MALARIA PARASITAEMIA LEVELS IN RELATION TO ANTIOXIDANT ENZYME LEVELS IN SEVERE MALARIA AMONGST GHANAIAN CHILDREN

BY

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DECLARATION

I do hereby declare that except for references to other people's work which I have duly acknowledged, the work embodied in this thesis was carried out by me at the Department of Microbiology, University of Ghana Medical School and at the Department of Medical laboratory Sciences of the School of Allied Health Sciences (SAHS), College of Health Sciences, University of Ghana, under the supervision of Prof. P. F. Ayeh-Kumi and Mr. Richard Harry Asmah, of the SAHS.

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DEDICATION

TO

My family and friends, Sylvia Afriyie Squire, the participants, Supervisors,

Collaborators and the Microbiology Department, UGMS

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CDC	Center for Disease Control and Prevention
CDD	Charge- Coupled Device
Cu/ZnSOD	Copper/ Zinc Superoxide dismutase
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic Acid
FP	Ferri/ Ferroprotophyrin
GDP	Gross domestic product
GrxS2	Oxidized Glutaredoxin
GrxSH2	Reduced Glutaredoxin
GSH- Px	Glutathione Peroxidase
GSSG	Glutathione
HETE	Hydroxy- arachidonic Acid
HOCL	Hypochlorous acid
IRS	Ischemic Reperfusion Syndrome
MOA	Malondialdehyde
NAD (H)	Nicotine Adenine Dinucleotide reductase
NADP (H)	Nicotine Adenine Dinucleotide Phosphate Oxidase
NMCP	National Malaria Control Programme
NO	Nitric Oxide
$O^{\bullet -}_2$	Superoxide radical
OH- PUFA	Polyenoic Fatty Acid

$ONOO^-$	Peroxynitrite
PfHRP2	Histidine- rich Protein- 2 Complex from *Plasmodium falciparum*
PMNs	Polymorphonuclear Cells
RBC	Red Blood Cell
RNS	Reactive Nitrogen Species
ROI	Reactive Oxygen Intermediate
ROS	Reactive oxygen species
SCGE	Single Cell Gel Electrophoresis
SD	Standard Deviation
SOD	Superoxide dismutase
TBE	Tris/ Borate Ethylene Diamine Tetra Acetic Acid
TNF- α	Tumour Necrotic factor- alpha
TRAP	Thrombopondin Related Anonymous Protein
Trx	Thioredoxin
TrxR	Thioredoxin Reductase
TrxS2	Oxidized Thioredoxin
TrxSH2	Reduced Thioredoxin
TWBC	Total White Blood Cell

ABSTRACT

Malaria is an important infectious disease in tropical and subtropical regions. The disease presents a major global health problem with over 40% of the world`s population exposed to varying degrees of infection risk in over 100 countries. About 1 to 2 million deaths occur annually, 90% of whom are children in sub-Saharan Africa. During the course of malarial infection, the parasites as well as the red blood cells (RBCs) come under oxidative stress and the host system responds in an attempt to protect the RBCs against the damage caused by Reactive Oxygen Species (ROS) by producing antioxidants. This study investigated the protective role of ROS in relation to malaria parasitaemia levels.

One hundred and fifty (150) structured questionnaires were administered to the guardians of participants (children up to 12 years old). Blood samples were collected to estimate the parasitaemia levels and to measure the haematological parameters. Activities of superoxide dismutase and DNA comet assay were used to evaluate extent of damage on parasite DNA as a result of oxidative stress.

Participants who tested positive for malaria parasitaemia were categorized either as severe (high parasitaemia; 56.75 x 10^3 ± 57.69/µl) or uncomplicated malaria (low parasitaemia; 5.87 x 10^3 ± 2.87/µl), whiles those who tested negative were categorized as controls. Quantitative analysis of the impact of SOD activity on malaria parasites showed that participants with severe malaria had low SOD activity (295.33 ± 211.40) U/ml while the uncomplicated had high SOD activity levels (520.69 ± 275.10) U/ml. Which was significant ($P<0.05$). There was also a significant difference ($P<0.05$) in the levels of haemoglobin (Hb), neutrophils and lymphocytes between the different study groups. The study indicated that, ROS plays a pivotal role as a first-line anti-parasitic defense in *P. falciparum* malaria infection. Hence, addition of anti-oxidant foods and fruits to the routine anti-malarial treatments should be recommended during the infection.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Malaria is an important infectious disease in tropical and subtropical regions, it is a major global health problem, with over 40% of the world`s population exposed to varying degrees of infection risk in over 100 countries (1). Over 500 million people suffer from the infection yearly, with about 1-2 million deaths occurring, of whom 90% are children in sub-Saharan Africa (1). Studies conducted suggest that reactive oxygen species (ROS) play an important role in many human disease pathology and in malaria infection (2).

Oxidative stress during malaria is considered useful to the patient in the fight against the intra-erythrocytic parasite (2). Studies have been described in which induction of oxidative stress by treatment with pro-oxidants proved to be effective against the infection (3). On the other hand ROS play a role in the pathology of malaria (4). Excessive oxidative stress particularly at unprescribed sited (e.g. vascular lining, blood brain barrier) can damage the defense system. This is however, controlled by intra- and extracellular anti-oxidants systems, which may fail during disease. Treatment with anti-oxidants reinforces these systems and protects the patient, especially during the life threatening phase of the disease (5, 6).

ROS are produced as a result of activated monocytes and by the enzyme NADPH oxidase in the membrane lining other leukocytes (neutrophils, lymphocytes etc), and this probably play an important role in the control of the parasitaemia (7, 8). It has been

suggested that phagocytic cells, like monocytes and polymorphonuclear cells (PMNs) damage the intra-erythrocytic parasites by their production of ROS (7, 8). Activated neutrophils and blood monocytes *in vitro*, partially inhibit maturation of *P. falciparum* (9) whereas an increase in lipid peroxidation and sensitivity to haemolysis normally occurs during parasite maturation.

1.2 PROBLEM STATEMENT

Malaria remains one of the leading causes of morbidity and mortality worldwide and in sub- Saharan Africa (10, 11). Mortality from malaria is due to complication arising as a result of severe infections usually caused by *P. falciparum.* Studies on mortality have shown that deaths occur predominantly among young children and mortality rates among patients with an illness severe enough to warrant hospitalization are consistently high with case fatality rates varying from 5% to 30% (12). In Ghana, malaria is hyper endemic and presents a serious health problem in the country (12). It is also a leading cause of deaths in the country and accounts for over 40% of out-patient attendance with annual reported cases and children less than five years are the most affected (14). A study conducted by Ministry of Health in 2006 showed that more than 17 million of Ghana's over twenty million people are infected with malaria every year, with cost of $85 million for treatment (15).

Despite the importance of *P. falciparum* as a human pathogen, the patho- physiologic basis of its infection is not well understood. Parasitic infections such as malaria in host organisms often lead to oxidative stress condition which is a disturbance in the balance between the production of ROS and antioxidant defenses (16, 17). The constant

generation of free radicals as a result of oxidative stress and other reactive species *in vivo* leads to extensive damage in parasite bio- molecules such as DNA, lipids and proteins (18, 19). It has also been shown that the parasites are vulnerable to oxidative stress during their erythrocytic life stages (18, 19, 20). In erythrocytes, *P. falciparum* encounters enhanced oxidative stress, resulting largely from its digestion of haemoglobin and thus, its redox balance becomes fragile (12). Superoxide (O_2^-) is normally produced when oxidized haemoglobin is exposed to the acid environment of the food vacuole, and can therefore be considered as the major source of ROS. Inside the parasite, regardless of its origin, O^{2-} is dismutated by superoxide dismutase (SOD) to H_2O_2 (21).

Even though studies have been carried out on *P. falciparum* and ROS, the focus has mainly been on the pathological effect of these radicals and there is paucity of documented work carried out on the beneficial role ROS plays to the host. It will therefore be of value to investigate whether ROS gives any beneficial role to the human host in its fight against the parasitaemia effects. Also what accounts for the host system's ability to overcome the parasite burden without damage to the host organs and how the host system accomplishes this. Understanding the parasite and host interaction during infection by studying malaria parasitaemia levels in relation to ROS levels in the host, will increase our knowledge of the protective benefits of ROS to the host and its significance. This will aid in the formulation of suitable antioxidant therapy in the control of ROS-mediated oxidative damage, and the disease processes and in the design of a diagnostic kit.

1.3 JUSTIFICATION

The high incidence of malaria morbidity and mortality is dependent largely on the complex pathogenesis of this parasitic infection. During the course of malarial infection, the host immune system is activated thereby causing release of ROS (22) resulting in the host cell coming under oxidative stress and one of the consequences of this is the development of malarial anaemia (23). Despite more than a century of efforts to eradicate or control the disease, it remains a major and growing threat to the public health and economic development of countries in the tropical and subtropical regions of the world.

The mortality levels are greatest in sub-Saharan Africa, where children under 5 years of age account for 90% of all deaths due to malaria (24), as a result of resistance to anti-malarial drugs and insecticides, the decay of public health infrastructure, population movements, political unrest, and environmental changes (25). Annual economic growth rates captured over a 25-year period in countries with endemic malaria has been found to be less 1.5% than in other countries (26). This could suggests that the collective outcome of the lower annual economic yield in these malaria-endemic countries was a 50% less in the per capita GDP as compared to a non-malarious country (26). Research finding suggests that the number of malaria cases may double in 20 years if new methods of control are not devised and implemented (24). Oxidative stress, when over expressed, plays a role in various clinical conditions such as malignant diseases, diabetes, atherosclerosis, chronic inflammation, viral infection, and ischemia-reperfusion injury (27-32). ROS causes DNA and protein damage, damages tumour suppressor genes and enhanced expression of proto- oncogenes when over expressed (33-35) as well as shown to induce malignant transformation of cells in culture (36). Nonetheless, the effects of

ROS on cell metabolism are well documented in a variety of species. These include not only roles in apoptosis (programmed cell damage) but also positive effects such as the function of host defense (37, 38) genes and mobilization of ion transport systems. This implicates them in redox signaling, also known as "oxidative signaling" (39). It has also been shown that the production of ROS is essential, depending on the dose, in carrying out some important beneficial functions. These include apoptosis, which eliminates precancerous and cancerous, microbial-infected and otherwise damaged cells (40).

1.4 AIM

The study aim was to estimate superoxide dismutase (an antioxidant enzyme) levels in relation to severe malaria.

1.4.1 Specific Objectives

- To determine ROS by assaying for SOD levels in children with severe malaria.
- To determine the extent of ROS damage on *Plasmodium* infected RBCs using comet assay.
- To establish correlations of SOD with parasitaemia in children with severe malaria.
- To determine white blood cells levels and any significance relationship to ROS and human host immunity during malaria infection.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY OF MALARIA

Malaria is one of the oldest diseases believed to have infected man over 50,000 years (41) and the evidence of this was first found in the Xian Dynasty and Medieval Europe through archeological studies (41). The name originated from the Italian word mal'aria meaning "bad air' and it is believed to have influenced to a great extent of human history (41).

Although the parasite was first noticed in the red blood cell in 1880, by Charles Louis Alphonse Laveran, a French Medical surgeon and proposed that it was a protozoan disease, it was not until 1886 that another scientist, Camillo Golgi, an Italian neurologist established that the parasite has at least two disease forms, the tertian periodicity (fever every other day) and the quartan periodicity (fever every third day). He also observed that these forms produced differing numbers of merozoites when they matured and fever always occurred with the release of the merozoites into blood circulation (41). In 1890, two other scientists, Giovanni Batista Grassi and Raimondo Filetti assigned the names *Plasmodium vivax and Plasmodium malariae* for two of the malaria parasites affecting humans. Also, in 1897, William H. Welch, an American, named *Plasmodium falciparum* as the parasite responsible for the malignant tertian form. In 1922, the fourth human malaria parasite was described by John William Watson Stephens (41). In 1897, Ronald Ross established that the malaria parasite was transmitted by infected mosquitoes. He did this by isolating the malaria parasite from the salivary glands of mosquitoes that bit malaria infected birds and then transmitted the parasite to healthy birds. This led to the

discovery of the transmission of the human malaria parasite *Plasmodium* between 1898-1899 by a team of Italian investigators led by Giovanni Batista Grassi (41)

2.2 GLOBAL VIEW OF MALARIA

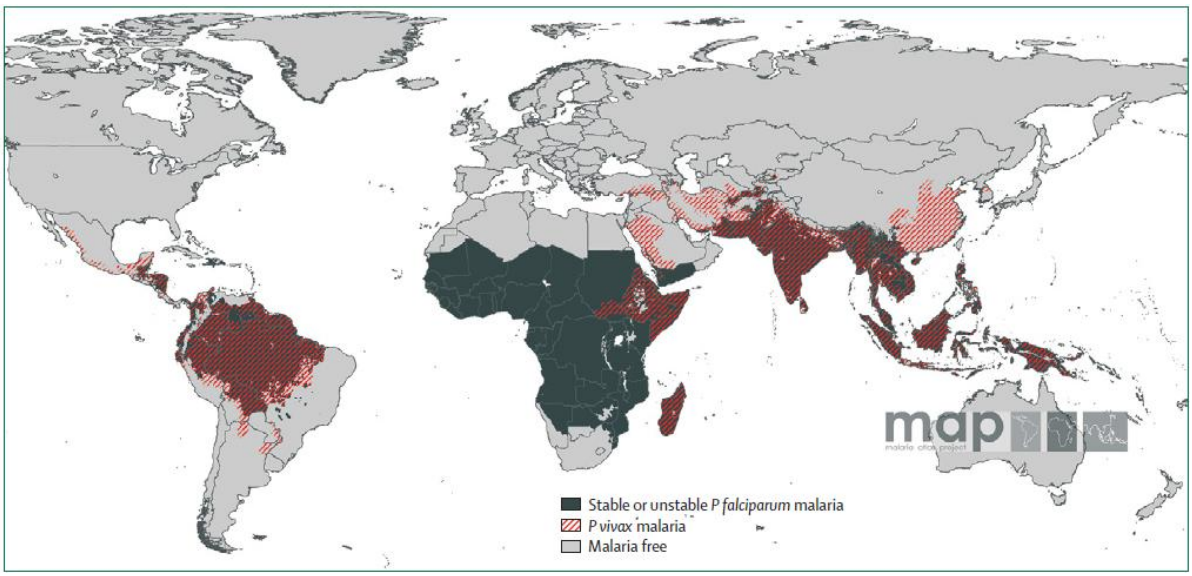

Figure 1: Global spatial distribution of Plasmodium falciparum and global distribution of Plasmodium vivax malaria (source: MAP. Malaria atlas project. http://www.map.ox.ac.uk (accessed 2010).

Malaria remains an important infectious disease in tropical and subtropical regions, and a major global health problem, with over 40% of the world`s population exposed to varying degrees of risk of this infection in over 100 countries (1). It is estimated that over 500 million people suffer from malaria infection annually, resulting in about 1-2 million deaths, of whom 90% are children in sub-Saharan Africa (1). Beside Sub- Saharan Africa, other developing countries most affected are South and Southeastern Asia, Oceania and Haiti, where *P. falciparum* malaria prevails. *P. vivax* has also been found to be prevalent in India, the Middle East and Central America. Though much less frequent in the United States, malaria cases do occur. According to the Centers of Disease Control and Prevention (CDC), in 2002, 1,337 malaria cases were diagnosed, all of which were

reported to have travelled to endemic areas (42). Even though it remains as the major cause morbidity and mortality, between 2000 and 2009, the number of estimated death due to malaria worldwide has been found to decrease from 985, 000 to 781,000 (43).

2.3 MALARIA BURDEN IN GHANA

Although malaria remains a major health burden in tropical and subtropical countries; with the majority of cases in sub-Saharan Africa, several regions show an impressive decline of malaria cases and a lower number of malaria-associated deaths (13). In Africa alone, the direct and indirect costs of malaria are estimated to exceed US$2 billion a year while accounting for a reduction of 1.3% of the annual economic growth rate (44). In Ghana however, statistics shown by the NMCP indicates that malaria is the leading cause of illness and it causes about 8,200 cases daily and 3,000,000 illnesses every year with over 3000 deaths in 2010; and the mortality even though high, represents a steady drop from the 40, 000 deaths reported ten years ago with the most vulnerable groups being children under five years of age, pregnant women and non-immunes. (44). A study in 2003 by Asante *et al.* showed that nine workdays were lost by economically active people who were ill with malaria; while more than five workdays were lost by their caretakers (12).

2.4 THE LIFE CYCLE OF THE MALARIA PARASITE

The Plasmodia parasite has a complex, multistage life cycle which involves an insect vector (the mosquito) and the vertebrate host (human). The presence of more than 5,000 parasite genes and their specialized proteins aid the parasite to invade and develop within multiple cell types and to evade host immune responses thus, ensuring the parasite

survival and development inside the invertebrate and vertebrate hosts, in intracellular and extracellular environments. (45,46). Four species are known to infect man: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*; all of which exhibit a similar life cycle with only minor variations. The parasite goes through various phases during its developmental cycles such as the sporozoites, merozoites, trophozoites, (asexual schizogony stage) and gametocytes (sexual sporogony stage) and all of these stages have been found to have their unique shapes and structures as well as protein complements. As the parasite goes through the different stages of its cycle the surface proteins and metabolic pathways change, making it possible for the parasite to elude the immune clearance, thereby creating problems for the development of drugs and vaccines (46).

2.4.1 The Asexual Schizogony Phase

Man is the intermediate host for malaria, in which the asexual phase of the life cycle occurs. This phase of the cycle is initiated from the liver when the infested anopheles mosquito inoculates the sporozoites during a blood meal, and the latter part continues inside the red blood cells, which results in the various clinical manifestations of the disease.

2.4.1.1 The Pre-erythrocytic Stage

During blood meal by the infested mosquito, hundreds of sporozoites are introduced into the intradermis. Some of these sporozoites are destroyed by the local macrophages, while others find a blood vessel (47–49). Some of the surviving sporozoites enter the lymphatic vessel into the draining lymph node where some of them partially develop into exoerythrocytic stages (47) and may also activate the T cells to build up a protective immune response (50).

