Glucose-6-Phosphate Dehydrogenase Status of Sickle Cell Disease Patients in Ibadan, Oyo State, Nigeria

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Certification

This is to certify that Bilikis KHARASHI-KAREEM with matriculation number LCU/PG/001514 carried out this research work titled "Glucose-6-Phosphate Dehydrogenase Status of Sickle Cell Disease Patients in Ibadan, Oyo State, Nigeria" in the Department of Biological Science, Faculty of Natural and Applied Sciences, Lead City University, Ibadan, Oyo State, for the award of Master Degree (MSc) in Molecular Biology and Genomics and that this has not been previously submitted.

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Dedication

This research work is dedicated to God Almighty

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme involved in the pentose monophosphate pathway. Deficiency of this enzyme leads to free radical-mediated oxidative damage to red blood cells, and in turn causes haemolysis. G6PD deficiency is the most common genetic enzymatic disorder of red blood cells, affecting 400 million people worldwide. Sickle cell disease (SCD) is a genetic disorder in which individual inherit two abnormal copies of β-globin subunits given rise to haemoglobin S. The HbS red cells fail to return to normal shape when normal oxygen tension is restored thereby leading to vessel occlusion, ischaemia, and destruction of the red blood cells (haemolysis). Sickle cell disease and glucose-6-phosphate dehydrogenase deficiency are inherited disorders associated with chronic haemolysis. Therefore, coinheritance of both disorders could worsen haemolysis in the former and compound a haemolytic crisis. This work aims at determining the glucose-6-phosphate dehydrogenase status of sickle cell disease patients in Ibadan. Blood samples were collected from 147 sickle cell anaemia patients recruited from The Haematology Day Care Unit UCH Ibadan and Sickle Cell Clinic Adeoyo Ibadan. Haemoglobin electrophoresis was carried out to determine the haemoglobin genotype. The haemoglobin variants obtained were 142 (96.6%) homozygous haemoglobin S (SS), 4 (2.7%) heterozygous haemoglobin SC and 1 (0.7%) homozygous haemoglobin CC with. Qualitative analysis of G6PD was carried out using fluorescent spot test resulting to 21 (14.3%) full deficient and 11 (7.5%) partial deficient. Quantitative analysis of G6PD was determined using G6PD RANDOX reagent and 21.8% were G6PD deficient. DNA extraction and amplification was carried out and the gel electrophoresis was used to determine the band size of 308bp of G6PD variants (rs1050829, 376 A→G, chrX:154535277; 156 asn → asp). All that were G6PD A variants were digested with FokI restriction enzyme to determine the G6PD A variant polymorphism in which none was observed. This study has shown that G6PD deficiency is highly prevalent among those with HbSS and that all SCD patients should be screened for G6PD deficiency to avoid the use of medications and agents that could aggravate haemolysis during the treatment and management. In view of the findings in this study, it is recommended that G6PD status of everyone should be determined in order to prevent haemolysis after exposure to oxidative agents.

Key words: Haemolysis, G6PD (glucose-6-phosphate dehydrogenase), FokI restriction enzymes, oxidative agent.

Word count: 260 words

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List of Acronyms

Abbreviation	Meaning
G6PD	Glucose-6-phosphate dehydrogenase
PPP	pentose phosphate pathway
NADPH	nicotinamide adenine dinucleotide phosphate
GSSG	oxidized glutathione
ROS	reactive oxygen species
AHA	acute haemolytic anaemia
WHO	World Health Organization
RBC	red blood cells
SCA	Sickle cell anaemia
SCD	Sickle cell disease
dbSNP	single nucleotide polymorphism
Hb	Haemoglobin
CBC	complete blood count
MCH	Mean corpuscular volume
PBF	Peripheral Blood Smear
RWD	red cell distribution width
SCT	sickle cells trait
CZE	Capillary Zone Electrophoresis
HPLC	High Performance Liquid Chromatography
PCR	Polymerase Chain Reaction
ARMS	The amplification-refractory mutation system
RFLP	Restriction Fragment Length Polymorphism
GWASs	genome-wide association studies
NGS	Next-generation sequencing
WES	whole-exome sequencings
SNVs	single-nucleotide variants

SGP	Sickle Genome Project
LFA	Lateral flow assays
QCM	Quartz Crystal Microbalance
EIS	electrochemical impedance spectroscopy
PQ	primaquine
TLS	tumor lysis syndrome
NNJ	Neonatal jaundice
CNSHA	Congenital non-spherocytic haemolytic anaemia
EDTA	ethylenediaminetetraacetic acids
DNA	Deoxylribonuceic acid
FST	Fluoroscent spot test
TBE	tris boric EDTA
PD	Partial deficiency

Chapter One

Introduction

1.1 Background of the Study

The G6PD (EC 1.1.1.49) is an X-linked cytosolic enzyme which is available in all forms of life from prokaryotes to animals[1]. The G6PD gene that span 18kb on X chromosome (Xq28) has 13 exons separated by 12 introns which is highly conserved throughout evolution[2]. Glucose-6-phosphate dehydrogenase (G6PD) the rate limiting enzyme as it channels glucose catabolism into pentose phosphate pathway (PPP) from glycolysis[3]. It has a key role in the pentose phosphate pathway which is to produce nicotinamide adenine dinucleotide phosphate (NADPH), a very important reducing agent that helps in the regeneration of oxidized glutathione (GSSG) and also confers protection against cellular oxidative stress in the cells[1]. Reactions involving NADPH produce compounds that prevent reactive oxygen species (ROS) from building up to toxic levels within cells[3]. The production of NADPH by glucose-6-phosphate dehydrogenase is essential in red blood cells due to their susceptibility to damage by reactive oxygen species as they lack another NADPH-producing enzyme[4]. Using crystallographic studies, the primary and tertiary structure of human G6PD has been determined from the nucleotide sequence of full-length cDNA clones[1]. G6PD activity physiologically reduces as red blood cells age due to the absence of protein synthesis in the matured red cells[5].

G6PD deficiency is a genetic disorder and the most common inherited enzymopathies globally which affects more than 400 million people with the prevalence based on the ethnic group. It is common among populations at risk of malaria, which may be the consequence of a protective effect of G6PD deficiency against some forms of malaria[6]. Different levels of

G6PD activities occur due to point mutation of the gene leading to a diverse range of biochemical and clinical phenotype[7]. Over 400 G6PD variants have been identified so far but considering the stability and activity only 186 variants are linked to G6PD deficiency[7]. The World Health Organization categorized G6PD variants into 5 classes according to the level of enzyme activity and severity of haemolysis.

Class I variants, such as G6PD volendam (C514T), characterize severe deficiency and are associated with chronic non spherocytic haemolytic anaemia.

Class II variants, such as G6PD Mediterranean (C563T), have enzyme activity values lower than 10%, leading to more frequent symptoms and crises.

Class III variants, including G6PD A (A376G), A- (G202A/A376G), G6PD Asahi (G202A), the enzyme activity ranges from 10 to 60%, and patients are usually asymptomatic, but the clinical relevance is the expression of medication-induced haemolytic crises.

Class IV variants, such as G6PD São Borja (G337A), have 60 to 150% of normal enzyme activity, with no clinical manifestations.

Class V variants have increased enzyme activity (>150%) and were described only once (G6PD Hektoen), in 1969[8].

The most common phenotypes are G6PD A (genotype A376G), which has normal enzyme activity; G6PD A- (genotype G202A), which presents from 10 to 60% of the normal enzyme activity; and B- or G6PD Mediterranean (genotype C563T), with 7% of normal activity[9] .

Taking account of these new variants, the number of reported G6PD mutations is 217, being as follows: 182 (83.9%) are single nucleotide substitutions (missense variants), 19 (8.7%) are multiple mutations (two or more substitutions), 11 (5.1%) are deletions, and five (2.3%) mutations affect the introns[10]. Interestingly, there are 16 mutations corresponding to single

nucleotide substitutions (missense variants) that were previously reported before in Minucchi's review and that were not considered as the G6PD Class I mutants: Zacatecas (Arg257Leu; exon 7), Palermo (Arg257Met, exon 7), Hamburg (Pro276Leu, exon 8), Veracruz (Arg365His, exon 10). Yucatan (Lys429Glu, exon 10), Tennessee (Leu422Val, exon 10), and one deletion named G6PD Taif (174Gly, exon 6)[11].

G6PD deficiency is mostly assymptomatic except when triggered by an exogenous agent which subjects the red blood cells to oxidative stress that causes acute haemolytic anaemia (AHA), which may be severe and even life threatening[12]. Exogenous agents that can cause AHA in G6PD deficient individuals include: (a) certain drugs (b) certain infectious diseases, (c) ingestion of fava beans and some chemicals[8].

Fava beans has been established to be the commonest trigger of haemolysis among people with G6PD deficiency[13]. *Vicia faba*, also known in the culinary sense as the broad bean, fava bean, or faba bean, is a species of vetch as a flowering plant in the pea and bean family *Fabaceae*. It is widely cultivated as a crop for human consumption, and also as a cover crop[14]. Varieties with smaller, harder seeds that are fed to horses or other animals are called field bean, tic bean or tick bean. Horse bean, *Vicia faba var. equina Pers.,* is a variety recognized as an accepted name. Broad beans have a long tradition of cultivation in Old World agriculture, being among the most ancient plants in cultivation and also among the easiest to grow[15]. Along with lentils, peas, and chickpeas, they are believed to have become part of the eastern Mediterranean diet around 6000 BCE or earlier, however their wild ancestor has not been identified and their origin is unknown. Some people suffer from favism, a haemolytic response to the consumption of broad beans, a condition linked to a metabolism disorder known as G6PD deficiency[16]. Otherwise the beans, with the outer seed coat removed, can be

eaten raw, other than beans, or cooked. In young plants, the outer seed coat can be eaten, and in very young plants, the seed pod can be eaten[12].

One of the drugs that pose a possible risk in some persons with G6PD deficiency include some antimalarial. Antimalarial drugs that csan cause acute haemolysis in people with G6PD deficiency includes primaquine (PQ), an 8-aminoquinoline, which is currently considered the most effective drug to prevent Plasmodium vivax clinical relapses as well as in *Plasmodium falciparum* infections to prevent gametocyte development and further parasite transmission to mosquitoes[17]. However, PQ use has been restricted because it can trigger variable degrees of haemolysis in individuals with G6PD deficiency, depending on the variant type. Due to intensification of malaria control programmes worldwide and the perspectives for malaria elimination in some regions where PQ could be massively deployed, it is important to better characterize prevalence of G6PD in malaria endemic communities in these area[18]. People with G6PD deficiency are about 20 to 30 times more sensitive to the haemolytic activity of primaquine than the people with normal G6PD[19]. Mechanism for induction of haemolytic anaemia was studied in rats. Toxic metabolites of primaquine cause reduction of GSH in red blood cells, formation of methemoglobin and Heinz bodies that induce haemolytic anaemia[20]. The primaquine metabolite, 6-methoxy-8-hydroxylaminoquinoline after N-hydroxylation from 6-methoxy-8-aminoquinoline, by peroxidation of lipids in red blood cells with significant GSH and oxidation of protein in GSH-depleted red blood cells can induce hemolytic response[21]. Other antimalarial drugs include pamaquine, mepacrine, quinine and chloroquine. There is evidence that other antimalarials may also exacerbate G6PD deficiency, but only at higher doses[22].

Infection is the most commonly cited cause of acute haemolytic anaemia in patients with G6PD deficiency, particularly in those with severe deficiency and cytomegalovirus, hepatitis A and B, pneumonia, or typhoid fever. Infection, whether bacterial, viral, or fungal, produces reactive oxygen species through the inflammatory response, to which deficient cells are particularly susceptible[23]. Haemolytic anaemia caused by infection and subsequent medication is a clinically important concern in patients with G6PD deficiency[24]. G6PD deficiency also may increase susceptibility to infection through these mechanisms: in leukocytes, a decrease in reactive oxygen species production within the pentose phosphate pathway reduces the microbicidal activity of phagocytes. Impaired formation of reactive oxygen species-dependent neutrophil extracellular traps reduces extracellular defense mechanisms against bacterial and fungal pathogens[25].

