the possibility of its emergence to be associated with malaria outbreak. Suggesting an association of FC-27 allelic family with virulence in this study.Several studies have investigated the association ofspecific *P. falciparum* genotypes with the clinicaldisease and virulence. A similar association was reported in Papua New Guinea, where the FC27 allele was linked to increased disease severity [330]. A case-control study also in Papua New Guinea, suggested that *msp2* FC27-type infections are more virulent than those of 3D7-type infections in the study area[344]. Conversely, another study reported that there was no association between FC27 or 3D7 alleles of *msp-2* and malaria symptoms[345]. In an earlier study by us on the MSP1 gene, we observed a similar finding of an association of an allele with virulence. A 380 base pair allele of RO33 allelic family of MSP1 gene was over represented in high transmission summer season and was seen frequently in isolates with high parasitaemia raising the possibility of its association with *P. falciparum* virulence [229].

Multiclonality of infections has been shown to be a common feature in most malaria endemic areas and was reported to vary with age, immune status, epidemiological settings and transmission seasons[346, 347]. In the present study, ethnicity, site and gender were seen as confounding factors in MOI. Though multiclonal infections were seen at both sites in this study, it was higher at Guabari where accessibility to health was poor and the socio-economic status of the population was low and drug non-compliance was rampant; precludes effective and complete treatment leading to a level of immunity that allows carriage of some clones, over time and also during the low transmission periods. We also observed a positive association of number of clones with age in winter at Guabari. The observation that a significantpercent of people in this region were found to be asymptomatic as observed by us in an earlier study and also by Mohapatra et al. (1998) [333, 329]. Increased MOI was also observed to be related to asymptomatic*P.falciparum* infection[349]. Some studieshave reported decrease in

multiplicity with age[350], others have observed a positive correlation in infantsand children but not in older individuals[351, 352]. Somereports have indicated decrease of MOI during adulthood to the levels found in infants, while others didnot observe any relationship between the two parameters[353,354].

Lower mean parasitaemia and multiclonality in Adivasis in comparison to Bodos could be related to higher antibody levels in Adivasis, as had been reported earlier by Lourembam et al. (2012) [355]. The males at Guabari were seen to have more number of clones (>2bands) than the females, the reason could be that Guabari is a forest fringe area and it's usually the males who venture into the forest for cutting wood and moreover the farms are also near the forest. *Anopheles dirus* is a vector of malaria in Asian forested zones and its geographical distribution is overlapping with areas of high malaria prevalence rates and the occurrence of drug resistant *P. falciparum* [356, 357].

In conclusion, we observed that there was temporal clonal fluctuation at two study sites related to transmission seasons. An increased frequency of an allele of 330 base pair of the FC-27 allelic family was seen at Kondoli which could be associated with malaria outbreaks therefore needs to be investigated further. Lower mean parasitaemia and MOI in Adivasis suggest that ethnic background of the host is important. Association of age with MOI was observed at Guabari. Guabari was also identified as a risk factor (p=0.014; Odds ratio=2.49), with the probability of having more than 2 clones per infection than Kondoli.The result of this study is important for vaccine-development program.It further emphasized the importance of determining genetic structures and population genetics studies of *P.falciparum*malaria whereby vaccine formulations must include all important allelic types.

CHAPTER 7: CONCLUSION

7. Conclusion

In this study, we have examined the association of some red blood cell polymorphisms (CR1, Fya, Fyb, K, Kpa, Kpb), immune receptor polymorphisms (TLR2, 4 and 9), proinflammatory cytokines (IL-1β,IL-8, IL-18, TNF-α) of the innate system with risk of malaria in two ethnic groups, the Austro-Asiatics and Tibeto-Burmans, and tried to understand the influence of malaria in selection of polymorphisms and the proinflammatory cytokines/chemokines in these genetically distinct populations.We have also examined here the allelic diversity of the MSP2 and attempted to understand its variation with transmission season and malaria epidemiology at the different sites.

Our data indicated TLR9 1486T/C heterozygosity to be protective, while it was negatively associated with complicated malaria, whereas TLR9 (T-1237C) was seen to predispose to complicated malaria in heterozygosity.While TLR9 over expression was favourable, that of TLR2 and 4 were risk factors for complicated malaria. In addition, CR1 981H/Q was also noted to be negatively associated with *falciparum* malaria. It was also observed that the alleles were not in HWE and our data suggests malaria could be playing a role in balancing selection in these genes.

