ASSESSMENT OF THE RELATIONSHIP BETWEEN FOETAL HAEMOGLOBIN LEVELS, TISSUE DESTRUCTION AND DISEASE SEVERITY IN INDIVIDUALS WITH SICKLE CELL ANAEMIA

BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE AWARD OF MASTER DEGREE IN BIOCHEMISTRY

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FEBUARY, 2018

DECLARATION

I declare that the work in this dissertation entitled "Assessment of the Relationship between Foetal Haemoglobin Levels, Tissue Destruction and Disease Severity in individuals with Sickle Cell Anaemia" has been carried out by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references is provided. No part of this dissertation was previously presented for the award of another degree or diploma at this or any other Institution.

_ IshayaDogonzo Yusuf _____ _____

Name Signature Date

CERTIFICATION

This dissertation entitled "Assessment of the Relationship between Foetal Haemoglobin Levels, Tissue Destruction and Disease Severity in individuals with Sickle Cell Anaemia" by Yusuf Dogonzo ISHAYA (P13SCBC8032) meets the regulations governing the award of the degree of Master of Science in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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To all well-wishers I am grateful. May God reward you all.

DEDICATION

I dedicate this work to God almighty for his mercies, kindness and favour upon me, to my family, who were with me throughout this period, sharing my burdens all the way and to all well-wishers for their support in various ways.

ABSTRACT

Sickle-cell Anaemia is a genetic disorder that leads to the production of Sickle haemoglobin, whichresults in abnormal functions of the red blood cell under hypoxic conditions.The aim of this study was to assess the relationship between Foetal haemoglobin, markers of tissue destruction and disease severity among individuals with sickle cell anaemia. Thirty seven adults with sickle cell anaemia with average age of 26.24±7.32 years were enrolled in this study. Twenty four of them were females while thirteen were males. Participants enrolled were individuals attending clinic at Haematology Department, Ahmadu Bello University Teaching Hospital Zaria who have been diagnosed with Sickle cell anaemia and were confirmed by cellulose acetate electrophoresis. Foetal haemoglobin was assayed using betke's alkali denaturation method and complete blood count was done using automated haematology analyzer. Lactate dehydrogenase (LDH) activity and bilirubin concentration were determined by spectrophotometric method while disease severity was computed based on the scores obtained from the clinical and haematological data. From our findings, there was a significant difference ($P<0.05$) in foetal haemoglobin (HbF) levels and LDH activity between individuals in steady state and those in crises condition. Also there was a significant inverse correlation between HbF levels and LDH activity ($P= 0.01$, $r=-0.59$) and between LDH activity and disease severity ($P=0.01$, $r=0.42$) but there was no significant ($P>0.05$) relationship between HbF levels & disease severity ($P = 0.69$, $r = -0.07$) and HbF levels & bilirubin concentration ($P=0.78$, $r=-0.05$).Therefore the present study suggested that HbF level has an inverse relationship with disease severity and tissue destruction. However, disease severity has a direct relationship with tissue destruction.

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ABBREVIATIONS, GLOSSARY AND SYMBOLS

ABUTH: Ahmadu Bello University Teaching Hospital

ATP: Adenosine-tri-phosphate

DMD: Disease Modifying Drugs

EDTA: ethylenediaminetetraacetic acid

GAG: Glutamic acid

GTG: Valine

HbA: Normal Haemoglobin

HbAA: Homozygous Normal Haemoglobin

HCT: Haematocrit

HbF: Foetal Haemoglobin

HbS: Sickle Haemoglobin

HbSS: Homozygous Sickle Haemoglobin

HBSF (HPFH): Hereditary Persistence of FoetalHaemoglobin.

LCR: Locus Control region

LDH: Lactate dehydrogenase

RBC: Red Blood Cell

R-form: Relax form of haemoglobin

SCA: Sickle cell anaemia

SCD: Sickle cell disease

T-form: Tense form of Haemoglobin

WBC: White Blood Count

CHAPTER ONE

1.0. Introduction

Sickle cell anaemia (SCA) is a disorder of the Haemoglobin caused by inheritance of a single abnormal haemoglobin (termed sickle haemoglobin- HbS) which results in physical and chemical modifications of the haemoglobin molecule (Damanhouri*et al.,* 2015). Under conditions of low oxygen concentration, the sickle haemoglobin causes the red blood cell to become fragile and prone to rupture. This condition causes the aggregation of haemoglobin (polymerization) and reduces the overall capacity of the haemoglobin to convey oxygen to tissues by impeding free passage of blood in the vessels (vaso-occlusion) to tissues. Increased rupturing of red blood cells leads to anaemia (reduction in number of red blood cells) (Elias *et al.,* 2012; Damanhouri*et al.,* 2015).

Lactate dehydrogenase (LDH), an enzyme of the glycolytic pathway that catalyzes the inter conversion of pyruvate to lactate is found in abundance in all tissues. It consist of five (5) isoenzymes forms classified according to their electrophoretic mobility. The concentration of these isoenzymes varies from one organ to the other, with LDH1 and LDH2 found primarily in RBCs and heart muscle (Ballas, 2013). There is increased level of LDH in the general blood circulation of individuals with SCA. This is due to the increased breakdown of diseased RBCs and other tissues and has been used as a marker of hemolysis and tissue damage. The haemolysis/tissue damage increases further in individuals with Sickle cell anaemia in crises state (Kato *et al.,* 2006; Ballas 2013). Numerous studies have associated SCA individuals with high LDH level to high prevalence of pulmonary hypertension, renal failure and early death (Kato *et al.,* 2013). High LDH observed in this individuals is as a result of haemolysis and tissue destruction (Mehdi*et al.,* 2013; Alzahri*et al.,*

2015). Despite the importance of LDH as a gold standard marker for tissue damage information about LDH in population suffering from SCA in Nigeria is scarce.

SCA studies have shown that individuals with HbF>10% are regarded as having high HbF (Moreira *et al.,* 2015). SCA Individuals with high HbF, like those from eastern Saudi Arabia and India have been found to experience mild clinical complications of the disease (Miller *et al.,* 1986). Foetal hemoglobin is a type of haemoglobin composed of two subunits of α- globin peptide chains, two subunits of γ globin peptides and a heme moieties necessary for this molecule's oxygen-carrying capacity. It is the dominant haemoglobin synthesized in the foetus (Sankaran and Orkin, 2013). It is produced by a fraction of RBCs called F-cells that are able to produce HbF alongside other haemoglobins. The F-cells have the advantage of longer life cycle compared to non-F-cells, and the higher the HbF content the longer the life span of the F-cells. The long life span of F-cell is associated with the characteristic of HbF which is able to resist polymerization caused by sickle haemoglobin (HbS) (Colella&Traina, 2015). This has prompted studies into HbF composition from other regions of the world, with the aim of understanding the role of HbF in ameliorating SCA complications. Although the HbF levels are known in several populations around the world, only fewstudies have examined HbF level in patients in Nigeria. Knowledge of the HbF levels will be important in assessing the possible impact of introducing disease modifying drugs (DMD) on the individuals with SCA in Nigeria.

SCA is known to exhibit great clinical diversity. Though the same disease, the effect of disease complications vary significantly from one individual to another. The clinical variation at one extreme are a group that may need close care and at the other end, are a group that may not require any care. Classification of individuals into disease severity group can help detect which severity group they belong and respective care given accordingly based on their need (Mpalampa*et al.,* 2012).

Unlike in the developed countries where advancement in technology can be used to identify who will need close attention, in developing countries these equipment's are not available (Okocha*et al.*, 2015). Because of this and many other factors the rate of morbidity and mortality is still high compared to in the developed world (Adewoyin, 2015). Therefore, this study intends to classify individuals with SCA based on some common clinical data into severity groups, and to find out if there is any relationship between this severity index, HbF level and markers of tissue damage.

1.1. Statement of Research Problem

- Sickle cell anaemiais one of themost common inheritable diseases in Africa. It affects more than 3million people worldwide with about 300,000 births recorded annually (Weatherall, 2010). In Nigeria alone, about 100,000 new births are recorded annually (CDC, 2012).
- It's a major cause of morbidity and mortality in the world (Zempsky, 2010). There is no comprehensive data from Africa on the death rate but survival rate is expected to be lower than that from Europe and North America who have a median survival rate of 45-55 years (Serjeant, 2005).
- Despite the numerous studies on SCA worldwide, there is presently no cure for this disease and a means to measure the severity of the disease (Rees *et al*., 2010).

1.2. Justification of the Study

- Information on the HbF levels, a modulator of disease severity in SCA individuals is scarce in Nigeria. This information if available will provide basis for the use of hydroxyurea to increase the HbF production for the management of SCA.
- Study on the relationship between HbF and markers of tissue damage will provide information on the levels of these markers and the role HbF have on tissue damage.
- Development of a tool that can measure the severity of a disease will help identify individuals likelyto develop a severe disease so that close care is given to them.

1.3. Alternative Hypothesis

There is no relationship between levels of foetal haemoglobin, tissue destruction and disease severity among individuals with sickle cell anaemia.

1.4 Research Questions

i. What is the age sex composition of the study population and the level of haematological parameters within Individual with Sickle cell Anaemia
ii. How do we classify individuals into severity groups and what is the composition of biochemical markers in the different classes of disease severity groups.
iii. What is the concentration of some biochemical markers among individuals with SCA in steady state and those in crises state?
iv. Is there any relationship between foetal haemoglobin levels, markers of tissue destruction and disease severity

1.5 Aim of the Study

The general aim of this study is to determine the relationship between Foetal Haemoglobin (HbF) level, tissue destruction and disease severity among Individuals with Sickle Cell Anaemia attending Clinic at ABUTH betweenApril to June 2016.

1.6 Specific objectives of the Study

I. To determine the age gender classification of the study population and examine the composition of theirhaematological parameters.
II. To classify individuals into disease severity groups.
III. To determine and compare the levels of HbF, LDH and bilirubin in individuals with Sickle cell anaemia.
IV. To determine the relationship between disease severity, markers in III above in individuals with SCA.