Henna, obtained from the crushed leaves of Lawsonia alba, has been used for centuries to dye the skin, hair and nails in many countries in south-east Asia and the Middle East[26]. The application of henna has resulted in life-threatening episodes of haemolytic anaemia in individuals with a genetic deficiency in G6PD activity. The use of L. alba (henna) for medicinal and cosmetic purposes is part of ancient and modern cultures and traditions of North Africa and Asia[27]. In some countries, it is used in ceremonial and social events, such as weddings and circumcisions, where henna is applied to the skin to create various patterns[22]. It is a commonly used traditional cosmetic agent, which also holds medical potentials and is used to treat skin lesions including seborrheic dermatitis or fungal infections and also has possible anti-inflammatory effects. The active dye ingredient in henna, lawsone (2-hydroxy-1,4-naphthoquinone) has been implicated as a cause of henna-induced haemolytic anaemia because of its structural similarity to other ortho-substituted 1,4-naphthoquinones, such as

menadione, which are known to induce oxidative injury in red blood cells. Henna extract, or lawsone, were found to induce severe haemolytic anaemia in various animal models after exposure of the skin or ingestion. This reaction is associated with oxidative damage to erythrocytes[28].

This deficiency express different spectrum of clinical manifestations including the following: favism, neonatal jaundice, drug induced haemolysis, chronic non-spherocytic haemolytic anaemia and infection induced haemolysis[29].

All patients with favism are G6PD deficient; however, not all G6PD deficient subjects are sensitive to fava beans, and even those who are sensitive show striking variability from one exposure to the next[30]. The reason for this discrepancy is not clear, and it seems likely that one or more factors in addition to G6PD deficiency are required for the development of favism and to determine the severity of the individual attack. Clinical favism presents characteristically with sudden onset of acute haemolytic anaemia within 24-48 hours of ingestion of the beans. Pallor, jaundice and haemoglobinuria are the hallmarks. Acute renal failure may supervene in adults, but it is very rare in children; however, fatalities in children were not uncommon prior to the availability of transfusion therapy[31].

A critical analysis of the data whereby individual drugs have been implicated in the causation of haemolysis in G6PD-deficient subjects has been conducted by Beutler who uncovered a discrepancy between the relatively small list of drugs for which there is strong evidence linking them to haemolytic anaemia and a much larger list of agents for which the evidence is less secure[32]. The degree of haemolysis is also influenced by the activity of the host G6PD activity, the dose and duration of therapy, and the presence of additional oxidant stress, for example, infection. Furthermore, clinical and haematological assessment of haemolysis has

notoriously low sensitivity, in that even a two- to three-fold increase in red cell destruction may not produce a significant anaemia or reticulocytosis. Clinical haemolysis and jaundice typically begin within 2 to 3 days of starting the drug[33].

There is genetic heterogeneity of G6PD deficiency which means that a drug found to be safe in some deficient subjects may not necessarily safe in all. The risk and severity of haemolysis is usually dose related[34]. Quinine, chloroquine and quinidine are all acceptable for the treatment of acute malaria, and chloroquine, mefloquine, halofantrine proguanil and pyrimethamine (but not Maloprim, which contains dapsone, or Fansidar, which contains a sulphonamide) are acceptable for malaria prophylaxis[35]. The haemolysis is largely intravascular and it is characteristically associated with haemoglobinuria. The anaemia worsens until the seventh to eighth day, a reticulocyte response then sets in, and the haemoglobin level begins to recover on the eighth to tenth day. Invitro tests have been developed aiming to predict whether a drug will cause haemolysis in vivo and they should be carried out before a new drug is introduced to a population in which G6PD deficiency is prevalent[36]. The haemolysis is largely intravascular and it is characteristically associated with haemoglobinuria. The anaemia worsens until the seventh to eighth day, a reticulocyte response then sets in, and the haemoglobin level begins to recover on the eighth to tenth day[36].

Infection is probably the most common cause of haemolysis in subjects with G6PD deficiency. Numerous bacterial, viral and rickettsial infections have been reported as precipitants, but particularly important are infectious hepatitis, pneumoniaand typhoid fever. Viral infections affecting either the upper respiratory tract or the gastrointestinal tract are reported to cause more severe haemolysis than bacterial infections in G6PD-deficient

children. Haemolysis is again largely intravascular and renal failure is a well-recognized complication in adults, whereas it is rare in children[37].

Frequency and severity of haemolysis vary depending on the G6PD variant and exposure to various oxidative agents. As an X-linked recessive trait, it is predominantly a disease of male[38]. However, females can be affected if they have a mutation in both copies of the G6PD gene (homozygous), or at times, if they have only one mutation(heterozygous)[7]. Affected male are called hemizygous due to the fact that they have one X chromosome[39]. All races and ethnic backgrounds are being affected by G6PD deficiency[40]. G6PD gene is highly polymorphic and the frequency of these mutations varies among populations and countries[41].

As earlier stated, G6PD deficiency is more commonly expressed in males compared to females with the highest prevalence rates found in Africa, the Middle East, certain parts of the Mediterranean, and certain areas in Asia. In these regions, the rate ranges from 5% to 30% of the population. The severity of G6PD deficiency can vary based upon specific racial groups[42]. Interestingly, the prevalence of G6PD deficiency correlates with the geographical distribution of malaria, leading to postulate that G6PD deficiency gives a partial protection against this infection[43]. The severe form of the disorders occurs more often in the Mediterranean population. In the United States, the incidence is much higher among the African-American population than in other sectors. The frequency of a carrier state in which one partner carries a normal gene and the other carries an abnormal variant is as high as 24%. About 10%-14% of African-American males are affected[44].

The prevalence of this deficiency varies around the globe, with frequencies ranging from 2% to 20% in Greece, Turkey, and Italy; but increased as much as 70% in groups of Kurdish Jews. Recently, a Bayesian geo-statistical model was used to estimate the prevalence of

G6PD deficiency worldwide. The results showed that in Latin America (LA), the prevalence of G6PD deficiency is lower compared with other regions such as sub-Saharan Africa or Asia[45]. In Asia, the deficiency prevalence ranges from 6.0% to 15.8%. In India, it is 10.5%, while in the Middle East the prevalence varies from 3% to 29%6. In Brazil, a few studies have found a prevalence between 1.7% and 6.0%109. In africa, the prevalence of G6PD deficiency has been reported as high as 28.1% in south-west Nigeria, 22.5% in Congo (Brazzaville), 15.7% in Mali (Bamako), 13.0% in Uganda and 9.0–15.5% in Gabon109. Previous reports in Nigeria showed that the prevalence of G6PD deficiency ranged from 4% to 26%. However, a study carried out in Sokoto (Nigeria) among 118 children visiting the Emergency Pediatrics unit of Usmanu Danfodiyo University Teaching Hospital for pediatrics related care indicated G6PD deficiency of 14.4%[46]. Also, an overall prevalence of 16.2% G6PD deficiency was reported in Katsina among children aged 0 – 5 years. It is most frequent among individuals of African descent, with a frequency ranging from 3.6%-28%.

According to the World Health Organization (WHO), 7.5-10% of the world population bears one or two genes variants for G6PD deficiency and about 2.9% out of the said number are G6PD deficient[47]. For instance, Mutation S188F, sometimes called the Mediterranean mutation, is most prevalent among individuals from the Middle East. Mutations C131G and G487A that were common in Dhaka, Bangladesh appear to be linked to G6PD deficiency by affecting NADP binding or disrupting the protein structure[15]. The G6PD genetic variants were relatively homogeneous in America, Africa, and Western Asia compared to those in East Asia and Oceania. In North America, Africa, Yemen and Saudi Arabia, G6PD*A-variant is predominant among populations[48]. For example, two common variants occur in many African-American males. Approximately 20 to 25 percent have the near normal G6PD

variant called "A+" (mutation A376G), while about 10 to 13 percent have another variant called "A-" (G202A arose on a G6PD A+ chromosome). Africa, the Middle East, certain parts of the Mediterranean, and certain areas in Asia has the highest prevalence rates of G6PD deficiency. In these regions, the rate ranges from 5% to 30% of the population[13]. Base on the residual enzyme activity and disease severity, World Health Organization (WHO) has classified the variants in five classes[8].

The most severe of all variants are the class I which show chronic haemolysis even in the absence of any triggering factor. Class II and III are variants with marked enzyme deficiency but no chronic haemolysis. Class IV are variants with normal enzyme activity; class V was designed for variants with increased enzyme activity[49,50]. In sub-Saharan Africa three variants occur with polymorphic frequencies (> 0.1%); G6PD*B, G6PD*A and G6PD*A-). The most common variants are the G6PD A which predominate among the African which has a single A→G substitution at nucleotide number 376 while G6PD B remains the wild type of the allele[15]. G6PD A- is a deficient variant with about 8-20% of the wild type enzyme activity and, in addition to the A376G mutation that describes the G6PD A variant, most commonly also involves a G→A substitution at nucleotide 202[16]. Also, researchers have identified other variants of G6PD A-with substitutions at 542 G→T, 680 G→T or 968 T→C in Africa[15].

There are multiple ways to determine individuals' G6PD status. Some methods are more suited for population studies (e.g. genetic tests) and others are more suited for case management decision-making (e.g. tests that measure enzyme activity). Tests for G6PD deficiency can be classified as either phenotyping assays, which measure the G6PD activity in the individual's blood or genotyping assays, which determine at the DNA level whether someone is G6PD deficient[51]. Phenotypic assays can be quantitative (providing a precise

measure of G6PD activity) or qualitative (indicating normal or abnormal activity). Qualitative test can be used to indicate whether an individual is above or below a threshold predetermined by the diagnostic test for G6PD activity (30–40% of normal activity)[52].

Deficient results (sometimes referred to as positive results) determine that the individual has G6PD activity below this threshold and should be considered G6PD deficient. Normal results (sometimes referred to as negative results) mean that the individual has G6PD activity above the threshold for G6PD normal activity, but this is a wide range. Qualitative tests are able to provide good performance characteristics (sensitivity and specificity) at a 30–40% G6PD activity threshold[53]. As males have either normal or deficient activity, qualitative tests have very good performance in males; however, qualitative tests have lower sensitivity and specificity in heterozygous females with variable activity (between 30–80%), which may result in "intermediate" classification by some qualitative tests[52].

Quantitative testing for G6PD is performed in reference or specialized laboratories using a complex assay on a temperature-regulated instrument because of the large temperature impact on enzyme activity18. Quantitative tests measure G6PD activity in a whole blood sample and provide a quantitative result for G6PD activity. Quantitative tests are able to accurately measure G6PD activity for all individuals, from those with severe G6PD deficiency (<10% normal) to those with high G6PD activity (>100% normal)[54]. Typically, quantitative tests normalize the G6PD activity per red blood cell count or haemoglobin concentration in order to account for varying individual haematocrit ranges at the time of sampling. Most importantly, quantitative tests can detect individuals with intermediate G6PD activity, such as females who are heterozygous for G6PD deficiency. Quantitative tests can be used to independently determine the threshold at which to exclude patients from the

standard daily dose of primaquine, based on an assessment of risks and benefits. Such tests are most suitable for making treatment decisions for heterozygous females with intermediate G6PD activity[55]. However, most currently available quantitative tests for G6PD activity require highly complex laboratory equipment and facilities, and therefore are only performed in newborn screening facilities or reference laboratories. New quantitative tests for use at the point of care are under development[56].

G6PD deficiency is usually caused by mutations in the G6PD gene that result in below-normal G6PD activity, a condition that particularly affects red blood cells[57]. Most genotyping studies perform polymerase chain reaction (PCR)-based single nucleotide polymorphism (SNP) analysis. PCR-SNP analysis is limited to identifying the presence or absence of known genotypes; therefore, it may not correctly identify G6PD deficiency in patients with mutations not included in the referensce panel[58]. DNA sequencing can determine if any mutation is present in the G6PD gene; in the case of females, sequencing can determine if one or both genes are affected in order to determine if the female is heterozygous or homozygous for G6PD deficiency. Genotypic assays are both expensive and technically complex. For these reasons, they cannot be used to provide accurate risk estimates of G6PD deficiency in individuals[57].

