ANTIOXIDANT STATUS OF SICKLE CELL DISEASE CHILDREN WITH AND WITHOUT MALARIA AT THE KORLE-BU TEACHING HOSPITAL

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DECLARATION

I hereby declare that except for references to other peoples work, which have been duly acknowledged, the thesis is a result of my own research conducted at the Department of Chemical Pathology (UGMS, Korle-Bu) and the Department of Clinical Pharmacology (UGMS, Korle-Bu) under the supervision of Dr George Obeng Adjei and Dr Sylvester Yaw Oppong.

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DEDICATION

I dedicate this project to God Almighty, to my husband Vannel, to my children; Manuel and Enoch and to my parents; Mr and Mrs Wilson.
ACKNOWLEDGEMENT

I appreciate God Almighty for his grace and favor through-out the duration of this course.

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<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>...i</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>BACKGROUND</td>
<td>1</td>
</tr>
<tr>
<td>PROBLEM STATEMENT</td>
<td>3</td>
</tr>
<tr>
<td>JUSTIFICATION</td>
<td>4</td>
</tr>
<tr>
<td>AIMS AND OBJECTIVES</td>
<td>4</td>
</tr>
<tr>
<td>HYPOTHESIS</td>
<td>4</td>
</tr>
<tr>
<td>CHAPTER 2: LITERATURE REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>SICKLE CELL DISEASE DISCOVERY</td>
<td>5</td>
</tr>
</tbody>
</table>
DESCRIPTION OF SICKLE CELL DISEASE.............................................6

HAEMATOLOGICAL PARAMETERS OF SICKLE CELL DISEASE.........13

MALARIA..........................................................................................13

HAEMATOLOGICAL PARAMETERS OF MALARIA SUBJECTS.........21

ANTIOXIDANT..................................................................................22

OXIDATIVE STRESS IN SICKLE CELL ERYTHROCYTES..............30

OXIDATIVE STRESS IN MALARIA......................................................31

MALARIA INFECTION IN SCD AND OXIDATIVE STRESS............41

BACTEREMIA IN SCD AND OXIDATIVE STRESS..........................42

CHAPTER 3: MATERIALS AND METHOD...........................................43

STUDY SITE DESCRIPTION............................................................43

STUDY DESIGN............................................................................44

SAMPLE SIZE DETERMINATION....................................................44

CASE DEFINITION.........................................................................45

PATIENTS AND SAMPLING..........................................................45

LABORATORY INVESTIGATION......................................................46

ETHICS.........................................................................................50
LIST OF FIGURES

FIGURE 2.0 Antioxidant defense mechanism against ROS.................................29

FIGURE 4.1 Bar chart comparing the mean activity of SOD for the various groups
.......................................................................................................................63

FIGURE 4.2 Bar chart comparing the mean activity of GPx for the various groups with
.......................................................................................................................63

FIGURE 4.3 Bar chart comparing the mean levels of GSH for the various groups ……..64

FIGURE 4.4 Bar chart comparing the mean levels of ASC for the various groups ……..64

LIST OF TABLES

Table 4.0: Selected demographic characteristics of subjects in the various groups……..53

Table 4.1a: Selected clinical characteristics of subjects in the various groups……………55

Table 4.1b: Selected clinical characteristics of subjects in the various groups……………56

Table 4.2: Mean values of haematological parameters of subjects in the various groups……59

Table 4.3: mean values of selected antioxidant concentration or activity of subjects in the various groups.........................................................................................................................62

Table 4.4: Pearson correlation between parasite density and antioxidant concentration or activity
...............................................................................................................................65
LIST OF ABBREVIATIONS

%-percentage

ACS - acute chest syndrome

ASC - ascorbic acid

ATP - Adenosine Tri Phosphate

CAT - catalase

Cu – copper

Cys - cysteine

DCH - Department of Child Health

DDT – dichlorodiphenyltrichloroethane

DNA - Deoxyribonucleic acid

e- -electron

ESR - erythrocyte sedimentation rate

FAD - Flavin Adenosine Phosphate

FADH - Reduced Flavin Adenosine di- Phosphate

Fe - iron

G6PD - glucose-6-phosphate dehydrogenase
GPx-glutathione peroxidase

GSH-reduced glutathione

GSR- glutathione reductase

GSSG-glutathione disulfide

H⁺- hydrogen ion

H₂O- water

H₂O₂-hydrogen peroxide

Hb- Haemoglobin concentration

HbAA- Haemoglobin AA

HbC- Haemoglobin C

HbS- haemoglobin S

HbSB+- Haemoglobin S Beta plus thalassaemia

His-histidine

HPLC- high-performance liquid chromatography

IRS-Indoor residual spraying

KBTH- Korle-Bu Teaching Hospital

MCH- mean corpuscular haemoglobin
MCHC- mean corpuscular haemoglobin concentration

MCV- mean corpuscular volume

Mn- manganese

NADP+- Nicotinamide Adenisine di-Phosphate

NADPH-Reduced Nicotinamide Adenisine di-Phosphate

Ni- nickel

NO Nitric oxide

O$_2$-oxygen

O$_{2}^{2-}$-superoxide radical

*P. falciparum* - *Plasmodium falciparum*

*P. malariae* - *Plasmodium malariae*

*P. ovale* - *Plasmodium ovale*

*P. vivax* - *Plasmodium vivax*

PCA- patient-controlled analgesia

PCR-polymerase chain reaction

PGluDH- P. falciparum glutamate dehydrogenase

PHD- pulmonary hypertension
pLDH - *Plasmodium* Lactate Dehydrogenase

POP’s-Persistent Organic Pollutants

RBC- red blood cell

ROS- reactive oxide species

RSeOH - seleninic acid

SCD- sickle cell disease

SCT- sickle cell trait

SH- thiol group

SOD-superoxide dismutase

TCD- trans-cranial Doppler

UGMS- University of Ghana Medical School

WBC- white blood cell

WHO- World Health Organization

α- alpha

β- beta
ABSTRACT

Background

Sickle cell disease (SCD) is a hereditary disorder prevalent in malaria endemic areas, in which there is state of chronic vasculopathy characterized by endothelial dysfunction, and increased oxidative stress. SCD patients with malaria may experience worse outcomes because malaria infection in SCD leads to depletion of antioxidants which may complicate the disease. Few studies, however, have evaluated the antioxidant status of SCD patients with discrete clinical syndromes. There is also little information on the extent to which malaria depletes antioxidants in SCD patients.

Setting

The study was carried out at the Department of Child Health, Korle-Bu Teaching Hospital and the Centre for Tropical Clinical Pharmacology and Therapeutics.

Aim

The objective of the study was to investigate the levels of selected antioxidants in acutely ill sickle cell disease children with and without malaria.

Methods

Blood samples of 121 children between ages of 6 months and 13 years presenting with sickle cell and acute febrile illness were recruited. Known SCD children with confirmed malaria (n=26) or bacteremia (n=21) were enrolled. SCD children in steady state (n=21) visiting clinic for routine check-up as well as children with haemoglobin genotype, HbAA with malaria (n=26) and without malaria (n=25) attending the Korle-Bu Polyclinic were recruited. Relevant
demographic data was recorded and clinical examination was undertaken. Haematological parameters as well as levels of the following antioxidants: reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and ascorbic acid (ASC) were determined for each group.

**Results**

The mean levels of the antioxidants SOD, GSH and ASC was higher in non-SCD children, who were malaria negative (AAM-) compared to SCD steady state children. The results for the antioxidants were: SOD (AAM- = 27.51, SCDSS = 25.85) U/ml, GSH (AAM- = 10800, SCDSS = 4465) mg/dl and ASC (AAM- = 3567, SCDSS = 2598) mg/dl. However, the reverse was found for GPx (AAM- = 29.8, SCDSS = 259) U/ml, where mean activity was lower for non-sickle cell malaria negative children. Also, the mean levels of SOD, GPx and ASC in confirmed malaria positive children (SCDM+, AAM+) had lower activity than the non-malaria children (SCDSS, AAM-). Results for the antioxidant s were: SOD (SCDSS =25.85, SCDM+ =18.51 / AAM- =27.51 , AAM+ = 23.99) U/ml, GPx (SCDSS =259, SCDM+ = 92.60 / AAM- =29.8, AAM+ = 7.05) U/ml and ASC (SCDSS = 2598, SCDM+ = 2401/ AAM- =3567, AAM+ = 2514) mg/dl. However, the mean GSH (SCDSS = 4465, SCDM+ = 7941/ AAM- =10800, AAM+ = 11700) mg/dl was higher for malaria positive compared to malaria negative groups.
Conclusion

Three of the four antioxidant investigated; SOD, GSH and GPx were higher in non-SCD malaria negative groups compared to SCD steady state group. Also, Three of the four antioxidant investigated; SOD, GPx and ASC were higher in malaria negative groups compared to malaria positive groups for both sickle cell and non-sickle cell children. It can however be concluded that, generally, levels of antioxidants in SCD and non-SCD malaria negative groups was higher than that of the malaria positive group.
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Sickle cell disease (SCD) is a hereditary disease condition resulting from substitution of glutamic acid for valine in position 6 of the β-globin gene chain (Chirico and Pialoux, 2012). A major and frequent complication faced by SCD patient is painful vaso-occlusive crisis, which may be due among others, to the interaction between activated blood and endothelial cells (Platt et al., 1994). Oxidative stress in SCD may result from multiple sources: “abnormal amounts or locations of bioactive iron, heme, and hemoglobin; several endothelial or vascular wall-based enzyme systems (xanthine oxidase, NADPH oxidase, myeloperoxidase, and electron leakage from mitochondria); and oxidant generation from circulating, or endothelial-bound, white cells or red cells” (Kato et al, 2009).

SCD patients have a chronic inflammatory condition that often results in low local oxygen tension leading to sickling of erythrocytes, increased blood viscosity, thrombosis with subsequent ischemic tissue breakdown (Arinola et al., 2008).

Patients with SCD are also susceptible to recurrent infections, as well as increased leukocyte counts even during the steady state. These elevated leucocyte counts may be due to the clogging of the spleen with sickled erythrocytes (Raphael et al., 2005). The combined effect of the inflammatory response and infections in SCD produce secondary disease states such as acute chest syndrome (ACS), pulmonary hypertension (PHT), and
indirectly, stroke (Chirico and Pialoux, 2012). Oxidative stress also promotes sickled blood cell adherence to the endothelium, this interaction is associated with a further exacerbated oxidative stress (Chirico and Pialoux, 2012). Furthermore, malaria is also known to induce the immune system which is known to boost the host’s oxidative stress capacity, thereby causing release of reactive oxide species (ROS) (Kilic et al., 2010).

The oxidant stress induced by malaria parasite on host results in depletion of host antioxidants (Mishra et al., 1994). SCD patients were also shown to have significantly lowered concentration of antioxidants compared with healthy patients due to oxidative stress (Chiroco and Pialoux, 2012). These antioxidants play a major role in the defense mechanism of erythrocytes of host (Mishra et al., 1994).

Antioxidants neutralize the effects of these ROS by cellular mechanisms such as; the enzymatic removal of ROS by glutathione peroxidase, catalase and superoxide dismutase and non-enzymatic defense against the ROS consists of endogenously produced scavenging antioxidants such as glutathione (GSH), NADH, NADPH and uric acid and the diet derived ones like ascorbic acid and vitamin E and A (Mishra et al., 1994).

Malaria is known to be the major cause of morbidity and mortality in SCD patients when infected and this may be related to reduced antioxidant levels (Mirshra et al, 1994). In spite of the existence of these co-morbidities and their possible additive effects, little literature is available on the extent to which malaria infection in SCD children influence the depletion of antioxidants. The study therefore seeks to bridge the gap in knowledge by investigating levels of antioxidants in SCD patients with discrete clinical syndromes and compare them to some selected clinical and demographic characteristics.
1.2 Problem Statement

Malaria is the major cause of mortality and morbidity in sickle cell disease (SCD) subjects in Sub-Saharan Africa (Aluoch, 1997). Subjects homozygous for the sickled mutant gene are at reduced risk of malaria infection, in spite of this risk, if infected, are at substantial risk of subsequent mortality than in normal subjects (HbAA) (Williams and Obaro, 2011).

Malaria is the most common precipitating cause of crises in sickle cell disease in countries where malaria is endemic (Konotey- Ahulu 1971a). A study reported malaria infection as the precipitating cause of about 15% of consecutive admissions for crises in homozygous sickle cell disease in a hospital in Ghana (konotey-Ahulu, 1971b). However, the bulk of the evidence on malaria in SCD is not recent, though unpublished data suggests malaria as one of the major presenting conditions in febrile SCD patients at the paediatric sickle cell clinic, KBTH (Adjei et al, unpublished data)

Antioxidants are protective elements that play a major role in the defense mechanism of erythrocytes of host (Mishra et al., 1994). SCD patients have been shown to have significantly lowered concentration of antioxidants compared with healthy patients due to oxidative stress (Chiroco and Pialoux, 2012). Malaria parasite also induces oxidative stress in host resulting in the depletion of host antioxidants (Mishra et al., 1994).