The sporozoites that enter the blood vessel invade the liver within a few hours. Recent studies have shown that the sporozoites travel by a continuous sequence of stick-and-slip motility, using the thrombospondin-related anonymous protein (TRAP) family and an actin–myosin motor (48, 51, 52). Inside the liver the sporozoites develop into schizonts, each containing 10,000–30,000 merozoites (or more in case of *P. falciparum*) (53–55). In the liver, the parasite growth and development is made easier by a conducive environment created by the circumsporozoite protein of the parasite (56, 57). The whole pre-erythrocytic phase lasts about 5–16 days depending on the parasite species on an average 5-6 days for *P. falciparum*, 8 days for *P. vivax*, 9 days for *P. ovale*, 13 days for *P. malariae* and 8-9 days for *P. knowlesi* (58). At maturity, the merozoites are released into the blood stream and invade red cells by multiple receptor–ligand interactions (49).

In *P. vivax* and *P. ovale* malaria, some of the sporozoites remain dormant for months within the liver. These are called hypnozoites, and develop into schizonts after some latent period, usually of a few weeks to months. It is suspected that these hypnozoites are genotypically different from the sporozoites that cause acute infection soon after the inoculation by a mosquito bite (59, 60), and in some patients cause relapses of the clinical infection after weeks to months.

2.4.1.2 Erythrocytic Stage

The asexual development of the malaria parasite occurs in the red blood cells. It is suspected that the disappearance of the parasite from circulation into the red cells reduces the exposure of its surface antigens, thus protecting it from the host immune response (45, 49, 61). Interaction between the parasite and the red cell causes alteration

across the red cell membrane, resulting in the formation of a stable parasite–host cell junction. As a result, the parasite enters the erythrocyte with the aid of the actin–myosin motor, proteins of the thrombospondin-related anonymous protein family (TRAP) and aldolase, forming a parasitophorous vacuole to seal itself from the host-cell cytoplasm, thus creating a hospitable environment for its development in the red cell. The parasite at this stage appears as an intracellular "ring" (61, 62, 63).

Within the red cells, the parasite multiplies rapidly. The parasite ingests the haemoglobin in the red cell into a food vacuole and degrades it. It utilizes the amino acids in the haemoglobin for protein biosynthesis and the heme is detoxified by heme polymerase and sequestrated as hemozoin (malaria pigment). The malaria parasite also depends on anaerobic glycolysis for energy, utilizing enzymes such as pLDH, plasmodium aldolase etc. (64, 65). As the cycle progresses, the merozoites develop and divide within the vacuole each into fresh merozoites, trophozoites, and schizonts. Some of the merozoites at this stage do not undergo schizogony but differentiate into the sexual stage male and female gametocytes. These forms are extracellular and nonpathogenic and help in transmission of the infection to others through the female anopheline mosquitoes, inside which they continue the sexual phase of the parasite's life cycle (66, 67).

2.4.2 Sexual Sporogony Phase

The sexual phase of the parasite's life cycle occurs in the mosquito which is the definitive hosts. The sexual phase results in the development of infecting forms of the parasite within the mosquito that stimulate disease in the human host following their injection during blood meal. During a blood meal the female *Anopheles* mosquito picks up the male and female gametocytes of the parasite which find their way into the gut of the

mosquito where they develop into the gamete forms. The male and female gametes fuse in the mosquito gut to form zygotes, which subsequently develop into actively moving ookinetes that moves into the mosquito's midgut wall to develop into oocysts. These further develop and divide into active haploid forms called sporozoites which find their way into the body cavity of the mosquito, from where they travel to and invade the mosquito salivary glands. When the mosquito at this stage takes another blood meal, the sporozoites get injected from its salivary glands into the human bloodstream, causing malaria infection in the human host (68-70).

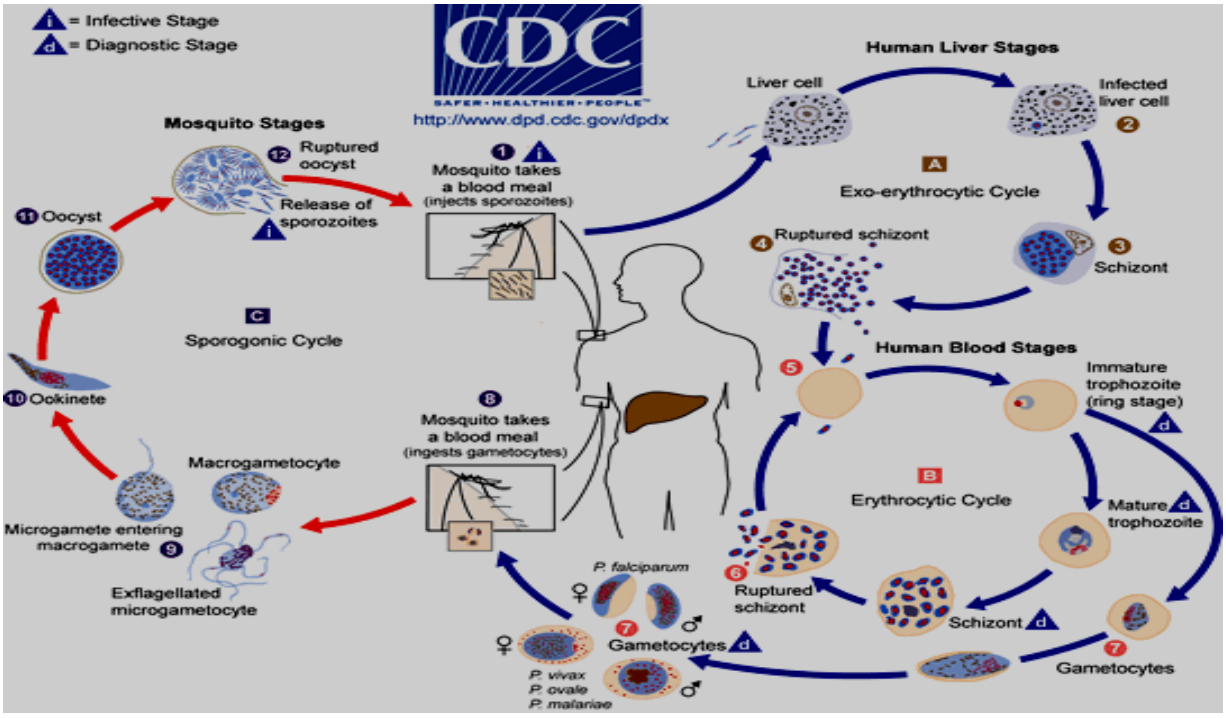

Figure 2: Life Cycle of *Plasmodium falciparum* (Adapted from http//www.dpd.cdc.gov/dpdx)

2.5 MALARIA DISEASE AND OXIDATIVE STRESS

The role of oxidative stress during malaria infection is still unclear; whiles some school of thoughts suggest a protective role, others claim a relation to the physiopathology of

the disease (71). However, recent studies have suggested that reactive oxygen and nitrogen species (ROS and RNS) associated with oxidative stress play an important role in the development of systemic complications caused by malaria (71). Malaria infection induce hydroxyl radicals ($OH^{\bullet}$) generation in the liver, which may probably be the main reason for the induction of oxidative stress and apoptosis (72). Furthermore, Atamna *et al*. (73) observed that erythrocytes infected with *P. falciparum* produced $OH^{\bullet}$ radicals and H_2O_2 twice as much compared to normal erythrocytes. The host's haemoglobin molecule is a possible source of free radical production in this disease, since the parasite utilizes it for its amino acid needs for its own nutrition during the erythrocytic stage of the disease, resulting in the release of large amounts of circulating haem. These haems have Fe^{2+}-associated groups which are able to induce intravascular oxidative stress, resulting in changes in erythrocytes and endothelial cells, thus, facilitating the internalization of the parasite in tissues such as the liver and brain (74). A free radical species, which appears to be involved in this disease, is nitric oxide (NO) (75–80, 81, 82). However, its role is still controversial. Oxygen radicals have been shown to be important for the clearance of disease in mice and humans (83). This therefore, suggests that reduced production of ROS by monocytes might aggravate infection and may contribute to the disease manifestation.

2.6 OXIDATIVE STRESS IN *FALCIPARUM* INFECTED ERYTHROCYTES

Malaria parasites are particularly vulnerable to oxidative stress during their erythrocytic life stages (84- 87). The parasites live in a pro-oxidant environment that contains oxygen and iron which are the key requirements for the formation of reactive oxygen species (ROS). The parasites take up haemoglobin into their acid food vacuole which leads to the oxidation of Fe^{2+} to Fe^{3+} and the formation of superoxide anions. This combination leads

to the generation of hydrogen peroxide and subsequently hydroxyl radicals, both highly reactive and toxic oxygen intermediates (88). Furthermore, toxic haem (ferri/ ferroprotoporphyrin IX; FP IX) is released upon haemoglobin digestion and this must be detoxified. Most of the released FP IX is biomineralized (up to 90%); (89) to form inert haemozoin. It is suggested that an appreciable amount of FP IX (even as much as 50%); (90, 91) escapes biomineralization and is degraded or sequestered by other means to prevent membrane damage and parasite death (92- 94). Apart from the parasite itself being under oxidative stress, the host cell also comes under oxidative alterations when infected with *Plasmodium*. Changes in erythrocyte membrane fluidity, most probably because of alterations of erythrocyte membrane lipid composition and protein cross-linking suggest that (95-98).

Oxidative stress is commonly observed to arise from five sources during disease physio-pathogeny: 1, Inflammatory process initiated in the host in response to infection; 2, transition metal catalysis, since in feeding on hemoglobin, the parasite releases significant amounts of free iron; 3, the occurrence of ischemia-reperfusion syndrome, resulting from cytoadherence processes and anemia triggered by infection; 4, direct reactive species production by the parasite; and 5, action of anti-malarial drugs (99). Oxidative stress has a protective role in malaria patients as possible agents capable of destroying the *Plasmodium*. Thus, H_2O_2 and $O_2^{\bullet-}$ can operate independently as cytotoxic agents or form other toxic molecules, including radical $OH^{\bullet}$, hypochlorous acid (HOCl) and peroxynitrite (ONOO−) in the presence of NO (99). ROS generated by macrophages are non-specific effectors molecules in the host's defense arsenal, which can contribute to oxidative damage in the parasite as well as parasitized erythrocytes, once ROS are able to diffuse through the membrane of red blood cells (100).

Also, neutrophils secrete proteolytic enzymes and ROS, which in low concentrations can trigger apoptosis of endothelial cells and necrosis in high concentrations (101). *P. falciparum* trophozoites increase the viscosity of red blood cells by causing changes in the parasitized cell surface thus, permitting its adhesion to the endothelial wall of capillaries, which seems to be a defense mechanism of the parasite, thereby preventing the passage of parasitized red blood cells through the spleen and their consequent destruction (102). However, the increased viscosity of the cells appears to be primarily responsible for the blocking of blood vessels, especially of kidney capillaries, pulmonary capillaries and brain capillaries, and cerebral malaria is the most common reason for coma and death in infected children (103,104).

Lipid peroxidation occurs on the surface of the infected red blood cells (105). The parasitized erythrocytes contain large amounts of monohydroxy derivatives of polyenoic fatty acids (OH-PUFA) in their lipids, which suggest that the episode of lipid peroxidation is as a result of the release of haem iron from non-enzymatic breakdown (105). One of the common OH-PUFA (12- and 15-hydroxy-arachidonic acid (HETE)) increases according to the evolutionary stage of the parasite. Low concentrations are found after phagocytosis of parasitized RBCs, suggesting that other lipid peroxidation products also may play a key role in this process (105). Additionally, accelerated aging of these cells is attributed to oxidative changes in *P. falciparum*-infected red blood cells and is reported to contribute to the development of anemia. (106). This promotes changes in the circulatory physiology, which results in moments of hypoxia alternating with the maintenance of tissue oxygenation at basal levels, favoring the participation of ischemia

and reperfusion syndrome (IRS) accountable for additional free radicals production (107).

2.7 OXIDATIVE CHANGES IN *PLASMODIUM*

In addition to the host producing ROS/RNS during infection, the parasite is also capable of producing free radicals, which in turn hinder the biochemistry of red blood cells and may facilitate the internalization of the parasite in hepatocytes and RBC (108). *Plasmodium* parasites are exposed to high levels of oxidative stress during development in host cells (109). Their ability to protect themselves against this hostility is important to their continued existence (109). As a result, these parasites have developed several antioxidant defense mechanisms (Fig. 3). Gene expression during the erythrocytic phase of infection by *Plasmodia* suggests there is a continuous cascade of gene expression at the early stages, and five different proteins with antioxidant properties are expressed (109). Additionally, *Plasmodium* reduces its own production of reactive oxygen species and adapts new mechanisms to prevent oxidative damage arising from the host. This, the parasite does to compensate for the oxidative stress suffered (110). One such mechanisms is the apicoplast; it is a symbiotic intracellular organelle located near the mitochondria which seems to synthesize lipoic acid, a potent antioxidant the parasite uses as a defense (110).

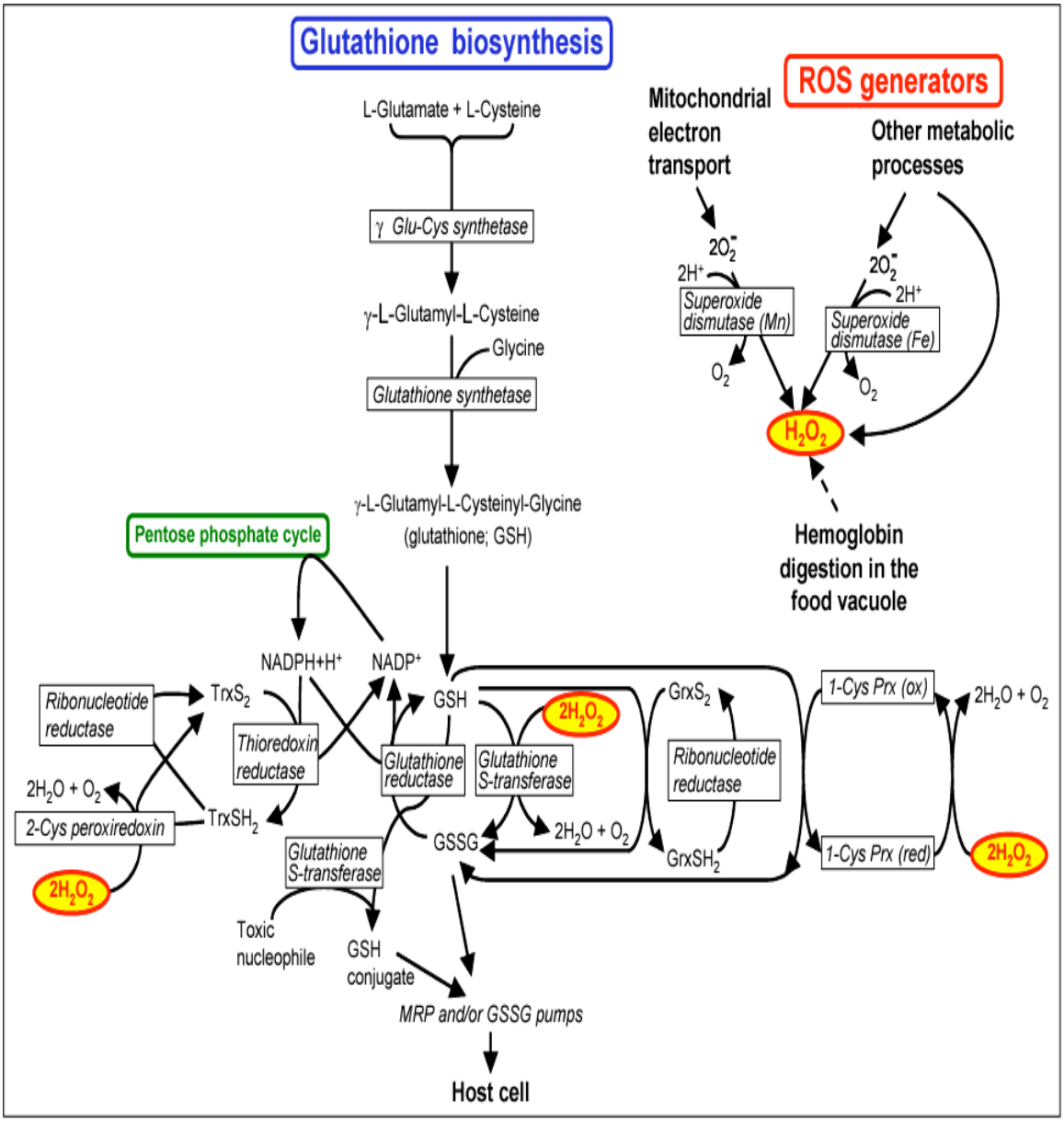

Figure 3: A schematic representation of the antioxidant defence in the Plasmodium-infected erythrocyte. TrxS2 and TrxSH2 are oxidized and reduced thioredoxin, respectively. GrxS2 and GrxSH2 are oxidized and reduced glutaredoxin, respectively (Adapted from Bozdech and Ginsburg, 2004).

Furthermore, in most *Plasmodium* cells, the redox homeostasis is thought to be based on the synthesis of reduced glutathione and thioredoxin system (Trx)/thioredoxin reductase (TrxR). The oxidized glutathione (GSSG) reduction is supported by the high proportion of the TrxR/Trx system in glutathione reductase-deficient cells, and is important for certain stages of the parasite cycle (80,111). The glutathione and thioredoxin redox

systems represent two powerful ways to detoxify reactive oxygen species in *Plasmodium falciparum* and these are efficient systems that prevent parasite development in the host cells (112). Also, peroxiredoxin (an enzyme) is suggested to be linked with chromatin in *P. falciparum*. The enzyme makes use of thioredoxin and glutaredoxin as reducing agents, thus, protecting the parasite against the oxidative abuse imposed by the host (113). The TrxR, plays a role in the continuance of redox homeostasis and antioxidant defense of *P. falciparum* (114). Interruption of the parasite antioxidant system is therefore a viable means of obstructing development during erythrocytic schizogony (115).