CHAPTER TWO

2.0. Literature review

2.1 Genetic Disorder

The human body is made up of cells, each containing genes responsible for making all the necessary chemicals and structural components needed for normal functioning of the body. These genes are packed into chromosomes. Human beings have 46 chromosomes arranged into 2 pair. The first 22 pairs are known as autosomal chromosome while the 23rd pair containing sex determining material is called the germ line/sex chromosome. Each copy of a pair of chromosome comes from our parents i.e. father and mother (Mahdieh&Rabbani. 2013). Like all our chromosomes, genes which are located on chromosomes come in pairs. Mutation in any genetic information carried in genes is dangerous and can lead to production of an abnormal protein (such as the abnormal haemoglobin called sickle haemoglobin) that has negative or life threatening effect on the whole individual (Krafts,2013). As old cells age out, new ones are synthesizes to replace the old ones and maintain their functions. If mutation has affected any of the old cells a mutated cell will be produced. Somatic mutations are not inherited, and are therefore limited to the descendants of the original cell that developed the mutation – it is not present in other cells in the patient's body. On the other hand, germ line mutations can be passed onto future generations. This kind of mutation is harmful because it doesn't just stop in the original person that had the mutation, but is passed from generation to generation through the germ cells; they are present at conception and therefore are passed down into every cell in the body (Lodish, *et al.*, 2000).

2.2 Haemoglobinopathies

Haemoglobinopathies are a group of red blood cell disorders (mutations) which occur when the haemoglobin produced inside the red blood cells is structurally different from the normal haemoglobin present in a healthy adult. Haemoglobinopathies can be broadly classified into two groups (Forget & Bunn 2013), i.eThose in which there is a quantitative defect in the production of one of the globin subunits(either total absence or marked reduction) and those in which there is a structural defect in one of the globin subunits e.g. Sickle cell anaemia.The focus of our present study wason defects caused by structural changes in globin subunits as seen in sickle haemoglobin (HbS).

2.3. Sickle cell anaemia

Sickle cell anaemia (HbSS) is a genetic disorder of the red blood cell (Rees *et al.,* 2010). It is caused by a mutation in the beta globin chain of haemoglobin. This mutation results in the production of a defective haemoglobin called sickle haemoglobin (HbS) that polymerizes under hypoxic conditions in the erythrocyte forming a rigid and deformed shaped erythrocyte instead of the normal flexible and biconcave disc shaped haemoglobin (Serjeant, 2010) as shown in figure2.1. The deformed shaped red blood cells have properties that differ from the normal erythrocyte, resulting ultimately to their shorter life span. Generally the disease is characterized by occlusion events in the blood vessels that results in pain, organ failure and, occasional death (Edoh*et al.,* 2006). Under ideal conditions where there is no infection or any complicating factor that can lead to sickling, individuals with sickle cell anaemia are normal just like individuals without SCA. If they remain in this state for at least four consecutive week without acute painful crises then, they are said to be in a steady state. However, if there is deviation from this state, and the individual suffers acute painful crises then the individual is said to be in a crises state (Ballas, 2011).

Sickle cell disease is more common in people who originate from Africa. It is also seen in people from the Middle East, Mediterranean, Central Asia and Southern America (Mpalampa, *et al.,* 2012; Zempsky, 2010). It affects more than 3 million people worldwide (Weatherall, 2010) with about 300,000 births recorded annually. In Africa where the highest number of individuals with SCA live (Piel*et al.,* 2010), it is estimated that about 10% to 40% of the population carry the sickle-cell gene, resulting in estimated SCD prevalence of at least 2% in some of these countries like Nigeria (SCAF, 2015).

Sickle cell anaemia is inherited as an autosomal recessive disorder; it affects both male and female equally and becomes clinically significant only when one inherits both copies of the mutated beta globin genes from the parents. Therefore, only homozygous individuals show signs and symptoms of the condition (Smith, 2015). Individuals who have one mutated β-globin gene (HbS) and one normal copy of the β-globin gene (HbA) do not show signs of this disease and are known as genetic carriers for sickle cell disease or sometimes referred to as having sickle cell trait (Serjeant, 2013). Genetic carriers for sickle cell disease are generally healthy but can pass the faulty gene onto their children (Smith, 2015; CGE 2015).

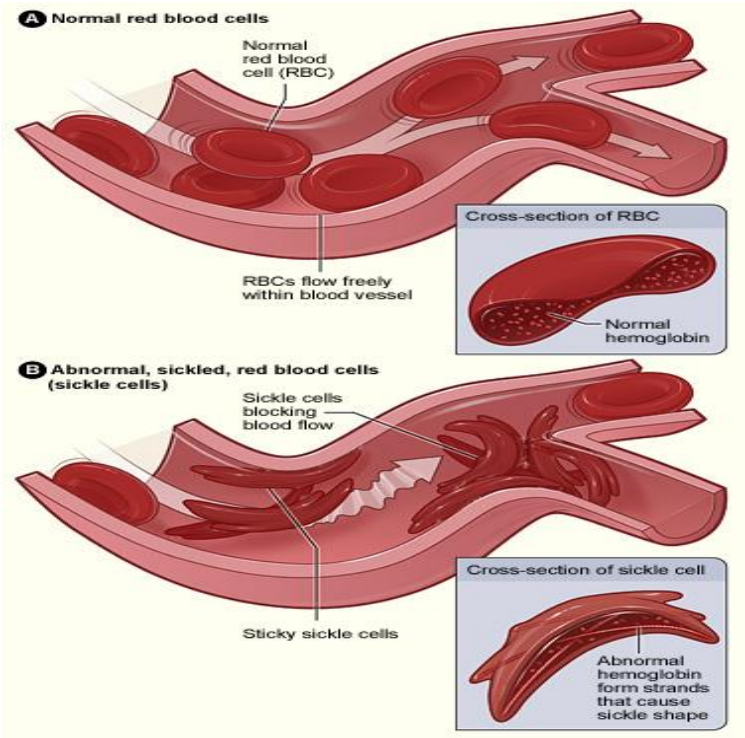

Figure 2.1: Normal and Sickle cell red blood cells under hypoxic condition.
Source:http://www.nhlbi.nih.gov/sites/www.nhlbi.nih.gov/files/images/anaemia.jpg-retrieved from this site on 5th/12/2016.

2.4. Sickle Cell Haemoglobin

Sickle cell haemoglobin (HbS) is a variant type of haemoglobin found in individuals with sickle cell anaemia. It results from a point mutation on chromosome 11 that produces valine (GTG) instead of glutamic acid (GAG) (Serjeant, 2010) as shown in Figure 2.2below. The concentration of HbS varies from one SCA individual to another depending on amount synthesized by the individual (Pauling *et al.,* 1949). Like the normal haemoglobin, HbS is structurally made up of $\alpha_2\beta_2$-like globin chains which have the same properties like HbA in the oxygenated state but unlike the HbA the 2 beta globin chains in HbS carry a mutation which affects the haemoglobin properties in hypoxic

conditions. This mutation on position 6 of chromosome 11 is the source of all complications being suffered by an individual with sickle cell anaemia (Marengo-Rowe, 2006). Because of this mutation the sickle haemoglobin losses most of the normal properties of haemoglobin under hypoxic conditions. For example a normal haemoglobin is flexible, biconcave in shape and lives an average 120 days before its being destroyed and other haemoglobins produced. The mutation which results in the formation of a non-ionic amino acid valine instead of the negatively charged ionic amino acid glutamic acid forms a rigid, sickle cell shaped RBC which has an average life span of less than 120 days. The fundamental cause of this differences is that under hypoxic conditions the glutamic acid at position 6 in normal adult's haemoglobin causes repulsion between adjacent haemoglobin molecules in the RBC but in individuals with sickle cell anaemia there is a slight covalent bond. This bond formation between the HbS molecules in the sickle cell individual's red blood cell leads to formation of a polymer of HbS in a process known as polymerization (Vekilov, 2007; Kaur *et al.*, 2013).

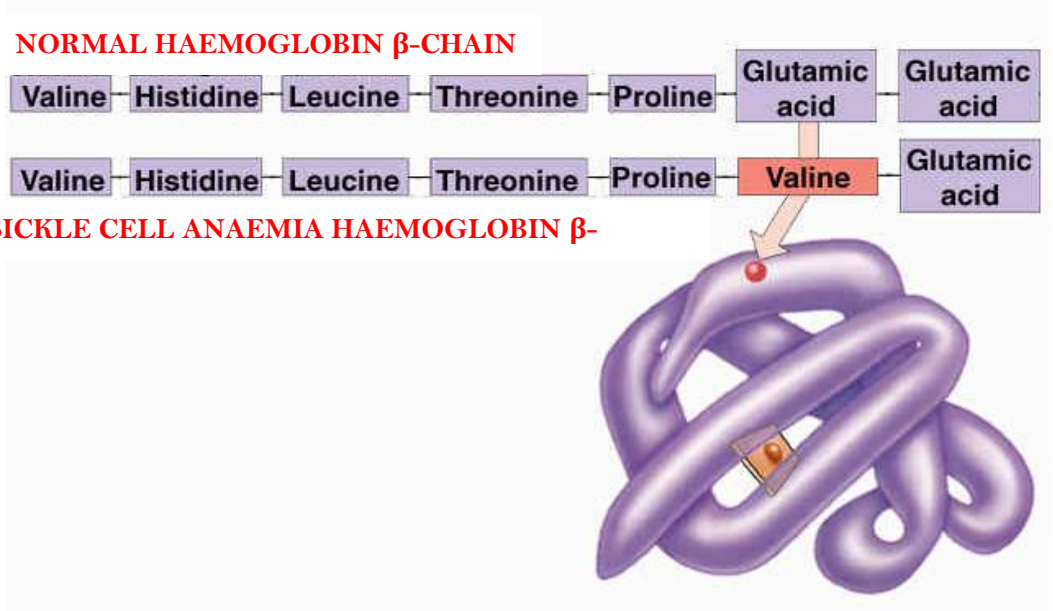

Figure 2.2: Effect of point mutation on Adult normal haemoglobin.
Source:www.mrhorrocks.com/wp-content/uploads/2016/04/HbS.jpg-retrieved from this site on 5/12/2016

2.5. Sickle Cell Polymerization

The hallmark of SCA complications are haemolysis and vaso-occlusive crises caused by polymerization of deoxy-HbS in the erythrocyte (vekilov, 2007). Polymerization of the HbS has the ability of changing the shape of the normal biconcave RBC to an irregular or sickle shaped red blood cell. In this irregular condition the RBC may get stuck in smaller blood vessels around the body, leading to blockage of the vessels (vaso-occlusion) supply of oxygen and other important nutrients. (Steinberg, 2017). The polymerization reaction can be reversed after re-oxygenation of the haemoglobin. Thus, depending on the rate of exposure of HbS to low oxygen concentrations, an RBC can undergo repeated cycles of sickling and un-sickling. Forget & Bunn (2013) categorized into two the effect of de-oxygenation and oxygenation on polymerization of sickle cells. They include;

i. Repeated cycles of sickling/unsickling damages the red blood cell membrane leading to abnormalities of permeability and cellular hydration, eventually causing premature destruction of RBCs and a chronic haemolytic anaemia.
ii. Rigidity of sickled RBCs can lead to increase in viscosity of blood. Increased viscosity will lead to obstruction of blood and nutrients flow through the capillary leading/resulting to hypoxia, necrosis and organ damage if obstruction is prolonged.