Sickle cell anaemia is an autosomal recessive genetic disorder caused by a variant of the beta-globin gene called sickle haemoglobin (HbS). The disease can be expressed in the presence of either two copies of Hb S or one copy of Hb S plus another beta-globin variant (such as Hb C)[59]. The most common subtypes of SCD is homozygous (two copies of the β-globin S (βS) mutation that codes for sickle cell hemoglobin (Hb S)) commonly called SS. The second common subtype of SCD is compound heterozygosity for βS and βC (SC).

Others are compound heterozygosity with β+-thalassemia (Sβ+-thalassemia). The rare subtypes are haemoglobin SB 0 (Beta-zero) thalassemia, Hemoglobin SD, hemoglobin SE, and hemoglobin SO[60].

In humans, there are haemoglobin A, (which consists of two alpha and two beta chains) haemoglobin A2, (which consists of two alpha and two delta chains) and haemoglobin F, (consisting of two alpha and two gamma chains) in their bodies. The one that dominate until about 6weeks of age is haemoglobin F then haemoglobin A takeovser throughout life. HbA1 comprises 2 chains of the alpha-globin and two chains of the beta-globin (a2b2) - This constitutes 95% of the adult hemoglobin. HbA2 comprises 2 chains of the alpha-globin and two chains of the delta-globin (a2d2) - This constitutes less than 4% of the adult hemoglobin. HbF- comprises 2 chains of the alpha-globin and two chains of the gamma-globin (a2g2). HbF is more prevalent in the fetus (due to high oxygen binding affinity that helps in extracting oxygen from maternal circulation).

Homozygosity of the beta-S (βS) allele (located on chromosome 11p15.5), which differs from the wild-type β-allele by a single nucleotide polymorphism dbSNP Rs334(T;T) in which GTG replaced GAG in the sixth codon of the β-globin gene is the major cause of sickle cell anaemia[61]. This replacement results into coding of valine instead of glutamate in position 6 of the Hb beta chain[17]. Red blood cells with HbS are rigid blood cells and are unable to deform as they pass through narrow capillaries unlike the normal red blood cells that are quite elastic and have a biconcave disc shape, which allows the cells to deform to pass through capillaries under low oxygen tension[62]. When red blood cells (RBCs) containing homozygous HbS are under deoxygenated conditions, red cells with HbS fail to return to normal shape when normal oxygen tension is restored thereby sickling process begins. HbS

undergoes marked decrease in solubility, increased viscosity, and results into gradual formation of polymer[63]. The sickling of the red blood cells results into breaking down of the red cells causing haemolysis, reduced oxygen carrying capacity and then results into episode of microvascular occlusion leading to tissue ischaemia and painful crises[64]. The compensation (erythropoesis) from the bone marrow does not meet up with the rate of destruction thereby leading to anaemia. These crises can lead to several diseases causing multiple organ damage from microinfarcts, including heart, skeleton, spleen, and central nervous system which are often life-threatening[19].

Patients with SCD may experience intense pain early in infancy, childhood and adulthood. Pain usually accounts for the majority of hospitalisations and overall negative impact in patients' health related quality of life[65]. Pain is the cardinal feature of SCD and it is characteristically unpredictable, episodic in nature, described as one of the most excruciating forms of pain that affects human beings[66]. Pain occurs due to stimulation of nociceptive nerve fibres caused by microvascular occlusion. The microcirculation is obstructed by sRBCs, thereby restricting the flow of blood to the organ and this results into (i) ischaemia, (ii) oedema, (iii) pain, (iv) necrosis, and (v) organ damage. In the first year of life, one of the cardinal features is the 'hand-foot syndrome' due to vaso-occlusion of post-capillary vasculature resulting in tissue oedema and pain of the extremities[67].

Chronic tower skull, bossing of the forehead and fish mouth deformity of vertebrae are the result of extended haematopoietic marrow causing widening of the medullary space, thinning of the trabeculae and cortices, and osteoporosis. The excruciating pain of bone infarction in the "hand- foot syndrome" that occurs around the age of 12 years is often the first symptom of sickle cell disease. This dactylitis resolves spontaneously and is treated with hydration and

analgesic. Necrosis occurs with equal frequency in the femoral and humeral heads, but the femoral heads more commonly undergo progressive joint destruction, as a result of chronic weight bearing. Arthritic pain swelling, and effusion may be related to periarticular infarction or to gouty arthritis. Bone marrow infarction causes reticulocytopenia, exacerbation of anaemia, a leukoerythroblastic picture, and sometimes pancytopenia[68].

SCD increases susceptibility to infections, notably bacterial sepsis and malaria in children under five years[69]. Respiratory infections can trigger the sickle-cell acute chest syndrome, with a high risk of death. Hyperhaemolysis is precipitated by infections, glucose-6-phosphate dehydrogenase (G6PD) deficiency, acidosis and dehydration. There is increased pallor, jaundice and hepatosplenoemgally. Risk factors for infections include: (i) functional asplenia/hyposplenia which present with reduced splenic immune response at a very young age, (ii) impaired fixation of complement, (iii) reduced oxidative burst capacity of chronically activated neutrophils, dysfunctional IgM and IgG antibody responses and defective opsonisation. The main pathogen of concern is Streptococcus pneumoniae, though severe and systemic infections arise with Haemophilus influenzae, Neisseria meningitides, and Salmonellae leads to osteomyelitis especially Salmonella due to bowel ischaemia and gut flora dissemination[70].

Aplastic crisis is characterized by an acute failure of erythropoiesis often following viral infections especially parvovirus B19. The patient will present with weakness, progressive pallor and pancytopenia[71]. During this crisis the patient may have associated bone and joint pain. Acute sequestration is caused by pooling of blood in the spleen and the liver characterized by sudden onset of progressive anemia, splenic enlargement, abdominal pain,

and shock. Various crises and increased susceptibility to infections are responsible for recurrent illness in patients with sickle cell disease[72].

Sickle cell anaemia (SCA) is a multisystem disorder with the highest prevalence among the people of Sub-Saharan Africa, South Asia, the Middle East, and the Mediterranean[73]. It is also a highly inherited disease in the United States, affecting 1 in 500 African Americans. About 1 in 12 African Americans have the autosomal recessive mutation, and approximately 300,000 infants are born with sickle cell anemia yearly[19]. Annually, about 300,000–400,000 babies with SCA are born and around three quarters of them are across a geographical band in Africa stretching from Senegal to Madagascar, mirroring the continent of malaria endemicity. Over 1% of all newborns in most of this region have SCA with Nigeria alone is having up to 25–35% of global SCD births[74]. About 50 and 90% of SCA children died before the age 5 as reported from a recent review of cross-sectional population surveys and cohort studies of SCD in Africa. This extrapolates to 150,000–300,000 annual SCA child deaths, potentially accounting for 5–10% of the total region child mortality[75]. Compare to healthy children, many surviving children with SCA, as well as tens of thousands of additional children born in Africa every year with other forms of SCD experience significant morbidity[76].

1.2 Statement of the Problem

Sickle cell anaemia (SCA) affects millions of people throughout the world and is especially high among those whose ancestors came from sub-Saharan Africa; Spanish-speaking regions in the Western Hemisphere (South America, the Caribbean, and Central America); Saudi Arabia; India; and Mediterranean countries such as Turkey, Greece and Italy[77]. Sickle cell disease (SCD) is common throughout much of sub-Saharan Africa, affecting up to 3% of

births in some parts of the continent. Nevertheless, it remains a low priority for many health sectors[78]. Haemolysis is a constant finding in sickle cell syndromes. Probably due to absence of membrane filaments during oxygenation and deoxygenation, about one third of the erythrocytes pass through intravascular haemolysis[79]. More than average of patients with SCD will experience an acute anaemic event, which can be fatal, at some point in their life. Life-threatening bacterial infections are a major cause of morbidity and mortality in patients with SCD as a result of chronic haemolytic anaemia[80].

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common inherited disorder of erythrocyte metabolism and can cause haemolysis in the presence of triggers. Incidence is higher in certain ethnic groups (American blacks, people from the Mediterranean basin, people with Asian ancestry). Triggers include acute illnesses (infections), drugs (salicylates) and other substances (fava beans) that cause oxidative stress[81]. The most common medical problem associated with glucose-6-phosphate dehydrogenase deficiency is haemolytic anaemia, which occurs when red blood cells are destroyed faster than the body can replace them[9].

1.3 Justification of the Study

Sickle cell anemia (SCA) and glucose-6-phosphate dehydrogenase (G6PD) deficiency are both hereditary diseases of the red blood cells that cause haemolysis. The impact of the interaction of both conditions on the clinical and laboratory presentations of the affected persons is sparse[82]. G6PD deficiency significantly contributes to recurrent painful vasoocclusive crisis in SCA persons in the steady state[29]. Determination of the enzyme activity and type of G6PD variants that is common among deficient and non-deficient SCA patients can help ameliorate haemolytic reaction in SCA patients.

1.4 Aim and Objectives of the Study

The aim of the study is to determine the Glucose-6-phosphate Dehydrogenase status in sickle cell disease patients

The Objectives are to

(i) study the frequency of the various sickle cell status (i.e. SS, SC and CC) among participants attending the selected sickle cell clinics in Ibadan

(ii) investigate the G6PD deficiency among the different sickle cell types in the participants

(iii) determine gene polymorphism (A376G) among participants with G6PD deficiency

(iv) evaluate gene polymorphism in the different abnormal haemoglobin Hb SS, SC, CC participants and G6PD Status

1.5 Research Questions

(a) What is the prevalence of haemoglobin variants present among sickle cell anaemia patients?

(b) What is the G6PD status of the sickle cell anaemia patients?

(c) What is the enzyme activity of those SCA patients deficient of G6PD?

(d) Which of the G6PD variants are present among G6PD-deficient SCA patients?

1.6 Significance of the Study

This study will provide the frequency of different types of haemoglobin variants among the SCD patients. It will also enable the determination of the G6PD status of sickle cell anaemia patients in Ibadan. Also, it will help to determine the enzyme activity and the G6PD variants. The Impact of G6PD deficiency in the haemolytic crisis can be prevented by avoiding the trigger agents. The knowledge of G6PD status of sickle cell anaemia patients will help in the diagnosis, treatment and management of aforementioned patients. This will also help in the

counseling of the patients and relations so as not to be exposed to agents that aggravate haemolysis.

1.7 Scope of the Study

The study was carried out on 147 sickle cell anaemia patients from both Haematology Daycare Unit of University College Hospital and sickle cell clinic, Adeoyo Hospital Ibadan.

1.8 Limitation of the Study

Most of the study participants were not ready to subject themselves for sample collection. It is also difficult to collect their sample due to the non-prominence of their veins. There are limited numbers of recent journals in the area of this study hence leading to paucity of data. Inaccessibility to case note of the sickle cell patients which has limit this study from getting more information about the past medical history of the patients. There are other variants of G6PD in our area but could not be investigated due to financial constraint.

1.9 Operational Definition of Terms

Hemolytic anemia is a blood condition that occurs when your red blood cells are destroyed faster than they can be replaced. Hemolytic anemia can develop quickly or slowly, and it can be mild or serious.

Glucose-6-phosphate dehydrogenase is an enzyme which protects red blood cells, which carry oxygen from the lungs to tissues throughout the body. A defect of the enzyme results in the premature breakdown of red blood cells.

Glucose-6-phosphate dehydrogenase deficiency is an X-linked recessive disorder that results in defective glucose-6-phosphate dehydrogenase enzyme.

Haemolysis is the destruction of red blood cells which could result into anaemia.

Pentose phosphate pathway is a metabolic pathway parallel to glycolysis. It generates NADPH and pentoses as well as ribose 5-phosphate, a precursor for the synthesis of nucleotides. While the pentose phosphate pathway does involve oxidation of glucose, its primary role is anabolic rather than catabolic.

