Assessment of Mutation Pattern in a Gene Associated with HbF Expression among Sickle Cell Disease in Ibadan South-west, Nigeria

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Certification

This is to certify that Elizabeth Oluwatimilehin **ABODUNRIN** with Matric No. **LCU/PG/001248** carried out this research work titled "Assessment of Mutation Pattern in a Gene Associated with HbF Expression among Sickle Cell Disease in Ibadan, South west, Nigeria" in the Department of Biological Science, Faculty of Natural and Applied Sciences, Lead City University Ibadan, Oyo state, for the award of a Master Degree (M.Sc) in Molecular Biology and Genomics and this has not been previously submitted.

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Dedication

This research work is dedicated to God Almighty, those who paved way for learning process in research and to my lovely PARENTS

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I want to acknowledge the Biological Science Department of Lead City University, University College Hospital, Adeoyo Teaching Hospital and Sickle Cell Foundation, for the unrestrained support and management.

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Even though the above-mentioned institutions and persons have assisted in the process of this research, I alone stand responsible for the errors, if any, found in the work.

Abstract

This study aimed at assessing the level of Foetal Haemoglobin (HbF) in among Sickle Cell Patients in the Ibadan, determined the occurrence of the SNP(rs66650371) responsible for elevated HbF levels and investigated the relationship between the SNP type, HbF level and SCA severity in the SCD patients. Relationship between foetal haemoglobin and SCD is poorly studied. Hence, this study provides the data needed for future research on HbF variants among SCD patients and its severity. This is a cross-sectional hospital based study, blood samples were collected from the university college hospital and Adeoyo state hospital in Ibadan respectively. The socio-demographic information and SCD severity was evaluated from 260 SCD patients, based on the frequency of significant painful episodes, blood transfusion, acute chest symptoms and leg ulcers in the past one year. HbF and haematocrit levels were determined. Amplification Refractory-Mutation System (ARMS) PCR was performed to determine the 3-bp deletion in the HBS1L-MYb gene. The mean HbF levels of 260 patients is 4.9% ± 2.4, about 50% of the population had low HbF level, patients with elevated levels of HbF had increased PCV counts, reduced vaso-occlusive crises in a year, reduced acute chest syndromes and reduced leg ulcer. The allelic frequency of the rs66650371 is three percent. rs66650371 SNP was associated with elevated level of HbF and reduced disease severity, The study demonstrated the beneficial effect of the rs66650371 SNP in Nigerian patients. Facilities for early and regular quantification of foetal haemoglobin should be made available in Sickle cell Clinics and Hospitals.

Keywords – Foetal Haemoglobin, Sickle Cell Disease (SCD), ARMS-PCR, rs66650371, SNP, vaso-occlusive, allelic frequency
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List of Acronyms

Abbreviation	**Meaning**
S.C.D	Sickle Cell Disease

S.C.A	Sickle Cell Anemia
H.B.F	Fetal Haemoglobin
ARMS - PCR	Amplification Refractory Mutation System-polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
P.C.R	Polymerase Chain Reaction
D.N.A	Deoxyribonucleic Acid
P.C.V	Packed Cell Volume
H.b.S.S	Sickle Haemoglobin
H.B	Haemoglobin
H.B.B	Hemoglobin beta-chain
S.C.T	Sickle Cell Trait
V.O.C	Vaso-Occlusive Crisis
R.B.C	Red Blood Cell
W.B.C	White Blood Cell
Q.T.L	Quantitative Trait Loci
M.C.V	Mean Corpuscular Volume
H.P.L.C	High Performance Liquid Chromatography
H.b.A	Haemoglobin A

Chapter One

1.1 Background to the Study

As a monogenetic blood disorder, sickle cell disease (SCD) is characterized by clinical heterogeneity, which can be influenced by a combination of genetic, epigenetic, and environmental factors [1]. More than 70% of cases of Sickle Cell Disease (SCD) worldwide are caused by sickle cell anaemia (SCA)[2]. Sickle-cell disease is a major global public health issue and the most common life-threatening genetic disorder worldwide. However, despite being the most prevalent genetic disease in Africa, the serious health and socio-demographic consequences are largely ignored[3].

In SCD, the oxygen-carrying protein haemoglobin is abnormally altered in red blood cells, resulting in an autosomal recessive genetic blood disorder. Those with HbS develop deformed red blood cells (erythrocytes), which become hard and sticky under stress and resemble a C-shaped farm tool called a 'sickle'. Due to the short lifespan of sickle cells, anaemia is often caused by the constant shortage of red blood cells[4,5].

In the sixth codon of the human β globin gene (HBB), the point mutation of GAG to GTG replaces glutamic acid with valine, leading to the formation of HbS[6]. The haemoglobin molecule on chromosome 11 is formed by substituting the hydrophilic amino acid glutamic acid with the hydrophobic amino acid valine at position six[7], HbS becomes less soluble and forms long fibers in the deoxygenated state.

It has been estimated that approximately 90 percent of the world's SCD population lives in three countries: Nigeria, India, and the Democratic Republic of Congo[8], where the disease affects 2% of the population and carriers (sickle cell trait) account for 10 to 30% of all cases[9]. In Nigeria alone, there are estimated to be 150,000 babies born with SCD every year, which is 20 per 1000 births. It is difficult to estimate the prevalence of SCD because there are no federally funded new-born screening programs; however, approximately 700,000 births occur

each year and the prevalence of SCD among new-borns was 3 percent in a regional new-born screening program[10].

During SCD, vaso-occlusion is a predominant pathophysiological feature. A major part of the mechanism for vaso-occlusion is the entrapment of erythrocytes and leucocytes in microcirculation following repeated polymerization of HbS, resulting in vascular obstructions and the release of inflammatory mediators, which stimulate afferent nerve fibers and cause pain[11]. Vaso-occlusion encompasses adherence of circulating blood elements to endothelial cells, endothelial dysfunction, hypercoagulability, hypoxemia, altered nitric oxide bioavailability, and ischemia-reperfusion injury[12].

These sequences of events lead to complications such as episodes of pain, acute chest syndrome, and cerebrovascular disease. These events usually evolve to involve multiple organs, including the bone, lungs, central nervous system, cardiovascular system, spleen, skin, and kidneys[13].

In order to establish a clinical classification based on severity, it is necessary to search for factors responsible for the clinical variability of SCD. By using this classification, management can be optimized and follow-up matched to each patient's actual risk[10]. As a result, understanding the genetics underlying the heritable sub-phenotypes of SCD, specifically for each population, would be useful for prognosis and could help to tailor therapeutic interventions. Numerous studies have been devoted to genetic modulating factors of SCD[13]. Foetal haemoglobin (HbF) concentration is one of the major modifier of the Sickle Cell Disease which directly affects the sickle erythrocyte and is a major modulator of the phenotype of the disease[14]. Foetal hemoglobin (HbF) concentration is the most powerful modifier of the clinical and haematological features of the sickle cell disease (SCD)[15]. Understanding the genetics underlying the heritable sub phenotypes of sickle cell anemia

would be prognostically useful. This could inform personalized therapeutics, and might help the discovery of new "drug gable" pathophysiologic targets[15].

Foetal haemoglobin levels are heritable and highly variable. HbF production peaks in utero following the first switch of haemoglobin from embryonic to foetal, and rapidly declines soon after birth to predominant adult haemoglobin[14]. The latter switch consists of a decline in the foetal haemoglobin tetramer (HbF: α2γ2) to an adult tetramer containing two α-like and two β-like globin subunits (HbA: α2β2). However, there are adults with hereditary persistence of foetal haemoglobin in whom HbF production is not switched off and present with apparently normal red cell morphology with pan cellular distribution of HbF in the erythrocytes[16].

In normal adults, HbF levels are distributed in a non-uniform way in the red cells with a range varying from 0.1 to 7% of total haemoglobin (Hb). In red cells producing higher HbF (termed F cells), HbF is elevated (around 25% of cellular Hb) and genetically determined[17]. In most adults with sickle cell anemia, HbF levels are increased; however, the magnitude of this increase is very variable. HbF production is restricted to a small number of erythroid precursors; their progeny in the blood are called F-cells. Both HbF concentration and its distribution among erythrocytes are heritable. One of the major ameliorating factors of clinical severity in SCD, is the persistence of HbF (α2γ2) production[18].

Foetal haemoglobin (HbF; α2γ2), encoded by two nearly identical -globin genes (HBG2, HBG1) that are part of the _-globin gene (HBB) cluster (11p15.4), comprises 70 to 90% of the hemolysate in new-borns, falling to <1% after 12 months[19]. The "switch" from foetal to adult haemoglobin synthesis in sickle cell anaemia (homozygosity for the sickle haemoglobin gene) takes 5 to 10 years and is rarely complete.

An indication that additional variation at the b globin cluster is responsible for some of this variability came from the discovery of the 'sickle b haplotypes', and that the β^s gene on certain β^s haplotypes are associated with higher HbF levels and a milder disease[10]. HbF

response is also variable in β thalassemia[13]. Although some of this variability can be explained by the specific b thalassemia mutation itself, and the b chromosomal background[9], a substantial proportion of the HbF increase is clearly unlinked to the HBB cluster[15]. In some cases, the levels of HbF increase is sufficient to compensate for the complete lack of HbA, resulting in mild disease, i.e. β^0 thalassemia intermedia and no transfusion dependence[8].

Foetal haemoglobin (HbF) one of the most characterized modulators of clinical expression of SCD indicates that elevated HbF levels have been associated with reduced rates of acute pain episodes, leg ulcers, less frequent acute chest syndromes, and reduced disease severity heritability[20], this is possibly because increased HbF level modified the polymerization kinetics and increase the delay time to polymerize the HbS-F mixture.

HbF levels have no clear association with other SCD clinical manifestations such as stroke and silent cerebral infarction, priapism, urine albumin excretion, and systemic blood pressure several other genetic modifiers[15] contributing to the variation in clinical expression of SCD have also been identified Homozygosity for HbS, or sickle cell anemia, is the most common genotype. Compound heterozygotes for HbS and other haemoglobin variants, like HbC (*HBB* glu6lys; HbSC disease), or with one of the many forms of β thalassemia (HbS- β thalassemia), usually have milder disease than HbS homozygotes because of the reduced intracellular concentration of HbS[21,22].

In sickle cell disease, deoxy sickle haemoglobin polymerization is essential to the disease's pathophysiology. In addition to reducing HbS concentration, HbF and its mixed hybrid tetramer (β2S) cannot enter the phase of deoxy sickle haemoglobin polymerization[19]. A hybrid tetramer containing both the βS and βA chains has only half the probability of entering the polymer as HbS, which explains the unique value of HbF compared to other haemoglobins. HbF inhibits polymerization primarily through the residues glycine β87 and

aspartic acid β80 of HBG (β-globin genes). As a result of inhibiting the polymerization of deoxy sickle hemoglobin, sufficient HbF inhibits the damage induced by HbS polymers[22].

In designing gene therapy vectors, additional substitutions can be added to recombinant HbF and HbA molecules to enhance their ability to inhibit polymerization. Alternatively, the natural mutant hemoglobin, HbS-Antilles (HBB glu6val; val23ile), has enhanced polymerization tendencies; whereas individuals with sickle cell trait are symptomatic, homozygotes have severe sickle cell disease[23].

In Sickle Cell Disease, high γ-globin expression inhibits HbS polymerization and α-globin precipitation, respectively[23]. Point mutations at the promoter of the γ-globin gene and deletions within the β-globin gene cluster can result in increased levels of HbF. However, HbF levels are also affected by genetic factors outside of the β-globin gene cluster.

Five typical haplotypes have been described across the β -globin gene cluster based upon the pattern of specific restriction fragment-length polymorphisms across the region[24]. Four haplotypes are associated with HbS in Africa (Benin, Bantu/Central African Republic, Senegal and Cameroon) and the fifth is thought to have arisen in India and/or the Arabian Peninsula (Arab/Hindu)[25].

It has been suggested that these haplotypes also have an effect on the severity of the disease through their genetically determined effect on HbF level[26]. Very low HbF and haematocrit are associated with SCD individuals carrying Bantu (Central African Republic) haplotype and they mostly present with severe clinical complications[25]. Meanwhile, clinical presentations of individuals with Senegal or Arab–India haplotype are usually mild due to high levels of HbF and haematocrit. Clinical course of persons with Benin haplotype is in between the Bantu and Arab/India haplotypes. Benin haplotype, associated with low Hb F level, is the predominant haplotype in Nigeria with a frequency of 93.2–97%[27]. The mean HbF among Nigerian SCD patients is about 6%[28].

Genetic association at the three principal loci have been identified, SNPs in major loci that are associated with different HbF levels in patients with SCD and in healthy adults. The BCL11A gene is located on chromosome 2 (2p16) and the HBS1L-MYB intergenic region on chromosome 6 (6q23)[29]. The polymorphisms of BCL11A gene that have been described and are associated with variable HbF levels that are located within the second intron of this gene. These genes have shown the influence HbF levels and disease severity in SCA. Taking into account these loci, there is still substantial residual variance in HbF levels, suggesting the importance of other quantitative trait loci (QTL) modulating HBG expression[30].

The HBS1L-MYB intergenic polymorphisms (HMIP) are present in three linkage disequilibrium (LD) blocks with most of the effect on HbF levels and numbers of F cells contributed by the second block[31]. Among the causes of unusually high HbF levels are deletions or single base substitutions in the HBB gene cluster and single nucleotide polymorphisms (SNPs) in the genes. *HBS1L-MYB*: known main HbF sub-loci in this region, *HMIP-2A* (tagged by *rs66650371*). The SNP rs66650371, the functional 3-bp deletion in the MYB enhancer, is one of the most significant SNPs in the HMIP region[32].

As a result of polymorphisms in the HMIP region, normal Europeans accounted for 19.4% of the variance in F-cell levels. In HMIP blocks 1, 2, and 3, SNPs were distributed; however, the sequences of HBS1 and MYB and other genes in these blocks did not indicate HbF regulation.45 However, MYB and HBS1L expressions were down-regulated in adults with HPFH non-gene deletion. Nevertheless, the expression profile of *MYB* and *HBS1L* in adults with non-gene deletion HPFH was down-regulated.

In K562 cells, overexpression of MYB inhibited HBG expression.46 Lower MYB levels were associated with reduced cell expansion and accelerated erythroid differentiation, suggesting that variations in MYB levels might affect HbF by affecting the cell cycle.

It was recently reported that the most significant functional motif accounting for HMIP modulation of HbF is a 3-bp deletion polymorphism, which is in complete linkage disequilibrium with the SNP rs9399137 shown by several GWASs to be most significantly associated with HbF in Africa. It is located near erythroid-specific DNase I hypersensitive site 2 within the HMIP block 2, 42.6 kb upstream of *HBS1L* and 83.8 kb upstream of *MYB*. In close proximity to the 3-bp deletion polymorphism, there is binding of 4 erythropoiesis-related transcription factors, *TAL1*, *E47*, *GATA2*, and *RUNX1*. Furthermore, the short DNA fragment encompassing the 3-bp deletion polymorphism appears to have enhancer-like activity based on in vitro transient transfection experiments

There is also a significant association between HbF and HBS1L-MYB intergenic polymorphism among sickle cell anaemia patients of African descent, but much less so than with European or Chinese individuals due to the low frequency of minor alleles[20]. In people of African descent, there may be other variants of HMIP associated with HbF level that are not well tracked by SNP rs9399137.

Currently, hydroxyurea (HU) is the only Food and Drug Administration (FDA USA)-approved[33] pharmacologic treatment for induction of HbF in patients with SCD. HU benefits primarily comes from its HbF-Producing effects[34] that reduces pain, acute chest episodes, the necessity for blood transfusions and mortality. Several studies have demonstrated variability in the response to HU treatment, with induced HbF levels ranging from 10% to greater than 30%[35]. Some of this variance has been attributed to variants at principal HbF-promoting loci like BCL11A and HMIP[36]. However, despite this, a complete understanding of the molecular mechanisms by which HU induces HbF are not fully understood. In the first randomized controlled trial with hydroxyurea therapy in Nigeria, high adherence rate to HU in children with abnormal TCD was demonstrated[37].

1.2 Statement of the Problem

A very high infant mortality due to the genetic disease occurs especially in rural areas. Poor availability of resources to the public health and welfare sectors and economic inflation are severely curtailing access to appropriate medical and social services. Up to now, most genetic research on the foetal haemoglobin of sickle cell disease has taken place in the US and Europe while the great majority of patients live in Nigeria[3]. More genetic and epidemiological studies can help to address the variability in the clinical severity of SCA and factors that affect HbF levels.

1.3 Justification of the Study

Foetal haemoglobin is a major contributor to the phenotypic heterogeneity of Sickle Cell Anaemia (SCA), a major ameliorating factor is an inherent ability to produce foetal haemoglobin. In patients with SCA, elevated levels are associated with reduced morbidity and mortality. Different approaches have been developed to reactivate HbF synthesis after HBF, but current therapeutic agents are toxic, and gene therapy is still pending. Identification of the genes and genetic variants would help to provide explanation for the following;

i. Variability of HbF production
ii. Variation of phenotypic severity of SCA
iii. Variation in response to therapeutic interventions
iv. Possible therapeutic framework/strategy which may lead to new improved pharmaceutical approaches for HbF augmentation.

1.4 Aim and Objectives of the Study

The aim of this study is to investigate the relationship between the 3-bp deletion of rs66650371, foetal haemoglobin levels and disease severity among individuals with sickle cell anaemia.

The Objectives are to:

i. Determine the foetal haemoglobin concentration of individuals with SCD
ii. Identify the number of SCD individuals with HBSIL-MYB gene
iii. Determine the severity of the HbSS disease in those with the gene type
iv. Identify the association of rs66650371 and HbF among SCD individuals

1.5 Research Questions

1. What is the HbF concentration of individuals with Sickle Cell Disease?
2. What is the frequency of HBSIL-MYB gene variant in Ibadan?
3. What is the severity of HbSS in individuals with HBSIL-MYB?
4. Is there an association between rs66650371 and HbF among Sickle Cell Disease patients in Ibadan, Nigeria?

1.6 Significance of the Study

This study makes some important contributions to providing necessary data important for future researches in identifying the mutation pattern of foetal haemoglobin (HBS1L-MYB gene) among sickle cell disease patients. Knowing the foetal haemoglobin levels of SCD patients in Nigeria will help provide rapid response in administering care in line with boosting HbF levels of patients with confirmed reduced decreased HbF. This will help in the diagnosis, clinical management and in the determination of the clinical course of sickle cell disease. There is need to build the capacity in resource poor countries to optimize the diagnosis of sickle cell disease and other haemoglobinopathies.

There is little genetic counselling available for prospective parents, unions between SCT (Sickle Cell Trait) carriers result in the birth of SCD children. Nigeria has inadequate national health policies and plans, scarce facilities, diagnostic tools, treatment services and trained personnel. There is therefore a need for urgent interventions to address this public health problem.

1.7 Scope of the Study

Elevated levels of foetal haemoglobin has been associated with reduced severity of sickle cell disease and reduced complications associated with high levels of HbF. This study determined the levels of foetal haemoglobin among sickle cell patients. The effect of HbF levels in respect to disease severity was determined. Also, the Amplification refractory mutation system (ARMs) PCR was used to detect the 3-bp deletion polymorphism within the HMIP gene. This 3-bp deletion polymorphism is most probably the functional motif accounting for the increase level of HbF[38]. Accordingly, elevated levels of HbF are associated with longer life expectancy, fewer painful crises, and fewer leg ulcers.

1.8 Limitation of the Study

A major limitation during the study was that continuous and one-on-one orientation had to be giving to each patient, the importance of the research had to be emphasized. Also, there were difficulties faced in the blood collection process, as many of the veins of many of the patients was not easily accessible.

Another limitation of this study was the self-report nature of some clinical variables such as VOC episodes, last Hospitalization date and so on, which can lack precision. In addition, pain tolerance and financial factors could have been limiting factors for hospital attendance[39].

information on the lifetime incidence of complications and frequency of significant pain episodes may have been subject to recall bias, despite the review of relevant

medical charts in addition to clinical histories to obtain that information. Secondly, the relatively small number of patients studied could also affect generalisability of this findings.

Despite the above limitations, this study represents an important step towards the understanding of molecular structure of foetal haemoglobin among SCD patient in Nigeria.

1.9 Operational Definition of Terms

Haemoglobinopathies – Haemoglobinopathies are a group of recessively inherited genetic conditions affecting the haemoglobin component of blood. They are caused by a genetic change (mutation) in the haemoglobin.

Red Blood Cell (RBc) - The red blood cells are highly specialized, well adapted for their primary function of transporting oxygen from the lungs to all of the body tissues. Red cells are approximately 7.8 μm (1 μm = 0.000039 inch) in diameter and have the form of biconcave disks, a shape that provides a large surface-to-volume ratio.

Anaemia - Anaemia is a condition in which you lack enough healthy red blood cells to carry adequate oxygen to your body's tissues. Having anaemia, also referred to as low haemoglobin, can make you feel tired and weak

Sickle Cell Trait - Sickle cell trait (SCT) is not a disease, but having it means that a person has inherited the sickle cell gene from one of his or her parents. People with SCT usually do not have any of the symptoms of sickle cell disease (SCD) and live a normal life. People who have inherited one sickle cell gene and one normal gene have SCT. This means the person won't have the disease, but will be a trait "carrier" and can pass it on to his or her children.

Sickle Cell Anaemia - Sickle cell anaemia is one of a group of inherited disorders known as sickle cell disease. It affects the shape of red blood cells, which carry oxygen to all parts of the body. Red blood cells are usually round and flexible, so they move easily through blood vessels. In sickle cell anaemia, some red blood cells are shaped like sickles or crescent moons. These sickle cells also become rigid and sticky, which can slow or block blood flow.

Sickle Cell Disease - SCD is a genetic condition that is present at birth. In SCD, the red blood cells become hard and sticky and look like a C-shaped farm tool called a “sickle.” The sickle cells die early, which causes a constant shortage of red blood cells. Also, when they travel through small blood vessels, they get stuck and clog the blood flow. This can cause pain and other serious problems. It is inherited when a child receives two sickle cell genes—one from each parent. A person with SCD can pass the disease or SCT on to his or her children.

Foetal Haemoglobin (HbF) - Foetal haemoglobin is the major haemoglobin present during gestation; it constitutes approximately 60 to 80 percent of total haemoglobin in the full-term new born. In individuals without hemoglobinopathies, it is almost completely replaced by adult haemoglobin (haemoglobin A, HbA, alpha2beta2) by approximately 6 to 12 months of age, and it amounts to less than 1 percent of total haemoglobin in adulthood.

Vaso-oclussive crises (VOC) - A vaso-occlusive crisis, occurs when sickled red blood cells block blood flow to the point that tissues become deprived of oxygen. This in turn sets in motion an inflammatory response as the body tries to rectify the problem

Mutation - Mutations are changes in the genetic sequence, which are responsible for diversity among organisms. These changes can occur at many levels, and they can have widely varying effects.

Polymorphism - A discontinuous genetic variation resulting in the occurrence of several different forms or types of individuals among the members of a single species. A discontinuous genetic variation divides the individuals of a population into two or more sharply distinct forms. The most obvious example of this is the separation of higher organisms into male and female sexes. Another example is the different blood types in humans. In continuous variation, by contrast, the individuals do not fall into sharp classes but instead are almost imperceptibly graded between wide extremes.

Monogenesis - Monogenic disorders (monogenic traits) are caused by variation in a single gene and are typically recognized by their striking familial inheritance patterns. Examples include sickle cell anaemia, cystic fibrosis, Huntington disease, and Duchenne muscular dystrophy. By contrast, complex disorders (complex traits) are those in which multiple genes play a role, often together with environmental factors.

Point Mutation - In a genome, point mutation occurs when a single base pair is added, deleted or changed. While most point mutations are benign, they can affect gene expression or encoded proteins in various ways. There are two types of point mutations: transition mutations and trans-version mutations. Transition mutations occur when a pyrimidine base (i.e., thymine [T] or cytosine [C]) substitutes for another pyrimidine base or when a purine base (i.e., adenine [A] or guanine [G]) substitutes for another purine base. In double-stranded DNA each of the bases pairs with a specific partner on the corresponding strand—A pairs with T and C pairs with G.

Amplification Refractory Mutation Systems (ARMS-PCR) - The Amplification Refractory Mutation System PCR (ARMS-PCR) is one of the most accurate tools in genetic disease diagnosis in recent days. It is a gold standard method for thalassemia and sickle cell anaemia. Unlike ARMS-PCR, restriction digestion is not applicable in all types of mutation or polymorphism because of lower accuracy and absence of restriction sites in some sequences.

Single Nucleotide Polymorphism (SNP) - A single nucleotide polymorphism (abbreviated SNP, pronounced snip) is a genomic variation at a single base position in a DNA sequence among individuals. Recall that the DNA sequence is formed from a chain of four nucleotide bases: A, C, G, and T. If more than 1% of a population does not carry the same nucleotide at a specific position in the DNA sequence, then this variation can be classified as a SNP. If a SNP occurs within a gene, then the gene is described as having more than one allele. In these

cases, SNPs may lead to variations in the amino acid sequence. SNPs, however, are not just associated with genes; they can also occur in noncoding regions of DNA. Scientists study if and how SNPs in a genome influence health, disease, drug response and other traits.

DNA - DNA, or deoxyribonucleic acid, is the hereditary material in humans and almost all other organisms. Nearly every cell in a person's body has the same DNA. Most DNA is located in the cell nucleus (where it is called nuclear DNA), but a small amount of DNA can also be found in the mitochondria (where it is called mitochondrial DNA or mtDNA). Mitochondria are structures within cells that convert the energy from food into a form that cells can use. The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). Human DNA consists of about 3 billion bases, and more than 99 percent of those bases are the same in all people. The order, or sequence, of these bases determines the information available for building and maintaining an organism, similar to the way in which letters of the alphabet appear in a certain order to form words and sentences.

Haemolysate - the disintegration of red blood cells, with the release of haemoglobin, occurring in a living organism or in a blood sample. It is the disruption of erythrocyte membranes, which causes the release of haemoglobin. Haemolysis is also defined as erythrocyte necrosis and occurs at the end of every erythrocyte's life.

Globin - The globins are a superfamily of heme-containing globular proteins, involved in binding and/or transporting oxygen. These proteins all incorporate the globin fold, a series of eight alpha helical segments. Two prominent member include myoglobin and haemoglobin. Both of these proteins reversibly bind oxygen via a heme prosthetic group. They are widely distributed in many organisms.

Pathophysiology – Pathophysiology, a convergence of pathology with physiology – is the study of the disordered physiological processes that cause, result from, or are otherwise

associated with a disease or injury. Pathology is the medical discipline that describes conditions typically observed during a disease state, whereas physiology is the biological discipline that describe processes or mechanisms *operating* within an organism. Pathology describes the abnormal or undesired condition, whereas pathophysiology seeks to explain the functional changes that are occurring within an individual due to a disease or pathologic state.

Epigenetics - Epigenetics is the study of how cells control gene activity without changing the DNA sequence."Epi-"means on or above in Greek,and "epigenetic" describes factors beyond the genetic code. Epigenetic changes are modifications to DNA that regulate whether genes are turned on or off. These modifications are attached to DNA and do not change the sequence of DNA building blocks. Within the complete set of DNA in a cell (genome), all of the modifications that regulate the activity (expression) of the genes is known as the epigenome.

Erythrocytes - Erythrocytes contain the pigment haemoglobin, which imparts the red colour to blood and the vertebrate's principal means of delivering oxygen to the body tissues—via blood flow through the circulatory system. They are the most common type of blood cell.

Leukocyte - A type of blood cell that is made in the bone marrow and found in the blood and lymph tissue. Leukocytes are part of the body's immune system. They help the body fight infection and other diseases. Types of leukocytes are granulocytes (neutrophils, eosinophils, and basophils), monocytes, and lymphocytes (T cells and B cells). Checking the number of leukocytes in the blood is usually part of a complete blood cell (CBC) test. It may be used to look for conditions such as infection, inflammation, allergies, and leukemia. Also called WBC and white blood cell.

Priapism - Priapism is a prolonged erection of the penis. The full or partial erection continues hours beyond or isn't caused by sexual stimulation. The main types of priapism are ischemic and non-ischemic. Ischemic priapism is a medical emergency. It is the result of blood not

being able to leave the penis. Blood is trapped in the penis because it cannot flow out of the veins of the penis or there is a problem with the contraction of smooth muscles within the erectile tissue of the penis.

Haematocrit - The haematocrit (Ht or HCT), also known by several other names, is the volume percentage (vol%) of red blood cells (RBCs) in blood, measured as part of a blood test. The measurement depends on the number and size of red blood cells. It is normally 40.7–50.3% for males and 36.1–44.3% for females. It is a part of a person's complete blood count results, along with haemoglobin concentration, white blood cell count and platelet count.