There is also very little information in literature on the extent to which malaria influence the antioxidants status in SCD children.
1.3 Justification

The data from the study may provide a better insight of the antioxidant status of malaria infection in sickle cell disease children that could serve as basis for further studies. It could also be of prognostic importance.

1.4 Aim

To investigate the levels of selected antioxidants in acutely ill sickle cell disease and non-sickle cell disease children with and without malaria

1.5 Specific Objectives

- To investigate the relationship between selected antioxidant levels and other possible risk factors in three cohorts of SCD patients and two cohorts of HbAA subjects.
- To determine the association between antioxidant levels and degree of plasmodium infestation in SCD patients.

1.6 Hypotheses

- There is no significant difference in the antioxidant status between sickle cell disease children with malaria and sickle cell disease children without malaria.
- There is no correlation between the antioxidant status and degree of malaria of infection (recorded as parasite count) in sickle cell disease children.
2.0 LITERATURE REVIEW

2.1 Discovery of sickle cell disease

Existence of sickle cell disease was first reported by the medical world in 1910 when they discovered microscopic oddly shaped red blood cells. Sickle Cell Disease” often abbreviated as SCD, had been present in Africa for at least five thousand years and has been known by many names in many tribal languages. In 1910, Dr. James Herrick described an anemia characterized by bizarre, sickle-shaped cells. Hahn and Gillespie discovered in 1927 that red blood cells from persons with the disease could be made to sickle by removing oxygen. The hereditary nature of the disease was suspected but not demonstrated until 1949 by Dr. James V. Neel., Dr. Linus Pauling in 1949 made a distinction between sickle cell haemoglobin (haemoglobin S) and normal haemoglobin (haemoglobin A) and the actual amino acid substitution by Vernon Ingram in 1956. The life span and the quality of life of patients were improved. Genetic counseling became an important tool for informing people about the risks of having a child with sickle cell disease. Today, over 100 years after discovery, physicians and scientists continue to move forward in new understanding of the disease and new ways to treat it. The goal of a total cure has not been reached but great progress has been made.
2.2 Description of Sickle Cell Disease

Sickle cell disease (SCD) is a group of haemoglobin disorders inherited in a Mendelian recessive manner in which a sickle $\beta$-globin gene $\beta_s$ is inherited. This change in amino acids allows deoxygenated HbS to become “sticky” and polymerize to form fibers that cause rigidity and sickle shape (Christoph et al., 2005). At times the stiff, sickled cells cannot squeeze through the narrow blood vessels and stack up to block blood flow. This blockage prevents oxygen from being transferred to tissue and organs which is known as ischemia. The loss of oxygen can result in tissue damage. Reperfusion after an ischemic event can be extremely painful and these events are common in SCD patients. The lifespan of sickle RBCs 10-20 days as compared to the 120-day lifespan of normal RBCs which results in chronic anemia in SCD patients (Ohnishi et al., 2000). This chronic hemolysis leads to the generation of many other molecules, some of them toxic. Symptoms of sickle cell disease include Hand-foot syndrome, anemia, fatigue and paleness, episodes of pain, chronic inflammation, eye problems, jaundice and delayed growth.

Sickle cell disease affects millions of people throughout the world. It is common in people whose origins are African, Mediterranean, Arabian and Indian (Asian)

Molecular difference between haemoglobin A(HbA) and haemoglobin S (HbS) is a substitution of glutamic acid by valine at position 6 on the beta chain of Hb (Browning et al., 2007). “There are 4 major genotypes of SCD: SS, SC, $\beta^+$, and $\beta^0$. Homozygosity for the sickle mutation, also known as sickle-cell disease SS, is the most prevalent and severe variant” (Erin LB, 2011, Nitin, 2010).
The distribution of the S allele has recently been shown to have close association with the historical distribution of *Plasmodium falciparum* malarial endemicity (Piel et al., 2010). Within Africa, the frequency of S, and accordingly SS, is highest in low-altitude equatorial regions. The second subtype of SCD common in Africa is compound heterozygosity for S and C (SC). The C allele is found almost exclusively among people of West African ancestry, being most common among those in Burkina Faso and northern Ghana. Compound heterozygosity with thalassemia (S-thalassemia) is a form of SCD that is believed to be rare in most of sub-Saharan Africa (Fleming AF, 1989). In Central, East, and Southern Africa, SCD is generally assumed to be synonymous with SS disease.

Homozygous inheritance of the abnormal haemoglobin genes usually leads to disease (Edington et al., 1955). Heterozygotic carriers HbAS (having the “sickle cell trait”), do not have disease symptoms and show significant resistance to severe malaria (Haque et al., 2011). SCD prevalence in Ghana has remained at 2% and the trait 25% in the population (Ohene-Frimpong et al., 2008).

Hemoglobin allows red blood cells to carry oxygen. It is made up of alpha chains and beta chains. A child with sickle cell disease has inherited two defective genes for the beta chain of hemoglobin.

Pathogenesis of sickle cell anaemia (SCA) develops gradually from polymerization of HbS resulting in chronic hemolytic anemia and vaso-occlusive phenomena and underlies the significant morbidity and mortality associated with the disease (Fasola et al., 2007).

Some clinical features include; anaemia, severe pain, chest pain, pallor, strokes joint pain and severe infections. Sickle cell disease (SCD) children have impaired immune response
and are uniquely vulnerable to infections such as malaria which is a common trigger of vaso-occlusive crisis in patients living in malaria endemic countries (Bolarinwa et al., 2010).

2.2.1 Ways of diagnosing sickle cell disease

Sickle cell disease usually is diagnosed at birth with a blood test during routine newborn screening tests. If a child tests positive on the screening test, a second blood test (called a hemoglobin electrophoresis) is performed to confirm the diagnosis.

There are several tests suitable for determining what type of hemoglobin is made by a person's red blood cells. These include: sickling test, haemoglobin electrophoresis, Isoelectric focusing and Chromatography. These tests can determine whether a person has a type of sickle cell disease or sickle cell trait. DNA analysis is also used to determine changes in the genes for making hemoglobin. This test indirectly predicts the type of hemoglobin made in the red cells.

In HbSS, the full blood count reveals haemoglobin levels in the range of 6–8 g/dL with a high reticulocyte count (as the bone marrow compensates for the destruction of sickle cells by producing more red blood cells). In other forms of sickle-cell disease, Hb levels tend to be higher. A blood film may show features of hyposplenism (target cells and Howell-Jolly bodies).

Sickling of the red blood cells on a blood film, can be induced by the addition of sodium metabisulfite. The presence of sickle haemoglobin can also be demonstrated with the "sickle solubility test". A mixture of haemoglobin S (Hb S) in a reducing solution (such
as sodium dithionite) gives a turbid appearance, whereas normal Hb gives a clear solution.

Abnormal haemoglobin forms can be detected on haemoglobin electrophoresis, a form of gel electrophoresis on which the various types of haemoglobin move at varying speeds. Sickle-cell haemoglobin (HgbS) and haemoglobin C with sickling (HgbSC)—the two most common forms—can be identified from there. The diagnosis can be confirmed with high-performance liquid chromatography (HPLC). Genetic testing is rarely performed, as other investigations are highly specific for HbS and HbC (Clarks and Higgin, 2000).

An acute sickle-cell crisis is often precipitated by infection. Therefore, a urinalysis to detect an occult urinary tract infection, and chest X-ray to look for occult pneumonia should be routinely performed.

People who are known carriers of the disease often undergo genetic counseling before they have a child. A test to see if an unborn child has the disease takes either a blood sample from the fetus or a sample of amniotic fluid. Since taking a blood sample from a fetus has greater risks, the latter test is usually used.

2.2.2 Management and treatment of sickle cell disease

2.2.2.1 Folic acid and prophylactic penicillin

Sickle cell disease patients are required to take a 1 mg dose of folic acid daily for life. By the age of two months, the child should begin receiving orally administered penicillin V in a dosage of 125 mg twice daily. When the child reaches three years of age, the dosage is increased to 250 mg twice daily (Gaston et al., 1986). Penicillin prophylaxis can be
discontinued after the age of five years (Falletta et al., 1995) except in the child who has had a splenectomy.

2.2.2.2 Malaria chemoprophylaxis

The protective effect of sickle cell trait does not apply to people with sickle cell disease; in fact, they are uniquely vulnerable to malaria, since the most common cause of painful crises in malarial countries is infection with malaria. It has therefore been recommended that people with sickle cell disease living in malarial countries should receive anti-malarial chemoprophylaxis for life (Oniyangi and Omari, 2006)

2.2.2.3 Vaso-occlusive crises

Most people with sickle-cell disease have intensely painful episodes called vaso-occlusive crises. The frequency, severity, and duration of these crises, however, vary tremendously. Painful crises are treated symptomatically with analgesics; pain management requires opioid administration at regular intervals until the crisis has settled. For milder crises, a subgroup of patients manage on NSAIDs (such as diclofenac or naproxen). For more severe crises, most patients require inpatient management for intravenous opioids; patient-controlled analgesia (PCA) devices are commonly used in this setting.

2.2.2.4 Acute chest crisis

Management is similar to vaso-occlusive crisis, with the addition of antibiotics (usually a quinolone or macrolide, since cell wall-deficient ["atypical"] bacteria are thought to contribute to the syndrome) (Aldrich and Nagel, 1998), oxygen supplementation for
hypoxia, and close observation. Should the pulmonary infiltrate worsen or the oxygen requirements increase, simple blood transfusion or exchange transfusion is indicated.

2.2.2.5 Hydroxyurea

The first approved drug for the causative treatment of sickle-cell anaemia, hydroxyurea, was shown to decrease the number and severity of attacks in a study in 1995 (Charache et al.,) and shown to possibly increase survival time in a study in 2003 (Steinberg et al.). This is achieved, in part, by reactivating fetal haemoglobin production in place of the haemoglobin S that causes sickle-cell anaemia. Hydroxyurea had previously been used as a chemotherapy agent, and there is some concern that long-term use may be harmful, but this risk has been shown to be either absent or very small and it is likely that the benefits outweigh the risks (Platt, 2008).

2.2.2.6 Transfusion therapy

Blood transfusions are often used in the management of sickle cell disease in acute cases and to prevent complications by decreasing the number of red blood cells (RBC) that can sickle by adding normal red blood cells (Drasar et al., 2011). In children prophylactic chronic red blood cell (RBC) transfusion therapy has been shown to be efficacious to a certain extent in reducing the risk of first stroke or silent stroke when trans-cranial Doppler (TCD) ultrasonography shows abnormal increased cerebral blood flow velocities. In those who have sustained a prior stroke event it also reduces the risk of recurrent stroke and additional silent strokes (Gyang et al., 2011).
2.2.2.7 Bone marrow transplants

Bone marrow transplants have proven to be effective in children. Bone marrow transplants are the only known cure for SCD (Walters at al., 1996). Transplants are complex and risky procedures and currently are an option only for a carefully selected subset of patients with severe complications.

2.2.2.7 Counseling and Education

The family physician should provide education and anticipatory guidance to help parents adjust to having a child with a chronic illness (Thompson et al., 1994). The affected child should not be overprotected or neglected. Spouses and other children in the family need to have their share of attention, and they should be involved with, but not overwhelmed by, the care of the child with sickle cell disease.

During the early months of the child's life, emphasis should be given to teaching the mother and other caregivers how to recognize early signs of serious complications. It is also important to ensure that prophylactic measures, mainly penicillin and routine and special immunizations are instituted in a timely fashion. Baseline laboratory values should be recorded during this period.

2.2.2.8 Immunization

The new heptavalent conjugated pneumococcal vaccine (Prevnar) should be given from two months of age (O'Brien et al., 1996). The 23-valent unconjugated pneumococcal vaccine is mandatory from the age of two years (it is not effective below this age), and it can be boosted at least once three years later. After six months of age, children can begin
receiving influenza virus vaccines yearly. The American Academy of Pediatrics recommends meningococcal vaccine for children with splenic dysfunction. All routine immunizations should be given in a timely fashion.

2.3 Haematological Parameters in sickle cell disease

SCD patients usually have low Hb and haematocrit due to the background rate of red cell sickling which drastically shortens the life span of red cells leading to chronic haemolytic anaemia and jaundice even in steady state (Kaul et al., 1996). High platelet count is the usual finding in most patients with SCD in asymptomatic steady state, except in crisis situation such as vaso occlusive crisis (Allen et al., 1988) this is associated with background haemolytic anaemia and the auto-splenectomy associated with the disease (Swartz 1972). A modest leucocytosis is a feature of SCD and may be due to redistribution of granulocytes from to the circulating pool (Boggs et al., 1973).