Moreover, glutaredoxin-1, thioredoxin-1 and plasmoredoxin catalyze protein deglutathionylation, a widely distributed important mechanism of post-translational modification of thiol groups with glutathione which functions as an intracellular redox signaling regulating device (116). Campanele *et al*. suggests that *P. falciparum* proteins interact with ferriprotoporphyrin IX, and that thioredoxin reductase appears much more sensitive to inhibition by FP than glutaredoxin (117). Conversely, the parasite's glutathione reductase has shown to be more resistant to being reduced by FP. Mashima *et al*. established in a study that the histidine-rich protein-2 complex from *Plasmodium falciparum* (P*f*HRP2) connected to ferriprotoporphyrin IX has antioxidant properties useful to the parasite, which may not have been earlier known by host antioxidants (118).

2.8 OXIDATIVE CHANGES IN THE HOST INDUCED BY *PLASMODIUM*

During malaria infection, the host natural defense machinery is activated with involvement of phagocytes (macrophages and neutrophils). These, sequentially, produce huge amounts of ROS and RNS, resulting in an imbalance between oxidizing species

formation and antioxidants activity. This imbalance triggers oxidative stress, which is an important machinery of human hosts in response to infections and can lead to the death of the parasites. The ability of oxidative stress to promote the killing of parasites has been established in *in vitro* studies (119). *Plasmodium yoelii* species grown in the presence of glucose and glucose oxidase generated H_2O_2, a reactive oxygen species, capable of killing the parasite; free radical superoxide ($O_2^{\bullet -}$) when grown in the presence of xanthine and xanthine oxidase, and an ensuing burst of further oxidative products, with subsequent damage to the parasites (119). Moreover, oxidative stress indicators are found to be high in infected humans (71, 72, 120–122). This result from increased free radicals production, suggested by increased malondialdehyde (MDA) which is an important lipid peroxidation marker, signifying that oxidative stress is an important machinery in parasite infection (74).

Oxidative stress according to studies can take part in the pathogenesis of thrombocytopenia associated to malaria. It has been suggested that *P. vivax* malaria infected individuals have reduced platelets number and antioxidant enzymes—superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities while lipid peroxidation of platelets (estimated by measuring the MDA), is elevated (123).

Beside the synthesis of radical species, organisms have developed several antioxidant defense mechanisms in response to increased oxidative stress. Antioxidant defense is an innate physiological mechanism of organisms against damage caused by free radicals and is dependent on the utilization of cellular and systemic antioxidant reserves. Endogenous synthesis of these antioxidant compounds usually consists of three interdependent systems: enzymatic, small molecules and metal chelation, which prevent

oxidation of biomolecules. The antioxidant defense system also halts oxidative species generation by scavenging or by free radicals reduction, which by self-oxidation generate less reactive compounds (124). Even though a number of antioxidant enzymes are essential in the defense system, GSH-Px, catalase and SOD are considered among the important ones. These enzymes act directly on some free radicals, making them less reactive. However, they are not able to act on the highly reactive free radicals that are chiefly responsible for oxidative pathological processes such as hydroxyl and perhydryl radicals or peroxynitrite (124). GSH molecule of all the antioxidant molecules stands out as the most powerful protector of eukaryotic cells in the host defense against oxidative stress, acting upon several diverse mechanisms (125). Autonomously, the secretion of tumor necrosis factor-alpha (TNF-α) seems to induce oxidative stress through modulation of GSH metabolism, playing a key function in malaria physiopathogenesis (126).

2.9 MECHANISMS OF OXIDATIVE STRESS IN THE HUMAN HOST

Oxidative stress is caused by an imbalance between the pro-oxidant attacks and the antioxidant defense in animals. Particularly, RBCs in particular are susceptible to oxidative damage: 1) because as an oxygen carrier, RBCs are uninterruptedly exposed to high oxygen tension, 2) because RBCs have no capacity to repair their damaged components, and 3) because their membrane components are susceptible of lipid peroxidation (127). On the other hand, normal RBCs have a series of antioxidants, such as superoxide dismutase, catalase, glutathione peroxide, nicotinamide-adenine-dinucleotide phosphate, nicotinamide-adeninucleotide, glutathione, glutathione reductase, capable of hydrolyzing the oxidatively modified proteins and preventing a degree of the adverse damages by the oxidative stress (127). The absence of these

antioxidants often makes many microbial pathogens more vulnerable to oxygen radicals induced by the immune response (from monocytes and neutrophils) to the infection. In contrast, there are reports that the parasitic infection also induces the oxidative stress in the RBC environment (128). The parasites are considered to induce the production of reactive oxygen species (129) by depleting these defense components of RBC as above (130). It would be important to investigate the roles increased oxidative stress serves in killing intraerythrocytic parasites or cause damage to host RBC or surrounding tissues (129). Free radicals and other reactive species are constantly generated in vivo and cause oxidative damage to biomolecules (131).

Deoxyribonucleic acid (DNA) is probably the most significant target of oxidative attack (131). Oxidative damage to DNA by reactive oxygen species (ROS) may result in base modification, sugar damage, strand break, and DNA protein cross links (132). DNA Comet assay, (alkaline version in particular), is a popular method for the analysis of DNA damage (133). DNA damages consist of strand breakage, alkali labile sites and incomplete excision repair sites (65). The direct DNA-breaking can be estimated by alkaline elution, nick translation and also by the alkaline single cell gel-electrophoresis (SCGE) (133). Of these, modification of guanine by hydroxyl radicals at the C-8 site, frequently estimated as 8-OhdG is the most commonly studied lesion. Urinary excretion of 8-OhdG repair product from oxidative DNA modification by excision enzymes is an *in vivo* measure of overall oxidative DNA damage (132). Alpha tocopherol (vitamin E) shows a high solubility to lipid and preferably locates in cell membranes, where it also prevents the propagation of free radicals reactions as an effective antioxidant (133, 134). Although tocopherol deficiency can contribute to anemia because lack of the

antioxidants increases potential oxidant damage to erythrocytes (135), several animal studies have reported an apparent protective role of vitamin E deficiency in malaria (134). The absence of this antioxidant makes the parasite more vulnerable to oxygen radicals' reactions from the immune response to *Plasmodium* infection (134).

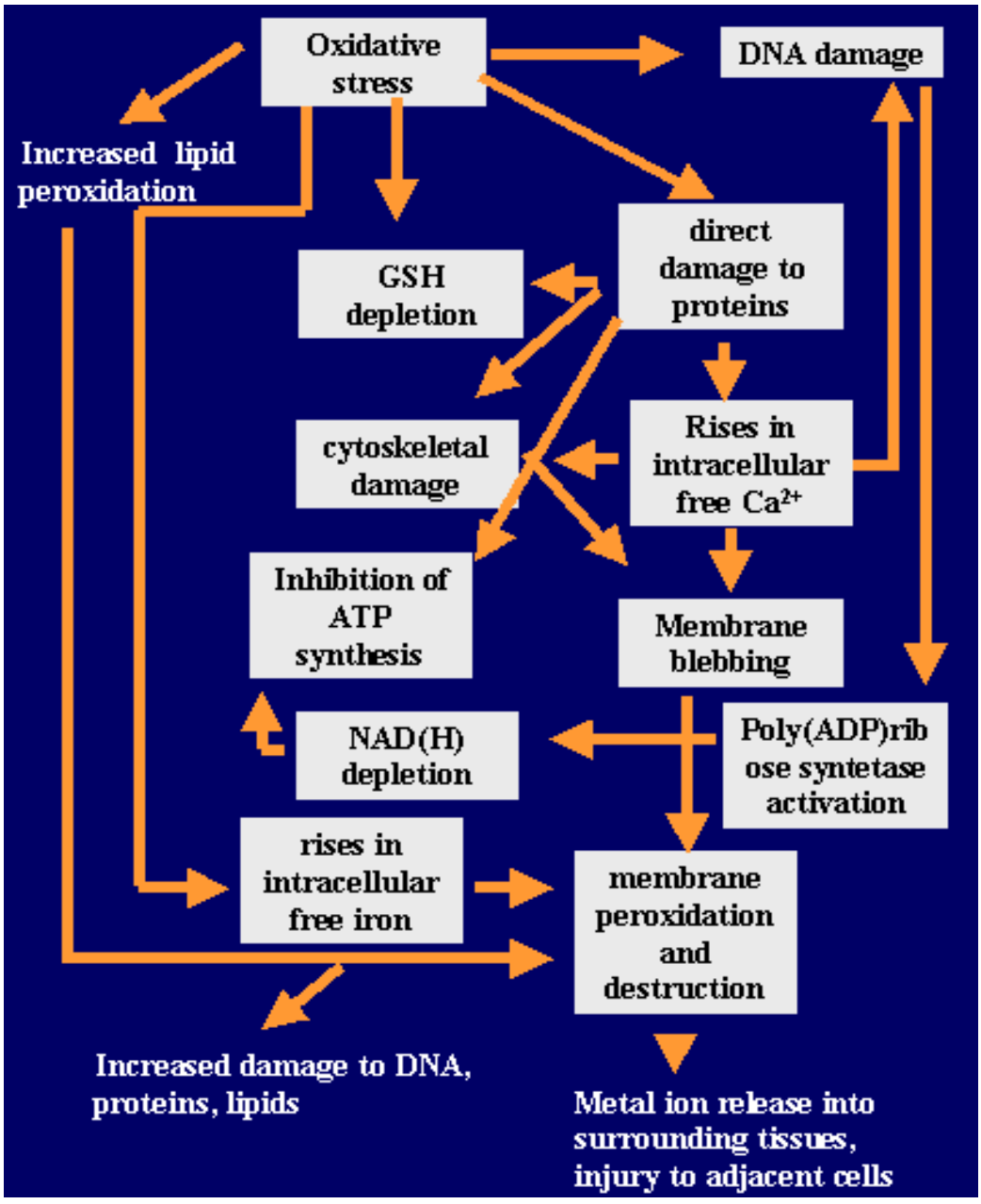

Figure 4: Mechanisms of cell damage by oxidative stress (Adapted from Halliwell, 1992).

CHAPTER THREE

3.0 METHODOLOGY

3.1 STUDY DESIGN

Case control study design was used for this investigation.

3.2 STUDY SITES

The study was conducted between June 2012 and June 2013 at Princess Marie Louis Hospital located in the Ashiedu Kete Sub-Metropolis in the coastal region of the capital city of Accra. This hospital serves as specialist hospital for children and provides service for the Ashiedu Kete township and its surrounding areas; the Achimota Hospital, located in the Achimota township which lies in the northern part of Accra, it serves the Achimota school as well as the local community; the Amasaman Hospital, located in the Ga West Municipality in the Greater Accra Region provides service to the Amasaman township and its surrounding areas. These study sites are all located within Accra which has a rainy period from April to October, followed by dry season from November to March. The transmission of malaria is perennial, but highest during the rainy season. Malaria transmission is perennial with distinct seasonal patterns. The peak malaria transmission season coincides with the period of major rains while the dry season has low rates of malaria infection (136). Infection is attributed mostly to *P. falciparum* with *Anopheles funestus* and *Anopheles gambiae* as the principal vectors (137). The estimated annual entomological inoculation rates (137), parasite prevalence (136) and malaria attack rates have been well studied (138).

3.3 INCLUSION AND EXCLUSION CRITERIA

3.3.1 Inclusion criteria

The study population in this investigation comprised of children (both male and female) up to 12 years old, admitted at the Achimota hospital, the Amasaman hospital and the Princess Marie Louis Hospital, all in the Accra metropolis presenting with severe malaria (parasitaemia level above 10,000/μL) and fever (i.e. axillary temperature > 37.5^{o}C), headache, vomiting, diarrhea, prostration, pallor, jaundice, respiratory distress and other clinical signs and symptoms, as documented earlier) but without cerebral malaria (68) (data collected by administering questionnaire, see Appendix 13). Those who satisfied the above- mentioned selection criteria and whose parents voluntarily gave informed consent (see Appendix 12) were enrolled.

3.3.2 Exclusion criteria

Subjects presenting with severe malaria (parasitaemia level above 10,000/ μL) and fever (i.e. axillary temperature > 37.5^{o}C), headache, vomiting, diarrhea, prostration, pallor, jaundice, respiratory distress and other clinical signs and symptoms, as documented earlier) but fell outside the age range of up to twelve (12) years as well as those who fell within the age range but did not present with malaria (i.e. blood film reporting negative for malaria parasites) were excluded from the study. Also, those who satisfied the inclusion criteria but whose parents did not give their consent were excluded from the study.

3.3.3 Control subjects

The control targets included non-malarious (as health controls) children (both male and female) up to 12 years of age as well as children who presented with conditions that mimicked malaria.

Parents were interviewed on the presenting symptoms and data on patients' documented clinical findings including vital signs were collected (with the express permission of physician).

3.4 SAMPLE COLLECTION AND LABORATORY ANALYSIS

After enrolling the participants, 2mls- 3mls of venous blood specimen were taken. Blood smears were done for all participants. Thick and thin blood films were made, thin film fixed with methanol and both thin and thick film stained with 10% Giemsa and examined for malaria parasites. Parasite density were documented as the number of parasites counted per 200 leucocytes on a thick film and converted into parasites per microlitre of blood based on the participants total white cell count obtained at enrolment and graded as low (1-999/µL), moderate (1000-9999/µL) and severe (>10,000/µL). Ten percent (10%) of both positive and negative slides were randomly selected and read by an independent microscopist as a quality control check. One hundred high power fields of the thick films were examined at 100× magnification before assigning a negative result.

3.5 HAEMATOLOGICAL AND PARASITOLOGICAL ANALYSIS

For haematological analysis whole blood specimens were analyzed for full blood count using automated haematology analyzer (Sysmex KX-21N, Japan).

3.5.1 Parasitological analysis

Working Giemsa stain was prepared by diluting one part stock Giemsa stain to 10 parts triton-buffered water. Both thick and thin films were prepared on a clean, dry, grease-free and labeled glass slide, with the thick film near to the frosted end and left to air- dry. The thin film was fixed by dipping that end of the slide in absolute methanol for 30 seconds. The whole slide (leaving the frosted end) was dipped in the working Giemsa stain for 10 minutes. The slide was then rinsed gently with phosphate buffer of pH 8.6. Stained slides were placed vertically on a draining rack to air- dry.

3.5.1.1 Microscopic Examination

Thick smear: minimum of 200 fields was examined for the presence of blood parasites under oil immersion objective. Thin smears: minimum of 200 fields under oil immersion objective was examined for species identification.

3.6 SUPEROXIDE DISMUTASE ACTIVITY DETECTION

The SOD activity levels were determined by a colorimetric method using SOD Assay kit (*Cayman Chemicals, Michigan- USA*). Whole blood obtained by venipuncture was centrifuged (3000rpm, 10mins at 4^{o}C), and plasma carefully separated. Two hundred and fifty (250) microlitres of erythrocytes was lysed with thousand (1,000) microlitres of ice-cold deionized distilled water (4^{o}C) and centrifuged at 6,000rpm for 20 minutes at 4^{o}C. Thousand (1,000) microlitres of the supernatant (erythrocyte lysate) was collected for assay and stored on ice. The supernatant was then diluted by a factor of 100 with sample buffer, and 10 µl of the diluted solution used to assay Cu/ZnSOD (Copper/Zinc Superoxide dismutase) activities as described by the manufacturer (*Cayman Chemicals, Michigan, USA*) (See Appendix 3)

3.7 COMET ASSAY

Fifty microlitres of erythrocytes from samples was collected and dispensed into individual cells and suspended in molten low-melting point agarose at 37°C. This mono-suspension was then cast on a comet microscope slide. The agarose was then gelled at 4°C.The slides were then immersed in a lysis solution to lyse the erythrocytes. After lysis of the cells (typically 1 hour at 4°C) the slides were washed in distilled water to remove all salts and immersed in a second solution - an electrophoresis solution (1x TBE). The slides were left for ~20 minutes in the electrophoresis solution prior to an electric field being applied. An electric field was applied (typically 1 V/cm) for ~20 minutes. The slides were then neutralized to pH 7, stained with a DNA-specific silver stain and analyzed using a microscope with an attached CCD (charge-coupled device - essentially a digital camera) that is connected to a computer with image analysis software (*Gaithersburg, MD, USA)* (see appendices 1and 2).

3.8 ETHICAL ISSUES

Ethical approval for this study was obtained from the Ethical and Research Review Committee of the University of Ghana Medical School, College of Health Sciences.

3.9 DATA AND STATISTICAL ANALYSIS

The data obtained in this investigation was subjected to statistical analysis. The parameters were presented as mean ± SD. Student's-'t' test was used to test differences between means. Pearson's correlation coefficients were used to examine the relationship between parameters. Statistical significant differences were indicated by $p<0.05$. All study data was captured on a structured case report form bearing subject demographic and identification numbers. All forms were reviewed before being double entered.

Statistical analyses were carried out with IBM SPSS statistical software (version 20.0) and Megastat (version 9.1; USA). Continuous and normal distributed data was compared by two-tailed student's t-test and proportions compared using chi- square (χ^2) tests with Fisher's exact test. Basic statistics was calculated for the baseline characteristics- fever, parasitaemia and presenting symptoms. Point estimates using proportions and means, and 95% confidence intervals was computed for the clinical and laboratory features.

