ALTERATION OF SIALIC ACID LEVELS DURING *PLASMODIUM FALCIPARUM* INFECTION IN HUMANS

BY

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AUGUST, 2018

Declaration

I, declare that the work in this dissertation entitled **"Alteration of Sialic Acid Levels During *Plasmodium falciparum* Infection in humans"** has been carried out by me in the Department of Biochemistry, Ahmadu Bello University. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

IKA DANJUMA MBATEUDI _____ . _____

Signature Date

Certification

This dissertation report entitled **"Alteration of Sialic Acid Levels During *Plasmodium falciparum* Infection in humans"** By IKA DANJUMA MBATEUDI meets the regulations governing the award of the degree of Master of Science in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dedication

This work is dedicated to GOD almighty for his mercy, love and grace.

Abstract

Malaria is still a major disease that causes millions of deaths, mostly among children under five years old and pregnant women, in tropical countries, especially sub-Saharan Africa. The present study was aimed at investigating the alteration in sialic acid level during *Plasmodium falciparum* infection in human and the presence of sialidase gene in the parasites genome. Individuals were grouped as > 5years male (A5-M), > 5years female (A5-F), 6 months to 60 months of age male (U5-M) and 6 months to 60 months of age female (U5-F) for both infected *P. falciparum* individuals and control respectively. Packed cell volume, hemoglobin concentration, white blood cell and red blood cell count were measured using an automated hematological analyzer. Free serum sialic acid (FSSA), total bound sialic acid (TBSA) and total sialic acid (TSA) concentrations were also analyzed using thiobarbituric acid method, while Basic Local Alignment Search Tool (BLAST) was used to investigate the presence of sialidase gene in the genome of *P. falciparum*. The results show a significant decrease ($P<0.05$) in packed cell volume and haemoglobin level and red blood cell in *P. falciparum* infected subjects when compared to their respective control groups. Changes observed in the concentrations of FSSA, BSA and TSA were not significant ($P>0.05$). The result further showed no significant ($P>0.05$) relationship between FSSA-TBSA, PCV - FSSA, FSSA-parasitemia during *P. falciparum* infection. BLAST analysis indicated non-existence of sialidase gene in *P. falciparum* owing to high expected value (E-value) and low Max scores confirming the low homology/similarity observed. In conclusion, no alteration was observed in sialic acid profile during P. falciparum infection and no sialidase gene was detected from the BLAST search.

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List of Abbreviations

1.	EBA	Erythrocyte Binding Antigen
2.	GPI	Glycosylphosphatidylinositol
3.	*Pf*Rh	*P. falciparum* Reticulocyte-like binding Homologue
4.	SA	Sialic Acid
5.	PCV	Packed cell volume.
6.	HGB	Haemoglobin
7.	WBC	White blood cells
8.	Neu5Ac	N-acetyl neuraminic acid
9.	CDC	Centre for Disease Control
10.	Kdn	2-keto-3-deoxynononic acid
11.	FSA	Free sialic acid
12.	BSA	Bound sialic acid
13.	TSA	Total sialic acid
14.	BLAST	Basic Local Alignment Search Tool

CHAPTER ONE

1.0 INTRODUCTION

Malaria is a disease caused by a group of intracellular parasites belonging to the *Plasmodium* genus (Marcilla *et al*, 2014). Different species of *Plasmodium* are known to cause malaria in mammals and among these species, *Plasmodium falciparum* is considered to be the most dangerous species that prevails mostly in Africa (CDC, 2015). This species of the parasite is found mostly in the tropical and subtropical areas of the world. Latest estimate released by World Health Organization (2016) on *P. falciparum* infection in Africa, showed that in 2015 alone approximately 212 million cases of malaria were recorded with about 429 000 deaths, mostly among under five children and pregnant women.

Sialic acid dependent and sialic acid independent pathways are the two major invasion pathways that have been implicated for the invasion of *P. falciparum* parasite into the erythrocyte (Satchwell, 2016). The sialic acid dependent pathway is being governed by ligands from the erythrocyte binding-like (EBL) family and the reticulocyte binding-like (Rh) family, except for Rh1 which mediates sialic acid independent invasion (Ord *et al*., 2012).

Sialic acid is a family of negatively charged monosaccharide which is a nine carbon acidic sugar, found as terminal residues in glycans of the glycoconjugates of eukaryotic cell surface or as components of capsular polysaccharides or lipooligosaccharides of some pathogenic bacteria (Li and Chen, 2012). It has been

considered as one of the most important molecule in life due to its peculiar strategic location and its structural diversity (Chen and Varki, 2010).

Sialidase is an enzyme that catalyses the removal of terminal sialic acid residues attached to glycoproteins and glycolipids on erythrocytes, which is the initial step taken in the breakdown of glycoconjugates. The enzyme is widely spread across different organisms such as fungi, bacteria and protozoa (Engstler *et al.,* 1993; Miyagi *et al.,* 2008; Hayre *et al*., 2012). Sialidase acts on and cleaves sialic acid found on the surface of erythrocytes by desialylating them, thereby exposing the masked galactosyl residues (Akin-Osanaiye *et al*, 2013).

Sialidase has significant roles in many physiological and pathological processes in biological system which includes; immunological regulation, viral infection, process and spread of disease, microbes binding that leads to infection among others (Varki and Varki, 2007). For invasion of pathogens into the cell, sialidase and trans-sialidase are involved in the cleavage of sialic acid from the surface of erythrocytes (Varki and Varki, 2007).

Female anopheles mosquitoes are responsible for the transmission of malaria into the mammalian host. Sporozoites from the vectors are injected into the host (vertebrate) via saliva during a blood meal (Cowman and Crabb, 2006) and after then, the sporozoites infect the host's liver cells where it undergoes rapid asexual reproduction of merozoites that invade circulating erythrocytes (Dhangadamajhi *et al*, 2010; Soulard *et al*. 2015). Multiple ligand proteins are expressed in *P. falciparum*, which are used to recognize specific targets on erythrocyte surface. The major targets found on the erythrocyte surface are terminal sialic acid on *O*-

linked glycan chains attached to glycophorins, which are the most abundant erythrocytes surface glycoproteins (Varki and Gagneux, 2009).

Specific ligand-receptor interactions are required between the parasite and the erythrocyte for the successful invasion of *P. falciparum* (Duraisingh *et al*., 2003). The two major ligand families that are implicated in these interactions, are the micronemal protein from the erythrocyte binding ligands (EBL) family (EBA-175, EBA-140, EBA-181 and EBL-1) and the rhoptry proteins from the reticulocyte binding homolog (*Pf*RH) (*Pf*RH1, *Pf*RH2a, *Pf*RH2b, *Pf*RH3, *Pf*RH4, and *Pf*RH5) (Ord *et al*., 2012; Koch and Baum, 2016). These families of ligand aid in the parasites invasion either through sialic acid dependent (in the case of EBL) or sialic acid independent pathways (Satchwell, 2016).

1.2 Statement of Problem

Malaria imposes a huge health burden on the world's most vulnerable populations, claiming the lives of nearly a million children and pregnant women each year in Africa alone (Crompton *et al*., 2014).

Multiple ligand interaction between parasite ligand and their receptors makes it successful for the parasite to invade erythrocyte. Sialic acid dependent and independent pathways are the two major pathways that the parasite utilizes during its invasion process into the erythrocytes (Crosnier *et al*., 2011, Ord *et al*., 2012).

Studies by Sharma *et al* (1992), indicated that *P. falciparum* utilizes the sialic acid dependent pathway due to its ability to synthesize sialidase, while Clough *et al* (1996) reported that *P. falciparum* lacks the ability to synthesize sialidase, making

the two studies seemingly contradictory. Hence, it is not yet fully ascertained whether or not the invasion of erythrocytes by the parasites involves cleavage of membrane bound sialic acid into the blood stream by a *P. falciparum* sialidase.

1.3 Justification

Pathogenic infections such as malaria have been a major health and socioeconomic setback in Nigeria and other countries where malaria exist. In the complex life cycle of the parasite, invasion of the erythrocyte is the most important step in its pathogenicity, where it utilizes the sialic acid dependent or independent pathways. In order to fully decipher whether these pathways involves hydrolysis of sialic acids, the changes in the serum sialic acid levels among malaria infected patients can be monitored.

Also, considering the crucial role of sialidase in the hydrolysis of sialic acid, there is need to investigate the presence of its gene in the genome of *P. falciparum*, which will pave a way in understanding more about the disease and the mechanism of its pathogenesis.

1.4 Aim

The aim of this study was to assess the changes in the sialic acid profile of *P. falciparum* infected patients and to investigate the presence of sialidase gene in the genome of the parasite using bioinformatics approach.

1.5 Objectives

1. Determination of the effect of malaria parasite density on some haematological indices (PCV, RBC, hemoglobin and WBC) in various categories of *P. falciparum*-infected patients.

2. Determination of the level of total bound sialic acid, free serum sialic acid and total sialic acid in various categories of *P. falciparum* infected individuals.

3. To asses for the presence of sialidase gene in the genome of *P. falciparum* using bioinformatics studies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Brief History of Malaria

Malaria is a disease that is caused by protozoan parasites which belong to the *Plasmodium* species. These parasites are transmitted to the host by bites of infected female *Anopheles* mosquito (Cox, 2010) during a blood meal. The understanding of parasites came into limelight in the 1880's, following its discovery by a French army surgeon Charles Louis Alphonse Laveran (Cox, 2010), who was stationed in Constantine, Algeria (CDC, 2015). He was able to discover the agent causing malaria while examining the blood of an infected patient that was febrile for 15 days (CDC, 2015).

Before the discovery of malaria parasite in 1880's by Charles Louis Alphonse Laveran, earlier theories were that bad air ("mala aria" in Italian) from marshlands were responsible for malaria infection. However, following the discoveries of Louis Pasteur that most infectious diseases are caused by microbial germs (the "germ theory"), the hypothesis of a bacterial origin of malaria became increasingly attractive (CDC, 2015).

The symptoms of malaria were described in ancient Chinese medical writings. Several characteristics symptoms of malaria were described in the *Nei Ching* (the canon of medicine) in the 2700 BCE. Malaria was widely recognized in Greece since 4th century BC, and it was accountable for the fall in the population of many cities. By the age of Pericles, there were extensive references to malaria in the

literature and depopulation of rural areas was recorded. In the *Susruta*, a Sanskrit medical treatise, the symptoms of malarial fever were described and attributed to the bites of certain insects. A number of Roman writers attributed malarial diseases to the swamps (CDC, 2015).

2.2 The Genus *Plasmodium*

Plasmodium belongs to the family Plasmodiidae, order Haemosporidia and phylum Apicomplexa, and is well known to be the causative agent of malaria. *Plasmodium* species responsible for humans' infection in nature includes: *P. vivax, P. malariae, P. ovale, P. falciparum, P. knowlesi* (WHO, 2015). In addition, another *Plasmodium* specie responsible for macaques' infection is now recognized to be a cause of zoonotic malaria in humans (Dhangadamajhi *et al*., 2010; CDC, 2015). Among these species, *P. falciparum* is known to cause severe form of malaria, termed malaria tropica, with a high mortality and morbidity rate worldwide (Cowman and Crabb, 2006). *P. vivax* and *P. ovale* cause malaria tertiana and *P. malariae* causes malaria quartana with lesser fatality (Cox, 2010).

Plasmodium falciparum is found throughout South East Asia and in parts of South America but is hyper-endemic in most of sub-Saharan Africa (Cowman and Crabb, 2006). As estimated, approximately 1 million cases of deaths are being recorded annually due to infections by *P. falciparum*, especially in countries where these species dominate. According to the center for disease control (CDC) 2015, *P. falciparum* can cause severe malaria due to its rapid multiplication in the blood, thereby causing severe blood loss (anemia).