2.4 Malaria

Malaria is a global disease prevalent in the tropics and dangerous diseases known to man, it is also by far the world’s most important tropical parasitic disease (Trigg and Kondrachin, 1993). Over 90% of deaths caused by malaria infection occur in sub-Saharan Africa where the disease is estimated to kill one child every 30 seconds (WHO, 2011). In Ghana, malaria is endemic throughout the country. It is an infection caused by parasites of the species plasmodium that are spread from person to person through the bites of infected mosquitoes and is prevalent in the tropics (Shudhanshu et al., 2012) and causes over 2 million deaths each year, mainly among African children.
Malaria is caused by a protozoa parasite from the genus plasmodium. The species of *plasmodia* that cause malaria in humans are *P.malariae, P.vivax, P. ovale* and *P.falcparum* of which the latter is the major cause of morbidity and mortality. The parasite is transmitted to human and animal hosts by the Anopheles mosquito. The parasite infects and destroys red blood cells (Taylor-Robinson, 1998). Infected red cells adhere to lining of small blood vessels providing considerable obstruction to tissue perfusion (Richard et al., 1998).

Through a process of natural selection, children with sickle cell trait (SCT) have relative higher survival advantage in malaria endemic zones. The mechanism of protection was thought to be largely related to innate factors such as the reduced ability of Plasmodium falciparum parasites to grow and multiply in SCT red cells (Aidoo et al., 2002).

Malaria and SCD affect the erythrocyte cycle, induce micro-thrombosis (including vaso-occlusion), organ dysfunction and necrosis (Mariethoz et al., 1999). Recently increased oxidative stress and impaired antioxidant defense have been suggested as a contributory factor for initiation and progression of complications in schizophrenia, diabetes, malaria and coronary artery diseases (D’Souza et al., 2008).

**2.4.1 Discovery of malaria**

The first evidence of malaria parasites was found in mosquitoes preserved in amber from the Palaeogene period that are approximately 30 million years old (Poinar G, 2005). Malaria may have been a human pathogen for the entire history of the species (Joy et al., 2003). Humans may have originally caught Plasmodium falciparum from gorillas (Liu et al., 2010). About 10,000 years ago malaria started having a major impact on human
survival which coincides with the start of agriculture (Neolithic revolution) (Hemplemann at al., 2009); a consequence was natural selection for sickle-cell disease, thalassaemias, glucose-6-phosphate dehydrogenase deficiency, ovalocytosis, elliptocytosis and loss of the Gerbich antigen (glycophorin C) and the Duffy antigen on the erythrocytes because such blood disorders confer a selective advantage against malaria infection (balancing selection) (Canalis, 2008). The three major types of inherited genetic resistance (sickle-cell disease, thalassaemias, and glucose-6-phosphate dehydrogenase deficiency) were present in the Mediterranean world by the time of the Romans. The name malaria derived from ‘mal’aria’ (bad air in Medieval Italian). This idea came from the Ancient Romans who thought that this disease came from the horrible fumes from the swamps. The idea that the disease came from the foul gasses released from soil, water and air persisted throughout the nineteenth century (Opeskin, 2009).

2.4.2 Discovery of the Mosquito vector

Vector means an organism that carries an infectious disease to another organism. For malaria, the vector is the anopheles mosquito. It carries Plasmodium to people. Patrick Manson identified mosquitoes as the vector for filariasis and in 1878 published the view that mosquitoes were likely to harbour malaria parasites (Gilles, 1993). In 1897, Ronald Ross first observed parasite forms in the cells of the stomach of a mosquito in India (Gilles, 1993). Subsequently, Italian researchers Bignami, Bastianelli and Grassi described the entire sporogenetic cycle of human Plasmodia in Anopheles mosquitoes (Grassi et al, 1899 as cited by Biggs and Brown, 2011). Years later the exo-erythrocytic cycle of the malaria parasite was defined and the dormant liver stages were discovered (Krotowski et al., 1982).
2.4.3 Vector treatment and management

Vector control is one way to stop malaria. The most used method of vector control is pesticides. These are chemicals that kill the mosquito. The first pesticide used for vector control was DDT (dichlorodiphenyltrichloroethane). DDT was first used in World War II (Bruce-Chwatt, 1981).

In many places mosquitos became resistant to DDT. This meant that DDT did not work anymore in these areas. Scientists also worried that DDT was making people and animals sick. Scientists think it might cause hormones to not work right. It might also make people and animals have trouble reproducing (getting pregnant and making babies.) It killed a lot of wildlife too. DDT also stays in the environment for a long time (Greenwood and Mutabingwa, 2002). For these reasons; people mostly use other chemicals for vector control. Organophosphate or carbamate pesticides are used, like malathion or bendiocarb.

2.4.4 Life cycle of malaria parasite

In the life cycle of Plasmodium, a female Anopheles mosquito (the definitive host) transmits a motile infective form (called the sporozoite) to a vertebrate host such as a human (the secondary host), thus acting as a transmission vector. A sporozoite travels through the blood vessels to liver cells (hepatocytes), where it reproduces asexually (tissue schizogony), producing thousands of merozoites. These infect new red blood cells and initiate a series of asexual multiplication cycles (blood schizogony) that produce 8 to 24 new infective merozoites, at which point the cells burst and the infective cycle begins anew (Schlagenhauf-Lawlor, 2008). Other merozoites develop into immature gametes,
or gametocytes. When a fertilised mosquito bites an infected person, gametocytes are
taken up with the blood and mature in the mosquito gut. The male and female
gametocytes fuse and form zygotes (ookinetes), which develop into new sporozoites. The
sporozoites migrate to the insect’s salivary glands, ready to infect a new vertebrate host.
The sporozoites are injected into the skin, alongside saliva, when the mosquito takes a
subsequent blood meal (Cowman et al., 2012).

Only female mosquitoes feed on blood; male mosquitoes feed on plant nectar, and thus
do not transmit the disease. The females of the Anopheles genus of mosquito prefer to
feed at night. They usually start searching for a meal at dusk, and will continue
throughout the night until taking a meal (Arrow et al., 2004). Malaria parasites can also
be transmitted by blood transfusions and transplacentally, although this is rare.

2.4.5 Diagnosis of malaria

The mainstay of malaria diagnosis has been the microscopic examination of blood,
utilizing blood films (Krafts et al., 2011). Although blood is the sample most frequently
used to make a diagnosis, both saliva and urine have been investigated as alternative, less
invasive specimens (Sutherland and Hallet, 2009). More recently, modern techniques
utilizing antigen tests or polymerase chain reaction have been discovered, though these
are not widely implemented in malaria endemic regions (Ling et al., 1986). Areas that
cannot afford laboratory diagnostic tests often use only a history of subjective fever as the
indication to treat for malaria

17
2.4.5.1 Blood films

The most economic, preferred, and reliable diagnosis of malaria is microscopic examination of blood films. Two sorts of blood film are traditionally used. Thin films allow species identification because the parasite's appearance is best preserved in this preparation. Thick films allow the microscopist to screen a larger volume of blood and are about eleven times more sensitive than the thin film, so picking up low levels of infection (Warhurst and Williams, 1996).

From the thick film, an experienced microscopist can detect parasite levels (or parasitemia) as few as 5 parasites/µL blood (Richard et al., 2006). Diagnosis of species can be difficult because the early trophozoites ("ring form") of all four species look identical and it is never possible to diagnose species on the basis of a single ring form; species identification is always based on several trophozoites.

2.4.5.2 Antigen tests

For areas where microscopy is not available, or where laboratory staff are not experienced at malaria diagnosis, there are commercial antigen detection tests that require only a drop of blood (Pattanasin et al., 2003). Immuno-chromatographic tests (also called: Malaria Rapid Diagnostic Tests, Antigen-Capture Assay or "Dipsticks") have been developed, distributed and fieldtested. These tests use finger-stick or venous blood, the completed test takes a total of 15–20 minutes, and the results are read visually as the presence or absence of colored stripes. The threshold of detection by these rapid diagnostic tests is in the range of 100 parasites/µl of blood (commercial kits can range
from about 0.002% to 0.1% parasitemia) compared to 5 by thick film microscopy. One disadvantage is that dipstick tests are qualitative but not quantitative.

The first rapid diagnostic tests were using *P. falciparum* glutamate dehydrogenase as antigen (Ling et al., 1986). PGluDH was soon replaced by *P.falciparum* lactate dehydrogenase, a 33 kDa oxidoreductase [EC 1.1.1.27]. It is the last enzyme of the glycolytic pathway, essential for ATP generation and one of the most abundant enzymes expressed by *P.falciparum*. PLDH does not persist in the blood but clears about the same time as the parasites following successful treatment. The lack of antigen persistence after treatment makes the pLDH test useful in predicting treatment failure. In this respect, pLDH is similar to pGluDH. Depending on which monoclonal antibodies are used, this type of assay can distinguish between all five different species of human malaria parasites, because of antigenic differences between their pLDH isoenzymes.

**2.4.5.3 Molecular methods**

Molecular methods are available in some clinical laboratories and rapid real-time assays (for example, QT-NASBA based on the polymerase chain reaction) (Mens et al., 2006) are being developed with the hope of being able to deploy them in endemic areas.

PCR (and other molecular methods) is more accurate than microscopy. However, it is expensive, and requires a specialized laboratory. Moreover, levels of parasitemia are not necessarily correlative with the progression of disease, particularly when the parasite is able to adhere to blood vessel walls. Therefore more sensitive, low-tech diagnosis tools need to be developed in order to detect low levels of parasitemia in the field (Redd et al., 2006).
2.4.6 Malaria Prevention and Control

Currently, the only means of prevention is by avoiding contact with mosquitoes that cause the disease (Miller et al., 1994). Methods used to prevent the spread of disease, or to protect individuals in areas where malaria is endemic, include prophylactic drugs, mosquito eradication, and the prevention of mosquito bites. There is currently no vaccine that will prevent malaria, but this is an active field of research (Pierce and Miller, 2009).

2.4.6.1 Prophylactic drugs

Several drugs, most of which are also used for treatment of malaria, can be taken preventively. Use of prophylactic drugs is seldom practical for full-time residents of malaria-endemic areas, and their use is usually restricted to short-term visitors and travelers to malarial regions. This is due to the cost of purchasing the drugs, negative side effects from long-term use, and because some effective anti-malarial drugs are difficult to obtain outside of wealthy nations.

2.4.6.2 Indoor residual spraying

Indoor residual spraying (IRS) is the practice of spraying insecticides on the interior walls of homes in malaria affected areas. After feeding, many mosquito species rest on a nearby surface while digesting the blood meal, so if the walls of dwellings have been coated with insecticides, the resting mosquitoes will be killed before they can bite another victim, transferring the malaria parasite.
2.4.6.3 Mosquito nets and bedclothes

Mosquito nets help keep mosquitoes away from people, and thus greatly reduce the infection and transmission of malaria. The nets are not a perfect barrier, so they are often treated with an insecticide designed to kill the mosquito before it has time to search for a way past the net.

Insecticide-treated nets (ITN) are estimated to be twice as effective as untreated nets, and offer greater than 70% protection compared with no net. ITNs have the advantage of protecting people sleeping under the net and simultaneously killing mosquitoes that contact the net (Briggs and Brown., 2001).

2.4.6.4 Vaccination

Vaccines for malaria are under development, with no completely effective vaccine yet available. Presently, there is a huge variety of vaccine candidates on the table (Pierce and Miller, 2009). This is because the genome of P. falciparum alone encodes 5,600 proteins, making it difficult to easily identify targets of protective immunity.

2.5 Haematological parameters of malaria subjects

“Haematological parameters are measurable indices of blood that serve as a marker for disease diagnosis” (Petel et al., 2004). Haematological parameters include haemoglobin level, total erythrocyte count, total and differential leucocyte counts, erythrocyte sedimentation rate (E.S.R), platelet count, packed cell (P.C.V), mean corpuscular volume (M.C.V), mean corpuscular haemoglobin (M.C.H) and mean corpuscular haemoglobin concentration (M.C.H.C).
Anaemia and thrombocytopenia are usual manifestations in patients infected with malaria (Ladhani et al., 2002). The anaemia that results from the parasites destruction of red blood cells further compromises oxygen delivery to cells. Severe anaemia could also arise from multiple processes such as acute haemolysis of uninfected RBC’s, dyserythropoiesis, interaction of malaria parasite infection with haemoglobinopathies and with nutritional deficiencies (Dondorp et al., 2000).

A study revealed a significant reduction in Haemoglobin concentration, total WBC count and platelets counts of children infected with malaria compared to healthy controls. However, white blood cell differentials were found to be normal in both cases and controls (George and Ewelike-Ezeani, 2011). It has also been reported that thrombocytopenia is an important predictor of the severity of childhood falciparum malaria. Platelet survival is reduced to 2-4 days in severe falciparum malaria.