CHAPTER FOUR

4.0 RESULTS

4.1 DEMOGRAPHIC DESCRIPTION OF STUDY PARTICIPANTS

Table 1: Demographic description of study participants

VARIABLES	MALE	FEMALE	UNCOMPLICATED MALARIA	SEVERE MALARIA
USSHER POLYCLINIC	5/150 (3.33%)	7/150 (4.67%)	4/105 (3.81%)	8/ 150 (7.62%)
CHILDREN'S HOSPITAL (PML)	30/150 (20.00%)	24/150 (16.00%)	9/ 105 (8.57%)	45/105 (42.85%)
ACHIMOTA HOSPITAL	44/150 (29.33%)	28/150 (18.67%)	7/ 105 (6.67%)	20/ 105 19.05%)
AMASAMAN HOSPITAL	3/150 (2.00%)	9/150 (6.00%)	6/ 105 (5.72)	6/ 105 (5.72)
TOTAL	83/150 (54.67%)	52/105 (45.33%)	26/105 (24.76%)	79/105 (75.24%)

A total of 150 children aged 6 months to 12 years that volunteered through the consent of their guardians to participate in the study were made available for study by questionnaire administration and venous blood sample collection. The average age of the children was 4.46 years at a standard deviation of 2.23 years. Out of the 150 children recruited as test subjects, 8.00% were recruited from Ussher polyclinic, 36.00% from PML, 48.00% from Achimota hospital, and 6.00% from the Amasaman hospital. Of these, 54.67% were males and 45.33% were females (see Table 1 and fig. 6). 24.8% (26/105) presented with uncomplicated malaria whiles 75.2% (79/105) presented with severe malaria (see table 1 and figure 6). Forty five (45) controls subjects were recruited in the study (see Table 1 and figure 5)

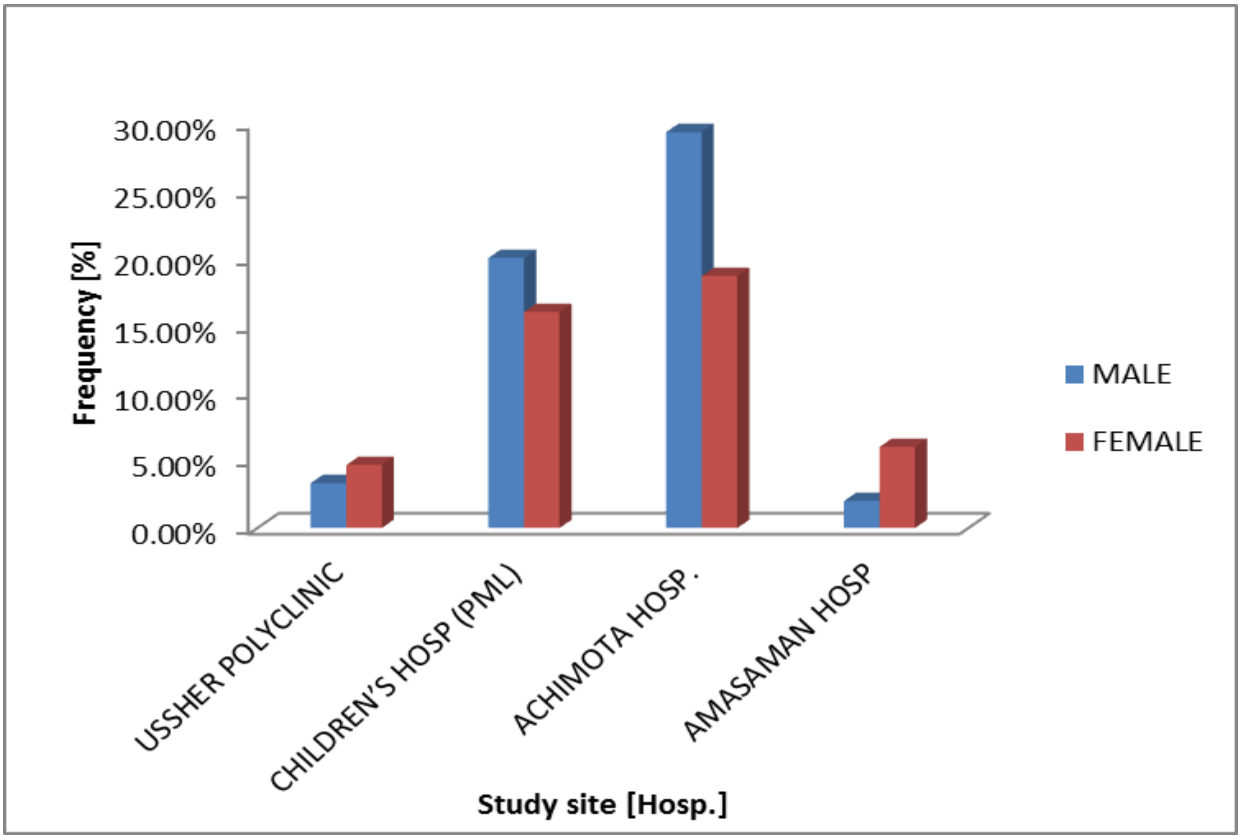

Figure 5: Demographic Distribution of Participants

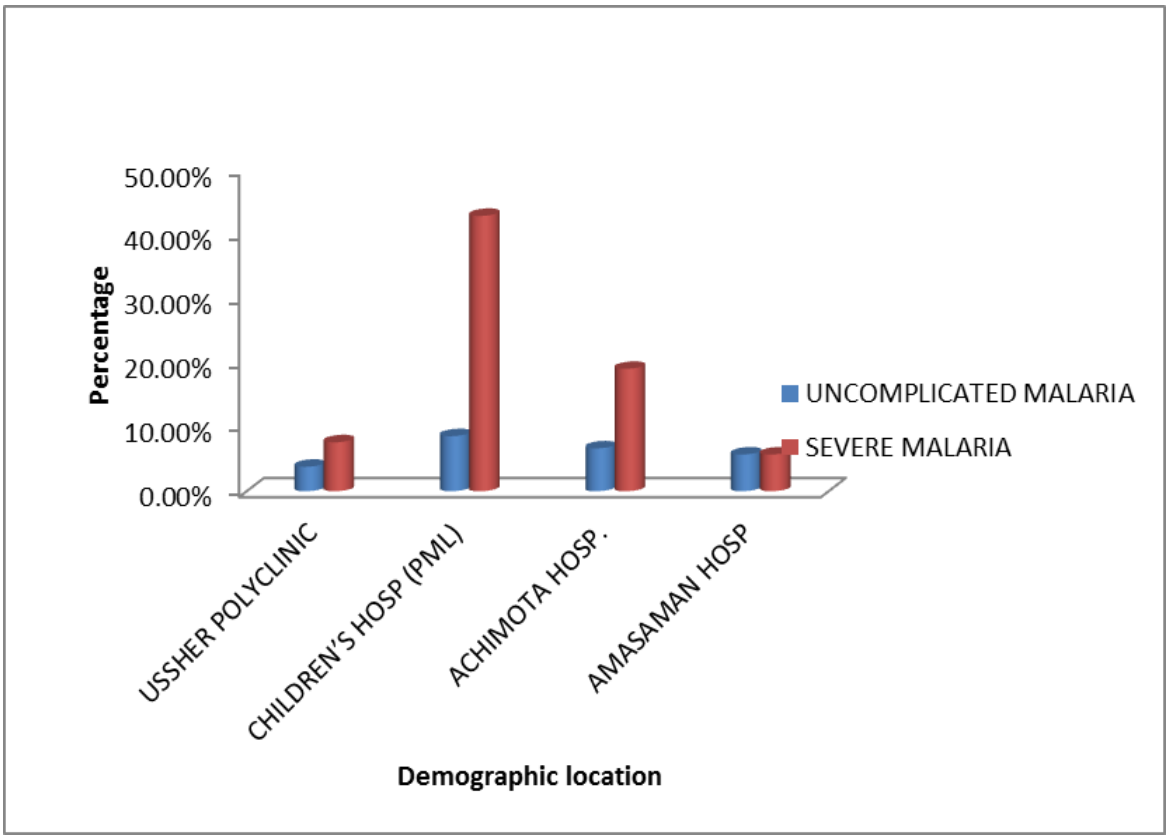

Figure 6: Demographic Distribution of Malaria Presentation

4.2 CLINICAL MANIFESTATIONS IN PARTICIPANTS

Table 2: Clinical Manifestation in Study Participants

CLINICAL MANIFESTATION	SEVERE MALARIA	UNCOMPLICATED MALARIA	CONTROL
	n= 79	**n= 26**	**n= 45**
FEVER	79 (**100%**)	26 (**100%**)	45 (**100%**)
DIARRHOEA	14 (**17.72%**)	4 (**15.38%**)	4 (**8.89%**)
RESPIRATORY DISTRESS	47 (**59.49%**)	8 (**30.77%**)	2 (**4.45%**)
ANAEMIA	52 (**65.82%**)	15 (**57.69%**)	13 (**28.89%**)
VOMITTING	71 (**89.87%**)	18 (**69.23%**)	0 (**0%**)
PALLOR	54 (**68.35%**)	14 (**53.85%**)	13 (**28.89%**)
PROSTRATION	50 (**56.96%**)	4 (**15.38%**)	0 (**0%**)
CONVULSION	15 (**18.99%**)	0 (**0%**)	0 (**0%**)

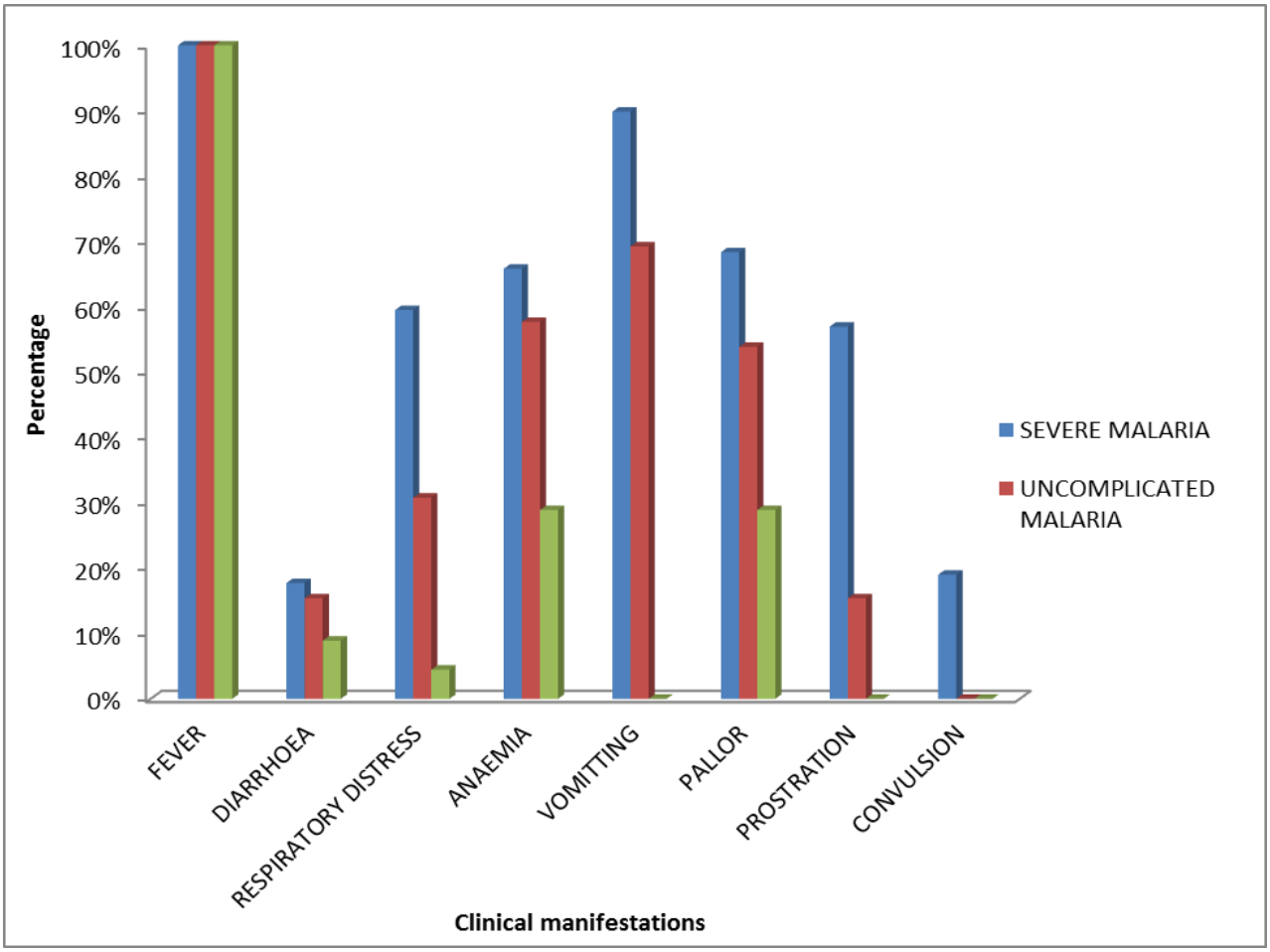

Figure 7: Clinical Manifestations in study participants

Of the 26 uncomplicated malaria subjects, 26/26 (100%) presented with fever; 4/26 (15.38%) diarrhoea; 8/26 (30.77%) respiratory distress; 15/26 (57.69%) anaemia; 18/26 (69.23%) vomiting; 14/26 (53.85%) pallor; 4/26 (15.38%) prostration and without convulsion. Of all these subjects, 5/26 (19.23%) presented only one clinical sign; 5/26 (19.23%) two clinical signs; 2/26 (7.69%) three clinical signs and 14/26 (53.85%) more than three clinical signs.

Out of the 45 control subjects mimicking malaria, 45/45 (100%) presented with fever, 4/45 (8.89%) diarrhoea, 2/45 (4.45%) respiratory distress, 13/45 (28.89%) anaemia and

13/45 (28.89%) pallor. None presented vomiting, prostration and convulsion. Of these, 28/45 (62.22%) presented only one clinical sign, 5/45 (11.11%) two clinical signs, 8/45 (17.78%) three clinical signs and 4/45 (8.89%) more than three clinical signs.

Of the 79 severe malaria subjects, 79/79 (100%) presented with fever, 14/79 (17.72%) diarrhoea, 47/79 (59.49%) respiratory distress, 52/79 (65.82%) anaemia, 71/79 (89.87%) vomiting, 54/79 (68.35%) pallor, 50/79 (56.96%) prostration and 15/79 (18.99%) convulsion. Of all these subjects, 3/79 (3.79%) presented only one clinical sign, 14/79 (17.72%) two clinical signs, 8/79 (10.13%) three clinical signs and 53/79 (67.09%) more than three clinical signs (see Table 2 and figure 7).

Table 2a: Comparison of clinical variables among control and uncomplicated malaria subjects

	Controls	Uncomplicated		
Symptoms	**%**	**%**	**Z-value**	**P-value**
Fever	100.0	100.0	0.000	1.000
Diarrhoea	11.1	15.4	0.492	0.623
Respiratory Distress	4.4	30.8	3.082	0.002*
Anaemia	28.9	57.7	2.391	0.017*
Vomiting	0.0	69.2	6.456	0.001*
Pallor	28.9	58.8	2.479	0.013*
Prostration	0.0	15.4	2.710	0.008*
Convulsion	0.0	0.0	0.000	1.000

***Significant at 5%**

Comparison of clinical manifestations between control and uncomplicated malaria subjects are shown in Table 2a. With the exception of fever convulsion and diarrhoea there were significant associations in the clinical signs presented between these groups with *p- values* < 0.05.

Table 2b: Comparison of clinical variables among control and severe malaria subjects

	Controls	Severe		
Symptoms	**%**	**%**	**Z-value**	**P-value**
Fever	100.0	100.0	0.000	1.000
Diarhoea	11.1	17.7	0.997	0.319
Respiratory Distress	4.4	59.5	6.040	0.001*
Anaemia	28.9	65.8	3.794	0.001*
Vomiting	0.0	88.6	7.041	0.001*
Pallor	28.9	68.4	3.752	0.001*
Prostration	0.0	57.0	7.582	0.001*
Convulsion	0.0	19.0	4.012	0.001*

***Significant at 5%**

Comparison of clinical manifestations between control and severe malaria subjects are shown in Table 2b. Besides fever and diarrhoea there were significant associations in the clinical signs presented between these groups with *p- values* < 0.05.

Table 2c: Comparison of clinical variables among uncomplicated and severe malaria subjects

	Uncomplicated	Severe		
Symptoms	**%**	**%**	**Z-value**	**P-value**
Fever	100	100	0.001	1.000
Diarhoea	15.4	17.7	0.671	0.787
Respiratory Distress	30.8	59.5	2.567	0.010*
Anaemia	57.7	65.8	0.461	0.769
Vomiting	69.2	88.6	2.525	0.024*
Pallor	58.8	68.4	0.937	0.348
Prostration	15.4	57.0	3.686	0.002*
Convulsion	0.0	19.0	2.418	0.016*

***Significant at 5%**

Comparison of clinical manifestations between uncomplicated and severe malaria subjects are shown in Table 2c. There were significant associations in the clinical signs (respiratory distress, vomiting, prostration and convulsion) presented between these groups with *p- values* < 0.05.

4.3 HAEMATOLOGICAL PARAMETERS IN STUDY PARTICIPANTS

Blood samples from the malaria patients showed positive blood smear for the malaria parasites, while no parasites were found in the healthy control subjects. The effects of malarial infection on the parameters measured are compared in Table 3.