The rate of polymerization differs within individuals with sickle cell anaemia. Factors such as genetic polymorphism (coinheritance of sickle haemoglobin(HbS) with other haemoglobin types) may affect the rate of polymerization (Lettre, 2012; Kaur *et al.,* 2013). In general, the concentration of HbS in the RBC determines the level of polymerization. Thus, a small increase in intracellular HbS concentration, as a result of cellular dehydration, can have a major effect on the rate of the polymerization reaction (Bookchin and Lew, 1996; Ilesanmi, 2010).

Presence of non-sickle haemoglobin in the red blood cell such as HaemoglobinC (HbC) increases polymerization of HbS. This is because the presence of HbC in the red blood cell greatly increases potassium efflux and red blood cell dehydration, which increases red blood cell haemoglobin concentration and promotes polymerization (Bunn *et al.*, 1982: Bookchin and Lew, 1996; Forget and Bunn, 2013). On the contrary, the presence of HbF in the RBC can reduce the rate of polymerization in the RBC. HbF does not polymerize with HbS, as a result will resist sickling which is the basis of crises in in SCA. In individuals with hereditary persistence of foetalhaemoglobin (HPFH), each RBC has a mixture of about 70% HbS and 30% HbF combined together. As a result clinical manifestation of SCA in these individuals is mild compared to those without these haemoglobin. Similarly unlike HBSF cells some individuals have a subset of the red blood cells producing HbF called F-cells. They also experienced less polymerization just like individuals with HBSF. The difference between HBSF and HbF is that HbF produced in F-cells are isolated from HbS containing cells while HBSF dominate in a cell containing a mixture of HbF and HbS (Sankaran&Orkin 2013).

2.6. Haemoglobin

The human haemoglobin molecules are a set of closely related metallo-proteins found in abundance in the red blood cells. A fully functional adult Haemoglobin is structurally, a hetero-tetramer formed by symmetric pairing of a dimer of the polypeptide chains, the α-globin and β-globin. Its main function in mammals is to transport oxygen (O_2) from the lungs to peripheral tissues to support oxidative metabolism. Other functions include transports of carbon dioxide (CO_2), carbon monoxide (CO) and nitric oxide (Schechter, 2008; Hardison, 2012). It is responsible for the red colouration of blood. Attached to the globin portion of haemoglobin is the haem portion. Each haem group is present on a globin chain and contains a porphyrin ring and a ferrous atom (Fe^{2+}) that is capable of reversibly binding one oxygen molecule at a time. (Hsia. 1998). The α-globin chain consists of 141

amino acids and the β-globin chain 146 amino acids (Marengo-Rowe, 2006). The four subunits of the haemoglobin molecule are held together mainly by non-covalent interactions and hydrogen bonds; There are no covalent bonds between the subunits. Hb molecule synthesis in humans occurs in erythroid cells of the bone marrow in adults (Hardison, 2012). It was discovered byHunefeld in 1840 at Liepzig University (Sheftel*et al.,* 2012). Each erythrocyte contains around 200–300 million molecules of haemoglobin (Thomas and Andrew, 2012). Three types of haemoglobin are synthesized in humans depending on the stage of development. Embryonic haemoglobin ($\alpha_2\varepsilon_2$), produced before birth, foetalhaemoglobin ($\alpha_2\gamma_2$) during foetal life, and adult haemoglobin ($\alpha_2\beta_2$) after birth. Foetalhaemoglobin production is switched off soon after birth, although the exact time of switch over is not known. Generally, haemoglobin is structurally made up of 2 alpha and 2 non-alpha chains (Edoh*et al.,* 2006).

The functional properties of haemoglobin molecules are primarily determined by its globular structure; the globular structure is formed by the association of the amino acids at specific position in the primary chain. These four (4) polypeptides of the haemoglobin tetramer each have a large central space into which a haem prosthetic group (an iron-protoporphyrin IX molecule) is bound by non-covalent forces, and thus the iron atom is protected from access of the surrounding aqueous solution (Schechter, 2008; Butcher *et al.,* 2014). Each haemoglobin has four (4) binding sites for oxygen present on each of the 4 globin sub-unit of the molecule (Hardison, 2012) as shown in Figure 2.3 below.

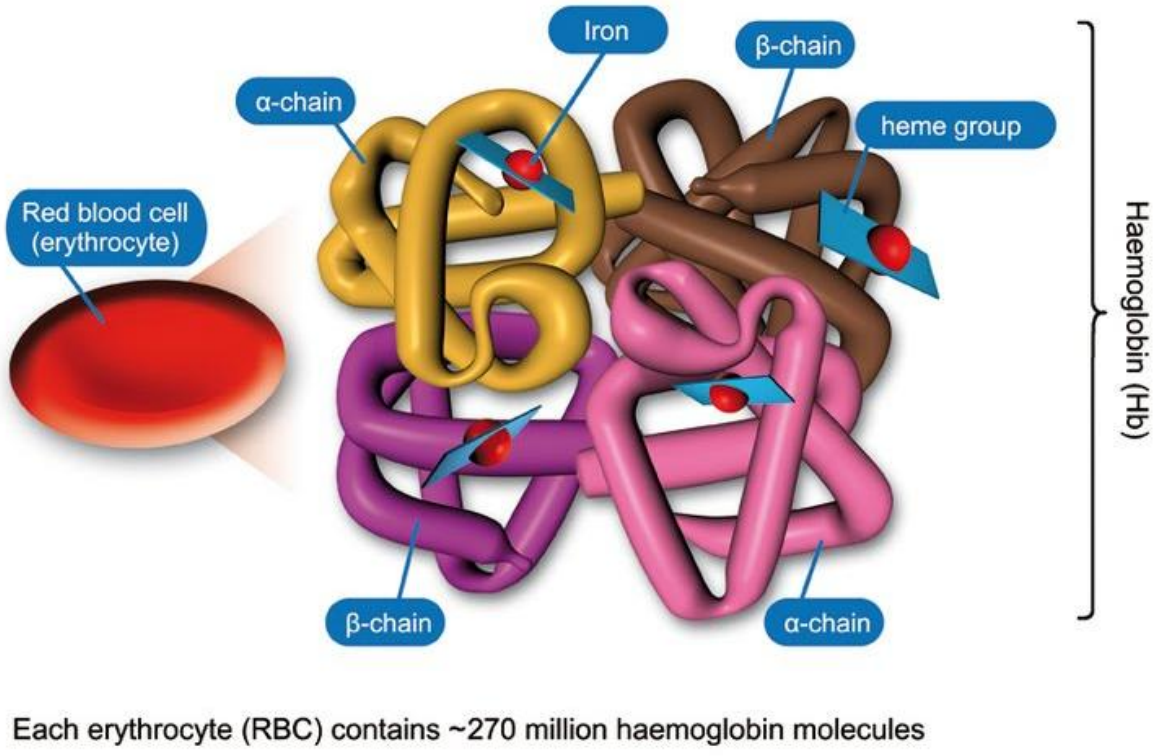

Figure 2.3: A Haemoglobin molecule.
Source: http://www.thealevelbiologist.co.uk/_/rsrc/1337700547906/haemoglobin/haemoglobin.jpg. Retrieved on 5th/12/2016.

2.7. Functions of Haemoglobin

A normal functional haemoglobin has several transport properties, which include transport of oxygen from the lungs to the tissues mostly to facilitate oxidative phosphorylation in the mitochondria, transport of carbon dioxide from tissues to the lungs, transport of carbon monoxide and buffering of hydrogen ions formed in the erythrocyte (Hardison, 2012).

I. Transport of oxygen and the Bohr effect: Every cell in the body needs oxygen to survive. This oxygen is obtained mostly from the atmosphere when an organism breaths in air. In the cells oxygen is needed as a terminal electron acceptor in the mitochondria for the production of energy as ATP. ATP is then used to power all cellular reactions and other metabolic functions

of the body (Cooper, 2000). For this process haemoglobin has to bind and transport oxygen to all tissues in the body. In an oxygen rich environment in the body such as in the arterial circulation, oxygen binds to ferrous iron (Fe^{2+}) of the four globin subunits of the haemoglobin molecule. The remaining five of the six coordination sites of the Fe are occupied by four porphyrin ring and one by the "proximal" histidine (His) of the surrounding globin polypeptide (Hardison, 2012). The binding of oxygen to the haem is determined by the shape of the crevice on globin. In fully deoxygenated state, the globin units of haemoglobin are tightly held by electrostatic bonds resulting in the Hb quaternary structure assuming a tense/contracted conformation called Tense structure (T conformation) with a relatively low affinity for oxygen as a result of reduction in size of the crevices on globin where oxygen normally binds to haem (Hsia. 1998). Binding of oxygen to haemoglobin helps break the electrostatic bonds within globin chains. This changes the structure of the haemoglobin gradually from the tense state to the relaxed state by enlarging the crevice on adjacent globin chains and increasing their oxygen affinity. When fully oxygenated with four oxygen molecules, the haemoglobin achieves its 'R' or 'relaxed' quaternary structure (R structure) (figure 2.4). In the R state the iron molecule becomes oxidized to Fe^{3+}. In this form it can't bind oxygen (Forget and Bunn.2013).

The Bohr effect describes the reduction in affinity of haemoglobin for oxygen by conditions that bring about low pH in blood (such as increase concentration of CO_2 and hydrogen ions) and the increase in affinity when pH is high. This effect helps with the offloading of oxygen from haemoglobin to provide a vital oxygen supply to tissues where it is needed. Similarly since haemoglobin is an allosteric protein: 2, 3-DPG molecule an allosteric effector of haemoglobin can bind to the crevice located at the center of haemoglobin molecule between the four globin molecules there by reducing its affinity for oxygen but promoting the release of oxygen to

tissues (Bunn and Briehl, 1970). The production of 2, 3-DPG is increased in anaemic tissues and as a result, the relative tissue hypoxia is partially corrected by the increased P_{50} (partial pressure of oxygen needed to saturate 50% of haemoglobin) which promotes more oxygen release to the tissues (Schecter, 2008: Forget and Bunn, 2013).

