RELATIONSHIP BETWEEN ANAEMIA, SOD AND G6PD DEFICEINCY ON SICKLE CELL PATIENTS

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APPROVAL PAGE

This project written under the direction of candidate project supervisor and approved by the members of project committee, has been presented and accepted by the department of Biotechnology, Godfrey Okoye university in partial fulfillment of the requirement for the award of Bachelor of science (B.Sc.) Degree in Biotechnology.

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This is to certify that this research work THE RELATIONSHIP BETWEEN ANEMIA, SOD AND G6PD DEFICIENCY is the original work done by UGWU ONYINYE MARYROSE. Matriculation number U14/NAS/BTG/021 and has never being carried out in any institution.

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DEDICATION

I dedicate this research work to the Almighty God because He is the maker and creator, of all living things and the greatest scientist ever. I also dedicate it to all those sickle cell patients out there, and want to all to know that it will get better. We all can still strive to live together and better. We will someday live without worry.

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ABSTRACT

The relationship between anemia, SOD (superoxide dismutase) and G6PD (glucose-6-phosphate) in SCD (sickle cell disease) patients were determined for the better understanding of their pathophysiology. Hemolytic anemia is one of the most common complications of sickle cell. It can be influenced by different factors, malaria infections, oxidative stress, dehydration, environmental stress and much more. SOD are enzymes that help with the regulation of oxidative stress and G6PD deficiency together with SCA are at high frequencies in malaria epidemic regions. Both SCA and G6PD deficiency patients suffer from anemia and can be said to coexist in some individuals. This study aims to find out the relationship between anemia, SOD and G6PD in SCD patients, if they coexist together or influence each other's manifestations. A total of 70 patients were used including AA and AS genotypes as control. Anemia indices including hemoglobin (Hb) concentration, hematocrit were tested for. The presence of SOD and G6PD deficiency were tested for in all the patients. G6PD non deficient SS patients were 32.9% and 7.1% were deficient. The hemoglobin (Hb) concentration and hematocrit in SS patients were normal 6.76mg/dL which normally ranges from 5.0-10.0mg/dL. The SOD levels in SS were also at normal levels (2.45 10^{-1} U). There was no relationship between anemia, SOD and G6PD deficiency in sickle patients. These parameters can exist independently but cannot influence their prevalence as there was no interaction between G6PD deficiency and sickle cell to influence SOD level and anemia indices. However, anemia is associated with sickle cell disease.

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND

Sickle cell disease (SCD) is a group of genetic disorder that is inherited in an autosomal recessive manner due to the homozygous or heterozygous state of the mutation. It is caused by a single base mutation in the β-globin gene of hemoglobin, where GAT is replaced by GTT in the 6^{th} codon of exon 1 resulting to valine instead of glutamic acid on the sixth position in chromosome 11. In the normal adult hemoglobin (HbA), there are 2 α-globin chains and 2 β-globin chains that form a globin tetramer. They are stabilized by intramolecular points of contact, without any interaction between them. When they bind or release oxygen they retain their normal shape but in the mutated β-globin there is a hydrophobic interaction between the adjacent valine amino acids which align into polymers and distort the shape of the red blood cells. These polymers, which are poorly soluble, distort the normal shape of the red blood cells, changing it to a sickle or crescent shape which prevents the normal flow of blood in the blood vessels (microcirculation) and increasing its adhesion to the endothelium of the vessels. This leads to vaso-occlusive crisis and hemolytic anemia which are the hallmark of the disease. SCD is a systemic pleiotropic disease that affects almost all the organs of the body or causes tissue infarction and a good number of other clinical manifestations throughout the affected individual's life as a result of the polymerization of the beta hemoglobin under deoxygenated, acidic or dehydrated conditions and hypoxia. Sickled RBCs are more readily destroyed or are broken down prematurely by the reticulo-endothelial system due to their rigidity makes them filtered by the spleen. Most of the clinical manifestations are protean in nature and vary in frequency and severity among patients. SCD is a hemoglobinopathy in which the single base substitution mutation in the β-globin chain can result to either hemoglobin S, C, β^+ or β^o thalassemia, D, E or O_{Arab} and are all known as hemoglobin variants but when they are

combined with HbS they are known as SCD variants. Individuals, who are affected with sickle cell anemia which is one of the variants of SCD, have two copies of the mutated gene (HbSS). Other heterozygote individuals have one copy of the Hb S and other variant which could be Hb C, Hb β^+ or β^o thalassemia. The mutation in HbSS is Glu6Val, in HbSC is Glu6Lys, the mutation in hemoglobin D glutamine replaces glutamic acid at position 121 of the gene and the mutations that cause the Sβ+ or the Sβº are deletions or additions of a single base substitute or more in the HBB gene (Serjeant 2013; Ashley-Koch *et al.,* 2000; Heiman and Greist, 2010; Bunn, 1997; Booth *et al.,* 2010; Al-Jafar *et al.,* 2016; Kaur *et al.,* 2013; Ballas, 2002; Ballas *et al* 2010; Wild and Bain, 2006 and Emecheba *et al.,* 2017).

Carrier individuals have one copy of the mutated hemoglobin and normal hemoglobin (Hb AS) and are said to have sickle cell trait. They are also said to be protected from malaria infection, resulting to the high frequency of the Hb S variants in malaria epidemic regions. Not all the variants of the mutated hemoglobin are detrimental, a concept known as genetic polymorphism. Millions of people worldwide are affected with this disease with the highest population in Africa but it can also be seen in Sub-Saharan Africa, Eastern Saudi Arabia, Central India Mediterranean, Afro-Caribbean, South and Central American, Arab and East Indian. Some of these variants are frequent in some of these populations than others. The two commonest hemoglobin variants in Nigeria are HbS and HbC. HbS is distributed fairly well in Nigeria but HbC is commonly seen among the westerns (Yoruba) and decreases from the west eastwards. It was estimated in Nigeria around 1982 that 30,000 infants are born each year with SCD as it is seen as the country with the highest affected individuals with the trait ranging from 20-30%. As of recent it is estimated that >40 million individuals are carriers, >150,000 infants are born each year with the disease and about 1 million survive past childhood (Galadanci *et al.,* 2013; Emecheba *et al.,* 2017 and Grosse *et al.,* 2011).

According to Robbins, (2014) the major cause of the symptoms in patients with SCD is the sickling of the red blood cells. The clinical or phenotypic manifestations of SCD are grouped into three, which include hemolytic anemia, pain episodes or crisis and severe organ damage. The sickled cells are unable to deliver oxygen to tissues in the body and this leads to tissue or organ damage. They also die faster than normal cells which lead to anemia, a blood condition that the red blood cells are lower than normal and it is a major symptom in patients with SCD. Due to their inflexibility they are unable to pass through small capillaries, causing blockage in the blood vessels leading to severe vaso-occlusive crisis. Other signs and symptoms of sickle cell disease which vary from person to person and can change over time include; acute pain (sickle cell or vaso-occlusive crisis), frequent infections, pulmonary complications, leg ulcers, priapism, brain complications (clinical stroke and silent stroke), eye problem, retarded growth and puberty, kidney problem (nocturnal enuresis), gallstones, liver complications (intrahepatic cholestasis), joint complications (avasualar or aseptic necrosis) and metal health. Lack of a large, readily accessible population for clinical studies has contributed to the absence of standard definitions and diagnostic criteria for the numerous complications of SCD and inadequate understanding of SCD pathophysiology (Ballas *et al.*, 2010).

Most of these complications found in SCD patients can be triggered by a lot of factors such as malaria infections, stress, temperature change (favorably warmth) and dehydration. As discoveries are been made, new body of evidence has shown that oxidative stress is a significant pathway sickle cell complications and morbidity as it enhances the sickling phenomenon of the cells. These could all contribute to the heterogeneous phenotypic expression of the disease. Oxidative stress is an imbalanced redox status caused by over production of oxidants (reactive oxygen specie) and depletion of antioxidants. This excess oxidant state leads to release of heme, auto-oxidation of

hemoglobin, uncoupling of nitric oxide synthase activity leading to a decrease in NO. It has been observed that the antioxidant defense systems in SCD are ineffective in neutralizing the excess oxidant specie. Normal erythrocytes counter oxidative stress using self-sustaining activities of antioxidant defense enzymes such as superoxide dismutase (SOD) which is a key enzyme in dismuting super radicals into hydrogen peroxide. The activity of this enzyme is seen to be higher in SCA patients.

Anemia is a medical condition where the red blood cells or hemoglobin level in the body is lower than the normal level. Sickle cell disorders are associated with variable degrees of anemia depending on genotype, with the most severe decrease in hemoglobin level seen in sickle cell anemia and the least severe in hemoglobin S-β+ thalassemia. Normal red blood cells live for 120 days while the sickle cells live for 10-30 days as a result of continuous breakage of the cells. When the body is short of red blood cells, the tissues do not receive adequate amount of oxygen and this leads to fatigue or weakness. Severe anemia episodes may result from a variety of causes, including hyperhemolysis, acute splenic sequestration, and aplastic crises (Ballas *et al.,* 2010). Although chronic hemolytic anemia is a major feature of sickle cell disorders, a marked drop in hemoglobin with an increased hemolytic rate is referred to as hyperhemolysis. Hemolytic anemia varies intensively among the genotypes of sickle cell disease and it may be the driving force behind some complications of sickle cell disease because of its effects on Nitric oxide (NO) bioavailability which its decrease is associated with pulmonary hypertension, priapism, leg ulceration, and possibly with non-hemorrhagic stroke (Kato *et al.,* 2007).