Table 3: Haematological Parameters for Severe Malaria, Uncomplicated Malaria and Control Groups

	HAEMOGLOBIN [g/dl]			TOTAL WBC [x 109/l]			NEUTROPHILS[%]			LYMPHOCYTES [%]		
	SEVERE MALARIA	UNCOMPLIC. MALARIA	CONTROL	SEVERE MALARIA	UNCOMPLIC. MALARIA	CONTROL	SEVERE MALARIA	UNCOMPLI. MALARIA	CONTROL	SEVERE MALARIA	UNCOMPLIC. MALARIA	CONTROL
Count	79	26	45	79	26	45	79	26	45	79	26	45
Mean ± SD	10.03 ± 2.36	9.92 ±2.87	11.09 ±1.39	8.90 ±3.86	9.99 ±10.73	8.85 ±4.96	63.48 ±16.65	58.64 ± 18.93	49.86 ± 20.32	36.65 ±16.59	41.36 ±18.94	50.14 ±20.31
Min.-Max.	4.7-15.0	1.03-10.36	6.9-14.0	1.0-20.6	1.5-59.7	1.0-22.4	21.6-90.6	27- 88.0	12.7-85.4	9.4-78.4	12.0-73.0	14.6-87.3
Sample Variance	5.57	8.22	1.93	14.86	115.06	24.57	227.18	358.51	412.71	275.29	358.73	412.65
S.E.M	0.27	9.33	0.21	0.43	58.2	0.74	1.87	61	3.03	1.87	61	3.03
C.I 95. %	9.5-10.6	0.5-8.8	10.7-11.5	8.0-9.8	2.1- 5.7	7.4-10.3	59.8-67.2	3.7-51.0	43.8-56.0	32.9-40.4	3.7-33.7	44.0-56.3

The mean haemoglobin levels for the subjects presenting with uncomplicated malaria, severe malaria and the control group were 9.92 g/dl ± 2.87, 10.03 g/dl ± 2.36 and 11.09 g/dl ± 1.39 respectively. The mean neutrophil levels for the subjects presenting with uncomplicated malaria, severe malaria and the control subjects were 58.6% ± 18.93, 63.5% ± 16.65 and 49.9% ± 20.32 respectively. The mean lymphocyte levels for the subjects presenting with uncomplicated malaria, severe malaria and the control subjects were 41.4% ± 18.94, 36.7% ± 16.59 and 50.1% ± 20.31 respectively (see Table 3).

4.4 VARIABLES IN DIFFERENT GENDER AMONG STUDY GROUPS

Table 4: Variables In Different Gender Among Study Groups

	UNCOMPLICATED MALARIA		SEVERE MALARIA		CONTROL	
VARIABLES	**MALE (MEAN)**	**FEMALE (MEAN)**	**MALE (MEAN)**	**FEMALE (MEAN)**	**MALE (MEAN)**	**FEMAL (MEAN**
SOD ACTIVITY (U/ml)	462.77	390.41	266.03	358.91	433.44	740.84
Parasite density ($x10^3$)	5.89	6.61	59.41	54.89	N/A	N/A
Haemoglobin (g/dl)	9.92	9.83	10.37	9.72	11.08	11.04
TWBC ($x10^9$/l)	10.00	11.30	8.90	8.60	9.15	8.26
Neutrophils (%)	58.70	61.00	63.4	61.9	49.02	49.40
Lymphocytes (%)	41.30	39.00	36.7	38.1	50.98	50.6

Key

N/A: Non applicable

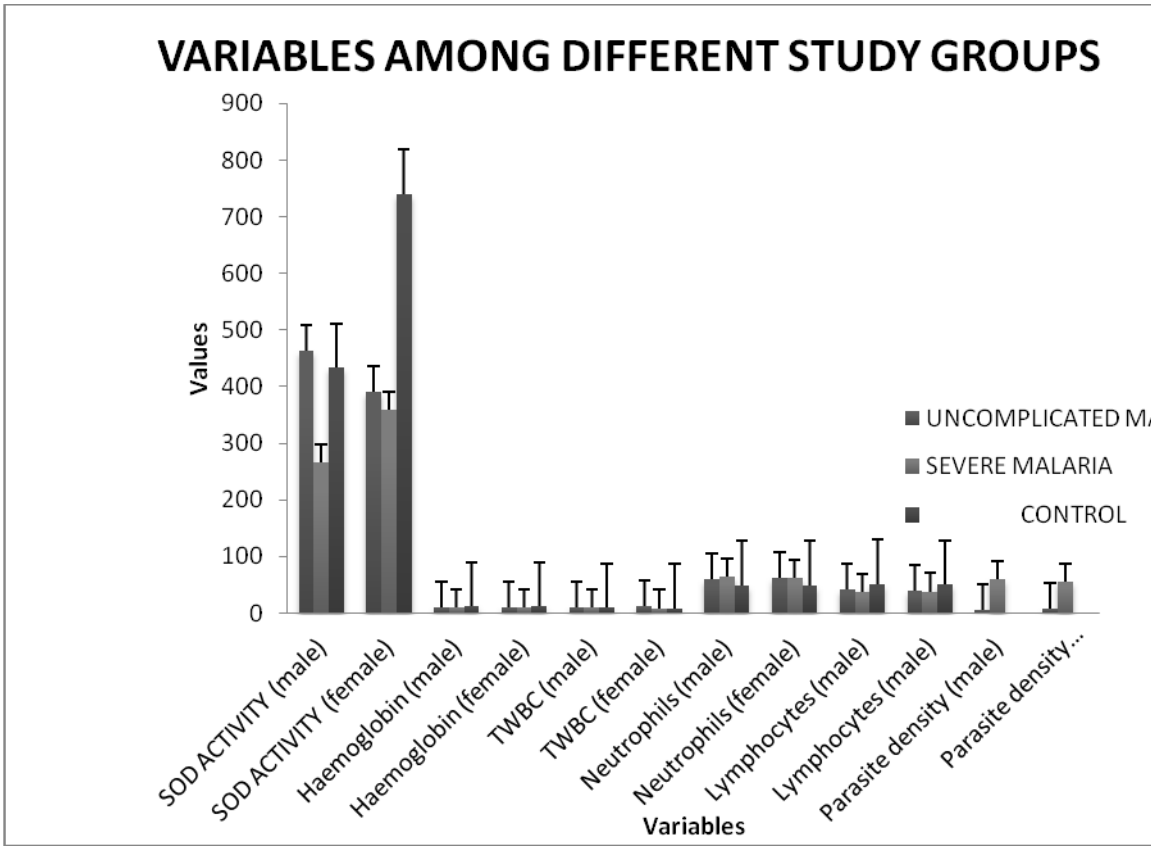

Figure 8. Variables In Different Sexes Among Study Groups

The mean SOD activity level for uncomplicated malaria subjects was 468.31 U/ml and 485.99 U/ml for male and female respectively. Male and female mean SOD levels in severe malaria subjects were 266.03 U/ml and 358.31 U/ml respectively. The mean SOD levels for the control subjects were 433.44 U/ml and 740.84 U/ml for males and females respectively (see Table 4 and figure 8).

4.5 SOD ACTIVITY LEVELS AND HAEMAMTOLOGICAL VARIABLES IN CASE SUBJECTS

Table 5: SOD Activity Levels and Haematological Parameters in Case Subjects

	Mean ± SD	Regression (r)	p- value
Haemoglobin [g/dl]	10.00 ± 2.43	0.001	0.001*
TWBC [x 10^9/l]	9.17 ± 6.25	-0.086	0.038
Neutrophils [%]	62.28 ± 17.28	-0.177	0.001*
Lymphocytes [%]	37.81 ± 17.23	0.174	0.001*

***Significant at 5%**

The mean ± SD haemoglobin (Hb) level was 10.00± 2.43 g/dl, the mean leucocytes (TWBC) level was 9.17 x 10^9± 6.25 /µl, the mean absolute neutrophils level was 62.28% ± 17.28, and the total absolute lymphocyte level was 37.81% ± 17.23. The relationship between the SOD activity and the haemoglobin level gave a positive regressive association, r= 0.001 at a *P-value*= 0.001. The relationship between the SOD activity and the total leucocytes level gave a negative regressive association, r= -0.086 at a *P-value*= 0.038. The relationship between the SOD activity and the absolute neutrophils level gave a negative regressive association, r= -0.177 at a *P-value*= 0.001. The relationship between the SOD activity and the absolute lymphocyte level gave a positive regressive association, r= 0.174 at a *P-value*= 0.001 (see table 5).

4.6 SOD ACTIVITY AND HAEMATOLOGICAL VARIABLES IN MALARIA SUBJECTS

Table 6: Correlation between variables and parasitaemia

Variables	Correlation Coefficient	P-value
SOD	-0.175	0.038*
Hb	-0.232	0.036*
WBC	0.163	0.046*
Neutrophils	-0.771	0.001*
Lymphocytes	0.659	0.001*

***Significant at 5%**

There was a significant (*p- value* 0.038) negative correlation (r= -0.175) between the SOD activity and the parasite density. There was also a significant (p- value < 0.05) association between parasite density and haemoglobin, white blood cells, neutrophils and lymphocytes (see Table.6). However, the correlation between parasite density and haemoglobin and neutrophils was negative.

4.7 SOD ACTIVITY AND HAEMATOLOGICAL VARIABLES IN UNCOMPLICATED MALARIA

Table 7: Correlation between Haematological Variables and SOD levels

	Mean ± SD	R	P- value
Haemoglobin [g/dl]	9.92 ± 2.68	0.068	**0.001***
TWBC [X10^9	9.99 ± 10.73	-0.170	**0.001***
Neutrophils [%]	58.64 ± 18.93	-0.072	**0.001***
Lymphocytes [%]	41.36 ± 18.94	0.072	**0.001***

***Significant at 5%**

The mean levels for haemoglobin, total leucocytes (TWBCs), neutrophils and lymphocytes in uncomplicated malaria were 9.92 ± 2.68 g/dl; 9.99 x 10^9 ± 10.73 /l; 58.64% ± 18.93 and 41.36% ± 18.94 respectively. The association between SOD and haemoglobin gave a positive correlation (r = 0.068) and a *P- value* of 0.001. The association between SOD and total leucocyte gave a negative correlation (r = -0.170) and a *P- value* of 0.001. The association between SOD and neutrophils gave a negative correlation (r = -0.072) and a *P- value* of 0.001, and the association between SOD and lymphocytes gave a positive correlation (r= 0.072) and a *P- value* of 0.001 (see table 7).

4.8 SOD ACTIVITY AND HAEMATOLOGICAL VARIABLES AMONG DIFFERENT PARASITAEMIA GROUPS

Table 8: Comparison of variables among the different parasitaemia (Anova)

Variables	Groups	n	Mean	S.D	F-value	P-value
SOD	Controls	45	546.42	776.22		
	Uncomplicated malaria	26	465.55	341.38		
	Severe malaria	79	256.55	241.46	5.713	0.004*
	Total	150	379.74	495.70		
Hb	Controls	45	11.08	1.38		
	Uncomplicated malaria	26	9.92	2.68	3.970	0.021*
	Severe malaria	79	10.02	2.36		
	Total	150	10.32	2.22		
TWBC	Controls	45	8.79	4.98		
	Uncomplicated malaria	26	9.98	2.70	2.276	0.106
	Severe malaria	79	10.11	2.31		
	Total	150	9.69	3.42		
NEUTROPHIL	Controls	45	49.87	20.27		
	Uncomplicated malaria	26	58.64	18.93	7.462	0.001*
	Severe malaria	79	63.48	16.65		
	Total	150	57.33	18.62		
LYMPHOCYTES	Controls	45	50.16	20.29		
	Uncomplicated malaria	26	58.68	18.91	7.704	0.001*
	Severe malaria	79	63.50	16.64		
	Total	150	58.66	18.99		

***Significant at 5%** **Anova**

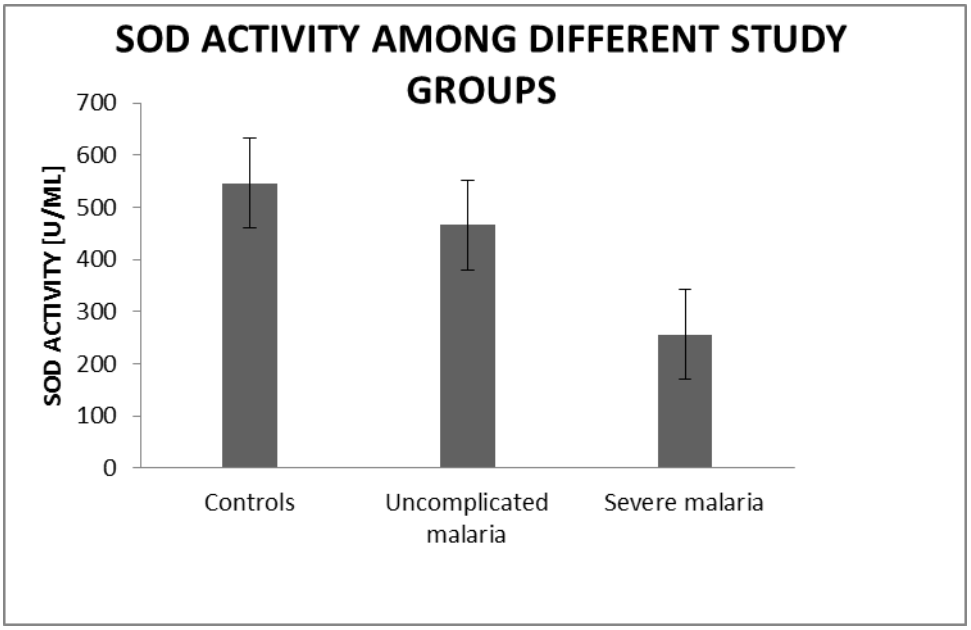

Figure 9: Chart showing the mean SOD activity levels among the different parasitaemia groups

The mean SOD activity levels for the uncomplicated malaria, severe malaria and the control subjects were 465.55 ± 341.38, 256.55 ± 241.46 and 546.42 ± 776.22 respectively. The association of the SOD activity with the different parasitaemia subjects was significant at a *p-value* of 0.004 (see table 8 and figure 9).

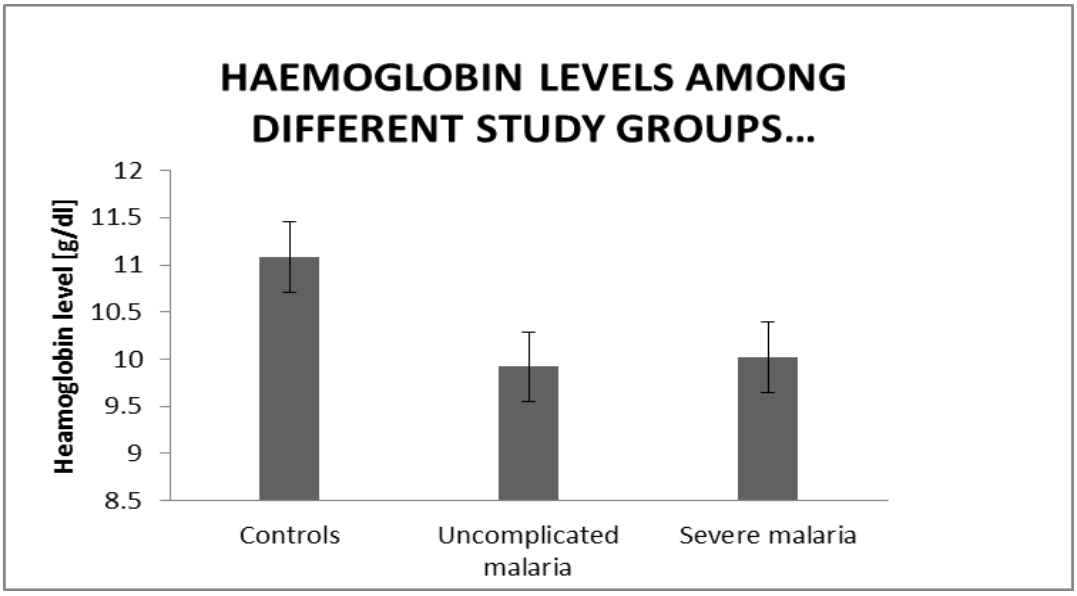

Figure 10: Chart showing mean Haemoglobin levels among different Study groups.

The mean haemoglobin levels for the severe malaria, uncomplicated malaria and control subjects were 5.87 g/dl ± 2.87, 10.03 g/dl ± 2.36, and 11.08 g/dl ± 1.37 respectively. The association of the haemoglobin with the different parasitaemia subjects was significant at a *p-value* of 0.001 (see Table 8 and figure 10).

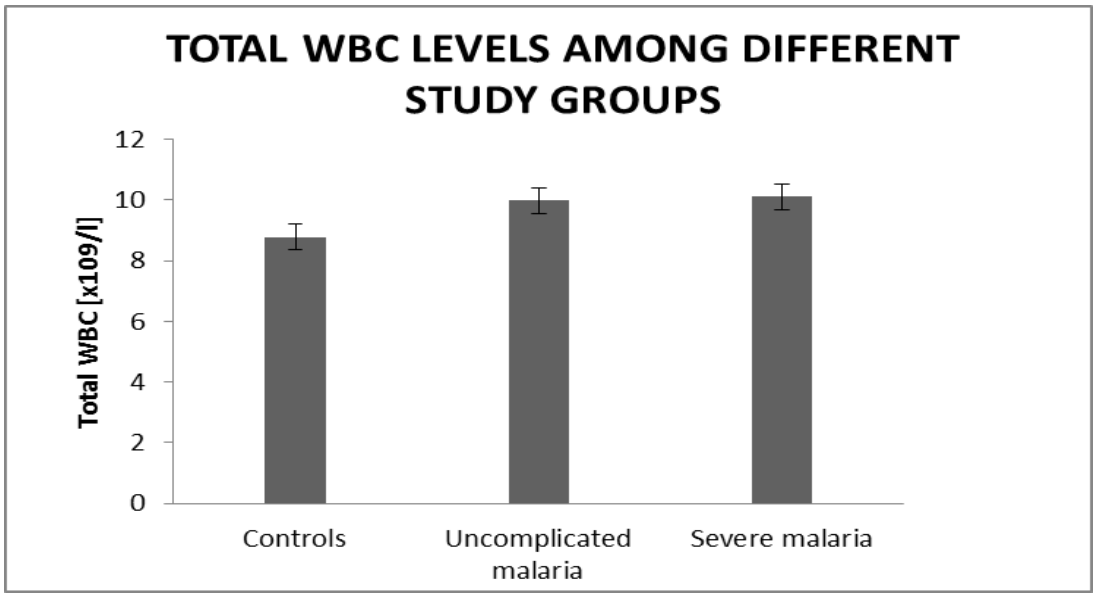

Figure 11: Chart showing mean TWBC levels among different parasitaemia groups

The mean total white blood cells level for the uncomplicated malaria, severe malaria and the control subjects were 9.98 x 10^9 ± 2.70 /l, 10.11 x 10^9 ± 2.31/l and 8.79 x 10^9/l ± 4.98/ l respectively. The association of the total white cells with the different parasitaemia subjects gave a *p- value* of 0.106 (see table 8 and figure 11).