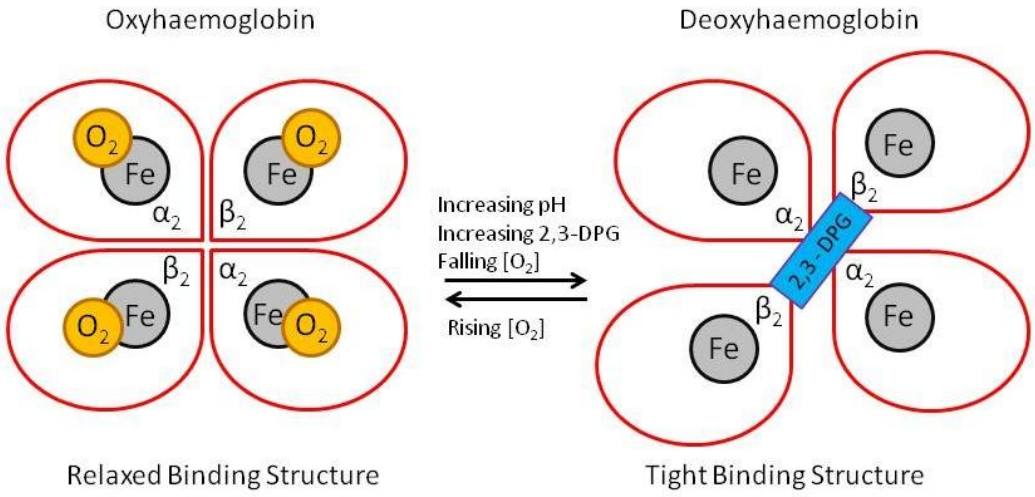

Figure2.4: Oxygen binding and unloading of haemoglobin
Source: http://www.fastbleep.com/assets/notes/image/8936_1.jpg. Retrieved on 5th/12/2016.

II. Transport of carbon dioxide and Hamburger effect: Carbon-dioxide, a product of various biochemical reactions (e.g. Tricarboxylic acid cycle)needs to be regulated, in order to avoid high concentration from accumulating in the body. It is mainly produced in the mitochondria by metabolism of carbohydrates, fats and proteins. Haemoglobin has the ability to bind and transport carbon-dioxide to the lungs where it is exhaled as waste. Carbon-dioxide transport is concentration dependent i.e. the higher the concentration of haemoglobin in the tissues the more carbon-dioxide will be attached to the haemoglobin, transported and get excreted (Hsia, 1998). Generally carbon-dioxide is transported in the blood in three forms namely; in blood solution,

buffered with water in the red blood cell as carbonic acid and bound to haemoglobin as carbamino compounds. High percentage of carbon-dioxide from tissues is transported in RBC and a few in plasma. The concentration of CO_2 gradually increases in blood as it passes through the venous capillary towards the lungs, thereby reducing the affinity of haemoglobin for oxygen.These rapid interactions between the bohr andhaldane effects promote the optimal transport of both oxygen and carbon dioxide by red cells (Quaye, 2015).

RBCs contain the enzyme carbonic anhydrase which is able to convert carbon-dioxide and water into carbonic acid. This enzyme is only found in the RBC but not in the plasma (Arthur &Sudhakar, 2005). CO_2 and water can freely permeate the RBCs membrane and be converted to carbonic acid. The carbonic acid freely dissociates into hydrogen ions and bicarbonate ions and is transported in the red blood cell to the lungs via the circulatory system. In the lungs where gaseous exchange occurs, a trans-membrane protein called band 3 protein facilitates the transport of bicarbonates out of the red blood cells and chloride ions from the blood into the cell to maintain ionic balance. This process of maintaining ionic balance by band 3 protein is known as chloride shift or Hamburger effect. The hydrogen ion producedsimultaneously during dissociation of carbonic acid gradually increases in concentration within the RBC. To regulate this increase, haemoglobin binds to the excess hydrogen ions, exchanging them for oxygen during gaseous exchange. This process regulate the concentration of free hydrogen ions in the cell so that the cell doesn't become highly protonated or acidic: Since acidic conditions support binding of CO_2 and release of oxygen (Arthur &Sudhakar, 2005: Thomas& Andrew, 2012).

2.8. Haemoglobin Switching

Haemoglobin switching is the process by which HbF is being replaced by HbA as the main haemoglobin component in the red blood cell of a normal individuals or HbS in individuals with sickle cell anaemia (Steinberg *et al.,* 2014). Chromosome 16 and 11 respectively harbour the genes responsible for the synthesis of alpha and beta globin chains of haemoglobin in adult humans. During development from foetus to adulthood, 3 types of haemoglobin are produced. When analyzed by electrophoresis, the different haemoglobins differ only in the beta globin subunits composition; the alpha globin subunit is consistent throughout the different haemoglobins. As a result there is a difference in physiologic properties of the haemoglobins (Sankaran*et al.,* 2010). The genes responsible for synthesis of these non-alpha HbS (ε, γ and β) are jointly regulated by a single locus control region. The arrangement from the 5' end region close to the locus control region LCR to the 3' end is in the order: embryonic (Hbε), foetal (Hbγ1 & Hbγ2), and Adult (Hbδ&Hbβ) as shown in Figure 4. From the arrangement, the haemoglobin first expressed is the embryonic Hb ($\alpha_2\varepsilon_2$), followed by the foetal Hb ($\alpha_2\gamma_2$) present in the foetus and then adult Hb ($\alpha_2\beta_2$)dorminant in the adult (Ginder, 2015).

The mechanism of switching between the different types of haemoglobin during development involves genetically switching on or switching off of various globin genes present on the same loci on chromosome 11(Sankaran&Orkin, 2013). The combined association of the cis-acting elements (chromatin remodelling complex) and trans-acting elements (transcription factors such as from BCL11A gene) regulates the haemoglobin switch (Ginder 2015; Steinberg *et al.,* 2014). Natural variation in the level of a particular haemoglobin among individuals appears to reflect differential levels of transcription of the haemoglobin gene (Thein*et al.,* 2009). The regulation of globin gene

expression during development serves as an important model for understanding higher eukaryotic transcriptional control mechanisms.

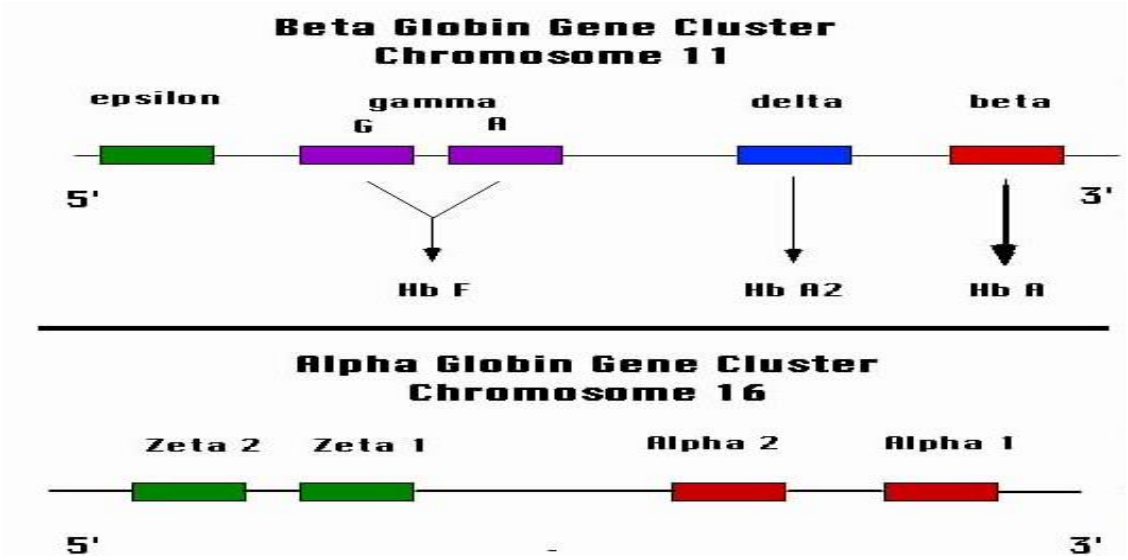

Figure 2.5: Beta globin cluster of haemoglobin 11 showing the haemoglobin genes expressed at different stages of development.
Source: http://sickle.bwh.harvard.edu/HbSynthesis retrieved on 5th/12/2016

2.9. Foetal Haemoglobin

Foetal haemoglobin (HbF) is the major type of haemoglobin expressed in the developing foetus and new born babies. It accounts for approximately 80% of their total haemoglobin. It is produced by RBCs called F-cells from the sixth week of gestation to the rest of foetal life (Franco, 2006). After birth, as one develops to an adult, the expression of HbF is replaced by HbA in normal adult or HbS in individuals with Sickle cell anaemia, making it the dorminantHb from few months after delivery to adulthood (Steinberg *et al.*, 2014). The structure of HbF just likeHbA in a normal adult is a tetramer consisting of two chains of 141 alpha amino acids, but unlike HbA, it is made up of two gamma globin chains of 146 amino acids just like HbA but differ from HbA by a sequence of 39 amino acids residues not arranged in a specific order (Chen, 2000). It can therefore be said that HbF

have the same α-globin subunits but highly homologous non-alpha subunits which differ significantly in structure to HbA (Schechter, 2008).

HbF is the most powerful modulator of the clinical severity features of sickle cell anaemia. Patients with high levels of HbF normally show mild clinical complications associated with SCA. The reason is that the increased expression of HbF in erythrocytes reduces the net concentration of HbS in the red blood cells (diluting the HbS, there-by reducing its concentration). Thus HbF does not participate in the polymerization that occur between molecules of HbS which usually occurs at low oxygen tension in patients with SCA, the polymerization reaction is thus inhibited depending on the levels of HbF present in the red blood cell (Mehdi*et al.,* 2013; Steinberg *et al.,* 2014). In addition, red blood cells with higher HbF content have prolonged survivalhence anaemic patients with high HbF level also tend to have higher overall Hb levels. These properties are possible because of the difference in structure between HbF and HbS (Steinberg *et al.,* 2014).

In a healthy adult, 2,3 -diphospho-glycerate (2,3-DPG), a negatively charge intermediate of glycolysis present in RBC, binds to the positively charge pocket of haemoglobin containing the amino acids, Histidine 2 ,143, asparagine 80 and lysine 82. This molecule helps stabilize the T conformation of haemoglobin, hence reducing its affinity for oxygen. These positively charged amino acids gives an overall positive charge to the haemoglobin pocket. In foetalhaemoglobin, histidine 143 and asparagine 80 present in adult haemoglobin are replaced by the neutral amino acids serine and negative amino acid aspartate at the same positions. This replacement reduces the overall positive charge in the center of the foetalhaemoglobin compared to adult haemoglobin. The net charges between the negative 2,3- DPG (COO^- and $2PO_3^{2-}$) and positive center of adult haemoglobin, increases the attraction between 2,3-DPG and HbA compared to that between 2,3-DPG and foetalhaemoglobin (Adachi *et al.,* 1997; Sears, 2011). This difference is as a result of a shortage of

one positive molecule and addition of a negative charge from aspartate, which reduces the net positive charge in HbF. The physiological consequence of this is that foetalhaemoglobin will have a higher oxygen affinity than adult haemoglobin because 2,3-DPG will bind less tightly to HbF compared to HbA. These effect discourages the change of Hb to the T-conformation which in-turn encourages oxygen binding to the Hb (Bunn and Briehl, 1970; Oski*et al.,* 1970).