Another clinical symptom that can be associated with SCD is G6PD deficiency. Glucose-6-phosphate dehydrogenase deficiency is a genetic disorder that results to an inadequate production of G6PD enzyme. This enzyme helps to regulate many biochemical processes in the body

including the proper and normal functioning of the red blood cells. This deficiency causes the red blood cells to break prematurely called hemolysis leading to a common medical problem called hemolytic anemia. This anemia could lead to paleness, jaundice, fatigue, rapid heart rate and so on. In individuals with this deficiency, hemolytic anemia can be triggered by bacterial or viral infections, antibiotics or antimalaria drugs, favism which is caused by eating fava beans. This deficiency occurs exclusively in males. This deficiency results from mutations in the G6PD gene. This gene provides the instruction for producing the enzyme which is involved with the chemical reactions that prevent reactive oxygen species from accumulating to toxic levels in the body. With these mutations occurring, the production or structure of the enzyme is altered leading to an accumulation of the reactive oxygen species and would be harmful to the red blood cells. This gene is found on the X-chromosome and since males have only one copy of this chromosome they are more affected then females that have 2 copies of the chromosome and it is very rare for the mutation to occur on both genes. G6PD deficiency just like SCD is prevalent where malaria is epidemic and very common among the black population with a protective role against malaria. The presence of the G6PD deficiency can lead to an increase in the severity of crisis in SCD patients. Studies have also shown that this deficiency is prevalent in SCD patients more than the general population but this could be otherwise in some other population. The coexistence of this relationship can lead to hemolytic anemia, acute splenic sequestration and vaso-occlusive crisis. Patients usually are asymptomatic, these disorders do not alter the hemoglobin (Hb) levels and RBC count in stable conditions (Genetic home reference, 2018; Benkerrou, *et al.,* 2013; Memon, *et al.,* 2016, Firempong, *et al*., 2016 and Al-Nood, 2011).

Over the years, measures like prenatal screening, parent education, better medical care, immunization and the use of penicillin prophylaxis have been used to increase the life expectancy

of affected individual, having the three basic therapeutics modalities as hydroxyurea, blood transfusion and bone marrow transplant. World Health Organization (WHO) has promoted several national screening programs with the goal of informing reproductive choice in other to reduce severely affected infants (Kaur *et al.,* 2013).

This study is designed to access the relationship between sickle cell disease and the factors that trigger their complications. Hemolytic anemia is the most common clinical manifestation found in each of the single genotype of this disorder. Oxidative stress is known to increase the anemia rate in SCD patients and leads to vaso-occlusive crisis and any other clinical complications and how it affects G6PD patients is quite unclear but antimalaria drugs can trigger hemolytic crisis. Individuals that have only G6PD deficiency tend to have hemolytic anemia as the main clinical symptom, and the relationship between SCD and G6PD is not definite for all population but they are common in black population and have a protective role against malaria. Thus, it will be of interest to evaluate the influence of oxidative stress, anemia and G6PD deficiency on SCD patients.

1.1 STATEMENT OF PROBLEM

Nigeria is said to have the highest number of SCD cases, having the two most common variants as SS and SC. Despite the high burden of SCD in Nigeria, it has been difficult to improve the care and management of diseases. Most of the new treatments, therapies, and creation of awareness is lacking or is not widely available especially in the rural regions. The pathophysiology of the diseases, to an extent is not really understood resulting from lack of assessable data which also leads to the inability of providing a permanent cure for the disease. Studying the various factors that triggers their crisis and the degree of their phenotypic manifestations would give a better understanding of the disease

pathophysiology and more data would be available in order to provide better improved treatment of the disease.

1.2 AIMS AND OBJECTIVES

This study intends to assess the relationship between anemia, SOD and G6PD deficiency and how they increases the vaso-occlusive crisis in SCD patients visiting ESUTH and UNTH in Enugu State, Nigeria

The objectives of this study are to determine:

- Screen patients for sickle cell anemia based on their genotype status-using questionnaire.
- Assess hemoglobin concentration and hematocrit level in patients.
- Quantify the level of superoxide dismutase (SOD) activity in patients.
- To determine the absence or presence of G6PD deficiency in SCD patients.

CHAPTER TWO

Literature Review

2.0 MOLECULAR PATHOGENESIS OF SICKLE CELL DISEASE

Sickle cell disease is a chronic blood disorder that is characterized by an abnormal, rigid, sickle shaped red blood cells. It can also be known as drepanocytosis, and is a quattuorvirate of anemia and its sequel, pain episodes infection and organ damage. The pathogenesis of the disease is due to the adherence of sickle cells to vascular endothelium that initiates and contributes to the microvascular occlusion and pain episodes. It is also a hereditary disorder that affects the hemoglobin when the sickle gene is inherited as a homozygous state from both parents. The mutation in the gene is a single base substitution of thymine instead of adenine in the 6^{th} codon of exon 1 of the β-globin gene which is responsible for the synthesis of β-globin. As a result of the single base mutation, the hydrophilic glutamic acid in a normal β-globin peptide is replaced by hydrophobic valine at the 6^{th} position of the amino acid chain. The hemoglobin S molecule is a protein with its quaternary structure being a tetramer consisting of 2 normal α-globin chains and 2 mutated β-globin chains and it is involved in the pathology that leads to the sickle shape of the red blood cells. The pathophysiological basis of SCD known as the sickling process is markedly accelerated by an increase in the intracellular concentration of hemoglobin S (HbS) and rapid deoxygenation. The hemoglobin S can carry oxygen like every other normal hemoglobin molecule but form semi-solid aggregate structures when deoxygenated which distort and decrease the flexibility of the RBC. These repeated cycles of deoxygenation could cause permanent red blood cell damage and these sickle RBCs are short lived and interact with endothelial cells, platelets and some other plasma components. After deoxygenation, the aggregated structures are polymers of

the HbS molecule which is formed by hydrophobic interactions between β-6 valine of one tetramer and β-85 phenylalanine together with β-88 leucine of an adjacent tetramer. These polymers align and lengthen into a complex helical fiber consisting of a bundle of 14 strands paired together that twist in a regular manner with a core of 4 strands surrounded by 10 outer strands in a roughly hexagonal packing which stiffens and induce the characteristic sickle shape of the RBCs. The polymerization occurs as the cells traverse the microvasculature which occurs within a specific time called 'delay time' and increases with time spent (transit time) and concentration of intracellular hemoglobin S, but reduces with high concentration of fetal or normal hemoglobin. Vaso-occlusion crisis (VOC) could occur when transit time of the red cells flowing through the capillaries exceeds the delay time for deoxygenation leading to polymerized hemoglobin. The erythrocytes that contain HbS are sticky, less soluble and adhere to the post-capillary venule, narrowing its lumen which decreases the velocity of blood flow and increases its transit time propagating VOC. It becomes wedged and occludes small vessels, causing ischemia and necrosis which initiate inflammatory response leading to painful crisis. This adhesion involves the interaction between the receptors on sickle erythrocytes (α4β1 intergrin complex or late antigen-4 (VLA-4) and plasma proteins, which binds to endothelial cell adhesion molecule VCAM-1 and fibronectin. CD36 also interacts with another CD36 molecule through a molecular bridge made from a molecule of plasmatic thrombospondin (TSP) and von Willebrand factor (vWF) on the endothelium. The main factors of this abnormal adhesion are a population of young red blood cells called 'stress reticulocytes' which comes prematurely from the bone marrow due to anemic stress as the proteins expressed on their surface adhesion do not maintain them in the bone marrow. The vaso-occlusive crisis therefore seems to be composed of two consecutive steps; the first one involves the adhesion of stress reticulocytes to the endothelium, retarding blow flow which induces

the sickling of the mature red cells in a hypoxic environment. This steps eventually leads to the second where the irreversible sickle cells form and the complete occlusion of the micro-vessels. An increase in the potassium-chloride co-transport and Ca^{+} activated K^{+} efflux (Gardos channel) in the sickle red blood cells increases dehydration and intra-erythrocyte hemoglobin S due to a loss in potassium ion leading to the polymerization of these cells. As the hemoglobin is denatured the hemichromes and cytoskeleton protein concentrate at the internal side of the membrane thereby losing the heme and the liberation of Fe^{3+}, therefore promoting an iron-mediated generation of oxidants or an oxidizing environment. These free hemoglobin react with nitric oxide (NO) and reduces is bioavailability in the plasma and this leads to hemolysis and other SCD complications. The equilibrium between the liquid and solid phases of hemoglobin S is determined by oxygen and hemoglobin S concentrations, temperature and other hemoglobin types (Ogedegbe, 2002; Rosse, 2000; Rodgers *et al.*, 1987; Ballas, 2002; Odièvre *et al.*, 2011; Darghouth *et al.*, 2010; Manwani and Frenette, 2013; and Ilesanmi, 2010).