Quantitative trait Loci (QTL) - A quantitative trait locus (QTL) is a region of DNA which is associated with a particular phenotypic trait, which varies in degree and which can be attributed to polygenic effects, i.e., the product of two or more genes, and their environment.[2] These QTLs are often found on different chromosomes. The number of QTLs which explain variation in the phenotypic trait indicates the genetic architecture of a trait. It may indicate that plant height is controlled by many genes of small effect, or by a few genes of large effect. Typically, QTLs underlie continuous traits (those traits which vary continuously, e.g. height) as opposed to discrete traits (traits that have two or several character values, e.g. red hair in humans, a recessive trait, or smooth vs. wrinkled peas used by Mendel in his experiments).

Mean Corpuscular Volume (MCV) - MCV stands for mean corpuscular volume. An MCV blood test measures the average size of your red blood cells. Red blood cells carry oxygen from your lungs to every cell in your body. Your cells need oxygen to grow, reproduce, and stay healthy. If your red blood cells are too small or too large, it could be a sign of a blood disorder such as anaemia, a lack of certain vitamins, or other medical conditions.

Mean corpuscular haemoglobin concentration (MCHC) – It is a measure of the average concentration of haemoglobin inside a single red blood cell. MCHC is commonly ordered as part of a complete blood count (CBC) panel. It is calculated by dividing the haemoglobin with the haematocrit. It is thus a mass or molar concentration. Still, many instances measure MCHC in percentage (%), as if it were a mass fraction (m_{Hb} / m_{RBC}). Numerically, however, the MCHC in g/dL and the mass fraction of haemoglobin in red blood cells in % are identical, assuming an RBC density of 1g/mL and negligible haemoglobin in plasma.

Haplotype - A haplotype is a physical grouping of genomic variants (or polymorphisms) that tend to be inherited together. A specific haplotype typically reflects a unique combination of variants that reside near each other on a chromosome. A haplotype is in its most general sense referring to a set of DNA variations along a chromosome that tend to be inherited together because they're very close together. They get inherited together because they're not generally crossovers or recombinations between these markers or between these different polymorphisms because they are very, very close. So a haplotype can refer to a combination of alleles in a single gene, or it could be alleles across multiple genes. It could be single nucleotide polymorphisms that are not in a gene but are in-between genes.

Variant - An alteration in the most common DNA nucleotide sequence. The term variant can be used to describe an alteration that may be benign, pathogenic, or of unknown significance. The term variant is increasingly being used in place of the term mutation.

Gene Therapy - Gene therapy is a medical approach that treats or prevents disease by correcting the underlying genetic problem. Gene therapy techniques allow doctors to treat a disorder by altering a person's genetic makeup instead of using drugs or surgery.

F-cells - True foetal erythrocytes containing foetal amounts of HbF can also occur in the adult circulation during the leakage of HbF-containing cells from the foetus to the maternal

circulation. In normal adults, HbF is restricted to a small proportion (3-7%) of red blood cells (RBC), termed 'F cells

Leucocytosis - Leukocytosis is usually an indicator of an underlying inflammatory, infectious, or neoplastic process. Although lymphocytosis is most often associated with chronic lymphatic leukaemia, neutrophilia or monocytosis of modest degree is most frequently associated with inflammation or infection. Marked elevation in the neutrophil and monocyte count raises the possibility of chronic leukemia. Moderate leucocytosis, involving mature white cells, carries no significant increase in perioperative risk.

Cross- sectional study - A cross-sectional study involves looking at data from a population at one specific point in time. The participants in this type of study are selected based on particular variables of interest. Cross-sectional studies are observational in nature and are known as descriptive research, not causal or relational, meaning that you can't use them to determine the cause of something, such as a disease. Researchers record the information that is present in a population, but they do not manipulate variables.

Vaso-Constriction - Vasoconstriction is the narrowing (constriction) of blood vessels by small muscles in their walls. When blood vessels constrict, blood flow is slowed or blocked. Vasoconstriction may be slight or severe. It may result from disease, drugs, or psychological conditions. Medicines that cause vasoconstriction include: Decongestants, including pseudoephedrine, Cough and cold combinations

Pathology - Pathology is a branch of medical science that involves the study and diagnosis of disease through the examination of surgically removed organs, tissues (biopsy samples), bodily fluids, and in some cases the whole body (autopsy). Aspects of a bodily specimen that may be considered include its gross anatomical make up, appearance of the cells using immunological markers and chemical signatures in the cells. Pathology also includes the

related scientific study of disease processes whereby the causes, mechanisms and extent of disease are examined.

Inflammation - Inflammation refers to a biological response to stimuli interpreted by the body to have a potentially harmful effect. Inflammation is a normal, healthy response to injury, infections or certain other medical conditions. An *inflammatory disorder*, however, is where the immune system mistakenly attacks your body's own cells or tissues. This causes abnormal inflammation that can result in chronic pain, redness, swelling, stiffness and damage to otherwise healthy body tissues

Quantitative trait Loci (QTL) - A quantitative trait locus (QTL) is a region of DNA which is associated with a particular phenotypic trait, which varies in degree and which can be attributed to polygenic effects, i.e., the product of two or more genes, and their environment.[2] These QTLs are often found on different chromosomes. The number of QTLs which explain variation in the phenotypic trait indicates the genetic architecture of a trait. It may indicate that plant height is controlled by many genes of small effect, or by a few genes of large effect. Typically, QTLs underlie continuous traits (those traits which vary continuously, e.g. height) as opposed to discrete traits (traits that have two or several character values, e.g. red hair in humans, a recessive trait, or smooth vs. wrinkled peas used by Mendel in his experiments).

Endnote

[1] O. Oyesola, A. Titilope, C. Oluseyi & A. Adekunle, *Current Perspectives of Sickle Cell Disease In Nigeria: Changing The Narratives*, **Expert Review Of Hematology,** 12(8), 2019, 609-620.

[2] F. Piel, M. Steinberg, M. Rees. *Sickle Cell Disease*. **N Engl J Med**, 376(16), 2017, 1561–1573.

[3] Michael. R & Najibah. A. *Sickle Cell Disease In Sub-Sahara Africa*, Available Online: Https://Www.Uptodate.Com/Contents/Sickle-Cell-Disease-In-Sub-Saharan-Africa/2021

[4] E. Wastnedge & I. Rudan, *The Global Burden Of Sickle Cell Disease In Under 5 Years Of Age: A Systematic Review And Meta-Analysis*". **Journal Of Global Health** 8(2), 2018: 2-10

[5] G. Kato, F. Piel, C. Reid Cd, & M. Gaston. *Sickle Cell Disease'*. **Nat Rev Dis Primers** 4(10), 2018 :18010-18017

[6] C. Guarda, S. Hou, R. Santiago, J. Neres, C. Fernandes, & M. Goncalve. *Sickle Cell Disease: A Distinction Of Two Most Frequent Genotypes (Hbss And Hbsc)*. **Plos One** 15(1), 2020: 1-15

[7] A. Wjdon. & A. Harri. *Techniques For The Detection Of Sickle Cell Disease: A Review*"**Micromachines Basel** 12 (5), 2021: 519-524.

[8] F. Piel, S. Hay, S. Gupta, D. Weatherall & T. Williams. *Global Burden Of Sickle Cell Anaemia In Children Under Five, 2010-2050: Modelling Based On Demographics, Excess Mortality, And Interventions*". **Plos Med**. 10(7), 2013; 1484-1490

[9] O. Chinwe, N. Obageli, C. Odoh. *Hsc and Mirna Regulation with Implication for Foetal Haemoglobin Induction In Beta Haemoglobinopathies,* **Ingenita**, 17(14), 2022, 339-347

[10] C. Okunmdi, *Sickle Cell Disease Child Mortality - A Silent Epidemic In Nigeria: Issues In Political Economy,* Available Online: Https://Gdc.Unicef.Org/Resource/Sickle-Cell-Disease-Child-Mortality-Silent-Epidemic-Nigeria-Issues-Political-Economy.2018

[11] S. Veluswamy, P. Shah, C. Christopher, C. Michael, D, Thomas, *Vaso-Occlusion In Sickle Cell Disease: Is Autonomic Dysregulation Of The Microvasculature The Trigger?* **Journal Of Clinical Medicine** 8(10), 2019, 1690-1701

[12] C. Brown, M. Tonda & Miguel R. Abboud. *Voxelotor For The Treatment Of Sickle Cell Disease In Pediatric Patients*, **Expert Review Of Hematology**, 15(6), 2022, 485-492

[13] L. Jennifer, K. Pharm, A. Koyfarm, D. Hayes, *High Risk And Low Risk Prevalence Disease: Acute Chest Syndrome In Sickle Cell Disease* **The American Journal Of Emergency Medicine** 58(10), 2022, 235-244.

[14] T. Qadah. "*Detection Of BCl11a And HBS1L-MYB Genotypes In Sickle Cell Anemia."* **Indian Journal Of Hematology & Blood Transfusion**, 36(4), 2020, 705-710.

[15] T. Adeyemo, O. Oyesola, C. David. & M. Stephen. *A Survey Of Genetic Foetal-Haemoglobin Modifiers In Nigeria Patients With Sickle Cell Anaemia*". **Plos One** 13(5), 2018, 6-10

[16] H. Pandey, R. Ranjan R, Singh K. *Contrasting Co-Inheritance Of Alpha And Beta Mutations In Delta Beta Thalassemia And Hereditary Persistence Of Fetal Hemoglobin: A Study From India*. **Haematology**.;23 (9), 2018, 692–696

[17] S. L. Thein & S. Menzel, *Discovering The Genetics Underlying Foetal Haemoglobin Production In Adults,* **British Journal Of Haematology** 145(10), 2019, 455-467

[18] G. Stamatoyannopoulos, *Control Of Globin Gene Expression During Development And Erythroid Differentiation*. **Exp Hematol**, 33(3), 2017, 259–271.

[19] R. Nagel, M. Steinberg,. *Hemoglobins Of The Embryo And Fetus And Minor Hemoglobins Of Adults. In Disorders Of Hemoglobin: Genetics, Pathophysiology And Clinical Management*, **Cambridge University Press: Cambridge, Uk**, 2010, 119-136.

[20] P. Huang, C. Keller, Giardine B. *"Comparative Analysis Of Three-Dimensional Chromosomal Architecture Identifies A Novel Fetal Hemoglobin Regulatory Element*". **Genes Dev** 31(16), 2017;1704-1713

[21] J. Olaniyi, O. Arinola & B. Ojetunde. *Foetal Haemoglobin (Hbf) Status In Adult Sickle Cell Anaemia Patients In Ibadan, Nigeria*. **Annals Of Ibadan Postgraduate Medicine** 8(1), 2010:1-5

[22] E. Wastnedge, W. Dornarld & R. Igor. *The Global Burden Of Sickle Cell Disease Of Children Under Five Years: A Systematic Review And Meta-Analysis*. **J Glob Health,** 8(2), 2018 : 1-10

[23] A. Paikari, V. Sheehan. *Fetal Haemoglobin Induction In Sickle Cell Disease*. **Br J Haematol**. 180(2), 2018, 189-200.

[24] S. Embury, N. Mohandas N, Paszty C. *In Vivo Blood Flow Abnormalities In The Transgenic Knockout Sickle Cell Mouse.* **J Clin Invest**. 103(6), 1999, 915–920.

[25] G. Kato, V. Mcgowan V, Machado Rf. *Lactate Dehydrogenase As A Biomarker Of Hemolysis-Associated In Nitric Oxide Resistance, Priapism, Leg Ulceration, Pulmonary Hypertension, And Death In Patients With Sickle Cell Disease*. **Blood,** 107(6),2016, 2279–2285.

[26] G. Kato, M. Gladwin, M. Steinberg. *Deconstructing Sickle Cell Disease: Reappraisal Of The Role Of Hemolysis In The Development Of Clinical Subphenotypes*. **Blood Rev**, 21(1), 2007, 37–47.

[27] A. Adabala, Makanjuola. S, Akinbami. A, Dosumu. A, Akanmu. A, Javid. F. *Frequency Of Beta S Globin Gene Haplotypes Among Sickle Cell Patients In Nigeria,* **J Int Med Res**, 49(6), 2021, 3418-31

[28] T. Adeyemo, O. Ojewunmi Oo, Oyetunji Ia. *A Survey Of Genetic Foetal-Haemoglobin Modifiers In Nigerian Patients With Sickle Cell Anaemia*. **Plos One**, 13(6), 2018, 6-10.

[29] J. Xu, V. Sankaran, T.F. Menne, R. Puram, W. Kim & Orkin Sh: *Transcriptional Silencing Of {Gamma}-Globin By BC11a Involves Long-Range Interactions And Cooperation With Sox6.* **Genes Dev,** 24(8), 2010, 783–798.

[30] I. Akinsheye, A. Alsultan, N. Solovieff "*Fetal Hemoglobin In Sickle Cell Anemia*," **Seminars In Haematology**,118(1), 2011: 19-27

[31] J. Xu, Bauer De, Kerenyi Ma, Vo Td, Hou S, Hsu Yj, Yao H, Trowbridge Jj, Mandel G, Orkin Sh: *Corepressor-Dependent Silencing Of Fetal Hemoglobin Expression By Bcl11a. Proc* **Natl Acad Sci Usa**, 110(16), 2013, 6518–6523.

[32] M. Shaikho, J. Farrell, *Cis- And Trans-Acting Expression Quantitative Trait Loci Differentially Regulate Gamma-Globin Gene Expression* **Biorxiv**, 3(12), 2018, 414-421

[33] U. S. Food And Drrug. *Fda Approves Hydroxyurea For Treatment Of Pediatric Patients With Sickle Cell Anaemis Available Online*: Https://Www.Fda.Gov/About-Fda/Fda-Organization/Center-Drug-Evaluation-And-Research-Cder Center For Drug Evaluation And Research, 2017

[34] S, Lanzkron, G. Sawicki, K. Hassell, M. Liem & S. Mccolley. *Transition To Adulthood And Adult Health Care Patients With Sickle Cell Disease Or Cystic Fibrosis: Current Practices And Research Priorities.* **Journal Of Clinical And Translational Science**, 2(5), 2018, 334-342

[35] G. Pule, S. Mowla, Novitzky N, Wiysonge Cs, Wonkam A. *A Systematic Review Of Known Mechanisms Of Hydroxyurea-Induced Fetal Hemoglobin For Treatment Of Sickle Cell Disease.* **Expert Rev Hematol**., 8(5), 2017, 669-79.

[36] S. Rahyssa Rodrigues, N. Bárbara Lisboa, Tosatti Jéssica Abdo Gonçalves, Gomes Karina Braga, Luizon & M. Rizzatti. *Do Genetic Polymorphisms Affect Fetal Hemoglobin (Hbf)*

Chapter Two

Literature Review

2.1 Conceptual Review

2.1.1 Sickle Cell Disease

Sickle Cell Disease (SCD) is a general term that defines a group of inherited diseases (including sickle cell anaemia (SCA), HbSC and HbSβ-thalassaemia) characterized by mutations in the gene encoding the haemoglobin subunit β (*HBB*)[1]. It is a group of inherited red blood cell disorders. Healthy red blood cells are round, and they move through small blood vessels to carry oxygen to all parts of the body[2](Fig 2.1).

SCD is inherited as an autosomal codominant trait; individuals who are heterozygous for the βS allele carry the sickle cell trait (HbAS) but do not have SCD, whereas individuals who are homozygous for the βS allele have SCA. SCA, the most common form of SCD, is a lifelong

disease characterized by chronic haemolytic anaemia, unpredictable episodes of pain and widespread organ damage[3]. It is a genetic blood disorder affecting red blood cells, with high morbidity and mortality rates. Sickle Haemoglobin (HbS) is a structural variant of normal adult haemoglobin (HbA)[4]. Patients with sickle cell anemia (SCA), or haemoglobin SS disease, suffer from lifelong complications, in part as a result of the markedly shortened life span of their RBCs. Sickle erythrocytes can lead to recurrent vaso-occlusive episodes that are the hallmark of SCD[5].

SCD causes significant morbidity and mortality, particularly in people of African and Mediterranean ancestry[6]. Morbidity, frequency of crisis, degree of anaemia, and the organ systems involved vary considerably from individual to individual.

Fig 2.1 Normal Red Blood Cell and a Sickle Shape Red Blood Cell

Source: Center for Disease Control[3]

2.1.2 History of Sickle Cell Disease

In medical history, 1910 is regarded as the date of the discovery of sickle cell disease, the disorder we call "Sickle Cell Disease" often abbreviated as SCD, had been present in Africa for at least five thousand years and has been known by many names in different tribal languages[7]. What we call its "discovery" in 1910 occurred, not in Africa, but in the United States. In 1904, The Scientist travelled from Grenada to the United States to start studying at the Chicago College of Dental Surgery[8]. A few months later he was admitted to the Presbyterian Hospital in Chicago when he developed severe respiratory distress and a leg ulcer, both of which we now know are symptoms of Sickle Cell. Dr. Earnest E. Irons, the intern who was on duty that day, performed a routine blood test and a urine analysis for Noel and was the first to observe these "pear-shaped, elongated" sickled blood cells[9].

It was not until 1910 that his article was published describing these "peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anaemia." This was the first documented and recorded case of Sickle Cell in western medicine. He subsequently published a paper in one of the medical journals in which he used the term "sickle shaped cells"[10].

The third case of Sickle Cell was described in 1915 by Cook and Meyer in a 21-year-old woman. Interestingly, blood samples from both the patient and her father, who displayed no symptoms, showed the sickling deformity of the red cells and three of her siblings had died from severe anaemia. These observations made by Dr. Emmel suggested a genetic basis for the disease but also led to a period of confusion with the genetics of the disease. Dr. Mason, who observed the fourth reported case of Sickle Cell, was also the first to call the disease "Sickle Cell Anaemia" and to notice the similarities between the cases. He also noted t—0 that all of these patients were black, inadvertently giving rise to the popular misconception that

Sickle Cell originated from people of African origin[11]

There are two main theories put forward to explain the origin of the Sickle Cell mutation.

a. Single Mutation Theory**:** A single mutation occurring in Neolithic times in the Arabian Peninsula was originally postulated where changing climatic conditions resulted in human migration to India, Saudi Arabia, and down to Equatorial Africa. Evidence for this theory included the distribution of certain agricultural practices and anthropological evidence. Evidence from blood groups and other genetic markers appeared to agree with an origin in Equatorial Africa and subsequent diffusion of the gene to India, Arabia, and the Mediterranean by the East African slave trade[12].

b. Multiple Mutation Theory**:** The multiple mutation theory has recently gained considerable support via genetic studies with the use of special enzymes called restriction endonucleases. These enzymes recognize and cut DNA at specific sequences. By using these enzymes to cut DNA from a normal individual and an individual with a disease and by looking at the differences between the patterns scientists are able to identify variations in DNA that are inherited. The technique is known as restriction fragment polymorphism mapping. One of the first marker to be looked at was the Beta-globulin gene (the one mutated in SCA) where a difference in the cutting patterns was observed between normal beta-globin genes and the samples that came from SCA patients. These variations identified within the beta-globin gene gives support to the multiple mutation theory. Since there were 4 different mutations, it is believed that these arose at 4 different geographical locations in Africa Benin, Senegal, Central Africa and Cameroon. There's a 5th mutation that is associated with Eastern provinces of Saudi Arabia and Central India. These observed differences suggest rather than originating from a single origin and spreading across the world that the beta-globin mutation arose at multiple geographical locations independently

of each other. The multiple mutation theory also refutes the misconception of African origin[13].

In 1927, it was discovered that red blood cells from persons with the disease could be made to sickle by removing oxygen. This was exciting because red cells are the oxygen transporters of the body. The trouble was, that there were people –often relatives of the patient – whose red cells had this trait of sickling when deprived of oxygen but who had no disease. This condition became known as "sickle trait".[14]

In the late 1940's and early 1950's the nature of the disease began to become clearer. In 1949, it was suggested that the Darwinian paradox of high frequencies of genetic blood disorders could result from a selective advantage conferred by such disorders in protecting against *Plasmodium falciparum* malaria infection in heterozygotes[10]. This balancing selection, commonly referred to as the 'malaria hypothesis', was originally suggested to explain the geographical correspondence between the distribution of thalassaemia and malaria in the Mediterranean region, and was later confirmed in many locations including Sardinia, Melanesia and Kenya. At the same time, a similar relationship between HbS and malaria was independently discovered in Africa. *In vitro* and *in vivo* studies have since added support for the protective role of HbS against malaria[15].

In 1951, the famous Nobel Prize-winning chemist, discovered that the red, oxygen-carrying protein called "haemoglobin" had a different chemical structure in persons with SCD. This led to the term "molecular disease" for disorders that resulted from proteins with abnormal chemical structures[14]. Pauling tested the haemoglobin samples from normal individuals, Sickle cell patients and people with Sickle cell trait using a technique called electrophoresis, which separated proteins based on their size and electrical charge. While normal individuals had haemoglobin of one type distinguishable from the haemoglobin from patients with sickle cell, the individuals with the sickle cell trait had both[11]. This was the first reported case

where a change in protein structure was shown to be inherited in a Mendalian fashion. Today, thousands of such diseases are known but in 1951, SCD was the first. The details of the abnormality were worked out by Dr. Vernon Ingram in 1956[16].

In the 1970's, more details of how this abnormal structure affects the red blood cells were revealed and better tests for the detection of the disease were developed. Development of DNA sequencing by Walter Gilbert and Frederick Sanger allowed the mapping of the sickle cell gene. DNA is made up of four different bases (these are the letters of the DNA alphabet A, T, C and G) and the genes coded in the DNA are identified (or read) by determining the order of these bases. The technique developed by Sanger and colleges allowed this ordered mapping of the bases using which the gene responsible for Sickle cell was mapped[17].

Loss of splenic function makes Sickle cell patients more susceptible to bacterial infections, especially pneumococci which results in Pneumococcal meningitis. In the early 1980s, it was noted that these Pneumococcal infections can be prevented by prophylactic penicillin in early childhood and by pneumococcal vaccines at later stages. Although with the rise of many pneumococci strains that are resistant to penicillin, these preventative measures might need to be replaced in the future. In the years following, better ways of treating sickle cell patients and potential treatments appeared. The life span and the quality of life of patients were improved. Genetic counselling became an important tool for informing people about the risks of having a child with sickle cell disease[18].

Today, physicians and scientists continue to move forward in new understanding of the disease and new ways to treat it. The goal of a total cure has not been reached but great progress has been made. Perhaps within the lifetime of some of us, that goal will be reached.

2.1.3 Inheritance

Sickle cell disease is inherited as an autosomal recessive condition whereas sickle cell trait is inherited as an autosomal dominant trait. This means that the gene can be passed on from a parent carrying it to male and female children. In order for sickle cell disease to occur, a sickle cell gene must be inherited from both the mother and the father, so that the child has two sickle cell genes[19].

The inheritance of just one sickle gene is called sickle cell trait or the "carrier" state. Sickle cell trait does not cause sickle cell anaemia. Patients with sickle cell trait usually do not have many symptoms of disease and have normal hospitalization rates and life expectancies.

2.1.4 Epidemiology of Sickle Cell Disease

Almost as soon as sickle cell anaemia was recognized as a blood-based disease, its higher frequency in families of African descent was noted. However, the first reports of cases in Africa itself did not come until the 1920s. In 1925, a 10-year old Arab boy was admitted to a hospital in Omdurman in the Sudan (on the Upper Nile, East central Africa, near Ethiopia) with severe weakness; later he was ascertained to have sickle cell disease (anaemia)[20]. In 1944, a pathologist at the West African Military Hospital, studied the blood of 600 men from Gambia, the Gold Coast, Nigeria and the Cameroons (all in western Africa on the Gulf of Guinea). He found about 20% of the population affected by the sickle-cell condition (disease + trait). However, a striking observation became apparent: while the frequency of sickle-cell trait in

Africa was three times that in the United States, sickle cell disease was much less common. Even as late as the 1950s it was still unclear why this discrepancy existed[21].

SCD is the most prevalent genetic disease in the African Region. There are different subtypes of SCD in which the abnormal S gene coexists with other abnormal haemoglobin genes.

Structural studies of the S gene suggest that the sickle-cell mutation arose in at least four different places in Africa and a fifth mutation occurred in the Arabian Peninsula[22]. The SCT is widespread in the WHO African Region the S gene prevalence in at least 40 countries varies between 2% and 30%, resulting in high SCD-related morbidity and mortality[20]. Deaths from SCD complications occur mostly in children under five years, adolescents and pregnant women. Because there is little genetic counselling available for prospective parents, unions between SCT carriers result in the birth of SCD children. Most countries have inadequate national health policies and plans, and scarce facilities, diagnostic tools, treatment services and trained personnel. There is therefore a need for urgent interventions to address this public health problem[23].

Three quarters of sickle-cell cases occur in Africa. About 2% of new-borns in Nigeria are affected by sickle cell anaemia, giving a total of 150,000 affected children born every year in Nigeria alone[22]. The carrier frequency ranges between 10% and 40% across equatorial Africa, decreasing to 1–2% on the North African coast and <1% in South Africa The highest frequency of sickle cell disease is found in tropical regions, particularly sub-Saharan Africa, India and the Middle-East. Migration of substantial populations from these high prevalence areas to low prevalence countries in Europe and America has resulted in a dramatic increase of sickle cell disease in some European countries and the United States[21]. In the US, the prevalence is approximately 1 in 5,000, affecting predominantly Americans of Sub-Saharan African descent In mainland France, 1/2,415 birth is affected with SCD. In the United Kingdom 1 baby in every 2,000 is born with SCD. Approximately 17% of the population in the Eastern province of Saudi Arabia 7 carry the gene and about 1.2% have sickle cell disease. The prevalence of SCD in endemic areas of India ranged from 9.4 to 22.2%. Sickle cell disease is associated with a variety of complications with attendant high mortality rate[24].

Sickle-cell disease prevalence depends on sickle-cell trait. Where the prevalence of SCT exceeds 20%, SCD is estimated to be at least 2%. The S gene concerns the population of at least 40 countries in the Region, and in about 23 countries of west and central Africa the prevalence of SCT varies between 20% and 30%; it is as high as 45% in some secluded areas in West Africa. Although more than 40 countries are affected, much of the data are still hospital-based and not population-based. Most SCD manifestations are readily amenable to treatment using available interventions; however, the interventions are not accessed by the majority of patients, specifically the vulnerable groups: children under five years, adolescents and pregnant women. In addition, laboratory facilities for accurate diagnosis are limited[25]. Adequately trained health professionals are few, specialized health care facilities are insufficient and effective medicines, vaccines and safe blood transfusion are very limited. Presently, even in developed countries where stem cell transplantation can be contemplated, there is no widely acceptable public health intervention for the clinical cure of SCD. Consequently, the median survival of SCD patients in Africa is less than five years; about 50%– 80% of the estimated 400 000 infants born yearly with SCD in Africa die before the age of five years. The survivors suffer end-organ damage which shortens their lifespan. Thus, to improve management of SCD there is a crucial need for early case identification and implementation of comprehensive health care management (CHCM)[26].

Malaria contributes not only to mortality but also to anaemia and other crises. While the lifespan of SCD Malaria contributes not only to mortality but also to anaemia and other crises. While the lifespan of SCD patients was also quite dismal in the USA prior to the 1970s, there has been a dramatic upturn in the last 30 years such that most patients now live into their 40s and 50s. The main factor responsible for this turnaround is the availability of comprehensive care with newborn screening for early diagnosis, penicillin, prophylaxis, pneumococcal vaccination and the use of transcranial Doppler (TCD) ultrasound to predict patients at risk of

stroke who are then treated on chronic transfusion programs[27]. Use of hydroxyurea, which is effective in reducing the frequency of painful crisis, acute chest syndrome and anaemia, has changed the outlook of the disease both in adults and children. Despite the high burden of SCD in Nigeria, a national policy to combat the disease is still awaiting ratification and many of the new modalities of management are not widely available. In 2010, the Nigerian SCD Network (NSCDN) was established as a co-operating body bringing together Nigerian physicians, nongovernmental organizations (NGOs) and other interested bodies both within the country and in the Diaspora. One of the first tasks of the network was a survey to document the available facilities and the prevalent management practices in SCD clinics in the country in order to put the current situation in perspective and to enable planning and rational research projections. Here we present the analysis of this survey and suggest ways of moving forward to improve the care of patients in the country[28].

Despite logistic and economic constraints, neonatal SCD screening along with CHCM have been successfully practised in some parts of Africa. For example, in Benin where neonatal screening and CHCM were practised, the under-five mortality rate of SCD was 15.5 per 10 000, which is ten times lower than the overall under-five mortality rate. These findings are consistent with those from developed countries, demonstrating the benefit of new-born screening and close follow-up of children using CHCM. Research has been done in several countries in the Region to achieve better understanding of SCD, but more remains to be done. The research includes issues related to efficacy of conventional and traditional medicines. The safety, efficacy and quality of some traditional medicines have been evaluated and appeared to be safe and effective in reducing crises associated with severe pain. However there is no substantive documented evidence to support the efficacy of traditional practice or herbal medications in curing SCD[29].