2.10. Markers of tissue destruction in Sickle cell anaemia

The Biomarkers Definitions Working Group has defined a ‘‘biomarker’’ as a ‘‘characteristic that is objectively measuredand evaluated as an indicator of normal biological or pathogenic processes, or pharmacological responses to a therapeuticintervention’’ (BDWG, 2001). The concentration of some substances in the general circulation is important and they aid in the diagnosis of several diseases. They serve as markers or indicators of disease development or severity. These substances are confined within the cells/tissues of normal individuals and if present in the general circulation, their concentration is limited to a small concentration. However, in some disease conditions the levels of these normal substances in the general circulation is increased beyond a threshold concentration. In such cases the increased substances serve as markers of that disease (Damanhouri*et al.,* 2014). In individuals with SCA, elevation in the general circulation of certain substances provides a hint that a particular organ is being damaged. In this study we used bilirubin and lactate dehydrogenase as markers to determine the extent of haemolysis(tissue destruction) in individuals with sickle cell anaemia since significant concentration of these substances in the blood of individuals with sickle cell anaemia arises as a result of haemolysis (Kato et al., 2006).

2.11 LactateDehydrogenasesand Sickle cell Anaemia

Lactate dehydrogenase (LDH) is an enzyme of the glycolytic pathway responsible for the catalytic conversion of pyruvate to lactate with concurrent conversion of NADH to NAD^+. It is a cytosolic

enzyme that belong to the oxido-reductase class of enzymes. Its concentration increases in the general circulation when the plasma membrane of worn out or diseased tissues is damaged (Kato *et al.,* 2013). The increased concentration in the blood serves as marker of tissue or organ damages. It is a tetrameric enzyme made up of H and M subunits. Based on the different subunits combination it exists in five (5) iso-enzyme forms in the body numbered 1-5. The isoenzymes are differentiated into different forms by their amino acid composition, detected by monitoring their mobility in electrophoretic field. Based on this criteria LDH1 travel faster in electrophoretic field while LDH5 is the slowest. It is found in many different cell types in the body, but the distribution of its isoforms is not uniform across body tissues. LDH1 and LDH2 are found primarily in RBCs and heart muscle; LDH3 is highest in the lungs; LDH4 is highest in the kidneys, placenta, and pancreas; and LDH5 is highest in skeletal muscle and liver (Ballas, 2013). Studies have associated SCA individuals with high LDH activity to high prevalence of pulmonary hypertension, renal failure and early death. These high LDH activity observed in this individuals was as a result of increased haemolysis and tissue destruction brought about vaso-occlusion(Mehdi *et al.,* 2013; Alzahri*et al.,* 2015)

2.11Bilirubin and sickle sell anamia

Bilirubin is the major final end-product from the breakdown of haem portion of haemoglobin, myoglobin and the cytochromes. Haemolysis accounts for the main stream of bilirubin circulating in the body, while the remaining comes from degradation of other sources such as myoglobin and cytochromes (Sticova and Jirsa, 2013). At the end of a cell's cycle, the senescent RBCs are haemolysed with the release of haemoglobin, which is subsequently broken down to the component globin and haem. The globin chains are recycled in proteins and amino acids pool while the haem is forwarded into the oxygenase pathway with subsequent production of bilirubin (Puppalwar*et al.,* 2012). The bilirubin formed is insoluble in water and is transported in the blood stream attached to

albumin to the liver to be where its converted to water soluble compound. In the liver, the albumin-bilirubin complex is transported into the hepatocytes by ligandin, a glutathione-1-transferase. In the hepatocytes the water soluble bilirubin-glucuronide complex is formed from the conjugation of bilirubin with glucuronic acid by the enzyme uridyldiphosphateglucuronyltransferase. This process is normally highly efficient, able to clear plasma unconjugated (indirect) bilirubin concentrations before they pile up and cause a problem in an individual (VanWagner& Green, 2015). The newly formed water soluble bilirubin can either be excreted by bile via the intestine or can be recycled for reuse in the body in a process known as entero-hepatic circulation (Hansen, 2010).

Moderate bilirubin concentration in the body serves a beneficial role as a strong antioxidant: destroy free radical (Hansen, 2013), but can be harmful when its concentration increases beyond the moderate concentration. The result of this accumulation in the various tissues and plasma is a condition called jaundice, which causes the yellowish colour of the skin and sclera. Other effects of high concentration of bilirubin are serving as uncouplers of oxidative phosphorylation and inhibitors of DNA synthesis (Sticova and Jirsa, 2013). In some disease condition such as SCA, the concentration of bilirubin in the circulation is increased via increase in haemolysis (Damanhouri*et al.*, 2014). At a point, the influx of this substance due to high rate of haemolysis may become too much for the cells to control leading to high concentration of bilirubin in circulation. Therefore the rate of haemolysis is always monitored to make sure bilirubin concentration does not reach an uncontrollable level in the body of individuals with SCA. Practically, the presence of conjugated bilirubin in the plasma serves as a marker of haemolysis and other pathological process in individuals with sickle cell anaemia (Hansen, 2013). Bilirubin is photosensitive, and specimens should be protected from light when highly accurate measurements are required.

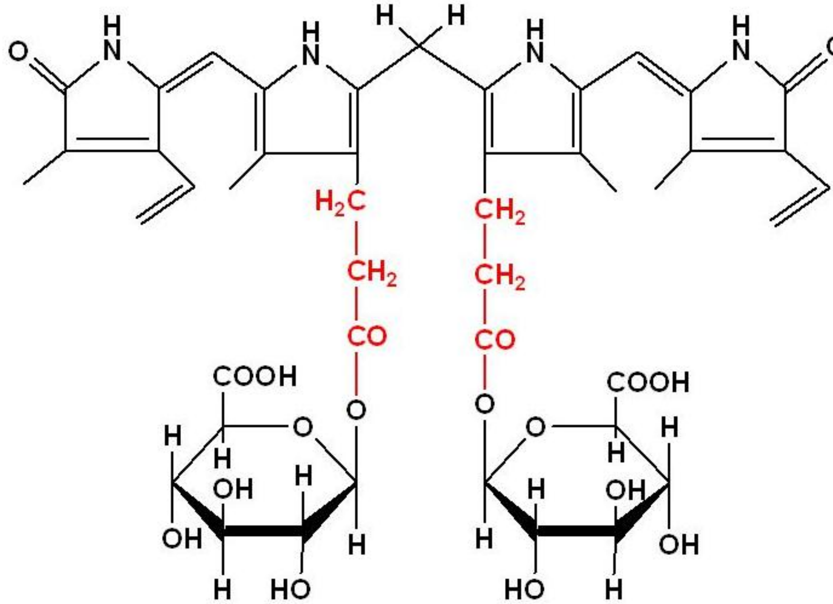

Figure 2.6: Structure of bilirubin conjugated to a glucuronic acid
Source: slideplayer.com/slide/5924010-retrieved from site on 5th/12/2016.

2.11. Disease severity

Van den Tweeldefined severity as "the rate and extent of reversible and irreversible damage to organs brought on by the sickle cell disease process, resulting in impairment that may require medical intervention" (Van den Tweel, 2009). Individuals with SCA present diverse clinical features associated with the disease (Sanjay *et al.*, 2014). Some of the clinical features generally presented by individuals with SCA include leg ulcer complication, renal complications, abdominal complications, ocular disease, pulmonary disease, stroke, musculoskeletal Complications, hepatobiliary complications, immunological and infectious complication etc. (Adewoyin,2015). These diverse clinical features have made patients stratification into severity groups particularly difficult (Adegoke and Kuti 2013; Coelhol*et al.*, 2012). But based on certain clinical features presented, individuals with sickle cell anaemia can be classified as having either a mild, moderate or a severe disease. Individuals who do not present any of these clinical features may be regarded as having a mild disease. On the other end, individuals who presented most or all of the clinical features were

regarded as having a severe form of the disease. there is an ongoing research from different parts of the globe to associate markers with severe disease (Coelho *et al.,* 2012), so that if found individuals capable of getting a severe disease can be identified and given a special and timely care before the disease gets to a critical period that may be difficult to manage. Markers like HbF have been found to moderate disease severity but more markers are needed because of the diverse symptoms exhibited by different SCA individuals (Steinberg, 2005). Another factor facing severity classification is the diverse markers used by different authors worldwide to classify severity. This has made result comparison between the different researchers all over the world difficult (Steinberg, 2005).

In developed countries like USA and England where there are sophisticated technology, SCA has been managed to a certain level reducing the number of morbidity and mortality associated with the disease. In Africa, morbidity and mortality due to SCA is still a big problem, partly due to inability to effectively manage the disease as those in the developed countries (Piel*et al.,* 2013). Patients with severe disease tend to present with more complications than others, hence identifying individuals who may develop a severe disease and giving them the necessary management may prevent/reduce the rate of morbidity and mortality as a result of SCA (Adewoyin, 2015). Previous studies carried out in Nigeria by Okocha*et al.*, 2015 focused on using simple clinical data to achieved classification of individuals with SCA into disease severity groups. This is with the hope that individuals with potential of developing severe form of the disease can be identified and given the appropriate treatment before it gets to a severe stage. For a better prognostic tool to be developed, it is essential to know which determinants are associated with severe disease, so that prognostic models can be constructed.

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

3.1.1. Chemicals and Reagents

Carbon tetrachloride

0.9% saline solution

Distilled water

Drabkins solution (Potassium ferricyanide-$K_3Fe(CN)_6$, potassium cyanide-KCN, potassium dihydrogen phosphate, Non-ionic detergent- Triton-X-100

1.2M sodium hydroxide (NaOH)-Alkali

Saturated ammonium sulphate $(NH_4)_2SO_4$- Acid

Whatman filter paper (Number 42 or 6)

LDH assay Kits from Agappe Diagnostic limited. Hills, Dist. Ernakulam, kerala, india, 683562

Bilirubin Kits from Chemelex, S.A Pol. Ind. 08420 Canovelles-Barcelona

EDTA tubes and Plain tubes (red head)

3.1.2 Equipment

Spectro UV/V-16-MRC UV/VIS Spectrophotometer. 3, Hagavishstreet. Isreal

Spectrolab 23 Spectrophotometer (LABOMED INC.) Los Angeles, CA 90034 USA

Centrifuge: HeraeusLabofuge 300 Centrifuge, (Thermo Fisher Scientific Company), RS-232C

Mettler Toledo NewClassic ME104 Analytical Balance. (Thermo Fisher scientific)

Stop watch

Population Study Participants

This study involved a cross sectional non-intervention based descriptive study in which thirty-seven persons with sickle cell anaemia who consented to participate were recruited for the study.