2.1 PHENOTYPIC MANIFESTATIONS OF SICKLE CELL DISEASE.

SCD is a systemic pleiotropic disease that affects almost all the organs of the body during the life time of the patient. Most of the clinical complications of SCD are protean in nature and vary in frequency and severity among patients. There are a good number of different clinical manifestations based on the different events that results from the pathophysiology of mutated sickle hemoglobin which is majorly the vaso-occlusion caused by intra-erythrocytic polymerization of deoxygenated sickle haemoglobin, poor solubility, non-delivery of oxygen to most organs by the red cells and hemolysis. The adhesion of the sickle blood cells to the vascular endothelium causes obstruction of the lumen of small blood vessels which leads to acute and chronic tissue ischemia and infarction, with multisystem or organ effect, especially in the bone,

lungs, brain, kidneys and spleen. It also results to the painful crisis episodes and other long term complications of the disease. Sickled red blood cells are destroyed readily by the reticulo-endothelial system due to their rigidity makes them to be easily filtered by the spleen and also the changes that occur in the structure of their lipid bilayer which promotes phagocytosis leading to chronic anemia (a steady state Hb of 6-8 g/dl). In infants these manifestations start to show after the first 6 months of their life and their fetal hemoglobin decreases as it inhibits the polymerization of the sickle red cells. The frequent symptoms in children are fever, respiratory infections caused by some common bacteria such as *Streptococcus pneumoniae* (pneumococcus), *Salmonella species* and *Haemophilus influenza*, jaundice, and pain in the joints, abdomen, chest and limbs. This pain is common to all patients both children and adults and it can be severe, acute or chronic in both. Other complications or symptoms include; fatigue, dyspnea, leg ulcer, priapism, acute complications (acute chest syndrome, acute multi-organ damage syndrome, watershed stroke), chronic complications (painful crisis, aseptic necrosis of the bone, ocular complications, hypertransfusion syndrome), pulmonary hypertension, retinopathy, central nervous system disorder, renal effects such as microalbuminuria and albuminuria, hyposthenuria, acute exacerbations of anemia (hyperhemolysis, acute splenic sequestration, aplastic crises), cardiac complications (cardiomyopathy, cardiomegaly or heart enlargement, congestive heart failure), neurological complications such as headache, neuropathic pain, transient ischemic attack, hemorrhagic stroke, silent cerebral infarction, moyamoya, brain atrophy, SCD psychosis and so on. Mostly the sickle cell crisis is grouped into vaso-occlusive pain crisis (VPC), recurrent crisis, aplastic crisis and splenic sequestration which are both forms of anemia. The hemoglobin is really an important protein which supplies oxygen and nutrients to all the body cells for its proper growth and development, so all the complications of SCD is due to the improper function of the abnormal

hemoglobin (Al-Jafar, *et al.,* 2016; Ballas, *et al.,* 2010, Booth, *et al.,* Kaur, *et al.,* 2013 and Rosse, *et al.,* 2000).

2.2 VARIANTS OF SICKLE CELL DISEASE AND ITS GEOGRAPHICAL DISTRIBUTION.

Sickle cell disease, a type of hemoglobinopathy, is the name given to a group of inherited disorders that is characterized by a hemoglobin variant called sickle hemoglobin (Hb S). They are variants of hemoglobin that can be inherited in an autosomal recessive manner together with the sickle hemoglobin to give the different variants of sickle cell disease. All these variants are located on the β-globin gene found on chromosome 11. It is a member of the globin gene family, involved in oxygen transport throughout the body. Other members included α, ζ, ε, δ and γ globin genes. The genes are regulated such that some are expressed at a particular period and are turned off later in life during human development. There are over 475 β-globin genes variants that exist and cause life threatening illness. The normal adult hemoglobin is a tetramer of 2 β-globin chains and 2 α-globin chains therefore any abnormal hemoglobin is due to various mutations that could occur in the β-globin gene. All the hemoglobin variants are characterized by a single base substitution mutation on the β-globin gene at different positions. Individuals whom are heterozygous for normal hemoglobin 'A' and any other hemoglobin variants, are known as carriers (Hb AS) and are protected against malaria with high frequencies in Sub-Sahara Africa, Eastern Saudi Arabia, Central India and the Mediterranean. It is also referred to as 'sickle cell trait'. In a case where individuals are homozygous for Hb S variant, they are known to have sickle cell anemia (Glu6Val). Other cases of SCD are the heterozygous form of hemoglobin S and other hemoglobin variants such as Hb C, Hb β-thalassemia (β^{+}/β^{0}), Hb E, Hb D, Hb O_{Arab} and so on. Hb S and Hb C are commonly found in Nigeria while Hb E, Hb D and Hb G are rarely seen in West Africa. In Hb C, lysine replaces glutamic acid at the 6^{th} position, with high frequencies in the Ghana, Burkina Faso

and Western Nigeria. The β-thalassemia variants are derived from many different molecular mutations and their clinical significance differ as β^+ produce little normal adult hemoglobin, commonly seen in Jamaicans with the mutations found in the promoter region (-88 C> T, -29 A>G) while β^0 doesn't produce any normal hemoglobin owing it to the mutation in the intervening sequence (IVSII-849 A>G). In Hb D Punjab or Los Angeles, glutamine replaces glutamic acid at the 121st position. It is seen mostly in Punjab regions of Northwestern India, Italy, Belgium, Austria and Turkey. Hb E (Glu26Lys) is commonly found in Sri Lanka, Eastern India, Southeast Asia and Southwest China. The heterozygous form of Hb O_{Arab} (Glu121Lys) have been reported in Saudi Arabia, North Africa, Yugoslavia, Bulgaria, Jamaica, Mediterranean and the United States. They are also high frequencies of these variants in the Caribbean, South and Central American, Southern Europe, Arab, and East Indian. More than 50,000 Americans are affected with sickle cell disease about 12,000 people in the UK and millions worldwide, making it one of the most prevalent genetic diseases in the world. The sickle cell mutation was as a result of the protection against malaria seen in sickle cell trait and it is widespread throughout Africa where malaria is prevalent, with higher frequency in the equatorial Africa. The mutation has shown to arise on 3 independent occasions in the African continent and are referred to as β-globin haplotypes, named after the areas where they were first described; Benin, Senegal and Central African Republic or Bantu. The 4th independent occurrence was seen in India and is known as Asian haplotypes and is associated with high level of fetal hemoglobin (Masiello *et al.,* 2007; Pandey *et al.,* 2012; Emechebe *et al.,* 2017; Serjeant 2013; Ashley-Koch *et al.,* 2000 and Zimmerman *et al.,* 1999).

2.3 SICKLE CELL DISEASE AND ANEMIA.

Anemia is a medical condition where the red blood cells or hemoglobin level in the body is lower than the normal level. Sickle cell disorders are associated with variable degrees of anemia depending on genotype, with the most severe decrease in hemoglobin level seen in sickle cell anemia and the least severe in hemoglobin S-β+ thalassemia. Normal red blood cells live for 120 days while the sickle cells live for 10-30 days as a result of continuous breakage of the cells. When the body is short of red blood cells, the tissues do not receive adequate amount of oxygen and this leads to fatigue or weakness. Severe anemia episodes may result from a variety of causes, including hyperhemolysis, acute splenic sequestration, and aplastic crises (Ballas*et al.,* 2010). Although chronic hemolytic anemia is a major feature of sickle cell disorders, a marked drop in hemoglobin with an increased hemolytic rate is referred to as hyperhemolysis. Hemolytic anemia varies intensively among the genotypes of sickle cell disease and it may be the driving force behind some complications of sickle cell disease because of its effects on Nitric oxide (NO) bioavailability which its decrease is associated with pulmonary hypertension, priapism, leg ulceration, and possibly with non-hemorrhagic stroke (Kato *et al.,* 2007).