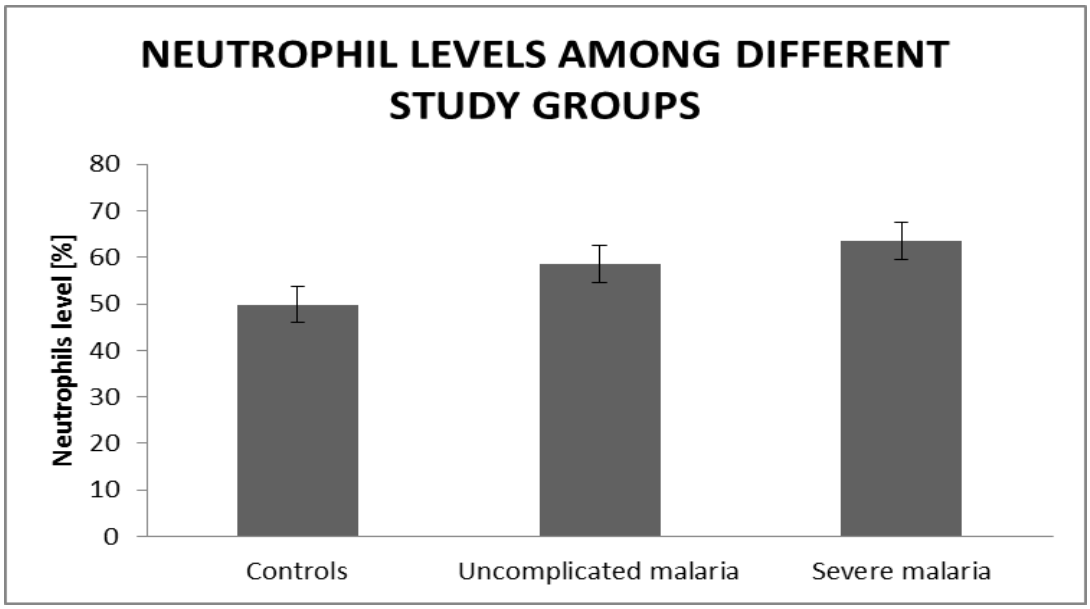

Figure 12: Chart showing mean Neutrophils level among the different parasitaemia groups

The mean neutrophils level for uncomplicated malaria, severe malaria and the control subjects were 58.64 % ± 18.93 %, 63.48 ± 16.65% and 49.86 ± 20.32% respectively. The association of the neutrophils level with the different parasitaemia groups gave a *p- value* of 0.001 (see table 8 and figure 12).

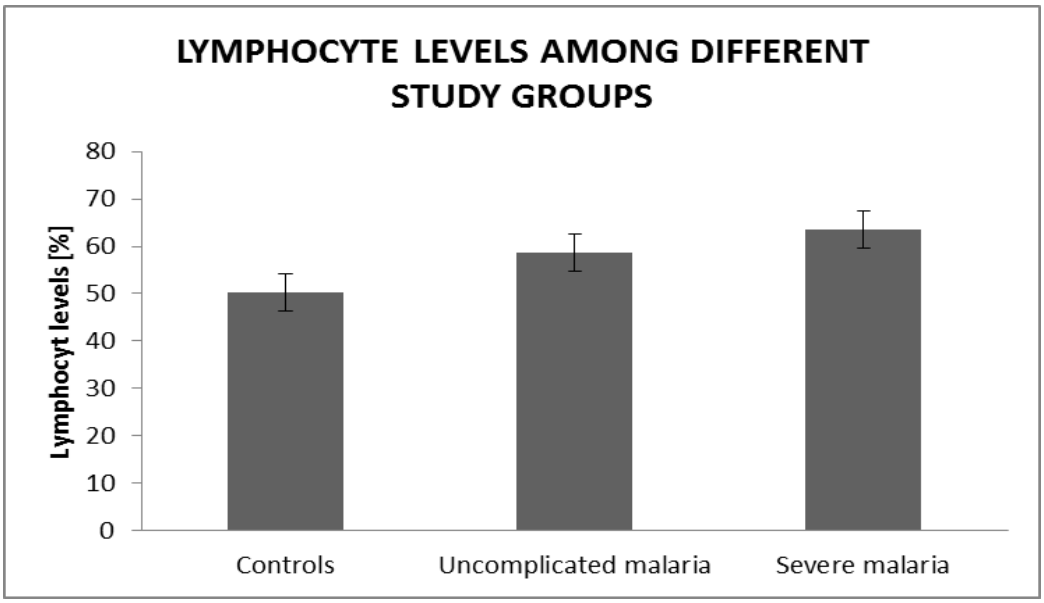

Figure 13: Chart showing mean Lymphocytes level among different parasitaemia groups

The mean Lymphocytes level for uncomplicated malaria, severe malaria and control groups were 41.36% ± 18.94, 36.65 %± 16.59 and 50.14% ± 20.31 respectively. The association of the lymphocytes level with the different parasitaemia groups gave a *p-value* of 0.001(see Table 8 and figure 13).

4.9 PARASITE DENSITY AND HAEMATOLOGICALVARIABLES AMONG STUDY GROUPS

Table 9: Comparison of variables among different parasitaemia (Post Hoc)

Dependent Variable	(I) GRP	(J) GRP	Mean Diff. (I-J)	Std. Error	P-value	95% C.I.	
SOD [U/ml]	Controls	Uncomplicated	80.87	118.42	0.496	-153.165	314.899
	Controls	Severe	289.86*	89.78	0.002*	112.435	467.297
	Uncomplicated	Complicated	209.00	108.69	0.056	-5.801	423.798
Hb [g/dl]	Controls	Uncomplicated	-1.170	0.537	0.031*	-2.231	-0.108
	Controls	Severe	-1.066	0.407	0.010*	-1.872	-2.615
	Uncomplicated	Complicated	0.103	0.493	0.834	-0.871	1.078
TWBC X [10^9 /l]	Controls	Uncomplicated	-1.19	0.84	0.155	-2.845	0.458
	Controls	Severe	-1.35	0.63	0.039*	-2.569	-0.064
	Uncomplicated	Severe	-0.12	0.77	0.873	-1.639	1.393
NEUTROPHILS [%]	Controls	Uncomplicated	39.88*	3.02	0.001*	33.911	45.854
	Controls	Severe	40.93*	2.29	0.001*	36.440	45.495
	Uncomplicated	Severe	1.08	2.77	0.696	-4.396	6.566
LYMPHOCYTES [%]	Controls	Uncomplicated	-8.51	4.48	0.059	-17.371	0.341
	Controls	Severe	-13.65	3.40	0.001*	-20.051	-6.622
	Uncomplicated	Severe	-4.82	4.11	0.243	-12.950	3.307

***Significant at 5%** **Post Hoc Analysis**

The mean difference and the *p-value* in the SOD levels between the control and the uncomplicated groups were 80.87 U/ml and 0.496 whiles the same for the control and severe subjects were 289.86 U/ml and 0.002 respectively. The mean difference between the uncomplicated and the severe subjects was 209.00 and a *p-value* of 0.056.

The mean difference and the *p-value* of the haemoglobin levels between the control and the uncomplicated groups were -1.170g/dl and 0.031 whiles the same for the control and severe subjects were -1.060 and 0.010 respectively. The mean difference between the uncomplicated and the severe subjects was 0.103 and a *p- value* of 0.834.

The mean total leucocyte (TWBC) between the control and uncomplicated malaria subjects was -1.19 $x10^9$ /l and a *p-value* of 0.155. The mean leucocyte difference between the severe and control subjects was -1.35 $x10^9$ /l and the p-value was 0.039 whiles the mean leucocyte difference between the uncomplicated and severe malaria subjects and the *p-value* were -0.12 $x10^9$ /l and 0.873 respectively.

The mean neutrophil and lymphocyte difference as well as the *p-value* between the control and uncomplicated subjects, severe and control subjects, as well as the uncomplicated and severe subjects were 39.88% , 0.001 and -8.51%, 0.059; 40.93%, 0.001 and -13.65%, 0.001, and1.08%, 0.696 and -4.82, 0.243 respectively (see table 9).

4.10 EVALUTION OF *P. FALCIPARUM* DNA TO REACTIVE OXYGEN SPECIES IN PARASITE INFECTED RED BLOOD CELLS USING DNA COMET ASSAY

The figures below show various picture micrographs of infected RBCs take through the DNA comet assay and taken through silver staining.

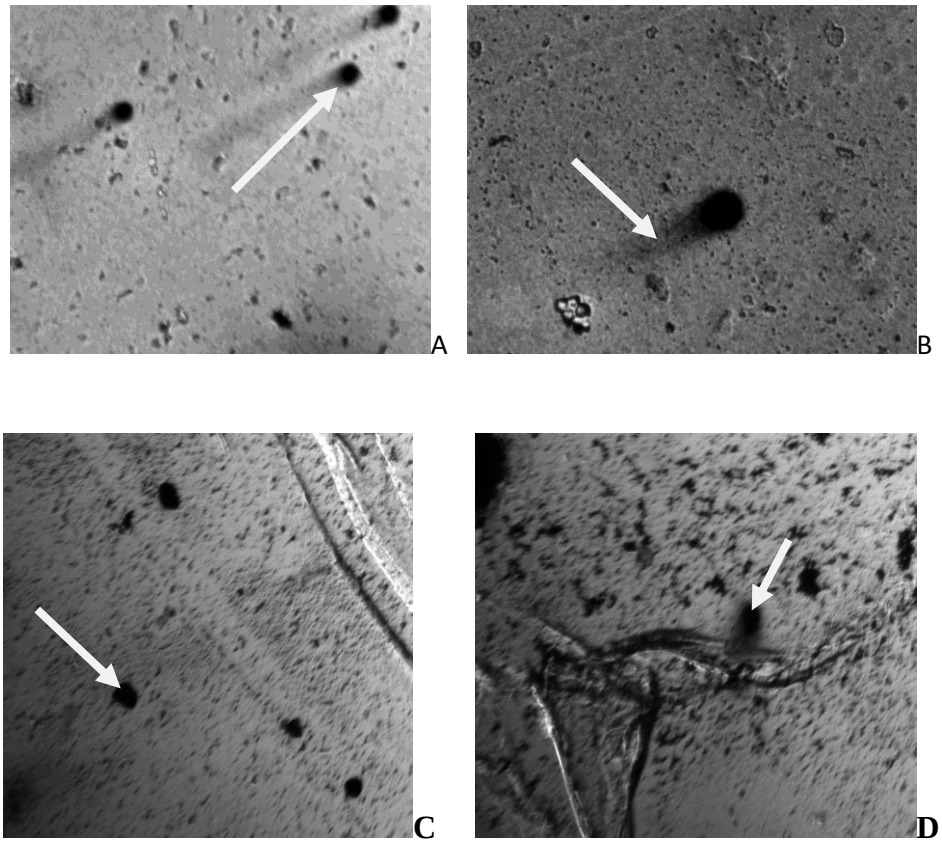

Figure 14: Silver stained parasite infected RBCs with comet tail, magnification X100**.** (Comet slides. A: IRBCs with comet tail; B: comet tail; C: Raptured IRBCs: D: comet head). (A-D) are images of silver stained comet assay slides prepared from packed cells (patient sample) demonstrating parasite infected RBCs with comet tails of varying degree indicating extend of parasite DNA damage by reactive oxygen species.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

During malaria infection, ROS (mainly superoxide anion) are produced in high levels, inducing parasite destruction and also tissue damage to the human host (139). To avoid biological injury, the antioxidant enzyme Cu/ Zn SOD in the human host converts these unstable radicals into H_2O_2 and water which is removed by catalase and glutathione (140). Previous reports have implicated *Plasmodium* parasites in the induction of oxidative stress in addition to its ability to cope with the oxidative stress generated during its erythrocytic stages (141, 142). This assertion was found to be in agreement with previous report that *Plasmodium* parasites produces active redox products, free haem, and H_2O_2 leading to oxidative stress in infected cells (143).The host builds up defense against the oxidative insults arising from the parasite's metabolism of haemoglobin which results in the formation of reactive oxygen species (ROS). In normal healthy conditions, ROS are mopped up from the host cell by the action of detoxification enzymes including SOD, GSH, and catalase (141) to the damaging effects of ROS build-up (oxidative stress) to the host system.

In this study, superoxide dismutase (SOD) activity was measured as a gauge of the level of ROS generated during malaria infection. The level of SOD was found to be significantly higher in subjects with uncomplicated malaria (low parasitaemia count) than those with high parasitaemia counts. This may be due to the utilization of SOD in the mopping up of high ROS generated during malaria infection This implied a higher oxidative stress status in high parasitaemic patients, confirming the findings in a similar study by Stocker *et al*. which showed that the activity of GSSG (oxidized glutathione)

reductase, an antioxidant enzyme was elevated in non- parasitized red blood cells but decreased with increasing parasite concentration (144). The findings in this study are also consistent with the suggestion in a study conducted by Kharazmi *et al*, that reactive oxygen intermediates (ROI) generated during malaria infection contribute to parasite death (145). The findings in this study are also in agreement with an inverse association obtained between parasite clearance time and oxygen radical production in a study results reported by Greve *et al.* (146). Previous studies have also reported that in parasitized erythrocytes, increase in the production of free oxygen radicals leads to a decrease in antioxidant enzymes (143, 144, 147). Thus, the significant reduction of erythrocyte antioxidant enzymes activity in high parasitaemia patients might be the predisposing factor to higher oxidative stress leading to damage on erythrocyte membranes (148).

The mean erythrocyte concentration of SOD was significantly lower in cases than control subjects which confirms results obtained in a study by Narsaria *et al.* (149). This affirms its role as an antioxidant, where levels decreased in an effort to offset the oxidant stress. SOD upgrades endothelial cell damage triggered by adherent parasitized RBCs, underscoring their probable therapeutic benefit as endothelial cell protectors (150). In malaria subjects, erythrocytic antioxidants have been shown to be lower (151) and this affirms the results obtained in this study.

A study findings showed that as the parasite load increases in the RBC, about 80% of the total haemoglobin is digested (152). The haemoglobin is degraded, within the food vacuoles of the parasite, from oxyhaemoglobin to methaemoglobin (153) with resultant production of ROS. This assertion was found to be in agreement with the findings in this

study: haemoglobin levels were found to be lower in malaria groups with higher SOD levels. Oxidative stress has been reported to play a major role in the development of malarial anaemia and Plasmodium falciparum-infected red cells are known to produce more ROS (164), thus making anaemia a reliable predictor of the severity of disease. Severe anaemia, besides fever and vomiting was the most common manifestation of uncomplicated and severe malaria in all the age groups as observed by other workers (165).

There was a negative association between parasite density and neutrophils in all the various study groups (*p- value*= 0.001) among case subjects, which suggests an occurrence of down- regulation of neutrophils as parasite density increases. This is because even though neutrophils are the first cells recruited from the bloodstream to fight invading microbes (154), these cells are activated during their interaction with parasites. This leads to the generation of NADPH which is a major subunit in the generation of ROS (mainly O_2^-) which is dismutated spontaneously by RBCs SOD or by antioxidant system of the host. Lambert and Brand, (155), reported that ROS produced by activated polymorphonuclear cells (PMNs), probably play an important role in the control of parasitaemia during malaria infection.

CD8+ T cells have been known to play a major role in protection against pre-erythrocytic stages of malaria infection (156). Rzepczyk *et al.* reported in their study that gamma delta T cells play meditative role in protective immunity, most probably through the production of Th1 cytokines such as TNF alpha, TNF delta and IFN gamma during malaria infection (157). In this study, lymphocyte levels were found to increase as the

parasitaemia level increased. This could be attributed to the activation of the lymphocytes during *P. falciparum* infection. The results agreed with the argument by Rzepczyk *et al.* that gamma delta T cells are activated during *P. falciparum* infection and the activated cells can persist for many weeks after treatment. (157). The results in this work showed T lymphocytes direct association with the SOD levels which meant that during malaria infection, the T lymphocytes respond to parasite antigens by releasing factors that stimulate the proliferation of effector cell precursors and their subsequent recruitment into the red pulp of the spleen. Here, and probably in the peripheral circulation, it is believed that these effector cells bind to the surface of the parasitized erythrocytes and are activated to release superoxide (O_2^-), resulting in oxidative stress build- up. The consequent exposure to this oxidant stress lead to the degeneration of parasites in erythrocytes (158).

The mean white cell levels were higher in the severe malaria subjects and lower in the control group which could be considered to be due to the presence of the *falciparum* infection as the white cells are known to be a major defense line in the host. Leucocytes are recruited and stimulated during *P. falciparum* infection and this leads to the release TNF which is correlated with clearance of the plasmodium parasite (159). Since TNF is known to activate the respiratory burst and degranulate neutrophils (160), the study results suggest that enhanced oxygen radical release is the underlying mechanism for parasite clearance. Nonetheless, while it has been shown that human phagocytes activated by *P. falciparum* antigens release ROI (145) and these may contribute to both pathology and parasite death in malaria (161), it remains uncertain to what extent

oxygen-dependent mechanisms participate in phagocyte-mediated killing of malaria parasites.

The use of the comet assay demonstrated parasite DNA damage which suggests an effective killing of the *falciparum* parasite by the generated ROS and this could result in effective protection of the human host from the falciparum parasite. It was observed from the study results that subjects who had less parasite DNA damage also had lower parasite density and higher SOD activity and vice versa. The characteristic comet tail was not obvious in most of the examined slides, which could be due to the hosts' inability to mount up immune response against the parasite. Also, most observed results from subjects with higher parasite density and lower SOD activity showed raptured RBCs. This finding could be attributed to the production of more ROS as a result of the parasite activity in the RBC leading to build of oxidative stress resulting in the cell rapture and parasite destruction in the process as shown by Fletcher *et al.* (162).