3.2 Methodology

3.2.1 Study Area

The study was conducted in the Departments of Biochemistry, Chemical pathology and Haematology of Ahmadu Bello University Teaching Hospital (ABUTH), Zaria from April to June 2016. Zaria is a local government in northern part of Kaduna state. It has a population of about 408,198 people recorded from 2006 censusbut is estimated to be around 700,000 at present. It has a land mass of about 33,000 sq.km and is situated on Latitude**:** 11° 06' 40.61"N and Longitude**:** 7° 43' 21.72" E (EB, 2018; Latlong.net, 2018). Participants were drawn from consenting individuals with sickle cell anaemia, who attend Clinic at the Haematology Department of Ahmadu Bello University Teaching Hospital (ABUTH) Zaria for management of sickle cell related emergencies and continued follow up care. Out-patient services for persons with SCA are provided in the clinic and the day care unit with an annual patronage of one thousand one hundred and four (1104) individuals (2006 case record estimate).

3.2.2. Ethical considerations

a. Approval for this study was sought and obtained from the Health Research Ethic Committee of ABUTH, Zaria.
b. All literate and English speaking participants were given a questionnaire for their response to participate in the research while Non literate participants had the questions translated to them in interview format by trained research assistants. The questionnaire was translated to the most frequently spoken local language (Hausa language).
c. The aim and the procedures of the study were explained to the participants.
d. The participants were informed that they could withdraw at any time without further obligation.
e. Anonymity of the participants was guaranteed and no personal details were recorded.
f. The researcher bore the costs of the study

3.2.3. Inclusion and Exclusion Criteria

Inclusion Criteria

All Individuals diagnosed with sickle cell anaemia.

All individuals with sickle cell anaemia that consented.

Sickle cell individuals from 18 years and above only were considered.

Exclusion Criteria

Individuals with other haemoglobin disorders such as thalassemia.

Persons who have had stem cell transplantation

SCA patients who had blood transfusion within the last 3 months

Persons who were on hydroxyurea therapy

3.2.4. Sample Size

Thirty-seven consenting individuals with sickle cell anaemia participated in the research. Systematic sampling technique was used to select the patients attending clinic until the desired sample size was attained. Briefly all patients available for clinic were recruited using lottery method of random sampling, where numbers (1 and 0) are written and folded in papers and participants are allowed to choose. The numbers corresponding to one (1) were recruited while those corresponding to zero were not selected. The sample size was calculated using formula for sample size estimation as suggested by Pourhoseingholi, *et al.,* (2013).

$$n = \frac{Z^2 P (1 - P)}{d^2}$$

Where n = Sample size

Z = standard normal deviate corresponding to level of confidence at 95%.

P = Expected prevalence (that can be obtained from same studies or a pilot study conducted by the researchers).

d = Precision (corresponding to effect size).

Where Z = 1.96

P = 2% = 0.02 (WHO Regional Office for Africa Report, 2015; SCAF Report, 2015)

d = 5% = 0.05

n = 30

Therefore at least 30 HbSS individuals will be recruited for this study.

3.2.6. Data Collection Procedure and Analysis

Informed consent was obtained from parents and guardians of the participants less than 18 years attending clinic in ABUTH. Questionnaires were issued out to the participants, after which a clinical examination by the attending physician was conducted to ascertain the presence or otherwise of complications. Four millilitres (4mls) of venous blood was obtained from each consenting participant by venepuncture in an EDTA container in compliance with the principles of asepsis and universal safety precaution. The blood samples were then taken to the laboratory for analysis.

3.2.7 Determination of Foetal haemoglobin by Alkali Denaturation

HbF is relatively resistant to denaturation by alkalis as compared to HbA. This method is able to give reliable results in the range of 0.8 to 25%, which are the levels most commonly found in carriers of most forms of red blood cell disorders such as sickle cell anaemia. Blood was collected in EDTA anticoagulant bottles, washed three times in 0.9% saline (centrifuged for 5-10 mins at 3000rpm) and then lyse by adding 2ml of water and 1 ml carbon Tetrachloride (CCl_4) to 2 mls of packed cells. The mixture was shaken vigorously for about 1min, and then centrifuged at 3000 rpm (1200g) for 30 min then, 0.6 ml of haemolysate was pipetted into 10ml of drabkin reagent. Exactly 2.8ml of the cyanmet haemoglobin was added to 0.2ml of 1.2M NaOH, the mixture was well agitated and kept for 2min. After 2min incubation, 2ml of saturated ammonium sulphate solution was added and again vigorously mixed and allowed to stand for about 5 mins. The mixture was then separated through a whatman no. 6 filter paper. Optical density of the filtrate which contains the alkali resistant HbF was read at 415nm using a spectrophotometer. A control solution was prepared by mixing 1.4 ml of

Cyanmet-Hb, 1.6ml of distilled H_2O and 2 ml of saturated ammonium sulphate. This solution was then diluted in the ratio 1:10 with distilled H_2O and optical density was measured at 415nm (Ithapedia, 2013).

Calculation:

$$\%HbF = \frac{OD\ test}{OD\ control} X\ \frac{100}{20}$$

3.2.8. Determination of Lactate Dehydrogenase activity by Spectrophotometry

Principle:

The enzyme LDH catalyses the reduction of Pyruvate by NADH to yield NAD^+. The LDH activity was determined based on the rate of decomposition of NADH, measured by the decrease in absorbance at 340 nm (Elias *et al.*, 2012). The equation for the reaction is as follows

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

The plasma isolated from the RBCs were collected and refrigerated until time for analysis. The LDH assay kit procedure from Agappe diagnostic ltd. India was used for this experiment. Briefly the working solution was prepared according to manufacturer's manual by adding 4ml of R1 solution (Pyruvate) to 1ml of R2 solution (NADH). The solutions were mixed in the ratio 4:1 (R1:R2). For the analysis, 1ml (1000 µl) of the working reagent was added to 10µl of the sample. The solution was then Mixed and incubated at 37^0C for 1 minute. The change in absorbance was measured per minute (ΔOD/min) during 3 minutes at 340nm. Distilled water was used as the blank (Agappe, 2008).

Calculation:

$$LDH - activity\ \left(\frac{U}{L}\right) = {\Delta OD}/{_{min}} \times 16030$$

(Factor =16030)

3.2.9. Determination of Bilirubin by Spectrophotometric Method

Principle:

Direct and total bilirubin were determined. Bilirubin present in plasma is converted to coloured azobilirubin by diazotised sulfanilic acid (R1) and measured spectrophotometrically. The intensity of the colour form is proportional to the bilirubin concentration measured at wavelength of 555nm.

Procedure: Bilirubin assay kits from chemelexind Barcelona were used for this experiment. Briefly the procedure taken is as follows. Plasma from HbSS individuals was used for this experiment for the determination of total bilirubin and direct bilirubin. For the determination of total bilirubin, 1.5ml of R2 (mixture of dimethylsulphoxide (DMSO), sulfanilic acid and HCL) were added to two test tubes labelled blank and test containing 100µl of sample. To the tube labelled test, 50µL of R3 (sodium nitrite) was further added. The solution were mix, incubated for 5mins at 25 ^{0}C and optical density was measured at 555nm. The same procedure was repeated for determination of direct bilirubin, the only difference being that R1 (mixture of sulfanilic acid and HCL) replaced R2 (Chemelex,2013).

Calculation:

$$Bilirubin\ concentration\left(\frac{mg}{dl}\right) = ODsample - ODblank\ \times factor$$

Factor for total bilirubin = 19.1, Factor for direct bilirubin =14

Conversion factor: mg/dl X 17.1 = µmol/L

3.2.10. Estimation of Disease Severity

Each sickle cell anaemia individual recruited was given a questionnaire to fill. Information such as the participant socio-demographic information (Age, Sex, BMI), clinical information (such as pulse,

blood pressure, Number of times he/she has visited the hospital in the last 1 year, number of Surgery he/she have had in the last 1 year, how he/she was feeling the day he visited the clinic, etc) along with information on haematological parameter (RBC, WBC, HCT, and LYM) were obtained. Theseparameters were assigned scores. Parameters whose value were normal were assigned no mark or score while those that were abnormal were assigned score based on their degree. The individual scores for each participant were summed up. This final score represented the disease severity status of each of the individuals.

3.2.11. Determination of individuals in steady state and crises state

Information from questionnaire on state of health of each individual on the day of sample collection were taken. According to Ballas (2011) 'The steady state refers to a point in time where the patient in question is not experiencing an acute painful crisis or any changes due to therapy'. Individuals that were feeling ill on the day of recruitment were considered to be under a kind of crises, while individuals who did notmade any complain of illness but came in for a regular routine check-up were regarded as being in a steady state.

3.3. Statistical analysis

Analyses of data were carried out with SPSSstatistical tool using both inferential and descriptive statistics with mean and standard error of mean (SEM) respectively. Skewness test was used to measure normality of data while Independent sample T test and spearman correlation were used to test for significant differences in means of various groups. All mean values with $P \leq 0.05$ were considered statistically significant.

CHAPTER FOUR

4.0. RESULTS

4.1 Haematocrit, RBCs and HbF levels in sickle cell anaemic patients

The findings from this study showed that the level of haematocrit, RBCs and HbF levels in SCA patients was low, while female population was higher than male population.

4.2 Age sex distribution of individuals with sickle cell anaemia

Females were the majority of the SCA patients with 64.9% out of thirty seven (37) participants whereas the males accounted for the remaining 35.1%. The Age sex distribution showed a modal age range of 16-25 years. The mean age for females was 25.64 years while that for males was 27.92 years. There was no significant ($P>0.05$) difference between the ages of females and males participants investigated.

Table 4.1: Age sex distribution of individuals with sickle cell anaemia

Sex	N (%)	16-25 Yrs. N (%)	26-35 Yrs. N (%)	36-45 Yrs. N (%)	Mean Age	P-Value
Female	24(64.9)	12 (32.4)	10 (27.0)	2 (5.4)	25.64±7.09	
Male	13(35.1)	7 (18.9)	4 (10.8)	2 (5.4)	27.92±7.97	0.43
Total	37(100)	19 (51.4)	14 (37.8)	4 (10.8)	26.24±7.32	

P value >0.05

4.3. Distribution of mean haematological parameters by sexin SCA patients

The distribution of haematological parameters in males and females showed that haematocrit, platelet and RBC were higher in females but WBC and lymphocytes were higher in males. There was no significant ($P>0.05$) difference in haematological indices between the sexes of the participants.