2.1.5 Haemolysis

Sickle erythrocytes are highly unstable, with a lifespan that is reduced by ≥75%[30]. Haemolysis is thought to occur principally via extravascular phagocytosis by macrophages, but a substantial fraction (roughly one-third) occurs through intravascular haemolysis[31]. It has been hypothesized that the rate of intravascular haemolysis in SCD is insufficient to produce a clinical phenotype, including pulmonary hypertension[32], the most serious consequence of intravascular haemolysis. However, the epidemiological, biochemical, genetic and physiological data supporting a link between intravascular haemolysis and vasculopathy continue to expand[33].

2.1.6 Oxidative Stress.

Haemolysis is both a cause and an effect of oxidative stress. The substantial levels of oxidative stress in sickle erythrocytes enhance HbS auto-oxidation, which could contribute to the damage of the cell membrane, premature erythrocyte ageing and haemolysis[36]. In addition to the accelerated auto-oxidation of HbS, oxygen radicals result from increased expression of oxidases, especially xanthine dehydrogenase and xanthine oxidase, and reduced NADPH oxidase[34], extracellular haem and Hb in plasma and probably also from recurrent ischaemia–reperfusion of tissues. Cytoskeletal proteins and membrane lipids become oxidized, and this chronic severe oxidative stress in sickle erythrocytes depletes the levels of catalytic antioxidants such as superoxide dismutase, peroxiredoxin 2 and peroxiredoxin 4. This issue is worsened by depletion of the endogenous reductant glutathione46, impaired antioxidant capacity probably contributes to haemolysis[35].

2.1.7 Free Plasma Hb and Haem.

Extracellular Hb (in plasma or in microparticles) and haem in plasma promote severe oxidative stress, especially to blood vessels and blood cells[36]. Continuous auto-oxidation of extracellular Hb produces superoxide, which dismutates into hydrogen peroxide (H2O2), a

source for additional potent oxidative species, including the ferryl ion, which promotes vasoconstriction. Extracellular Hb scavenges nitric oxide (NO; which is generated by NO synthase (NOS) in endothelial cells and promotes vasodilation) ~1,000-fold more rapidly than cytoplasmic Hb, thereby decreasing NO bioavailability[36]. This decreased bioavailability of NO results in vascular dysfunction, indicated by impaired vasodilatory response to NO donors, activation of endothelial cells (producing cell-surface expression of endothelial adhesion molecules and detected by elaboration of soluble ectodomains of the adhesion molecules into plasma) and haemostatic activation of platelets, indicated by cell-surface expression of P-selectin (which mediates the interaction between activated platelets and leukocytes) and activated αIIbβ3 integrin. Markers of haemolytic severity (such as low Hb or high serum lactate dehydrogenase) predict the clinical risk of developing vascular disease complications[38].

2.1.8 Pathophysiology of SCD

Under low oxygen tension, HbS chains form a hydrophobic contact between valine on one chain and other amino acids such as alanine, phenylalanine, and leucine on the other chain[37]. This crystallization gives rise to a polymer nucleus, which grows and fills the red blood cell, unsettling its architecture and elasticity and promoting cellular dehydration, with physical and oxidative cellular stress[38]. HbS tends to aggregate into rod-like polymers, alters the normal, biconcave disc shape resulting in the deformed sickle shape and rigidity of red blood cells (RBCs) characteristic of this condition[25].

The polymerization of deoxygenated HbS is the primary indispensable event in the molecular pathogenesis of sickle-cell disease. It is dependent on intra erythrocytic HbS concentration, extent and duration of cell deoxygenation, pH, and the intracellular concentration of HbF[39]. Haemolytic anaemia has been proposed as a major factor causing the vasculopathy and endothelial dysfunction that occurs in SCD[40]. Intravascular haemolysis which releases free

haemoglobin into the plasma inhibits nitric oxide (NO) signalling. In SCD, NO bioavailability is limited through

(i) increase in the free radical superoxide

(ii) the products of haemolysis (free haem and arginase), and

(iii) Through the uncoupling of endothelial NOS[29].

Haemolysis is associated with a clinical sub-phenotype of pulmonary hypertension, leg ulceration, priapism, and risk of death in patients with sickle cell disease[41]. Several complications such as cutaneous leg ulceration, cholelithiasis, priapism, and pulmonary hypertension are associated with low steady state haemoglobin concentrations and an increased rate of intravascular haemolysis.

The predominant pathophysiological feature of SCD is vaso-occlusion. An important mechanism of vaso-occlusion is thought to involve entrapment of erythrocytes and leucocytes in the microcirculation following the repeated polymerization of HbS, causing vascular obstruction and release of inflammatory mediators that in turn stimulate afferent nerve fibers and cause pain[27]. Vaso-occlusion encompasses adherence of circulating blood elements to endothelial cells, endothelial dysfunction, hypercoagulability, hypoxemia, altered nitric oxide bioavailability, and ischemia-reperfusion injury[30]. These sequences of events lead to complications such as episodes of pain, acute chest syndrome, and cerebrovascular disease. These events usually evolve to involve multiple organs, including the bone, lungs, central nervous system, cardiovascular system, spleen, skin, and kidneys[26].

2.1.9 The Symptoms of Sickle Cell Anaemia:

2.1.9.1 Pain in Sickle Cell Disease

Recurrent episodes of acute, severe pain are the hallmark of SCD. The pain is highly variable both within and among patients, and is the result of complex and poorly understood interactions between biological and psychosocial factors. Vaso-occlusion within the bone

marrow vasculature leads to bone infarction, which in turn results in the release of inflammatory mediators that activate afferent nerve fibres and cause pain. Although the basic mechanism is simple, the precise details of the vaso-occlusion are poorly understood, involving complex interactions between red cells, endothelium, white cells and platelets. The unpredictability of the pain is a major factor in undermining the patient's ability to cope[42].

Acute pain frequently occurs spontaneously, but may be precipitated by infections, skin cooling, dehydration or stress. Acute pain in SCD is described as throbbing, sharp or gnawing, and patients can usually recognize whether it is typical of their SCD. If the patient thinks the pain is atypical, then other causes of pain should be sought. Acute painful episodes may occur on top of chronic pain, or be precipitated by other painful events, such as cholecystitis. Hospital admissions for acute pain in SCD typically last 4–10 days, but this varies widely. If persist more than 3 months it's considered as chronic, chronic pain may be an extension of recurrent acute painful episodes or in a specific tissue or organ, such as avascular necrosis of the hips, or leg ulcers. Chronic pain is often associated with other conditions that enhance its chronicity. These include psychosocial factors such as depression, anxiety, feelings of despair, insomnia, loneliness, helplessness and dependence on pain medications, chronic pain can be hard and may lead in mental drain[27].

2.1.9.2 Sickle Cell Crisis

The term "sickle cell crisis" is used to describe several independent acute conditions occurring in patients with sickle cell disease**,** the most common complication of SCD is an acute episode of severe pain referred to as an acute vaso-occlusive crisis (VOC). A VOC is defined as pain resulting from tissue ischemia caused by vaso-occlusion most commonly in the bone(s) and bone marrow[27].

2.1.9.3 Vaso-occlusive Crisis (VOC)

A VOC is the hallmark acute complication for SCD and manifests as acute severe pain. The sickled erythrocytes block the flow of blood through the small blood vessels (capillaries) resulting in ischemia. Sudden episodes of pain throughout the body are a common symptom of SCD. This sudden pain can range from mild to very severe form and usually lasts from hours to a few days. VOCs and their accompanying pain most commonly occur in the extremities, chest, and back. When they occur in other sites, they can be confused with, or can be the prodromal stage of other acute complications (e.g., head (stroke), flank (papillary necrosis), and abdomen (hepatic or splenic sequestration, constipation from opioid toxicity, or another Hepatobiliary complication). Patients with more than three hospitalizations for a VOC in a year are considered to be at an increased risk of early death[43].

2.1.9.4 Hand-Foot Syndrome

When sickle cells block the small blood vessels at hands or feet, pain and swelling along with fever may occur. The first VOC may appear as early as at 6 months of age, often presenting as dactylitis, but there after VOCs occur with variable frequency. This may be the first sign of sickle cell anaemia in infants[44].

2.1.9.5 Fever- Infections

Patients with SCD have an increased risk for severe bacterial infection, resulting primarily from reduced or absent splenic function[45]. The result is an extremely high risk of septicemia and meningitis, primarily due to Streptococcus pneumonia. The risk of such infections continues throughout childhood and to a lesser extent in adults. Fever, as a presenting symptom, heralds many acute and sometimes life-threatening conditions, such as ACS and osteomyelitis. It is critical that fever alone is taken seriously in these patients and considered a potential emergency situation. Fever associated with pain should not be considered a VOC until infection is ruled out. Acute osteomyelitis, another complication associated with fever,

may be unifocal or multifocal and may be caused by Staphylococcus aurous, salmonella, or other enteric pathogens[31].

Pneumonia is the most common cause of death in young children with sickle cell disease. Meningitis, influenza, and hepatitis are other infections that are common in people with sickle cell disease[46].

2.1.9.6 Acute Renal Failure

Acute renal failure is defined as a rapid reduction in renal function manifested by a rise in serum creatinine and reduction in glomerular filtration rate (GFR), with or without a decline in urine output. Acute renal failure may be due to pre-renal (e.g., dehydration) or post-renal (e.g., obstruction) insults, or result from intrinsic renal disease (e.g., glomerular injury). It may occur during an acute VOC, most often in association with ACS or acute multisystem organ failure. Renal papillary necrosis due to medullary infarction from obstruction of the blood supply in the vasa recta affects up to 15–30 percent of individuals with SCD[32]. Signs and symptoms include pain and haematuria. When present, fever suggests possible infection. The serum creatinine levels are generally low or low-normal in patients with SCD and the values in acute renal function may still be within normal limits even if serum creatinine level increase two times from baseline[31].

Identification of early renal disease in people with SCD is important as these patients hyper secrete creatinine through the proximal tubules, thus making significant renal impairment before the serum creatinine rises. Micro albuminuria is most often the first manifestation of chronic kidney disease in SCD. Proteinuria due to glomerular injury is also common, but both micro albuminuria and macro albuminuria are typically asymptomatic. The most common

renal complication in people with SCD is hyposthenuria, or the Inability to concentrate the urine, which is progressive with age[29].

2.1.9.7 Hepatobiliary Complications

Biliary tract abnormalities are common in SCD patients. These abnormalities include cholelithiasis, acute cholecystitis, and biliary sludge. Haemolysis of any etiology results in increased secreted unconjugated bilirubin that tends to precipitate and leads to gallstones and sludge. Acute hepatic sequestration of red blood cells in the liver often develops over a few hours to a few days, and the resultant stretching of the hepatic capsule is usually painful. Acute intrahepatic cholestasis (also called sickle cell hepatopathy or "drepanocyte" liver) is also associated with SCD. It is characterized by the sudden onset of pain, increasing jaundice, a progressively enlarging and extremely tender liver, light-colored stools, and extreme hyperbilirubinemia (both conjugated and Unconjugated). This complication may prove fatal if not recognized and treated promptly[47].

2.1.9.8 Splenic Sequestration

Splenic sequestration is defined as sudden enlargement of the spleen and reduction in haemoglobin concentration by at least 2 g/dL below the baseline value. Splenic sequestration (pooling) - crises are a result of sickle cells pooling in the spleen. This can cause a sudden drop in haemoglobin and can be life threatening if not treated promptly. The spleen can also become enlarged and painful from the increase in blood volume. After repeated episodes of splenic sequestration, the spleen becomes scarred, and permanently damaged. Most children, by the age of 8 years old, do not have a functioning spleen from repeated episodes of splenic sequestration. The risk of infection is a major concern of children without a functioning spleen. Infection is the major cause of death in children under the age of 5 years in this population. It is a major cause of acute anaemia and it may present acutely accompanied by

severe anaemia and hypovolemic shock. The reticulocyte count and circulating nucleated red blood cells are usually elevated[33].

2.1.9.9 Acute Chest Syndrome (ACS)

Acute chest syndrome is a life-threatening condition for SCD patients. It is the second most frequent reason for hospitalization in children and adults with SCD and the most common cause of death. It's similar to pneumonia and is caused by an infection or by sickle cells trapped in the lungs. Patients with this condition usually have chest pain, fever, and an abnormal chest xray. Over time, lung damage may lead to pulmonary hypertension[30].

2.1.9.10 Acute Stroke

Stroke is one of the most common and devastating complications of SCD. Sickle-shaped red blood cells may stick to the walls of the tiny blood vessels in the brain. This type of stroke occurs mainly in children. This complication presents as sudden onset of weakness, aphasia, and sometimes seizures or coma and results in adverse motor and cognitive squeal. In the absence of secondary prevention measures such as a chronic transfusion program or hematopoietic stem cell transplantation, recurrence rates have been shown to range between 46 and 90 percent in children with SCD. Brain haemorrhage occurs more often in adult's patients[48].

2.1.9.11 Priapism

Males with sickle cell disease may have painful and unwanted erections lasting about 4 hours, called priapism. This happens because the sickle cells stop blood flow out of an erect penis. Priapism is a common complication of SCD, affecting 35 percent of male patients, over time, priapism can damage the penis and lead to impotence[49].

2.1.9.12 Multisystem Organ Failure

Multisystem organ failure is a severe, rare and life-threatening complication usually associated with a VOC and characterized by failure of the lungs, liver, and/or kidneys. Symptoms linked to this complication are fever and changes in mental status such as sudden tiredness and loss of interest in their surroundings. The incidence of chronic complications appears to increase with age and understanding of the pathophysiology and the involved factors is necessary to prevent or reduce long-term morbidity[34].

2.1.9.13 Avascular Necrosis

Avascular or aseptic necrosis can occur when capillaries are occluded by sickled erythrocytes at distal portions of a bone, near a joint, where hypoxia is maximal and collateral circulation is inadequate, the femoral neck is the most common site of aseptic necrosis. It causes chronic severe pain and disability[33].

2.1.9.14 Leg Ulcers

Leg ulcers are a common complication of SCD. Sickle cell ulcers usually begin as small sores on the lower third of the leg. Leg ulcers occur more often in males than in females and usually appear between the ages of 10 and 50. The cause of leg ulcers is not clear. Some heal rapidly, but others persist for years or recur[34].

2.1.9.15 Pulmonary Hypertension

Pulmonary hypertension (PH) is defined as an elevation of the resting mean pulmonary arterial pressure (>25 mmHg) as determined by right heart catheterization (RHC). PH can occur in chronic hemolytic anemia and in the setting of chronic lung disease, chronic thromboembolic disease, or can be due to unclear and multiple mechanisms. Initial testing for PH has been done with an echocardiography assessment to estimate pulmonary artery pressure using tricuspid regurgitant jet velocity (TRV), but diagnosis requires right heart

catheterization and direct measurement of the pulmonary arterial pressure and vaso-reactivity of the vessels. Excessive shortness of breath is an important symptom of PH[30].

2.1.9.16 Ophthalmologic Complications

Chronic ophthalmological complications of SCD include proliferative sickle retinopathy and vitreous haemorrhage. They occur in up to 50 percent of patients with SCD and are associated with significant visual loss[30]. The symptoms and complications of sickle cell disease (SCA) arise mainly from the crisis, activation and damage of endothelial cells with activation of adhesion molecules lead to inflammation, release of C-reactive protein (CRP) and other inflammatory mediators and subsequent enhancement of ischemia[32].

The above and other lines of evidence suggest that SCD is associated with a chronic inflammatory state, in which inflammation, oxidative stress and tissue oxidative damage occur, leading to various degrees of disease severity and end-organ dysfunction. Exploring the role of CRP in this chronic inflammatory state is very important as we search for therapeutic targets in this disease[29].

2.1.10 Techniques for the Detection of Sickle Cell Disease

Several techniques and assays are used for the detection and monitoring of the sickle disease. These techniques can be divided into two main categories:

1. Currently used methods in the diagnosis of SCD; and
2. Innovative techniques which are mostly still in the research stage. Several reviews have been published related to the development of point of care (POC) SCD detection

2.1.10.1 Complete Blood Cell Count

The complete blood count (CBC) is a primary test to characterize the different types of anaemia. However, the haemoglobin mutation will affect the haematological parameters, showing a variable change[50]. Patients with homozygous SS and heterozygous S/β° mutations usually present with haemolytic anaemia where the red blood cells (RBCs), haemoglobin and

haematocrit are low. In contrast, the counts of white blood cells (WBC) and platelet are elevated, and they can fluctuate. However, reticulocyte counts are variable and depend on different factors such as the degree of anaemia caused by the cells haemolysis, sequestration, and bone marrow response to anemia[50]. Mean corpuscular volume (MCV) is usually elevated in SCD patients receiving hydroxyurea. Moreover, elevated red cell distribution width (RDW) is seen in SCD patients because of RBCs' different subpopulations. Although CBC is widely used to describe the haematological parameter as valuable information, it is insufficient to give a complete picture of patients' diagnoses.

2.1.10.2 Peripheral Blood Smear

The peripheral blood smear (PBF) is usually done after spotting abnormality in the automation counts and is considered a landmark of any hematological evaluation. PBF examines the morphology of the blood cell and evaluates any microscopic changes, which can provide valuable information that helps in the diagnoses of the different types of anaemia[51]. In sickle cell anaemia, moderate to severe anisopoikilocyte is seen with a variable number of elongated sickle cells, which is best observed when the red blood cells are deprived of oxygen. The preparation of these blood smear slides is relatively simple, rapid, and inexpensive. Although peripheral blood smear is an informative hematological test, it relies on the pathologist's skills, and the availability of trained pathologists is limited. Furthermore, the blood film analysis is too complicated due to the changes in the cell's edge, location, shape, and size. As a result, a computerized system has been developed to provide a more accessible way to recognize the type of anaemia[52].

2.1.10.3 Solubility Sickling Test

Sickling tests are mainly based on the polymerization of HbS in the deoxygenated state. The solubility test is the most widely used nowadays; its principle is based on the insolubility of

Hb-S in the presence of concentrated phosphate buffer, a haemolysing agent, and sodium dithionate. These agents crystalize the HbS and precipitate the cells, which refract the light and cause solution turbidity. The result is compared with negative and positive controls[53]. This test is easy to perform and inexpensive. It suffers from a false-negative result when utilized for new-borns, due to the presence of a high amount of haemoglobin F and when the HbS is less than 10% of the total haemoglobin[52]. Furthermore, false-negative results are observed in patients with coinheritance of α-thalassemia trait and severe anaemia. In contrast, false positives are observed in patients with high serum viscosity, erythrocytosis, highly marked leukocytosis and in some cases of anaemia. Moreover, the sickle solubility tests cannot differentiate between sickle cells trait (SCT) and SCD, and they are insensitive to the detection of haemoglobin AS (HbAS).These disadvantages make them difficult to use in screening programs[54].

2.1.10.4 Hemoglobin Electrophoresis

Electrophoresis is a type of chromatography techniques, and it is considered as one of the important tests used to detect Hb variants[50]. In this test, an electrical field is applied to facilitate the migration of electrically charged molecules. The first described haemoglobin variant Hb-S by using electrophoresis was in 1949. To identify haemoglobin variants, different pH and mediums are used, either cellulose acetate electrophoreses at alkaline pH or citrate agar at acidic pH[55].

Alkaline electrophoresis is a diagnostic tool that has been used to detect thalassemia and sickle cell anaemia at pH 8.4. First, a hemolysate is prepared from the red blood cells; then, it is added to a cellulose strip and run-in buffer at a constant voltage in an electrophoresis chamber[54]. As a result, the different haemoglobin types with different net charges are separated into various bands depending on their mobility. Hemoglobin electrophoresis can differentiate between HbS and HbC, which are the most clinically significant variants.

However, electrophoresis does not distinguish between hemoglobin variants with the same electrical charges and gives the same migration patterns, such as HbD and HbG, which migrate with HbS; HbE and Hb0-Arab have similar migration to the HbC molecules[56]. Furthermore, alkaline electrophoresis can be affected by the presence of large amounts of hemoglobin F in new-borns, which can dominate the smaller electrophoresis band. Therefore, extra care should be taken to reliably detect the HbS.

In addition, smaller bands such as HbA2, HbH, and Hb Bart's may be missed. Therefore, a more efficient test should be used as a diagnostic test to overcome these limitations[54]. Citrate agar electrophoresis is performed in acidic pH 6.0–6.2, and it depends on the interaction of the agaropectin in the gel mixture with the structural changes of the Hb[55]. Most haemoglobin variants that co-migrate at alkaline pH can be separated effectively using citrate agar electrophoresis. Citrate agar electrophoresis is not affected by the high amount of hemoglobin F in new-borns; thus, it can be used as a diagnostic test for sickle cell disease at birth. However, it is laborious and challenging to perform in limited resources areas[57].

Capillary electrophoresis has been documented to separate Hb fractions and diagnose sickle cell disease and thalassemia. The capillary electrophoresis separates the protein in an untreated fused-silica column reliably[58]. Fully automated methods such as CAPILLARYS 2 system has been available in the market since the early 2000s. This method has eight parallel fused silica columns where multiple samples can be analyzed, and each column can be used for at least 3000 runs. The hemolysates are prepared automatically from red cell pellets.

The reference ranges for HbA2 are adapted to be 2.1–3.2% and <0.8% for HbF. However, in the presence of different Hb variants, Capillary Zone Electrophoresis (CZE) is better than HPLC for quantifying HbA2 except in the presence of HbC. Moreover, a fully automated Neonat Fast Hb device with CAPILLARYS cord blood mode can analyze dried blood spots

on filter paper and liquid cord blood. Thus, it can be used in the neonate screening test. These advantages make the CAPILLARYS instrument the first-line test for screening hemoglobinopathies in new-born and adult patients[58].

2.1.10.5 Isoelectric Focusing

Isoelectric focusing (IEF) is a high-resolution method for separating proteins depends on their isoelectric points (PI). The Hb molecules travel across a pH gradient until they reach their isoelectric points where the net charge is zero. The Hb molecules precipitate and appear as a sharp band. This technique can detect HbS and HbA easily in a high concentration of HbF. Moreover, it separates Hb D-Punjab from HbS. Generally, it can provide the result within 45 min[59]. Although IEF is relatively expensive and requires highly trained personnel to interpret the results due to the larger number of bands, it is still considered the standard test for newborn screening, as it needs a very small volume of sample and can be used with a dried blood spot[58].

2.1.10.6 High Performance Liquid Chromatography

HPLC is documented to separate the haemoglobin fractions as they have different interaction with the stationary phase. HPLC detects different types of haemoglobin based on the retention time and shape of the peak[60]. Each haemoglobin has a specific retention time and can be compared with the retention time of the known haemoglobin fractions. HPLC is used to detect and quantify HbF, Hb A2, HbS, HbC, Hb Barts, and other Hb variants. Developing a fully automated HPLC would be useful in testing a large number of samples accurately. HPLC shows better sensitivity in separation of hemoglobin variants than electrophoresis[54]. HPLC is much less labor-intensive and more reliable for monitoring patients under blood transfusion or hydroxyurea. However, HPLC is an expensive machine and cannot differentiate among all variants with the same retention time. For example, all Hb variants with a similar retention time to HbS are eluted out with the HbS peak. Therefore, it can

misdiagnose new variants that mimic HbS. Thus, HPLC cannot stand alone as a diagnostic test and should be done along with a confirmatory test such as DNA analysis before giving a final diagnosis[61].

2.1.10.7 Genetic Test

The genetic study is important for the precise detection of the various types of sickle cell disease, based on the detection of β-globin mutations that lead to sickle cell disease development[61].

2.1.10.8 Polymerase Chain Reaction (PCR)-Based Techniques

Polymerase chain reaction is one of the most powerful diagnostic techniques, where special enzymes are used to amplify specific parts of the genetic materials to millions of copies, using specific primers. PCR can detect well known single genes or several genes in a single tube[62]. The PCR program involves denaturation, annealing, and elongation, which is repeated for 20– 40 thermal cycles. Then, the result can be detected by gel electrophoresis, sequencing, melting curve analysis, or monitoring the change in the fluorescence. PCR sensitivity and specificity have revolutionized the prenatal and neonatal diagnostic field. Several PCR-based techniques are documented to detect βs mutations, such as high-resolution melting (HRM) analysis, which is simple, sensitive, and cost-effective for use in mass screening of SCD genotypes[61]. Another simple, low-cost PCR-based technique has been developed using bi-directional allelespecific amplification (ASA) and a hot star system to provide more specific single-tube genotyping, where the point mutation of sickle cell anaemia is used as the SNP model. In addition, discriminatory conditions have enabled the determination of homozygous and heterozygous states based on the different band sizes on the agarose gel electrophoresis[63]. The amplification-refractory mutation system (ARMS) is a simple technique for detecting point mutation or small deletion. The ARMS principle is to use primers with specific sequences to allow the amplification of DNA in the presence of the target allele. Therefore,

the detection of the target allele is based on the presence of the PCR product. The alleles can then be differentiated on agarose gel with different band sizes. ARMS has been mostly used in prenatal diagnosis by detection of sickle cell mutation in the foetal sample. The ARMS's sensitivity has been measured by comparing the result to identify the presence of haemoglobin variants by HPLC[64]. An allele-specific oligonucleotide (ASO) hybridization to detect sickle cell mutation using two PCR primers was. One primer was used for the normal allele and the other one for the mutated allele. The primer is joined to the complementary sequence and amplified, which in turn releases the fluorescent label that determines the amount of the target. This method can differentiate between the allelic variations[63].

2.1.10.9 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) is used to detect sickle cell disease based on restriction enzymes, which remove the recognition site at the βs mutated gene. For example,

MstII is one of the first described restriction enzymes; it cuts the DNA in the sequence CCTNAGG (where N represents any nucleotide). Therefore, when thymine replaces the adenine, it removes the recognition site for MstII restrictase. After separation, the number of bands resulting from the enzyme cutting indicates the number of mutations. In a healthy individual with (βA βA), the gene is cut by the MstII restrictase and yields two bands. In homozygotes, the restrictase cuts both genes, and two short bands appear. In the sickle cell trait (βAβS), no cut is made in the βS, so a single band appears; however, the βA gene is cleaved, and two bands appear. In sickle cell anemia homozygous (βSβS), there is no enzyme cutting due to the mutation in both genes, so a single wide band appears[65]. Another restriction enzyme has been used in sickle cell detection is Ddel I. The mutation caused sickle cells Anaemia(SCA) removes the restriction site of Ddel I, 5′-GTNAG-3′. As a result, bands with different lengths appear depending on the presence of sickle cell anaemia mutation[64].

2.1.10.10 DNA Microarrays and Sequencing Techniques

DNA microarrays consist of a large number of immobilized DNA oligonucleotide spots on the array surface, where hybridization events occur with complementary sequences, which in turn indicate the concentrations of the nucleic acids[66]. Microarrays have been used in genome-wide association studies (GWASs) to identify the presence of single nucleotide polymorphisms (SNPs) in a single run, as well as the copy number of variants. A novel database that combines the gene expression with genome-wide association study (GWAS) was developed, using homozygous SS microarray datasets to determine SCD transcriptomic profile[63].

Next-generation sequencing (NGS), which is deep DNA sequencing, has been used to identify different types of mutation. NGS can be run for the whole-exome sequencing (WES) or wholegenome sequencing (WGS). These techniques have been used widely for genetic analyses to predict sickle cell disease's severity and progression, which can help make a treatment decision, discover new therapies, and develop novel diagnostic assays[67]. WES is performed to determine single-nucleotide variants (SNVs) in sickle cell mutation by sequencing the coding region of the β-globin gene. This procedure gives a full description of the β-globin gene accurately[63]. Few studies have utilized this approach to identify genetic modifications in the SCD severity. One study reported an increase in the number of strokes in African Americans due to mutation in GOLGB1 and ENPP1[68].

Another study pointed out that mutation in SALL2 is associated with a high level of HbF in response to hydroxyurea. Variants in MBL2 and KLRC3 were observed more frequently among adult SCD with hyperhemolysis syndrome than controls. Whole-genome sequencing helps analyse the entire genome, identify the genomic modification of SCD, and create the Sickle Genome Project (SGP). It helped develop a robust pipeline for the correct identification of SNPs Furthermore, it confirmed the association of the SCD phenotypes with

common genetic modifiers, including foetal haemoglobin BCL11A, HBB, UGT1A1, and APOL1. This technique will help in precision medicine to make better treatment decisions and discover new treatments[64].

2.1.10.11 Image Processing Techniques

Image processing techniques play an essential role in the analysis of red blood cells. Blood cell disorders can be classified based on different features: the cell shape, central pallor diameter, target flag, etc. The cells can also be classified based on the image features by using segmentation and artificial neural network[69].