2.2.1 Life Cycle of *Plasmodium falciparum* Parasite

The complex life cycle of *P. falciparum* involves some critical developmental stages that encompasses both invertebrate (mosquito) vector and a vertebrate (human) host as seen in Figure 2. 1 (Crompton *et al*., 2014). This complex life cycle begins when sporozoites (a highly motile differentiated form of the parasite) are injected into the bloodstream by infected female *Anopheles* mosquito during a blood meal (Dhangadamajhi *et al*., 2010; Crompton *et al*., 2014). The injected sporozoites then migrates into liver, where it invade the hepatocyte (Mota *et al*., 2001). In the hepatocyte, the sporozoites undergo mitotic division to produce thousands of merozoites that are released into the blood stream to invade circulating erythrocytes (marking the clinical manifestations of the disease) (Cowman and Crabb, 2006). In the blood stream, free merozoites invade the erythrocytes and undergo replication to form schizont for the asexual stage of the parasite (Kumaratilake *et al*., 1992; Crompton *et al*., 2014), which is associated with some clinical symptoms observed during malaria infection (Schmidt *et al*., 2015).

The life cycle of *P. falciparum* in the human host is completed when the asexual blood-stage parasites differentiate into male and female gametocytes that are taken up by a mosquito during a blood meal. The male and female gametes undergo fusion in the mosquito's midgut to form ookinetes which crosses the midgut epithelium and eventually differentiate to form sporozoites that invade the mosquito's salivary glands, thereby completing the life cycle of the parasite in its invertebrate host (Crompton *et al*., 2014).

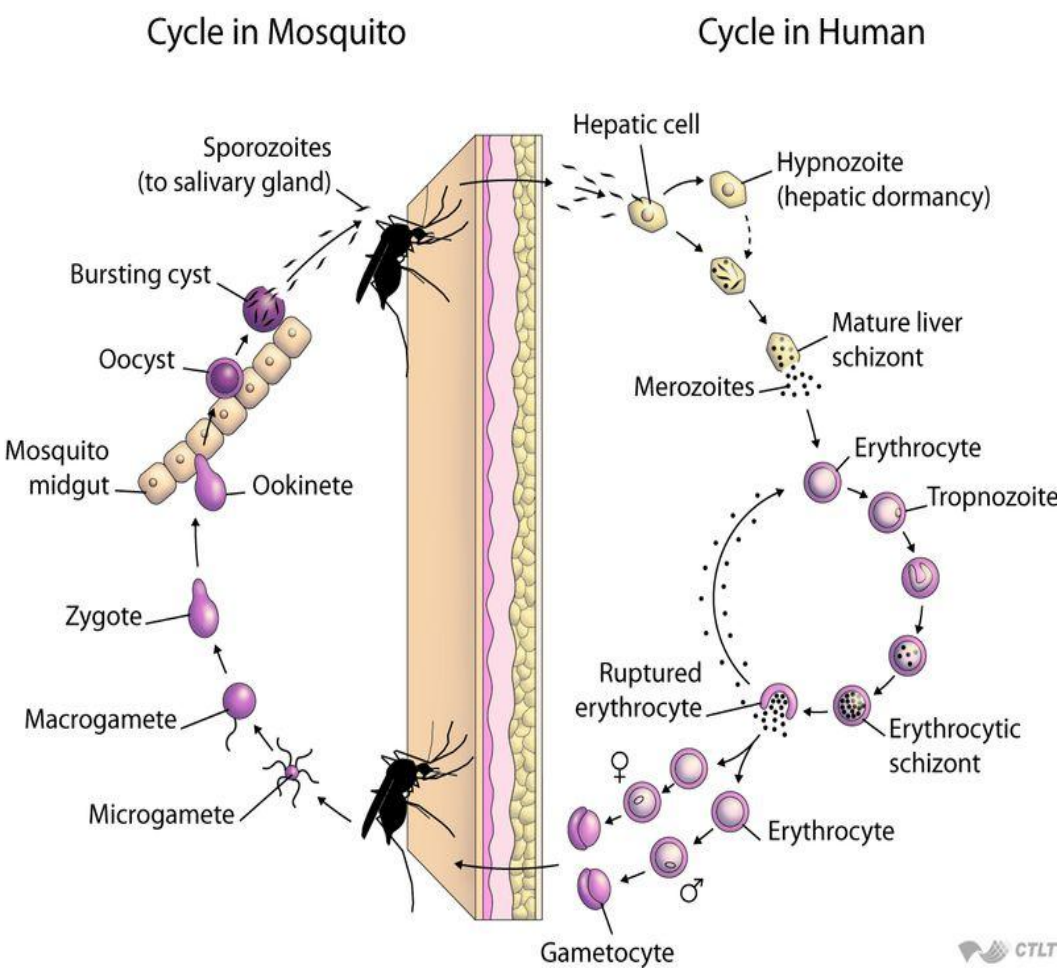


Figure 2. 1 Life cycle of *Plasmodium* parasite. The life cycle of parasite *P. falciparum* starts in human with inoculation of parasite through mosquito. Sporozoites are injected into the liver and infect hepatocytes. The sporozoite multiplies and produces thousands of blood stage infective merozoites. These merozoites enter the blood stream and infect the erythrocyte to start the erythrocytic cycle of parasite. In erythrocyte, they go through different stages of development before merozoites are released into the blood stream to infect new erythrocytes. A small percentage of asexual parasite transforms into sexual form, i.e., gametocytes, which finally develop into sporozoites in the mosquitoes. (Source: Morrow, 2007)

2.3 Pathogenesis of Malaria Infection

The pathogenesis of malaria infection is complex, and the spectrum of its manifestation differs from children to adult (Wassmer *et al*., 2015). Some unique features seen during malaria infection include; cytoadherence of infected red blood cells (IRBC) to the vascular endothelium and rosetting of the erythrocytes. These features contribute partly to vascular damage and consequent excessive inflammatory/immune response of the host organism. These features can explain some clinical manifestation that occur during infection process (Treeratanapiboon *et al*., 2005).

Acute anemia is said to occur due to an increase in parasitemia level which could lead to a massive destruction of circulating erythrocytes (Ghosh and Ghosh, 2007). Increase in the destruction of both parasitized and non-parasitized erythrocyte due to splenic sequestration also serve as features leading to anemia during malaria infection (Wassmer *et al*., 2015).

2.4 Merozoite Invasion Process

Erythrocyte invasion by merozoite is central to the pathogenesis of malaria infection (Crosnier *et al*., 2011). This process is complex and rapid (Haldar and Mohandas, 2007; Wright and Rayner, 2014). It involves four major steps that include primary contact and adhesion, reorientation of the apical portion for direct contact with the erythrocyte surface, formation of the tight junction complex between the erythrocyte surface and the merozoite apical region, and finally, ingress and migration of the tight junction complex (Farrow *et al*., 2011). The

formation of tight junction complex is a secondary ligand interaction that is mediated by specific parasite ligand-erythrocyte receptor interactions (Gaur and Chitnis, 2011; Crosnier *et al*., 2011), which aid in the parasites invasion into the erythrocytes. This cascade of interactions that permit parasites merozoite to invade uninfected erythrocyte serve as prerequisite for the survival of the parasite inside its human host (Cowman and Crabb, 2006).

The existence of alternative pathway for a successful invasion of erythrocyte by *P. falciparum* has long been established (Gomez-Escobar et al., 2010). Two families of parasite molecules (ligands) are utilized during merozoites invasion into the erythrocytes. These molecules include the micronemal proteins from the erythrocyte binding ligand (EBL) family ;EBA-140, EBA-175, EBA-181 and EBL-1, and the rhoptry proteins from the reticulocyte binding homolog (*Pf*Rh) family; *Pf*Rh1, *Pf*Rh2a, *Pf*Rh2b, *Pf*Rh4, and *Pf*Rh5 (Ord *et al*., 2012). The release of these protein ligands (EBA & *Pf*Rh) from the micronemes and rhoptries to the apical surface of the parasite merozoites is crucial to the parasite survival and the invasion process (Gaur and Chitnis, 2011).

For successful merozoites invasion of the erythrocytes, there is need for the formation of a complex between merozoites ligands (EBA & *Pf*RH) and some specific receptors on the erythrocyte surface. For example, glycophorin A serves as a receptor for EBA-175 (Wanaguru *et al*., 2013), glycophorin B for EBL-1, glycophorin C for BAEBL (EBA-140), complement receptor 1 for *Pf*RH4 (Spadafora *et al*., 2010; Tham *et al*., 2010), and basigin for *Pf*RH5 (Crosnier *et al*., 2012; Chen *et al*., 2014). From previous studies, it has been well established that

glycophorins play a vital role in merozoites attachment on to the erythrocyte surface during invasion. These glycophorins are relatively abundant on erythrocyte surface and also serve as an anchor for sialic acid (Baldwin *et al*., 2015).

The merozoites ligands invade erythrocyte by utilizing the different alternative invasion pathways that have been established (Persson *et al*., 2013). Parasite ligands in the EBL family associate with sialic acid residues on the surface of erythrocyte which facilitate parasites invasion into the erythrocyte; this process is termed sialic acid dependent pathway due to their dependence on sialic acid for invasion. Parasite ligands EBA-175, EBL-1 and EBA-140 are known to interact with glycophorins A, B and C respectively in a sialic acid-dependent manner. EBA-181 and *Pf*Rh1 also invade erythrocyte via sialic acid-dependent pathway, but their specific receptors on erythrocyte surface still remain unknown (Ord *et al*., 2012.,Koch, 2016., Beeson et al., 2016).

Erythrocyte invasion assays on different isolates of *P. falciparum* showed differences in their dependence on sialic acid for invasion (Gomez-Escobar *et al*., 2010), indicating that invasion by *P. falciparum* merozoites on erythrocytes surface is not solely dependent on sialic acid but through multiple invasion pathways which either depend on sialic acid or are independent of sialic acid. These differences predict the presence of another alternate invasion pathway that is independent of sialic acid. Ligands from Rh; *Pf*Rh2a, *Pf*Rh2b, *Pf*Rh4, and *Pf*Rh5 appear to play pivotal roles in sialic acid-independent invasion of erythrocytes (Gunalan *et al*., 2011., Sahar *et al*., 2011). *Pf*Rh4 has been shown to interact with complement receptor 1 (CR1) (Tham *et al.*, 2010) while *Pf*Rh5 binds to basigin (BSG) (Crosnier

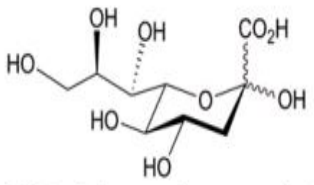
et al., 2011). Due to the ample collections of ligands that the parasite possesses, EBL and Rh3 proteins show functional redundancy, giving the parasite the opportunity to switch between sialic acid-dependent and sialic acid-independent pathway (Ord *et al*., 2012).

2.5 Sialic acid

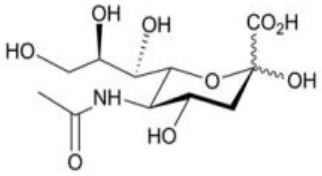
Sialic acid is a family of structurally unique *N*- or *O*-substituted derivatives of neuraminic acid (Mahajan and Pillai, 2016) that are composed of negatively charged 9-carbon monosaccharaide (Kim., 2014). It is expressed as terminal buildup on eukaryotic cell surface attached to glycoconjugates and some higher invertebrates (Chen and Varki 2010; Varki *et al*., 2011). More than 50 different sialic acid derivatives are found in nature (Li and Chen, 2012). N-acetyl neuraminic acid (Neu5Ac) (Varki and Schauer, 2009) and 2-keto-3-deoxynononic acid (Kdn) (Figure 2.3) are the two most abundant forms of sialic acid found in nature, which also serve as derivatives of other sialic acid (Chen and Varki, 2010). These derivatives differ by structural modification at position 5 (C-5), with an acetamido or a hydroxyacetamido moiety respectively (Freire-de-Lima *et al.*, 2012).