Red blood cell, also called erythrocyte, cellular component of blood, millions of which in the circulation of vertebrates give the blood its characteristic colour and carry oxygen from the lungs to the tissues. The mature human red blood cell is small, round, and biconcave; it appears dumbbell-shaped in profile. The cell is flexible and assumes a bell shape as it passes through extremely small blood vessels. It is covered with a membrane composed of lipids and proteins, lacks a nucleus, and contains hemoglobin—a red iron-rich protein that binds oxygen.

Glutathione, oxidized (GSSG) is the oxidized version of the naturally occurring and very important detoxification agent glutathione (GSH). When used in vivo, GSSG will not remain oxidized, but rather be reduced back to GSH. A ratio of GSH to GSSG can be used to determine the level of oxidative stress that has occurred in cells. When a cell has higher levels of GSSG, then it can be implied that more oxidative stress has occurred within the cell.

Reactive oxygen species (ROS) are oxygen-containing radicals that are capable of independent existence with one or more unpaired electrons. However, the term ROS is most often expanded to include reactive oxygen-containing compounds without unpaired electrons, such as hydrogen peroxide (H2O2) and singlet oxygen (O2).

Sickle cell disease (SCD) is a group of blood disorders typically inherited from a person's parents. The most common type is known as sickle cell anaemia. It results in an abnormality

in the oxygen-carrying protein haemoglobin found in red blood cells. This leads to a rigid, sickle-like shape under certain circumstances.

Single nucleotide polymorphism (SNP), variation in a genetic sequence that affects only one of the basic building blocks—adenine (A), guanine (G), thymine (T), or cytosine (C)—in a segment of a DNA molecule and that occurs in more than 1 percent of a population.

Hemoglobin is a protein in your red blood cells that carries oxygen to your body's organs and tissues and transports carbon dioxide from your organs and tissues back to your lungs.

High-performance liquid chromatography (HPLC), formerly referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

Fetal hemoglobin (HbF) is the form of hemoglobin that is dominant during fetal life and is also markedly increased in concentration for several weeks after birth. HbF is a form of Hg that has a stronger oxygen affinity as compared to adult Hg. This greater affinity towards oxygen increases its transport to the fetus within the uterus by capturing oxygen from the placental vasculature, which has much lower oxygen tension than in the lungs. The decline in its production by about 6 months after birth reduces oxygen exchange between the blood vessels and other parts of the body, including the brain.

Electrophoresis is a technique that separates molecules in their liquid state, based on their ability to move in an electric field.

PCR is a very sensitive technique that allows rapid amplification of a specific segment of DNA. It makes billions of copies of a specific DNA fragment or gene, which allows

detection and identification of gene sequences using visual techniques based on size and charge.

Complete blood count (CBC) is a blood test used to evaluate your overall health and detect a wide range of disorders, including anemia, infection and leukemia by investigating the white blood cells, red blood cells plasma cells and platelets.

Restriction fragment length polymorphism is a technique that exploits variations in homologous DNA sequences, known as polymorphisms, in order to distinguish individuals, populations, or species or to pinpoint the locations of genes within a sequence.

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Chapter Two

Literature Review

2.1 Sickle Cell Disease

Sickle cell disease (SCD) is a life-threatening genetic disorder due to the formation of sickle haemoglobin molecule (HbS) with low affinity to oxygen. In the presence of hypoxia, HbS polymerizes and ultimately results in sickled red blood cells that rupture leading to haemolytic anaemia[82]. Sickle cell disease, one of the most common inherited diseases worldwide, is now understood to be a disorder of global importance and economic as well as clinical significance. Those affected by the disease live in areas of sub-Saharan Africa, the Middle East, India, the Caribbean, South and Central America, some countries along the Mediterranean Sea, as well as in the United States and Europe[82]. Sickle Cell Disease (SCD) is one of the most prevalent genetic diseases globally, affects mainly Africans or their descendants, and is responsible for more than 300,000 live births per year. It is characterized by a pathological haemoglobin S that differs from the normal one by the presence of the amino acid valine at position 6 of the β-globin subunit instead of glutamic acid (Glu6Val mutation). Sickle cell disease is associated with severe complications that adversely impact the quality of life and survival[82]. This complication includes stunting and delayed puberty, particularly in homozygous patients, mainly because of increased basal metabolism related to hemolysis and chronic inflammation, endocrine disorders related to free iron toxicity on endocrine organs, multiple morbid episodes, micronutrient deficiency, and probably low socioeconomic level[82]. Herrick's first published account of SCA was in 1910 when he described the clinical and haematologic manifestations of the disease in a 20-year-old dental student from Grenada. Later, it was determined that a point mutation in the gene coding the β

chain of the haemoglobin molecule resulted in a single amino acid substitution (valine for glutamic acid) in the β globin chain, whi+ch, when present on both chromosomes in a patient, resulted in sickle cell haemoglobin or HbS[82].

2.1.1 Classification of Sickle Cell Anaemia

Sickle cell anaemia is caused by homozygosity of the beta-S (βS) allele (located on chromosome 11p15.5), which differs from the wild-type β-allele by a single nucleotide polymorphism dbSNP Rs334(T;T) in which GTG is substituted for GAG in the sixth codon of the β-globin gene[82]. This leads to the replacement of a hydrophilic glutamic acid residue (Glu) with a hydrophobic valine residue (Val) at the sixth position in the β-globin chain, resulting in a mutated haemoglobin tetramer HbS (α2βs2) in the erythrocytes of individuals with sickle cell anaemia[7]. Homozygous inheritance of the βS mutation (HbSS) or coinheritance of βS with other mutations such as βC (HbSC), βD (HbSD), βO (HbSO/Arab), βE (HbSE), or a β-thalassemia allele (HbS/β-thal0 or HbS/β-thal+) leads to other forms of SCD via multiple interlinked molecular and cellular mechanisms[82].

Sickle cell anemia is inherited in an autosomal recessive pattern, whi/ch means that both copies of the gene in each cell have mutations. The parents of an individual with an autosomal recessive condition carry one copy of the mutated gene, but they typically do not show signs and symptoms. In regards to sickle cell anemia, a person who carries one copy of the mutated gene is said to be a carrier for the condition, or to have sickle cell trait. When two people who are carriers of an autosomal recessive condition have a child, there is a 25% (1 in 4) chance that the child will have the condition, a 50% (1 in 2) chance that the child will be a carrier like each of the parents, and a 25% (1 in 4) chance that the child will not have the condition and not be a carrier.

2.1.2 Pathophysiology of Sickle Cell Anaemia

When exposed to deoxygenated environment, red blood cells (RBCs) that contain HbS or HbS in combination with other abnormal β alleles, undergo polymerization and become rigid. The rigid RBCs are liable to haemolysis, and due to increased density may affect blood flow and endothelial vessel wall integrity. The dense rigid RBC's lead to vaso-occlusion, tissue ischaemia, infarction as well as haemolysis[82]. The consequence of haemolysis is a complex cascade of events including nitric oxide consumption as seen in figure 2.1 below. Haemolysis linked nitric oxide dysregulation and endothelial dysfunction which result into complications such as leg ulceration, stroke, pulmonary hypertension and priapism[82].

Unlike normal RBC's with half-life of approximately 120 days, sickle RBC's (sRBC) may survive just 10–20days due to increased haemolysis[82]. During deoxygenation; healthy haemoglobin rearranges itself into a different conformation, enabling binding with carbon dioxide molecules which reverts to normal when released[8]. In contrast, HbS tends to polymerise into rigid insoluble strands and tactoids, which are gel-like substances containing Hb crystals. During acute sickling, intravascular haemolysis results in free haemoglobin in the serum, while RBC's gaining Na+, Ca2+ with corresponding loss of K+[82]. The increase in the concentration of Ca2+ leads to dysfunction in the calcium pump. The calcium depends on ATPase. Furthermore, hypoxia also inhibits the production of nitric oxide, thereby causing the adhesion of sickle cells to the vascular endothelium[9]. The lysis of erythrocytes leads to increase in extracellular haemoglobin, thus increasing affinity and binding to available nitric oxide or precursors of nitric oxide; thereby reducing its levels and further contributing to vasoconstriction[8]. These changes result in endothelial damage. The sickled red cells also

become dehydrated as a result of abnormalities in the Gardos channel. Haemolysis contributes to oxidative stress and dysregulation of arginine metabolism, both of which lead to a decrease in nitric oxide (NO) that, in turn, contributes to the vasculopathy that characterizes SCD[8].

2.1.3 Etiology of SCD

Haemoglobin (Hb) is a major protein within the red blood cell (RBC). It is made up of four globin chains, two of which are derived from alpha-globin (locus on chromosome 16) and two from beta-globin (locus on chromosome 11). There are many subtypes of Hb. The most common ones that are found in adults without haemoglobinopathies are listed here:

HbA1- comprises 2 chains of the alpha-globin and two chains of the beta-globin (a2b2) - This constitutes 95% of the adult hemoglobin.

HbA2- comprises 2 chains of the alpha-globin and two chains of the delta-globin (a2d2) - This constitutes less than 4% of the adult hemoglobin.

HbF- comprises 2 chains of the alpha-globin and two chains of the gamma-globin (a2g2) - This Hb is more prevalent in the fetus (due to high oxygen binding affinity that helps in extracting oxygen from maternal circulation).

The sickle cell mutation occurs when negatively charged glutamine is replaced by a neutral valine at the sixth position of the beta-globin chain. The mutation is transmitted via Mendelian genetics and is inherited in an autosomal codominant fashion. A homozygous mutation leads to the severest form of SCD, i.e., SCA- also called HBSS disease. The coinheritance of beta-naught thalassemia and sickle cell mutation leads to HBS-Beta-0 disease, which phenotypically behaves like HBSS disease.

A heterozygous inheritance leads to HbAS. Patients with HbAS are not considered within the spectrum of SCD as most of them never present with typical symptoms of SCA but have heterozygote advantage of resistance against malaria[12].

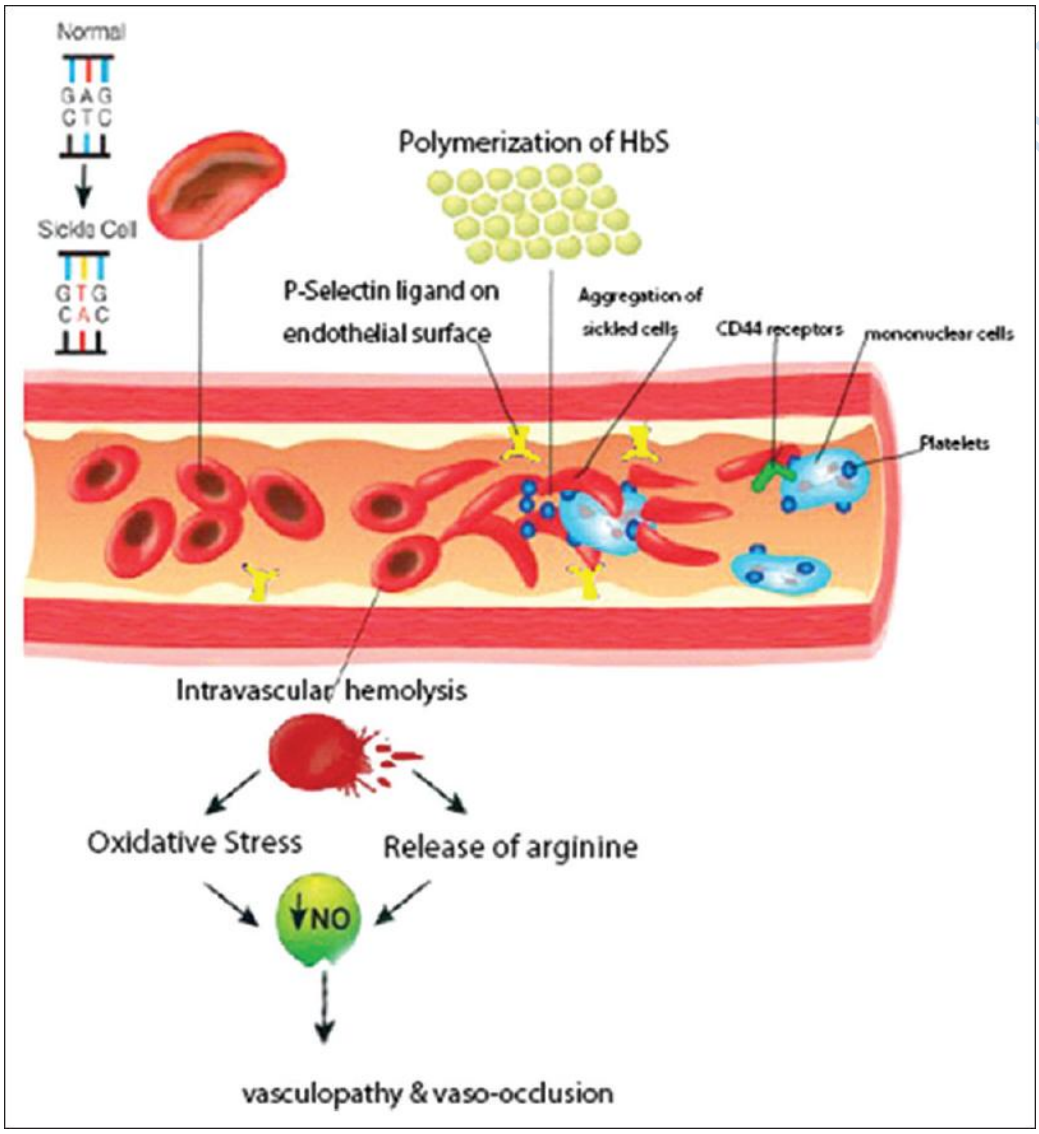

Figure 2.1: Schematic representation of the pathophysiology of sickle cell anemia[8].