Table 4.2. Distribution of mean haematological parameters by sex in SCA patients.

Sex	Haematocrit	WBC	Platelet	RBC	Lymphocyte
Male	22.80±5.28	12.30±5.90	331.09±99.31	2.53±0.70	5.92±4.56
Female	23.12±4.56	12.09±3.64	378.92±164.95	2.71±0.66	4.56±1.63
P-Value	0.85	0.92	0.38	0.46	0.34

4.4. Concentration of some biochemical markers in individuals with Sickle cell anaemia

The HbF level, LDH activity and bilirubin concentration of individuals with SCA were significantly ($P<0.05$) higer than their corresponding reference values of 1.04±0.44%, 120-240IU/L and 1-18.81µmol/L respectively.

Table 4.3: Concentration of some biochemical markers in individuals with sickle cell anaemia

Biochemical markers	Concentration (Mean±SD)	P-Value
HBF (%)	4.35 ± 2.62	0.001*
Bilirubin (µmol/l)	37.18 ± 23.81	0.001*
LDH activity (IU/L)	423.80 ± 178.97	0.001*

*significant at P <0.05

LDH: Lactate dehydrogenase, HbF: foetal haemoglobin

LDH reference value: 120-240IU/L (Obtained from Chemelex diagnostic Kit)

Bilirubin reference value: 1-18.81µmol/L (Obtained from Agappe diagnostic kit).

HbFvalue of HbA individuals: 1.04±0.44% (Obtained from previous Studies by Olaniyi*et al.*, 2010)

4.5. Comparison of some selected biochemical markers between individuals with SCA in steady and in crises state

Table 4.2 showed a higher bilirubin level and LDH activity in the crises state participants compared to those in steady state, whereas HbF levels is higher in the steady state. Moreover the difference in the HbF level and LDH activity between the steady state and crises state participants issignificant ($P<0.05$) while there is no significant ($P>0.05$) difference in bilirubin concentration in both steady states and crises state participants.

Table 4.4: Comparison of some selected biochemical markers between individual with SCA in steady and in crises state

Biochemical markers	Steady state (N=25)	Crises state (N=12)	P-Value
HbF (%)	5.25±2.57	2.47±1.54	0.01*
Bilirubin Conc. (µmol/L)	37.10 ± 24.90	37.22 ± 23.79	0.99
LDH Activity (IU/L)	320.55 ± 64.47	638.92 ± 145.08	0.01*

LDH: Lactate dehydrogenase, HbF: Foetal Haemoglobin
*significant differnce at P<0.05

4.6. Classification of participants into different groups based on disease severity

The participants with moderate disease complications were the majority in the present study with about 73%. While the participants with mild complications have higher HbF levels whereas participants with severe complications showed higher bilirubin concentration and LDH activity when compared to either mild or moderate complications.

Table 4.5. Classification of disease severity

Disease Severity	Percentage Composition	HbF Level (%)	LDH Activity (IU/L)	Bilirubin (µmol/L)
Mild	13.5% (5)	4.60±2.80	328.24±154.17	27.54±6.50
Moderate	78.4%(29)	4.18±1.70	433.58±188.07	37.38±24.46
Severe	8.1%(3)	2.19±0.61	488.54±57.80	51.39±34.73
P-Value		0.32	0.38	0.40

4.7. Correlation between disease severity and markers

There was a negative but significant ($P<0.05$) correlation between diseases severity on the one hand and RBC and haematocrit. The relationship between disease severity and WBC and lymphocytes counts is positive and moderately significantly ($P<0.05$) correlated. However, pulse rate, platelet count, systolic blood pressure and diastolic blood pressure have a positive and weak relationship with disease severity but there was no significant ($P>0.05$) difference amongs the participants investigated.

Table 4.6: Correlationship between disease severity and markers

Disease Severity Markers	Correlation Coefficient	P-Value
Disease severity – RBC	-0.58	0.01*
Disease severity – WBC	0.74	0.01*
Disease severity – Haematocrit	-0.40	0.01*
Disease severity – Lymphocytes	0.75	0.01*
Disease severity – Platelets	0.10	0.56
Disease severity – Pulse	0.22	0.19
Disease severity – Systolic Blood Pressure	0.26	0.12
Disease severity – Diastolic Blood Pressure	0.06	0.71

*significant at P<0.05
Values are mean±SEM.

4.8. Relationship between HbF Levels, LDH, bilirubin and disease severity in individulas with sickle cell anaemia

There was a weak negative but signifant ($P<0.05$) relationship between HbF leveland LDH activity among the participants. HbF level has a weak and negative relationship that is not significant ($P<0.05$) with bilirubin and disease severity. The relationship between LDH activity and disease severity is a weak positive and significant ($P<0.05$) while the relationship between bilirubin concentration and disease severity is positive, weak and not significant ($P>0.05$).

Table 4.7. Correlation between disease severity, HbF Level, LDH activity and bilirubin concentration

Parameter	Correlation Coefficient	P-Value
HBF – LDH	-0.59*	0.01*
HBF – Bilirubin	-0.05	0.78
HBF – Disease Severity	-0.07	0.69
LDH - Disease Severity	0.50*	0.01*
Bilirubin - Disease Severity	0.02	0.91

LDH: Lactate Dehydrogenase, HbF: Foetal Haemoglobin
* Significant at P=0.05

CHAPTER FIVE

5.0. DISCUSSION

Majority of the participants in this study are less than forty years (40yrs) old, having a moderate disease, low haematocrit count, foetal haemoglobin and red blood cells count while lactate dehydrogenase activity and bilirubin concentration were lower in steady state than in crises state participants.

In addition, most of the participants in our present study were females, a pattern similar to that was observed in a previous study carried out by Okocha*et al*., 2015. This is probably due to a better health seeking behaviour among females especially for the purpose of reproductive health challenges. However, men were the lowest participants in this study. This may be due to low life expectancy in males with SCA compared with females (Platt et al., 1994; Lanzkron et al., 2013). However, there was no significant difference in age between males and female participants (Table 4.1). The mean age of the participants shows that they are young as represented by age distribution (18-43 years). This is attributable to the shorter life expectancy of individuals with sickle cell anaemiain Africa (Serjeant, 2005 and Mpalampa*et al*, 2012). However individuals with SCA can still live a longer life with better management. Infections which are a significant contributor to morbidity and mortality in sickle cell disease needs to be tackled seriously. Hence, the need for introducing Pneumovax ® and Hi-B ®vaccinations against the most common type of infections causing agents like streptococcus and human influenza. (Shaina *et al*., 2014 and Booth *et al*., 2009)**.** Additional measures include provision of potable drinking water, environmental and food sanitation, safe housing and addressing the health and psychological needs of individuals with sickle cell anaemia.

All the participants were anaemic considering the normal levels haematocrit count of 35-55% and red blood cell count of 3.5-5.5% (obtained from haematological analyser). Low haematocrit and red

blood count are a characteristic of individuals with SCA due to lifelong haemolysis. (Individuals with life threatening anaemia will require blood transfusion). Both males and females participants in this study had a low Haematocrit values and red blood cell count which were not significantly different (Table 4.2). The present data is in agreement with previous studies (Akinbami*et al*., 2012). Moreover, WBC and Lymphocytes levels are elevated in people with SCA in the presence of bacterial infections (staphylococcus spp) or virus especially parvo-virus B19 which is specific to humans (Aliyu*et al*., 2008, Ujo*et al*., 2012). Infections is one of the factors that triggers vaso-occlusion in SCA leading to crises (Booth *et al*., 2009). In the present study, WBC and haematocrit counts in both males and females were higher than the normal values of 3.5-10% and 0.5-5.0% respectively for WBC and haematocrit counts (reference values were obtained from haematological analyser). However the values were not significantly different between the two groups. Similar findings was also observed in previous studies conducted by Akinbami*et al*., 2012 and Iheanacho, 2015. The platelet count which plays a key role in blood clothing and vaso-occlusion was normal in both male and female participants and there was no significant difference between the two sex groups. This result is in agreement with the findings by Iheanacho, 2015 and Akinbanmi*et al*., 2012. The implication of these results is that sex has no effect on sickle cell anaemia.

The clinical presentation of Sickle cell anaemia start manifesting around the sixth month of life when HbF production is switched to HbA production (Forget and Bunn, 2013). Individuals who still retained the ability for production of reasonable amount of HbF tend to have a milder disease because of its ability to resist polymerization with HbS. High HbF levels has been defined in earlier studies by Fatunde& Scott-Emuakpor, (1992), El-Hazmi (1992) and by Mpalampa*et al.,* (2012) as foetal haemoglobin levels ≥ 10%. Based on the foregoing definition, individuals from this study can be classified as having a low HbF level (Table 4.3). There was a significant difference between the

persons with sickle cell anaemia in this study and a reference values. This finding was supported by previous findings(Olaniyi*et al*., 2010 and Mikobi*et al*., 2015). Thus individuals with sickle cell anaemia of African originare linkedwith production of low HbF levels and suffered froma relatively severe form of sickle cell anaemia. Unlike individuals of African descent,people with SCA from parts of Asia experience a milder form of sickle cell disease because of relatively higher HbF levels in their red blood cells (Miller *et al.,* 1986; Damanhouri*et al.,* 2015). We reported that HbF level was significantly higher among individuals in steady state than their counter parts in crises state (Table 4.4). This finding agrees with a previous finding of Omoti, 2005.Although the difference between the steady state participants and those in crises state were not significant. This suggest a likely utilization of HbF during crises state by SCA patients. Furthermore,HbF has a higher affinity for oxygen than HbS, which implies that the presence of HbF within a red blood cell reduces the likelihood of sickling and its attendant consequences.