2.4 SICKLE CELL DISEASE AND OXIDATIVE STRESS

Oxidative stress is an imbalance redox status caused due to the over production of oxidants and depletion of antioxidants. Oxidative stress has a significant impact on the complications of sickle cell anemia due to having higher potential for oxidative damage due to chronic redox imbalance in red cells which leads to the sickling phenomenon of the disease. The mutated hemoglobin in SCA alters the delicate balance of the generation of free radicals and the antioxidant defense systems in the red blood cells which are important in the regulation of free radicals in biological

systems. SCA has an association with increased serum activity of several oxidases such as nicotinamide adenine dinucleotide phosphate oxidase, and endothelial xanthine oxidase which are major sources of super oxides. This leads to auto-oxidation of hemoglobin, heme iron release, increased levels of asymmetric dimethyl arginine, uncoupling of nitric oxide (NO) synthase activity, and decreased NO levels. Studies have shown that sickle erythrocytes generate twice as much more superoxide, peroxide and hydroxyl radicals than normal cells. As a result they become more vulnerable during sickling, and the denatured hemoglobin releases its iron, which may produce free radical through Fenton's reaction. These free radicals initiates lipid peroxidation on the erythrocyte membrane leading to an increase in sickling, converting reversible sickle cells to irreversible sickle cells, thereby vaso-occlusion and hemolytic anemia. The antioxidant defense systems in SCA is ineffective in neutralizing the excess pro-oxidant species produced and consequently a state of chronic oxidative stress is established leading to endothelial dysfunction, peroxidation of membrane phospholipids as well as multiple organ damage. Normal erythrocytes counter oxidative stress using self-sustaining activities of such as antioxidant defense enzymes; glutathione peroxidase and superoxide dismutase (SOD) which is a key enzyme in the dismutation of superoxide radicals resulting from cellular oxidative metabolism into hydrogen peroxide. They are an important antioxidant defense system especially in aerobic cellular metabolism and are seen in higher concentrations in SCA's serum. SODs such as Copper (Cu) SOD, Zinc (Zn) SOD, Manganese (Mn) SOD and extracellular SOD are protective enzymes against oxidative stress. They scavenge for reactive oxygen- O_2 (ROS) by catalyzing its dismutation to hydrogen peroxide and molecular oxygen. Mn-SOD is a mitochondrial form of SOD and is involved in controlling O_2 toxicity in the mitochondria which is of extreme oxidative load. Other antioxidants include vitamin C, a potent water-soluble micro-molecule whose antioxidant function is its ability to act as an

electron donor, ameliorating the adverse effects of reactive oxygen and nitrogen radicals (Hundekar, *et al.,* 2011; Sogut, *et al.,* 2011 and Okocha, *et al.,* 2017).

2.5 SICKLE CELL DISEASE AND G6PD DEFICIENCY.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an incomplete hereditary X-linked hemolytic disease which overlaps with malaria and predisposes carriers to hemolysis (anemia). It is widely spread throughout the tropics and subtropics where malaria is endemic. In these areas, malaria is treated with drugs that can cause severe hemolysis in G6PD deficient individuals. Although it is mostly seen in men, the diagnosis of the homozygous deficient is complicated and hardly seen in women. G6PD catalyzes a reaction in the pentose phosphate pathway that generates reduced NADPH, which is responsible for glutathione (GSH) homeostasis, an antioxidant, making cells to resist and control oxidative stress. The GSH keeps sulfhydryl groups on hemoglobin and other proteins in a reduced state by preventing the formation of disulfide bonds which is really important to prevent hemoglobin precipitation and hemolysis. The inability for erythrocytes to maintain GSH homeostasis leads to oxidative stress and affects the red blood cells due to rigidity from crosslinking the spectrin that decreases membrane flexibility, in turn giving rise to anemia. In patients with SCD, together with G6PD deficiency are normally seen as having no effect but its severity increases occasionally. One of the manifestations of SCD due to hemoglobin S polymerization is hemolytic anemia and G6PD deficiency is also another cause of hemolytic anemia. Both G6PD deficiency and hemoglobin S confer protection against malaria and are both prevalent in geographical regions were malaria is endemic especially in the Sub-Saharan Africa, Arabian Peninsula and central India. Most studies investigating the effect of G6PD deficiency on the severity of SCD have produced conflicting results as some found no effect of G6PD deficiency on the phenotypic manifestations, Hb levels, or reticulocyte count of SCD patients while some

others found lower Hb levels in patients suffering from both disorders. High rates of frequent anemic episodes and low steady-state hemoglobin levels were also observed in children with both disorders. Interaction of SCD with thalassemia and G6PD deficiency have been reported in most studies to occur in patients as these 3 disorders share the same clinical importance of hemolytic anemia and that their carriers are also protected against malaria infection. Studies in Burkina Faso, Senegal, Turkey, Kenya and Ghana confirm to the high association of SCD and G6PD deficiency in patients than the general population but studies in the Saudi population showed no relationship (Benkerrou, *et al.*, 2013; Mbanefo, *et al.*,2017; Peters and Van, 2009; Firempong, *et al*., 2016 and Al-Nood, 2011).

2.6 TREATMENT OF SICKLE CELL DISEASE.

Most people with sickle cell disease hardly survived past the childhood age, but recently due to preventive drugs and treatment, better medical care and continuous research patients have been able live longer. SCD patients are treated by hematologist (doctors that specialized for different hemoglobinopathies) or pediatricians mostly for children. The treatment and management of the disease in majority of the patients is palliative in nature which is done mostly to prevent painful crisis episodes and numerous clinical complications during their life time because they are not responsive to curative therapy. It includes love and care from family and friends, family counselling, medical follow up and management of the clinical complications. Other major treatment approaches include symptomatic, preventative, abortive, supportive and curative approaches. Supportive approach involves empowering the patients to be the authorities over the manifestations of the disease that apply to them individually through educative and family counseling, providing them with proper nourishment, constant rehydration and basic supplements. Symptomatic approach involves blood transfusion and antibiotics which help reduce the effects of

some clinical manifestations and symptoms. The main goal of preventative therapy is to reduce the re-occurrence of the disease complications, mostly the painful episodes, using an anti-sickling agent to prevent the polymerization of hemoglobin S. Some of the promising approaches include, vaccination which can be used against pneumonia, hydroxyurea (HU) is taken to induce fetal hemoglobin while blood transfusion averts primary and secondary stoke episodes. Nitric oxide (NO) is the only accepted agent for the abortive approach, reported to completely terminate of chronic pain episodes in some SCD patients. Lastly, the curative approach is the ultimate treatment for all genetic disorders, due to the fact that it tends to correct the mutation causing the disorder and to prevent all the complications accompanying it. Currently, transplantation of hematopoietic stem cells (HSCs) or bone marrow transplant is the only accepted curative treatment for SCD. Antibiotics such as prophylactic oral penicillin and aspirin are used to prevent infections (Ballas 2002; Pule and Wonkam 2014).

A significant collective knowledge on the treatment of SCD is lacking due to the complex pathology of the condition. Researchers are still going on to find better clinical pharmaceutical products to ameliorate neurological complications, crisis episodes, organ damage, infections and some many more complications of SCD. Clinical trials are ongoing to moderate RBC dehydration by blocking the Gardos channel which is inhibited by clotrimazole and magnesium retards K^+ efflux, preventing erythrocyte dehydration and thereby inhibiting sickling. Sodium cromoglycate has been noted to also have some anti-sickling effect. Azacytidine and decitabine are hypomethylating agents which increase hemoglobin-oxygen affinity. It is safe and well tolerated in adults with varying doses. GBT 440 is an oral agent, which acts as a direct hemoglobin modifier to prevent sickling of the red blood cells. It is increases hemoglobin-oxygen affinity, inhibits polymerization of sickled hemoglobin, restores normal RBC function and is normally taken ones

daily. GMI-1070, pentosanpoly sulfate sodium, tinzaparin, sevuparin, sulfated non-anticoagulant heparins, arginine, green tea, aged garlic, and herbal extracts, gene therapy, are all been used for the improved treatment of SCD. Some of these are used on investigational basis and reports of success in few patients are been documented (Ballas 2002; Kaur *et al.,* 2013 and Al-Jafar*et al.,* 2016).

Chapter Three

Methodology

3.0 REAGENT AND APPARATUS/EQUIPMENT FOR LABORATORY ANALYSIS

Consumables

- micropipette tips (50µl, 200µl and 1000µl)
- Cotton wool
- Hand gloves
- Test strips for glucometer
- Methylated spirit
- Eppendoff tubes (1.5ml)
- Test tubes
- EDTA tubes

Reagents

- 0.18M Sodium nitrate solution
- 0.28M Glucose solution
- 0.08M Phosphate buffer pH7.0
- 8mM Pyrogallol
- 0.0004M Methylene blue

Equipments

- Electrical Weighing Balance (Model no:Yp.502N)
- Spectrophotometer (spectrum lab 23A,England)

- Adjusted Micropipette (Perfect,U.S.A)
- Refrigerator (Kelvinator,Germany)
- Beakers
- Reagent bottles

3.1 STUDY POPULATION AND DESIGN.

This study will be a prospective cross-sectional carried out at the University of Nigeria Teaching Hospital (UNTH) Enugu involving Sickle cell disease patients visiting the hospital. A total of 100 patients or less with the disease will be recruited for the study. The study will be conducted in accordance to the Helsinki declaration. Written informed consent will be collected from patients willing to participate in the study. The privacy of patience will be kept confidential and patients are free to withdraw from the study at any point in time. Outpatients will be screened for sickle cell disease and only patients with the disease will be recruited for the study. Patients with severe malaria or critical health complication who are admitted at the hospital will be excluded from the study. Also, pregnant women, or breast feeding women will be excluded from the study.

3.2 DATA COLLECTION

Questionnaire will be used for the collection of baseline information of patients including age, sex, genotype, blood group, the duration of the disease, ethnic group, location, treatment administered and progress of treatment.

3.2 BLOOD COLLECTION.

A total volume of 5ml of blood will be collected from each patient of which 2.5ml will be transferred into anticoagulant tube (EDTA) while the remain 2.5ml will be transferred into anticoagulant free tubes (EDTA free tubes) for various analysis.

3.3 LABORATORY ANALYSIS.

Anemia and haemological parameters

Anemia indices including hemoglobin (Hb) concentration, haematocrit, mean cell volume (MCV), mean cell hemoglobin (MCH), pack cell volume (PCV) and blood cell counts (WBC & RBC counts, and WBC differential count) will be done using autoanalyser.