An automated method to detect sickle cell anaemia (SCA) using an image processing technique. An algorithm is used to automate the detection of sickle cells found in thin blood smears. The first step in this technique is to take blood images using a camera connected to a light microscope. Then, a pre-processing step converts the images into grayscale, enhances the image, and passes it through the median filter to reduce the noise. After that, the RBCs are segmented through a segmentation threshold, followed by a morphological operation for the image to remove the unwanted objects. The features of the images are created based on color, texture, and the cells' geometry. As a final step, the computer classifier is trained to assess the picture. In total, 120 photos were used to assess this technique: 80 for training and 40 images for testing. The authors reported 95% accuracy and 96.55% sensitivity[65].

Deep learning models to detect SCA and classify the red blood cells based on the microscopic images was employed[64]. The models were able to extract and implement the classification functions automatically in one shot. Moreover, they developed three deep learning models to determine and categorize the red blood cells based on the shapes: round shape indicating normal cells, elongated shape indicating sickle cells, and other blood shapes. The researcher focused on resolving the lack of training data, where they used the transfer learning technique. The study employed 626 images; 202 were classified as circular; 211 images were identified

as elongated; and 213 as other cell shapes. The model achieved 99.54% accuracy and 99.98% when they used the same model plus a multi-class support vector machine[70].

A smartphone microchip, a microscope, and machine learning algorithms to develop an affordable, portable, and rapid screening test for sickle cell anaemia was combined. This module uses two deep neural networks: The first one enhances the picture taken by the smartphone microscope. The second one complements the first neural network by enhancing the picture and performing semantic segmentation between the normal RBCs and sickle RBCs within the blood film. Finally, these segmented images are used to help the diagnoses of the sickle cell disease patients. This method achieved around 98% accuracy using 96 samples; 32 were SCD thin blood smears and 64 normal thin blood smears[54]. A smartphone-based image acquisition process has been developed for imaging the RBCs from the SCD patients under oxygen control. This method can automatically distinguish the normal RBCs from the sickled RBCs based on the morphology change, using image processing (MATLAB R2019a) to analyze the image and quantify the sickled cells. This advanced technique is cheap and easy to use[70].

The image processing methods provide automated interpreting of the blood cell images, minimizing errors, which can effectively monitor the SCD patient's status[69]. However, the image processing techniques have some drawbacks: they cannot distinguish between the different types of the SCD; a high concentration of HbF can affect the polymerization of HbS, which can exclude the application of these tests to new-born screening[71]; they cannot classify RBCs accurately because they rely on binary classification, which ignores other blood cells shapes; and they are time consuming and require special equipment such as digital camera or smartphones[70].

2.1.10.12 Emerging Flow Cytometry

Conventional flow cytometry techniques have been used to detect sickle cells based on fluorescent markers or cellular morphology[72]. Advanced flow cytometry based on imaging techniques has been demonstrated to enhance the sensitivity by combining cell population analysis and morphological data. Beers et al. developed an imaging flow cytometry assay (SIFCA) and software algorithm to distinguish between sickle RBCs and normal RBCs based on their morphology. SIFCA is performed by diluting the peripheral blood sample, deoxygenating the cells by reducing the oxygen to 2% for 2 hours, and then analyse it using imaging flow cytometry. Finally, the cells are classified based on the morphology into sickled and normal cells by using algorithm software. The authors analyzed 100 images of normal cells and 100 images of sickle cell, and they reported 100% sensitivity and 99.1% specificity. The study proved that SIFCA can assess sickling tendency in SCA patients to identify the severity of the disease and drug monitoring[73].

In-vitro photoacoustic flow cytometry (PAFC) for morphological detection of sickle cells containing haemoglobin S. They employed photo thermal and photoacoustic spectra for determination of haemoglobin heterogeneity and accumulation of the HbS in sickle RBCs was developed. The sickled RBCs showed 2–4-fold lower linear mode than normal RBCs. This method is useful in monitoring the sickling states[72]. Microfluidic flow cytometry based on the electrical impedance spectroscopy.

This technique detects the changes in the electrical impedance resulted from the change in the cells' shape from the round soluble cells to sickle rigid cells under hypoxic condition. In this study, the cells were obtained from a healthy donor and three sickle cell patients, and the difference in the electrical impedance was measured to show the difference between normal cells and sickled cells. They showed that the electrical impedance signal can be used as an indicator of the cell sickling events. However, it is still unclear if these novel flow cytometry

techniques can be used to monitor disease severity or if they can distinguish between sickle cell trait and sickle cell disease[73].

2.1.10.12 Mechanical Differentiation of Sickle Cells

The deformability of red blood cells is a crucial determinant of blood flow in circulation. In sickle cell disease, RBCs are mechanically fragile and poorly deformable, resulting in impaired blood flow. This feature of the sickle cell can be used to monitor the disease severity and the sickling event[74].

Brandao et al. developed an optical tweezer to capture red blood cells (RBC) by dragging them through a viscous fluid (human AB plasma) to measure the elasticity of the cells. In this study, the RBC deformability was measured in 10 homozygous patients (HbSS), 5 patients taking hydroxyurea (HU) for six months (HbSS/HU), 10 patients with sickle cell trait (HbAS), and 35 normal controls. The RBCs deformability was lower in the patients with HbSS and HbAS; however, in patients taking hydroxyurea HbSS/HU, the cells' deformability was found to be similar to the normal control cells.

These results show that optical tweezers have the potential to be used to monitor hydroxyurea response in sickle cell disease, but they cannot be used to distinguish between different types of haemoglobin diseases[74].

An amplitude-modulated electrode formation in microfluidic for identifying the mechanical fatigue in single cells demonstrated. This method depends on the cell's mechanical fatigue, which leads to deterioration of the physical properties by subjecting the cells to static loads. In this method, a constant amplitude fatigue load is applied to deform the RBCs, and, with more fatigue cycles, the cells progressively lose their ability to stretch. Moreover, cyclic deformation was shown to be a fast method to deform RBCs under static deformation at the same maximum load. This testing platform can provide the possibility of flexible detection of sickle cells, but it is not yet validated in sickle cell detection[75].

A microfluidic-based method to detect and quantify sickle cells by modulating the disease's pathophysiology. In this study, the kinetics of cell sickling, unsickling, and cell rheology were investigated by exposing the cells to different hypoxic conditions to mimic microvasculature scenarios, sickling events, and hydroxyurea therapy was developed[74].

The microfluidic chip is a double-layer device consisting of a cell channel, polydimethylsiloxane film in the middle, and a gas channel where the O_2 concentration is controlled. In the deoxygenated state, when the oxygen is less than 5%, the shape of the RBCs that contain HbS change, and form sickled cells within 12 secs. This method has been used to monitor sickling events and hydroxyurea therapy[75].

A spatiotemporal analysis of cell membrane fluctuations for the diagnosis of SCD was developed. The test is based on using a hologram video containing either normal RBCs or sickled RBCs. The video was recorded using a low-cost, compact, 3D-printed shearing interferometer. Each hologram film was reconstructed and formed a spatiotemporal data cube. These data were extracted by calculating the standard deviations and the mean of the cell membrane fluctuations at every location over time. This resulted in a two-dimensional map of the standard deviation and mean. This method can be considered as low-cost, fast, and it does not need trained personnel to run. The accuracy of the results could be enhanced by combining it with the machine learning approach[75].

Techniques that depend on the detection of sickle cells' mechanical deformability can only be used as a monitoring test in SCA, as they cannot indicate the disease's severity in heterozygous states.

2.1.10.13 Lateral Flow Immunoassay

Lateral flow assays (LFAs) are widely used as portable platforms in biomedical detection. a sensing platform called Sickle SCAN. It is used to detect normal haemoglobin HbAA, sickle cell trait HbAS, haemoglobin C trait HbAC, sickle-haemoglobin C disease HbSC, and

haemoglobin C disease HbCC was demonstrated. The test used polyclonal antibodies on lateral flow chromatographic immunoassay against haemoglobin S, haemoglobin C, and haemoglobin A to detect the different types of SCD qualitatively. The polyclonal antibodies are attached on the test strip; the sample migrates in the absorbent pads, where the antibody conjugated to the nanoparticles binds to the haemoglobin; and then both migrate to the test strip.

The haemoglobin binds with the corresponding antibody and produces blue lines. The Sickle SCAN cartridge contains four detection bands: the control band, normal HbA, HbS band, and HbC band. The test can be performed within minutes and costs a few dollars per test[76]. The validation of the Sickle SCAN assay to detect different haemoglobin. The test was performed using 139 whole blood samples (venous samples, dried blood spots, and spiked blood samples), and the results were compared to capillary electrophoresis (CZE) was tested[76]. The test's accumulative sensitivity and specificity for HbSS were 98.4% and 98.6%, respectively. The cumulative sensitivity and specificity for the diagnosis of HbSC disease were 100%. A neonate sample with a high amount of HbF was tested and demonstrated that the detection of HbS or

HbC was not affected by the high concentration of HbF. Furthermore, they examined the test's storage condition and documented that the device can be stored at 37 °C for 30 days. However, the test suffers from some limitations such as misinterpretation of the result due to visual reading, cross-reactivity of the polyclonal antibody, and false-positive results in detecting the

HbA heterozygous with HbS. The authors recommended another validation for the sickle SCAN in primary healthcare centers[76].

2.1.11 Current Management of SCD in Nigeria

2.1.11.1 Prophylaxis and Vaccinations

Persons with SCD have impaired immunity and are prone to infections. This occurs mainly due to poor splenic function with 28% and 94% of SCD children being affected before age 1 and 5 years, respectively[77]. Spleen in SCD is generally normal at birth but its susceptibility to injury after haemoglobin switch initiates vaso-occlusion followed by ischemia. These events favour RBC adhesion to the spleen matrix owing to increased expression and activation of adhesion molecules, leading to fibrosis, atrophy, and finally auto splenectomy. Children with SCD are often more susceptible to fatal infection by encapsulated organisms such as Streptococcus pneumoniae, Haemophilus influenza, and Salmonella species while older children with SCD are affected more often by Gram-negative enteric organisms such as Escherichia coli[78]. In a multicentre, randomized, double-blind, placebo controlled clinical trial, daily administration of oral penicillin reduced the incidence of septicaemia due to S. pneumoniae in children (younger than three years) with sickle cell anaemia by 84% as compared with the group given placebo with no deaths from pneumococcal septicaemia occurring in the penicillin group but three deaths from the infection occurring in the placebo group[79]. Thus, twice daily oral administration of penicillin prophylaxis until age 5 was introduced into SCD standard care and has significantly reduced morbidity and mortality in high-income countries. In Nigeria, only 50% of paediatric sickle cell clinics in major cities routinely offer penicillin prophylaxis while Pneumococcal vaccines administration is not a commonplace except in Lagos. High cost and inaccessibility of these prophylactic interventions coupled with inadequate knowledge of the efficacy of the prophylaxis have limited the widespread implementation in Nigeria[80]. The importance of malaria prophylaxis for SCD persons in malaria-endemic regions cannot be overemphasized

2.1.11.2 Blood Transfusion

Blood transfusion is a life-saving therapy for the management of complications of SCD especially in the treatment and prevention of stroke and acute chest syndrome. Significant progress has been made in the prevention of first stroke occurrence with the use of Transcranial Doppler screening and chronic blood transfusion for SCD patients with abnormal cerebral blood flow. The STOP trial provided evidence that chronic blood transfusion to reduce haemoglobin S to ≤ 30% is effective in reducing the risk of a first stroke by 90% in children with sickle cell anaemia who have abnormal TCD scans. However, the major impediments to successful chronic blood transfusion therapy particularly in countries in sub- Saharan Africa are the unavailability of blood for regular transfusion, poor access to comprehensive treatment centers, cost of transfusion, socio-economic burden on the families, cultural and religious beliefs, risk of allo-immunisation, iron overload and transfusion transmissible infections. It was reported that all of the children with abnormal TCD declined chronic blood transfusion[81]. Diaku-Akinwumi et al. revealed that 78% of hospitals surveyed in a Nigerian study were unable to transfuse patients regularly due to blood scarcity. Problems of blood transfusion in Nigeria also contribute to the devastating complications of SCD and the eventual high incidence of morbidity and mortality.

2.1.11.3. Hydroxyurea Therapy

Hydroxyurea is a well-known HbF inducing agent. The proposed mechanism of action of hydroxyurea involves inhibition of ribonucleotide reductase, the enzyme that converts ribonucleotides into deoxyribonucleosides for DNA synthesis. The cytotoxic influence of hydroxyurea allows differentiation of erythroid progenitors for the production of red cells containing a high foetal haemoglobin level (F cells)[82]. This drug also lowers lactate dehydrogenase and bilirubin levels to improve red blood cell rheology. It also reduces the

leukocytes, reticulocytes, and platelets, thus decreasing their contributory effects on endothelial dysfunction. HU also increases haemoglobin and mean corpuscular volume (MCV).

Hydroxyurea appears to act as a donor of nitric oxide (NO) and may, in turn (or directly), stimulate guanylate cyclase, resulting in increased foetal haemoglobin production in erythroid lineage cells[83]. NO generated from HU may also compensate reduced NO bioavailability due to haemolysis and diminish vascular inflammation. The clinical effects of HU are seen in fewer vaso-occlusive crises and hospital admissions, decreased anaemia and haemolysis, and significantly improved quality of life of affected persons. In a multicentre BABY HUG trial involving daily oral use of hydroxyurea in children with sickle cell anaemia (9–18 months of age), hydroxyurea considerably correlated with lower incidence of acute chest syndrome, dactylitis, hospitalization and episodes of pain[84].

Based on the observed significant clinical findings, it was concluded that hydroxyurea therapy can commence at a younger age for all SCD patients. This, however, is not without some drawbacks due to misconceptions of health professionals, patients, and caregivers about the drug toxicity[55]. Hydroxyurea therapy was recommended in a cohort of Nigerian SCD children with elevated velocities on TCD scan but 38% of the children declined[55]. Nevertheless, children on HU showed a significant reduction in TCD velocities, thus providing an operational strategy for primary stroke prevention in Nigeria. Hydroxyurea treatment is feasible and seems safe for SCD children living in malaria endemic sub-Saharan Africa, without increased severe malaria, infections, or adverse events[85]. In the first randomized controlled trial with hydroxyurea therapy in Nigeria, high adherence rate to HU in children with abnormal TCD was demonstrated[56]. This indicates that compliance to HU therapy can be achieved when the doctors embrace the drug and give adequate counselling to parents/patients. It is therefore imperative to develop a workable protocol for primary stroke

prevention in Nigeria in order to reduce the burden of stroke among SCD children. Where chronic blood transfusion therapy is not feasible especially in many countries in sub-Saharan Africa, alternatives such as the use of hydroxyurea (HU) should be encouraged.

2.1.10.4 Haematopoietic Stem Cell Transplantation (HSCT)

Allogeneic HSCT is a highly specialized technique which involves replacing the patient's hematopoietic stem cell (HSC) with donor's HSCs containing a normal beta-globin genotype. Apart from the bone marrow-derived stem cells which have been used for most of the transplantation in

SCD, recently, peripheral blood stem cells and umbilical cord stem cells have been used successfully for transplantation[57]. SCD children with devastating complications such as stroke, acute chest syndrome, sickle nephropathy, recurrent priapism, and frequent vaso-occlusive episodes are those who have mostly benefited from the transplant due to the post-transplant risks including death; some, however, are of the opinion that transplant should be offered to all SCD children with matched sibling donors irrespective of whether or not they have less severe SCD complications. In the developed countries, HSCT has played a significant role in the management of SCD and remains the only cure for SCD, although gene therapy also holds great potential in future going by recent advances in the procedure. The first successful allogeneic HSCT in Nigeria was done in 2011 for a-7-year-old boy with SCD and stroke who received stem cells from his 14-year-old HLA matched sibling[86]. Thereafter, eight other successful HSCT had been done in the country. Donor availability, high cost of the transplant, graft versus host disease (GVHD), graft rejection, and infertility are major limiting factors and complications associated with HSCT program[58]. There are ongoing efforts to establish an

HSCT program in sub-Sahara Africa countries but the much-needed expertise, infrastructural development, and funding are lacking. Nonetheless, collaborative efforts between teaching

hospitals, governmental and non-governmental organizations can establish a well-coordinated, cost efficient and sustainable HSCT program in Nigeria.

The number of patients with sickle cell disease is expected to increase, both in high-income and lower-income countries. In high-income countries, this increase largely reflects gains in life expectancy among affected persons as a result of interventions such as newborn screening, penicillin prophylaxis, primary stroke prevention, and hydroxyurea treatment[87]. Life expectancy has improved significantly in high-income countries over the past 40 years, with childhood mortality now close to that in the general population and an observed median survival of more than 60 years[88].

Despite these remarkable achievements, life expectancy for patients with sickle cell disease is reduced by about 30 years, even with the best medical care, and the quality of life is often poor. Hydroxyurea treatment the sole approved pharmacologic therapy for sickle cell disease is increasingly used in both adults and children. However, treatment and management of the disease remain costly[89], making full access to care available only for the most privileged; otherwise, access is very limited because of increasing pressures on public health services. New developments in the management of sickle cell disease are highlighted by many recent and ongoing phase 3 clinical trials and by the increasing numbers of patients who are benefiting from hematopoietic stem-cell transplantation.

In lower-income countries, where childhood mortality from all causes has been substantially reduced in the past two decades, increased numbers of affected babies and young children now survive to adulthood, requiring diagnosis and treatment. In Africa, where there is a lack of newborn screening and routine childhood vaccinations and where malaria, malnutrition, and poverty remain important challenges, the mortality among children with sickle cell disease who are younger than 5 years of age can be as high as 90%[61]. Although a few large-scale screening programs have been successfully launched relatively recently, the lack of a

basic health care infrastructure in many regions makes the prevention and management of sickle cell disease extremely difficult.

2.2 Haemoglobin Structure and Function

Haemoglobin, iron-containing protein molecule in the red blood cells (erythrocytes) of vertebrates that transports oxygen to the tissues[90]. The haemoglobin molecule is a tetrameric protein composed of 2-alpha-globin and 2 gamma globin molecules in HbF and 2alpha-globin and two 2 beta-globin molecules in HbA[91], with each of the four chains attached to a haem group composed of porphyrin and an Iron (Fe) atom[37]. Haem, which accounts for only 4 percent of the weight of the molecule, is composed of a ring like organic compound known as a porphyrin to which an iron atom is attached. It is the iron atom that binds oxygen as the blood travels between the lungs and the tissues. There are four iron atoms in each molecule of haemoglobin, which accordingly can bind four molecules of oxygen. Globin consists of two linked pairs of polypeptide chains (Fig 2.2)[92]. Haemoglobin is what gives RBCs their shape. RBCs usually look like donuts, but with a thin centre instead of a hole.

Haemoglobin is a two-way respiratory carrier, transporting oxygen from the lungs to the tissues and facilitating the return transport of carbon dioxide[93]. Haemoglobin is a two-way respiratory carrier, transporting oxygen from the lungs to the tissues and facilitating the return transport of carbon dioxide. In the arterial circulation, haemoglobin has a high affinity for oxygen and a low affinity for carbon dioxide, organic phosphates, and hydrogen and chloride ions. In the venous circulation, these relative affinities are reversed[51].

Haemoglobins forms an unstable reversible bond with oxygen. In the oxygenated state, it is called oxyhemoglobin and is bright red; in the reduced state, it is purplish blue. Haemoglobin is produced in bone marrow by erythrocytes and is circulated with them until their destruction[94]. It is then broken down in the spleen, and some of its components, such as iron, are recycled to the bone marrow. Other components, such as the heme groups, are broken

down into bilirubin, transported to the liver, and secreted with the bile into the intestine for eventual elimination from the body[95].

Nitric oxide and carbon monoxide can also bind with haemoglobin. Carbon monoxide binds to haemoglobin much more strongly than oxygen[96]. Its presence keeps oxygen from binding to haemoglobin. Some normal haemoglobin types are; Haemoglobin A (Hb A), which is 95–98% of haemoglobin found in adults, Haemoglobin A2 (Hb A2), which is 2–3% of haemoglobin found in adults, and Haemoglobin F (Hb F), which is found in adults up to 2.5% and is the primary haemoglobin that is produced by the foetus during pregnancy[97].

The haemoglobin loading and unloading of oxygen can be expressed by an oxygen dissociation curve. The physiologic consequences of the abnormal haemoglobins depend on the oxygen affinity, which defines the point of 50% saturation (p50)[98]. The oxygen dissociation curve of normal haemoglobin represents the reaction of haemoglobin with oxygen as modified by hydrogen ions (Bohr effect) and 2,3-bisphosphoglycerate (BPG). Haemoglobin oxygen affinity increases with falling temperature and decreases with rising pH and 2,3-BPG. Hence, red blood cells containing such an abnormal haemoglobin may have an abnormal oxygen dissociation curve because of;

1. An intrinsic abnormality of haemoglobin-oxygen dissociation,
2. An altered interaction of haemoglobin with BPG,
3. An altered Bohr Effect, or
4. A combination of any or all of the above. It is common to speak of the oxygen-dissociation curve as being shifted to the left (increased oxygen affinity) or to the right (decreased oxygen affinity).

Several haemoglobins with increased oxygen affinity have substitutions affecting the $\alpha_1\beta_2$ contact of the tetramer. Others have substitutions involving the C-terminal residues of the beta chain or of the BPG binding sites. All these substitutions favour the oxygenated

conformation and cause a left shift of the oxygen dissociation curve, which reflects an increased blood affinity for oxygen. Therefore, it follows that the red cells of such individuals give up less oxygen to the tissues. The relative anoxia increases erythropoietin production and causes polycythaemia[55].

Most of the abnormal haemoglobins with increased oxygen affinity manifest themselves by causing polycythaemia in the carrier. The increased oxygen affinity reduces tissue oxygen delivery, causing an increase in erythropoietin secretion and in red cell mass[57]. The possibility of an abnormal haemoglobin with high oxygen affinity should be considered in those atypical patients with polycythaemia in which the white blood cell and platelet counts are not elevated and splenomegaly is absent. The importance of establishing the correct diagnosis is mainly to protect the patient from the chemotherapeutic treatment of polycythaemia. Family members should be advised that their children may be affected. The life expectancy of affected individuals is essentially normal, and most patients are symptom free. However, if such patients become symptomatic and their haematocrit rises towards 60%, then phlebotomy may be necessary to reduce blood viscosity[58].

2.2.1 Foetal Haemoglobin

Foetal haemoglobin (also haemoglobin F, HbF, or $\alpha_2\gamma_2$) is the main oxygen carrier protein in the human foetus. Haemoglobin F is found in foetal red blood cells, and is involved in transporting oxygen from the mother's bloodstream to organs and tissues in the foetus. It is produced at around 6 weeks of preg1ancy[99] and the levels remain high after birth until the baby is roughly 2–4 months old[100]. Haemoglobin F has a different composition from the adult forms of haemoglobin, which allows it to bind (or attach to) oxygen more strongly. This way, the developing foetus is able to retrieve oxygen from the mother's bloodstream, which occurs through the placenta found in the mother's uterus[50].

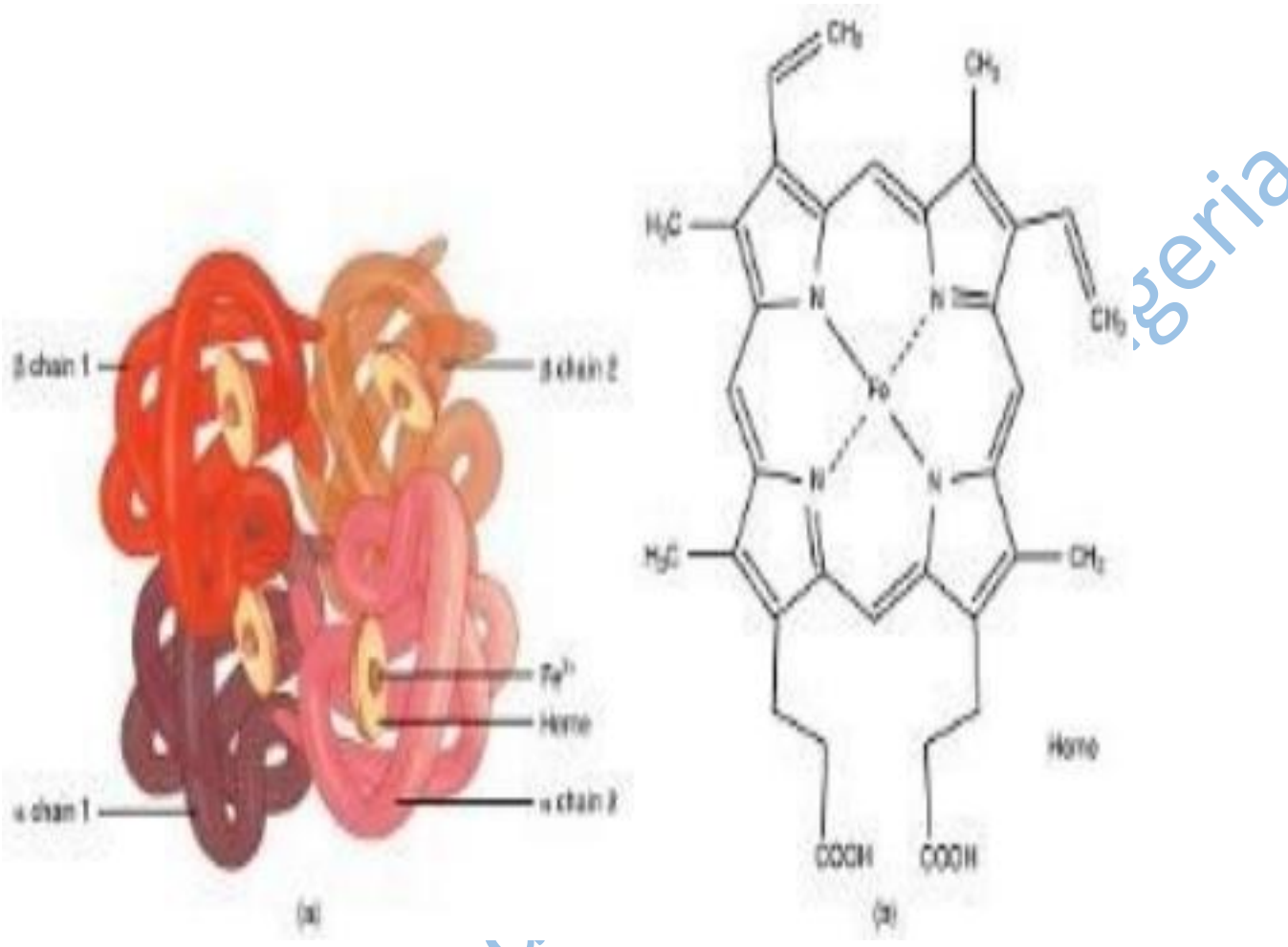

Figure 2.2 Structure of Haemoglobin

After birth, the HbF- gamma-gene is switched down and the HbA beta-gene is switched on so that adults mainly produce HbA (α2β2). After this developmental switch, low levels of HbF are still produced, and this is distributed heterogeneously with some red cells (F cells) expressing more HbF than others[101].

The level of HbF, and the associated proportion of F cells, is a highly heritable trait[102], and within a normal population the distribution of HbF is much skewed. Most healthy individuals produce <0.6% HbF distributed among 1–7% F cells, but a small proportion (about 2%) produce up to 5% HbF and 25% F cells and such individuals are said to have heterocellular hereditary persistence of foetal Hb. SCA patients with high HbF levels not only have less severe clinical course, but also have mild clinical complications because an increase in haemoglobin F inhibits polymerization of sickle haemoglobin[52]. The varying levels of foetal haemoglobin in erythrocytes account for a larger part of clinical heterogeneity observed in patients with sickle cell anaemia[103]. It is also a major prognostic factor for several clinical complications[104].

2.2.2 Biochemical and Clinical Evidence of HbF Amelioration in SCD

The first evidence that HbF plays a protective role in SCD is that affected infants are asymptomatic until after ~ 6 months of life[105]. Biochemical and clinical studies have confirmed the role of HbF in ameliorating the sickling process. The heterotetramer (α2γβS) that is formed in the presence of γ globin chains is much more soluble than the homotetramer (α2βS2); thus, there is an increase in the minimum gelling concentration of mixtures of HbF and HbS. Moreover, the polymerization kinetics are modified with consequent increase in the delay time to gelation[106]. Irreversibly sickled cells have lower levels of HbF, and greater numbers of these cells produce increased viscosity of oxygenated whole SS blood[107]. Probably, the earliest clinical study of the influence of HbF on SCD phenotype was by

Jackson[108], who, in 1961, showed that their SS patients with HbF > 12% had a significantly lower pathology index than those with levels <10%.