They might only be detected during screening procedures conducted during childbirth, blood donation, etc. Several other compound heterozygotes exist where a single copy of the mutated beta-globin gene is co-inherited with a single copy of another mutated gene resulting in haemoglobin S, C, D or E depending on amino acid substitution. Haeamoglobin S and C are present in Nigeria while haemoglobin G (HbG), haemoglobin D (HbD) and haemoglobin E (HbE) rarely exist in West Africa[82].

The second most common variant of SCD is the HbSC disease, where the sickle cell gene is coinherited with a single copy of the mutated hemoglobin C gene. HbC is formed when glutamine is replaced by lysine at the sixth position on the beta-globin chain. HbSC disease accounts for 30% of patients in the United States[82].

2.1.4 Epidemiology of SCD

SCD is one of the most common inherited life-threatening disorders in human, it predominantly affects people of African, Indiana and Arab ancestry. It is estimated that over 80% of over 300,000 annual births occur in sub-Saharan Africa (SSA)[82]. The gene frequency is highest in West African countries with 1 in 4 to 3 (25–30%) being carriers of HbS compared to 1/400 African Americans and is variable in European population[82]. The prevalence of SCD in developed countries is increasing partly due to migration from high prevalent countries[82]. It is estimated that over 14,000 people live with SCD in the UK, similar to France, while countries like Italy, Germany have seen increasing numbers from Africa[82]. With increasing survival, the age distribution of SCD is changing from a childhood

disorder pattern that patients now survive into adulthood and old age. It is now reported that over 94% of those born with SCD now survive into adulthood in the US, France and UK in contrast to the high mortality in SSA where 50–90% may die in the first five years of life[82]. In low resource settings and countries where newborn screening is not yet standard care, patients may die young even before diagnosis is confirmed[16]. Among the common causes of death in the absence of early diagnosis followed by education and preventive therapies such as penicillin prophylaxis and regular surveillance include infections, severe anaemia (acute splenic sequestration, aplastic anaemia) and multi-organ failure[17]. It is essential therefore that Newborn and Early Infant diagnosis is given the priority it deserves by those countries where SCD is a public health problem[8,82]. The implementation of early infant diagnosis remains out of reach for the majority of countries in SSA despite multiple declarations by international organizations and public statements by politicians to honour such commitments[17]. The benefits of screening can only become meaningful when such practice is embraced by policy-makers across the continent and India where the majority of SCD are born and live.

2.1.5 Clinical Manifestations of SCD

Sickle cell disease is characterised by exceedingly variable manifestations ranging from anaemia and acute generalised pain to early onset stroke, leg ulcers and the risk of premature deaths from multi-organ failure[82]. As a result of the effect of HbF, clinical features do not begin until the middle to second part of the first year of post-natal life when this has predominantly switched to adult haemoglobin[82].

(i) Vasoocclusive Crisis and Pains

Patients with SCD may experience intense pain early in infancy, childhood and adulthood. Pain usually accounts for the majority of hospitalisations and overall negative impact in

patients' health related quality of life[82]. Pain is the cardinal feature of SCD and it is characteristically unpredictable, episodic in nature, described as one of the most excruciating forms of pain that affects human beings[21,82]. Pain occurs due to stimulation of nociceptive nerve fibres caused by microvascular occlusion. The microcirculation is obstructed by sRBCs, thereby restricting the flow of blood to the organ and this results in (i) ischaemia, (ii) oedema, (iii) pain, (iv) necrosis, and (v) organ damage[82]. In the first year of life, one of the cardinal features is the 'hand-foot syndrome' due to vaso-occlusion of post-capillary vasculature resulting in tissue oedema and pain of the extremities[24]. Infants display their pain non-verbally with irritability and apparent 'regression' tendencies such as inability to weight bear, walk or crawl. In older children and adults, vaso-occlusive pain can affect any part of the body. The onset of pain is spontaneous, usually no precipitating factors; well-known triggers include infections, fever, dehydration, acidosis, sudden change in weather including wind speed, cold, rain and air pollution. Resolution of pain is unpredictable[82]. Acute pain might lead to chronic pain.

(ii) Bone Complications

Chronic tower skull, bossing of the forehead and fish mouth deformity of vertebrae are the result of extended haematopoietic marrow causing widening of the medullary space, thinning of the trabeculae and cortices, and osteoporosis[18]. The excruciating pain of bone infarction in the "hand- foot syndrome" that occurs around the age of 12 years is often the first symptom of sickle cell disease. This dactylitis resolves spontaneously and is treated with hydration and analgesic. Necrosis occurs with equal frequency in the femoral and humeral heads, but the femoral heads more commonly undergo progressive joint destruction, as a result of chronic weight bearing. Arthritic pain swelling, and effusion may be related to periarticular infarction

or to gouty arthritis. Bone marrow infarction causes reticulocytopenia, exacerbation of anaemia, a leukoerythroblastic picture, and sometimes pancytopenia[82].

(iii) Anaemia

Sickle erythrocytes are destroyed randomly with a mean life span of 17 days. Symptomatic anaemia is the commonest symptom in SCD generally more common in SCA (Homozygous S), which usually runs the lowest haemoglobin level common to double heterozygous states[18]. The steady state haemoglobin for asymptomatic patients varies according to the phenotype, ranging from levels as low as 60–80 g/L for homozygous S and /Sβo to 100–110 g/L in double heterozygous SC and Sβ+ forms. However, the rate of fall from individual steady state haemoglobin level may trigger symptoms of hypoxia which may include hyperhaemolysis, aplastic crisis and acute sequestration crisis [26].

(iv) Infection

SCD increases susceptibility to infections, notably bacterial sepsis and malaria in children under five years[82]. Respiratory infections can trigger the sickle-cell acute chest syndrome, with a high risk of death. Hyperhaemolysis is precipitated by infections, glucose-6-phosphate dehydrogenase (G6PD) deficiency, acidosis and dehydration. There is increased pallor, jaundice and hepatosplenoemgally. Risk factors for infections include: (i) functional asplenia/hyposplenia which present with reduced splenic immune response at a very young age, (ii) impaired fixation of complement, (iii) reduced oxidative burst capacity of chronically activated neutrophils, dysfunctional IgM and IgG antibody responses and defective opsonisation. The main pathogen of concern is Streptococcus pneumoniae, though severe and systemic infections arise with Haemophilus influenzae, Neisseria meningitides,

and Salmonellae leads to osteomyelitis especially Salmonella due to bowel ischaemia and gut flora dissemination[82].

(v) Acute Aplastic Crisis

Aplastic crisis is characterized by an acute failure of erythropoiesis often following viral infections especially parvovirus B19. The patient will present with weakness, progressive pallor and pancytopenia[82]. During this crisis the patient may have associated bone and joint pain. Acute sequestration is caused by pooling of blood in the spleen and the liver characterized by sudden onset of progressisve anemia, splenic enlargement, abdominal pain, and shock. Various crises and increased susceptibility to infections are responsible for recurrent illness in patients with sickle cell disease[29].

2.1.6 Techniques and Assays to Diagnose and Monitor SCD

Several techniques and assays are used for the detection and monitoring of the sickle cell disease. These techniques can be divided into two main categories: (i) currently used methods in the diagnosis of SCD; and (ii) 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[82].

(i) Current Techniques to Diagnose and Monitor SCD

(a) The complete blood count (CBC): This is a primary test to characterize the different types of anaemia. However, the hemoglobin mutation will affect the hematological parameters, showing a variable change[30]. Patients with homozygous SS and heterozygous S/β◦ mutations usually present with hemolytic anemia where the red blood cells (RBCs), hemoglobin and hematocrit are low. In contrast, the counts of white blood cells (WBC) and platelets are

elevated, and they can fluctuate. However, reticulocyte counts are variable and depend on different factors such as the degree of anemia caused by the cell hemolysis, sequestration, and bone marrow response to anemia[82]. 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 hematological parameter as valuable information, it is insufficient to give a complete picture of patients' diagnoses[31].

(b) Peripheral Blood Smear (PBF): this 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 anemia. In sickle cell anemia, 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[82]. The preparation of these blood smear slides is relatively simple, rapid, and inexpensive. 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 anemia[82].

(c) 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 hemolyzing 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[34]. This test is easy to perform and inexpensive. It suffers from a false-negative result when utilized for newborns, due to the presence of a high amount of hemoglobin F and when the HbS is less than 10% of the total hemoglobin[82]. Furthermore, false-negative results are observed in patients with coinheritance of α-thalassemia trait and severe anemia. In contrast, false positives are observed in patients with high serum viscosity, erythrocytosis, highly marked leukocytosis and in some cases of anemia. Moreover, the sickle solubility tests cannot differentiate between sickle cells trait (SCT) and SCD, and they are insensitive to the detection of hemoglobin AS (HbAS). These disadvantages make them difficult to use in screening programs[33].

(d) Haemoglobin Electrophoresis: Electrophoresis is a type of chromatography techniques, and it is considered as one of the important tests used to detect Hb variants. In this test, an electrical field is applied to facilitate the migration of electrically charged molecules. The first described hemoglobin variant Hb-S by using electrophoresis was in 1949. To identify hemoglobin variants, different pH and mediums are used, either cellulose acetate electrophoresis at alkaline pH or citrate agar at acidic pH[82].

Alkaline electrophoresis is a diagnostic tool that has been used to detect thalassemia and sickle cell anemia 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[82]. As a result, the different hemoglobin 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, it is a little difficult for electrophoresis to distinguish between hemoglobin variants with the same electrical charges and gives the same migration patterns, such as HbD and

HbG, which comigrate with HbS; HbE and Hb0-Arab have similar migration to the HbC molecules[82]. Furthermore, alkaline electrophoresis can be affected by the presence of large amounts of hemoglobin F in newborns, 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.

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[82]. Most hemoglobin variants that comigrate at alkaline pH can be separated effectively using citrate agar electrophoresis[35]. Citrate agar electrophoresis is not affected by the high amount of hemoglobin F in newborns; 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[35].

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. 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[82]. 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[39]. 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 newborn and adult patients[82].

(e) Isoelectric Focusing (IEF): it 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[82]. 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[82].