Superoxide dismutase (SOD) activity

SOD activity will be quantified according to Marklund and Marklund (1974) method with slight modifications. Homogenized blood sample (50 µl) will be transferred into a tube containing 450 µl of 4 mM pyrogallol and 500 µl of 20 mM phosphate buffer (pH 6.6). The absorbance will be taken at 450 nm for 3 minutes against a blank preparation containing distilled water instead of sample in pyrogallol and phosphate buffer. SOD activity will be expressed as U/mg. One unit of SOD activity is defined as that amount of SOD required to cause 50% inhibition of pyrogallol autooxidation per 3ml of assay mixture.

Determination of G6PD deficiency

G6PD deficiency will be assayed by the methaemoglobin reduction test. Blood sample (500 µl) collected in EDTA tubes and 50 µl of 0.28M of glucose will be added to a set of three tubes labeled as Test, Deficient-Control and Normal-Control. Sodium nitrite (0.18 M, 50 µl) and methylene blue (0.4 mM, 50µl) solutions will be transferred into the tubes labelled "Test". A volume of sodium nitrite (50 µl) and normal saline 50 µl) solutions will be added to the tubes labeled "Deficient-Control" while 100 µl of normal saline solution only will be transferred to the tubes labeled "Normal-Control". All of the tubes are well mixed, corked with cotton wool and then incubated at 37^{o}C for 3 hours. After incubation, 100 µL of the incubated mixture will be transferred to new set of tubes labelled as before then 5 ml of 0.02M (pH 6.6) phosphate buffer will be added and colour

visualized. A dark brown or grey is indicative of G6PD Deficient while a red colour like the Normal-Control is considered as Non-Deficient.

3.5 STATISTICAL ANALYSIS.

Data will be analyzed with Statistical Package for Social Science (SPSS) version 16. Genotype and allele frequencies will be compared using the $\chi 2$ statistics or the fisher's exact test. Continuous variables will be compared using parametric tests (independent sample t-test and ANOVA with post hoc multiple comparison by Dunn-Sidak test). Odd ratios will be calculated by logistic regression adjusting for age. A p-value less than 0.05 will be considered statistically significant.

Chapter Four

Results

The results of the study reflect data collected through the use of questionnaire and the biochemical tests done using the blood samples. Table 1 reflects the gender population used. A total of 70 patients, 22 males and 48 females. For the control population, AA had a total of 6 males and 28 females, AS had 2 males and 6 females, SS had 14 males and 14 females. Figure 1 is a bar chart of the frequency of crisis in sickle cell anemia participants. The highest frequency were patients who had crisis mostly 1-3 times in a year and the lowest were those that had crisis 6 and above in a year. The second highest were those that don't have frequent crisis for a year and more. Figure 2 shows the level of crisis among the sickle cell participants, were a higher number of participants had mild crisis. Figure 3 shows the level of hospitalization among sickle cell patients. A higher number of patients are not hospitalized within the space of a year. The second highest are those hospitalized once in a year. This might be as a result of the mild crisis encountered in the patients. Figure 4 shows the frequency of blood transfusion among the sickle cell patients and this shows that a higher population of them are not transfused in the space of one year and more. Table 2 shows the demographic and clinical characteristics among patients, their age hemoglobin and hematocrit level and SOD status. Table 3 further explains table 2 as it differentiate between those that are anemic and those that have normal hemoglobin. Table 4 shows the relationship between G6PD deficiency and the genotype status of patients. A higher percentage of all patient were deficient. Table 5 shows the interaction between genotype status and G6PD deficiency in SOD and the anemia indices in all patients. Figures 5-7 further explains this relationship. Figure 5 is a graph that displays the relationship between genotype, SOD and G6PD deficiency. The G6PD

deficient patients had higher SOD levels than the non-deficient patients. In figure 6, it shows that the hemoglobin level of G6PD deficient for all genotypes is higher but decreases in SS patients but in non-deficient the hemoglobin level decreases from AA-AS then SS. The same relationship is also shown in figure 7 with the hematocrit level

Table 1: Gender distribution of study population

Characteristics		**AA**	**AS**	**SS**	*Total*	*χ2*	*p-value*
	Male	6 (8.6%)	2 (2.9%)	14 (20.0%)	22 (31.4%)		
Sex	*female*	28 (40%)	6(8.6%)	14(20.0%)	48 (68.6 %)	*7.631*	0.022
	Total	34 (48.6%)	8 (11.4%)	28 (40%)	70 (100%)		