Thus during the course of malarial infection, both the parasites and the RBCs come under oxidative stress and the host system responds in an attempt to protect these RBCs against the damage caused by ROS by producing antioxidants. Furthermore, the membranes surrounding the parasite might contribute greatly to the increased lipid peroxidation observed during parasite development in the presence and absence of exogenous ROS. This would explain why, during the natural course of an infection or after administration of chemicals that generate oxygen derived free radicals, parasites with damaged membranes are observed inside host RBCs that retain intact plasma membranes.

5.2 CONCLUSIONS

In this study we have demonstrated dual function of oxidative stress, i.e., first, it is involved in tissue damage and secondly, it contributes to malaria parasite DNA destruction. These mechanisms lead to the decrease of the antioxidant capacity of the body differentiating between severities of *P. falciparum* malaria in this study.

The results from the study sustain the assertion that ROS plays a pivotal role as a first-line anti-parasitic defence in *P. falciparum* malaria infection in the population of Ghanaian children studied.

5.3 RECOMMENDATIONS

- Further research is needed in the area of malaria parasitaemia levels in relation to ROS levels in the host;

a) To help increase knowledge of the protective benefits of ROS to the human host, and

b) To aid in the design of a suitable antioxidant therapy for the control of ROS-mediated damage and the disease process.

In addition to the routine drug therapy, foods and fruits high in antioxidants should be prescribed for the treatment of malaria in children and in adults.

5.4 STUDY LIMITATIONS

Financial and time constraints were limitations in this work, affecting the sample size used which could have been increased and the different assays that would have been used.

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APPENDIX 1

COMET ASSAY PROTOCOL

Materials and Equipments

- Low melting agarose (LMA)
- Lysis buffer
- Trevigen comet slide
- 200mM EDTA (pH 10)
- 10X PBS, Ca^{2+} and Mg^{2+}
- NaOH pellets
- Dimethylsulfoxide (DMSO)
- 10X TBE Buffer
- Silver Staining kit
- Methanol
- Deionised water
- Temperature- regulated water bath
- Eppendorf tubes
- Refrigerator
- Horizontal electrophoresis apparatus
- Improved Neubauer counting chamber
- Light microscope
- Pipettes and pipette tips

APPENDIX 2

REAGENT PREPARATION

Principle of Comet assay

The principle is based on the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of electric field: undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the resulting DNA "comet" tail shape and migration patterns allow for assessment of DNA damage.

In this assay, cells are immobilized in a bed of low melting agarose on a Trevigen comet slide. Following a gentle cell lysis, samples are treated with alkali to denature the DNA and hydrolyze sites of damage. The samples are then visualized after silver staining which allows standard light microscopy analysis. The comet or single cell gel electrophoresis (SCGE) assay provides a simple and effective procedure for assessing DNA damage in cells.

PREPARATION OF SOLUTIONS

1. **TBE (1X)**

   100mls of TBE (10X) was added to 900mls if distilled water.

   To prepare 10X TBE:

   Tris Base = 108g

Boric acid = 55g

EDTA = 9.3g

Tris base was dissolved in 900mls of distilled water; the volume was adjusted to 1 litre and stored at room temperature.

2. 5% acetic acid w/v

25mls of acetic acid was added to 475mls of distilled water to obtain the needed total volume of 500mls.

3. 70% ethanol

280mls of absolute ethanol was added to 120mls of distilled water to obtain the required 400mls.

4. PBS (1X) (Ca^{2+} and Mg^{2+} free)

Weigh 9.55g of PBS and dissolved in 1 litre of distilled water; it is homogenized and then autoclaved.

LYSIS SOLUTION

40mls of lysis solution (from manufacturer)/ 4mls of DMSO

40mls of the lysis solution is added to 4mls of DMSO and chilled at $4^{o}C$ (to prevent damage) or on ice at least for 20 mins before use. (DMSO addition is optional and is required only for sample containing harem, such as blood and tissue samples)

SAMPLE PREPARATION

1. Melt LM Agarose in a beaker or boiling water (100^{o}C) for 5 mins (loosened cap)
2. Transfer to water bath (37^{o}C) for at least 20 mins to cool.
3. Add LM Agarose (37 ^{o}C) 500µl + 50µl PBS + cells 1:10
4. 1: 10 cells (PBS) + 50µl agarose
5. Pipette 75µl immediately onto comet slide and spread evenly. (when working on many samples, place aliquots of molten in a pre-warmed micro-centrifuge tubes placed at 37 ^{o}C to prevent hardening)
6. Place slide flat at 4 ^{o}C in the dark (refrigerator) for 10 mins. A 0.5mm clear ring appears at the edge of Comet slide area. Increasing gelling to 30 mins improves adherence of samples in high humidity environments.
7. Prepare lysis solution 20 mins after chilled on ice before use.
8. Immerse slide in pre- chilled lysis solution and leave on ice for 30- 60 mins.
9. Tap excess buffer from slide and immerse in freshly prepared alkaline solution, pH □ 13. (Alkaline solution is prepared by dissolving 0.6g NaOH a mixture of EDTA (200mM, 250 µl) and distilled water (49.75mls).
10. Leave comet slide in alkaline solution in the dark for 20- 60 mins at RT.

TBE ELECTROPHORESIS

11. Remove slide from alkaline solution and gently tap excess buffer from slide; wash by immersing in 1X TBE buffer for 5mins, twice.
12. Transfer slide from 1X TBE buffer to a horizontal electrophoresis apparatus. Place slides flat onto gel tray and align equidistant from the electrodes. Pour 1X

TBE buffer until level just covers samples. Set power supply to 1 volt per cm. Apply voltage for 10mins.

13. Gently tap off excess TBE, and dip slide in 70% ethanol for 5mins.
14. Air- dry slides. Store at RT with desiccant. Samples must be well dried before staining.

COMET ASSAY SILVER STAINING

Trevigen's Comet Assay ™ Silver staining kit (Gaithersburg, MD, USA) is designed for the convenient staining of Comet Assay or Single cell gel electrophoresis results. Using the silver staining kit, permanent records that can be visualized using standard light microscopy are prepared, thereby avoiding the problems associated with fluorescent stains and epiflourescence microscopy. The silver staining kit is designed specifically for use with comet slides to minimize unwanted background and the amount of hazardous waste generated by silver nitrate. It is used for research only, not for use in diagnostic procedures.

REAGENT PREPARATION

Fixation Solution

Prepare immediately before fixation. Mix per sample. Mix per sample:

10µl 10x fixation Additive

30µl de-ionized water

50µl methanol

10µl glacial acetic acid

2X Staining Reagent ♯ 4

Before first use, 12ml of de-ionized water is added to the bottle and stirred to dissolve completely. Store at 4^{o}C and pre-warm at room temperature before each use.

Staining solution

Prepare immediately before staining. The staining reagents 1, 2 and 3 are ready to use in the staining solution as described:

Per sample, mix in a micro-centrifuge tube:

35µl de-ionized water

5µl 20x staining reagent ♯ 1

5µl 20x staining reagent ♯ 2

5µl 20x staining reagent ♯ 3

Mix by tapping tube then add 50µl 2x staining reagent ♯ 4 (at room temperature)

Stop solution

Prepare 5% acetic acid solution; 100µl per sample area

ASSAY PROTOCOL

To reduce assay-to-assay variability, slides are dried, fixed and then silver stained.

1. Drying: slides should be dried completely before the fixation step. To accelerate the drying step, simply immerse the slides into cold 80% ethanol for 5 minutes, gently tap off excess and air dry.

2. Fixing: fixation is recommended as it improves repeatability of staining between assays. After electrophoresis and drying, samples are covered in fixation solution.
    a. Cover the sample with 100µl of fixation solution
    b. Incubate for 20 minutes at room temperature.
    c. Rinse in de-ionized water for 30 minutes
3. Staining Reaction
    a. Cover the sample area with 100µl of staining solution.
    b. Incubate at room temperature for 5 to 20 minutes (intensity of staining can be visualized under the microscope using 10X objective and reaction stopped when comets are visible).
    c. Stop reaction by covering samples with 100µl of 5% acetic acid and incubate for 15 minutes.
    d. Rinse in de-ionized water
    e. Air dry
    f. Store in the dark

APPENDIX 3

SUPEROXIDE DISMUTASE (SOD) PROTOCOL FOR CELL LYSATE ASSAY

Reagents

Assay Buffer (10X)

Sample Buffer (10X)

Radical Detector

SOD standard

Reagent Preparation

1. **Assay Buffer (10x)**

   Dilute 3mls of assay buffer concentrate with 27mls of HPLC-grade water (or de-ionized distilled water) for assaying 96 wells. This final assay buffer should be used to dilute the radical detector. Store at 4^{o}C, this is stable for at least two months.

2. **Sample Buffer (10x)**

   Dilute 2mls of sample buffer concentrate with 1.8mls of HPLC-grade water (or de-ionized distilled water) for assaying 96 wells. This is used to prepare the SOD standard and dilute the xanthine oxidase and SOD samples prior to assaying. Store at 4^{o}C, this is stable for at least two months.

3. **Radical Detector**

   Prior to use, transfer 50 µl of radical detector to another vial and dilute with 19.95mls of diluted assay buffer for 96 wells. Cover with foil. The diluted radical detector is stable for two hours. Store unused radical detector at -20^{o}C.

4. **SOD Standard**

   Dilute 20µl of the SOD Standard with 1.98ml of sample buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G. Add the

amount of SOD stock and sample buffer (dilute) to each tube as described in manufacturer's manual.

5. Xanthine Oxidase

Prior to use, thaw one vial and transfer 50µl of the supplied enzyme to another vial and dilute with 1.95ml of sample buffer (dilute) for 96 wells. Store on ice. (Stable for one hour).

Sample preparation

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA

2. Centrifuge the blood at 700- 1,000 x g for 10 minutes at 4^{o}C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80^{o}C. The plasma sample is stable for at least one month. Plasma should be diluted 1:5 with Sample buffer before assaying for SOD activity.

3. Remove the white buffy layer (leucocytes) and store at -80^{o}C.

4. Lyse the erythrocytes (red blood cells) in four times its volume of ice- cold HPLC- grade water (or de-ionized distilled water).

5. Centrifuge at 10,000 x g for 15 minutes at 4^{o}C.

6. Collect supernatant (erythrocyte lysate) for assaying and store on ice. Store at -80^{o}C if not assaying same day. Sample is stable for at least one month. The erythrocyte lysate should be diluted 1:100 with sample buffer before assaying or SOD activity.

Performing the Assay

1. **SOD Standard Wells:** add 200 µl of the diluted Radical Detector and 10 µl of Standard (tubes A-G) per well in the designated wells on the plate.

2. **Sample Wells:** add 200 µl of the diluted Radical Detector and 10 µl of Sample to the wells

3. Initiate the reaction by adding 20 µl of diluted Xanthine Oxidase to all the wells you are using. *Note*: *If assaying sample backgrounds, add 20 µl of Sample buffer instead of xanthine oxidase.*

4. Carefully shake the 96- well plate for a few seconds to mix. Cover with the plate cover.

5. Incubate the plate on a shaker for 20 minutes at room temperature. Read the absorbance at 440- 460 nm using a plate reader.

Calculations

1. Calculate the average absorbance of each standard and sample (if assay was done in duplicates). If assayed, subtract sample absorbance from the sample.

2. Divide standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR)

3. Plot the linearized SOD standard rate (LR) (from step 2 above) as a function of final SOD activity (U/ml).

4. Calculate the SOD activity of the samples using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample.

$$\text{SOD (U/ml)} \left[\left(\frac{\text{sample LR} - \text{y-intercept}}{\text{Slope}} \right) \times \frac{0.23\text{ml}}{0.01} \right] \times \text{sample dilution}$$

APPENDIX 4

Demographic data of participants (case group data)

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X 10^3/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
UPC 001	6	F	469.98310529	3+	28.277	7.9	5.2	53.0	47.0
UPC 002	8	F	567.92627547	2+	21.663	5.5	5.9	44.0	56.0
UPC 003	12	F	546.18109457	1+	9.120	7.1	6.9	35.0	65.0
UPC 004	12	M	214.03855712	3+	27.640	14.0	3.7	82.0	18.0
UPC 005	12	F	481.61557144	1+	9.960	12.2	4.1	65.0	35.0
UPC 006	2	F	493.64572874	4+	85.744	11.3	8.0	60.0	40.0
UPC 007	9	F	207.32798500	4+	82.297	11.9	5.3	41.0	59.0
UPC 008	3	M	525.60083407	3+	34.485	12.4	8.3	62.0	38.0
UPC 009	3	M	163.78657696	3+	28.600	12.6	5.2	50.6	49.4
UPC 010	12	M	216.32225967	3+	35.406	10.8	6.6	75.8	34.2
UPC 011	12	F	607.03889458	1+	7.127	10.8	5.9	76.0	24.0

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
LAG 001/UPC 012	6	M	590.93816594	1+	2.504	10.8	6.2	88.0	12.0
PML L001	5	F	384.72050014	3+	27.791	9.1	11.4	88.0	12.0
PML L002	6	M	332.47348889	2+	12.178	6.7	12.8	67.0	33.0
PML L003	5	M	363.50129977	3+	36.269	6.6	9.9	82.0	18.0
PML L004	2	M	291.64241260	2+	16.595	9.5	8.1	45.0	55.0
PML L005	6	F	740.47136990	1+	4.982	9.8	9.6	43.0	57.0
PML L006	3	F	453.23798265	1+	3.795	7.8	7.9	50.0	50.0
PML L007	1	M	261.94350837	4+	92.700	14.7	20.6	87.0	13.0
PML L008	5	F	321.66916188	3+	28.695	9.5	6.0	62.0	38.0
PML L009	1	F	200.82076355	4+	152.571	10.3	14.6	79.0	21.0
PML L010	2	F	318.16066000	4+	89.181	5.9	18.0	57.0	43.0
PML L011	4	M	167.37667192	3+	43.199	8.6	8.4	49.0	51.0
PML L012	3	M	216.32225967	3+	71.941	10.0	9.4	82.0	18.0

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
PML L013	4	F	248.34541458	2+	16.761	10.2	8.0	30.0	70.0
PML L014	4	M	235.49038602	4+	106.470	7.4	8.9	73.0	27.0
PML L015	3	M	233.00321745	2+	17.042	10.5	9.3	72.0	28.0
PML L016	3	F	328.82465915	2+	22.965	11.0	12.0	78.0	22.0
PML L017	2	M	192.44513198	4+	125.474	5.5	10.5	41.0	59.0
PML L018	3	F	328.82465915	1+	8.650	11.9	15.5	79.0	21.0
PML L019	4	M	228.10911156	3+	47.288	11.5	8.4	72.6	27.4
PML L020	3	M	184.39476766	3+	35.705	15.0	3.7	59.2	40.8
PML L021	4	F	273.40131048	1+	6.506	13.1	5.3	74.9	25.1
PML L022	4	M	261.94350837	2+	21.158	7.2	9.9	80.9	19.1
PML L023	6	M	1725.47788207	1+	8.907	8.9	5.5	36.0	64.0
PML L024	4	F	1638.91670372	3+	34.318	10.6	8.2	47.0	53.0
PML L025	4	F	1046.75955189	3+	34.400	12.5	8.2	82.0	18.0

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
PML L026	5	M	304.56797344	1+	5.214	6.0	6.5	27.0	73.0
PML L027	3	M	437.28396816	1+	6.205	11.1	9.3	86.0	14.0
PML L028	9	F	200.82076355	3+	55.285	11.5	8.6	64.0	36.0
PML L029	6	F	253.69193782	3+	55.432	9.8	8.3	79.3	20.7
PML L030	5	F	198.69528921	4+	88.560	10.6	10.8	69.0	31.0
PML L031	5	F	253.69193782	3+	44.354	4.7	6.7	38.0	62.0
PML L032	1	M	211.77851987	2+	10.362	4.7	8.9	65.3	34.7
PML L033	5	F	165.57331403	2+	18.360	11.9	5.1	49.0	51.0
PML L034	7	F	184.39476766	3+	63.304	11.4	8.2	82.0	18.0
PML L035	5	M	150.06443625	2+	16.800	9.7	5.6	57.0	43.0
PML L036	5	M	279.33604031	4+	153.366	9.3	8.6	72.0	28.0
PML L037	1	F	253.69193782	3+	56.000	9.9	12.0	54.0	46.0
PML L038	4	M	153.40267003	3+	37.159	10.3	7.5	58.0	42.0

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
PML W001	5	M	314.69713890	2+	16.275	9.1	4.8	56.0	44.0
PML W002	2	M	174.76103784	4+	125.207	7.0	9.2	63.0	37.0
PML W003	4	F	186.37790619	4+	194.769	6.8	8.9	58.0	42.0
PML W004	5	F	314.69713890	3+	62.857	10.2	11.8	66.0	34.0
PML W005	2	M	288.50954039	2+	17.296	12.6	8.7	43.0	57.0
PML W006	2	F	211.77851987	3+	55.568	4.7	15.1	66.0	34.0
PML W007	2	M	198.69528921	4+	93.130	10.2	6.7	62.0	38.0
PML W008	4	M	393.64504618	3+	28.695	9.0	5.9	45.4	54.6
PML W009	3	M	148.41747578	3+	30.543	7.7	8.9	81.4	18.6
PML W010	2	M	138.83386958	3+	62.382	9.7	4.5	57.0	43.0
PML W011	3	M	553.29525869	1+	6.174	11.2	6.3	40.0	60.0
PML W012	3	M	248.34541458	2+	21.166	12.5	6.4	37.0	63.0
PML W013	3	M	119.67969610	3+	31.058	11.5	11.1	55.9	44.1