2.6 Antioxidants

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Helmut, 1997).
Antioxidant mechanism include; enzymic removal by glutathione peroxidase, catalase and superoxide dismutase and nonenzymic defence consist of endogenously produced scavenging antioxidants such as glutathione (GSH), NADH, NADPH and uric acid and the diet derived ones like ascorbic acid and vitamin E and A (Wood and Granger, 2007, Aslan et al., 2000).

Detrimental effects caused by reactive oxygen species occur as a consequence of an imbalance between the formation and inactivation of these species (Knight JA, 1995). Inactivation and removal of reactive oxygen species depend on reactions involving the antioxidative defense system (Jenner P, 1994). The most important enzymatic antioxidants are superoxide dismutase (SOD), which catalyzes dismutation of the superoxide anion (O\(^2^-\)) into H\(_2\)O\(_2\), which is then deactivated to H\(_2\)O by catalase (CAT) and glutathione peroxidase (GPx). GPx also reduces organic peroxides into their corresponding alcohols. GPx uses reduced glutathione (GSH) as a hydrogen donor whereby GSH is oxidized. The regeneration of GSH is catalyzed by glutathione reductase (GR) (Anderson et al., 1997).

2.6.1 Superoxide dismutase

Superoxide dismutases (SOD, EC 1.15.1.1) are enzymes that catalyze the dismutation of superoxide (O\(^2^-\)) into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen.

The SOD-catalysed dismutation of superoxide may be written with the following half-reactions:

- \(M^{(n+1)+}\)-SOD + O\(_2^-\) \(\rightarrow\) \(M^{n+}\)-SOD + O\(_2\)
- \(M^{n+}\)-SOD + O\(_2^-\) + 2H\(^+\) \(\rightarrow\) \(M^{(n+1)+}\)-SOD + H\(_2\)O\(_2\).
Where $M = \text{Cu} \ (n=1) ; \text{Mn} \ (n=2) ; \text{Fe} \ (n=2) ; \text{Ni} \ (n=2)$.

In this reaction the oxidation state of the metal cation oscillates between $n$ and $n+1$.

Three forms of superoxide dismutase are present in humans, in all other mammals, and most chordates. SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. SOD1 and SOD3 contain copper and zinc, whereas SOD2, the mitochondrial enzyme, has manganese in its reactive centre. The genes are located on chromosomes 21, 6, and 4, respectively (21q22.1, 6q25.3 and 4p15.3-p15.1).

SOD out-competes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. Its main reactions are with itself (dismutation) or with another biological radical such as nitric oxide (NO) or with a transition-series metal. The superoxide anion radical ($O_2^-$) spontaneously dismutes to $O_2$ and hydrogen peroxide ($H_2O_2$) quite rapidly (~105 M$^{-1}$s$^{-1}$ at pH 7). SOD is necessary because superoxide reacts with sensitive and critical cellular targets. For example, it reacts with the NO radical, and makes toxic peroxynitrite. At subnanomolar concentrations SOD within cells, superoxide inactivates the citric acid cycle enzyme aconitase, can poison energy metabolism, and releases potentially toxic iron.

SOD has powerful anti-inflammatory activity. Treatment with SOD decreases reactive oxygen species generation and oxidative stress and, thus, inhibits endothelial activation and indicates that modulation of factors that govern adhesion molecule expression and leukocyte-endothelial interactions. Therefore, such antioxidants may be important new therapies for the treatment of inflammatory bowel disease (Segui et al., 2004).
2.6.2 Glutathione peroxidase

Glutathione peroxidase (GPx) (EC 1.11.1.9) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.

The main reaction that glutathione peroxidase catalyzes is:

\[ 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS–SG} + 2\text{H}_2\text{O} \]

Where, GSH represents reduced monomeric glutathione, and GS–SG represents glutathione disulfide. The mechanism involves oxidation of the selenol of a selenocysteine residue by hydrogen peroxide. This process gives the derivative with a seleninic acid (RSeOH) group. The selenenic acid is then converted back to the selenol by a two-step process that begins with reaction with GSH to form the GS–SeR and water.

A second GSH molecule reduces the GS–SeR intermediate back to the selenol, releasing GS–SG as the by-product. A simplified representation is shown below:

\[ \text{RSeH} + \text{H}_2\text{O}_2 \rightarrow \text{RSeOH} + \text{H}_2\text{O} \]
\[ \text{RSeOH} + \text{GSH} \rightarrow \text{GS–SeR} + \text{H}_2\text{O} \]
\[ \text{GS–SeR} + \text{GSH} \rightarrow \text{GS–SG} + \text{RSeH} \]

Glutathione reductase then reduces the oxidized glutathione to complete the cycle:

\[ \text{GS–SG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+. \]
Several isozymes are encoded by different genes, which vary in cellular location and substrate specificity. Glutathione peroxidase 1 (GPx1) is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 (GPx4) has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. Glutathione peroxidase 2 is an intestinal and extracellular enzyme, while glutathione peroxidase 3 is extracellular, especially abundant in plasma (Muller et al., 2007). So far, eight different isoforms of glutathione peroxidase (GPx1-8) have been identified in humans.

2.6.3 Glutathione

Glutathione (GSH) is a tripeptide with a gamma peptide linkage between the amine group of cysteine (which is attached by normal peptide linkage to a glycine) and the carboxyl group of the glutamate side-chain. It is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Prompella et al., 2003).

Thiol groups are reducing agents, existing at a concentration of approximately 5 mM in animal cells. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG), also called L-(−)-glutathione.
Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor (Couto et al., 2013). The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity (Pastore et al., 2003).

Glutathione is not an essential nutrient (meaning it does not have to be obtained via food), since it can be synthesized in the body from the amino acids L-cysteine, L-glutamic acid, and glycine. The sulfhydryl (thiol) group (SH) of cysteine serves as a proton donor and is responsible for the biological activity of glutathione. Cysteine is the rate-limiting factor in cellular glutathione synthesis, since this amino acid is relatively rare in foodstuffs.

Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent (H\(^+\) + e\(^-\)) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Such a reaction is probable due to the relatively high concentration of glutathione in cells (up to 5 mM in the liver).

GSH can be regenerated from GSSG by the enzyme glutathione reductase (GSR) (Couto et al., 2013): NADPH reduces FAD present in GSR to produce a transient FADH-anion.

In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-
to-GSH ratio is considered indicative of oxidative stress. Glutathione has multiple functions:

It is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms (Scholz et al., 1989). Regulation of the nitric oxide cycle, which is critical for life but can be problematic if unregulated (Clementi et al., 1999).

### 2.6.4 Ascorbic acid

Ascorbic acid is a naturally occurring organic compound with antioxidant properties. It is a white solid, but impure samples can appear yellowish. It dissolves well in water to give mildly acidic solutions. Ascorbic acid is one form ("vitamer") of vitamin C. It was originally called L-hexuronic acid, but when it was found to have vitamin C activity in animals ("vitamin C" being defined as a vitamin activity, not then a specific substance), the suggestion was made to rename L-hexuronic acid.

Ascorbate usually acts as an antioxidant. It typically reacts with oxidants of the reactive oxygen species, such as the hydroxyl radical formed from hydrogen peroxide. Ascorbate can terminate chain radical reactions by electron transfer. Ascorbic acid is special because it can transfer a single electron, owing to the stability of its own radical ion called "semidehydroascorbate", dehydroascorbate. The net reaction is:
The oxidized forms of ascorbate are relatively unreactive, and do not cause cellular damage. However, being a good electron donor, excess ascorbate in the presence of free metal ions cannot only promote but also initiate free radical reactions, thus making it a potentially dangerous pro-oxidative compound in certain metabolic contexts.

**Fig 2.0 Antioxidant defense mechanism against ROS**
2.7 Oxidative stress in sickle cell erythrocytes

Reactive oxygen species (ROS) such as superoxide (O\(^{2-}\)) and hydrogen (H\(_2\)O\(_2\)) are naturally generated in RBCs. The heme iron of Hb transfers an electron to oxygen during the binding process of oxygenated Hb. Sickle Hb is instable and is especially prone to auto-oxidation resulting in production of methemoglobin (metHb) and superoxide. This process is responsible for the steady state levels of superoxide in RBCs. The rate of hemoglobin auto-oxidation is 1.7 times greater in sickle RBCs than in normal RBCs. The auto-oxidation of sickle RBCs denatures the protein often resulting in large amounts of free iron which can catalyze membrane lipid peroxidation (Aslan et al., 2000). Normal red blood cells reduce metHb back to active Hb with energy from nicotinamide adenine dinucleotide (NADH). However, sickled RBCs have an impaired ability to reduce metHb to Hb because of insufficient levels of NADH (Zerez et al., 1990).

Studies indicate that there is abnormal and intimate association of iron (both heme and nonheme) with the membrane of the intact sickle red blood cells (RBCs) and that this iron is bioavailable for participation in peroxidative biochemistry (Sugihara et al., 1992). ROS may greatly increase the inflammatory response and may also contribute to tissue damage. Studies have shown increased serum levels of acute phase protein and oxidative stress parameters in SCD patients in a steady state (Burmerster at al., 1970) and sickle cell crisis.

A study by Fasola et al., (2007), in 40 steady state sickle cell anaemic patients and 30 age-matched, confirmed healthy HbA subjects serving as controls at Ibadan, they found
out that the total antioxidants status in this steady state SCA patients were 50% lower than their controls.

Oxidative stress results in the production of reactive oxide species responsible for secondary disease conditions in SCD patients (Kilic at al., 2010).

Rice-Evans C et al. measured the oxidative damage to sickle erythrocytes and found that the endogenous free radical mediated oxidative damage correlated with the proportion of irreversibly sickled red blood cell.

Ascorbate levels in SCD patients are not significantly different from those with normal RBCs. Nevertheless, SCD patient serum ascorbate levels were much lower than control patients and urine-excreted ascorbate was 36% higher in SCD patients than control patients (Westerman et al., 2000).

Westerman and colleagues postulated that the normal levels of ascorbate in sickle RBCs is due to the recycling of ascorbate in the presence of free radicals. Redox balance in sickle RBCs is also very important. Levels of other non-enzymatic antioxidants are such as glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GRx), carotenoids and zinc are significantly lower in sickle cells than in normal RBCs (Chan et al., 1999).
2.8 Oxidative stress in malaria

Host-parasite interactions promote constant changes in the balance between pro-oxidant and antioxidant molecules since the host and parasite are capable of producing both (Gramaglia et al., 2006).

Malaria infection induces the host’s natural defense mechanism to activate phagocytes (macrophages and neutrophils). These, in turn, generate large amounts of ROS and RNS (reactive nitrites species), leading to an imbalance between the formation of oxidizing species and the activity of antioxidants. This imbalance is what triggers oxidative stress.

The implication of free radicals through oxidative stress is related to the pathogenic mechanism triggered by the parasite (Potter et al., 2005) as well as free radical production (Keller et al., 2004) and antioxidant defenses in host cells to fight the infection (Sohail et al., 2007).

The production of free radical in the host’s haemoglobin molecule is due to the use haemoglobin as a source of amino acids for the parasite’s nutrition during the erythrocytic stage of the disease. This results in liberation of large amounts of circulating heme. labile Fe$^{2+}$ of the exposed heme groups induce intravascular oxidative stress, causing changes in erythrocytes and endothelial cells and facilitating the internalization of the parasite in tissues such as the liver and brain (Kumar and Bandyopadhyay, 2005).

Malaria infection induces the generation of hydroxyl radicals (OH$^•$) in the liver. Atamna et al. (1993) observed that erythrocytes infected with *P. falciparum* produced OH$^•$ radicals and H$_2$O$_2$ about twice as much compared to normal erythrocytes.
Oxidative stress is commonly observed to arise from five sources during disease physiopathogeny:

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 cyto-adherence processes and anemia triggered by infection;

4. Direct reactive species production by the parasite;

5. Action of antimalarial drugs

Oxidative stress has been demonstrated to promote the killing of parasites. Incubation of Plasmodium yoelii species in the presence of glucose and glucose oxidase generated $\text{H}_2\text{O}_2$ which is capable of killing the parasite. Likewise, when incubated in the presence of xanthine and xanthine oxidase, it generated free radical superoxide ($\text{O}_2^{-}$) and a subsequent burst of other oxidative products, with consequent destruction of parasites (Dockrell and Playfair, 1984)

Studies suggest that oxidative stress can take part in the pathogenesis of thrombocytopenia associated to malaria. Erel et al. (2001) showed that the number of platelets and the activities of antioxidant enzymes, SOD and GPx in patients with vivax malaria were reduced while lipid peroxidation of platelets was elevated in infected individuals, suggesting a negative correlation between platelet count and platelet level of
lipid peroxidation. This suggests that oxidative stress plays a role in the genesis of thrombocytopenia present in malaria through loss of elasticity of membranes and by increasing brittleness and causing dysfunction in receptors, resulting in considerable functional impairment of thrombocytes (Percario et al 2012).