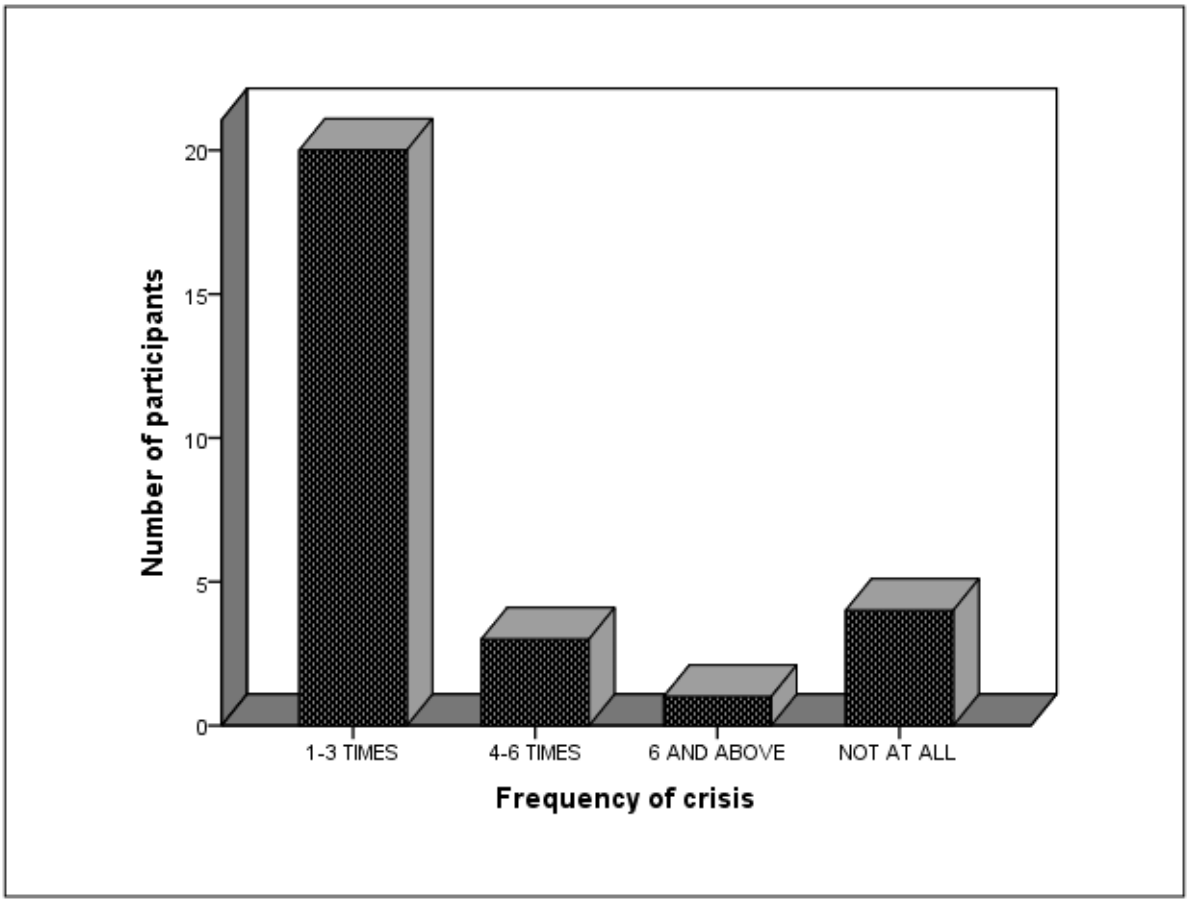

Figure 1: Frequency of crisis in sickle cell anaemia participants

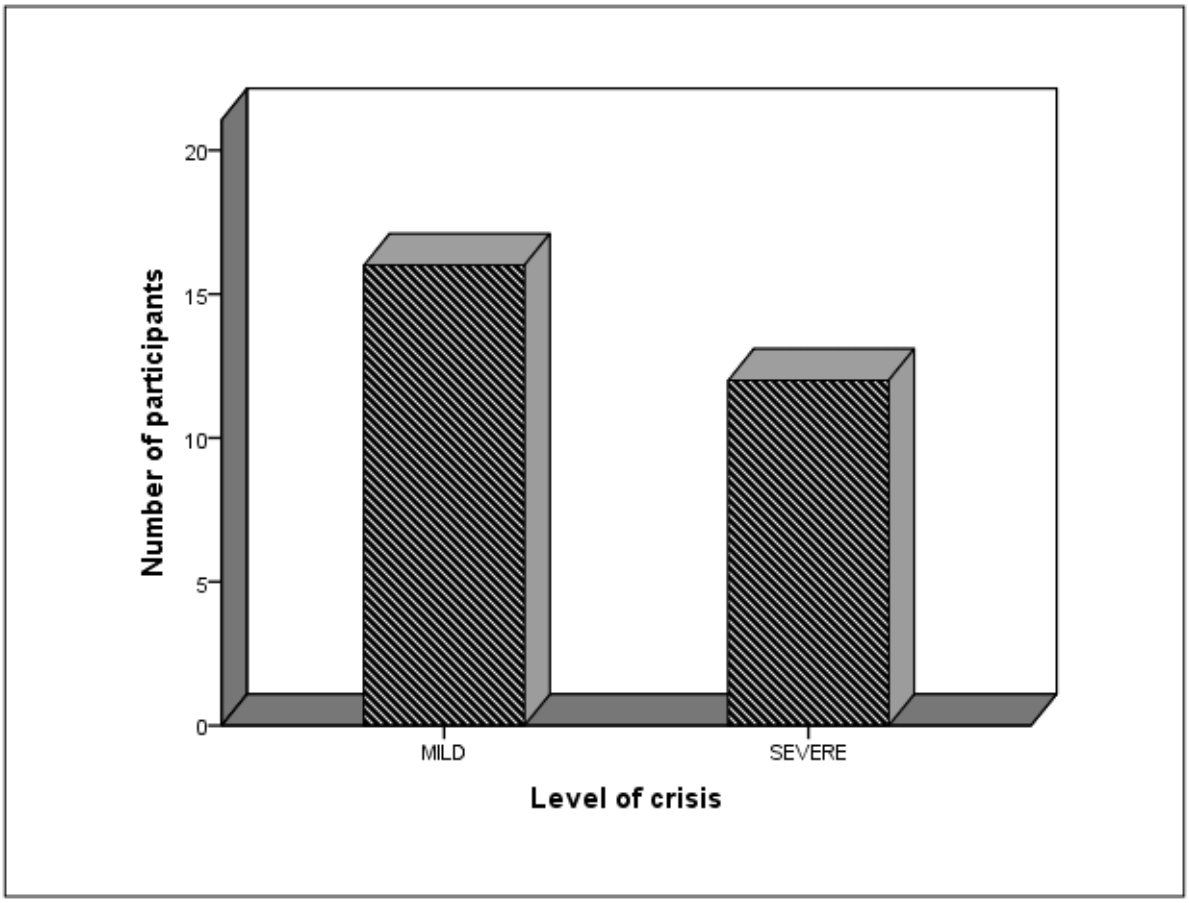

Figure 2: Level of crisis among sickle cell anaemia participants

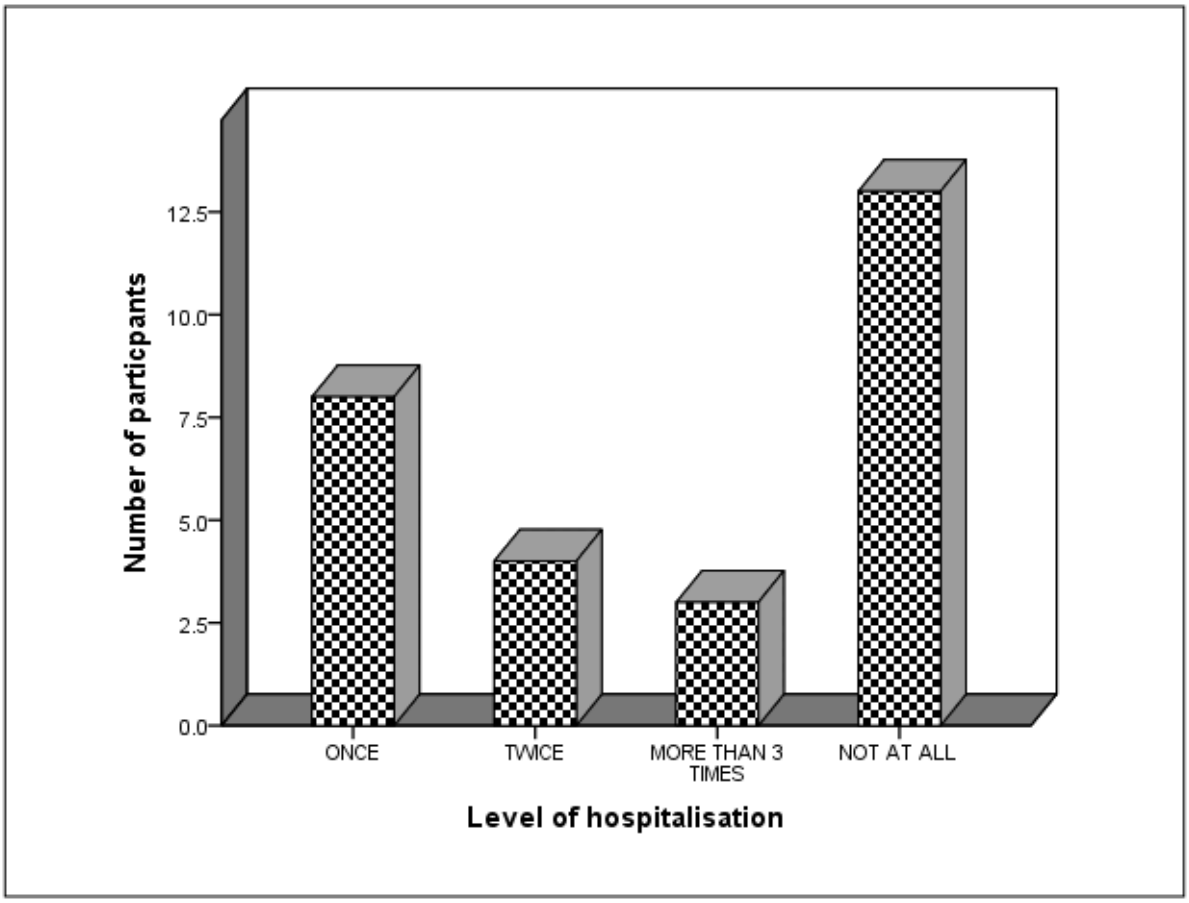

Figure 3: Level of hospitalization among sickle cell anemia participants

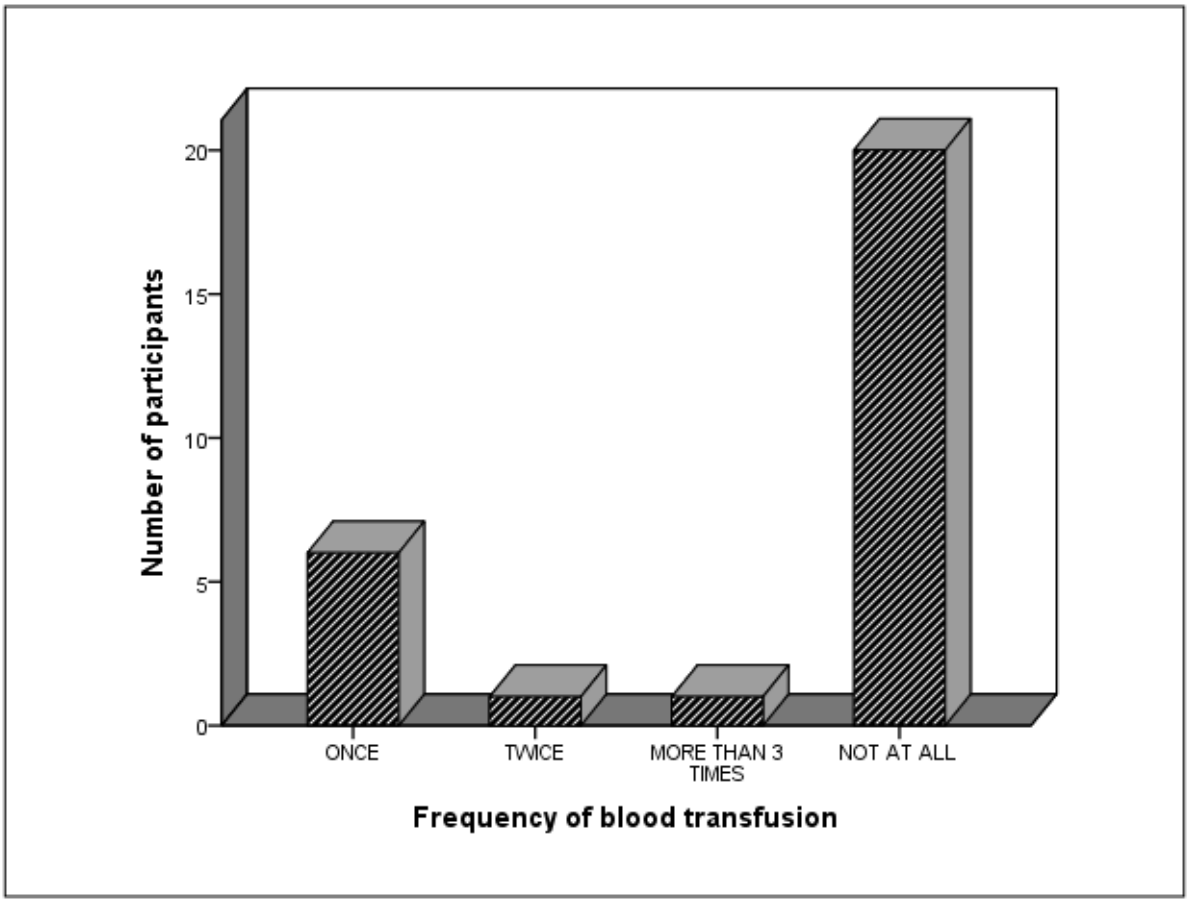

Figure 4: Frequency of blood transfusion among sickle cell anemia participants

Table 2: Demographic, clinical characteristics among patients

Characteristics (n =70)	**AA** **(n =34)**	**AS** **(n=8)**	**SS** **(n =28)**	*p-value*
Age (years)	31.12±2.58	36.88±3.92	20.59±2.32	0.02
Hemoglobin (mg/dL)	11.69±0.42	11.58±0.87	6.76±0.49	<0.001
Hematocrit (mg/dL)	34.38±1.23	34.00±2.54	19.96±1.43	<0.001
SOD (10^{-1} U)	3.22±0.50	2.43±0.72	2.45±0.47	0.47