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
PML W014	4	F	160.26205452	3+	49.892	6.6	1.2	52.0	48.0
PML W015	2	M	248.34541458	2+	23.970	11.6	6.3	81.8	18.2
PML W016	1	F	202.96781631	2+	11.311	4.7	16.2	62.2	37.8
ACH 001	1	M	583.12002366	1+	3.857	10.3	9.0	51.8	48.2
ACH 002	12	F	363.50129977	2+	21.700	9.8	5.1	56.7	43.3
ACH 003	1	M	546.18109457	1+	2.220	12.8	1.5	57.5	42.5
ACH 004	2	M	245.71695623	3+	28.560	10.4	8.4	21.6	78.4
ACH 005	1	M	125.35319529	4+	170.300	9.1	12.0	63.8	36.2
ACH 006	10	F	518.98358217	1+	8.763	10.6	9.4	31.6	68.4
ACH 007	11	F	427.06020694	2+	15.817	12	9.2	90.6	9.4
ACH 008	12	F	442.51756022	2+	15.500	11.6	1.0	30.9	69.1
ACH 009	4	F	261.94350837	4+	161.200	9.5	3.1	57.0	43.0
ACH 010	4	M	464.30989434	3+	63.380	7.2	4.5	33.6	66.4

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
ACH 011	1	F	351.46739709	3+	35.956	11.4	4.8	46.5	53.5
ACH 012	3	M	380.35453517	3+	33.923	12.8	17.0	46.2	53.8
ACH 013	11	F	332.47348889	3+	35.420	14.5	1.4	41.5	58.5
ACH 014	3	M	259.16136964	3+	56.967	11.0	8.4	89.3	10.7
ACH 015	3	F	525.60083407	1+	1.029	14.8	4.9	31.7	68.4
ACH 016	7	F	253.69193782	2+	21.054	9.2	9.1	50.2	49.8
ACH 017	5	M	205.13677777	2+	17.465	11.9	16.8	81.0	19.0
ACH 018	4	M	218.63000120	2+	16.046	7.5	9.1	63.9	36.1
ACH 019	6	M	282.35703230	2+	2.753	13.3	9.0	66.4	33.6
ACH 020	8	F	567.92627547	3+	27.317	7.9	12.9	83.5	16.5
ACH 021	1	F	499.81636977	3+	70.656	9.6	9.2	81.1	18.9
ACH 022	1	M	103.71884037	2+	13.609	12.9	7.9	51.8	48.2
ACH 023	1	M	525.60083407	1+	1.202	11.6	8.1	49.4	50.6

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
ACH 024	0.5	M	487.57967485	1+	4.036	9.8	19.9	42.8	57.2
ACH 025	1	M	88.02892510	4+	405.976	11.7	8.9	86.3	13.7
ACH 026	8	M	198.69528921	3+	33.003	10.7	12.9	78.7	21.3
ACH 027	10	M	125.35319529	3+	58.435	11.3	13.9	83.8	16.2
AMH 001	5	M	79.10295228	4+	91.651	11.9	16.8	81.0	19.0
AMH 002	8	F	267.60540473	3+	28.247	11.9	8.9	70.0	30.0
AMH 003	5	F	328.82465915	2+	15.327	12.6	8.9	80.0	20.0
AMH 004	9	F	560.54239784	1+	6.968	10.1	6.9	75.0	25.0
AMH 005	2	F	158.52382413	4+	147.600	11.3	12.3	78.2	21.8
AMH 006	3	M	607.03889458	3+	62.949	9.8	10.5	73.9	26.1
AMH 007	4	F	590.93816594	1+	8.799	7.0	7.7	79.6	20.4
AMH 008	5	M	216.32225967	3+	45.760	10.9	8.8	82.8	17.2
AMH 009	2	F	447.83488974	1+	3.443	5.1	7.4	57.1	42.9

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	PARASITE GRADING	PARASITE DENSITY X103/µL	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPH OCYTES %
AMH 010	5	F	380.35453517	1+	3.000	9.7	9.7	85.5	14.5
AMH 011	8	F	347.56524724	1+	8.763	5.7	59.7	57.8	42.2
AMH 012	9	F	525.60083407	1+	8.256	11.8	8.6	73.3	26.7

APPENDIX 5

Demographic data of control subjects

SPECIMEN NUMBER	AGE	SEX	SOD ACTIVITY[U/ml]	Hb [g/dl]	TWBC X 10^9/L	NEUTROPHILS %	LYMPHOCYTES %
PML M001	1	F	532.33731574	8.4	6.3	59.9	40.1
PML M002	2	M	598.90960512	12.2	9.6	81.2	18.8
PML M003	4	M	402.83599658	12.5	13.8	53.3	46.7
PML M004	3	M	145.16689589	9.4	16.8	81.7	18.3
PML M005	5	F	158.52382413	9.7	10.0	79.2	20.8
PML M006	4	F	546.18109457	6.9	11.7	37.6	62.4
PML M007	6	M	211.77851987	10.5	6.9	81.8	18.2
PML M008	5	M	282.35703230	10.8	8.6	85.4	14.6
ACH M001	7	M	186.37790619	9.1	15.8	32.5	67.5
ACH M002	1	M	525.60083407	11.6	15.6	63.3	36.8
ACH M003	1	M	205.13677777	9.1	11.7	28.8	71.2
ACH M004	4	M	276.35101251	13.0	10.0	51.4	48.6
ACH M005	3	F	256.41102678	11.1	5.3	24.5	75.5
ACH M006	1	M	1173.65037014	12.2	10.2	12.7	87.3
ACH M007	2	M	598.90960512	12.0	10.7	44.0	56.0
ACH M008	7	M	427.06020694	11.0	2.8	13.7	86.3
ACH M009	3	M	264.75799755	11.9	2.3	51.4	48.6
ACH M010	4	M	432.13215098	9.8	1.0	29.6	70.4
ACH M011	6	F	417.14862164	12.5	4.9	48.1	51.9

SPEC.No.	AGE	SEX	SOD ACTIVITY[U/ml]	Hb [g/dl]	TWBC x 10^9/l	NEUTROPHILS %	LYMPHOCYTES %
ACH M012	5	F	251.00357303	11.6	3.3	31.4	68.6
ACH M013	5	M	245.71695623	13.4	7.3	25.7	74.3
ACH M014	2	M	291.64241260	11.6	3.8	26.7	73.3
ACH M015	3	F	151.72610173	11.7	2.3	36.0	64.0
ACH M016	12	F	351.46739709	12.1	2.7	28.5	71.5
ACH M017	0.2	M	138.83386958	11.1	8.1	33.8	66.2
ACH M018	1	M	1452.81017029	9.9	3.3	37.0	63.0
ACH M019	6	F	2170.64965639	11.1	12.2	63.2	36.8
ACH M020	3	M	104.99176018	10.9	11.2	31.8	68.2
ACH M021	2	M	205.13677777	9.7	13.2	54.8	45.2
ACH M022	1	F	96.28460149	14.0	8.2	47.7	52.3
ACH M023	0.2	M	240.54717074	9.6	6.4	56.1	43.9
ACH M024	2	M	70.67633457	12.2	15.6	78.0	22.0
ACH M025	1	M	1522.60012033	9.0	19.9	76.8	23.2
ACH M026	10	M	2879.62692662	12.5	6.1	54.5	45.5
ACH M027	1	F	2879.62692662	11.3	5.3	46.7	53.3
ACH M028	5	M	-43.96152414	11.5	3.9	25.8	74.2
ACH M029	4	F	-33.52714403	10.8	5.2	79.3	20.7
ACH M030	1	F	-47.30950735	10.7	11.8	59.8	40.2
ACH M031	3	F	-34.37298029	10.6	13.8	30.9	69.1
ACH M032	5	F	1388.38867795	12.5	6.8	44.4	55.6
ACH M033	7	F	2768.84922815	11.7	22.4	72.6	27.4
ACH M034	12	M	-19.36911477	11.9	7.7	73.5	26.5
ACH M035	2	M	-11.19664058	11.3	6.0	61.4	38.6
ACH M036	1	M	-37.27459079	10.8	12.4	43.0	57.0
ACH M037	11	M	-36.45475025	12.0	5.2	64.1	35.9

APPENDIX 6

Descriptive Statistics of Severe Malaria Subjects

	SOD ACTIVITY x10^{-2}[U/ml]	***PARASITE DENSITY X10^{3}/µL***	***Hb [g/dl]***	***TWBC X 10^{9}/l***	***NEUTROPHILS %***	***LYMPHOCYTES %***
Count	79	79	79		79	79
mean	**295.33**	**56.752**	**10.03**	**8.90**	**63.48**	**36.65**
sample standard deviation	**211.40**	**57.699**	**2.36**	**3.86**	**16.65**	**16.59**
minimum	79.10	11.311	4.7	1	21.6	9.4
maximum	1638.92	405.976	15	20.6	90.6	78.4
sample variance	44,691.02	3,329.142	5.57	14.86	277.18	275.29
standard error of the mean	**23.79**	**6.492**	**0.27**	**0.43**	**1.87**	**1.87**
C. I 95.% lower	**247.98**	**43.827**	**9.49**	**8.04**	**59.75**	**32.93**
C. I 95.% upper	**342.68**	**69.675**	**10.56**	**9.77**	**67.21**	**40.36**

APPENDIX 7

Descriptive Statistics of Uncomplicated Malaria Subjects

	SOD ACTIVITY x10^{-2}[U/ml]	*PARASITE DENSITY X10^3/µL*	*Hb [g/dl]*	*TWBC X 10^9 /l*	*NEUTROPHILS %*	*LYMPHOCYTES %*
count	26	26	26	26	26	26
mean	**520.69**	**5.87**	**9.92**	**9.99**	**58.64**	**41.36**
sample standard deviation	**275.10**	**2.87**	**2.68**	**10.73**	**18.93**	**18.94**
minimum	211.78	1.03	4.7	1.5	27	12
maximum	1725.48	10.36	14.8	59.7	88	73
sample variance	75,680.82	8.22	7.20	115.06	358.51	358.729
range	1513.69	9.33	10.1	58.2	61	61
standard error of the mean	53.95	0.56	0.53	2.10	3.71	3.71
C. I 95.% lower	409.58	4.71	8.84	5.66	50.99	33.71
C.I 95% upper	631.81	7.03	11.00 7	14.32	66.29	49.01

APPENDIX 8

Descriptive Statistics of Control Subjects

	SOD ACTIVITY x10^{-2}[U/ml]	*Hb [g/dl]*	*TWBC X 10^{9}/l*	*NEUTROPHILS %*	*LYMPHOCYTES %*
Count	45	45	45	45	45
Mean	**546.42**	**11.09**	**8.85**	**49.86**	**50.14**
sample standard deviation	**776.22**	**1.39**	**4.96**	**20.32**	**20.31**
minimum	-47.31	6.9	1	12.7	14.6
maximum	2879.63	14	22.4	85.4	87.3
sample variance	602,519.31	1.93	24.57	412.71	412.65
standard error of the mean	115.71	0.21	0.74	3.03	3.03
confidence interval 95.% lower	313.21	10.68	7.36	43.75	44.04
confidence interval 95.% upper	779.62	11.51	10.34	55.96	56.25

APPENDIX 9

SOD activity level in Case And Control Group

	SOD ACTIVITY [CASE] x10^{-2}[U/ml]	***SOD ACTIVITY [CONTROL] x10^{-2}[U/ml]***
Count	105	45
Mean	**351.50**	**546.42**
sample variance	61,379.57	602,519.31
sample standard deviation	**247.75**	**776.22**
Minimum	79.10	-47.31
Maximum	1725.48	2879.63
standard error of the mean	**24.18**	**115.71**
confidence interval 95.%		
lower	303.56	313.21
confidence interval 95.%		
upper	399.45	779.62

APPENDIX 10

Descriptive statistics of SOD activity levels in study participants

	SEVERE MALARIA	**UNCOMPLICATED MALARIA**	**CONTROL GROUP**
	SOD ACTIVITY[U/ml]	*SOD ACTIVITY[U/ml]*	*SOD ACTIVITY[U/ml]*
count	79	26	45
mean	**295.33**	**520.69**	546.42
sample variance	44,691.02	75,680.82	602,519.31
sample standard deviation	**211.40**	**275.10**	776.22
minimum	79.10	211.78	-47.31
maximum	1638.92	1725.48	2879.63
range	1559.81	1513.69	2926.94
population variance	44,125.31	72,770.02	589,129.99
population standard deviation	210.06	269.76	767.55
standard error of the mean	**23.79**	**53.95**	115.71
confidence interval 95.% lower	247.98	409.58	313.21
confidence interval 95.% upper	342.68	631.81	779.62

APPENDIX 11

Descriptive statistics of variables in study participants

	SOD ACTIVITY[U/ml]	*PARASITE DENSITY X103/µL*	*Hb*	*TWBC X 109*	*NEUTROPHILS %*	*LYMPHOCYTES %*
count	105	105	105	105	105	105
mean	**351.5001692387**	**44.15205**	**10.001**	**9.172**	**62.283**	**37.813**
sample variance	**61,379.5676466984**	**2,985.81584**	**5.913**	**39.025**	**298.469**	**296.881**
sample standard deviation	**247.7490013031**	**54.64262**	**2.432**	**6.247**	**17.276**	**17.230**
minimum	79.10295228	1.029	4.7	1	21.6	9.4
maximum	1725.477882	405.976	15	59.7	90.6	78.4
range	1646.37493	404.947	10.3	58.7	69	69
population variance	60,795.0003357775	2,957.37950	5.856	38.653	295.626	294.053
population standard deviation	**246.5664217524**	**54.38179**	**2.420**	**6.217**	**17.194**	**17.148**
standard error of the mean	**24.1778268445**	**5.33257**	**0.237**	**0.610**	**1.686**	**1.681**
confidence interval 95.% lower	**303.5546312973**	**33.57736**	**9.530**	**7.963**	**58.939**	**34.479**
confidence interval 95.% upper	**399.4457071801**	**54.72675**	**10.472**	**10.381**	**65.626**	**41.148**

APPENDIX 12

CONSENT AND SIGNING FORM

Consent Form

Consent form for ward participation in a study conducted by **MR. DANIEL SAI SQUIRE**, an M.Phil Microbiology student of the College of Health Sciences, University of Ghana titled **"Malaria parasitaemia levels in relation to reactive oxygen species among Ghanaian children up to twelve years of age".**

Dear Sir/Madam,

Your consent is kindly sought to for your ward part-take in this study, which will be explained in details below. You are free to decline without any consequence.

Purpose of study: To evaluate the parasitaemia levels in relation to reactive oxygen species activity in malarial infections.

Study Background: Malaria is a disease caused by a parasite and is responsible for millions of human death. The parasites belong to the group *Plasmodium*, and transmitted by the bite of the infective female Anopheline mosquito. Malaria is the most important infectious disease in tropical and subtropical regions, and continues to be a major health problem in the world, with over 40% of the world`s population exposed to varying degrees of malaria risk in some 100 countries. It is estimated that over 500 million people suffer from malaria infection annually, resulting in about 1-2 million deaths, of which 90% are children in sub-Saharan Africa in whom the disease can exist in a severe form, often with destructive consequences.

During a malaria infection oxidative stress is increased by the increased production of reactive oxygen species (ROS), both inside the red blood cells and outside the parasitized red

blood cells. ROS are produced as a result of the oxidation and breakdown of ingested haemoglobin in the acid environment of the parasite`s food vacuole and these lead to an increased production of ROS such as H_2O_2 (Hydrogen Peroxide) and Hydroxyl radicals that may cause molecular and cellular damage.

How it will be done: You and other women whose children are going to be involved in this study will be educated about the study procedure by the investigator. You may ask any question(s) and if you are satisfied with the responses and would want your child to participate in the study, you will write you and your child's name, his/ her age and signature or thumbprint to confirm your consent.

Risks: You and your ward will not be exposed to any form of minimal risk because of your participation in this study. All that will be done is 2mls of blood sample will be taken from your ward.

Benefits: The study may provide information on the correlation of parasitaemia levels with the intensity of reactive oxygen species activity in malarial infection.

Privacy and confidentiality: Your personal information including your name will be kept confidential for reference purposes by investigators and not disclosed to anyone. All samples will be given identification codes.

Compensation: You will not be required to pay anything for the study.

Contacts: If you have any questions about the research study, you may contact any of the following people:

1. Rev. Prof. Patrick F. Ayeh-Kumi (Dept of Microbiology University of Ghana Medical School).
2. Mr. Richard Harry Asmah (School of Allied Health Sciences; 0244 266 529)

Yours sincerely,

Squire, Daniel Sai

APPENDIX 13

Signing Form

Malaria parasitaemia levels in relation to reactive oxygen species among Ghanaian children up to twelve years of age

I have fully understood the information of the consent form. I know what is required of me if I consent for my ward to take part in this study.

I willingly agree for my ward to partake in this study.

Name of Parent/ Guardian ----------

Signature/ Thumbprint----------

Name of participant ----------

Telephone number----------

Date----------

Witnessed by---------- (Field Worker)

MALARIA PARASITAEMIA LEVELS IN RELATION TO REACTIVE OXYGEN SPECIES AMONG GHANAIAN CHILDREN UP TO TWELVE YEARS OF AGE

STUDY QUESTIONNAIRE FORM

Target Number: …….

A: Personal data:

Name of Patient: ……

Age: [ ] **(0- 2) years** [ ] **(3- 5) years** [ ] **(6- 8) years**

[ ] **(9- 10) years** [ ] **(11- 12) years**

Sex: [ ] **1**: Male **2**: Female

B: Clinical data:

Fever: **[ ]** **1:** Yes **2:** No **Vomittng:** [ ] **1**: yes **2**: No

Diarrhoea: [ ] **1**: Yes **2**: No **Pallor:** [ ] **1**: Yes **2**: No

Respiratory distress: **[ ]** **1:** Yes **2:** No **Prostration:** **[ ]** **1:** Yes **2:** No

Anaemia: [ ] **1**: Yes 2: No **Convulsion:** **[ ]** **1:** Yes **2:** No