The parasite has different antioxidant defense mechanisms in response to increased oxidative stress. Endogenous synthesis of these antioxidant compounds typically consists of three interdependent systems: enzymatic, small molecules and metal chelation, which retards or prevents oxidation of biomolecules. The antioxidant defense system also avoids oxidative species generation by scavenging or by free radicals reduction, which by self-oxidation form less reactive compounds (Sales et al., 2001).

Several antioxidant enzymes are important in the defense system; the most important include GSPx, catalase and SOD. 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 (Percario et al., 2012).

As a result, the host uses small molecules that reduce the reactivity of various reactive radicals as an auxiliary antioxidant defense system. This group contains a large number of molecules, such as vitamins A, C and E, beta-carotene, uric acid and reduced glutathione molecule (GSH) (Percario et al., 2012).

In addition, our organism has proteins that bind to transition metals preventing them from catalyzing the Fenton and Haber-Weiss reactions, important sources of reactive species
production. These metal chelators include ferritin, transferrin and lacto-ferrin (chelating iron), ceruloplasmin and albumin (copper chelators) and metallothioneins having thiol groups capable of binding several heavy metals (Halliwell and Gutteridge, 2007).

Secretion of tumor necrosis factor-alpha (TNF-α) induce oxidative stress through modulation of GSH metabolism, playing a role in malaria physio-pathogenesis. In studies with rats, the administration of TNF-α induced decreased GSH levels, whereas in CD4+ and CD8+ splenic T cells, a significant increase occurred in oxidized glutathione (GSSG) (Gosli et al., 2002), thus both behaviors suggest oxidative stress increase. Several authors have reported decreased GSH in malaria patients (Balla et al., 1992).

Lower levels of various antioxidants are found in malaria patients caused by *Plasmodium vivax*. These are: antioxidant enzymes and glutathione S-transferase (GST) (Sohail et al., 2007), catalase, GSPx, SOD (Becker et al., 2004), NADPH-methemoglobin reductase [Becker et al., 2004]; heavy metal chelators desferrioxamine, salicylaldehyde isonicotinoyl (Golensen et al., 2007) and ferritin (Balla et al., 1992); small molecules such as vitamins A, E, C (Metzger et al., 2001); the pro-vitamins α- and β-carotene, lycopene, lutein and zeaxanthin (Metzger et al., 2001), among others.

Similarly, *Plasmodium falciparum* malaria patients presented lower levels of antioxidants, such as ascorbate, which correlated with disease severity. Mice infected with *P. vinckei* exhibit erythrocytic protection against reactive oxygen species by the enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, NADPH and NADH-methemoglobin reductase in red blood cells (Stocker et al., 1985).
A study conducted with 273 children between the ages of 1–10 with acute uncomplicated P. falciparum malaria in Kampala, Uganda, verified the antioxidant status in the pathogenesis of the disease. Children with acute malaria had low antioxidant plasma concentrations.

2.8.1 Oxidative Stress as Host Defense Mechanism against Plasmodium Infection

It has been shown that oxidative stress is related to a protective role in malaria patients, as possible agents capable of destroying the Plasmodium (Percario et al., 2012).

The increased production of ROS by phagocytes, as part of the host defense, is a primary event. ROS generated by macrophages are known as 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 (Das and Nanda 1999). Likewise, neutrophils secrete proteolytic enzymes and ROS, which in low concentrations can trigger apoptosis of endothelial cells and necrosis in high concentrations (Hemmer et al., 2005).

In fact, it is generally accepted that ROS, including O2− and ONOO−, can destroy the parasite intraerythrocytically (Precario et al., 2012). It has been demonstrated that the free heme can stimulate both neutrophil migration and ROS/RNS production by a G protein-coupled receptor, more specifically the inhibitory Gα receptor, which in turn activates protein kinase C, increasing the inflammatory response (Porto et al., 2008) and delaying apoptosis, thereby most probably contributing to the immune-suppression induced by malaria (Precario et al., 2012).
2.8.2 Hemolysis as an Oxidative Stress Induction Factor in Malaria

During the erythrocytic phase of malaria, red blood cell lysis and the release of hemozoin occurs, which consists primarily of ferriprotoporphyrin IX dimers and monomers (FP) and methemoglobin in plasmodial proteins. Free heme is a powerful free radical generator, which can cause serious molecular damage to both host and parasite; the heme group contains Fe$^{2+}$ atoms that can catalyze Fenton and Haber-Weiss reactions, generating free radicals. As a result of oxidative stress, lipid peroxidation occurs, promoting functional and structural changes of the plasma membrane that lead to hemolysis. Whereas heme induces neutrophil chemotaxis (Porto et al., 2007).

Heme activates neutrophils through chemo-attractant signaling and that mesoporphyrins may be important in the treatment of inflammatory consequences such as bleeding and hemolytic disorders. Additionally, the cellular response to hemozoin entails cytokine release (Parikh et al., 2004) and generation of reactive oxygen species such as NO (Moore et al., 2004). Research on oxidative stress induced by hemozoin/heme is exciting the scientific community because supports the development of new drugs, as is the case with the potential antimalarial [(aryl)arylsulfanyl]methyl] Pyridine (AASMP) (Kumar et al., 2008).

2.8.3 Oxidative stress in *Plasmodium falciparum*–infected erythrocytes

It has been shown that malaria parasites are particularly vulnerable to oxidative stress during their erythrocytic life stages (Müller et al., 2003; Becker et al., 2004). This is because parasites live in an environment that contains oxygen and iron, the key
prerequisite for the formation of ROS via the Fenton reaction. Haemoglobin is taken up by the parasites into their acid food vacuole which leads to the spontaneous oxidation of Fe\(^{2+}\) to Fe\(^{3+}\) and the formation of superoxide anions. This combination inevitably leads to the generation of hydrogen peroxide and subsequently hydroxyl radicals, both highly reactive and toxic oxygen intermediates (Liochev and Fridovich, 1999).

During the development of the blood esquizogeny, *P. falciparum* trophozoites increase the viscosity of red blood cells by causing changes in the parasitized cell surface permitting its adhesion to the endothelial wall of capillaries, which seems to be a defense mechanism of the parasite, preventing the passage of parasitized red blood cells through the spleen and their consequent destruction (Precario et al., 2012).

Increased lipid peroxidation and oxidative stress in human malaria can affect the membrane of infected erythrocytes and promote the reduction of the deformity of these cells. This has been linked to increased mortality of adults and children with malaria. The deleterious consequences of increased cell rigidity include microcirculatory obstruction (exacerbating tissue hypo-perfusion) and cell stiffness with subsequent removal by the spleen, increasing anemia (Becker et al., 2004).

Not only is the parasite itself under oxidative stress but the host cell also shows oxidative alterations when infected with *Plasmodium*. This is demonstrated by changes in erythrocyte membrane fluidity, most probably because of alterations of erythrocyte membrane lipid composition and protein cross-linking (Muller, 2004). Haemichrome accumulation on the innersurface of the parasitized erythrocytes as well as the aggregation of erythrocyte band 3 and the increasing occurrence of auto-anti-band 3
antibodies suggest that the host erythrocytes are severely oxidatively damaged by the
*Plasmodium* infection (Parker et al., 2004).

Red blood cell disorders such as SCD confer a certain degree of resistance to an infection
with *Plasmodium* and often limit the severity of the disease (Roberts and Williams,
2003). One hypothesis to explain this is that the increased oxidative stress within the
defective erythrocyte causes an impaired infection and growth rate of the parasites (Eaton
et al., 1976; Friedman, 1978; Pasvol et al., 1978). Another hypothesis (Cappadoro et al.,
1998) suggests that defective erythrocytes infected with early stages of *P. falciparum* are
more efficiently phagocytized by the host’s immune system. Thus, the parasitized
erythrocytes are recognized by the host’s immune system at an early stage of the
infection and the parasitaemia of the infected individuals is kept low.

Similar changes also occur in normal *Plasmodium* infected erythrocytes but at a much
later stage such that they are not recognized by the host’s immune system efficiently.

### 2.8.4 Redox and antioxidant systems in *P. falciparum*

Erythrocytic stages superoxide dismutases are metalloproteins that use copper/zinc
(Cu/Zn), iron (Fe) or manganese (Mn) as metal cofactors. Protozoan parasites generally
appear to contain only Fe-dependent SODs (Fridovich, 1995). *P. falciparum* possesses
two distinct genes that encode different SODs. One of the proteins is a cytosolic, Fe-
dependent SOD, called SOD-1 that appears to be transcribed and expressed throughout
the erythrocytic cycle of the parasite (Gratepanche et al., 2002; Sienkiewicz et al., 2004).

39
As SOD-1 is a cytosolic protein, it is unlikely that it acts on superoxide anions produced during haemoglobin digestion in the parasite’s food vacuole. Thus, it still remains a possibility that the large amount of Cu/Zn-SOD taken up by the parasite from the host erythrocyte contributes to the detoxification of superoxide anions in this organelle (Fairfield et al., 1983). Furthermore, it cannot be excluded that a large proportion of the superoxide anions generated in the food vacuole spontaneously dismutate because of the acidic pH in this parasite organelle.

The second \textit{P. falciparum} SOD, named SOD-2, is a mitochondrial protein. The presence of a mitochondrial SOD seems imperative in \textit{Plasmodium} as the parasites clearly possess an active respiratory chain (Kita et al., 2002) which unavoidably leaks superoxide anions. These have to be detoxified by a mitochondrial SOD to prevent damage to the metabolic functions, nucleic acids, proteins and membranes of the organelle (Inoue et al., 2003).

So far, no apparent apicoplast SOD gene has been identified in the genome of \textit{P. falciparum}, possibly implying that the organelle does not require this enzymatic activity because little or no superoxide anions are generated by the metabolic reactions present in the organelle.

The tripeptide glutathione (GSH; g-glutamyl-cysteinylglycine) is the major low-molecular-weight thiol redox buffer in almost all aerobic cells (Sies, 1999). The ratio between GSH and GSSG is usually between 10:1 and 100:1 – it is maintained far on the side of the reduced form of glutathione. This is mainly achieved by the action of glutathione reductase (GR) but there also exist GSSGefflux pumps that export excess GSSG in order to maintain an adequate intracellular redox balance (Muller, 2004). It has
been shown that the reversible formation of protein-GS-mixed disulphides also aids to maintain an adequate GSH/GSSG ratio (Thomas et al., 1995; Sies, 1999). In addition to these reactions, the de novo synthesis of the tripeptide also contributes to sustaining sufficiently high intracellular GSH levels (Griffith, 1999). Apart from its role as a general thiol redox buffer, GSH acts as a cofactor for a variety of proteins including glutathione-dependent peroxidases, glutathione S-transferases (GSTs), glutaredoxins and glyoxalases (Sies, 1999). It is also directly involved in antioxidant reactions – for instance, the termination of radical-based chain reactions where single electrons are transferred from thyl radicals or disulphide radicals (Frey, 1997).

Glutathione reductase is the enzyme responsible for keeping glutathione in its reduced state. GR is a member of a group of enzymes known as flavo disulphide oxidoreductases which also contains thioredoxin reductase, mercuric ion reductase and trypanothione reductase (Williams, 1992). Plasmodium GR has attracted a lot of attention as a potential therapeutic target against malaria (Muller 2004). The primary structure of Plasmodium GR contains parasite-specific insertions in the FAD domain (residues 123–134), the central domain (residues 314–347) and the interface domain (residues 496–499). The first two insertions have been experimentally deleted and this affected the stability and FAD cofactor binding capacity, respectively, of the protein (Gilberger et al., 2000). This strongly suggests that the compound has additional effects that lead to parasite death (Sarma et al., 2003). In fact, it has been shown that it inhibits the formation of hemozoin which obviously can have deleterious effects for the parasites (Atamna et al., 1996).
2.9 Malaria infection in SCD and oxidative stress

Increased oxidative damage to red cell has been associated with both malaria and SCD and attributed to increased red cell destruction (Arinola et al., 2008).

Malaria infection results in the activation of the host immune responses by the malaria parasite; thereby, causing release of reactive oxygen species (ROS) accompanied by significant reduction in the levels of some antioxidants in malaria positive patients. Remero et al. (1995) stated that malaria is a pro-oxidant agent that causes cell death, tissue damage and reduction in total antioxidant levels.

Several reports support the slow rate of multiplication of *Plasmodium* in erythrocytes of haemoglobin AS, SS and SC (Orijih 2005; Ntoumi et al., 2005). But if parasites are able to survive the initial inhibition, they become spontaneous pro-oxidant agents and cause cell death/tissue damage (Orijih, 2005). Another study showed that co-existence of both malaria and HbSS could generate disastrous effect in the affected individuals (Arinola and Ezeh, 2007).