2.2.3 Globin Switching Regulation

The β-like globin genes are linearly arranged in the order in which they are expressed during development (*HBE1*, *HBG1*/*HBG2*, *HBD*/*HBB*). The 16-kb-long locus control region (LCR) consists of four erythroid specific DNase I hypersensitive sites (DHS) that are made up of clusters of binding sites for transcription activators. The LCR is located 40–60 kb upstream of the β-like genes cluster, and regulates the expression of the β-like genes by looping and direct interaction with the β-like globin promoters[109]. A complex combination of factors, including GATA1, TAL1, E2A, LMO2 and LDB1, is thought to mediate formation of the loop between the LCR and the globin promoters[110]. β-like globin gene expression is regulated by geneautonomous control, competition between the globin genes for the LCR[57], and repressive elements, prominently trans-acting factors binding to the γ-δ intergenic region, such as BCL11A[60].

Genome-wide association (GWAS) and functional follow up studies have identified *BCL11A* (B-cell lymphoma/leukaemia 11A) as a key negative regulator of *HBG* (*HBG1*/*HBG2*) expression[111]. The BCL11A protein, a zinc-finger transcriptional repressor, occupies critical sites within the β-like globin gene cluster and promotes long-range physical interactions between the LCR and the *HBB* promoter at the expense of the *HBG* promoter. It is thought that

BCL11A exerts this role by interacting with the erythroid master regulators GATA1, SOX6, ZFPM1/FOG1 and the NuRD repressor complex, which includes HDAC1 and HDAC2[61]. In the absence of BCL11A, upstream enhancers of the β-globin gene cluster, the LCR, co-locate with the transcriptionally activated *HBG*[112], and γ–globin is expressed. Inhibition or reduction of BCL11A, therefore, is an excellent strategy for HbF induction. However, BCL11A has

multiple functions outside of HbF regulation in non-erythroid cells**.** Indeed, it is essential that the erythroid-specific BCL11A enhancer be used as a target for HbF induction[113].

Genomic studies have also identified common variants within the intergenic region between GTP-binding elongation factor *HBS1L* and myeloblastosis oncogene *MYB* on chromosome 6q that are associated with elevated HbF levels[114]. The MYB transcription factor is a key regulator of haematopoiesis, erythropoiesis, and HbF levels, and modulates the erythroid traits via two mechanisms: first, directly via activation of *KLF1* and through other repressors of *HBG*, such as the nuclear receptors TR2/TR4; and second, indirectly through alteration of the kinetics of erythroid differentiation. Low MYB levels accelerate erythroid differentiation, leading to release of early erythroid progenitor cells that are larger, and still predominantly express γglobin[62].

KLF1 (Kruppel-like factor 1) is an essential erythroid-specific transcription factor that plays a key role in erythropoiesis. KLF1 influences haemoglobin switching both by directly activating β-globin gene expression in the adult stage through interacting with the *HBB* promoter, and by increasing the expression of the γ-globin silencer BCL11A by occupying the *BCL11A* promoter. Naturally occurring heterozygous *KLF1* mutations are reported to cause hereditary persistence of fetal haemoglobin (HPFH), and lentiviral knockdown of *KLF1* in cultured adult erythroblasts results in increased γ-globin and decreased BCL11A. It is, therefore, possible that pharmacological or gene manipulation of KLF1 levels may induce γ-globin, and be a practical therapy for sickle cell disease (SCD)[115]. Several other transcription factors have been implicated in *HBE/HBG* silencing. These include GATA1 in association with FOG1 and the NuRD complex, NF-E4, LRF/ZBTB7A, the TR2/TR4/DRED complex, and Ikaros in

association with the PYR co-regulatory complex[116].

Developmental haemoglobin switching is also regulated epigenetically, including alterations to higher-order chromatin structure, histone modifications, and DNA methylation. In adult erythroid cells, *HBG* is associated with increased cytosine methylation, loss of surrounding active histone modifications (such as H3K36me3, H3K27ac), and a decrease in chromatin accessibility compared with fetal erythroid cells. Elevation of γ-globin expression is observed following genetic or chemical inhibition of DNA methylation[117] by the methyl-cytosine binding protein MBD2, the histone arginine methyltransferase PRMT5, the histone methyltransferases EHMT1 and EHMT2[64] and histone deacetylases (HDACs). Furthermore, chromatin regulators are observed to occupy the *HBG* promoter in the form of repressive complexes including the DNA methylating enzyme DNMT3A, the lysine methyltransferase SUV4-20 h1, the serine/threonine kinase CK2alpha, and components of NuRD[67].

Lysine-specific demethylases (LSDs) are also involved in *HBG* silencing. LSD1 demethylates lysine 4 and lysine 9 in histone H3K4 and H3K9 respectively, and diminished *HBG* promoter

DNA methylation[66]. In addition, LSD1, along with several other co-repressors, is involved in TR2/TR4/DRED critical role in silencing *HBG*[68]. Inhibition of LSD1 results in increased γglobin gene expression in transgenic mice, primate models, and cultured primary human erythroid cells. However, since LSD1 is required for normal erythroid maturation, its inhibition can cause adverse effects, such as neutropenia[118].

MicroRNAs (miRNAs) have also been implicated in globin gene switching through interactions with known γ–globin regulators and through unknown mechanisms. Lin28B proteins and its known target let-7 miRNA family are involved in foetal to adult erythroid development; at least part via the inhibitory effect of LIN28B on BCL11A expression[119]. MIR486-1-3p has been shown to bind to the *BCL11A* mRNA and down regulate its expression concomitant with upregulation of γ-globin expression in cultured human erythroid

cells[67] also have shown that miR-15a and miR-16-1 can elevate HbF expression by acting via MYB. Several other miRNAs such as miR-221/222, miR-26b, miR-146a and miR-96, are implicated in the regulation of γ-globin gene expression and globin gene switching, although their mechanism of action may not be well known. Well-described molecular mechanisms of HbF regulation are summarized in Fig 2.2.

2.2.4 Importance of HbF in Pathophysiology of SCD

Sickle cell anaemia is a prototypical monogenic disorder, caused by the autosomal recessive inheritance of a single base substitution (A-T) in the first exon of *HBB*. This substitution results in the replacement of negatively charged, hydrophilic glutamic acid by a hydrophobic amino acid, valine, at position 6 (*HBB*; glu(E)6 val(A); G*A*G-G*T*G; rs334), which leads to defective haemoglobin tetramers that polymerize and aggregate upon deoxygenation, changing flexible, soft discoid red blood cells into stiff, sickle-shaped cells. Biochemical studies have demonstrated that the presence of HbF profoundly delays the polymerization and increases the solubility of HbS under deoxygenated conditions. HbF is even more potent than HbA in terms of polymer interference. X-ray crystallography shows that the sickle haemoglobin polymer is stabilized by the hydrophobic β 6 Val of sickle haemoglobin on one strand binding to a hydrophobic patch at β 85–88 on the adjacent strand. γ-globin has a glutamine rather than a threonine at position 87, which makes this hydrophobic interaction weaker[60]. Consequently, HbF tetramers containing γ-chains have a much lower probability of co-polymerizing with the sickle haemoglobin tetramers containing two β^S peptides.

Clinically, the importance of HbF levels in SCD was theorized by a paediatrician, Janet Watson, in 1948, well before the mechanistic work summarized above, when she noted that SCD clinical complications were rare before the age of 1 year of age, while HbF levels were still elevated[66]. The role of HbF was confirmed by studies describing essentially asymptomatic patients with SCD who co-inherited a HPFH phenotype marked by a

substantially elevated HbF level in adulthood. HPFH can be caused by *cis*-acting factors, such as deletions in the βglobin gene cluster, leading to compensatory increases in γ-globin synthesis in response to decreased or absent β-globin synthesis, and mutations in the *HBG* promoter regions or inheritance of HbF modulating quantitative trait loci (QTL), such as *HBS1L-MYB* intergenic region (6q23) and *BCL11A* (2p16)[120].

As a representation of patients' ethnic background or geographical area of origin, five major SCD haplotypes have been described, namely Benin (BEN), Bantu or Central African Republic (CAR), Cameroon (CAM), Arab-Indian (ARAB) and Senegal (SEN), in addition to atypical haplotypes[69]. Each haplotype is associated with a characteristic average level of HbF (ArabIndian>Senegal>Benin>Bantu); however, HbF levels varied among patients homozygous for any haplotype. For example, in carriers of Senegal and Saudi-Indian haplotypes, Xmn1 C-T restriction site polymorphism (158 bps upstream of *HBG2*) is associated with high HbF and Gγ-globin (HBG2) levels[66].

Although the pathophysiology of β-thalassaemia, insufficient or absent production of β-globin chains, is different from SCD, induction of HbF can ameliorate the symptoms at least by alleviating imbalanced non-α to α-globin chain synthesis and its consequent ineffective erythropoiesis and haemolysis caused by precipitation of unpaired α-globin chains. More mechanistic details and studies related to HbF induction in β-thalassaemia can be found in other reviews[121].

Hereditary persistence of foetal haemoglobin, or HPFH, a term perhaps first coined in 1958, is defined by deletions or point mutations within the HBB gene cluster. Large deletions that result in higher than normal levels of HbF persisting into adulthood cause the most frequently recognized type of HPFH. β thalassemia is also caused by HBB gene cluster deletions but has some hematologic features of thalassemia. HPFH and β thalassemia are overlapping phenotypes; their distinction is historical and semantic. The sizes of the causative deletions

overlap while their 5^{1} and 3^{1} breakpoints differ. Deletions of different sizes with diverse breakpoints might deferentially change the interactions among -globin genes and their upstream and downstream regulatory elements, such as the locus control region (LCR), and transcription factor complexes. This could account for the wide variation in HbF levels in heterozygous carriers of these mutations and in compound heterozygotes with the HbS gene. Identical HPFH-causing mutations, can be associated with dissimilar HbF levels. Although the causes of this are unknown, this might be a result of the inheritance of the minor alleles of the quantitative trait loci (QTL) modulating HbF production that are associated with higher HbF.

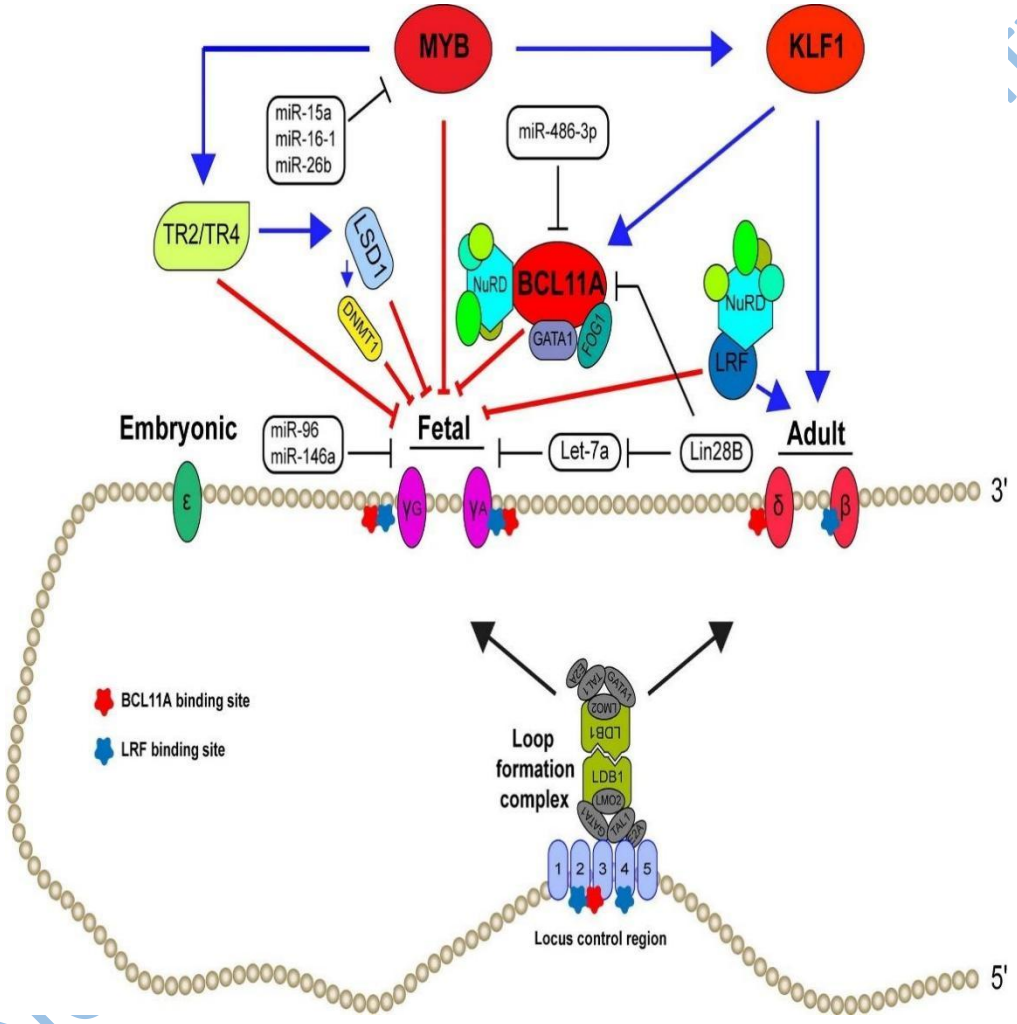

Fig 2.3 Regulation of Foetal Haemoglobin (HbF) Production[68].

2.3 Theoretical Review

The residual amounts of HbF in adults are distributed unevenly among the red blood cells; those that contain measurable amounts, are termed F cells (FC)[122]. The levels of both HbF and

F cells (erythrocytes with measurable amounts of HbF) are highly heritable traits[123] with up to 89% of variation being influenced by genetic factors. The remaining proportion is accounted for by age, sex and environmental factors. It is now clear that HbF is a quantitative trait which is shaped by genetic factors both linked and unlinked to the β-globin gene. Three main loci, namely BCL11A on chromosome 2, HMIP on chromosome 6, and HBG on chromosome 11,

have been identified across populations as associated with HbF levels[124]. The variants in these loci have been reported to contribute 20-50% of HbF variation in non-African populations, however the impact of these variants is different from one population to another. An example is a strong variant at HMIP, which is rare in the Tanzanian population and hence has a smaller impact on HbF levels there[125]. HbF levels in SCD, as a quantitative trait, is expected to be influenced by other polymorphisms, including insertions/deletions, rare mutations or copy number variations[126].

2.3.1 Positional Cloning and the Discovery of the HMIP locus on Chromosome 6q23.3

Positional cloning and the discovery of the HMIP locus on chromosome 6q23.3Early studies

suggested that high HbF determinants segregated independently of the HBB in some families with thalassemia and SCA; these families were often discovered through the probands who had unexpectedly mild disease[127]. In one such extended family of Asian-Indian origin, complex segregation analysis showed strong evidence for a major HbF gene that was inherited independently of the HBB cluster[76]. Using a regressive model that included the major QTL genotype and the effects of age, thalassemia and the Xmn1-HBG2site, a genome-wide linkage analysis of the Asian-Indian kindred identified a major locus on chromo-some 6q23–q24[128].

Follow-up haplotype mapping narrowed the candidate interval to 1.5 Mb[79] but re-sequencing of the five known genes in the region (ALDH8A1, HBS1L, MYB, AHI1 and PDE7B) revealed no mutations that could be implicated based on functional significance and segregation within the family[72]. High resolution association mapping was then carried out on a sample of North European ancestry[79]. A set of common SNPs spanning a nearly contiguous segment of 79 kb within HBS1L, the intergenic region 50 to HBS1L and the MYB oncogene, showed very strong association with F cell levels (p-value 10275 at the most significantly associated SNP)[77]. The SNPs were distributed in three linkage disequilibrium blocks referred to as HBS1L-MYB intergenic polymorphism (HMIP) blocks 1, 2 and 3.

2.3.2 Association with theXmn1-HBG2 Polymorphism at the HBB Locus

In 1985, a polymorphism (C/T at position2158 of HBG2, later termed Xmn1-HBG2 or rs7482144) was identified from re-sequencing of the HBG genes[78], and shown to promote the expression ofHBG2, and to contribute to HbF variability. Subsequent independent studies confirmed the association between theXmn1-HBG2T allele and increased HbF and FC, as well as milder disease among individuals with SCA and βthalassemia from different population groups[129]. The T allele was also shown to be associated with the Swiss-type HPFH[79]. Statistical analyses have identified no evidence for dominance at the locus,

suggesting an additive effect of theXmn1-HBG2polymorphism T allele. In a non-anaemic North European population, theXmn1-HBG2genotype was estimated to account for 13–32% of the total F-cell phenotypic variation[77]. The QTL identified with theXmn1-HBG2does not show Mendelian segregation and the distributions of the trait within each genotype class overlap considerably[79,80]. Presence of the 'T' allele does not always dictate the presence of a high HbF phenotype, and high HbF has been associated with βhaplotypes that do not include this allele[80]. The quantitative trait is characterized by genetic heterogeneity such that to produce a full high-HbF phenotype, Xmn1HBG2must exist on a genetic background requiring the presence of additional factors.

TheXmn1-HBG2site achieves its importance through its large impact on the trait variance and its high frequency (≈30%) in most population groups, including Europeans, Africans and Asian

Indians.

2.3.3 Genome-wide Association Studies and BCL11A on Chromosome 2

By early 2006, developments in genetic tools and genotyping platforms expedited by the International Human HapMap Project, led to a dramatic extension of the scope of genetic association studies, resulting in the genome-wide association study (GWAS) replacing the genome-wide linkage study as the most popular agnostic approach to whole-genome analysis[130]. Two GWAS of HbF/FC have been reported. The first GWAS utilized a selected genotyping study design, targeting 179 individuals with contrasting extreme FC values (FC values above 95th or below fifth percentile points), chosen from a phenotyped cohort of 5184 individuals[131]. Not only were the $^{\gamma}$globin gene region (with the strongest signal at theXmn1-HBG2site) and the chromosome 6 locus identified, but the study also found a new F-cell locus in intron 2 of theoncogeneBCL11A on chromosome 2p16. All SNPs implicated at this locus are common polymorphisms (with.10% minor allele frequency); the BCL11A locus

accounts for 15.1%; 6q, 19.4% and ᵞglobin region, 10.2% of the F-cell variability in Northern Europeans[81]. Statistical genetic theory predicts that association studies using the selected genotyping approach will be more powerful and cost effective than a design based on unselected individuals[81]. A criticism of the study design has been that it will be powerful for identifying genotypes that underlie the rare and extreme trait values, which are different from the genotypes responsible for normal variation in the trait. This criticism was put to rest by the second GWAS of 4000 individuals from Sardinia with unselected HbF, which replicated significant association at the same three loci[80]. The fact that both approaches, using FC or HbF as a quantitative trait, have identified the same set of three major loci is an additional argument that HbF and F cell are closely related traits.

2.3.4 Impact of the HbF QTLs`

Influence of the HBB locus and theXmn1-HBG2 site on HbF levels in SCA and βthalassemia has been validated by many studies in several populations. In the Asian Indian family in which the chromosome 6q23.3locus was first identified, the QTL affects individuals with and without βthalassemia[75]. Among βthalassemia heterozygotes, those who were homozygous for the 6q23.3 high F-cell QTL allele had trait values ranging from 10 to 24% HbF compared with a range of 0.3–3.6% in the individuals that were homozygous for the alternative allele. In family members without the βthalassemia mutation, high F cell 6q23.3 QTL homozygotes had 1.1– 3.0% HbF compared with 0.1–1.0% HbF in homozygotes for the alternative allele. Systematic studies of other patient groups have subsequently shown that the 6q QTL is also important in healthy individuals and in African American and British sickle cell patients with predominantly African admixed ancestry[132] and sickle cell patients from Brazil[82]. The chromosome 6q23.3 QTL contributes 3–7% of the trait variance in these populations[80].

The comparatively small contribution of the QTL to the trait variance in sickle patients compared with northern Europeans could be attributed to lower allele frequencies in African

populations[133]. Strong association with the locus was shown in Chinese βthalassemia heterozygotes, despite the confounding influence of the variable underlying ineffective erythropoiesis due to the wide spectrum of thalassemia mutations[134]. TheBCL11AQTL has shown the strongest effect on HbF/F-cell levels to date. In patients with SCA, the QTL accounts for7–12%[81] of the trait variance. The locus has also been shown to influence HbF or F cell levels in individuals of Chinese and Thai descent with βthalassemia or HbE[82]. In the Sardinian population the C alleles of the rs11886868 SNP at the BCL11A locus is strongly associated with high HbF levels and it is significantly more frequent in patients with milder βthalassemia disease (thalassemia intermedia) compared with the transfusion-dependent thalassemia major patients [81]. Both groups have identical band a genotypes, the Hb pattern comprised only HbF and trace amount of HbA2with no HbA, suggesting that variation at theBCL11Alocus can alleviate disease severity through raising HbF levels.

In SCA, the three known Hb/F cell loci contribute.20% to the HbF variance with a corresponding reduction in the frequency of acute pain associated with sickling[83]. High HbF expression clearly has ameliorating effects on sickle cell disease and βthalassemia, however, it is unknown if the effects translate to a selective advantage for carriers of the high HbF alleles in regions with a high incidence of the diseases. Genetic studies have shown that individuals with hemoglobinopathies concurrent with high expression of HbF can maintain normal fitness levels that would other-wise be severely limited by the debilitating consequences of their disease. The hemoglobinopathies are not present in the ancestral European population yet the alleles at the known major genes remain at high frequency and variation in the HbF expression persists. Strong evidence for natural selection has not been reported around the three major HbF/F cell QTLs[135] and similar allele frequencies have been observed for the QTL in different ethnic groups, suggesting that a selective sweep favouring genes for HbF persistence has not occurred in the relatively recent past.

It is possible that the pleiotropic effect of the major genes for HbF expression (for example, HMIP effect on other haematological variables)[83] ensure the persistence of the alleles in the absence of strong selection from the hemoglobinopathies. After the transition to expression of adult haemoglobin, there is no known biological role for HbF. Genetic studies of HbF/FC in varied populations will allow for more powerful analysis of the evolutionary history of the alleles at the known QTLs and a better under-standing of their biological importance.om Brazil[79].

2.4 Review of Empirical Studies

The variants most strongly associated in the 6q QTL reside in a 24 kb non-protein coding region between HBS1L and MYB oncogene (HMIP block 2)[78]. Recent studies show that HMIP 2 contains a distal regulatory locus evidenced by the presence of several prominent erythroidspecific GATA-1 signals that coincided with DNaseI hypersensitive sites, and the presence of intergenic transcripts in erythroid precursor cells[79]. It is suggested that the HMIP 2 regulatory elements distally control MYB expression that in turn influences erythroid differentiation, and indirectly, the control of HbF levels. MYB is a quantitative trait gene[136]; erythroid precursor cells from individuals with higher HbF and higher F cell levels have lower MYB expression that was also associated with lower erythrocyte count but higher erythrocyte volume, and higher platelet counts. Further, genotype variability at HMIP 2 has a pleiotropic impact on several types of peripheral blood cells: erythrocytes counts and volume, haemoglobin concentration, platelet and monocyte counts in healthy individuals of European ancestry[137]. Thus, the biological effect of the QTLs on HbF expression includes two plausible

mechanisms: (1) direct effect on HBG expression (activation or repression of HBG transcription) thereby increasing or decreasing the amount of HbF per cell; and

(2) alteration of the kinetics of erythroid maturation and differentiation, mimicking a situation encountered in stress erythropoiesis that results in accelerated erythropoiesis with the release of more erythroid progenitors that synthesize predominantly HbF i.e. FC, leading to an increase in circulating HbF.

The 3-bp deleted allele of *rs66650371* was also associated with increased haemoglobin levels[138]. This 3-bp deletion polymorphism is probably the most significant functional motif accounting for HMIP modulation of HbF in all 3 populations[88]. It was the only variant studied that had a significant effect on the red blood cell count[89]. This marker was also strongly associated with lower platelet counts. HbF-increasing alleles at *HBS1L-MYB* had low frequencies (3%), as is characteristic for African populations. Similar to the *BCL11A* locus, HbF-increasing alleles at the two sub-loci occurred within different haplotypes, which is typical for individuals of African descent[139]. This stands in contrast to the situation in European populations, where HbF-increasing alleles usually appear to be combined into a single haplotype (*HMIP-2AB*)[87]. Accordingly, for an ancestry informative marker tagging this haplotype, *rs9376090*, the 'G' allele, was not detected, indicating the absence of *HMIP-2AB* haplotypes and suggesting a lack of European, Asian, or North African admixture in the patient cohort[88].

2.5 Conceptual Framework

Ground-breaking studies have been conducted in Tanzania and Cameroon and some major steps have been taken to build African SCD subject cohorts and high-quality genetic datasets[140]. The studies also established the effects of the three HbF genetic modifier loci on clinical and laboratory measures of SCD severity. A significant point to note in the studies from the two countries is the remarkable genetic diversity of African populations, which

shows the need for genetic studies from individual African countries. There is a paucity of genetic data that give accurate characteristics of Nigerian SCD patients.

Based on recent studies, a hitherto unreported association between HbF expression and a 3-bp deletion, between 135 460 326 and 135 460 328 bp on chromosome 6q23 was found (*rs66650371)*[86]. This 3-bp deletion is in complete linkage disequilibrium with *rs9399137*, which is the single nucleotide polymorphism in HMIP, most significantly associated with HbF among Chinese, Europeans, and Africans[89].

The frequency of this ancestral sequence in African populations is comparable with the frequency of the sequence with the 3-bp deletion found in Chinese and European populations[89]. The rs7775698(T)-rs9399137(C) haplotype that tags the 3-bp deletion is very common in both the CHB and CEU 1000 Genomes Project samples with frequency of 31.5% and 21.4%, respectively[88]. In the YRI population, this 3-bp deletion haplotype is much less common with a frequency of 5.1%[89]. To examine the difference between non-African and African populations, we calculated the r2 between rs7775698 and rs9399137 in the 11 HapMap populations. All the non-African populations had a mean r2 (0.94) much higher than that in African populations (0.17). The frequency of the rs7775698 (T)-rs9399137(C) haplotype is 0.23 in non-African HapMap populations but only 0.05 in African Hap-Map populations. The difference in the r2 is the result of the presence of the ancestral sequence containing rs7775698 (T) without the 3-bp deletion that is present in 17% of the African populations but is rarely found in non-African populations (_ 0.01). The rs9399137 (C) tags the sequence containing rs7775698 (T) with the 3-bp deletion in the Chinese and European populations examined. As a result of this LD pattern between rs9399137 (C) and the 3-bp deletion, the deletion is as highly significantly associated with HbF levels as the nearby (383 bp) rs9399137 (C) allele[141].

2.6 Summary of Gaps in Literature Reviewed

Ground-breaking studies have been conducted in Tanzania and Cameroon and some major steps have been taken to build African SCD subject cohorts and high-quality genetic datasets[142],[143]. The studies also established the effects of the three HbF genetic modifier loci on clinical and laboratory measures of SCD severity. A significant point to note in the studies from the two countries is the remarkable genetic diversity of African populations, which shows the need for genetic studies from individual African countries. There is a paucity of genetic data that give accurate characteristics of SCD severity among Nigerian patients. In a recent study carried out in Nigeria[144], the first systematic evaluation of the known major HbF QTLs in Nigerian patients with sickle cell disease and demonstrated the beneficial effects of two quantitative-trait loci for foetal haemoglobin expression, BCL11A and HMIP[98]. The study presented the initial survey for subsequent large-scale genetic study. Another research group observed an association between a high-genetic risk profile (defined as co-inheritance of α-thalassemia and the BCL11A rs1427407 T allele) and increased haemolysis and stroke in the SCD cohorts in Ibadan[145]. It is important that researchers should take advantage of the teeming population of persons with SCD in Nigeria to carry out large sample size genotype–phenotype studies in order to have a clearer understanding and a good description of the patients. Such studies may also identify novel genetic modifiers with therapeutic potentials. Up to now, most research on the genetics of foetal haemoglobin concentration and its disease severity has taken place in the US and Europe while the great majority of patients live in Africa. Genetic and epidemiological studies can help to address this imbalance.

Endnotes

[1] B. Frédéric, B. Piel, H. Martin & David C. Rees, *Sickle Cell Disease*, **The New England Journal Of Medicine,** 376 (16), 2017, 1561 - 1573

[2] Centre For Disease Control (Cdc) *What Is Sickle Cell Disease* Available Online:

Https://Www.Cdc.Gov/Ncbddd/Sicklecell/Features/What-Is-Scd.Html, 2020

[3] Asnani Mr, Knight Madden J, Reid M,Greene Lg, Lyew-Ayee P. *Socio-Environmental Exposures And Health Outcomes Among Persons With Sickle Cell Disease*. **Plos One**, 12(4), 2017, 1-20

[4] S. Chakravorty, T.N. Williams. *Sickle Cell Disease: A Neglected Chronic Disease Of Increasing Global Health Importance*. **Archives Of Disease In Childhood**, 100 (1), 2018, 4853

[5] *Robert Resnik, In Creasy & Resnik's. Maternal-Fetal Medicine: Principles And Practice,* ***Elsevier Health,*** *(8th Edition), 2019, 999-1006*

[6] L. Pecker, S. Lankrazo., *Sickle Cell Disease* **Annintern Med** 174(1), 2021; 1-5

[7] P.S Frenette, G. Atweh.. *Sickle Cell Disease: Old Discoveries, New Concepts, And Future Promise*. **Journal Clinical Invest**. 117(4), 2007, - 850-858.