Sialic acid has long been discovered by Gunnar Blix, Ernst Klenk, and other investigators as a major product released by mild acid hydrolysis of brain glycolipids or salivary mucins (Varki and Schauer, 2009). The structure, chemistry, and biosynthesis of the compound that they obtained (*N*-acetylneuraminic acid, a

9-carbon, acidic α-keto sugar) were later unraveled in the 1950s and 1960s by multiple groups of researchers (Varki and Schauer, 2009).



A. 2-keto-3-deoxynononic acid (Kdn)



B. N-acetyl neuraminic acid (Neu5Ac)

Figure 2.3. Structure of the two common primary sialic acid.**A**. 2-keto-3-deoxynononic acid, (Kdn) and **B**. N-acetylneuraminic acid, (Neu5Ac) are the two common primary sialic acid found in nature. **C**. (Adopted from Deng et al., 2013)

2.5.1 Diversity of Sialic Acids

High structural and functional diversity is one of the fundamental characteristics of the sialic acid family. These molecule is usually found as terminal branches of N-glycans, O-glycans and glycophospholipids (Varki and Schauer, 2009). In nature, more than 50 structurally different sialic acid derivatives exist (Chokhawala *et al*., 2008; Li and Chen, 2012). These diversities are seen in two different levels (Varki and Schauer, 2009), which include diversity in structural modification and linkage.

Some of the biochemical properties that confer special structural properties on sialic acid molecules include its chemical structure, terminal position on cell surface and the negative charge of sialic acid residues. These account for their biological relevance, serving as key regulators of molecular and cellular interactions (Schauer, 2009).

The occurrence of these molecule in nature have been extensively studied. This group of 9-carbon monosaccharide are being synthesized in deuterostome lineage of the echinoderms and in higher mammals, which are being considered to express the highest structural diversity of sialic acid (Angata and Varki., 2002). Low level of sialic acid are detected in insects and gastropod, while in protostomes these molecules are not produced in glycoconjugate forms (Muchmore et al., 1987; Kim, 2014). In humans, sialic acid has been found as component of milk oligosaccharides, as component of the outer layer of erythrocytes, in lysosomal membranes, etc.

Studies have shown the presence of sialic acid on many parasites such as *Trypanosoma cruzi* (cause of Chagas' disease). However, these organisms do not have the mechanisms to synthesize sialic acid on their own, but rather acquire it from their host organism, by the expression of a unique enzyme called *trans*-sialidase. These organisms use this molecule in order to protect themselves from immune detection and attack by the host defense systems (Angata and Varki., 2002; Buschiazzo *et al*., 2012).

2.5.3 Biological Functions of Sialic Acid

Due to the structural diversity and terminal location of sialic acid on cell surface, these molecules play some significant roles in the physiological and pathological processes in biological system (Traving and Schauer, 1998; Kim, 2014). These include: cellular recognition, cell signaling, nervous system, cancer, regulation of the immune system, and during infections by microorganisms (bacteria, parasite and virus), etc. (Angata and Varki 2002; Chen and Varki 2010). Sialic acid serves as receptor for pathogens, antibodies and other molecules, due to its position on the cell surface (Kim, 2014).

Sialic acid play pivotal roles in the stabilization of molecules on the cell membrane, and also in the regulation of interaction with the environment, due to its relative abundance and location on cell surface, which aids in the prediction of some biological events in the body (Varki, 1992; Schauer, 2004). This also suggests the reason why numerous microorganisms such as virus, parasites and some bacteria utilize sialic acid from host's cells as targets for binding and recognition, which aid in the survival and invasion of these microorganisms (Varki and Gagneux., 2012).

Sialic acid has a dualistic function, where it serves as a receptor and as a biological mask. These functions can be seen during immune evasion, where the immune system can differentiate between self and non-self due to the differences in the pattern of sialic acid structures. Absence of sialic acid on membranes, make molecules and cells to be recognized as foreign body by macrophages, via Gal - recognizing receptors, which are then taken up and degraded. This dualistic role of sialic acid in recognition and degradation of sialic acid is best studied in influenza viruses (Von, 2007; Varki and Gagneux, 2012). Several eukaryotic pathogens also employ sialic acid recognition as part of interactions with hosts (Orlandi., *et al.*, 1992., Dolan *et al*., 1994).

The location of sialic acid on the cell surface makes it to be the first target for recognition and attachment by most pathogens, many microbial host first target the sialic acid on the cell surface to aid in its survival and pathogenesis it the host organism, especially in the recognition of airway epithelial sialic acid by influenza viruses, binding of *P. falciparum* ligand EBA-175 to glycophorin A on erythrocyte surface during parasite invasion process via the sialic acid dependent pathway (Varki and Varki, 2007; Varki and Schauer, 2009).

Sialic acid also function in the transportation of molecules across the cell membrane (positively charged molecules), due to its relatively high negative charge. It also function during the attraction and repulsion phenomena that takes place between cells and molecules, which can be seen in erythrocte (Schauer, 2004).

The position of this sugar molecules at the terminal of glycoconjugates, coupled to their relative negative charge and size, aid in the protection of macromolecules and

cells from enzymatic (protease degradation on glycoproteins) and immunological attack as in the case of the mucous layer of the respiratory epithelium (Schauer, 2004). Another fascinating phenomenon of this molecule is the spreading effect exerted on molecules containing sialic acid. This spreading effect prevent the aggregation of blood components, stabilization of protein conformations (enzymes), and enhancing the viscosity of mucins (Schauer and Kamerling, 1997; Schauer, 2004; Varki, 2008). Furthermore, sialic acid can regulate the affinity of receptors and are reported to modulate processes involved in trans-membrane signaling, fertilization, growth, and differentiation (Varki and Schauer, 2009).

Sialic acid play a major role in the protection of infective agent from the host's immune response through molecular mimicry. The evolutionary advantage of this molecular mimicry is so evident that several parasites exploit cell-surface sialylation to survive within the host environment and establish the infection by the use of enzymes responsible for hydrolyzing sialic acid from the cell surface and transferring it onto itself (Vimr and Lichtensteiger, 2002). Example of such parasite is *Trypanosoma cruzi* that uses trans-sialidase to remove host's cell sialic acid molecules and transfer onto its own glycan units in order to prevent recognition and elimination by host immune system (Varki and Schauer, 2009). These microbial sialidase and trans-sialidase are powerful virulence factors that may assist in the invasion, nutrients for some bacteria (Varki and Schauer, 2009).

2.6 Sialidases

Sialidases (EC 3.2.1.18) are family of glycohydrolases that catalyze the hydrolysis of terminal sialic acid from glycans of glycoproteins or glycolipids. They catalyze

the cleavage of sialic acid linked to galactose via an α-2,3 or α-2,6 linkage, and furthermore to glucose via a β-1,4 linkage (Roy *et al*., 2010). These enzymes are synthesized in most vertebrates, as well as some microorganisms (e.g trypanosomes) that lack the mechanism to synthese sialic acid (Monti and Miyagi, 2012). In microorganisms, sialidase have different functions which aid in the survival and pathogenesis of organisms, especially in the process of attachment and invasion of host cells and also in protection against host immune system, etc (Monti and Miyagi, 2012).

Sialidase have been identified in mammals where it is involved in the modulation of many biological processes. These mammalian sialidase have also been implicated in lysosomal catabolism since sialidase deficient individuals develop lysosomal storage diseases such as sialidosis and galactosialidosis (Monti *et al*., 2002; Miyagi *et al*., 2004; Monti and Miyagi., 2012). Four mammalian sialidase have been identified and characterized (Monti and Miyagi., 2012) with diversity in their subcellular localization, physiological functions (Miyagi, 2008), and enzymatic properties, including substrate specificity. These group of enzymes include the lysosomal sialidase NEU1 (Pshezhetsky *et al*,. 1997), the cytosolic sialidase NEU2 (Monti *et al*., 1999), the plasma membrane associated sialidase NEU3 (Wada *et al*., 1999) and the lysosomal or mitochondrial membrane-associated sialidase NEU4 (Monti *et al*., 2004).

2.6.1 Sialidase from Protozoans

Sialidase is widely distributed among microorganisms in nature, with its existence in virus, bacteria, fungi, and parasites. The existence of sialidase and its

pathological role during viral and bacterial infections have been well studied and documented. In parasites, studies have shown the existence of sialidase in some Trypanosome species: *Trypanosoma rangeli, T. evansi, T. brucei* (Engstler *et al*., 1993) and in *Trypanosoma cruzi*, where it plays a role during invasion processes (Schenkman *et al.*, 1991). Another form of sialidase also exist in *Trypanosomes* known as trans-sialidase which aid in the transfer of sialic acid from the host organism onto the parasite in order to escape immune evasion (Nardy *et al*., 2016). In *Plasmodium* species, many studies have been conducted to check for sialidase activity which is involved in disease progression and in the survival of many microorganisms in the host's cell (Makler, 1987; Akin-Osanaiye *et al*., 2015).

2.7 Sialic Acid and Sialidase in *P. falciparum*

Studies on the sialic acid dependent pathway of *P. falciparum* invasion have been widely conducted, but there is no much information on the alteration of this molecule during invasion process. For a successful invasion, a ligand-receptor complex must be formed between *P. falciparum* merozoites and receptors (sialic acid attached to glycophorin A, B and C) on erythrocyte surface. Invasion Inhibition Assay shows a reduction in merozoites invasion when uninfected erythrocytes were treated with sialidase (neuraminidase) so as to remove the terminal sialic acid (Ord *et al*., 2012). This reduction in merozoites invasion was attributed to the lack of sialic acids on the cell membrane which were cleaved during neuraminidase treatment. Earlier studies conducted by Makler (1987) show sialidase-like activity during *P. falciparum* invasion, which is used in the

modification of the terminal sialic acids on glycophorin A during invasion process. In a similar study by Akin-Osanaiye *et al*. (2015), a sialidase activity was detected in *P. berghei* during its invasion process and also an increase in free sialic acid level during *P. berghei* infection.

Irregular distribution of sialidase gene among organisms of same species, makes its interesting for evolutionary studies (Makler, 1987). Studies conducted by Sharma *et al* (1992) reported that *P. falciparum* produces a neuraminidase like activity on invasion of erythrocytes in culture on the basis of biochemical and immunological investigations. On the other hand, Clough *et al.*, (1996) show that *P. falciparum* lack both sialidase and trans-sialidase activity. In their work, they try to examined the sensitivity of *P. falciparum* with 4-guanidino-2, 3-didehydro-5-acetyl neuraminic acid (4-guanidino Neu5Ac2en), a potent inhibitor of influenza virus sialidase. They observed that invasion and subsequent development of the parasite was not affected by the sialidase inhibitor. The inhibitor did not affect rosette formation of parasite-infected erythrocytes with uninfected cells nor their cytoadherence to C32 melanoma cells. Little has been conducted at molecular level to ascertain the presence of sialidase and trans-sialidase gene in *Plasmodium* species.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

Giemsa stain, thiobarbituric acid, periodate, 2% sodium arsenite, butanol, phosphate buffered saline (PBS).

3.1.2 Equipment

Spectrophotometer (MODEL. 6305) JENWAY, UK, Centrifuge (Labofuge 300, Heraeus), Auto Hematology Analyzer (BC-3600) Mindray, USA.

3.2 Methods

3.2.1 Study Area

This study was carried out in Zaria, North-West Nigeria. Zaria lies on longitude 11.066°N and latiute 7.7000°E. Blood samples were obtained from consenting patients who present themselves for malaria test at the University Medical Center and other Primary Health Care centers in Samaru and Hayin-dogo (Sabon Gari Local Government, Kaduna State).