Table 3: Relationship between anemia and genotype status of patients

Characteristics		**AA**	**AS**	**SS**	*Total*	*χ2*	*p-value*
	Present	19 (27.1%)	4 (5.7%)	27 (38.6%)	50 (71.4%)		
Anemia	*Absent*	15 (21.4%)	4 (5.7%)	1 (1.4%)	20 (28.6%)	*14.401*	0.01
	Total	34 (48.6%)	8 (11.4%)	28 (40.0%)	70 (100.0%)		

Table 4: Relationship between G6PD deficiency and genotype status of patients

Characteristics		**AA**	**AS**	**SS**	*Total*	*χ2*	*p-value*
G6PD	*Deficient*	10 (14.3%)	3 (4.3%)	5 (7.1%)	18 (25.7%)	*1.730*	0.421
	Non-deficient	24 (34.3%)	5 (7.1%)	23 (32.9%)	18.0 (74.3%)		
	Total	34 (48.6%)	8 (11.4%)	28 (40.0%)	70 (100.0%)		

Table 5: Interaction between genotype status and G6PD deficiency on SOD and Anemia indices in patients

Characteristics		**AA**		**AS**		**SS**	*p-value*
	G6PD Deficient	**G6PD Non-deficient**	**G6PD deficient**	**G6PD Non-deficient**	**G6PD Deficient**	**G6PD Non-deficient**	Genotype x G6PD
SOD (10^{-1}U)	3.66±0.82	3.02±0.565	3.99±1.49	1.48±1.16	3.18±1.29	2.41±0.552	0.675
Hemoglobin (mg/dL)	11.83±0.75	11.75±0.52	9.2±1.38	13.0±1.06	7.70±1.19	6.55±0.51	0.074
Hematocrit (%)	34.80±2.22	34.52±1.53	27.0±4.06	38.20±3.15	22.75±3.52	19.32±1.49	0.073

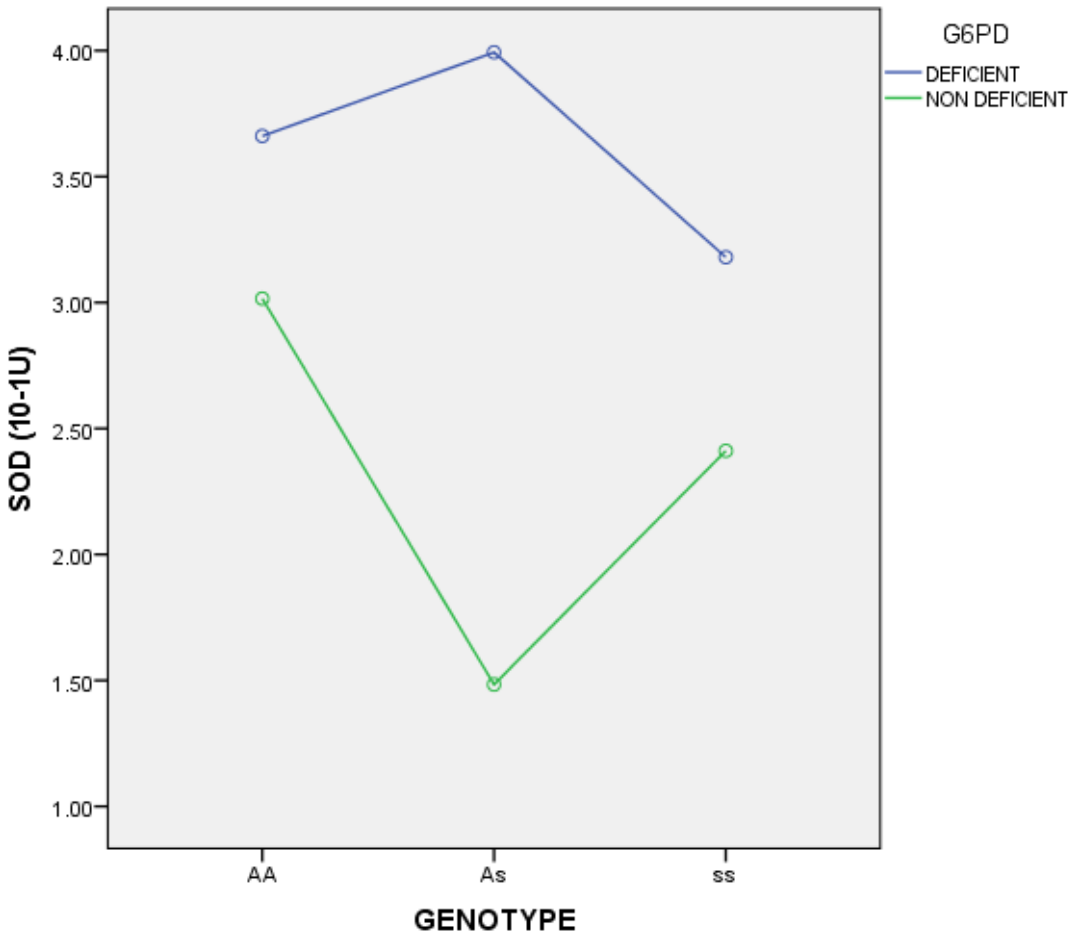

Figure 5: Interaction between genotype and G6PD status on SOD level

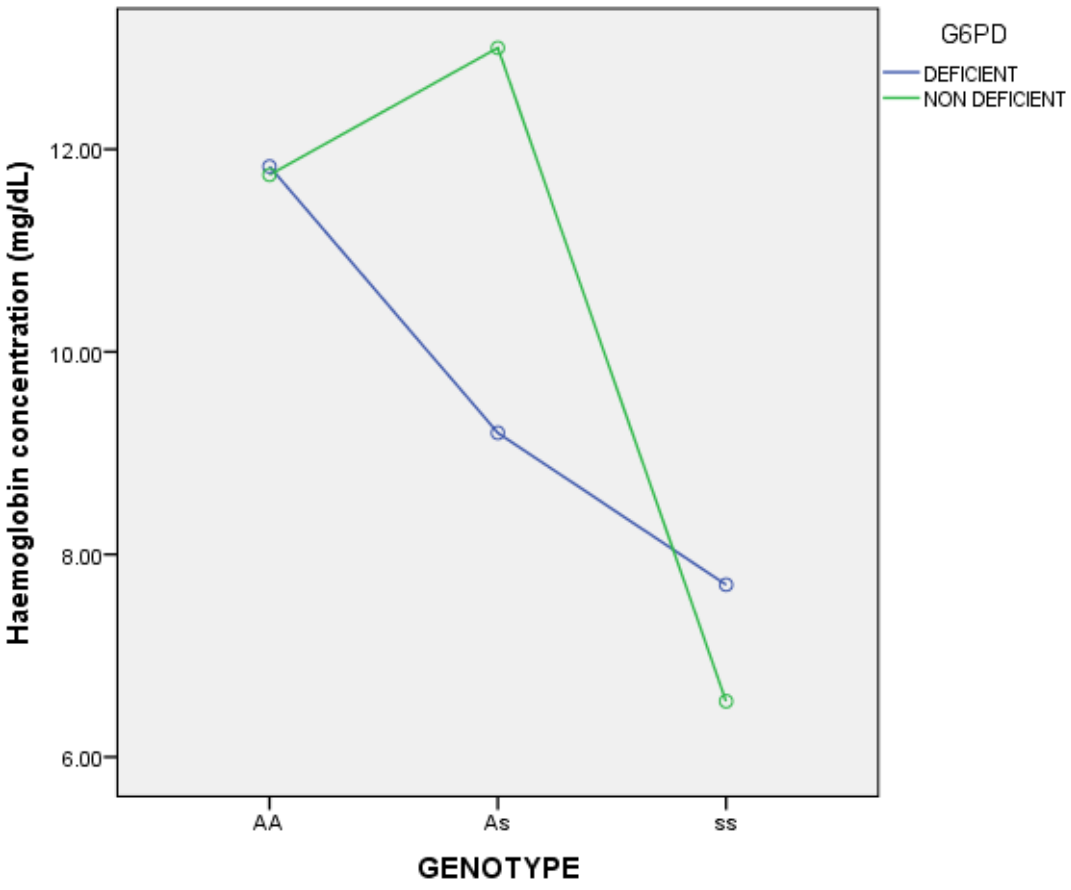

Figure 6: Interaction between genotype and G6PD status on hemoglobin level.