[8] L. Marathon *The Sickle Cell Association Of New Jersey*, **Inc.*Located In Newark At* 1016** Broad Street Newark, New Jersey 07102, 2016

[9] S. Embury. *The Contribution Of Endothelial Cell P-Selectin To The Microvascular Flow Of Mouse Sickle Erythrocytes In Vivo. Blood*. **Pubmed**, 104(25), 2014 :3378–3385.

R. Graham. "*The Natual History Of Sickle Cell Disease". Cold Spring Harb Prospect*, 3(19) **Pubmed**, 2013, 10-21

[10] S. Paul, Frenette & George F. Atweh. *Sickle Cell Disease: Old Discoveries, New Concepts, And Future Promise,* **Science In Medicine**, 17(4), 2007; 850-861

[11] B. Inusa, M. Casale & N, Ward Introductory Chapter: Introduction To The History, Pathology And Clinical Management Of Sickle Cell Disease, **Intechopen**, 5th Edition, 2017

[12] E. Joseph, M. Maakaron; Chief Editor: Emmanuel C Besa, *Sickle Cell Disease Clinical Presentation,* Updated: Nov 02, 2021.

[13] Centre For Disease Control (CDC) *What Is Sickle Cell Disease,* Available Online: Https://Www.Cdc.Gov/Ncbddd/Sicklecell/Features/What-Is-Scd.Html, 2020

[14] P. Mcgann. *Time To Invest In Sickle Cell Anemia As A Global Health Priority*. **Pediatrics,** 137(7), 2016, 40-59

[15] E. Gluckman, B. Cappelli, *Sickle Cell Disease: An International Survey Of Results Of HlaIdentical Sibling Hematopoietic Stem Cell Transplantation,* **Blood**, 129 (11), 2017, 1548–1556

[16] B. Modell B & M. Darlison. *Global Epidemiology Of Haemoglobin Disorders And Derived Service Indicators*. **Bull World Health Organ**, 86(8), 2008, 480–487

[17] V. Cumming, King L, Fraser R, Serjeant G & Reid M. "*Venous Incompetence, Poverty And Lactate Dehydrogenase In Jamaica Are Important Predictors Of Leg Ulceration In Sickle Cell Anaemia"*. **British Journal Haematol**, 142(208), 2008 119–125

[18] S. Ballas & D. Darbari. *Review/Overview Of Pain In Sickle Cell Disease*. **Complement Ther Med**. 49(3),2020, 1080-1117

[19] F. Piel, Hay Si & Gupta. S. *Global Burden Of Sickle Cell Anaemia In Children Under Five, 2010-2050: Modelling Based On Demographics,Excess Mortality, And Interventions*. **Plos Med.** 10(5), 2010, 154-67

[20] N. I. Ugwu , E.O.Nna , C. N. Ugwu, O. E. Ogah , C. Okike, R. C. Ikeagwulonu , N. U. Uzodin, A. J. Madu, H. C. Okoye, I. C. Uzoma, C. Alo, G. C. Ugwu , V. N. Ekpeagu, U. I. Okeke. *Evaluation Of Foetal Haemoglobin Status Among Nigerian Patients With Sickle Cell Anaemia Using High Performance Liquid Chromatography*, **Wjam,** 38(3), 2021, 222-227

[21] I. Osunkwo, Andemariam. B, Caterina.P, Inusa. B, Rassi. F, Nero. A, Colombatti. R, Debonnett. L, Bailey. T & John. J, *Impact Of Sickle Cell Disease On Patients' Daily Lives, Sympoms Reported, And Disease Management Strategies: Results From International Sickle Cell World Assessment Survey,* **American Journal Of Hematology** 96(4), 2021, 403-417

[22] D. J. Weatherall, Clegg Jb. *Inherited Haemoglobin Disorders: An Increasing Global Health Problem*. **Bull World Health Organ**.79 (1), 2001, 704–712

[23] B. Modell B & M. Darlison. *Global Epidemiology Of Haemoglobin Disorders And Derived Service Indicators*. **Bull World Health Organ**, 86(8), 2018, 480–487

[24] M. E. Odunvbun, A. A. Okolo, C. Rahimy. *Implementing Newborn Screening For Sickle Cell Disease As Part Of Immunisation Programmes In Nigeria: A Feasibility Study*, **The Lancet Haematology,** 7(7), 2020,1111–1116.

[25] G. Pule, S. Mowla, N . Novitzky, C. Wiysonge, A . Wonkam. *A Systematic Review Of Known Mechanisms Of Hydroxyurea-Induced Fetal Hemoglobin For Treatment Of Sickle Cell Disease.* **Expert Rev Hematol**., 8(5), 2017, 669-79.

[27]I. Lagunju. & Brown. J. T*ranscranial Doppler Screening In Nigerian Children With Sickle Cell Disease: A 10-Year Longitudinal Study On The Sppiba Cohort*, **Int J Hematol**. 68 (4), 2021, 710–718

[28] M. Kehinde, E. Temiye, M. Danesi. *Neurological Complications Of Sickle Cell Anaemia In Nigerian Africans–A Case-Control Study*. **J Natl Med Assoc**. 100(20), 2018, 394–399.

[29] S. A Adegoke, O. Adeodu N. Adekile. *Sickle Cell Disease Clinical Phenotypes In Children From South-Western, Nigeria.* **Niger J Clin Pract**. 18. (8), 2018, 95–101.

[30] V. Niel, G., D'angelo, G. & Raposo, G. *Shedding Light On The Cell Biology Of Extracellular Vesicles*. Nat. Rev. Mol. Cell Biol, Available Online:. Https://Doi.Org/10.1038/Nrm.2017.125 2018.

[31] C. T . Quinn,.. *Biochemical Surrogate Markers Of Hemolysis Do Not Correlate With Directly Measured Erythrocyte Survival In Sickle Sell Anaemia*. **Am. J. Hematol**. 91, 2016, 1195– 1201

[32] H. F. Bunn,. . *Pulmonary Hypertension And Citric Oxide Depletion In Sickle Cell Disease.* **Blood** 116, 2010, 687–692

[33] G. J. Kato, Steinberg, M. H. & Gladwin, M. T. *Intravascular Hemolysis And The Pathophysiology Of Sickle Cell Disease*. **J. Clin. Invest**. 127, 2017, 750–760.

[34] M. Aslan. & B. A Freeman. *Redox-Dependent Impairment Of Vascular Function In Sickle Cell Disease.* **Free Radic. Biol. Med**. 43(5), 2007 1469–1483.

[35] M. Lucky And Lynda.W. "*Sickle Cell Disease Among Children In Africa: An Integrative Literature Review And Global Recommendations*". **Internal Journal Of Africa Nursing Sciences** 3(5), 2018, 56-64

[36] L. Miller-Fleming., V. Olin-Sandoval, V., Campbell, K. & Ralser, M. *Remaining Mysteries Of Molecular Biology: The Role Of Polyamines In The Cell.* **J. Mol. Biol**. 427, 2015, 3389– 3406.

[37] J. Teixeira, K.Cristina Ribeiro Malmegrim, *Immune Mechanisms Involved In Sickle Cell Disease Pathogenesis: Current Knowledge And Perspectives*, **Immunology Letters**,224, 2020, 1-11,

[38] S. Chaturvedi, M. Debaun. *Evolution Of Sickle Cell Disease From A Life-Threatening Disease Of Children To A Chronic Disease Of Adults: The Last 40 Years*. **Am J Hematol**. 91(1), 2016;5–14.

[39] G. J. Kato, Mcgowan V, Machado Rf, . *Lactate Dehydrogenase As A Biomarker Of Hemolysis-Associated Nitric Oxide Resistance, Priapism, Leg Ulceration, Pulmonary Hypertension, And Death In Patients With Sickle Cell Disease*. **Blood**.;107(6), 2016, 2279–2285

[40] G. Kato, Gladwin Mt, Steinberg Mh. *Deconstructing Sickle Cell Disease: Reappraisal Of The Role Of Hemolysis In The Development Of Clinical Subphenotypes*. **Blood Rev**. 21(1), 2007; 37–47.

[41] R. Claud, A. Frans . Kuypers, R. Hagar, Sandra Larkin, Lisa Lavrisha, Augusta Saulys, Elliott P. Vichinsky, Jung H. Suh, *Implications For The Metabolic Fate Of Oral Glutamine Supplementation Within Plasma And Erythrocytes Of Patients With Sickle Cell Disease: A Pharmacokinetics Study,Complementary* **Therapies In Medicine**, 64(5), 2022, 102803,

[42] P. Baba, L. Lewis, K. Neeraj, A. Patel, Kilali Ominu-Evbota, Kofi A. Anie & W. Atoyebi, *Sickle Cell Disease—Genetics, Pathophysiology, Clinical Presentation And Treatment,* **International Journal Of Neonatal Screening**, 5(20) 2019, 1-15

[43] S. Ballas, *How I Treat Acute & Persistent And Persistent Sickle Cell Pain,* **Mediterranean Journal Of Haematology And Infectious Diesease**, 12(1), 2020, 064-083

[44] N. Aeddula, M. Bardhan, Baradhi Km. *Sickle Cell Nephropathy*. National Centre Of Biotechnology Information, Available Online: Https://Www.Ncbi.Nlm.Nih.Gov/Books/Nbk526017/, 2021

[45] D. Ochocinski, M. Dalal, L. Black, Silvana Carr, Judy Lew, Kevin Sullivan & N. Kissoon, *Life-Threatening Infectious Complications In Sickle Cell Disease: A Concise Narrative Review*, **Frontiers**, 4(1); 2020, 78-102

[46] E. Joseph, Maakaron, C. Besa, *Sickle Cell Disease,* **Medscape,** 2(4), 2021, 30-49

[47] R. Ware., D. Montalembert, M., Tshilolo, L. & Abboud, M. R. *Sickle Cell Disease*. **Lancet**, 309(10), 2017, 311–323.

[48] E. Anietie. "*Sickle Cell Disease*." **Innovait** 10(2), 2017: 73–81

[49] A. Ade A. Adebanji, Anne Akinyanju, Olu, *A Prospective Diary Study Of Stuttering Priapism In Adolescents And Young Men With Sickle Cell Anemia: Report Of An International Randomized Control Trial--The Priapism In Sickle Cell Study*, **Journal Of Andrology**, 32(10), , 2011, 85- 100

[50] D. Greene., C. Vaugn., B. Crews., M. Agarwal. *Advances In Detection Of Hemoglobinopathies*. **Clin. Chim. Acta**; 439(7), 2015, 50–57.

[51] S. Ilyas., Simonson A.E., Asghar W. *Emerging Point-Of-Care Technologies For Sickle Cell Disease Diagnostics*. **Clin. Chim. Acta**. ;501(1), 2020, 85–91

[52] V. Acharya V., Prakasha K. *Computer Aided Technique To Separate The Red Blood Cells, Categorize Them And Diagnose Sickle Cell Anemia*. **J. Eng. Sci. Technol. Rev**. ;12(5), 2019, 67–80.

[53] T. Ubman., Field J.J. *Sickle Solubility Test To Screen For Sickle Cell Trait: What's The Harm?* **Hematol. Am. Soc. Hematol. Educ. Progr,** 14(2). 2015, 433–435.

[54] A. Okwi., Byarugaba W., Parkes A.T.R. Of S. & P. Ocaido, M. *The Reliability Of Sickling And Solubility Tests And Peripheral Blood Film Method For Sickle Cell Disease Screening At District Health Centers In Uganda* **A. Clin. Mother Child Heal.,** 7(5), 2010:1–5.

[55] T. Kotila. *Guidelines For The Diagnosis Of The Haemoglobinopathies In Nigeria*. **Ann. Ibadan Postgrad. Med**. 8(2), 2011, 25–29.

[56] B. Rentapalli., Gade J., Md D., Ganji S.B., Kiranmai P., Revupalli S., Kavali S. & A. Ramtenki . *Agar Gel Alkaline Electrophoresis As A Cost Effective Screening Modality For Abnormal Hemoglobins*. **Iosr J. Dent. Med. Sci**. 18(5), 2017, 39–43.

[57] W. Arishi, H. Alhadrami, Zourob M. *Techniques For The Detection Of Sickle Cell Disease: A Review*. ***Micromachines.*** 12(5), 2021, 519

[58] F.A Chen., C. Liu., Y. Hsieh., Sternberg J.C. *Capillary Electrophoresis—A New Clinical Tool.* **Clin. Chem**. 37(6), 1991, 14–19

[59] C. Frömmel. *Newborn Screening For Sickle Cell Disease And Other Hemoglobinopathies: A Short Review On Classical Laboratory Methods-Isoelectric Focusing, Hplc, And Capillary Electrophoresis.* **Int. J. Neonatal Screen**.5(10), 2018;4:39.

[60] L. Pant., Kalita D., Singh S., Kudesia M., Mendiratta S., Mittal M., Mathur A. *Detection Of Abnormal Hemoglobin Variants By Hplc Method: Common Problems With Suggested Solutions*. **Int. Sch. Res. Not.** 2(10), 2014, 1–10.

[61] S. Nair S. *Potential Pithfalls In Using Hplc And Its Interpretation In Diagnosing Hbs*. **J. Rare Dis. Res. Treat.** 3(4) 2018, 9–12.

[62] S. Nair. *Potential Pithfalls In Using Hplc And Its Interpretation In Diagnosing Hbs*. **J. Rare Dis. Res. Treat.** 5(7), 2018, 9–12.

[63] E. Toye., G. Marle, W. Hutchins, O Abgabiaje., Okpuzor J. *Single Tube Allele Specific Pcr: A Low Cost Technique For Molecular Screening Of Sickle Cell Anaemia In Nigeria*. **Afr. Health Sci.** 18(5), 2018; 995–1002

[64] P.J Singh., Shrivastava A.C., Shrikhande A. V *Prenatal Diagnosis Of Sickle Cell Disease By The Technique Of Pcr.* **Indian J. Hematol. Blood Transfus**. 31(5), 2015, 233–241

[65] G.R Tripathi. *A Simplified And Cheapest Method For The Diagnosis Of Sickle Cell Using Whole Blood Pcr And Rflp In Nepal*. **Tribhuvan Univ. J.** 30(8), 2016, 57–64

[66] , T.K. Boy., Belding., Margolet, L.& Noyes, A.N. *Foetal Haemoglobin Restriction To A Few Erythrocytes (F Cells) In Normal Human Adults*.**Science**,188, 1995, 55-67

[67] B. Hamda C., Sangeda R., Mwita L., Meintjes A., Nkya S., Panji S., Mulder N., GuizaniTabbane L., Benkahla A., Makani J., *A Common Molecular Signature Of Patients With Sickle Cell Disease Revealed By Microarray Meta-Analysis And A Genome-Wide Association Study*. **Plos One**. 13(7), 2018, 125-35

[68] J. Flanagan., Sheehan V., Linder H., Howard T.A., Wang Y.-D., Hoppe C.C., Aygun B., Adams R.J., Neale G.A., Ware R.E. *Genetic Mapping And Exome Sequencing Identify 2 Mutations Associated With Stroke Protection In Pediatric Patients With Sickle Cell Anaemia.* **Blood**., 121(5), 2013, 3237–3245

[69] M. Abdulraheemfadhel, A. Humaidi., Razzaqoleiwi S. *Image Processing-Based Diagnosis Of Sickle Cell Anemia In Erythrocyteimages.* **Appl. Iraq Ntict**. , 5(1), 2017, 203–207

[70] L. Alzubaidi., Fadhel M.A., Al-Shamma O., Zhang J., Duan Y. *Deep Learning Models For Classification Of Red Blood Cells In Microscopy Images To Aid In Sickle Cell Anemia Diagnosis*. **Electron**. 9(7), 2020, 427-35

[71] L.L Mulumba., Wilson L. *Sickle Cell Disease Among Children In Africa: An Integrative Literature Review And Global Recommendations Lucky*. **Int. J. Afr. Nurs. Sci**. 3(5), 2015, 56– 64.

[72] L .Samsel., Mccoy J.P., Jr. *Imaging Flow Cytometry For The Study Of Erythroid Cell Biology And Pathology*. ***J. Immunol. Methods.*** 2016;59-65

[73] K. Fertrin., Samsel L., Van Beers E.J., Mendelsohn L., Kato G.J., Mccoy J.P. Imaging Flow Cytometry. Humana Press; New York, Ny, Usa: . Sickle Cell Imaging Flow Cytometry **Assay (Sifca) Pp**. 2016, 279–292.

[74] M. Brandao., Fontes A., Barjas-Castro M.L., Barbosa L.C., Costa F.F., Cesar C.L., Saad S.T.O. *Optical Tweezers For Measuring Red Blood Cell Elasticity: Application To The Study Of Drug Response In Sickle Cell Disease*. **Eur. J. Haematol**. 70(5), 2003, 207–211.

[75] Y. Qiang, Liu J., Dao M., Suresh S., Du E. *Mechanical Fatigue Of Human Red Blood Cells.* **Proc. Natl. Acad. Sci. Usa.** 116(5), 2019, 19828–19834

[76] P. Mcgann., Schaefer B.A., Paniagua M., Howard T.A., Ware R.E. *Characteristics Of A Rapid, Point-Of-Care Lateral Flow Immunoassay For The Diagnosis Of Sickle Cell Disease*. **Am. J. Hematol**.91(7), 2016, 205–210.

[77] A. Brown, Sleeper La, Miller St. *Reference Values And Hematologic Changes From Birth To 5 Years In Patients With Sickle Cell Disease*. *Cooperative Study Of Sickle Cell Disease*. **Arch Pediatr Adolesc Med**.;148(8), 1994, 796–804

[78] V. Brousse, Buffet P, Rees D. *The Spleen And Sickle Cell Disease: Thesick(Led) Spleen*. **Br J Haematol**.;166(2), 2018, 165–176.

[79] O. Ojewunmi , Adeyemo Ta, Osuntoki Aa. *Haemoglobin Oxygen Saturation, Leucocyte Count And Lactate Dehydrogenase Are Predictors Of Elevated Cerebral Blood Flow Velocity In Nigerian Children With Sickle Cell Anaemia*. **Paediatr Int Child Health**.;38(1), 2018, 34–39.

[80] O. Vincent O, Oluwaseyi B, James B. *Coinheritance Of B-Thalassemia And Sickle Cell Anaemia In Southwestern Nigeria.* **Ethiop J Health Sci.**;26(6), 2016, 517–522.

[81] I. Lagunju I, Sodeinde O, Telfer P, Brown. J.*Annual Stroke Incidence In Nigerian Children With Sickle Cell Disease And Elevated Tcd Velocities Treated With Hydroxyurea*. **Am J Hematol**. 68(3), 2018, 544–547

[82] O. Platt. *Hydroxyurea For The Treatment Of Sickle Cell Anaemia*. **N Engl J Med**.;358(13), 2018, 1362–1369.

[83] E. Nader, Marijke Grau, Romain Fort, Bianca Collins, Giovanna Cannas, Alexandra Gauthier, Katja Walpurgis, Cyril Martin, Wilhelm Bloch, Solène Poutrel, Arnaud Hot, Céline Renoux, Mario Thevis, Philippe Joly, Marc Romana, Nicolas Guillot, Philippe Connes, *Hydroxyurea Therapy Modulates Sickle Cell Anemia Red Blood Cell Physiology: Impact On Rbc Deformability, Oxidative Stress, Nitrite Levels And Nitric Oxide Synthase Signalling Pathway*, **Nitric Oxide**, 81, 2018, 28-35

[84] S. Rahyssa Rodrigues, N. Bárbara Lisboa, Tosatti Jéssica Abdo Gonçalves, Gomes Karina Braga, Luizon & M. Rizzatti. *Do Genetic Polymorphisms Affect Fetal Hemoglobin (Hbf)*

Levels In Patients With Sickle Cell Anemia Treated With Hydroxyurea? A Systematic Review And Pathway Analysis , **Frontiers In Pharmacology** , 12(5), 2022, 853-66

[85] J. Ansari J, Moufarrej Ye, Pawlinski R, *Sickle Cell Disease: A Malady Beyond A Hemoglobin Defect In Cerebrovascular Disease*. **Expert Rev Hematol**.;11(1), 2018, 45–55.

[86] A. Kassim Aa, Savani Bn. *Hematopoietic Stem Cell Transplantation For Acute Myeloid Leukemia: A Review*. **Hematol Oncol Stem Cell Ther**, 10(4), 2017, 245–251.

[87] H. Bello-Manga H, Debaun Mr, Kassim Aa. *Epidemiology And Treatment Of Relative Anemia In Children With Sickle Cell Disease In Sub-Saharan Africa*. **Expert Rev Hematol** 9(5): 2016,1031-1042

[88] K. Gardner, Douiri A, Drasar E. *Survival In Adults With Sickle Cell Disease In A HighIncome Setting*. **Blood**;128(5), 2016, 1436-1438

[89]T. Kauf, Coates Td, Huazhi L, Mody-Patel N, Hartzema Ag. *The Cost Of Health Care For Children And Adults With Sickle Cell Disease*. **Am J Hematol**;84(6), 2019, 323-327

[90] I. Isah, F. Udomah, O. Erhabor, I. Iwueke & T. Adias, *Foetal Haemoglobin Levels In Sickle Cell Disease (Scd) Patients In Sokoto, Nigeria*. **British Journal Of Medical And Health Sciences**, ,1(6), 2013, 36-47

[91] L. Manning, Russell Je, Padovan Jc, Chait Bt, Popowicz A, Manning Rs. *Human Embryonic, Foetal, And Adult Haemoglobins Have Different Subunit Interface Strengths. Correlation With Lifespan In The Red Cell.* **Protein Sci**. 16(5), 2017;1641–58.

[92] L. Manning, Russell Je, Padovan Jc, Chait Bt, Popowicz A, Manning Rs. "*Human Embryonic, Fetal, And Adult Hemoglobins Have Different Subunit Interface Strengths. Correlation With Lifespan In The Red Cell*". **Protein Sci**. 16(10), 2017, 1641–58.

[93] A. Marengo-Lowen**.** *Structure-Function Relations Of Human Hemoglobins* **Pubmed Central**.; 19(3), 2016, 239-245

[94] Britannica, T. Editors Of Encyclopaedia. "*Haemoglobin.Functions*" Available Online:. Https://Www.Britannica.Com/Science/Hemoglobin. 2021

[95] M. Lucky And Lynda.W. "*Sickle Cell Disease Among Children In Africa: An Integrative Literature Review And Global Recommendations*". **Internal Journal Of Africa Nursing Sciences** 2018; 3: 56-64

[96] C. Karakochuk, Hess Sy, Moorthy D. *Measurement And Interpretation Of Hemoglobin Concentration In Clinical And Field Settings: A Narrative Review*. ***Ann N Y Acad Sci***.;1450(1), 2019:126-146

[97] M. *Casale.; Cozzolino, F.; Scianguetta, S.; Pucci, P.; Monaco, V.; Sanchez, G.; Santoro, C.; Rubino, R.; Cannata, M.; Perrotta, S. "Hb Vanvitelli: A New Unstable A-Globin Chain Variant Causes Undiagnosed Chronic Haemolytic Anaemia When Co-Inherited With Deletion - A$^{3.7}$". **Clinical Biochemistry**., 74(7): , 2019, 80–85.*

[98] M. F. Perutz. *Stereochemistry Of Cooperative Effects In Haemoglobin*. **Nature**. ; 228(5273), 1970. 726–739.

[99] A. Oluwagbemiga, M. Akinlosotu & S. Adegoke. *Foetal Haemoglobin And Disease Severity In Nigerian Children With Sickle Cell Anaemia.* **Mediterranean Journal Of Hematology And Infectious Diseases**, 9(5), 2017, 1-5

[100] K. Gardner, T. Fulford, N. Silver, *G(Hbf): A Genetic Model Of Fetal Hemoglobin In Sickle Cell Disease*, **Bloodadvances,** 2(3), 2018, 253-259

[101] M. Odunvbun, A. Okolo, Rahimy Cm. *Newborn Screening For Sickle Cell Disease In A Nigerian Hospital*. **Public Health**. 122 (10), 2018, 1111–1116

[102] T. Adeyemo, O. Ojewunmi, A. Oyetunji, & S. Akanmu, *A Survey Of Genetic FetalHaemoglobin Modifiers In Nigerian Patients With Sickle Cell Anaemia,* **Plos One**, 13(6): 2018, 357-380

[103] O. Ojewunmi, A. Adeyemo, C. Ayinde, B. Iwalokun & A. Adekile, *Current Perspectives Of Sickle Cell Disease In Nigeria: Changing The Narratives*, **Expert Review Of Hematology**, 12(8), 2019, 609-620

[104] T. Adeyemo, O. Oyetunji Ia, *A Survey Of Genetic Fetal-Haemoglobin Modifiers In Nigerian Patients With Sickle Cell Anaemia***. Plos One**. 13(6): 2018; 30-50

[105] M. R Asnani, Knight Madden J, Reid M, Greene Lg, P. Lyew-Ayee. *Socio-Environmenta Exposures And Health Outcomes Among Persons With Sickle Cell Disease.* **Plos One**. 12(4): 2017; 295-301

[106] P. Sebastiani, N. Solovieff , Hartley Sw, Milton Jn, Riva A, Dworkis Da, *Genetic Modifiers Of The Severity Of Sickle Cell Anemia Identified Through A Genome-Wide Association Study.* **Am J Hematol**. 85(1), 2010; 29–35.

[107] G. Galarneau G, Palmer Cd, Sankaran Vg, Orkin Sh, Hirschhorn Jn, Lettre G. *Fine-Mapping At Three Loci Known To Affect Fetal Hemoglobin Levels Explains Additional Genetic Variation.* **Nat Genet**ics. 42(12), 2010; 1049–51.

[108] A. Sedgewick, Timofeev N, Sebastiani P, So Jcc, Ma Esk, Chan Lc, *Bcl11a Is A Major Hbf Quantitative Trait Locus In Three Different Populations With Beta Hemoglobinopathies***. Blood Cells Mol Dis**. 41(3), 2008;255–8.

[109] S. Demirci, Bjorg Gudmundsdottir, Quan L, Fayaz Seifuddin & F. John. *Tisdal Bt87qGlobin Gene Therapy Reduces Sickle Hemoglobin Production, Allowing For Ex Vivo AntiSickling Activity In Human Erythroid Cells,* **Molecular Therapy**, 7(3), 2020, 12-21

[110] L. Breda, Irene Motta, Silvia Lourenco, *Forced Chromatin Looping Raises Fetal Hemoglobin In Adult Sickle Cells To Higher Levels Than Pharmacologic Inducers* , ***Blood,*** 128 (8), 2016, 1139–1143

[111] M. Uda, R. Galanello,G. Lettre, *Genome-Wide Association Study Shows Bcl11a Associated*

With Persistent Foetal Haemoglobin And Amelioration Of The Phenotype Of BetaThalassemia. **Proc Natl Acad Sci U S A,** 105(5), 2008, 1620-5

[112] A. Basak, V. Sankaran. *Regulation Of The Foetal Haemoglobin Silencing Factor Bcl11a.* **Annny Acad Sci**, 1368(1), 2016, 25-30

[113] M. Canver, E. Smith, N. Sanjana, *Bcl11a Enhancer Dissection By Cas9-Mediated In Situ Saturating Mutagenesis* **Nature**. 527(7577), 2015, 192-197

[114] H. Kerstin, J. Kleylein-Sohn, A. Erich, *Astrin Is Required For The Maintenance Of Sister Chromatid Cohesion And Centrosome*, **Journal Of Cell Biology,** 178(3), 2017, 345-354

[115] S. Vinjamur, E. Bauer, H. Orkin, *Recent Progress In Understanding And Manipulating Haemoglobin Switching For The Haemoglobinopathies.* **British Journal Of Haematology,** 180(5), 2017, 630-643

[116] A. Masuda, Y. Kobayashi, N. Kogo, *S. Cognitive Deficits In Single App Knock-In Mouse Models.* Neurobiol Learn Mem. 135(6), 2016, 73-82

[117] A. Paikari & A. Sheehan**,** *Fetal Haemoglobin Induction In Sickle Cell Disease* **British Journal Of Haematology,** 180(2). 2017, 189-200

[118] P. Sundd, T. Mark, & M. Enrico, *Pathophysiology Of Sickle Cell Disease* **Annu Rev Pathol,** 14(24): 2019, 263-292

[119] L. Lee, K. Whitley, S. Banks, G. Puckrein, *Reducing Health Care Disparities In Sickle Cell Disease: A Review.* **Public Health Reports ,** 134(6), 2019, 599-607

[120] I. Akinseye, A. Alsultan & M. Steinberg. *Foetal Haemoglobin In Sickle Cell,* **Blood** 118(1), 2011; 19–27

[121] S.L Thein. *Genetic Basis And Genetic Modifiers Of B-Thalassemia And Sickle Cell Disease*, **Blood,** 3(1) , 2017, 27-57

[122] E. Khandro, P. Huang, S. Peshak, O. Abdulmalik, B. Giardine, Z. Zhang, C. Hardison. *Understanding Heterogeneity Of Foetal Haemoglobin Induction Through Comparative Analysis Of F And A Erythroblast*. **Blood**, 135(22), 2020, 1957-1968

[123] C. Garner, Tatu T, Reittie Je, Littlewood T, Darley J, Cervino S, . *Genetic Influences On F Cells And Other Hematologic Variables: A Twin Heritability Study*. **Blood** 95(10), 2000; 342– 346

[124] S. L Thein, Menzel S, Lathrop M, Garner C. *Control Of Foetal Haemoglobin: New Insights Emerging From Genomics And Clinical Implications*. **Human Molecular Genetics** 18, 2019, 216–223

[125] S. N Mtatiro, Singh T, Rooks H, Mgaya J, Mariki H, Soka D, *Genome Wide Association Study Of Foetal Haemoglobin In Sickle Cell Anaemia In Tanzania*. **Plos One**; 9(11), 2017, 125-137

[126] J. Makani, Menzel S, Nkya S, Cox Se, Drasar E, Soka D, *Genetics Of Fetal Hemoglobin In Tanzanian And British Patients With Sickle Cell Anaemia.* **Blood** 117(5), 2011, 1390–1392 [127] J. Farrell, M. Sherva, Z. Chen *A 3-Bp Deletion In The Hbs1l-Myb Intergenic Region On Chromosome 6q23 Is Associated With Hbf Expression* **Blood First Edition Paper**, 119(18): 2011, 4835-4946

[128] J. Lose., Game, L., Clark, B.E., Bergounioux, J., Gerovassili, A. & Thein, S.L., *Genome Annotation Of A 1.5 Mb Region Of Humanchromosome 6q23 Encompassing A Quantitative Trait Locus For Fetalhemoglobin Expression In Adults*. **Bmc Genomics**, 5(15), 2018, 33-38.