Sickle cell anaemia, a lifelong condition of public health significance is characterised by tissue damage. The higher bilirubin level and LDH activity observed in this study is indicative of a chronic tissue destruction in persons with sickle cell anaemia(Hansen, 2010: damanhouri*et al*., 2014**).** This marked increase of bilirubin level and LDH activity observed in persons with sickle cell anaemia from this studywere significantly different from the reference control values and agrees with a previous studies by Mikobi*et al*, 2015 and Adefehinti*et al*., 2015 (Table 4.3*).* Elevated bilirubin concentration observed in this study has the potential of forming gall stones, this calls for periodic monitoring of liver and gall bladder functions for the proactive diagnosis of gall bladder stones in these individuals with sickle cell anaemia. The increased LDH activity observed is not onlypeculiar to individuals with sickle cell anaemia but also in individuals with heart diseases. A differentiation which can be made by gel electrophoresis which separates the different isomers using their

electrophoretic mobilityin an electric field. Increases in LDH activityis not only an indicator of haemolysis but a pointer toward increased pulmonary hypertension (Aliyu*et al*., 2008, Kato *et al*., 2006). This study is however limited due to its inability to define the sources of the lactate dehydrogenase. Period of ill health (crises state) interrupts periods of relative wellness among individuals with sickle cell anaemia (Steady state) (Ballas, 2011 and Bookchin& Lew, 1996). Crises triggered by stress (physical and emotional) infection, extreme weather conditions etc. trigger haemolysis with an attendant rise in bilirubin concentration and LDH activity (Kato *et al*., 2013). Increase in bilirubin concentration clinically manifest in jaundice typically called hyper-haemolysis (Hansen, 2010). There was a significant difference in the LDH activity between the steady state individuals and the crises state individuals. In addition, there was an increase in bilirubin concentration between the steady state individuals and crises state individuals but, it was not significantly different (Table 4.4).The increased in the levels of these markers showed that tissue destruction increased further during crises.Hence, factors such as hypoxia, dehydration etc. which may have the tendency of triggering crises should be avoided.This pattern of marked increase in bilirubin concentration and LDH activity observed in crises state individuals the present study was in agreement with the findings of Adefehinti*et al*., 2015 and Kato *et al*., 2013.

Although intermittent ill health, Jaundice and anaemia dominate the clinical consequences of sickle cell anaemia; the clinical picture ranges from asymptomatic, well looking individuals to one of a repeatedly ill hospital care seeking individual. Thus, individuals were classified into severity groups by a similar tool used by Okocha*et al*., 2015 and Mikobi*et al*., 2015(Appendix 1). Clinical data was used in the classification of the individuals into different severity groups. Fromour result there was a significant reduction in theRBC and haematocrit values. This could confirm the presence of anaemia caused by haemolysis of sickle RBCs there by reducing the number of the red blood cells (Mikobi*et*

al., 2015). There was also a significant increase in WBC. This could possibly be due to increase production of antibodies against various infections. The pulse rate, blood pressure and BMI were all normal suggesting normal heart functions. From the disease severity classification tool, we found out that about 73% of the study individuals have moderate disease severity, 8.1% had have a mild severe disease while 18.9% had a severe form of sickle cell disease (Table 4.5). We observed also that the HbF level was highest in the mild group and it decreases as the severity of the disease increases from mild to severe. Likewise, the tissue destruction markers showed a similar progressionas severity increases. However the level of the LDH and bilirubin increased from the mild group to the severe group, indicatingthat tissue destruction was highest in the severe group and least in the mild group. Similarly SCA individuals with high HbF level like those from eastern parts of Saudi Arabia are known to experience a mild disease compared to individuals with SCA from African origin(Al-Sultan*et al.,* 2014).

In view of the gaseous transport functions of red cells, there was a weak, negative but significant relationship between disease severity and red blood cell count (RBC) which confirms the fact that oxygen delivery not only sustain life but also assures the well-being of the individual. Worsening anaemia on the other hand is an indicator of poor disease outcome. Tissue damage from a combination of hypoxia and infection (on account of splenic dysfunction) triggers an inflammatory response with consequentincrease in white blood cell count (WBC), lymphocyte counts and platelet count significant manner for WBC and lymphocyte only. Elevation in WBC, platelet count and lymphocyte count are of negative prognostic outcomes as they are pointers to emergence of a severe complications like pulmonary complication (Afzal*et al.,* 1999).

Drugs such as hydroxyurea which can increase HbF level and reduce the WBC count, lymphocyte count and platelet count. hydroxyurea is currently used for the management of this disease (Davies

and Gilmore, 2003 and Platt, 2008). The pulse rate, systolic and diastolic blood pressure have a weak positive relationship with disease severity that was not significantly different. These indicators reflect normal cardiovascular and renal functional state of the individual.

Foetalhaemoglobin is known to be the most important modulator of crises in individuals with sickle cell anemia (Aimola*et al*., 2014;Steinberg, 2014).The HbF level differ among individuals with SCA: some have low HbF level while others have high.This variationcan be traced to differences in the ability to express this HbF. Individuals with a high level of HbF, like individuals from southern part of Saudi Arabia are known to experience mild symtoms of the disease compared with other SCA individuals from other part of the world like Africa (Abdullah *et al*., 2017). Tissue destruction also varies between individuals with SCA. Concentration of HbS alongside HbF level in a red blood cell determine the degree of polymerizationin SCA. HbF containing cells are known to resist polymerization caused by HbS during anorexic conditions (Steinberg *et al*., 2005). Anaemia and tissue damages which are hallmark of sickle cell anaemia can be influenced by the level of HbF in the red blood cell. Our findings showed that there was a significant and inverse relationship between HbF level and LDH activity in the study population (Table 4.7). ThisHbF has the ability of reducing tissue damages brought about by hypoxia and vaso-occlusion. This observation is in harmony with findings by Collela and Traina, 2015. However we observed that there was a weak and inverse relationship between HbF level and bilirubin concentration that was not significantly different. The relationship between HbF level and severity of disease was weak, inversely related and not significant. Though the relationship between HbF level and disease severity was weak, our findings showed that increase in HbF level tend to decrease the severity of the disease. This is similar to previous study carried out byAdegokeand Kuti, 2013 who reported that HbF level was significantly high in individuals with mild disease.

Severity of sickle cell anaemia varies from one individual to another. Some suffer mild disease while others suffer a moderate or severe disease. BesidesHbF, other factors such as tissue damage determines the severity of diseases in individuals. We observed that there was a direct and significant relationship between LDH activity and disease severity. This means tissue damages tends to increase as disease severity increases. This finding agrees with the previous study(Adefehinti, 2015). There was also a weak and direct relationship between bilirubin concentration and disease severity which was not significant. The implication of thisfinding is that severity of the diseases increases with increase in bilirubin concentration and tissue destruction.

CHAPTER SIX

6.0. CONCLUSION AND RECOMMENDATION

6.1. CONCLUSION

It can be concluded therefore from this study that, disease severity and tissue destruction show an inverse relation to HbF level;there is a decrease in tissue damage and severity of disease in the presence of increase level of HbF and vice versa in the presence of decrease level of HbF. Similarly disease severity and tissue destruction showed a direct relationship: Severity of disease increases as tissue destruction increases in the individual.

6.2. RECOMMENDATION

- There is a need to standardize the protocol for the determination of HbF level by alkali denaturation method. This method when compared to HPLC technique is cheaper and also sensitive.
- Standard value for LDH activity for individuals with SCA which is a good marker of tissue destruction and disease severity should be developed and standardized in Nigeria.
- A uniform clinical markers that can be used to measure disease severity of individuals with sickle cell anaemia in Nigeria should be developed.

APPENDIX

Table 8. Disease Severity Classification Tool

Parameter/marker of severity	Standard value	Value for scoring parameters
Pulse (beats/min)	60 – 100	> 100= 2
Systolic blood Pressure(mmHg)	< 120	> 120 = 2
Diastolic blood Pressure(mmHg)	< 80	> 80 = 2
BMI(kg/m^2)	19 – 27	< 19 = 2
RBC ($x10^{12}/L$)	3.50 – 5.50	3.5 – 5.5 = 0, < 3.5 = 2
WBC ($x10^9/L$)	3.50 – 10.0	3.50 -10.0 = 0, >10 = 2
Haematocrit (%)	35.0 – 55.0	35 - 55 = 0, < 35.0 = 2
Lymphocyte ($x10^9/L$)	0.5 – 5.0	0.5 - 5.0 = 0, > 5.0 =2
Platelet ($x10^9/L$)	100 – 400	100 -400 =0, >400=2
Type of Complication suffered from birth.		General pain = 1 Leg ulcer = 2 Aseptic necrosis = 2
Number of Hospital Visits within a year		1-3 = 1, 4-5 = 2 >5 = 3
Disease Severity		**1-7 = mild** **8- 12 = moderate** **13- 23 = severe**

HEALTH RESEARCH ETHICS COMMITTEE

AHMADU BELLO UNIVERSITY TEACHING HOSPITAL

SHIKA - ZARIA, NIGERIA.

E-mail: abuthshika@yahoo.com website: www.abuth.org

ABUTH/HREC/TRG/36 18th April, 2016

Our Ref: *Date:*

Your Ref:

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

RE: ***"Assessment of the Relationship Between foetal Haemoglobin Levels, Lactate Dehydrogenase activity and Clinical Status of Sickle Cell Patients"***

ABUTH Ethics Committee assigned number:	-	**ABUTH/HREC/ U 12 /2016**
Name of the principal Investigator:	-	**Mr. Ishaya Dogonza Yusuf**
Address of the Principal Investigator:	-	**Department of Biochemistry**
		A.B.U-Zaria
Date of receipt of valid application:	-	**18/3/16**
Date of meeting when final determination		
On ethical approval was made:	-	**18/4/16**

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

Please note: this approval dates from ***18th April, 2016*** **to18th April, *2017***. No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: http://www.nhrec.net.

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

Prof. Aisha.I.Mamman,MBBS,FMCpath.
Chairperson HREC.

QUESTIONNAIRE

SECTION A: BIODATA

Participant identification number……..
Sex : Male ☐ Female ☐
Age……
Occupation……

SECTION B: CLINICAL DATA

I. How many times have you visited the hospital in the last one year? ……..

II. Have you had surgery in the last one year? Yes ☐ No ☐

III. If yes, what type of surgery ……..

IV. Are your eyes yellow today? Yes ☐ No. ☐

V. Do you use hydroxyurea? Yes ☐ No ☐

VI. Are you on any traditional medication? Yes ☐ No. ☐

VII. Do you take any pain medication between crises? Yes ☐ No. ☐

VIII. Are you ill today? Yes ☐ No. ☐

IX. Have you have a history of blood transfusion in the last one year? Yes ☐ No ☐

X. Do you have a history of sickle cell anaemia complications? Yes ☐ No. ☐

If yes, please tick type of complication you have previously encountered.

Painful crises	Aseptic necrosis	Leg ulcers
Enlarge heart	Joint pains	Others

If others, please state types of complications……

Part of body pain mostly affects…….

XI. Do you smoke cigarette or any other form of tobacco? Yes ☐ No. ☐

XII. Do you have any other major health challenges (e.g. hepatitis, etc.)? Yes ☐ No. ☐

If yes state type of health challenge.....

XIII. Other parameters.

Weight…… Height…….

Pulse rate…… Blood Pressure……..

Organ enlargement…….

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