3.2.2 Ethical Approval and Informed Consent

Ethical approval for this research was obtained from the ethical committee of Kaduna State Ministry of Health with reference number MOH/ADM/744/VOL.1/522. (Appendix 1.0).Written informed consent was obtained from each study volunteer or from the study participant's parents/legal guardian for study volunteers under 18 years of age.

3.2.3 Inclusion Criteria

Volunteer patients with positive result for malaria test and attend Ahmadu Bello University Medical Center, Samaru and Hayin dogo Primary Health Care Centers in Sabon Gari LGA, Kaduna State.

3.2.4 Exclusion Criteria

Patients with malaria complications, typhoid fever and other persistent infections and those that are under treatment for malaria infection.

3.6 Sample Size

The sample size was determined according to Ipsen *et al* 1970 as follows:

Sample size (n) $=\frac{Z^2 p(1-p)}{d^2}$

Z = standard normal deviation at 95% confidence interval 1.96

P= prevalence. 28.93% Bachemagbor, (2010)

d= allowable error 5% (0.05)

n= $\frac{3.8416x0.2893(1-0.2893)}{(0.05)^2}$

n= 316

3.7 Experimental Grouping

Samples were grouped into eight different groups based on sex and age

1. **A5-M**: *P. falciparum* infected patients > 5 years (male) (60)
2. **A5-F**; *P. falciparum* infected patients >5 years of age (female) (60)
3. **U5-M**: *P. falciparum* infected patients 6-60 months of age (male) (60)
4. **U5-F**: *P. falciparum* infected patients 6-60 months of age (female) (60)
5. **A5-Mc**: Apparently healthy individuals (control) >5 years of age (male) (20)
6. **A5-Fc**: Apparently healthy individuals (control) >5 years of age (female) (20)
7. **U5-Mc**: Apparently healthy individuals (control) 6-60 months (male) (10)
8. **U5-Fc**: Apparently healthy individuals (control) 6-60 months (female) (10)

3.8 Blood Sample Collection

Five (5) ml of blood were collected via venipuncture from *P. falciparum* infected patients and non-infected individuals and were then divided into two (2) separate tubes. Exactly 3 ml of the blood were placed into an EDTA tube for hematological evaluation and the other 2 ml were placed into anticoagulant-free tube for collection of serum.

3.9 Estimation of Parasitemia

Levels of parasitemia in the blood sample were determined by Giemsa stain as described by Garcia (2001). A thin and blood smear of whole blood were made on glass slides and allowed to dry in an incubator at 37°C for 1hour, the slides containing the dried blood smears were placed horizontally on the staining rack. A

10% dilution of Giemsa stain was used to flood the blood smear and allowed to stay for 30 minutes. The stain was then washed off gently using normal tap water until the excess stain was completely removed. The slides were air dried and made ready for microscopy. For the thin films containing the blood smears, the slides were first fixed with methyl alcohol for 2 minutes before the same staining process used in the thick film were then applied.. The slides were then allowed to dry again and observed with immersion oil at magnification of 100 X, (Cheesbrough, 2009). The parasite density of each parasitized sample were then calculated using the formula below,

$$\text{parasite density per } \mu L = \frac{\text{number of parasites counted x WBC per } \mu L(8000)}{\text{number of WBCs counted}}$$

3.10 Haematological Evaluation

Packed cell volume, red blood cell count and white blood cell count and haemoglobin concentration were all analysed using Auto Haematology Analyzer (BC-3600) at the Ahmadu Bello University Medical Center.

3.10.1 Principles of Operation Auto Haematology Analyzer

The BC-3600 Auto Hematology Analyzer (hereafter referred to as BC-3600) uses the impedance method to count and measure the size of WBC, RBC and PLT in human whole blood. The WBC, RBC and PLT are counted in a dedicated channel using the measurement of changes in electrical resistance. These blood cells are suspended in a conductive diluent and hydrodynamic technology is used to ensure

the cells pass through an aperture of known dimensions. The intensity of the electronic pulse from the cells passing through the aperture is proportional to the cell volume. HGB is determined by the colorimetric method. In addition, the analyzer uses a volumetric metering unit to control the count cycle and to ensure that a precise volume of sample is analyzed for the measurement.

3.11 Determination of Sialic Acid Concentration.

Free serum sialic acid was determined using the thiobarbituric acid method described by Aminoff (1961). Briefly, 0.5 ml of serum was mixed with 0.25 ml of 25 mM periodate in 0.125 N H_2 SO_4 and heated for 30 minutes in a water-bath at 37°C. Excess periodate was reduced with 0.2 ml 2% sodium arsenite in 0.5N HCl. As the yellow colour of the liberated iodine disappeared (1-2 minutes), 2ml of 0.1M of thiobarbituric acid solution was added. The tubes were then caped and heated in a boiling water-bath for 7.5 minutes. The colored solution was extracted with 5ml of acid butanol (n-butanol : conc. HCl, 95:5 % v/v) and then centrifuged. Absorbance of the butanol extract was measured using a spectrophotometer at 549 nm against a control blank. Total sialic acid was quantified using the same procedure after hydrolysis of the blood sample in 0.25ml of 0.1 N H_2SO_4 at 80^0C for 1 hour. The concentration of the total sialic acid and free sialic acid were calculated using a standard calibration curve. The total bound sialic acid was determined as the difference between the total sialic acid and free sialic acid. The distribution is thus;

$[SA]_B = [SA]_T - [SA]_F$

Where $[SA]_B$= Total Bound Sialic acid, $[SA]_F$ = Free Sialic acid, $[SA]_T$ = Total Sialic acid.

3.12 Multiple Alignment and BLAST Search for Sialidase Gene

Basic Local Alignment Search Tool (BLAST) was used to conduct a search on the genome of *P. falciparum.* The genome of *P. falciparum* and homologues of sialidase gene from different organisms were retrieved from plasmodb and NCBI gene bank (www.ncbi.nim.nih.gov) and queried against the *P. falciparum* genome (taxid: 5833), and multiple alignment using sialidase enzymes (protein sequence) from different organism was also performed using multiple sequence alignment with hierarchical clustering (Corpet, 1988).

Sialidase gene, mRNA and protein sequences from *Homo sapiens* (CAB41449.1), *Trypanosoma cruzi* (AAA30245.1) and *Mus musculus* (BAA92868.1), *Trypanosoma rangeli* (AAC95493.1), influenza A virus (AAA43413.1) and *Clostridium chauvoei* (CAR82361.1) retrieved from NCBI were used for blastp and tblastn search in the genome of *P. falciparum* (taxid:36329).

3.13 Statistical Analysis

Data were analyzed using Pearson correlation and sample t-test with the aid of SPSS version 20 for windows. Data obtained were expressed as mean ± standard deviation. *P* value less than 0.05 was considered significant ($P<0.05$).

CHAPTER FOUR

4.0 RESULTS

4.1 Parasite Density in *Plasmodium falciparum* Infected Patients

The parasitemia level in A5-M, A5-F, U5-M and U5-F are shown in Figure 4.1. The data shows higher level of parasitemia in U5-M and U-5F groups with 93.6 x 10^3 and 64.3 x10^3 parasite/µL of blood respectively, while the A5-M and A5-F had a lower parasitemia level of 13.3 x 10^3 and 12.0 x 10^3 parasite/µL of blood respectively.

4.2 Hematological Parameters

Hematological parameters in *P. falciparum* infected individuals for A5-M, A5-F, U5-M and U5-F showed a significant decrease ($P<0.05$) in White Blood Cell count when compared to their respective control groups (apparently healthy) as shown on Figure 4.2.

Figure 4.3 presents the packed cell volume (PCV) level in both *P. falciparum* infected individuals and control groups (apparently healthy). The mean PCV levels of infected individuals for A5-M, A5-F, U5-M and U5-F groups were 42.29±0.94%, 37.73±0.63%, 34.33±1.41% and 31.04±1.39% respectively. These values were significantly lower ($P<0.05$) when compared to their respective control 48.39±1.37%, 42.12±1.10%, 41.92±0.91%, 39.44±1.12%. Considering the difference in age for infected individuals, there was an observed decrease ($P<0.05$) in the PCV level for under 5 years when compared to above 5 years.

A significant (P<0.05) decrease in the hemoglobin concentration among *P. falciparum* infected individuals relative to the control groups was observed as shown in Figure 4.4.

The changes in red blood cell count (RBC) during *P. falciparum* infection among infected individuals was not significant (P>0.05) when compared to their control groups as shown in Figure 4.5.

4.3 Sialic Acid Profile of *P. falciparum* Infected Individuals and Apparently Healthy Individuals.

The sialic acid profile among *P. falciparum* infected individuals and control gave no significant (P > 0.05) difference (Figure 4.6). However, the concentration of free serum sialic acid for U5-M and U5-F infected groups were observed to be higher (292.73±13.06µg/mL and 296.34±8.85µg/mL) than A5-M and A5-F 261.37±5.56 and 257.83±17.29µg/mL across the age group respectively.

The total bound sialic acid concentrations during *P. falciparum* infection are presented in Figure 4.7. The decrease observed was not significant (P > 0.05) when compared to the control groups. Figure 4.8 shows the level of total sialic acid (TSA) in *P. falciparum* infected individuals when compared to the respective control groups (apparently healthy). The results show no significant (P > 0.05) difference between the infected and control groups.

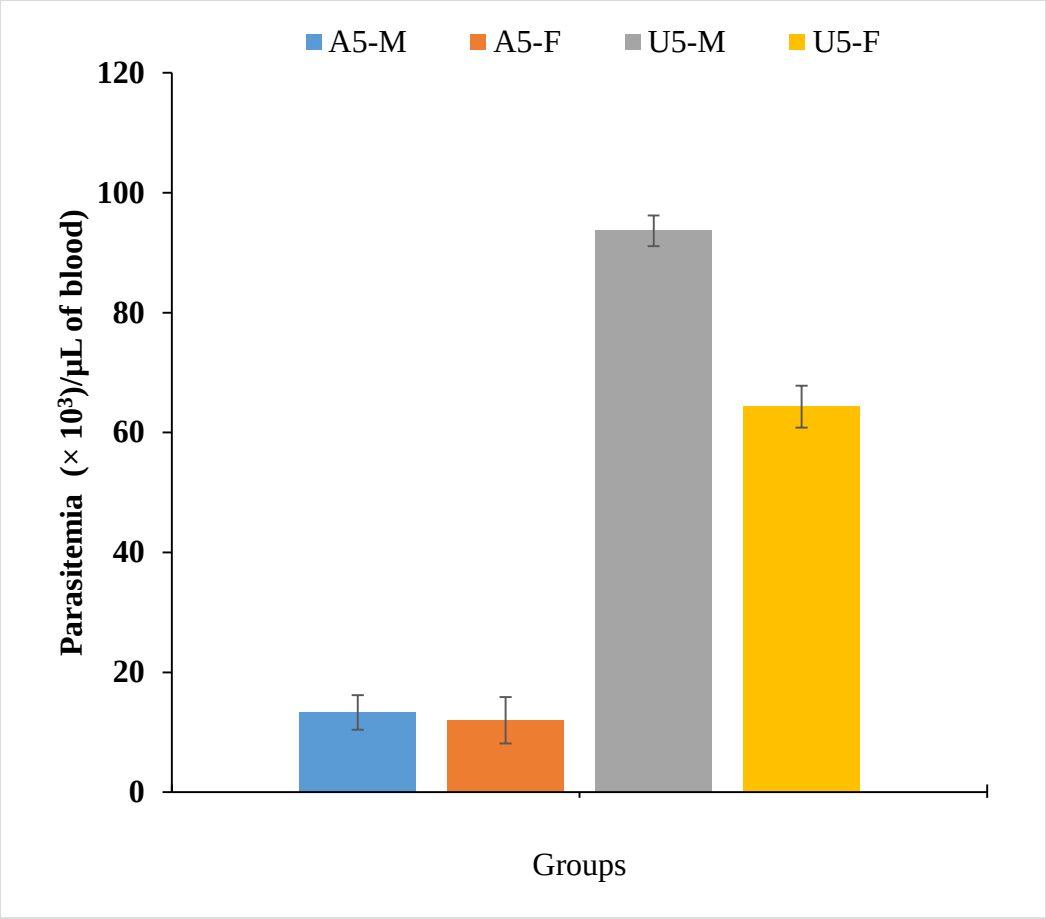


Figure 4.1 Parasitemia Level in Blood Samples of Malaria in Infected Individuals.