2.10 Bacteremia in SCD and oxidative stress

Sickle cell anemia predisposes children to bacteremia infections; in a study by (Platt et al., 1994) it was found that among SCD patients age 20 years and below, mortality peaked at one and three years and the primary cause of death was infection predominantly due to *staphylococcus* pneumonia sepsis.
Bacteria are known to release toxins that produce inflammation in host tissues. This inflammatory response results in the mopping up of L-arginine which is a precursor of nitric oxide (NO). Nitric oxide (NO) is a potent vasodilator and is known to help prevent vaso-occlusive crisis in SCD patients thus preventing oxidative stress. L-arginine has been found to be low in SCD adults in the steady state and appears to decrease to even lower levels during crisis. Thus a reduction in L-arginine will lead to a reduced NO bioavailability in SCD, enhancing oxidative stress with a consequent decrease in antioxidant levels (Paul et al., 2011).

McAuley et al., (2010) found out that SCD is strongly associated with bacteremia, with a diagnosis that was accompanied by an overall mortality of more than 20%.
CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study Site Description

The study was conducted at the Paediatric Out-patients Department (OPD) and Sickle Cell Clinic of the Department of Child Health (DCH), Korle-Bu Teaching Hospital (KBTH) as well as Korle-Bu Polyclinic between October 2012 and April 2013.

KBTH is situated in the nation’s capital, Accra. It is a leading tertiary hospital and serves as a major referral centre mainly for the southern sector of the country. It is affiliated to the University of Ghana Medical School. Currently, the Hospital has 2,000 beds and 17 clinical and diagnostic Departments/Units. It has an average daily attendance of 1,500 patients and about 250 patient admissions.

The Paediatric Department of the KBTH has sub-specialty clinics in areas including Sickle cell disease, Cardiology, Nephrology, Oncology, Asthma, Neuro-developmental challenges etc. The Paediatric Sickle Cell Clinic is one of the specialty clinics at the DCH. The Paediatric Sickle Cell Clinic, since its inception in 1963, served over 24,000 patients. About seventy five percent of this population is registered on attendance. It is aimed at keeping the children in steady state and at picking up early signs of complication of the disease. Children 13 years and younger, with confirmed diagnosis of SCD are seen at the clinic, held once a week at the Child Health department. About 60 patients are seen each clinic day. SCD children older than years are transferred to the adult Sickle Cell Clinic (Centre for Clinical Genetics) to continue routine check-up.
3.2 Study Design

The study design is a quantitative cross sectional study with matched control. This study was conducted on known SCD children reporting with acute febrile illness, SCD children in steady state reporting for routine check-up, and known HbAA children with acute febrile illness with and without malaria. SCD children were recruited if they had microscopically confirmed malaria and bacteremia.

3.3 Sample Size Determination

Sample size was calculated with an expected mean difference of 48% for GSH between SCD malaria positive group and non SCD malaria negative group with a power of 80% at 0.05 level of significance (two sided).

Minimum sample size used for this study was determined with the formula;

\[ N = \frac{16\delta^2}{\Delta^2} + 1 \]

\( N = \) sample size per group
\( \delta = \) expected mean difference
\( \Delta = \) standard deviation of the variable

\[ N = \frac{16(988)^2}{(1166)^2} + 1 \]

\[ = 11.5 \]

Therefore the minimum sample size for each group is 12.
3.4 Case Definition

Children aged between 6 months and 13 years who presented with acute febrile illness were screened for inclusion. Known SCD children with confirmed malaria or bacteremia were enrolled. SCD children in steady state visiting clinic for routine check-up as well as children with haemoglobin genotype, HbAA with and without malaria attending the Korle-Bu Polyclinic were recruited.

3.4.1 Inclusion Criteria

- Known SCD children confirmed for malaria or bacteremia
- Steady state SCD children visiting clinic for routine check-up and children with HbAA genotype with or without malaria

3.4.2 Exclusion Criteria.

- Children who have been transfused within the past 3 months
- Children diagnosed with other infections apart from malaria or bacteremia
- Children below 6 months and above 13 years

3.5 Patients recruitment and sampling

The sampling procedure involved collecting the samples of all children whose parents had given approval following their informed consent.

Eligible children for the study were recruited from the Paediatric sickle cell clinic and controls recruited consecutively from the OPD and the Korle-Bu Polyclinic (is a primary health care facility for both children and adult attached to KBTH).
This study was a sub study of a previous larger study that was conducted between October 2012 and April 2013. Physical examination was conducted to elicit measured variables like: pallor, jaundice and hepatic enlargement below the costal margin. Selected demographics (eg. age and gender) and selected clinical information from recruited subjects were recorded on standardized questionnaires. Two millilitres of venous blood was collected into EDTA tubes for laboratory investigations described below.

3.6 Laboratory investigations

3.6.1 Haematology and Antioxidant Estimation

Complete blood count was determined on EDTA collected whole blood. The remainder of the blood was centrifuged at 1,000g for 10 min at 4°C. Plasma was collected and stored at -20°C while awaiting further test.

Red blood cells were washed thrice with cold saline phosphate buffer, pH 7.4 (sodium phosphate buffer containing 0.15 mol/l NaCl). The erythrocytes were then suspended in an equal volume of physiological saline. Diluted haemolysates were prepared for the estimation of reduced glutathione (GSH), glutathione peroxidase and superoxide dismutase (SOD). The plasma was used for the estimation of ascorbic acid.

Thick and thin film were prepared and examined for identification of the presence and species of malaria parasite respectively.

3.6.2 Parasitological measurement

Thick and thin blood films were stained with Giemsa and examined for malaria parasites by light microscopy. Malaria parasites were counted against 200 WBCs. Parasite density
was obtained by multiplying the count by the total WBC counts, obtained from a haematological analyzer, by parasites/200 WBCs. Three microscopists independently examined the blood slides and the counts that were closest from at least two of them were accepted as the true value.

3.6.3 Complete Blood Count

Complete blood count with red cell indices was performed on heparinized blood samples using Sysmex corporation Germany KX 21N automated haematology analyzer. The Sysmex KX 21N is an automatic multi-parameter blood cell counter for in-vitro diagnostic use in clinical laboratories. It employs 3 detector blocks and two kinds of reagent for blood analysis. The WBC is measured by the WBC detector block using the DC detection method; the RBC count and the platelets count are taken by the RBC detector block, also using DC detecting method. The Hb detector block measures the haemoglobin concentration using non cyanide haemoglobin method.

3.6.4 Estimation of Antioxidants

3.6.4.1 Materials and reagents used for estimation of activity and concentration of Antioxidants

- Clear flat-bottom uncoated 96-well plate
- Human Plate Reader
- Centrifuge
- Vortex
- Pipetting devices
3.6.4.2 Colorimetric Determination of Reduced Glutathione at 412nm using QuantiChromTM Glutathione Assay Kit (DIGT-250)

GLUTATHIONE, a tripeptide of glycine, glutamic acid and cysteine, is one of the key antioxidants involved in protecting cells from damage by reactive oxygen species. Glutathione (GSH) reduces disulfide bonds in cytoplasmic proteins to cysteines, in which it is converted to its oxidized form GSSG. QuantiChromTM Glutathione Assay Kit is designed to accurately measure reduced glutathione in biological samples. The improved 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) method combines deproteination and detection (Reagent A) into one reagent. DTNB reacts with reduced glutathione to form a yellow product. The optical density, measured at 412 nm, is directly proportional to glutathione concentration in the sample. The optimized formulation has a long shelf life and is completely free of interference due to sample turbidity.

3.6.4.3 Quantitative Colorimetric Glutathione Peroxidase Determination using EnzyChromTM Glutathione Peroxidase Assay Kit (EGPX-100)
GLUTATHIONE PEROXIDASE (GPX, EC 1.11.1.9) represents an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. It helps prevent lipid peroxidation of cellular membranes by removing free peroxide in the cell. GPX catalyzes the following reaction with glutathione reductase (GR),

\[
\text{GPX} + \text{GR} \\
2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{SH} + 2 \text{H}_2\text{O}, \quad \text{GS-SG} + \text{NADPH} \rightarrow 2 \text{GSH} + \text{NADP}^+
\]

Simple, direct and high-output assays for GPX activity find wide applications. Assay directly measures NADPH consumption in the enzyme coupled reactions. The measured decrease in optical density at 340nm is directly proportional to the enzyme activity in the sample.

3.6.4.4 Quantitative Colorimetric Determination of SOD Activity using EnzyChrom™ Superoxide Dismutase Assay Kit (ESOD-100)

SUPEROXIDE DISMUTASES (SOD, EC1.15.1.1) are enzymes that catalyze the dismutation of superoxide into \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \). They are an important antioxidant defense in all cells exposed to \( \text{O}_2 \). There are three major families of superoxide dismutase: Cu/Zn, Fe/Mn, and the Ni type. Aberrant SOD activities have been linked to diseases such as amyotrophic lateral sclerosis, perinatal lethality, neural disorders and cancer. SOD assay provides a convenient colorimetric means for the quantitative determination of SOD enzyme activity in biological samples. In the assay, superoxide (\( \text{O}^2^- \)) is provided by xanthine oxidase (XO) catalyzed reaction. \( \text{O}^2^- \) reacts with a WST-1 dye to form a colored
product. SOD scavenges the $O^{2-}$ thus less $O^{2-}$ is available for the chromogenic reaction. The color intensity (OD440nm) is used to determine the SOD activity in a sample.

3.6.4.5 Quantitative Colorimetric/Fluorometric Ascorbic Acid Determination using EnzyChrom™ Ascorbic Acid Assay Kit (EASC-100)

Ascorbic acid (the L-enantiomer commonly known as vitamin C) is an important antioxidant found in living organisms and applied as additives in food and other industrial processes. By reacting with reactive oxygen species, it protects the cell from oxidative damages. The method provides a simple, direct and high-throughput assay for measuring ascorbic acid. In this assay, ascorbic acid is oxidized by ascorbate oxidase resulting in the production of $H_2O_2$ which reacts with a specific dye to form a pink colored product. The color intensity at 570nm or fluorescence intensity (530/585 nm) is directly proportional to the ascorbic acid concentration in the sample.

3.7 Ethics

This work received ethical clearance from the Ethical and Protocol Review Committee of the University of Ghana Medical School (UGMS).

3.8 Statistical analysis

Results were entered into SPSS (software version 16) and analyzed to address the objectives of the study. Basic descriptive statistics were calculated for baseline characteristics- age, clinical characteristics and haematological parameters. One-way analysis of variance (ANOVA) was used for multiple comparison of means with the post hoc analysis to confirm significance between groups. P-values less than 0.05 were
considered significant. Pearson product moment correlation coefficient (r) was used to find the association between parasite density and antioxidant levels or activities. Data obtained was tabulated and the chi-square test for hypothesis was employed for significant ‘p’ values (P <0.05).
CHAPTER FOUR

4.0 RESULTS

4.1 Patient Characteristics according to Groups

A total of 121 children between the ages of 6 months and 13 years were recruited; SCD with parasitological confirmed malaria (N = 26, mean age 9.64 years), sickle cell bacteria positive controls (N = 23, mean age 6.02 years), steady state sickle cell controls (N = 21, mean age 6.31 years), HbAA malaria positive controls (N = 26, mean age 5.96 years) and HbAA malaria negative controls (N = 25, mean age 4.53 years) were included in the analyses for this study.

Further analysis showed that the mean age of sickle cell malaria positive was significantly higher than all the other groups (p < 0.05) except the steady state sickle cell group. The mean weights between the groups also showed significant differences (p < 0.05) (Table 4.0).