(f) High Performance Liquid Chromatography (HPLC): it is documented to separate the hemoglobin fractions as they have different interactions with the stationary phase. HPLC detects different types of hemoglobin based on the retention time and shape of the peak. Each hemoglobin has a specific retention time and can be compared with the retention time of the known hemoglobin fractions[82]. HPLC is used to detect and quantify HbF, Hb A2, HbS, HbC, Hb Barts, and other Hb variants[39]. 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. 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[82].

(g) 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. The following are examples of genetic tests

(i) Polymerase Chain Reaction (PCR)-Based Techniques: PCR 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. 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[82]. Another simple, low-cost PCR-based technique has been developed using bi-directional allele-specific amplification (ASA) and a hot star system to provide more specific single-tube genotyping, where the point mutation of sickle cell anemia 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[82]. 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[82]. ARMS has been mostly used in prenatal diagnosis by detection of sickle cell mutation in the fetal sample. The ARMS's sensitivity has been measured by comparing the result to identify the presence of hemoglobin variants by HPLC[47]. An allele-specific oligonucleotide (ASO) hybridization was demonstrated to detect sickle cell mutation using two PCR primers. 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 variation[82].

(ii) Restriction Fragment Length Polymorphism (RFLP): it 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, as shown in Figure 2.2. 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, as shown in Figure 2.2(a). 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, as shown in Figure 2.2(b). In sickle cell anaemia homozygous (βSβS), there is no enzyme cutting due to the mutation in both genes, so a single wide band appears, as shown in Figure 2.2(c)[82]. 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 anemia mutation[82].

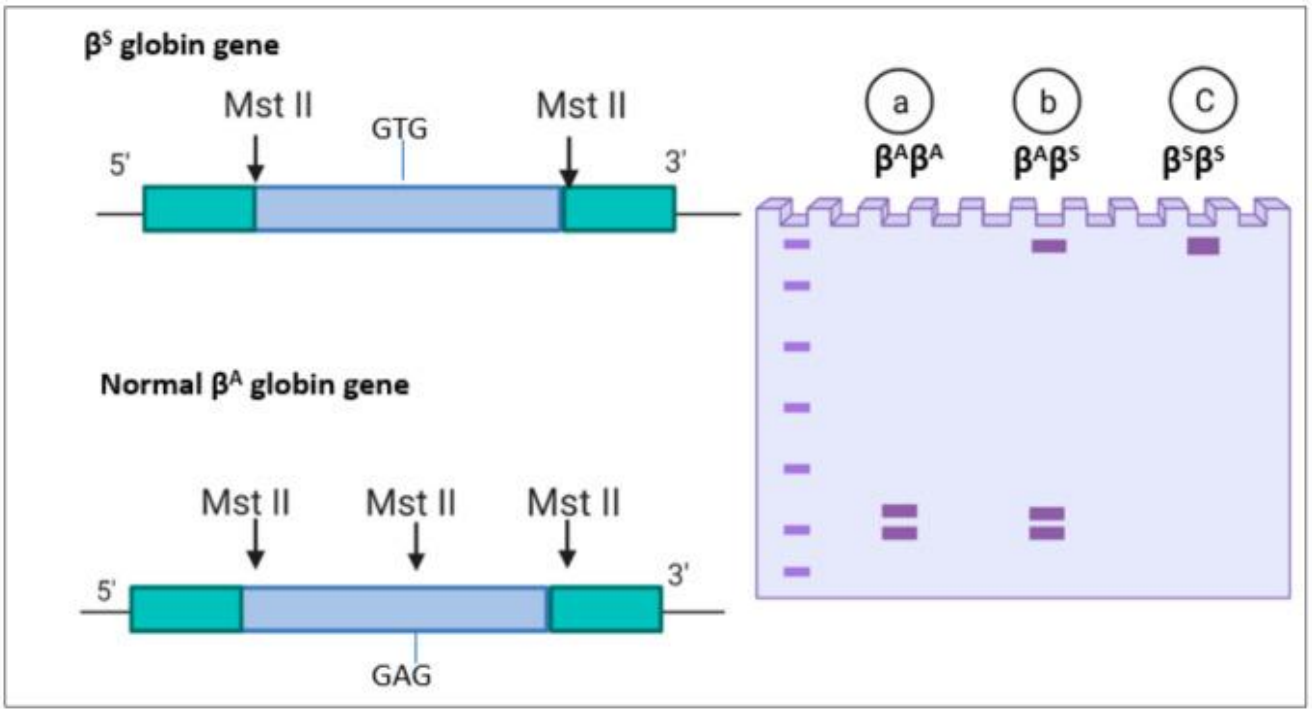

Figure 2.2: Restriction fragment length polymorphism (RFLP) for Sickle cell anemia: (a) normal gene(βAβA); (b) sickle cell trait (βAβS); and (c) sickle cell anemia (βSβS)[50].

(iii) 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. 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[82]. A novel database that combines the gene expression with genome-wide association study (GWAS), using homozygous SS microarray datasets to determine SCD transcriptomic profile was developed[82].

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 whole-genome 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[82]. 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[82]. 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. Another study pointed out that mutation in SALL2 is associated with a high level of HbF in response to hydroxyurea[82]. Variants in MBL2 and KLRC3 were observed more frequently among adult SCD with hyper hemolysis syndrome than controls[82]. Whole-genome sequencing helps analyze 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[82]. Furthermore, it confirmed the association of the SCD phenotypes with common genetic modifiers, including fetal hemoglobin BCL11A, HBB, UGT1A1, and APOL1. This technique will help in precision medicine to make better treatment decisions and discover new treatments[82].

(ii) Innovative Techniques for the Diagnosis and Monitoring of SCD

(a) 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.[82]. The cells can also be classified based on the image features by using segmentation and artificial neural network[59]. An automated method to detect sickle cell anemia (SCA) using an image processing technique was developed. 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, and at the end 95% accuracy and 96.55% sensitivity were reported[82]. Deep learning models have been employed to detect SCA and classify the red blood cells based on microscopic images. The models were able to

extract and implement the classification functions automatically in one shot. Moreover, three deep learning models were deployed to determine and categorize the red blood cells based on their shapes: round shape indicating normal cells, elongated shape indicating sickle cells, and other blood shapes. There were 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 the same model plus a multi-class support vector machine was used[82].

A smartphone microchip, a microscope, and machine learning algorithms were combined to develop an affordable, portable, and rapid screening test for sickle cell anemia. 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 diagnosis of 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[82]. A smartphone-based image acquisition process has been developed for imaging the RBCs from 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[82].

The image processing methods provide automated interpreting of the blood cell images, minimizing errors, which can effectively monitor the SCD patient's status[82]. 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 newborn screening; 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[82].

(b) Emerging Flow Cytometry: Conventional flow cytometry techniques have been used to detect sickle cells based on fluorescent markers or cellular morphology[82]. Advanced flow cytometry based on imaging techniques has been demonstrated to enhance the sensitivity by combining cell population analysis and morphological data. An imaging flow cytometry assay (SIFCA) and software algorithm was developed 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 h, and then analyze it using imaging flow cytometry. Finally, the cells are classified based on the morphology into sickled and normal cells by using algorithm software. One hundred images of normal cells and one hundred images of sickle cell were analyzed, and 100% sensitivity and 99.1% specificity were reported. It was proved that SIFCA can assess sickling tendency in SCA patients to identify the severity of the disease and drug monitoring[82]. In-vitro photoacoustic flow cytometry (PAFC) for morphological detection of sickle cells containing hemoglobin S was developed. Photothermic and photoacoustic spectra were employed for determination of haemoglobin heterogeneity and accumulation of the HbS in sickle RBCs. The sickled RBCs showed 2–4-fold lower linear mode than normal RBCs. This method is useful in monitoring the sickling states[82]. Microfluidic flow cytometry based on the electrical

impedance spectroscopy was also developed. 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[82].

(c) 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[82]. 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 was developed. 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 hemoglobin diseases[82]. An amplitude-modulated electrode formation in microfluidic for identifying the mechanical fatigue in single cells was 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[82].

A microfluidic-based method to detect and quantify sickle cells by modulating the disease's pathophysiology was developed. 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. 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 12secs. This method has been used to monitor sickling events and hydroxyurea therapy[82].

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, 3Dprinted 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[82]. 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.

(d) Lateral Flow Immunoassay: Lateral flow assays (LFAs) are widely used as portable platforms in biomedical detection. A sensing platform called Sickle SCAN was demonstrated as it is used to detect normal haemoglobin HbAA, sickle cell trait HbAS, haemoglobin C trait HbAC, sickle-hemoglobin C disease HbSC, and hemoglobin C disease HbCC, as shown in Figure 2.3. The test used polyclonal antibodies on lateral flow chromatographic immunoassay against hemoglobin S, hemoglobin C, and hemoglobin 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 hemoglobin; and then both migrate to the test strip. The hemoglobin binds with the corresponding antibody and produces blue lines, as shown in Figure 2.3. The SickleSCAN cartridge contains four detection bands: the control band, normal HbA, HbS band, and HbC band[82]. The validation of the Sickle SCAN assay to detect different hemoglobin was tested. 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)[82]. 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[76].

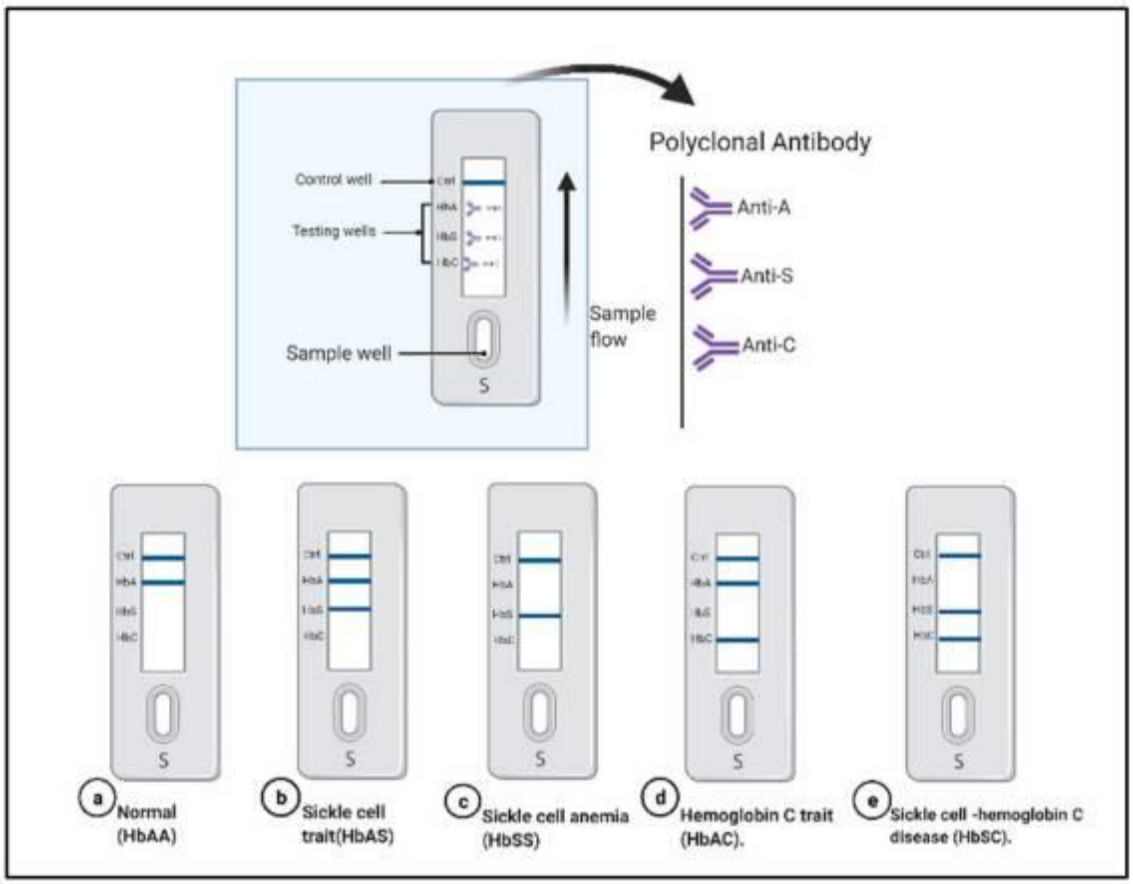

Figure 2.3 The Sickle SCAN based on lateral flow immunoassay to detect sickle-cell disease (SCD): normal haaemoglobin HbAA (a); sickle cell trait HbAS (b); sickle cell anaemia HbSS (c); haemoglobin Ctrait (d); and sickle cell-haemoglobin C disease HbSC (e)[75].