[129] G. Pule, S. Mowla, Novitzky N, Wiysonge Cs, Wonkam A. *A Systematic Review Of Known Mechanisms Of Hydroxyurea-Induced Fetal Hemoglobin For Treatment Of Sickle Cell Disease.* **Expert Rev Hematol**., 8(5), 2017, 669-79.

[130] J. Hardy. & Singleton, A. *Genome Wide Association Studies And Human Disease*. **N. Engl. J. Med**., 360, 2019, 1759–1768

[131] S .Menzel., Garner, C., Gut, I., Matsuda, F., Yamaguchi, M., Heath, S.,Foglio, M., Zelenika, D., Boland, A., Rooks, H.. *A Qtl Influencing F Cell Production Maps To A Gene Encoding A Zinc-Finger Protein On Chromosome*. **Nat. Genet**., 2(15), 2017, 1197–1199.

[132] R. Sales., Nogueira, B.L., Belisário, A.R. . *Fetal Hemoglobin-Boosting Haplotypes Of Bcl11a Gene And Hbs1l-Myb Intergenic Region In The Prediction Of Clinical And Hematological Outcomes In A Cohort Of Children With Sickle Cell Anemia*. **J Hum Genet** , 234(3), 2022. 130-145

[133] L.E Creary,., Mckenzie, C.A., Menzel, S., Hanchard, N.A., Taylor, V.,Hambleton, I., Spector, T.D., Forrester, T.E. & Thein, S.L. *Ethnic Differences In F Cell Levels In Jamaica: A Potential Tool Foridentifying New Genetic Loci Controlling Fetal Haemoglobin*.**Br. J. Haematol**.,144(5), 2019, 954–960.

[134] A. Tasha. Morrison, Ibifiri Wilcox, Hong-Yuan Luo, John J. Farrell, Ryo Kurita, Yukio Nakamura, George J. Murphy, Shuaiying Cui, Martin H. Steinberg, David H.K. Chui, *A Long Noncoding Rna From The Hbs1l-Myb Intergenic Region On Chr6q23 Regulates Human Fetal Hemoglobin Expression*, **Blood Cells, Molecules, And Diseases**, 69(5), 2018, 1-9

[135] J.K Pickrell.,. G., Novembre, J., Kudaravalli, S., Li, J.Z., Absher,D., Srinivasan, B.S., Barsh, G.S., Myers, R.M., Feldman, *Signals Of Recent Positive Selection In A Worldwide Sample Of Human Populations*. **Genome Res**., 19(5), 2019. 826–837.

[136] J. Jiang., Best, S., Menzel, S., Silver, N., Lai, M.I., Surdulescu, G.L., Spector, T.D. & Thein, S.L. *Cmyb Is Involved In The Regulation Of Fetal Hemoglobin Production In Adults*. **Blood,** 108(5), 2016, 1077–1083.

[137] S. Menzel,. J, Jiang, ., Silver, N., Gallagher, J., Cunningham, J., Surdulescu, G., Lathrop, M., Farrall, M., Spector, T.D. & S. Thein, *The Hbs1l-Myb Intergenic Region On Chromosome 6q23.3influences Erythrocyte, Platelet, And Monocyte Counts In Humans*. **Blood**, 110(7), 2017, 3624–3626.

[138] T. Adeyemo, O. Oyesola. *A Survey Of Genetic Fetal-Haemoglobin Modifiers In Nigerian Patients With Sickle Cell Anaemia*, **Plos One**, 13(16), 2018, 111-119

[139] S. Lanzkron, G. Sawicki, K. Hassell, M. Liem & S. Mccolley. *Transition To Adulthood And Adult Health Care Patients With Sickle Cell Disease Or Cystic Fibrosis: Current Practices And Research Priorities.* **Journal Of Clinical And Translational Science**, 2(5), 2018, 334342

[140] S. Saraf, T. Akingbola Ts, Shah. *Associations Of Alpha-Thalassemia And Bcl11a With Stroke In Nigerian, United States, And United Kingdom Sickle Cell Anemia Cohorts*. **Blood Adv.** 1(11), 2017, 693–698.

[141] R. Naik,., Galande, S. *Satb Family Chromatin Organizers As Master Regulators Of Tumor Progression*. **Oncogene** 38, 2019, 1989–2004

[142] M. Shaikho, J. Farrell, *Cis- And Trans-Acting Expression Quantitative Trait Loci Differentially Regulate Gamma-Globin Gene Expression,* **Biorxiv,** 3(12), 2018, 414-421

[143] A. Wonkam, ,N. Bitoungui, Vorster Aa, *Association Of Variants At Bcl11a And Hbs1lMyb With Hemoglobin F And Hospitalization Rates Among Sickle Cell Patients In Cameroon*. **Plos One**. 9(3): 2014;506-525

[144] T. Adeyemo, O. Oyesola, *A Survey Of Genetic Fetal-Haemoglobin Modifiers In Nigerian Patients With Sickle Cell Anaemia* **Plos One** , 13(6), 2018, 1111-1125

[145] S. Saraf, T. Akingbola, B. Shah, *Associations Of Alpha-Thalassemia And Bcl11a With Stroke In Nigerian, United States, And United Kingdom Sickle Cell Anemia Cohorts*. **Blood Adv**. 1(11), 2017, 693–698. *Levels In Patients With Sickle Cell Anemia Treated With Hydroxyurea? A Systematic Review And Pathway Analysis* , **Frontiers In Pharmacology** , 12(5), 2022, 853-66

Chapter Three

Methodology

3.1 Research Design

This was a cross-sectional hospital based study to investigate the foetal haemoglobin variant HBSIL-MYB in individuals with sickle cell anaemia and their phenotypic presentations with a view of providing explanations on strategy for reducing the burden of the disease in Nigeria. This work was conducted at the University College Hospital and Adeoyo state Hospital, Ibadan, Oyo state Ibadan is the capital and most populous city of Oyo state, Nigeria. By geographical area, Ibadan is the largest city in Nigeria, with a population of 3,552,000i.

3.2 Population of the Study

All suspected homozygous sickle cell patients attending Sickle cell clinics of Adeoyo Hospital and University College Hospital from age 13 above was enrolled from January to March, for this study, 260 blood samples of Sickle cell Patients were collected for this study.

3.2.1 Sample Size Determination for Cross-sectional Study

The minimum number of subjects required for the study is calculated using the formula;

$$N = \frac{Z_2\, P(1-P)}{d^2}$$

Where z = standard normal deviate set at 1.96, corresponding to[37] 95% confidence interval

p = estimated SCA prevalence of 25% [12]

d = degree of precision set at 0.05 (95% confidence interval)

$$N = \frac{(1.96)^2 * 0.25 * 0.955}{0.05^2} = 230$$

Estimate for non-response = 10% of the study size

10% attrition = 23 : total = 23+230= 253 HbSS participants

N was calculated at 253.

A total of 260 participants were recruited.

3.2.2 Inclusion Criteria

I. Intending participants must be sickle cell individuals attending the participating hospitals/clinics

II. They must be willing to give informed consent

III. Under 18 years intending participants must give assent while their parents/legal guidance will give consent to be part of the study

IV. Be willing to donate 7ml of blood sample for research

3.2.3 Exclusion Criteria

I. Refusal of consents/assents

II. Participants < 10 years

III. Participants who used Hydroxyurea in the last 3 months, hydroxyurea has been proven to boost HbF levels

3.3 Sample and Sampling Techniques

A consecutive non randomized Sampling of all SCD patients that attend the sickle cell clinics of the University college Hospital and Adeoyo State Hospital, was used to recruit participants into the study.

Questionnaires were administered to voluntary patients to collect information about demographic data and to identify the rate of acute painful episodes, fewer leg ulcers, less osteonecrosis, less frequent acute chest syndromes and reduced disease severity. The questionnaire was established according to international guidelines, using standardised questions and amendments were made.

3.3.1 Sampling Collection

A total of 7 ml blood sample was collected from each participant and dispensed into ethylene diamine tetra acetic acid (EDTA), for laboratory analysis to determine the Packed Cell Volume (PCV), Foetal Haemoglobin Concentration (HbF) and HMIP-2B variant of HbF.

3.3.2 PCV (Packed Cell Volume)

The packed cell volume (PCV) was determined to measure the proportion of the volume occupied by the Red Blood Cells to the volume of the whole blood. The PCV was determined using the haematocrit reading device.

3.3.2.1 Principle

Anticoagulated whole blood is centrifuged and the volume occupied by the erythrocytes is expressed as a percentage of the total volume (packed cell volume, PCV)iii

3.3.2.2 Reagents and Equipment

1. Capillary tubes (75 + 0.5 mm in length, 1.155 + 0.085 mm in bore)
2. Clay or critoseal
3. Microhematocrit centrifuge

Specifications:

a. radius should be greater than 8.0 cm

b. capable of reaching maximum speed in 30 seconds

c. sustains a RCF of 15,000 x g for five minutes without exceeding a temperature of 45°C

4. Micro haematocrit reading device.

3.3.2.3 Procedure

1. The capillary tube was filled approximately 2/3 to 3/4 full with the well-mixed blood sample.

2. The end of the capillary tube was sealed by placing it into the sealing clay (critoseal) at a 900 angle.

3. The capillary tubes were placed in the micro haematocrit centrifuge with the sealed end toward the periphery. Duplicate tubes were placed opposite each other for balance.

4. Then Centrifuged for five minutes. After five minutes of centrifugation the hematocrit was measured while the tubes were still kept in a horizontal position. The distinct column of packed erythrocytes was visible in one end of the capillary tube. The packed erythrocytes are followed by first a small turbid layer – the buffy coat layer – and then a clear column of plasma. Hematocrit is estimated by calculating the ratio of the column of packed erythrocytes to the total length of the sample in the capillary tube, measured with a graphic reading device.

5. Using a micro haematocrit reading device, the haematocrit results determined was recorded to the nearest whole number. The measurement was performed within 10mins to avoid merging of the layers.

3.3.2.4 Reference Intervals

	Conventional Units (%)	SI Units (L/L)
Adult Males:	41-53%	0.41-0.53
Adult Females:	36-46%	0.36-0.46

3.4 Foetal Haemoglobin Concentration

Foetal Haemoglobin Concentration was determined using betke methodiv. This is based on the fact that foetal haemoglobin is more resistant to strong alkali than the other haemoglobins.

3.4.1 Principle of Betke Method (Alkali Denaturation test)

To measure the percentage of HbF in a mixture of haemoglobin, Sodium hydroxide is added to a lysate and after the set time, denaturation is stopped by adding saturated ammonium sulphate. The ammonium sulphate lowers the PH and precipitates the denatured Hb. After filtration, the quantity of haemoglobin is measured, the proportion of alkali-resistant (foetal) haemoglobin is then calculated as a percentage of the foetal haemoglobin present.

3.4.2 Reagents

1. Drabkin's solution (KCN, 0.05 g: K3Fe(CN), 0.2g; distilled water, 1L)
2. Sodium Hydroxide 1.2N
3. Saturated ammonium sulphate solution
4. Carbon tetrachloride

3.4.3 Procedure

1. Red cell lysates was prepared from 2ml or more of whole blood collected in any standard anti-coagulant. The cells were washed three times with 0.85% sodium chloride (Nacl), lysed with 2-3 volumes of distilled water and half a volume of carbon tetrachloride, and centrifuged at 3000rpm for 30 mins at room temperature. The lysate was prepared in this way the final concentration is in the range 8-10g/100ml.

2. The solution of cyanmethaemoglobin (approximately 500 mg/100 ml) was prepared by adding 0.25ml of haemolysate to 4.75ml Drabkin's solution. Then 0.2ml of 1.2N sodium hydroxide was added to 2.8ml cyanmethaemoglobin solution. The solutions were mixed immediately by inversion and a stop watch is started. The reaction is stopped after exactly two minutes by the addition of 2.0ml of saturated ammonium sulphate with vigorous mixing. The mixture is allowed to stand for five minutes and then filtered twice through the same whatman number 42 filter paper, using a clean test tube to collect the filterate each time. An un-denatured sample for comparison is prepared by mixing 1.6ml distilled water, 1.4 ml cyanmethaemoglobin solution and 2.0 ml saturated ammonium sulphate. This solution is

distilled 1 in 10 with distilled water and the test and comparison solutions are read at 415nm on a suitable spectrophotometer. The whole procedure was carried out at a room temperature (18°C – 26°C).

3. The percentage of alkali-resistant haemoglobin was derived from the following formula:

OD415nm of test solution × 100 /

OD415nm of undenatured solution ×20

3.4.4 Reference Interval

In normal Adults: HbF = 0.8% to 2% (0.008 to 0.02)

In Sickle Cell Patients: HbF = > 2%

3.5 Molecular Analysis

3.5.1 DNA Extraction

DNA extraction is an important technique that involves the isolation of DNA from tissue. DNA extraction was done from archived buffy coat by using Da An Gene Kit, following the manufacturers protocol.

3.5.1.1 Reagents

1. Absolute ethyl alcohol
2. Eluent
3. Lysis solution
4. Proteinase K
5. Inhibitor removal
6. Deionized solution

3.5.1.2 Procedure

1. Added 50µl of proteinase K into sterile centrifuge tube of 1.5µl

2. Took 200µl specimen and add it into the centrifuge tube.

3. Added 200µl lysis working solution(i.e lysis solution containing carrier RNA), fasten down the tube cover, oscillate it in vortex for 15 seconds to mix the solution sufficiently, then conduct high-speed centrifugation for 10seconds (prevent bubbles produced during incubation period), 10 minutes at temperature of 720C; at the same time, prevent the eluent with temperature of 720C.

4. Added 250µl ethanol, fasten down the tube cover, oscillated it in a vortex for 15 seconds

5. Drew the whole mixture into the spin column, conducted 12,000g centrifugation for 1 minute at room temperature, then fit the spin column in a new collection tube

6. Added 500µl inhibitor remover into the spin column, conduct 12,000g centrifugation for 1 minute at room temperature, then fit the spin column into a new collection tube

7. Added 500µl deionized solution into the spin column, conduct 12,000g centrifugation for 1 minute at room temperature, then fit the spin column into a new collection tube

8. Added 500µl deionized solution into the spin column, conduct 12,000g centrifugation for 1 minute at room temperature, then fit the spin column into a new collection tube

9. Place the spin column and collection tube at room temperature, conducted 14,000g centrifugation for 3 minute in order to remove residual ethanol

10. Took out the spin column, fitted it in a new 1.5ml centrifuge tube, opened the cover of the spin column, laid it aside for 2 minutes at temperature of 720C

11. Carefully add 50µl eluent preheated at 720C right above the membrane of spin column, fastened down the tube cover, after standing for 1 minute at room temperature, conducted 14,000g centrifugation for 1 minute. Then the solution in the centrifuge tube is nucleic acid solution. The DNA extract was stored in -20oC freezer

3.5.1.3 DNA Quantification

Quantifying the DNA concentration and purity was done before the PCR amplification to avoid false positive results. This was done using the Nano drop spectrophotometer.

1. Before measuring any samples, the first step was to 'blank' the spectrophotometer using the solution the DNA is resuspended in (elusion fluid), but with no DNA added. 'Blanking' measures the background inherent to the machine and the solvent.

2. When using the NanoDrop to measure the samples, 1-2μL of mini-prepped DNA was placed onto the pedestal.

3. Close the lid and click measure, the concentration and purity was recorded.

Note: Purity was measured under the 260/280 column (Fig 3.1).

4. Repeat for each sample.

3.5.2 PCR (Polymerase Chain Reaction)

The PCR technique is based on the enzymatic replication of DNA. In PCR, a short segment of DNA was amplified using primer mediated enzymes. DNA polymerase synthesises new strands of DNA complementary to the template DNA. The DNA polymerase can add a nucleotide to the pre-existing31-OH group only.

ARMS-PCR was used to amplify the DNA segmentv. ARMS is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. The amplification-refractory mutation system (ARMS) is a simple method for detecting any mutation involving single base change or small deletions.

3.5.2.1 Components of PCR

1. DNA Template – The DNA of interest extracted from the sample

2. DNA Polymerase – Taq polymerase was used, because of its thermal stability and it doesn't denature at a high temperature.

3. Deoxyribonucleotide triphosphate – These provide energy for polymerization and are the buiding blocks for the synthesis of DNA.

4. Buffer System – Magnesium and Potassium provide Optimum conditions for DNA denaruration and renaturation.

5. Oligoucleotide Primers - Short stretches of oligonucleotide DNA sequences complementary to the 31 ends of sense and anti-sense codon

6. Q-solution – It is important in Multiplex PCR, which facilitates templates with high degrees by modifying the melting behaviour or high GC-contents of the DNA.

The DNA polymerase, Deoxyribonucleotide trisphosphate and Buffer System are all in the QIAGEN Master Mix which was used in this study.

3.5.2.2 Amplification Refractory Mutation System Test for the 3-bp Deletion

A bidirectional polymerase chain reaction (PCR) test using 2 pairs of allele-specific oligonucleotide primers was designed to detect the TAC 3-bp deletion on chromosome 6q23(Table 3.1). The primer pairs were TAC-1 and TAC-2 were to detect the sequence with with a PCR product of 207 bp and TAC-3 and TAC-4 to detect the sequence with the 3-bp deletion with a PCR product of 276 bp. PCR reaction was carried out in 25 µL total volume.

The total reaction, containing 100 ng (2µl) of target DNA; 12.5 µL of multiplex PCR master mix (QIAGEN), 1 µL of Q solution, 1 µL of each primer, and 5.5 µL of molecular grade water. Using GeneAmp PCR System 9700 (Applied Biosystems) at 98°C for 15 minutes, followed by 30 cycles of 98°C for 20 seconds, 58°C for 40 seconds, and 72°C for 40 seconds, and ended with the last cycle of 72°C for 10 minutes.

Fig 3.1 DNA Quality Display with a Nano drop

Source; Laboratory, 2022

Table 3.1 Primer Design with Melting Temperature

Primers	**Sequence**	**T_m(°C)**	**Primer concentration**
TAC-1(F)	TCACTCTGGACAGCAGATGTTACTAT	53°C	100µM
TAC-2(R)	CTCAGTGATGGTATTTCTGGAGAC	51°C	100µM
TAC-3(F)	AGCCCGTCCAGACACTCATTGTT	59°C	100µM
TAC-4(R)	GCCCTGATAACATTTTGTGGTTTTCATTTAA CAT	64°C	100µM

Source: Laboratory, 2022

1.5.1.3 Gel Electrophoresis

The basic steps followed to run the gel electrophoresis is described by; 1) Pouring the gel, 2) Preparing the samples, 3) Loading the gel, 4) Running the gel (exposing it to an electric field) and 5) Staining the gel.

1. The agarose gel was prepared at 2% (1% = 1g per 100ml). After it has boiled it was poured into a mold in the gel electrophoresis tank and allowed to cool for about 40 mins. After it has cooled it solidify into a matrix. When the gel was poured into the mold a comb is placed in the mold at one end so that the gel contains several wells. This step will be completed before you come to lab.

2. Preparing the sample means that the loading dye was added to the DNA sample(s). The loading dye serves two purposes. The first is that it allows to visualize the migration of the samples through the gel and secondly, it allows the PCR reaction sink well. Although, the DNA molecules cannot be seen with the naked eyes themselves but the progress can be monitored by observing how far the dye (which has a negative charge) has migrated. The most typical dye is bromophenol blue which migrates through a gel at the same rate as a DNA molecule that is 300 nucleotides in length.

3. Loading the gel means adding your samples to the gel. The first step was to remove the comb so that the wells are exposed. Each sample will be transferred into one of the wells so that it is essentially within the gel itself. The loading dye that was added also contains a sugar which makes the solution fairly dense so that is sinks into the well quite easily. A 100 bp DNA ladder was loaded at the first well of the agarose gel to guide in reading the band sizes of the separated SNPs

4. Running the gel refers to exposing it to an electric field. The agarose gels was ran at 120v for 40 mins. The higher the current the faster the molecules will migrate and the sooner you can analyse the gel. However, the slower the migration the easier it is to obtain clear results, reason for the chosen volts. The agarose gel was run until the dye has migrated about halfway from the wells to the end of the gel.

5. staining the gel refers to staining the DNA molecules so that we can determine how far they migrated from the origin (the wells). Ethidium bromide is the most commonly used stain but it is also a suspected carcinogen, EZ-VISION was the stain used in this work. The stain will bind to DNA and bands will appear on the gel wherever there is DNA. Once it had been stained the gel can be analyzed.

(Note, the stain is different than the dye from step 2. The dye did not bind DNA, it simply migrated through the gel so that we could monitor progress.)

The gel, which contains a series of wells at the cathode end, is placed inside the chamber and covered with a buffer solution (TBE). The chamber is connected to a power supply that, when turned on, applies an electric field to the buffer. The electric field causes negatively charged molecules to migrate through the gel toward the anode. (DNA and RNA are negatively charged; proteins must be treated with a detergent to give them a negative charge). The DNA was visualized under the ultraviolet light using a Gel Doc system which was also used to take photographs of the gel.

3.6 Statistical Analysis

Data obtained was entered in Microsoft excel sheet and statistically analysed using statistical package for the social sciences (SPSS®) software version 24.0. Chi- square was used to determine the association between

HbF and the genetic variants. P values ≤ 0.05 at 95 % confidence interval was considered significant. Descriptive analysis was used to present the frequencies of outcomes. Genetic association analysis was performed by multiple regression, with age and sex as covariates (SPSS v. 20).

3.7 Ethics Processing

Ethical approval was collected from the ministry of health, Oyo state, Nigeria. A written consent form was given to the parent or guardian for approval of each child to partake in the study by signing. Also adult participants signed the consent form before they were recruited for this study.

Endnotes

1. United Nations- *World Population Project, Ibadan, Nigeria Metro Area Population 1950-2022*, Available Online: 'Https://Www.Macrotrends.Net/Cities/21990/Ibadan/Population' </A>. 2022.

2. K. Jae-Hoon & C. In. *Choosing Level Of Significance: A Decision-Theoretic Approach.* **Abacus**, 57 (10), 2019, 1217-22

3. M. Himel. & S. Lotfollahzadeh. *Haematocrit*, **Treasure Island**, 2(18), 2022, 276-87

4. I. Isah, F. Udoma, & F. Aghedo, *Foetal Haemoglobin Levels Of Sickle Cell Disease (SCD) Patients I Sokoto, Nigeria*. **British Journal Of Medical And Health Sciences.** 1(6), 2013, 36-47

Chapter Four

Results and Discussion of Findings

4.1 Demographic Data

A total of 260 patients were recruited into the study. The age range between 10-56 years. Most of the study subjects were aged 16–21 years (38.5%) followed by those who were 22-27 years,(21.7%) then above 51 years been the smallest group (1.5%). The mean age is 23 ± 0.6 (SE) (Figure 4.1). Sex distribution of the SCD patients showed that there is an increased frequency in female patients (55.4%), compared to male patients (44.6%).

Table 4.1 shows the distribution of participants having crises on a yearly basis. Half (50%) have crises at least once in a year, 10% have crises at least twice in a year and the remaining 40 % experiences crises at least once in three months. The most common complications among SCD was fever (59.2%) followed by acute chest syndrome, seen in (30.5%) and leg ulcer,

(10.3%).

4.2 Clinical Data of the Study Patients

4.2.1 Haematological Phenotype Data

Statistical analysis of the patients sample in vaso-oclusive crisis and steady state in a year showed that The HbF Concentration ranged from 1.8% - 12 %, with no statistical significant difference in the mean of the levels of HbF in patients (4.9% ± 2.4). The mean levels of HbF for males was 4.61 ± 3.45, while females were 4.5 ± 3.1, the means were statistically similar ($p = 0.81$). Patients with Higher HbF Concentration > 4.5%, had milder crises, compared to patients with low HbF concentration of < 4.5% (Table 4.2).