A-5M= *P. falciparum* infected patients > 5 years (male), **A5-F**= *P. falciparum* infected patients >5 years of age (female), **U5-M**= *P. falciparum* infected patients 6- 60 months of age (male), **U5-F**= *P. falciparum* infected patients 6-60 months of age (female).

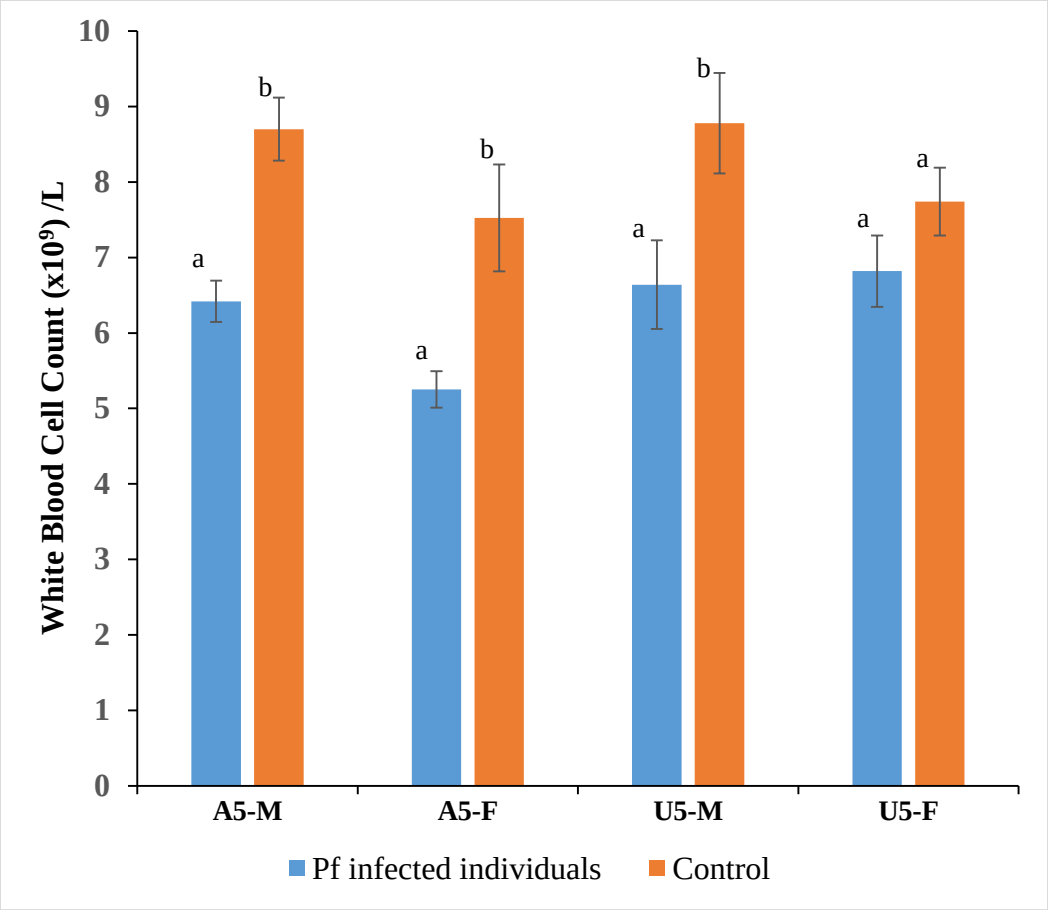


Figure 4.2 White Blood Cell Count in *P. falciparum* Infected Subjects and Control.

Bars with Different letters in each group are significantly different at ($P < 0.05$). **A5-M**= Male > 5 years, **A5-F**= Female > 5 years, **U5-M**= Male 6-60 months of age, **U5-F**= Female 6-60 months of age.

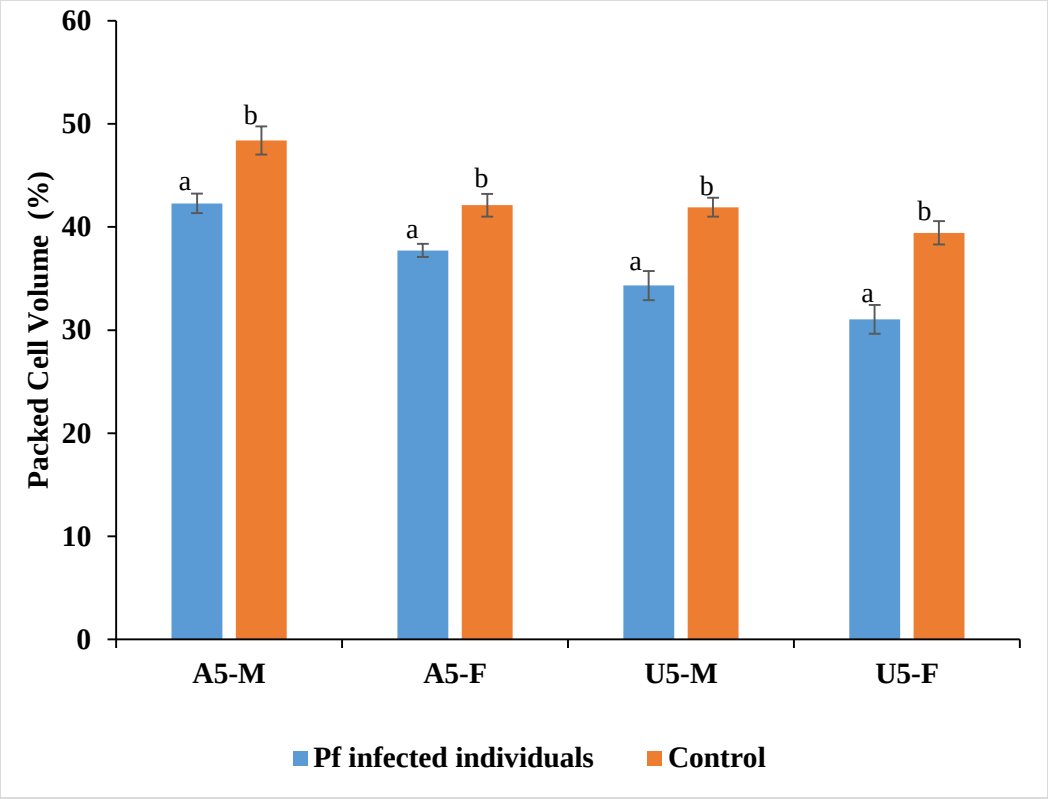


Figure 4.3 Packed Cell Volume in *P. falciparum* Infected Individuals and control.

Bars with Different letters in each group are significantly different at ($P < 0.05$). **A5-M**= Male > 5years, **A5-F**= Female > 5years, **U5-M**= Male 6-60 months of age, **U5-F**= Female 6-60 months of age.

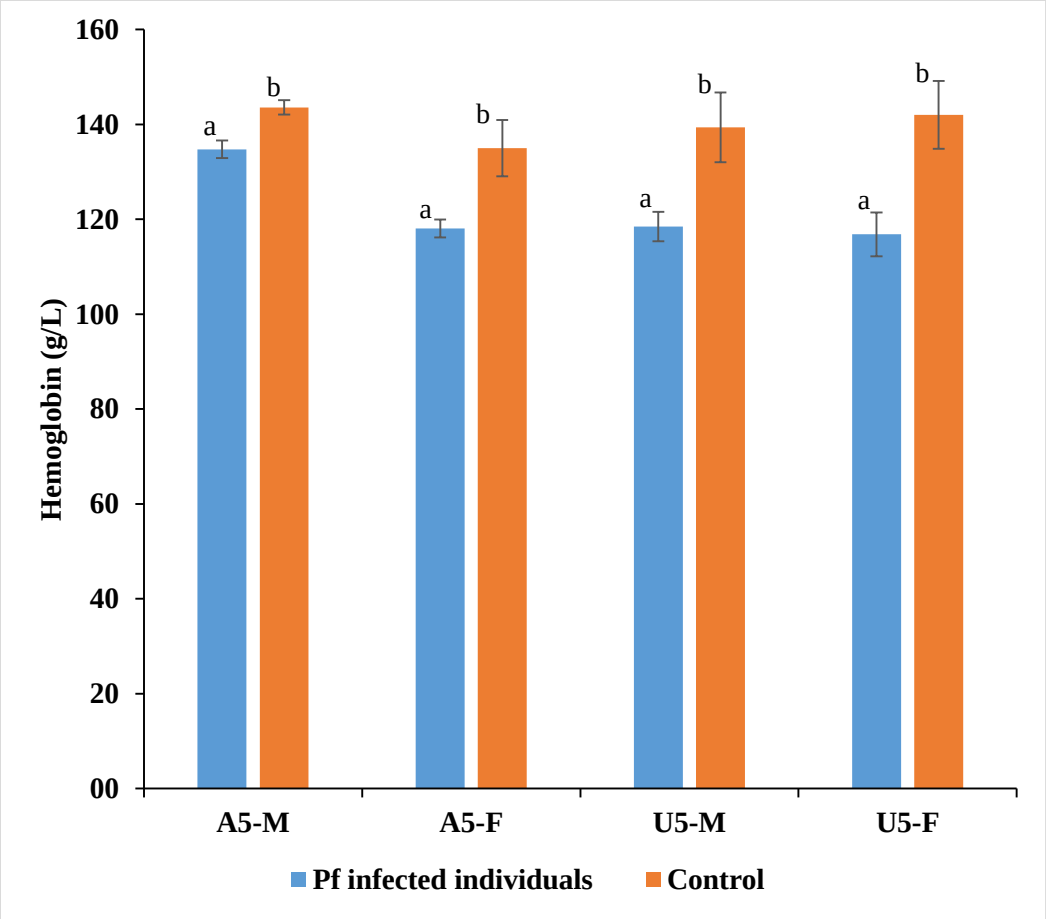


Figure 4.4 Hemoglobin Concentration in *P. falciparum* Infected Individuals and control.

Bars with Different letters in each group are significantly different at ($P < 0.05$). **A5-M**= Male > 5 years, **A5-F**= Female > 5 years, **U5-M**= Male 6-60 months of age, **U5-F**= Female 6-60 months of age.