(e) Density-Based Separation: Density-based separation detects sickled RBCs using aqueous multi-phase systems based on cell density measurements (AMPS). The dense sickle cells can be distinguished from normal cells by the two-phase AMPS system with 90% sensitivity and 97% specificity. In contrast, the three-phase systems reported 91% sensitivity and 88% specificity[82]. The test only requires around 5 μL of the blood sample, which is mixed in capillary tubes with aqueous polymeric solutions. Upon 10 min centrifugation, the dense RBC precipitates and forms a layer in the bottom of the tube, which indicates the SCD[77]. The three-phase systems in were integrated with an optical reader to enable the distinction between HbSS and HbSC. The test is rapid and straightforward; however, this technique cannot distinguish between HbAA and HbAS. In addition, the accuracy of the test was compromised in cases with elevated HbF levels, such as in newborns and the Arab-Indian haplotype, due to the absence of dense RBCs. Furthermore, many health conditions, treatments, and medications can affect the RBC density and the test's validity[82]. The AMPS test was validated after the testing of 505 samples where a sensitivity of 86% and 60% specificity for SCD-AMPS-2 was reported. However, the sensitivity was dropped in children from 6 months to 1 year of age to 84%. Furthermore, the study documented 75% sensitivity and 60% specificity for SCD-AMPS-3, while the diagnostic accuracy was 69% for SCD-AMPS-3 and 77% for SCD-AMPS-2[47].

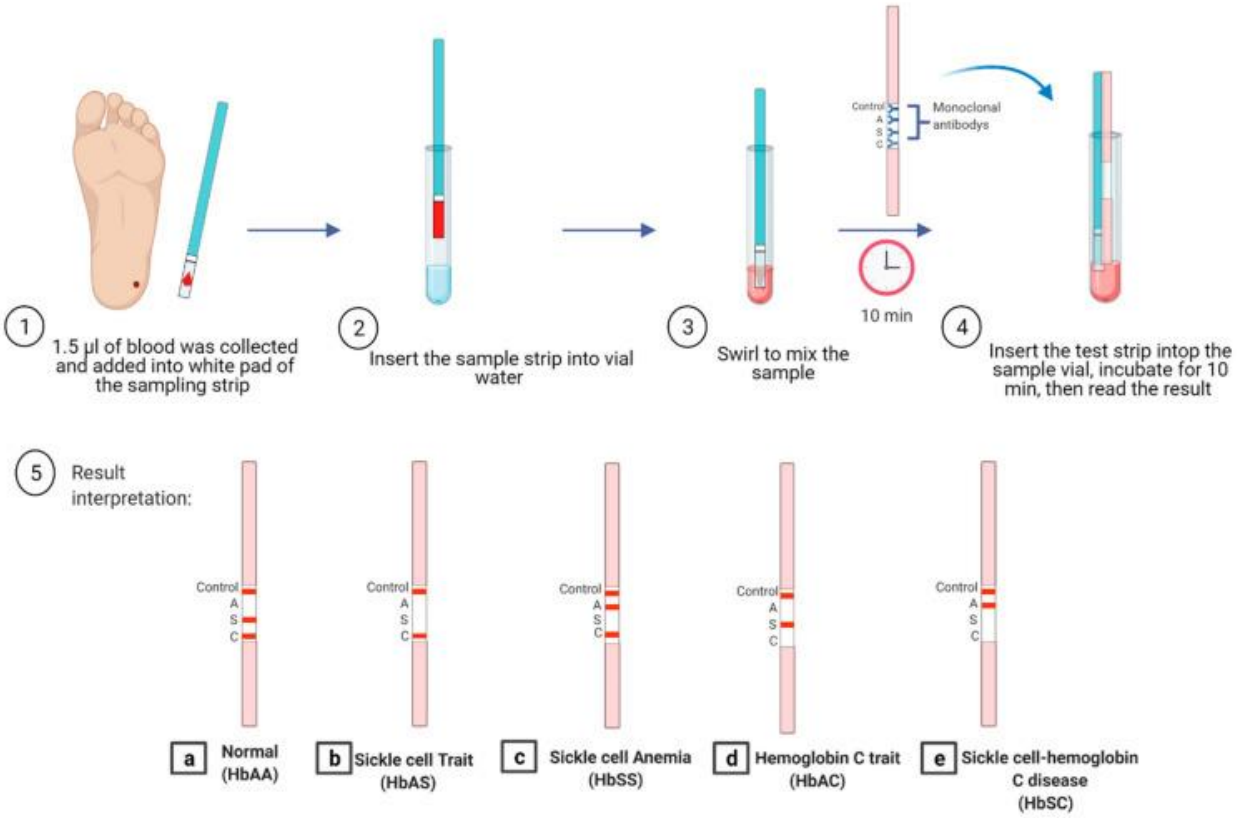

Figure 2.4: HemoTypeSC based on lateral flow immunoassay to detect SCD: normal hemoglobin HbAA (a); sickle cell trait HbAS (b); sickle cell anemia HbSS (c); hemoglobin C trait HbAC (d); and hemoglobin SC disease HbSC (e)[76].

(f) Paper-Based Haemoglobin Solubility Test: It is a technique that depends on the filtration properties of the paper substrate and on the insolubility of the HbS, where it can be visually interpreted to detect the presence of haemoglobin S. In this test, one drop of the blood sample is mixed with a hemoglobin solubility agent with a ratio of 1:10. Then, the mixture is placed onto chromatography paper followed by staining. A different stained blood pattern forms based on the hemoglobin, as shown in figure 2.5 below. These stains are used to determine HbSS (Figure 2.5a), the carrier HbAS (Figure 2.5b), and normal hemoglobin HbAA (Figure 2.5c). The test shows 94.2% sensitivity and 97.7% specificity for the visual detection of the HbS. Furthermore, the test can be performed within 20 min[82]. Moreover, the test's sensitivity is increased when combined with an image analysis algorithm, which quantifies the Hb in the sample based on the intensity of the color in the center spot. This test has several advantages: simple fabrication, easy to use as it needs single step, affordable, and low-cost (costs less than a dollar per test)[82]. However, this test is affected by the blood clotting, preventing the blood from wicking through the paper substrate. Furthermore, the test is unable to differentiate between HbSC and HbAS. Finally, this test is not reliable for newborns due to a high level of HbF, which prevents the polymerization and precipitation of hemoglobin S[80]. Validation of the paper-based screening test was performed in which 226 samples were tested and compared the results with IEF electrophoresis to distinguish HbAS and HbSS from HbAA patients. The study reported 94.2% sensitivity, 97.7% specificity, and 96.9% accuracy[80]. The automated image analysis C-index was employed to distinguish between HbSC and HbAS. They demonstrated 100% sensitivity and 59% specificity. The high

sensitivity but relatively low specificity indicated that the C-index could be used to exclude patients without the disease. Furthermore, this test cannot detect sickle cell disease in neonates due to the high level of HbF[80].

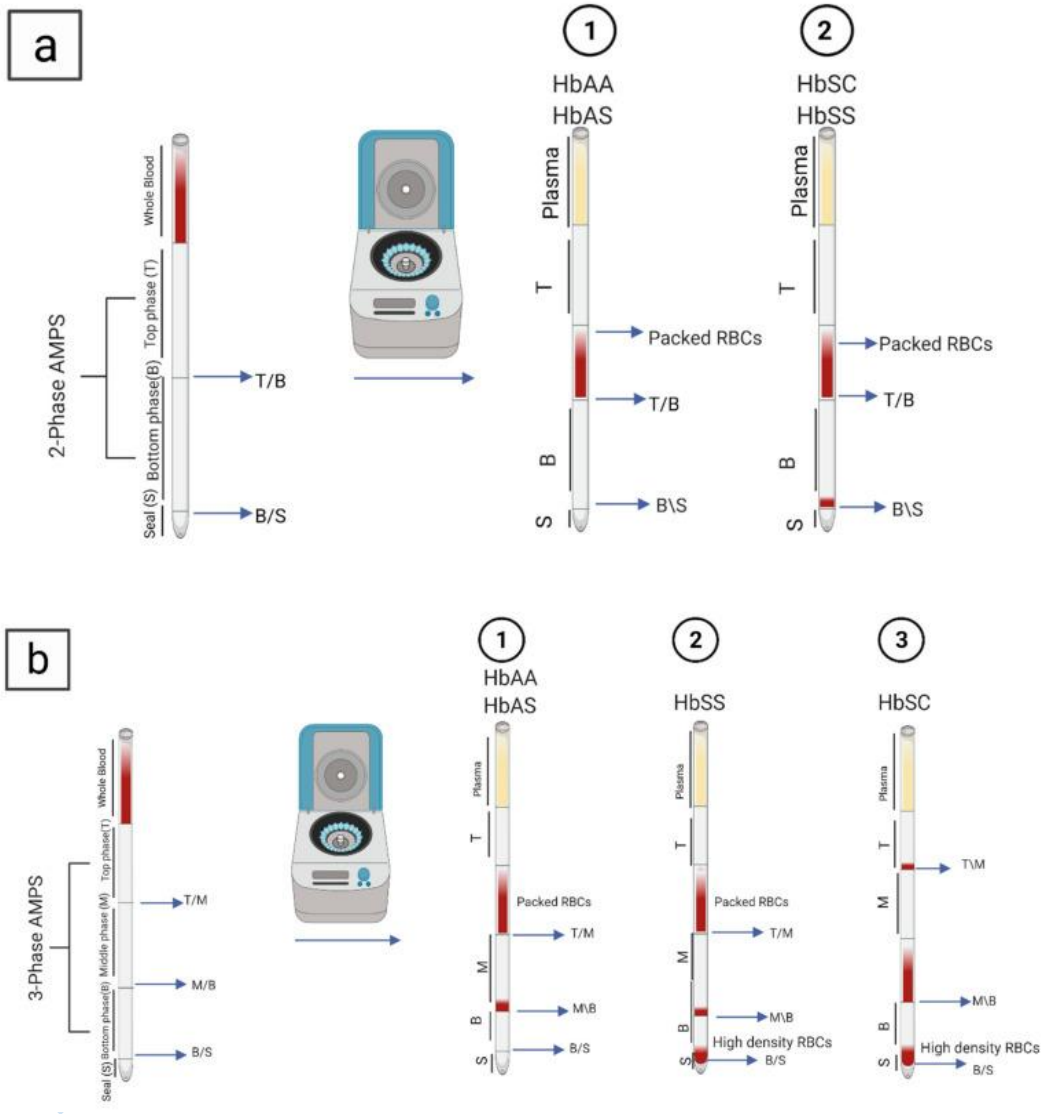

Figure 2.5: Multi-phase systems by cell density measurements (AMPS) to detects sickled RBCs: (a) two-phase AMPS HbAA, with HbAS (1) and HbSS and HbSC (2); and (b) three-phase AMPS, with HbAA and HbAS (1), HbSS (2), and HbSC (3)[77].