The genotype distribution for children in the various groups was consistent with findings from Fleming AF, 1989. It was observed that the SS genotype was most dominant in Sub Saharan Africa, followed by the SC and then other genotypes such as the SB+ and SD.
Table 4.0: selected demographic characteristics of subjects in the various groups

<table>
<thead>
<tr>
<th>Physical characteristics</th>
<th>SCD-SS</th>
<th>SCD-M⁺</th>
<th>SCD-B⁺</th>
<th>AA-M⁺</th>
<th>AA-M⁻</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>21</td>
<td>26</td>
<td>23</td>
<td>26</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>AGE (yrs) mean±SD</td>
<td>6.31±4</td>
<td>9.64±3</td>
<td>6.02±4</td>
<td>5.96±3</td>
<td>4.53±3</td>
<td>0.00</td>
</tr>
<tr>
<td>Male(%) / Female(%)</td>
<td>14(66.7)/7(32.3)</td>
<td>11(42.3)/15(57.7)</td>
<td>14(60.9)/9(39.1)</td>
<td>10(37)/</td>
<td>15(60)/10(40)</td>
<td></td>
</tr>
<tr>
<td>Weight (Kg) mean±SD</td>
<td>23.04±12</td>
<td>23.75±6</td>
<td>16.9±7</td>
<td>20.17±5</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Haemoglobin genotype</td>
<td>SC-4(19%)</td>
<td>SC-7(27%)</td>
<td>SC-8(35%)</td>
<td>AA-26 (100%)</td>
<td>AA-25 (100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SS-15(71%)</td>
<td>SS-18(69%)</td>
<td>SS-14(61%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SS/SD-2 (10%)</td>
<td>S/SD-1 (4%)</td>
<td>SF-1(4%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.2 Clinical Characteristics of Disease Groups

Temperature recorded showed that the disease groups i.e. sickle cell malaria positive, sickle cell bacteria positive and HbAA children malaria positive had relatively higher temperature than the steady state sickle cell group (P < 0.001). There was no significant difference between sickle malaria positive and sickle cell bacteria positive group with respect to conditions such as bone pain, urine colour, dysuria, cough and chest pain as shown in Table 4.1.

There was no significant difference between the two sickle cell groups with respect to the degree of pallor but this group differed from the HbAA group by showing significantly higher degree of pallor.

There was no significant difference (p value > 0.05) between HbAA group and SCD bacteria positive group with respect to jaundice. However, the two above mentioned groups were significantly lower than the SCD malaria positive group.

One hundred percent of HbAA group were found to have normal liver size (<2cm below costal margin) and was significantly different (p value <0.05) from the two groups which had ≤ 70% with normal(<2cm below costal margin) and 30% with abnormal liver size (>2cm below costal margin). There were no significant difference between the HbAA group, and SCD groups. (p value > 0.05).
Table 4.1a: Selected clinical characteristics of subjects in the various groups

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>SCD-SS</th>
<th>SCD-M⁺</th>
<th>SCD-B⁺</th>
<th>AA-M⁺</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>21</td>
<td>26</td>
<td>23</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>34.77 ± 11</td>
<td>37.79 ± 1</td>
<td>37.77 ± 1</td>
<td>38.75 ± 1</td>
<td>0.00</td>
</tr>
<tr>
<td>Pulse (bpm)</td>
<td>104 ± 33</td>
<td>125 ± 24</td>
<td>108 ± 15</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Respiratory rate (min)</td>
<td>29 ± 9</td>
<td>35 ± 15</td>
<td>21 ± 1</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Other morbidity count (%)</td>
<td>1(4%)</td>
<td>1(4%)</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Bone pain count (%)</td>
<td>17(65%)</td>
<td>13(56%)</td>
<td></td>
<td></td>
<td>0.72</td>
</tr>
<tr>
<td>Dark urine count (%)</td>
<td>11(42%)</td>
<td>3(13%)</td>
<td></td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td>Dysuria count (%)</td>
<td>1(4%)</td>
<td>0(0.0%)</td>
<td></td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>Chest pain count (%)</td>
<td>5(19%)</td>
<td>3(13%)</td>
<td></td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>Cough count (%)</td>
<td>2(8%)</td>
<td>12(52%)</td>
<td></td>
<td></td>
<td>0.14</td>
</tr>
</tbody>
</table>
Table 4.1b: Selected clinical characteristics of subjects in the various groups

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>SCD-SS</th>
<th>SCD-M(^+)</th>
<th>SCD-B(^+)</th>
<th>AA-M(^+)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pallor count(%)</td>
<td></td>
<td>(0)5(18%)</td>
<td>(0)7(30%)</td>
<td>(0)18(65%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+)10(40%)</td>
<td>(+)8(35%)</td>
<td>(+)8(35%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(++)7(27%)</td>
<td>(++)5(22%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+++)+4(15%)</td>
<td>(+++)+3(13%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jaundice count(%)</td>
<td></td>
<td>22(85%)</td>
<td>7(30%)</td>
<td>0(0%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Liver size(cm below costal margin) count(%)</td>
<td></td>
<td>(&lt;2cm)-15(58%)</td>
<td>(&lt;2cm)-16(70%)</td>
<td>(&lt;2cm)-26(100%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&gt;2cm)-11(42%)</td>
<td>(&gt;2cm)-7(30%)</td>
<td>(&gt;2cm)-0(0%)</td>
<td></td>
</tr>
</tbody>
</table>

Values reported as number of counts of subjects recruited who also presented with symptoms and mean. P-values obtained after ANOVA for continuous variables and chi square test for nominal variables. °C- Degrees Celsius, min - minutes, cm - centimetres, bpm - beats per minutes. Values reported in 2 decimal places. SCD-SS- steady state sickle cell disease children, SCDM\(^+\) -sickle cell disease children with malaria, SCD-B\(^+\) - sickle cell disease children with bacteremia. AA-M\(^+\) -Hb AA children with malaria, AA-M\(^-\) -HbAA children negative for malaria.
4.3 Haematological parameters of groups

Haemoglobin concentration (Hb) and hematocrit were highest in the HbAA groups followed by the SCD steady state group then the SCD bacteria positive and lowest in the SCD malaria positive group respectively. In descending order of magnitude for WBC, SCD malaria positive greater than SCD bacteria positive greater than SCD steady state greater than HbAA malaria positive greater than HbAA malaria negative (Table 4.2).

Haemoglobin concentration (Hb), white blood cell count (WBC) and Hematocrit (Hct) were significantly higher in HbAA groups than in sickle cell groups (p value < 0.05). However, there was no significant difference between the means of the three variables in SCD groups or HbAA groups (p value > 0.05).

Absolute neutrophil count (ANC) in HbAA malaria positive was significantly lower than SCD malaria positive and SCD bacteria positive groups. However, there was no significant difference between HbAA malaria negative subjects and all three SCD groups. Absolute lymphocyte count (ALC) in HbAA malaria positive was significantly lower than the three SCD groups. There was no significant difference in the means of ALC between the HbAA groups or among the SCD groups (p value > 0.05).

Platelet counts in SCD groups were relatively higher than those of the non-SCD groups. The mean platelet count for HbAA malaria positive was significantly lower than all other groups (p value < 0.05) meanwhile, mean platelet count for the remaining groups had no significant differences (P value > 0.05).
Parasite count in SCD malaria positive group was significantly lower than that of HbAA malaria positive group (p value < 0.05) (Table 4.2).
Table 4.2: Mean values of haematological parameters of subjects in the various groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>reference range</th>
<th>SCD-SS</th>
<th>SCD-M⁺</th>
<th>SCD-B⁺</th>
<th>AA-M⁺</th>
<th>AA-M⁻</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td></td>
<td>21</td>
<td>26</td>
<td>23</td>
<td>26</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Hb (g/dl) mean±SD</td>
<td>13-18</td>
<td>7.71 ± 2</td>
<td>6.71 ± 2</td>
<td>7.75 ± 2</td>
<td>10.08 ± 1</td>
<td>10.90 ± 1</td>
<td>.000</td>
</tr>
<tr>
<td>WBC(10⁹/L) mean±SD</td>
<td>4.3-10.5</td>
<td>17.64 ± 8</td>
<td>22.90 ± 13</td>
<td>19.05 ± 8</td>
<td>8.88 ± 3</td>
<td>10.94 ± 7</td>
<td>.000</td>
</tr>
<tr>
<td>ANC(10⁹/L) mean±SD</td>
<td></td>
<td>9.85 ± 1.3</td>
<td>10.67 ± 1.2</td>
<td>11.73 ± 1.2</td>
<td>5.61 ± 1.2</td>
<td>6.87 ± 1.2</td>
<td>.000</td>
</tr>
<tr>
<td>ALC(10⁹/L) mean±SD</td>
<td></td>
<td>6.03 ± 1.1</td>
<td>8.63 ± 9.9</td>
<td>5.38 ± 1.1</td>
<td>2.27 ± 9.9</td>
<td>2.84 ± 1.0</td>
<td>.000</td>
</tr>
<tr>
<td>Hct(%) mean±SD</td>
<td>42-16</td>
<td>24.01 ± 5</td>
<td>21.13 ± 7</td>
<td>23.20 ± 5</td>
<td>31.03 ± 4</td>
<td>34.57 ± 3</td>
<td>.000</td>
</tr>
<tr>
<td>Platelets(10⁹/L) mean±SD</td>
<td>150-350</td>
<td>319 ± 151</td>
<td>310 ± 150</td>
<td>347 ± 176</td>
<td>166 ± 134</td>
<td>308 ± 92</td>
<td>.000</td>
</tr>
<tr>
<td>Parasite density/uL</td>
<td></td>
<td>3318 ± 7993</td>
<td>29900 ± 37568</td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
</tr>
</tbody>
</table>

Values reported as mean. P-values obtained after ANOVA for > two groups and one sample t-test for two groups. Hb – Haemoglobin Concentration, WBC- White Blood cell Count, ANC- absolute neutrophil count, ALC- absolute lymphocyte count. Values recorded in 2 decimal places.
4.4 Antioxidant levels among groups

Superoxide dismutase (SOD) activity between groups in order of descending magnitude is as follows; HbAA malaria positive greater than SCD steady state greater than HbAA malaria positive greater than SCD bacteria positive greater than SCD malaria positive group (Table 4.3). There was no significant difference (p value > 0.05) between the means of all five groups for superoxide dismutase (SOD) activity. Numerically, mean SOD for SCDSS was lower than the HbAA malaria negative group (25.85, 27.51) U/ml (fig 4.1) and that of SCDSS was higher than the SCD malaria positive group (25.85, 18.51) U/ml, while the mean of HbAA malaria negative was higher than that of HbAA malaria positive group (27.51, 23.99) U/ml (fig 4.3).

The mean activity of Glutathione peroxidase (GPx) in SCD steady state was found to be significantly higher than the remaining four groups. The mean activity of HbAA malaria was significantly lower than the all three sickle cell disease groups (p value < 0.05). However, the mean GPx for SCD steady state was higher than that of SCD malaria positive group (fig 4.3) and that of HbAA malaria negative was higher than HbAA malaria positive.

Mean ascorbic acid concentration (ASC) for HbAA malaria negative subjects was significantly higher than the remaining groups (p value < 0.05) however; the differences between the means of the remaining groups was not statistically significant (p value > 0.05). However, the mean ASC was higher in HbAA malaria negative groups compared
to SCD steady state. Also the mean SCD as expected was higher in non- malaria groups compared to malaria groups for both SCD and non SCD.

The mean reduced glutathione (GSH) concentration for the HbAA groups was significantly higher than the SCD groups. There was no significant difference between the means of HbAA groups or the SCD groups (Table 4.3). GSH was not consistent with the trend followed by the other antioxidants. GSH was lower in SCD steady state than in SCD malaria positive groups and lower in HbAA malaria negative group compared to HbAA malaria positive group.
Table 4.3: mean values of selected antioxidant concentration or activity of subjects in the various groups

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>SCD-SS</th>
<th>SCD-M⁺</th>
<th>SCD-B⁺</th>
<th>AA-M⁺</th>
<th>AA-M⁻</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD(U/ml) mean±SD</td>
<td>21</td>
<td>26</td>
<td>23</td>
<td>26</td>
<td>25</td>
<td>0.43</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>18.2-33.4</td>
<td>11.7-25.3</td>
<td>15.3-29.9</td>
<td>17.1-30.8</td>
<td>20.5-34.4</td>
<td></td>
</tr>
<tr>
<td>GPx(U/ml) mean±SD</td>
<td>259± 82</td>
<td>92.60± 61</td>
<td>79.3± 68</td>
<td>7.05± 9</td>
<td>29.8± 149</td>
<td>0.00</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>221.1-295.9</td>
<td>58.9-126.2</td>
<td>43.5-115.0</td>
<td>-26.6-40.7</td>
<td>-4.5-64.0</td>
<td></td>
</tr>
<tr>
<td>GSH(mg/dl) mean±SD</td>
<td>4465±1655</td>
<td>7941±6385</td>
<td>5486±2158</td>
<td>11700±9056</td>
<td>10800±8524</td>
<td>0.00</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>1636-7294</td>
<td>5398-10483</td>
<td>2782-8189</td>
<td>9171-14257</td>
<td>8.217-13403</td>
<td></td>
</tr>
<tr>
<td>ASC(mg/dl) mean±SD</td>
<td>2598±941</td>
<td>2401±988</td>
<td>2455±847</td>
<td>2514±645</td>
<td>3567±877</td>
<td>0.00</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>87619-100094</td>
<td>-3205-8006</td>
<td>-3505-8415</td>
<td>-3091-8120</td>
<td>-2150-9284</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.1; bar chart comparing the mean activity of SOD for the various groups with error bars as one standard deviation

Figure 4.2; bar chart comparing the mean activity of GPx for the various groups with error bars as one standard deviation
Figure 4.3; bar chart comparing the mean levels of GSH for the various groups with error bars as one standard deviation

![GSH bar chart](chart1)

Figure 4.4; bar chart comparing the mean levels of ASC for the various groups with error bars as one standard deviation

![ASC bar chart](chart2)
4.5 Comparison of Antioxidant levels with Parasite density

There was no significant association between the parasite density and antioxidants (Table 4.4). There was a trend towards a weak negative correlation between the GPx and parasite density but association was not significant (P value > 0.05) (Table 4.4).