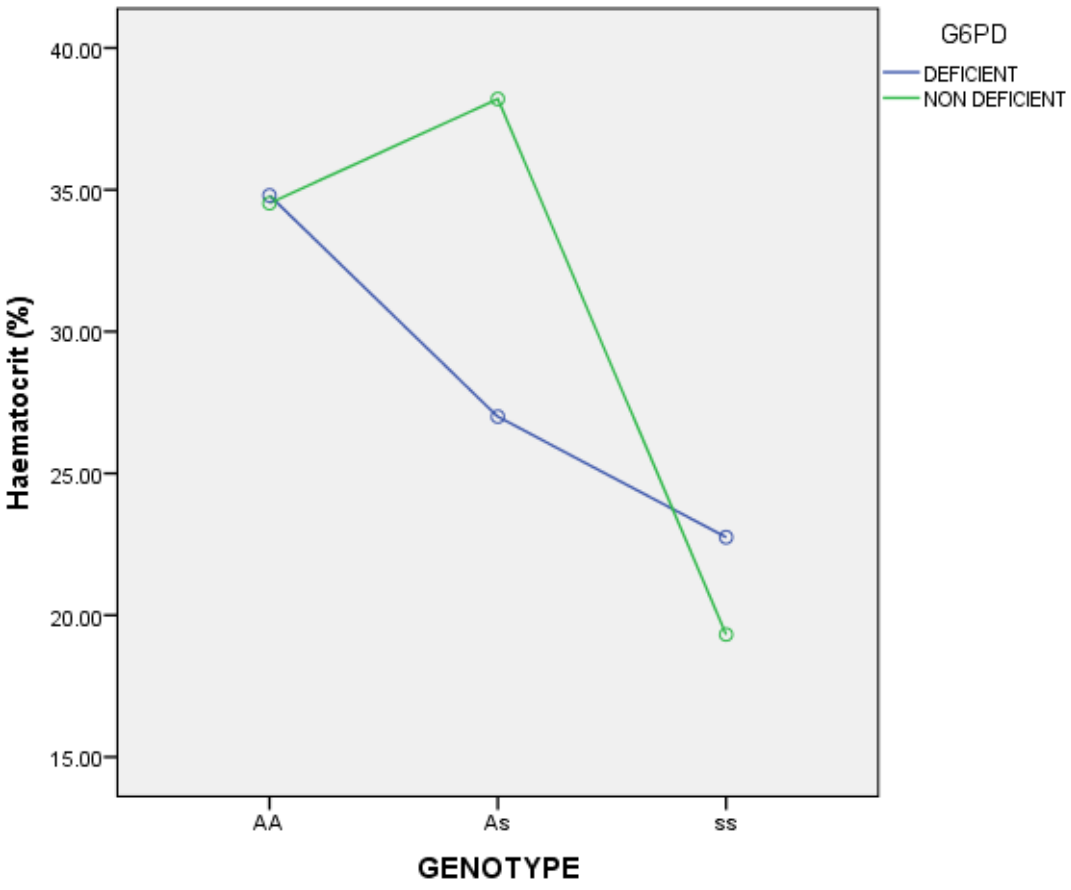

Figure 7: Interaction between genotype and G6PD status on hemotocrit level

Chapter Five

Discussion

In this study, the relationship between anemia, SOD and G6PD deficiency in sickle cell patients were analyzed. These factors in one way or the order increase, decrease or have no effect the pathophysiology of sickle cell disease. These factors could have an effect independently, could influence the effect of another by coexisting together or could not have any relationship at all. The total population size used for this study was 70 patients. All sickle cell patients which were a total of 28 were recruited from UNTH and the control genotype patients, 42 were recruited from ESUT. Questionnaires were distributed to these patients for information concerning their health. The questionnaire contained relatable parameters such as; age, sex, ethnic group, genotype, crisis rate, severity of the crisis, rate of hospitalization as well as blood transfusion. The relationships between these parameters were shown in Table 1, Figure 1, 2, 3 and 4. Table 1 shows the gender distribution of the population study consisting of both male and female of all genotypes with a p-value of 0.022. Figure 1 shows a bar chart representing the frequency of crisis in SCD patients. In a year according to the questionnaire, a higher percentage of patients do not have frequent crisis, that is, most of them just have crisis ranging from 1-3 times a year. The next high percentage do not have crisis in a year or more while the lowest percentage ranges from 4-6 times and above. Figure 2 displays with the use of a bar chat the level of the crisis rate in this sickle cell patients and it shows that the severity of the crisis in these patients is mild but a significant percentage is said to have severe crisis. In figure 3, it shows the level of hospitalization of these patients using a bar chat. Since most of them do not have frequent crisis and the severity of the crisis is mild, a good number of them are not hospitalized within the space of one year or more. Most of them are still

hospitalized, usually 1-3 times a year. Figure 4 displays using a bar chat level of transfusion which shows that a good percentage of them are not transfused due to no frequent or mild crisis. The relationship between these parameters shows that most SCD patients residing from the east part of Nigeria have little or mild complications. Although the population size is too small to draw conclusions, most literature have shown that the population of SCD in Nigeria is about 2-4% with little or no proper sickle cell medical associations, with most of their complications been triggered by infections, malaria or poor environmental conditions (Adewoyin 2015 and Diwe *et al.*, 2016). The parameters for estimating the relation between anemia, SOD and G6PD in SCD patients are analyzed in Table 2, 3, 4, 5, and Figure 5, 6 and 7. In table 2, the anemic status of the patients were shown using their hemoglobin and hematocrit levels against the control genotypes AA and AS. The ages of the patients were considered having a p-value of 0.02. The hemoglobin and hematocrit levels of the control genotypes were higher compared to that of the SCD patients having a p-value each of <0.001. This was compared against SOD values which showed to be higher in the control genotypes than in SCD patients having a p-value of 0.47 that is AA= 3.22±0.50, AS= 2.43±0.72 and SS= 2.45±0.47. Although they are not statically significantly different, other works have shown to have the same higher values in respective genotypes. These two parameters are compared because lecture have shown that oxidative stress leads to anemia causing higher amount of SOD enzymes in their serum levels. They also tell us that the level of these enzymes is higher in SCA than in normal genotypes (Hundekar, *et al.*, 2011; Sogut, *et al.*, 2011 and Okocha, *et al.*, 2017). Table 3 shows the genotypes and their anemia status. From the 70 participants included in the study 50 (71.4%) of them were anemic, that is, in a total of all the genotypes and 20 (28.6%) were not having a p-value of 0.01. Lecture shows that the normal state hemoglobin ranges from 5.0-10.0 with an average of 7.0mg/dL. From the study we have recorded 6.76±0.49 mg/dL which is

within the normal range and all the patients used were steady state patients (Juwah, *et al.*, 2004). This value is statically significant with higher values in normal genotypes (11.69±0.42). Table 4 shows the relationship of G6PD deficiency in the genotypes. In the SCD population 7.1% were deficient and 32.9% were non deficient. The population of G6PD deficiency in SCD was quiet low compared to the general population of 41.4% so there is a high statically difference. Based on previous lecture it is quiet unlikely that SCD patients suffer from G6PD deficiency because they are unfit to tolerate increased hemolysis and if the two problems should coexist, particular care should be taken on drug administration to prevent the initiation of hemolysis (Benkerrou, *et al.*, 2013; Mbanefo, *et al.*, 2017; Peters and Van, 2009; Firempong, *et al*., 2016 and Al-Nood, 2011). Table 5 displays the relationship between all the parameters anemia, SOD and G6PD deficiency in SCD patients and control genotypes. The values between hemoglobin, SOD and G6PD deficiency in SS patients were significantly lower than the control genotypes. Figure 5, 6, 7 are graphs that independently showing the relationships between them. The p-value for SOD and G6PD deficiency shows that there is a significant difference and no relationship between the two. The p-value for anemia and G6PD deficiency has no significant difference showing that there could be a relationship between the two since anemia is also a major complication in G6PD deficient patients.

CONCLUSION

There was no relationship between anemia, SOD and G6PD deficiency in sickle patients, that is, they could occur independently in individuals but not influencing the effect of each other in a patient. Both SOD and G6PD are enzymes that regulate the free radicals in the body system, so if an individual has either SOD or G6PD deficiency there would be an unregulated concentration of free radicals resulting to the abnormal breakdown of red blood cells thereby resulting to an anemic condition. There was no interaction between G6PD deficiency and sickle cell to influence SOD level and anemia indices. An individual could be both G6PD deficient and a sickle cell anemic patient but the two doesn't influence the effect of the other to result to crisis suffered at a particular time. They both have a different factors that influence the rate of their crisis respectively. These factors could trigger their crisis respectively and at the same time, leading to severe crisis, so proper attention has to be given to the individual but fortunately they are very rare. However, anemia was associated with sickle cell and G6PD patients.

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