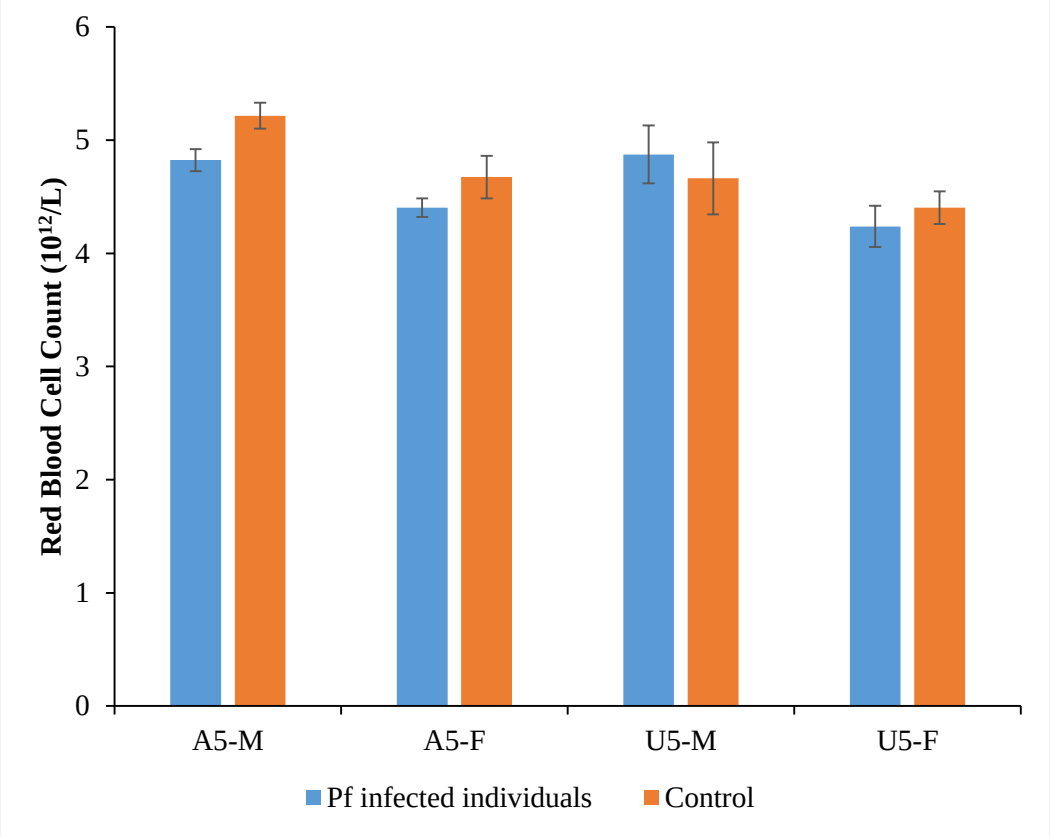


Figure 4.5 Levels of Red Blood Cell Count in *P. falciparum* Infected Individuals and control.

A5M= Male > 5 years, **A5F**=Female > 5 years, **U5M**= Male 6-60 months of age, **U5F**= Female 6-60 months of age.

4.4 Relationship between Free Serum Sialic Acid Concentration and parasitemia level among groups of *Plasmodium falciparum* Infected Individuals.

A weak positive (P>0.05) relationship between parasitemia level and free serum sialic acid concentration was observed among *P. falciparum* infected individuals during infection as presented in Table 4.1.

4.5 Relationship between total bound sialic acid and parasitemia level among groups of *Plasmodium falciparum* Infected Individuals.

The relationship between bound sialic acid at different wave of parasitemia is presented in Table 4.2. The results show a weak negative relationship between the concentration of total bound sialic acid and parasitemia level.

4.6 Relationship between Free Serum Sialic Acid Concentration and Parasitemia Level among groups of *P. falciparum* Infected Individuals by Age.

A correlation analysis between free serum sialic acid concentration and parasite density in *P. falciparum* infected individuals is presented in Table 4.3. The results show non corresponding relationship for all the groups.

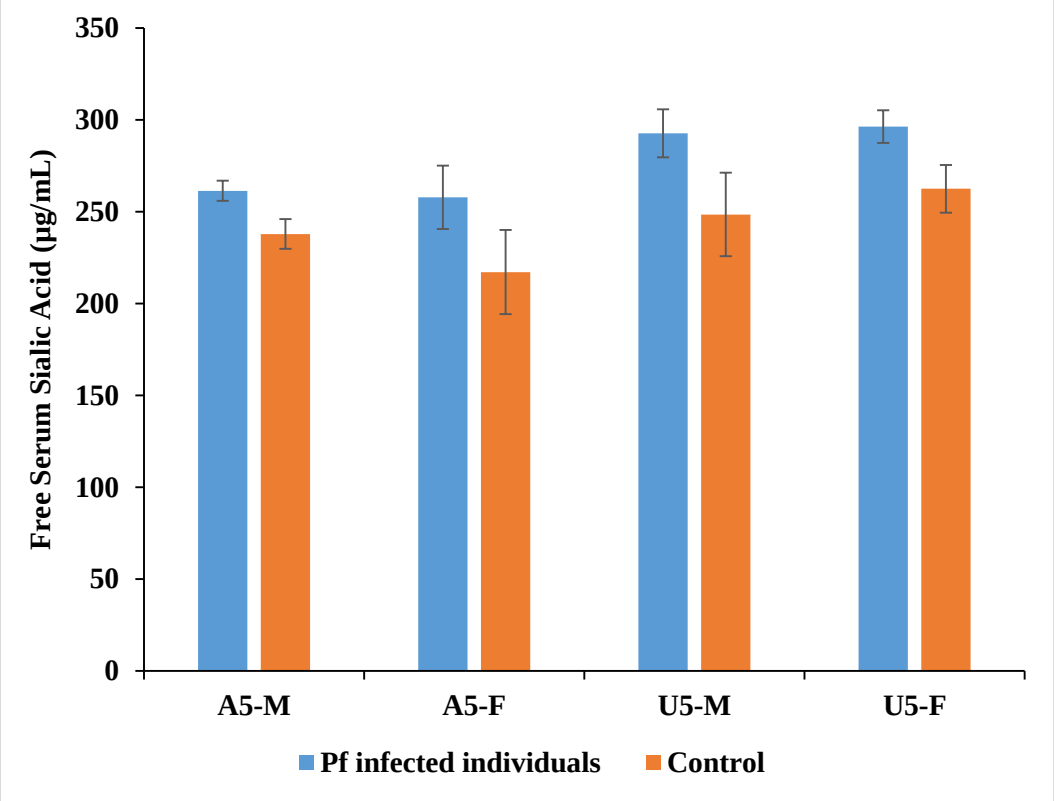


Figure 4.6 Level of Free Sialic Acid Concentration in both *P. falciparum* Infected Individuals and control group.

A5-M= Male > 5 years, **A5-F**=Female > 5 years, **U5-M**= Male 6-60 months of age,

U5-F= Female 6-60 months of age.

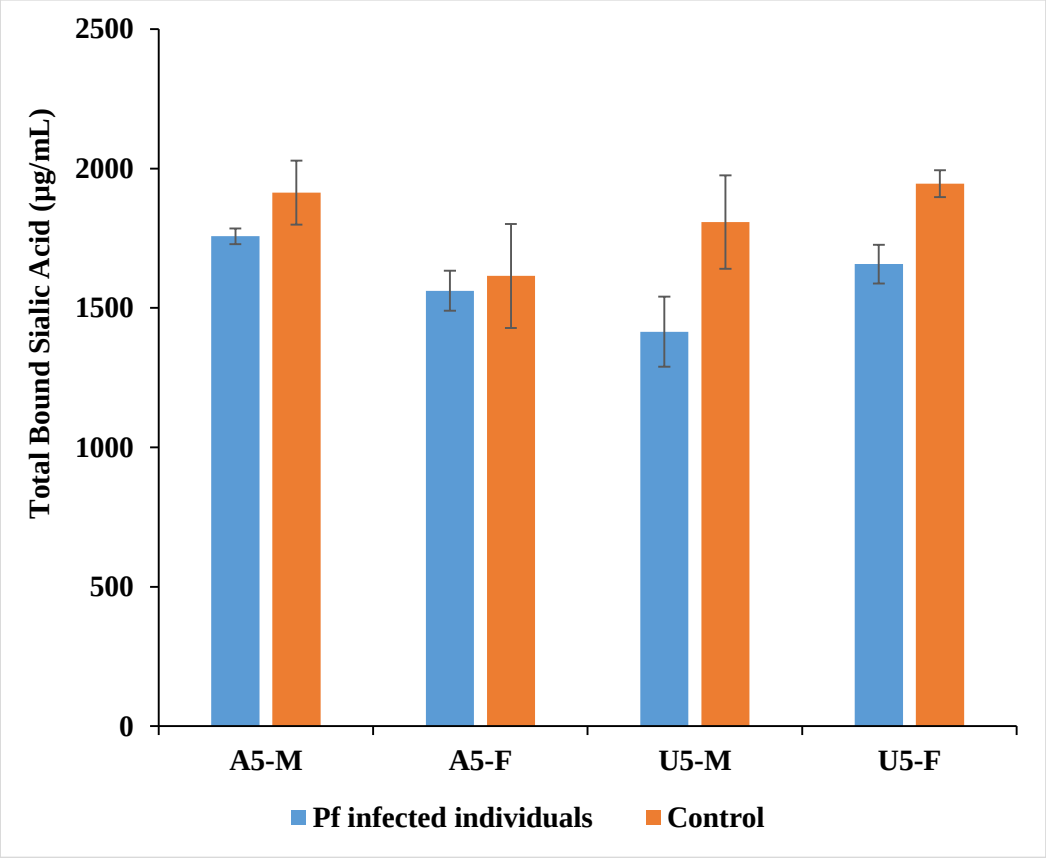


Figure 4.7 Level of Total Bound Sialic Acid Concentration in both *P. falciparum* Infected Individuals and Apparently Healthy Individuals.

A5-M= Male > 5 years, **A5-F**=Female > 5 years, **U5-M**= Male 6-60 months of age,

U5-F= Female 6-60 months of age.

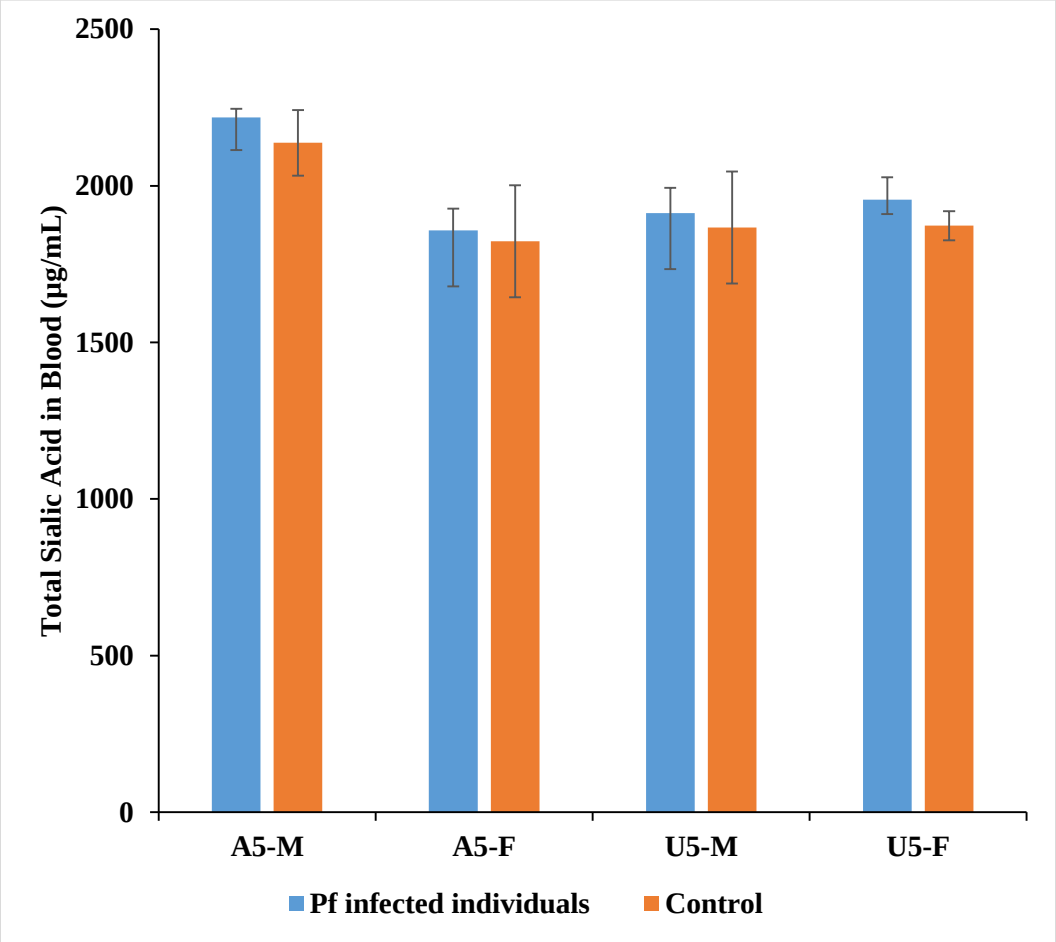


Figure 4.8 Level of Total Sialic Acid Concentration in both *P. falciparum* Infected Individuals and control groups.