Table 4.3 also showed the mean HbF concentration between each age group of SCD patients, there was no significant difference between HbF levels and age group, $P = 0.51$

The PCV value ranged from 18%-44% with a mean (24.3% ± 1.8). The mean PCV for male was 30.5 ± 2.2 while the mean PCV for female was 25.5 ± 1.7. There is a significant difference between the PCV of males and females ($P = 0.023$). Relationship between the Foetal haemoglobin Concentration and PCV, shows that the mean PCV counts was significantly higher in patients who had high foetal haemoglobin concentration, $P = 0.006$ (Table 4.4). The HbF-boosting allele frequency of rs66650371 in Chromosome 6 (HMIP) showed that 15 out of 260 SCD patients showed the rs66650371 gene, which is a 3bp deletion in position 276bp in gel electrophoresis, while the remaining 245 showed insertion

at position 207bp. This indicates an allelic frequency of 3% in the total population (II = 245, DI = 15). The deletion at position 276bp confirms the SNP rs66650371 (Figure 4.3)

10 (66.7%) out of the total population of 15, who showed the deletion in position 276bp were females (rs66650371) and 5 (33.3%) males showed the deletion at position 276bp. (P = 0.365) using chi-squared there is no significant difference between the rs66650371 SNP with the Gender (fig 4.2).Table 4.5 shows that the total number of crises of SCD patients with rs66650371 was significantly lower to the other patients who do not have the rs66650371 SNP, (P = 0.023)

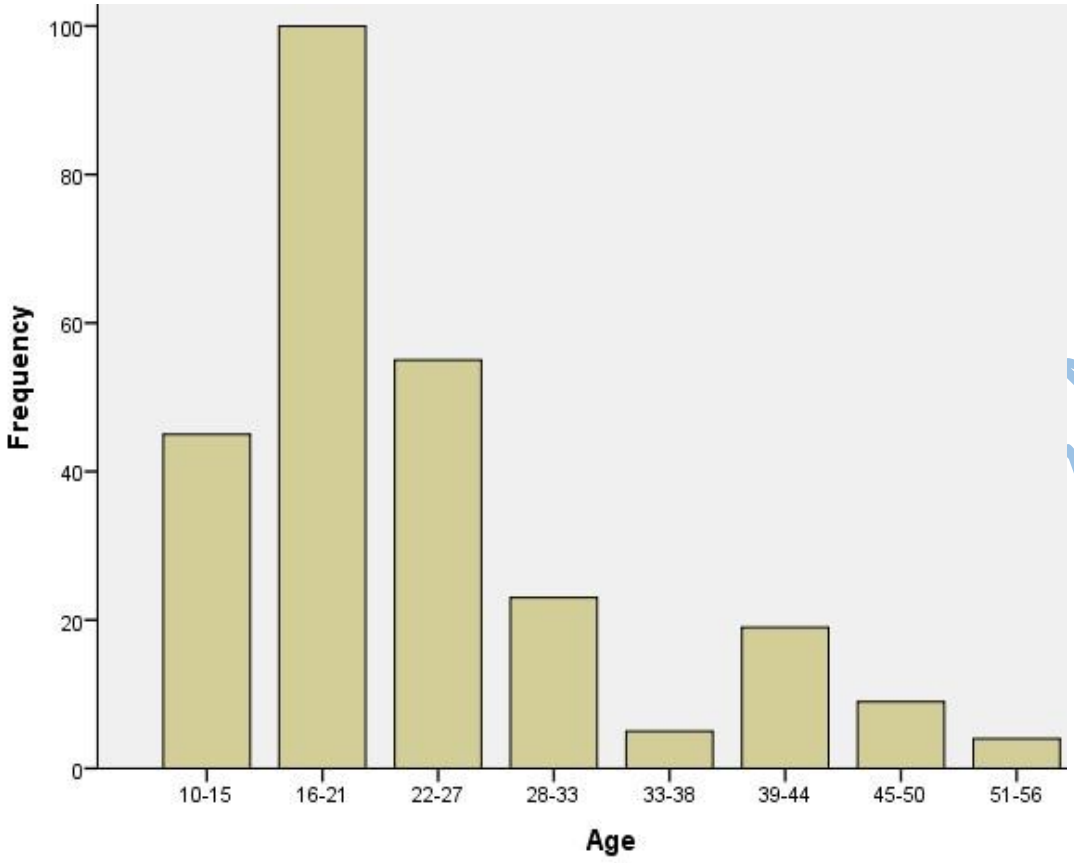

Figure 4.1 Age Distribution of SCD patients in Ibadan, South West

Source: Laboratory, 2022

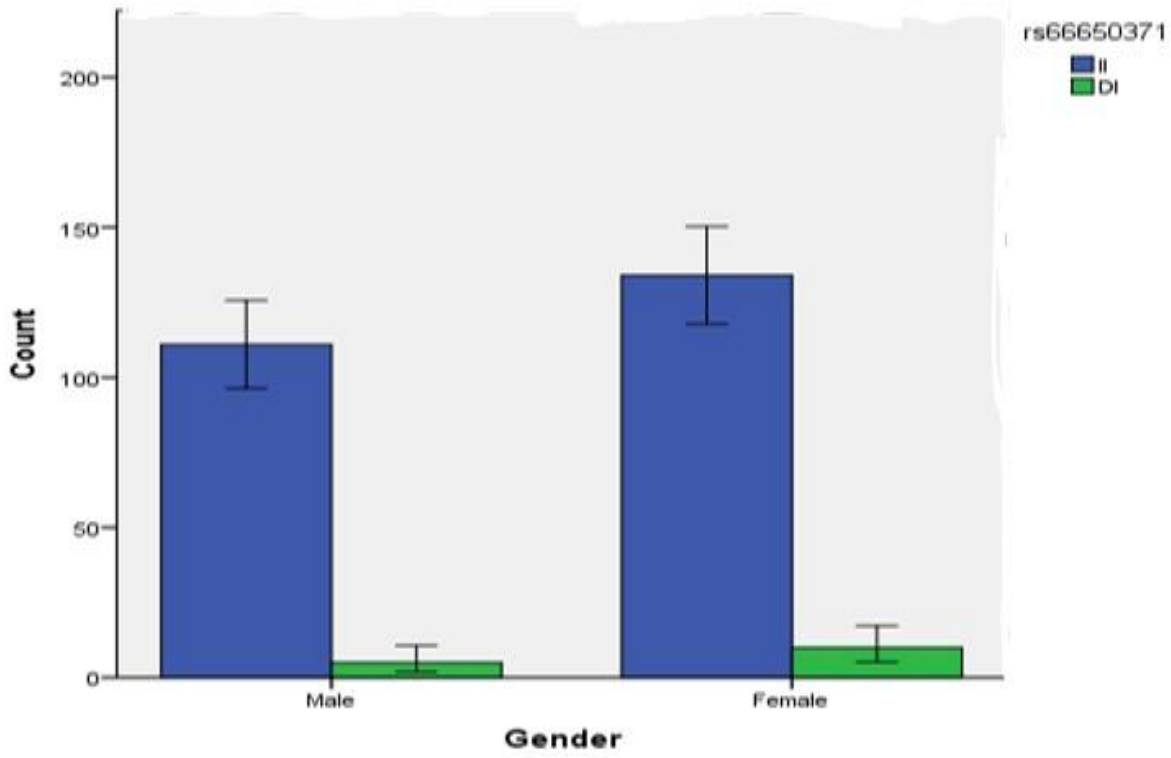

Figure 4.2 Relationship between Gender and rs66650371

Source: Laboratory, 2022

Table 4.1 Prevalence of Vaso-oclussive crises in SCD Patients

Number of Crises in a year, N = 260	Chest pain N =260	Leg Ulcer N = 260
Once = 130 (50%)	Yes = 154 (59.2%)	Yes = 41 (15.8%)
Twice = 26 (10%)	No = 106 (40.7%)	No = 219 (84.2%)
Quarterly = 104 (40%)		

Source: Laboratory,2022

Table 4.2 Relationship between Vaso-oclusive Variables and HbF

Vaso-oclussive Variables	Low HbF	High HbF
Leg Ulcer, Yes	28 (68.3%)	13 (31.7%)
No	116 (53.0%)	103 (47.0%)
Chest Pain, Yes	85 (71.4%)	34 (28.5%)
No	58 (41.1)	83 (58.8%)

Source: Laboratory, 2022

Table 4.3 Relationship between HbF and Age

HbF	10-15	16-21	22-27	28-33	34-39	40-45	46-51	52-57
Low HbF	34	57	31	7	2	7	2	4
High HbF	11	43	24	16	3	12	7	0
Total	45	100	55	23	5	19	9	4

Source; Laboratory, 2022

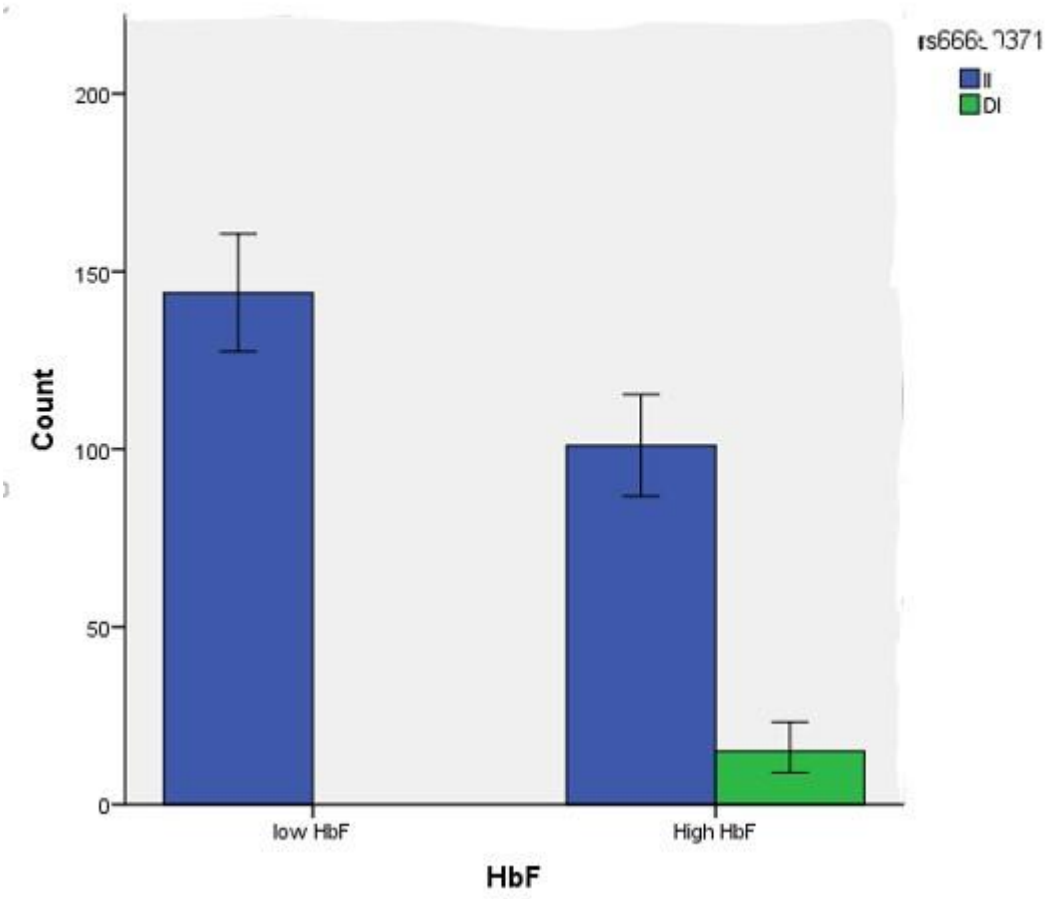

Fig 4.3 Relationship between HbF and rs66650371

Source; Laboratory, 2022

Table 4.4 Relationship between HbF Concentration and PCV

HbF Concentration	PCV(%)	
	Low	High
Low	80.8	19.2
High	4.3	95.7

Source: Laboratory,2022

Table 4.5 Relationship between rs66650371 and Number of Crises of SCD Patients

	Number of	Crises in a year		
	Once	Twice	Quaterly	Total
II	119	24	102	245
DI	11	4	0	15
Total	130	26	104	260

Source; Laboratory, 2022

Table 4.6 Relationship of rs66650371 with PCV

rs66650371	Low PCV	High PCV	Total
II	24	221	245
DI	2	13	15
Total	26	234	260

Source; Laboratory, 2022

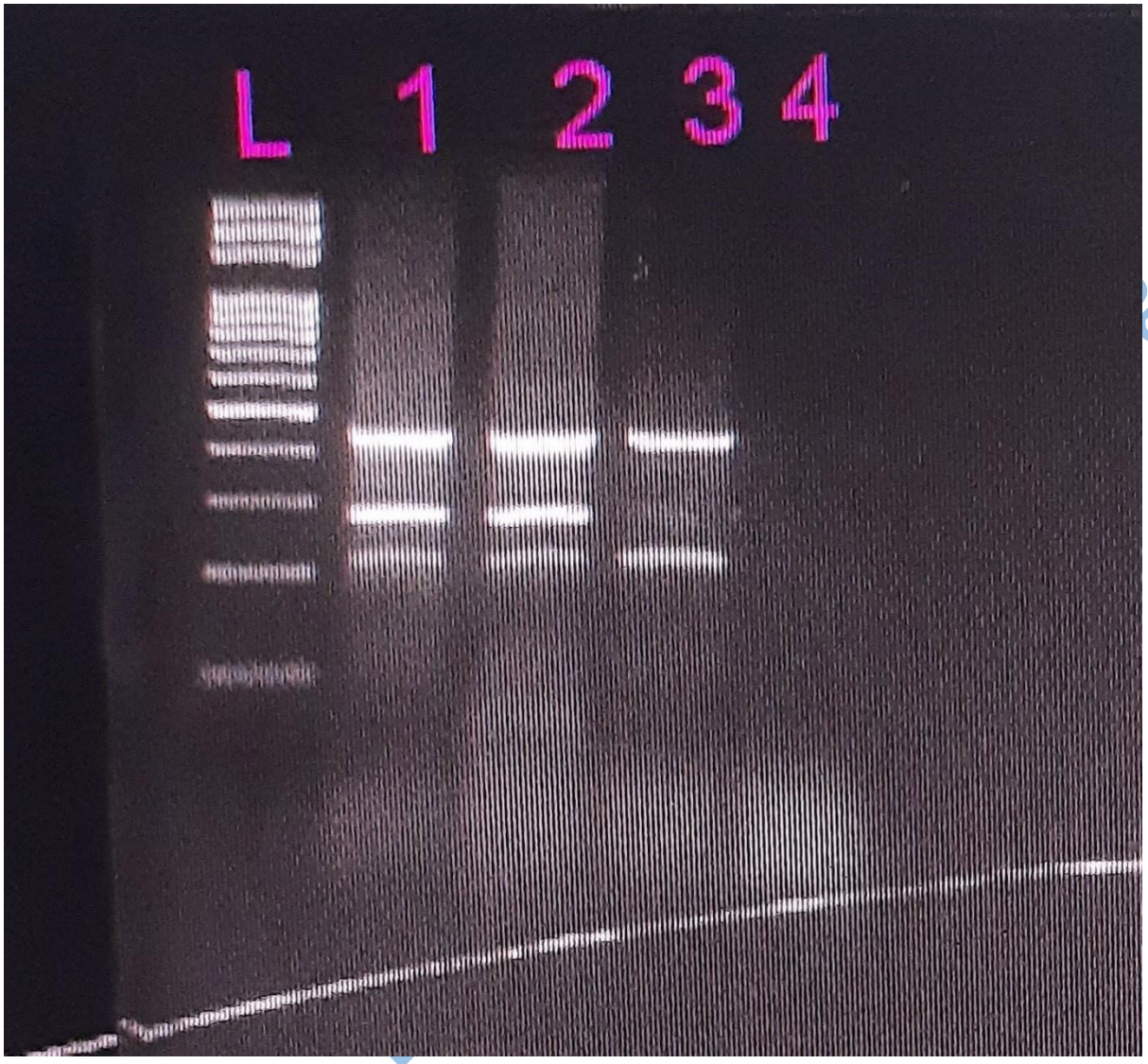

Fig 4.4 Gel Electrophoresis Image of rs66650371

Source; Laboratory, 2022

4.3 Discussion of Findings

The genetic study of HbF variation relative to the phenotypic expression of Sickle cell patients have been investigated during the past few years. These have included: ´A survey of genetic foetal-haemoglobin modifiers in Nigerian patients with sickle cell anaemia'[1] (Where the first systematic evaluation of the known HbF modifier loci in Nigerian SCA population was presented), 'The 3bp-deletion in HBSIL_MYB'[2] and the Genome Wide Association Study (GWAS) genotyping HbF in Tanzanian individuals with SCD[3].

A total of 260 Nigerian Sickle Cell Patients attended the Sickle Cell Centres during the period of this study. All study Participants included Children, Youths and Adults, with the highest frequency seen in Youths. Children less than 10 years of age were excluded from this study, because HbF levels are not stable at this stage. There was an increased frequency in the number of females recruited for this study than males, females had a total population of 144 while males were 116. The most common disease severity among the population was fever followed by acute chest symptoms and only few experiences leg ulcers. All study participants have not been blood transfused in the last one year, to prevent the alteration of foetal haemoglobin concentration.

The number of people who experienced sickle cell crises at least once a year is about 50% of the total population, while 10% experiences crises at least twice in a year, and 40% experience crises at least once in three months. Higher HbF levels have been associated to benefit some complications of the disease than others, examples of some of its benefit include, reduced rate of acute painful episodes, fewer leg ulcers, less frequent acute chest syndromes and reduced disease severity[4]. These vaso-occlusive variables were the bench mark used to determine how severe and how frequent participants experience crises in this study.

About 59.2% of the population experiences chest symptoms at least once in a year while only

15.2% experiences leg ulcers. The high frequency of sickle crises amongst the participants is similar to what has been reported in a previous study in Nigeria, which revealed that there is an overall increase in the frequency of vaso-occlusive crises among SCD patients[5]. This situation is consistent with the trend in some countries such as Britain and Saudi Arabia[6,7]. As a result of widespread use of hydroxyurea in developed nations, the frequency of pain and hospitalizations has significantly reduced. However, in Nigeria and other sub-Saharan African countries where the highest burden of SCD is been recorded, this drug is not readily available, affordable and accessible.

The HbF Concentration ranged from 1.8 – 12 percent, the mean HbF level of patients in this study was 4.9 ± 2.4 percent, which is lower than what has been reported in a study in Ife[8], Nigeria. The mean HbF was 9.9 ± 6.0 percent. This difference may be as a result of the different methods used in the estimation of foetal haemoglobin levels in both studies, or the mean age of the populations, for instance, report from Ife showed the age range recruited for their study was 1-15 years, at this stage, foetal haemoglobin levels are not yet stable, this might account for the discrepancy in the mean foetal haemoglobin levels of both studies. The mean Hbf of this study is close to a study that evaluated the foetal haemoglobin concentration levels of individuals in Ibadan (5.16 ± 4.04)[9]. There was no statistical difference in the mean HbF levels of males and females. Although, males had higher HbF levels compared to females. The study showed that about 55.4% of the total population had reduced level foetal haemoglobin concentration. The mean HbF levels were significantly lower in patients with moderate crises than those with milder disease severity and also with those who show leg ulcers and acute chest symptoms. This is similar to a report in O.A.U, Ile-Ife[10], that Increased polymerisation of the sickle cell haemoglobin in the presence of

lower foetal haemoglobin may account for these unfavourable events among children with SCA. Although, they did not find a relationship between foetal haemoglobin levels and leg ulcer, which is contrary to this study. This study showed that about 30% of those with high HbF levels experiences leg ulcers only, while the remaining 70% that experiences leg ulcer expressed reduced foetal haemoglobin levels, this conformed to previous studies that stated 'Threshold levels' above which HbF could be an ameliorating factor[11,12].

From this study, there is no significant relationship between HbF levels and the various age group recruited for this study, which is in contract to a findings among SCD patient in Kuwait and Ibadan, which stated that the mean HbF is inversely related to age and significantly reduces with increasing age[13]. This disparity could have been as a result of the wide range of age group recruited for this study.

PCV count is also significantly increased in patients with high HbF levels than in patients with low HbF levels, which agrees to a study done in Tanzania[14], how the beneficial effects of elevated HbF seen in their study reduced anaemia, leucocytosis and thrombosis. Increased PCV counts reduces anaemia, which is common to SCD patients. Also, the average mean of males PCV was higher compared to that of females.

In agreement to the first genetic study on foetal haemoglobin findings in Nigeria[15], the 3bpdeletion of rs66650371 on chromosome 6 showed an allelic frequency of 3% in the population. Although, about 100 participants had an elevated HbF level but do not express the rs66650371 SNP, accordingly, the inability to evaluate other genetic variant associated to the elevated levels of HbF. Additional loci may be identified through independent genome-wide association studies in African populations. Other active components have been proposed and might have a more important role in Nigerian patients[1].

There is a significant difference between the haematological factors (HbF and PCV) with rs66650371 SNP, patients with rs66650371 SNP had an increased level of foetal

haemoglobin, and about 80 percent of them also had increased level of PCV counts, this is agreement to the genetic study of foetal haemoglobin in SCD in Nigeria[15]. Also there was a reduced severity of the disease in patients with rs66650371, more than two-third of the patients with rs66650371 experiences mild crises about once a year, while the others experiences about twice in a year In this study, more females were recruited than the males, this shows the rs66650371 has a higher frequency in the female cohort compared to the males, although, there was no significant difference. Hence gender is not likely of direct functional significance in the rs66650371 SNP.

Although, there are no local data to corroborate or refute this observations in this study.

Endnotes

[1] T. Adeyemo, O. Ojewunmi, *A Survey of Genetic Fetal-Haemoglobin Modifiers in Nigerian Patients with Sickle Cell Anaemia.* **PLoS One,** 13(6),2018, 6-14.

[2] J. Farrell, M. Sherva, *A 3-bp Deletion in HBSIL-MYB Intergenic Region on Chromosome 6q23 is associated with HbF expression.* **BLOOD,** 117(18), 2011`4935-4950

[3] N. Skya, L. Mwita, *Identifying Genetic Variants and Pathways Associated with Extreme Levels of Fetal Hemoglobin in Sickle Cell Disease in Tanzania.* **BLOOD**, 238(1), 2019, 9 - 19

[4] I. Akinsheye, A. Abdulrahman, *Foetal Haemoglobin in Sickle Cell Anaemia,* **BLOOD**, 118(1), 2011, 19-27

[5] O. Amoran, A. Babatunde, *Prevention Practices Influencing Frequency off Occurrences Of Vao-Occlusive Crises Among Sickle Cell Patients In Abeokuta South Local Government Area Of Ogun State, Nigeria,* **BMC Hematology**, 17(6), 2017, 77-99

[6] A. Hamzzazi, H. Ageeli., *Epidemiology and characteristics of Sickle Cell Patients admitted to hospitals in jazan region, of Saudi Arabia,* **Journal of applied Haematology,** 11(1), 2020, 10-14

[7] A . Alsultan , N. Solovieff , Aleem A, AlGahtani FH, Al-Shehri A, Osman ME. *Fetal hemoglobin in sickle cell anemia: Saudi patients from the Southwestern province have similar HBB haplotypes but higher HbF levels than African Americans.* **Am J Hematol,** 86(7), 2011, 612-4

[8] O. Adeodu, M. Akinsolu, *Foetal Haemoglobin and disease severity I Nigerian Children with Sickle Cell Anaemia.* **J. Haem**, 9(1),2017, 20-3

[9] J. Olaniyi, O.Ojetunde & O. Arinola. *Foetal Haemoglobin status in adult sickle cell anaemia patients in Ibadan.* **Annals of Ibadan Postgraduate Medicine**, 8(1), 2010, 1-6

[10] O. Adeodu, M. Akinsolu, *Foetal Haemoglobin and disease severity I Nigerian Children with Sickle Cell Anaemia.* **J. Haem**, 9(1), 2017, 20-3

[11] C. Antiwi-Boasiako, E. Frimpong , *Sickle cell Disease:Reappraisal of the role of foetal Haemoglobin levels in the frequencyof vaso-occlusive crises.* **Ghana Medical Journal,** 49(2), 2015, 102-106

[12] S. Deepika, A. Sheehan & K. Samir, *The vaso-occlusive pain crises in sickle cell disease: Definition, pathophysiology, and management.* **European Journal of Haematology**, 105(3), 2020, 237-246

[13] L. Shaban, A. Al-Taiar., *Anaemia and its associated factors among adolescents in Kuwait,* **PubMed**, 10(20), 2020, 5857-61

[14] S. Mtatiro, J. Makani J, Mmbando B, Thein SL, Menzel S, Cox SE. *Genetic variants at HbFmodifier loci moderate anemia and leukocytosis in sickle cell disease in Tanzania*. **Am J Hematol.** 90(1), 2015, 1–4.

[15] K. Gardner, T. Fulford, *g(HbF): a genetic model of foetal haemoglobin in Sickle Cell Disease*. **Blood adv,** 2(3), 2018, 235-239.

Chapter Five

5.1 Summary of Findings

Interest in foetal haemoglobin among patients with SCA has been on the increase in the last six decades. However, in Nigeria, and most parts of sub-Saharan Africa, where the burden of the disease is highest, studies on foetal haemoglobin and its role on clinical manifestations and disease severity in children are scanty.

This study followed the ARMS-PCR method to determine the 3-bp deletion region in HBSIL-MYB among Nigerian population. This is achieved, using four primers in a single PCR, followed by gel electrophoresis. The tetra-primer amplification refractory mutation system–polymerase chain (ARMS–PCR) reaction is a simple and economical method that was used to identify Insertions (II) and Deletions (DI) in chromosome 6q23 of the rs66650371 SNP. The allelic frequency of the gene in the population was 3%, with the 3-bp deletion at 207bp on gel-electrophoresis, which is in agreement with the ground breaking study done in Nigeria using Real-Time PCR, Tanzania and Cameroon.

This study reveals that majority of the participants have high frequency of crises in a year, despite adequate knowledge in the prevention of crises of patients attending sickle cell clinics regularly. It was also found in this study that more half of the patients had more at least four episodes of vaso-occlusive crisis in a year and indeed this was responsible for most of the hospital admissions among them.

Infants and babies were excluded from this study, because of their unstable foetal haemoglobin level at this time. Also, patients who received blood in the past six months were

excluded from this study to prevent false positive results that can alter the foetal haemoglobin level. From this study, more than half of the population had reduced level of foetal haemoglobin concentration. It was also observed that a significantly higher proportion of patients with moderate disease severity compared to those with mild disease severity had low levels of HbF. On the other hand, more of the patients with mild SCD severity than those with moderate disease had high levels of HbF. The mean HbF in females is not significantly different from males in this study, which is similar to a study in Nigeria, and contrary to studies in Saudi Arabia, Congo. Although, the reasons remains unclear from the studies. The foetal haemoglobin levels was also not inversely proportional to age from this study

The study showed there was a relationship between the HbF concentration and Vaso-oclussive crises. Patients who showed higher foetal haemoglobin levels significantly show decreased vaso-occlusive crises in a year. The high levels of HbF may suggest that increased foetal Haemoglobin concentration may have beneficial effects in SCD. This conformed to previous studies in Nigeria and Tanzania, that stated 'Threshold levels' above which HbF could be an ameliorating factor.

The PCV count is also significantly increased in patients with high HbF levels than in patients with low HbF levels agreed with another study done in Tanzania, how the beneficial effects of elevated HbF seen in their study reduced anaemia, leucocytosis and thrombosis. The significant increase of foetal haemoglobin levels to PCV will reduce the rate of anaemia, thereby reducing sickle cell crises which has also been confirmed in this study. Also, the average mean of males PCV was higher compared to that of females.

About 30 percent of participants from this study who had elevated level of HbF but also had an increase in vaso-occlusive crises might be as a result of the un-even distribution of HbF among F-cells, or some cells might have insufficient concentrations to inhibit HbS

polymerization. Only when the total foetal haemoglobin concentration is near 30%, is it possible for the number of protected cells to approach 70%.

In agreement to findings in a study in Nigeria, the 3bp-deletion of rs66650371 on chromosome 6 showed an allelic frequency of 3% in the study population. HBSL-MYB gene, rs66650371 gene is common in the African cohort. All patients with the gene present showed an elevated level of HbF, high PCV and reduced vaso-occlusive crises, this confirms that rs66650371 is responsible for elevation of HbF levels in SCD. There is no significant relationship between the SNP and gender

Although, about 100 participants had an elevated HbF level but do not express the rs66650371 SNP, accordingly, the inability to evaluate other genetic variant associated to the elevated levels of HbF. Other active components have been proposed and might have a more important role in Nigerian patients.

5.2 Conclusion

The present study demonstrated the presence and beneficial effect of foetal haemoglobin, and HBSIL-MYB in Nigerian patients with SCD. Patients with an elevated level of foetal haemoglobin concentration expresses reduced or no vaso-occlusive crises in a year. Also, the number of vaso-occlusive crises, such as acute chest pain, Leg ulcer is significantly indirectly proportional to elevated HbF levels. Patients with reduced HbF levels, have repetitive number of vaso-occlusive crises in a year.

High HbF levels is directly proportional the increase in PCV (Packed Cell Volume) counts. Patients with High HbF experience little or no anaemia. HbF levels does not decrease with age.

The allelic frequency of the rs66650371 gene in the study population is 3%. Patients with the deleted 3-bp gene, expresses an increase in the HbF levels. This shows that the 3-bp gene is responsible for the elevated HbF levels in SCD. The rs66650371 is not determined by gender

or age. The HMIP intergenic polymorphism is associated with HbF levels of SCD patients in Africa, which is also confirmed in this study. Although much less significantly compared with Europeans or Chinese because of their much lower minor allele frequencies. It could be that there are other HMIP variants associated with HbF level among people of African descent that are not tracked well by SNP rs9399137

5.3 Recommendations

I. Facilities for early and regular quantification of foetal haemoglobin should be made available in Sickle cell Clinics and Hospitals

II. Access to HbF inducing agents, specifically hydroxyurea should be encouraged in order to reduce the morbidity and mortality among these patients. Induction of high levels of HbF is the most promising approach to the pharmacologic treatment of sickle cell anaemia because it targets the proximal pathophysiologic trigger of disease. Hydroxyurea can induce HbF in most patients and is clinically beneficial in many.

III. Genome-wide association testing to discover novel disease and additional modifier loci.

IV. Active community medical education about the sickle cell disease through medical personnel and public societies

5.4 Contribution to knowledge

In Nigeria, there is paucity of data on the influence of foetal haemoglobin levels on disease severity among sickle cell disease in Nigeria, despite been the highest burden of SCD globally. Hence, this study is aimed at providing data needed for future research on foetal haemoglobin among SCD patients and its severity.

The present study expands the knowledge of the frequency of the genetic modifier (HMIP) and the independent effect on HbF levels in Nigerian patients with Sickle Cell Disease. Also,

it demonstrated the presence and beneficial effect of the quantitative-trait loci for foetal haemoglobin levels. The results make a case for the development of further, extended studies in identifying additional loci, responsible for elevated levels of foetal haemoglobin among SCD patients in Nigeria.

5.5 Suggested Areas for Further Research

Genome-wide association testing to discover novel disease modifier loci should be considered for extended studies. Up to now, most research on sickle cell Disease has taken place in Europe and in the US while the great majority of patients reside in Africa. Genetic and epidemiology studies can also help to address this imbalance.

Future studies should be multi-centred, longitudinal in design and involve a larger population to avoid survival biases.

SNPs in these QTL and in the *HBB* gene-like cluster explain approximately one-third to one-half of HbF variation in sickle cell anaemia, leaving much of the variance in HbF level unexplained. Rare variants probably explain this "missing" heritability but are difficult to detect using genome-wide association studies (GWASs) and will require genomic sequencing