Table 4.4: Pearson correlation between parasite density and antioxidant concentration or activity

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Pearson correlation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>0.013</td>
<td>.893</td>
</tr>
<tr>
<td>GPx</td>
<td>0.174</td>
<td>.063</td>
</tr>
<tr>
<td>GSH</td>
<td>.097</td>
<td>.302</td>
</tr>
<tr>
<td>ASC</td>
<td>.075</td>
<td>.428</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

5.0 DISCUSSION

The above data from the study is to determine antioxidant levels in sickle cell disease and non-sickle disease children with and without malaria.

There was no significant difference between the mean values of all five groups for superoxide dismutase (SOD) activity. Activity between the groups in order of descending magnitude is as follows; HbAA malaria negative greater than SCD steady state greater than HbAA malaria positive greater than SCD bacteria positive greater than SCD malaria positive group. SOD was lower in malaria positive subjects than the negative subjects for both SCD and non-SCD groups as shown on figure 4.1. In vitro studies have demonstrated the ability of oxidative stress to promote the killing of parasites. Plasmodium yoelii species when incubated in the presence of xanthine and xanthine oxidase, it generated free radical superoxide (O$_2^-$) and a subsequent burst of other oxidative products, with consequent destruction of parasites (Dockrell anf Playfair, 1984) SOD provides the efficient dismutation of O$_2^-$ leading to the formation of H$_2$O$_2$ resulting its reduced activity.

Also mean SOD in SCD steady state was lower than non-SCD malaria negative groups. This could be as a result of sickled haemoglobin undergoing constant oxidative stress as a result of the existent of radicals or groups. This auto-oxidation reaction by HbS results in the production O$_2^-$ . SOD in an attempt to dismutate the O$_2^-$ may lose its activity (Aslan et al., 2000).
The mean activity of Glutathione peroxidase (GPx) in SCD steady state was found to be significantly higher than the remaining four groups. The mean activity of HbAA malaria was significantly lower than the all three SCD groups (p value < 0.05). This was in consistent with findings from Beretta L et al. (1983) who reported significantly higher GPx levels in sickle erythrocytes compared to non-sickled ones. GPx with a number of enzymes convert \( \text{H}_2\text{O}_2 \) to water (Wood et al., 2003). This is however inconsistent with the trend of activity for the other antioxidants. It could be that the enzyme has exceeded the threshold time at which the activity peaks and had dropped before the reading was taken.

Mean ascorbic acid concentration (ASC) for HbAA malaria negative subjects was significantly higher than the remaining groups however; the difference between the means of the remaining groups was not statistically significant. A study by Akanbi et al., 2010 reported that, Ascorbic acid was significantly reduced in malaria positive participants. Chou PT, Khan AU (1983) proposed that the anti-oxidant effect of vitamin C may be due to quenching of singlet oxygen in the aqueous medium produced as a result of oxidative stress. However,

The mean reduced glutathione (GSH) concentration for the HbAA groups was significantly higher than the SCD groups. There was no significant difference between the means of HbAA groups or the SCD groups (Table 4.3). HbS-containing red cells auto-oxidize faster, generating superoxide, hydrogen peroxide, hydroxyl radicals and lipid oxidation products when compared with HbA-containing red cells (Hebel et al., 1982). GSH plays a pivotal role in protection of cells against oxidative stress. It acts as a
non-enzymatic antioxidant by direct interactions of SH group with ROS or involved in the enzymatic detoxification reactions for ROS as a coenzyme.

Haemoglobin concentration (Hb) and hematocrit were highest in the HbAA groups followed by the SCD steady state group and the SCD bacteria positive and lowest in the SCD malaria positive group respectively. This is a similar trend in publications. In descending order of magnitude for WBC, SCD malaria positive greater than SCD bacteria positive greater than SCD steady state greater than HbAA malaria positive greater than HbAA malaria negative. During the erythrocytic phase of malaria, red blood cell lysis. Free heme is a powerful free radical generator, which can cause serious molecular damage to both host and parasite; the heme group contains Fe2+ atoms that can catalyze Fenton and Haber-Weiss reactions, generating free radicals. As a result of oxidative stress, lipid peroxidation occurs, promoting functional and structural changes of the plasma membrane that lead to further hemolysis (Porto et al., 2007).

Haemoglobin concentration (Hb), white blood cell count (WBC) and Hematocrit (Hct) were significantly higher in HbAA groups than in sickle cell groups (p value < 0.05). Low Hb and Hct in SCD may be due to the background rate of red cell sickling which drastically shortens the life span of red cells leading to chronic hemolytic anaemia and jaundice even in steady state (Kaul et al., 1996).

Absolute lymphocyte count (ALC) in HbAA malaria positive was significantly lower than the three SCD groups. A modest leucocytosis is a feature of SCD and may be due to redistribution of granulocytes to the circulating pool (Boggs et al., 1973).
Platelet count in SCD groups was relatively higher than that of the HbAA groups. High platelets count in SCD is associated with hemolytic anaemia and the auto-splenectomy associated with the disease (Swartz 1972). The mean platelet count for HbAA malaria positive was significantly lower than all other groups (p value < 0.05). Although the mean platelet count for the remaining groups had no significant differences, SCD malaria positive group had a lower mean platelet than the remaining SCD groups.

This is because oxidative stress plays an important role in the genesis of thrombocytopenia in malaria through loss of elasticity of membranes and by increasing brittleness and causing dysfunction in receptors, resulting in considerable functional impairment of thrombocytes. Erel et al., 2001, showed that the number of platelets and the activities of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx)—in patients with Falciparum malaria were reduced.

Parasite count in SCD malaria positive group was significantly lower than that of HbAA malaria positive group (Table 4.2). Red blood cell disorders such as SCD confer a certain degree of resistance to an infection with Plasmodium and often limit the severity of the disease (Roberts and Williams, 2003), increased oxidative stress within the defective erythrocyte causes an impaired infection and growth rate of the parasites (Eaton et al., 1976).

There was a weak association between the Parasite count and antioxidant activity or concentration but association was not significant. There was a trend towards a weak negative correlation between the GPx and parasite count but association was not significant (Table 4.4).
From the above discussion it is evident that for three of the four antioxidants investigated (ie SOD, GSH and ASC) non-SCD malaria negative subjects had higher antioxidant activity than the SCD steady state children (figure 4.1-4.4). This accounts for the fact that Hb S genotype in SCD subjects generates ROS due to rapid oxidative stress thus mopping up antioxidants. GPx was not consistent with the others because it may have exceeded the maximum time when its activity peaks before the reading was taken.

Also for three of the four antioxidants investigated (ie SOD, GPx and ASC) malaria positive subjects had lower activity than the non-malaria subjects for both SCD and non SCD children (figure 4.1-4.4). The inconsistent nature of GSH with the other antioxidant could not be accounted for.

But generally, we could say SCD steady state had a lower antioxidant status than the non-SCD malaria negative group and malaria positive group had lower antioxidant status than malaria negative groups for both SCD and non-SCD children. Malaria and sickle cell disease may results in an overall decreased antioxidant level compared to the effect of only malaria or sickle cell disease. This could be as a result of the pivotal role played by antioxidants in protecting serum lipid from the attack of reactive oxygen species (ROS) produced by these disease conditions. Hence the antioxidant status in SCD and non-SCD with malaria is generally lower than SCD children and non- SCD children without malaria. But it cannot be concluded yet if the disease condition imparts on the result or not or whether outcome is due to other internal factors.
5.1 Limitation

Diet and Medication could play an important role as oxidative stress predictor. However, they were not included in the research questionnaire because samples were taken from a previous study.

With reference to the seeming “unexpected” finding of no statistical difference between some of the groups could be attributed to the wide assumptions for potential expected inter-group difference estimates for some of the antioxidants used to calculate sample size. These big differences resulted in relatively small sample sizes for the groups which could have accounted for the lack of difference. These big differences resulted in relatively small sample sizes for the groups, which could have accounted for lack of differences.

Furthermore, since most of the children (including malaria negative ones) presented with acute febrile illness to the hospital, it is conceivable that the presence of other (undiagnosed) illness (es) with effect on oxidant status could also have contributed especially to those that showed no difference.
CHAPTER SIX

6.0 Conclusion

From the results apart from GPx, all the other three antioxidants were higher in non-SCD malaria negative groups compared to SCD steady state group. The inconsistent nature of GPx from the other antioxidants was attributed to the fact that threshold time at which activity peaks may have been exceeded before reading was taken. Also, apart from GSH, the other three antioxidants investigated had means higher in malaria negative groups compared to malaria positive groups. The reason for deviation of GSH from the others could not be accounted for.

It can however be concluded that generally, antioxidant status in SCD and non-SCD malaria negative groups is higher than that of the malaria positive group.

6.1 Recommendation

The study recommends carrying out;

- An extensive study involving a larger sample size that will minimize error
- A future study design to incorporate an extensive research on diet and medication in relation and how they can affect result of the study
- Also a prospective cohort study could be designed to follow up antioxidant status of diseased state groups i.e. Both SCD and non-SCD till the end of the disease conditions. This will clarify the impact of the disease on the results obtained.
- A molecular study on antioxidants to give a wider understanding and explanation for outcome.
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Pattanasin S, Proux S, Chompasuk D et al. (2003). Evaluation of a new Plasmodium lactate dehydrogenase assay (OptiMAL-IT) for the detection of malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene; 97: 672–674


APPENDIX 1

CONSENT FORM: Antioxidant status of sickle cell disease children with and without malaria

INTRODUCTION/PURPOSE

Information: To be read to parent/guardians in their own mother tongue or their preferred language

Dear Parent/Guardian:

I kindly ask your permission to participate in a research study which I will proceed to describe. In order to ensure that you are informed about being in this research, the consent form will be read to you in your preferred mother tongue. You will also be asked to sign it (or thumbprint). We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understand.

Why this study:

Your child is a confirmed sickle cell disease patient and has malaria. Certain substances called antioxidants are going to be measured in your child’s blood sample. An antioxidant is a molecule that is produced by the body or taken through the body through our diet to help defend the body against harmful substances known as reactive oxide species. It has been suggested that, levels of antioxidants in sickle cell disease children is markedly reduced compared to children without sickle cell. Also level of certain antioxidants in normal children affected with malaria is also reduced. It is very common in Ghana to find
children with sickle cell disease suffering from malaria which usually leads to death if not well treated. But there is no data on the antioxidants status of malaria infected sickle cell disease children. It is hoped that the study will help determine the impact malaria has on the antioxidant status of sickle cell disease children and if antioxidant status determines how a sickle cell child is predisposed to malaria.

**General Information and your part in the study:**

Two millilitres (about one teaspoonful) of venous blood will be taken from your child to check for the presence of malaria parasites and all other laboratory tests. Certain measurements will also be taken such as height and weight.

**Possible Risks**

A small amount of blood is going to be collected which is equivalent to the amount collected during routine analysis. When the needle is inserted to draw blood your child may feel moderate pain or only a prick or stinging sensation that may cause him or her to cry. Your child will receive appropriate treatment as necessary. Sterile techniques and disposable, single-use equipment will be used at all times.

**Withdrawal from study:**

Participation is strictly voluntary and abstaining from participation will have no consequences for your child or yourself. Even after agreeing to participate, you will still be free to withdraw from further participation at any time without consequences.
whatsoever; and in such a case, all available and standard care possible will be given to you and your child

Confidentiality

All information gathered would be treated in strict confidentiality. When results of this study are reported in medical journals or at medical meetings, identities will be kept anonymous. All medical records will be stored by the researchers in safe cabinets.

Contacts

If you have any questions about this study or study-related problems, you may contact Mrs Priscilla Dzigba (0243719506) of the Chemical Pathology Department, University of Ghana Medical School. You may also contact Dr. George Obeng Adjei (Tel: 0208114973) of the Centre for Tropical Clinical Pharmacology and Therapeutics (CTCPT), and Dr S.Y. Oppong (Tel: 0244267404) Department of Chemical Pathology.

You are free to ask any questions.

Thank you

I hereby agree to allow my ward ---------------------------------------- to be enrolled into the study. The full details of the study have been explained to me and I fully understand all the details and risks involved, as explained.

Name of parent/guardian ---------------------------------------------

Signature/thumbprint of parent/guardian-----------------------------
Witness

Signature of Principal Investigator