A5-M= Male > 5 years, **A5-F**=Female > 5 years, **U5-M**= Male 6-60 months of age, **U5-F**= Female 6-60 months of age.

.

Table 4. 1 Relationship between Parasitemia level and Free Serum Sialic Acid Concentration.

Number of parasite/µL (n=316)	Pearson Correlation (*r*)	*P*-Value
>5000	0.005	0.988
<5000	0.203	0.197
10000	0.077	0.520
<100000	0.015	0.968

Correlation is not significant (P>0.05).

Table 4. 2 Relationship between Parasitemia level and Total Bound Sialic Acid Concentration

Number of parasite/µL (n=316)	Pearson Correlation (*r*)	*P*-Value
> 5000	-0.064	0.828
< 5000	-0.044	0.780
10000	-0.168	0.163
<100000	-0.155	0.670

Correlation is not significant (P>0.05).

Table 4. 3 Relationship between Free Serum Sialic Acid Concentration and parasitemia level among groups of *P. falciparum* Infected Individuals according to age.

GROUPS (n=316)	Pearson Correlation (*r*)	*P*-Value
A5-M	0.224	0.126
A5-F	0.420	0.051
U5-M	0.530	0.070
U5-F	0.116	0.543

Correlation is not significant (P>0.05).

A5-M= *P. falciparum* infected patients > 5 years (male), A5-F= *P. falciparum* infected patients >5 years of age (female), U5-M= *P. falciparum* infected patients 6-60 months of age (male), U5-F= *P. falciparum* infected patients 6-60 months of age (female).

4.7 Relationship between Free Serum Sialic Acid Concentration and PCV among *P. falciparum* Infected Individuals and Control.

The relationship between free serum sialic acid concentration and PCV in *P. falciparum* infected and apparently healthy individuals in all the experimental groups is presented in Table 4.4. From the correlation analysis, a weak positive relationship was observed across the experimental groups. The *r* values for infected group were A5-M (0.079), A5-F (0.311), U5-M (0.246), U5-F (0.067).

4.8 Relationship between Free Serum Sialic Acid Concentration and Total Bound Sialic Acid Concentration among *P. falciparum* Infected Individuals and Control.

Correlation analysis between concentration of free serum sialic acid and total bound sialic acid is presented in Table 4.5. The result did not give a corresponding relationship between the two variables at $P > 0.05$.

Table 4. 4 Relationship between Free Serum Sialic Acid Concentration (FSSA) and PCV among *P. falciparum* Infected Individuals and Control.

GROUPS (n= 316)	**Pearson Correlation (*r*)**	***P*-Value**
A5-M	0.079	0.676
A5-F	0.311	0.154
U5-M	0.246	0.458
U5-F	0.067	0.793
A5-Mc	0.041	0.916
A5-Fc	0.014	0.969
U5-Mc	0.353	0.560
U5-Fc	0.458	0.438

Correlation is not significant (P>0.05).

A5-M: *P. falciparum* infected patients > 5 years (male), **A5-F**; *P. falciparum* infected patients >5 years of age (female), **U5-M**: *P. falciparum* infected patients 6-60 months of age (male), **U5-F**: *P. falciparum* infected patients 6-60 months of age (female), **A5-Mc**: Apparently healthy individuals (control) >5 years of age (male), **A5-Fc**: Apparently healthy individuals (control) >5 years of age (female) , **U5-Mc**: Apparently healthy individuals (control) 6-60 months (male), **U5-Fc**: Apparently healthy individuals (control) 6-60 months (female).

Table 4. 5 Relationship between Free Serum Sialic Acid Concentration and Total Bound Sialic Acid Concentration among *P. falciparum* Infected Individuals and Control.

GROUPS(n=316)	Pearson Correlation (*r*)	*P*-Value
A5-M	-0.074	0.693
A5-F	-0.123	0.490
U5-M	-0.536	0.137
U5-F	-0.103	0.695
A5-Mc	0.230	0.523
A5-Fc	0.088	0.852
U5-Mc	0.427	0.473
U5-Fc	0.514	0.375

Correlation is not significant (P>0.05).

A5-M: *P. falciparum* infected patients > 5 years (male), **A5-F**; *P. falciparum* infected patients >5 years of age (female), **U5-M**: *P. falciparum* infected patients 6-60 months of age (male), **U5-F**: *P. falciparum* infected patients 6-60 months of age (female), **A5-Mc**: Apparently healthy individuals (control) >5 years of age (male), **A5-Fc**: Apparently healthy individuals (control) >5 years of age (female) , **U5-Mc**: Apparently healthy individuals (control) 6-60 months (male), **U5-Fc**: Apparently healthy individuals (control) 6-60 months (female).

4.9 Bioinformatics Analysis for Sialidase Gene in the Genome of *P. falciparum*

In furtherance with assessing the presence of sialidase in the genome of *P. falciparum,* BLAST search was conducted with other known sialidase protein sequences. Table 4.6 is a summary of the BLAST result, indicating the maximum scores, E-value, percentage identity and the covered query area. The results of the multiple sequence alignment conducted for sialidase protein sequence from *Homo sapiens* (CAB41449.1), *Trypanosoma cruzi* (AAA30245.1), *Mus musculus* (BAA92868.1), *Trypanosoma rangeli* (AAC95493.1), influenza A virus (AAA43413.1) and *Clostridium chauvoei* (CAR82361.1), *Trypanosoma grayi* (XP_009315754.1), *alpha proteobacterium* (WP_054127450.1), *Streptococcus pneumoniae* (WP_050079028.1), *Porphyromonas gingivalis* (ATS03463.1), *Draconibacterium sp*. (PIF06554.1), show no area of homology among the sialidase sequence used from different organisms (see Appendix 6).

Table 4. 6 BLAST Search Result for Sialidase Gene from the Genome of *P. falciparum*.

Organism	**Protein, gene or transcript name**	**Accession number**	**NCBI data type queried**	**Max Scores**	**E-value**	**i.d (%)**	**Cover query (%)**
Homo sapiens	Neuraminidase	CAB41449.1	Protein	23.1	2.5	35	6
	Homo sapiens mRNA for sialidase (NEU4 gene)	AJ277883.1	Transcripts	33.7	2.3	100	1
Trypanosoma cruzi	Sialidase	AAA30245.1	Protein	NILL	NILL		
	*T.cruzi*sialidase (SA85-1.1) mRNA, complete cds	M62735.1	Transcripts	37.4	0.30	83	1
Clostridium chauvoei	precursor sialidase	CAR82361.1	Protein	32.7	4.6	21	46
Mus musculus	Sialidase	BAA92868.1	protien	26.6	7.4	65	4
	Mus musculus mRNA for sialidase, complete cds	AB026842.1	Transcripts	46.4	9e-04	82	6
Trypanosome rangeli	Sialidase	AAC95493.1	protein	28.1	8.0	40	3
Influenza A virus	Neuraminidase	AAA43413.1	Protein	NILL			
	Influenza A virus (A/Swine/Korea/ CY02/02(H1N2)) neuraminidase (NA) mRNA, complete cds	AY129157.1	Transcripts	46.4	0.008	94	2

CHAPTER FIVE

5.0 DISCUSSION

Malaria is a major public health hazard affecting a vast population of the world today especially in the tropical countries. In Nigeria, malaria accounts for most of the cases of infection and deaths among young children and pregnant women (Francis *et al*., 2014). Sialic acid on the erythrocyte is known to aid in the invasion of this parasite via the sialic acid dependent pathway. In an attempt to study the pathway, it became necessary to evaluate the alteration in sialic acid concentration during *P. falciparum* infection and to assess the existence of sialidase gene in *P. falciparum*, which will help shade more light in the pathogenesis of this parasite.

Haematological parameters are measurable indices of the blood that serve as markers for disease diagnosis. Changes in some of these heamatological indices are common observations that are attributed in cases of malaria infection (Muwonge *et al*., 2013). White blood cells (leucocytes) play a pivotal role in the defense mechanisms against disease infections. Changes observed in WBC level in malaria infection are variable, these depend on many factors such as parasite species responsible for the infection, nature of infection, density of the parasite, disease severity, state of the host immunity to malaria, and concurrent infections (Abdalla and Pasvol, 2004; Muwonge *et al*., 2013). In this study, significant decrease was ($P<0.05$) observed in the WBC count of *P. falciparum* infected individuals when compared to their respective control. This was found to be in concordance with previous studies (Kassa *et al*. 2005; Igbenegbu and odaibo, 2013; Al-Salahy *et al*.,

2016; Garba *et al*., 2015). Although Ladhani *et al*. (2002), Ani *et al.* (2016) in their own studies found a slight increase in WBC count of infected individuals. Several studies have shown that decline in WBC count (leucopenia) is more associated with mild malaria, which is linked to the depletion in lymphocyte sub-set through apoptosis or due to the sequestration of the cells in the lymph nodes or other tissues (Rojanasthien *et al.*, 1992).

In this study, a significant decrease ($P<0.05$) in the level of PCV and haemoglobin in malaria infected individuals were observed which was due to erythrocyte depletion by the parasite (Maina, *et al*., 2010, George and Ewelike-Ezeani., 2011 and Osaro, *et al*., 2014). An observed decrease in the PCV and haemoglobin level in malaria infected individuals could be a reflection of anaemia (Ifeanyichukwu and Esan, 2014) which is mostly due to destruction of infected (parasitized) erythrocytes coupled with spleenic clearance of parasitized and defected erythrocytes (Francis *et al*., 2014). A number of factors have been implicated to be responsible in the prevalence and severity of anemia in malaria infection especially in malaria endemic areas. These factors include level of parasites, age of host, species of parasite, host genetic factor and many others (Muwonge *et al*., 2013). In this study, a significant ($P<0.05$) decrease in the level of PCV observed especially among under 5 years (with high parasitemia level) might be as a result of low immunity against the parasite and species involved in the infection. Similar findings by George and Ewelike-Ezean, (2011) and Imoru *et al*., (2013) on children showed that this parasite has more effect on low immune individuals.

In many pathological conditions, some observable changes that occur in the expression of sialic acid serve in the prediction of disease severity in clinical pathology (Varki, 2008). Akin-Osanaiye *et al*., (2015) observed an increase in the concentration of free serum sialic acid which coincides to the decrease in the concentration of total bound sialic acid coupled with an increase in the parasitemia in blood of mice infected with *P. berghei*. These findings did not correlate with the present study. In this study, an increase in the concentration of free serum sialic acid in *P. falciparum* infected individuals was observed when compared to the control groups, although the increase was not significant ($P>0.05$). This might imply that the enzyme (sialidase) responsible for the hydrolysis of sialic acid might be absent or not expressed and no significant sialic acid hydrolysis during infection by *P. falciparum* occur. Also, increase in the parasitemia level did not yield a corresponding increase in the level of free serum sialic acid during infection by *P. falciparum*.

A weak positive correlation (Table 4.4) observed between free serum sialic acid and PCV among *P. falciparum* infected individuals is an indication that sialic acid hydrolysis might not be responsible for the decline in erythrocyte during *P. falciparum* infection. Table 4.5 show a non-corresponding relationship ($P>0.05$) between free serum sialic acid and total bound sialic acid. These means that no hydrolysis of sialic acid took place during infection. This can further be explained by Table 4.3 and Table 4.1, where increase in parasitemia among infected individuals did not give a corresponding increase in the concentration of free sialic acid in infected.