When proinflammatory cytokines were studied in relation to malaria, elevated level of IL-18 was implicated in complicated malaria and therefore, IL-18 can be used as a marker of complicated and symptomatic malaria. Also, increased levels of IL-18 along with TNF-α was seen to discriminate between malaria and non malaria fever controls. Combination of cytokines IL-1β*IL18*TNF-α were noted to be negatively associated with complicated malaria ($p<0.0001$). Cytokine profiles were also seen to change with age and with malaria disease.

Role of Fya and Fyb antigens with *Plasmodium vivax*infections have been well

documented, however limited work has been done in their relation with *P.falciparum* infections. It was noted that there was an association of Fya and Fyb antigens with malaria symptomology. Interestingly, combination of Fya*Kpa was observedwhere they were positively associated with frequent malaria episodes.Fya by itself was seen to be negatively associated with frequent malaria, however, together with Kpa i.e. Fya*Kpa, they were positively associated with frequent malaria episodes indicating that Kpa is the deciding antigen. It is worthwhile to mention here that kell proteins have been known to possess proteolytic activity and hence may facilitate invasion processby *falciparum* malaria parasite.

Diverse and extensive allelic polymorphism was seen for the Merozoite surface protein (MSP) 2 locus at both the study sites of *Plasmodium falciparum*was observed.Our data indicated that allele distribution was site dependent with some alleles common to the two sites but some were unique to a site. Ethnicity, site and gender were seen as confounding factors in MOI. Appearance of an allele of 330 base pair of FC-27 allelic family in summer of 2006, the period where malaria outbreak occurred, encourages us to speculate on the possibility of its emergence to be associated with malaria outbreakand this need to be investigated further.

Ethnicity was seen to be an important factor in the present study and their association with malaria pathogenesis. Consistent with their history of migration, the Austro-Asiatics and Tibeto-Burmans differed in their promoter region of as confirmed by sequencing. Our study demonstrated that AA (plains) had the favourable genotype with respect to TLR9 polymorphisms which protected them from malaria pathogenesis. In addition, AA were also observed to have lesser number of clones and this could be due to them having a better immune response to *falciparum* malaria and also may be related to their longer exposure to malaria.Since the presence of Kpa along with Fya was associated with frequent episodes of malaria, AA had the least

prevalence of Kpa and thus protected from frequent malaria episodes. Elevated levels of IL-18 along with TNF-α predisposes Tibeto-Burmans to suffer from complicated malaria and this was not seen for AA.From our observations, we can finally conclude that, the Austro-Asiatics have appeared to have accumulated favourable variationswhich protected them from malaria pathogenesis as compared to the Tibeto-Burmans of our study population.

CHAPTER 8:

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CHAPTER 9: LIST OF PUBLICATIONS and CONFERENCES

PUBLICATIONS FROM THESIS:

1. Sawian, C.E., Lourembam, S.D., Banerjee, A., Baruah, S. 2013. Polymorphisms and expression of TLR4 and 9 in malaria in two ethnic groups of Assam, northeast India *InnateImmunity* 19 (2) , pp. 174-183
2. Lourembam, S.D., Sawian, C.E., Baruah, S, 2013. Dysregulation of cytokines expression in complicated falciparum malaria with increased TGF-β and IFN-γ and decreased IL-2 and IL-12 *Cytokine* 64 (2) , pp. 503-508
3. "Association of a 330base pair allele of FC-27 allelic family of MSP2 gene with malaria outbreak in Assam, India" 2013; (Communicated).

International Conference (Poster presentation)

1. "NK Receptor polymorphism and RBC antigens in malaria"was presented at Indo-Australia conference- "Human variation and Pharmacogenomics, 2007" held at Manipal University, Manipal, India.

OTHER PUBLICATIONS:

1. Lourembam S.D, Sawian C.E. Baruah S,2011 Differential association of KIR gene loci to risk of malaria in thnic groups of Assam, Northeast India Infection Genetics and Evaluation, Volume 11, Issue 8, December, Pages 1921–1928

2. Kumar V, Sawian C.E, Mohanta D, Baruah S, Islam S, 2011, Physical and Biophysical Characteristics of Nanoscale Tungsten Oxide Particles and Their interaction with Human Genomic DNA, Journal of Nanoscience and Nanotechnology, Volume 11
3. Konwarh,R., Karak N., Sawian C, Baruah S and Mandal M 2010. Effect of sonication and aging on the templating attribute of starch for green silver nanoparticles and their interactions at bio-interface. Carbohydrate Polymers
4. Baruah S, Lourembam SD, Sawian CE, Baruah I, Goswami D, 2009, Temporal and Spatial variation in MSP1 clonal composition of Plasmodium falciparum in districts of Assam, Northeast India. Infection, Genetics and Evolution Vol 9:853 859