The results of the BLAST on the genome of *P. falciparum* gave a high expected value (E-value) and a low Max scores (less than 46.4%), with a low query length for all the hits. Exploring the annotation from these hits (Table 4.6) would find that they have significantly different functions from sialidase. These simply imply that *P. falciparum* may not have the gene responsible for the production of sialidase or a sialidase like enzyme due to low homology of sialidase homologues of the query sequence (*P. falciparum*). However, Akin-Osanaiye et *al*., (2015) reported the existence of sialidase activity in *P. berghei*, where they were able to observe an increase in the level of serum free sialic acid (due to hydrolysis) as the parasitemia level increase (which also leads to an increase in sialidase activity).

Grobe *et al*., (1998) gave some possible explanation for existence of sialidase in *P. berghei* and its absence in *P. falciparum* which could be due to the irregular distribution of sialidase gene among microorganisms of same species or even among single strain of a given species. This could be the reason why efforts to search for sialidase gene in the genome of *P. falciparum* was not significant. Clough *et al*., (1996) and other authors also reported the absence of sialidase/trans-sialidase activity in *P. falciparum* using sensitive fluorometric and haemagglutination assays. Interaction of *P. falciparum* ligands were examined for their sensitivity to 4-guanidino-2,3-didehydro-N-acetyl neuraminic acid (4-guanidino Neu5Ac2en), a potent inhibitor of influenza virus sialidase. This inhibitor did not affect subsequent parasite invasion into the erythrocyte and its pathogenesis.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

Conclusion

From the present study, the sialic acid profile show no significant (P>0.05) increase in the concentration of free serum sialic acid during infection by *Plasmodium falciparum,* decrease in total bound sialic acid was also not significant(P>0.05). The result also showed no significant relationship (P>0.05) between increase in free serum sialic acid and PCV level and also level of parasitemia during infection and sialic acid level. More so, BLAST search conducted on the genome of *Plasmodium falciparum* (taxid: 5833), did not reveal the presence of a sialidase gene in the genome of the parasite.

Limitation

In this present study, the concentration of erythrocyte bound sialic acid was not determined due to inability to prepare ghost erythrocyte, instead total bound sialic acid was measured.

Recommendation

From the present study, the following future researches are recommended;

1. More research should also be conducted on the sialic acid independent pathway for *P. falciparum* invasion so as to identify the receptors responsible.

2. More research should be conducted to shed more light on ligand receptor complex formation during *P. falciparum* invasion, which might aid in the reduction of parasite invasion into the merozoites which is the key stage in *P. falciparum* infection.
3. The presence of sialidase activity/gene should also be investigated in other Plasmodium species.

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APPENDICES

Appendix 1 Ethical Approval



MINISTRY OF HEALTH AND HUMAN SERVICES
KADUNA STATE, NIGERIA

MOH/ADM/744/VOL.1/522 21st MARCH, 2017

NOTICE OF APPROVAL AFTER FULL COMMITTEE REVIEW

STUDIES ON SIALIC ACID CHANGES IN PLASMODIUM FALCIPANUM INFECTION

Name of Principal Investigator:	IKA MBATEUDI DANJUMA
Address of principal Investigator:	DEPARTMENT OF BIOCHEMISTRY AHMADU BELLO UNIVERSITY, ZARIA
Date of receipt of application:	8TH MARCH, 2017
Date of Ethical Approval:	20TH MARCH, 2017

This is to inform you that the research described in the submitted protocol, the consent forms, advertisements and other participant information materials have been reviewed and given full approval by Health research Ethics Committee (HREC).

If there is delay in starting the research or any change, inform the HREC so that the dates of approval can be adjusted accordingly.

However, Researcher is kindly requested to submit a copy of his/her findings to the state Ministry of Health, please.

DR. BUTAWA NN
Secretary.
For: Chairman

21/3/17

Appendix 2. Level of Parasitemia in *Plasmodium falciparum* Infected Patients

Groups	Parasitemia/µL (10^3)
group 1 (male >5 years)	13.3
group 2 (female > 5years)	12.0
group 3 (male 6-60 months)	93.6
group 4 (female 6-60 months	64.3

Appendix 3. Comparison of Hematological Parameters between the cases of *Plasmodium falciparum* Infected and Apparently Healthy Individuals (Control).

Group	parameters	Malaria infected patients	Apparently Healthy subjects
A5M*Pf*I	WBC(10^9/L)	6.42±0.27[a]	8.70±0.42[b]
	HGB(g/L)	134.76±1.86[a]	143.60±1.52[b]
	PCV(%)	42.29±0.94	48.39±1.37[b]
	RBC(10^{12}/L)	4.82±0.09[a]	5.22±0.11[a]
A5F*Pf*I			
	WBC(10^9/L)	5.25±0.27[a]	7.23±0.71[b]
	HGB(g/L)	118.09±1.89[a]	131.00±9.93[b]
	PCV(%)	37.27±0.63[a]	42.12±1.10[b]
	RBC(10^{12}/L)	4.40±0.08[a]	4.67±0.19[a]
U5M*Pf*I			
	WBC(10^9/L)	6.64±0.59[a]	8.78±0.66[b]
	HGB(g/L)	118.45±3.11[a]	139.40±7.33[a]
	PCV(%)	34.33±1.41[a]	41.92±0.91[b]
	RBC(10^{12}/L)	4.87±0.26[a]	4.66±0.32[a]
U5F*Pf*I			
	WBC(10^9/L)	6.82±0.47[a]	7.74±0.45[b]
	HGB(g/L)	116.83±4.62[a]	142.00±7.16[b]
	PCV(%)	31.04±1.39[a]	39.44±1.12[b]
	RBC(10^{12}/L)	4.24±0.18[a]	4.40±0.14[a]

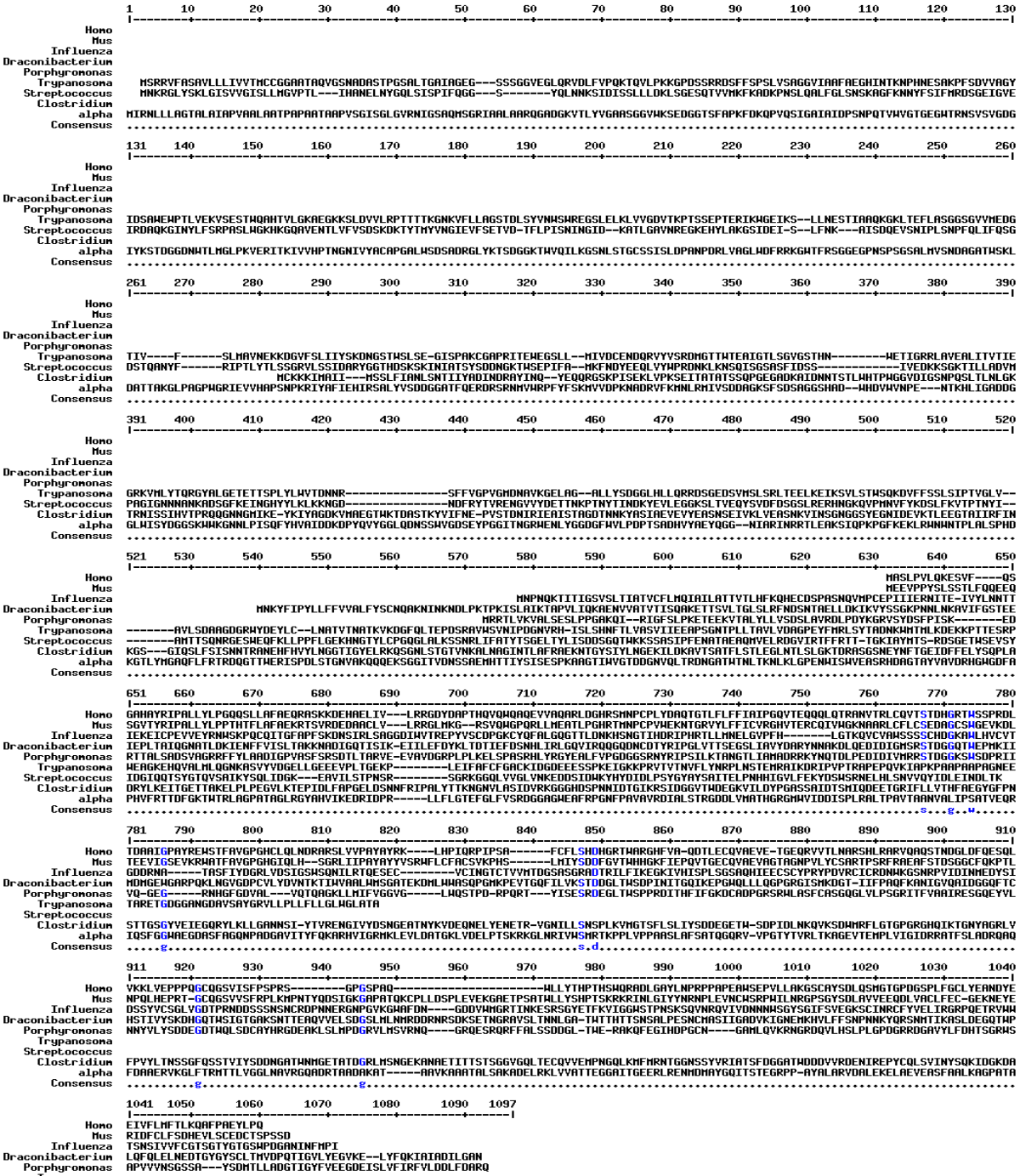
Paired Sample t-test comparison of hematological parameters between the cases of *P. falciparum* infection and apparently healthy subjects. Values are represented as mean±SD. Values with different superscript along the column are statically different ($p< 0.05$)

Appendix 4. Comparison of Sialic Acid Levels (FSSA, BSA & TSA) between the cases of *Plasmodium falciparum* Infected and apparently healthy Individuals (control).

Group	parameters	Malaria infected patients	Apparently Healthy subjects
A5M*Pf*I	FSSA(µg/ml)	261.37±5.56[a]	227.83±8.15[a]
	TSA(µg/ml)	2218.39±27.31[a]	2137.22±104.80[a]
	BSA(µg/ml)	1757.42±28.89[a]	1913.65±114.85[a]
A5F*Pf*I	FSSA(µg/ml)	257.83±17.29[a]	217.12±22.94[a]
	TSA(µg/ml)	1857.72±69.42[a]	1822.82±178.85[a]
	BSA(µg/ml)	1561.82±72.14[a]	1614.75±186.53[a]
U5M*Pf*I	FSSA(µg/ml)	292.72±13.06[a]	248.49±22.65[a]
	TSA(µg/ml)	1912.94±80.51[a]	1867.09±178.88[a]
	BSA(µg/ml)	1415.00±125.93[a]	1808.29±167.22[a]
U5F*Pf*I	FSSA(µg/ml)	296.34±8.85[a]	262.51±12.97[a]
	TSA(µg/ml)	1955.64±71.13[a]	1872.49±46.34[a]
	BSA(µg/ml)	1657.33±69.41[a]	1945.69±48.49[a]

Paired Sample t-test for comparison of sialic acid profile between the cases of *P. falciparum* infected and apparently healthy subjects. Values are represented as mean±SD. Values with different superscript along the column are statically different ($p< 0.05$)

Appendix. 5. Multiple alignment of sialidase protein from different organisms



Multiple alignment of sialidase proteins from; *Homo sapiens*, *Mus musculus*, Influenza A virus, *Draconibacterium sp.*, *Porphyromonas gingivalis*, *Trypanosoma rangeli*, *Streptococcus pneumoniae*, *Clostridium chauvoei*, alpha proteobacterium AAP81b.