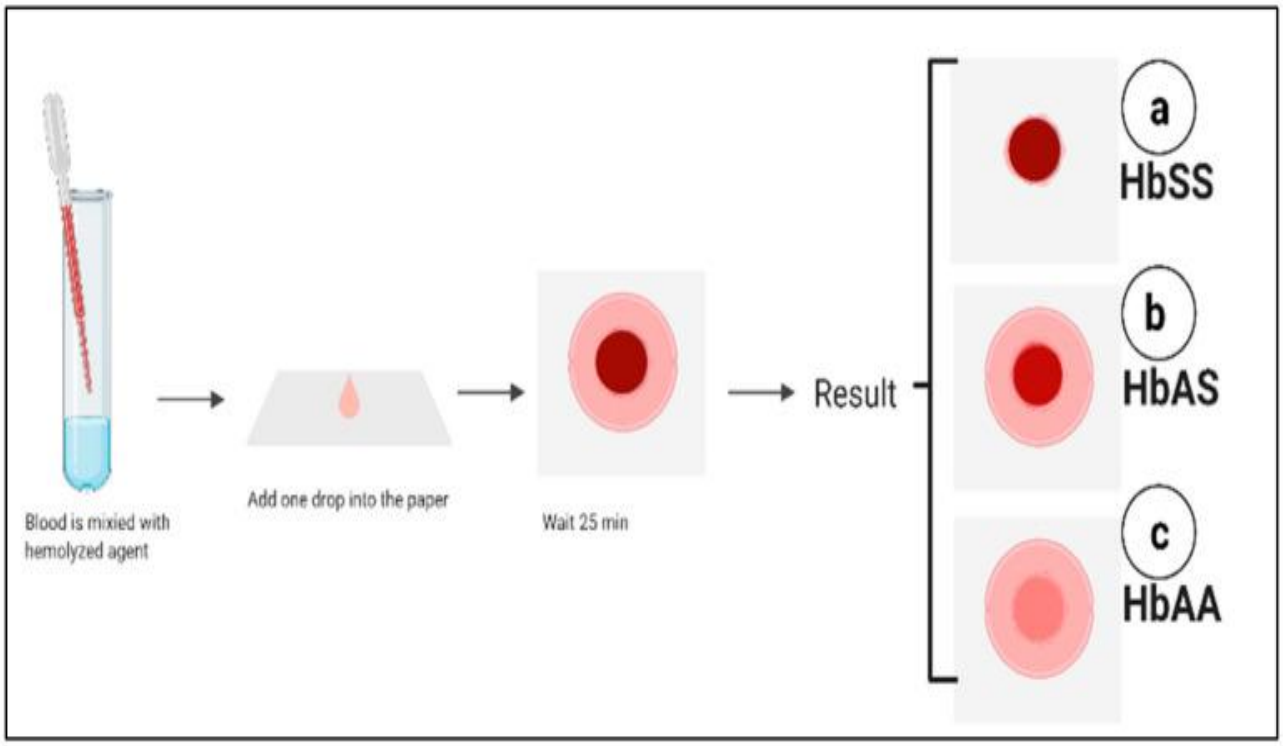

Figure 2.5: Paper-based hemoglobin solubility test[79].

A microchip electrophoresis paper-based has been developed to identify and quantify hemoglobin types, including HbA, A2, F, S, and C[82]. This microchip is named HemeChip platform, and it consists of a cartridge where the hemoglobin variants are isolated, pictured, and tracked during electrophoresis. HemeChip is based on separating the hemoglobin using cellulose acetate electrophoresis[81]. The HemeChip test is performed by mixing the blood sample with deionized water, and then the microchip is placed inside the HemeChip reader and an electrical field is applied, which results in separating the Hb. As a result, each Hb moves a unique distance across the paper strip. Real-time images are taken during the test and analyzed automatically using custom built-in software[81]. This test has a number of advantages such as low-cost, disposable, portable, high accuracy, easy to perform, and can be completed in less than 10 min.

(g) Sensors Based Techniques

(i) Fluorescence Based Optofluidic Resonator: An optofluidic resonator based on the waveguide has been used to identify the interaction of Fe2+ and Fe3+ within protoporphyrin IX in phosphate buffer saline. The metal clad waveguide optofluidic resonator is used for the real-time detection using a small sample volume. The Fe2+ molecule shows a fluorescent peak which corresponds to the normal hemoglobin, while the Fe3+ fluorescent peaks are correlated with the S hemoglobin of sickle-cell disease patients[82].

(ii) Sensors Based on Electrical Impedance Signal: The electrical impedance signal is a sensitive indicator of the disease severity and red blood cell sickling events. Combining the microfluidic chip with the electrical impedance provides a promising method for identifying sickling and unsickling processes in SCD patients[82]. A microfluidics-based electrochemical impedance sensor to determine the SCD red blood cells' electrical properties under oxygen

control conditions. The electrical impedance of sickle cells shows a significant difference between normoxic and hypoxic conditions[82]. These methods have been developed to monitor the cell sickling, and they do not accurately describe the severity of heterozygosis SCD.

(iii) Quartz Crystal Microbalance (QCM): The quartz crystal microbalance (QCM) sensor mainly depends on the change in the frequency of a quartz crystal resonator due to the mass variation at the crystal surface[82]. Quartz crystal microbalance has been used to determine the morphological change of RBCs. Combining quartz crystal microbalance and a novel mathematical model provides complete information about the changes in the RBC's elasticity. This sensor can differentiate between normal biconcave discoid RBCs and sickled cells and can be used as monitoring test[82].

(iv) Genosensors: An electrochemical nanosensor to detect SCA trait based on the immobilization of mutated single-strand DNA on a gold electrode has been demonstrated. The hybridization between the probe and target DNA is measured using electrochemical impedance spectroscopy (EIS). The sensor allows the detection of the SCA trait individuals and provides genetic consulting[82]. An oligonucleotide sensor has been developed for the detection of β-globin point mutation caused by sickle cell anemia. The sensor employs luminescence resonance energy transfers between photon up converting nanoparticles (donor) and conventional fluorophore (acceptor). The sensor can determine the matched target in random oligonucleotides sequences with high sensitivity and specificity[87]. SPR biosensor has been documented to detect point mutations in the HBB gene. The technology depends on the immobilization of wild-type and βS mutated probes. The probes can differentiate between wild-type alleles and mutated ones. This technique is affordable and allows real-time detection of single-point mutations, responsible for sickle cell disease, making it suitable for

effective prenatal diagnosis. This sensor needs a PCR product, which limits the ability to use it at POC[82]. The specificity of the genosensors depends on the probe design and the immobilization methods. These sensors can be improved to detect different types of SCD accurately and not be affected by the presence of different hemoglobin fractions.

(h) The Pyrosequencing Technique: The pyrosequencing technique (PyS) has been used to identify different types of homozygous or heterozygous SCD. This test aims to sequence hemoglobin of a small number of patients accurately to differentiate between βSβS and Sβ0 thalassemia and detect HbC mutations. The difference in the mutations is only in a single nucleotide, which is hard to detect with conventional tests. PyS was compared with sanger sequencing, and it was found to be able to correctly identify βSβS with 98.7% accuracy, sickle cell hemoglobin C disease with 98.7%, and the heterozygous with 92.2%. It was also proven to be satisfactory for β+ and β0 mutations found in SCD patients. However, several samples were wrongly classified by PyS, due to the low level of the pyrogram peaks[82]. This sequencing technique has significant potential to provide an accurate diagnosis of SCD disease and differentiate between the types of the disease, and it can be used in neonatal screening.

2.2 Glucose-6-Phosphate Dehydrogenase Deficiency

Based on the findings of world health organization (WHO), 7.5% of the world populations have one or two genes for G6PD deficiency and 2.9% of the world populations are G6PD deficient[82]. Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme involved in the pentose monophosphate pathway[82]. Deficiency of this enzyme leads to free radical-mediated oxidative damage to red blood cells, and in turn causes haemolysis. G6PD deficiency is the most common enzymatic disorder of red blood cells, affecting 400 million people

worldwide[82]. The highest prevalence rates with gene frequencies in the range of 5-25% are found in tropical Africa, the Middle East, tropical and sub-tropical Asia, some parts of the Mediterranean, and in Papua New Guinea[82].

The spectacular advances in molecular biology of the last decade are helping to unravel the molecular basis of its biochemical and clinical diversity[82]. It serves as a model for the clinician of the importance of the environment in determining the clinical expression of genetic disease[82]. For the population biologist, its study has yielded important insights into the interaction of host red cells with malaria parasite and the influence of this interaction on human genetic polymorphism[82].

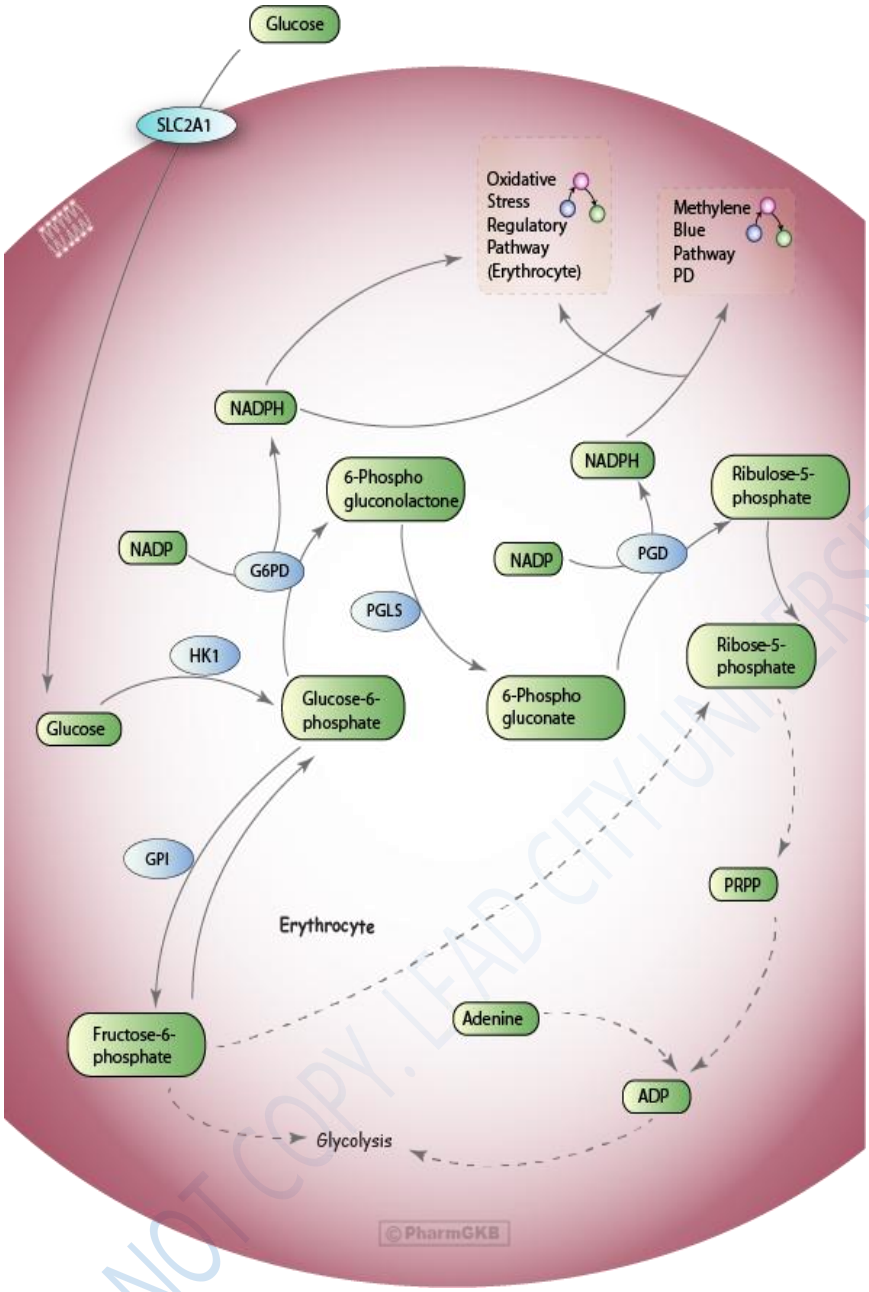

Figure 2.6: The Pentose Phosphate Pathway (PPP) and production of NADPH in red blood cells[82]

Most human pathology due to G6PD deficiency is preventable by population screening and avoidance of precipitants[82].

Population screening in regions where the prevalence of G6PD deficiency is 3–5% or greater (in males) is recommended by the World Health Organization (WHO)[82]. Due to cost and lack of infrastructure, this has yet to become regular practice in many parts of the world. The regional prevalence of G6PD deficiency in Africa ranges from 15 to 26%. G6PD deficiency contributes to hyperbilirubinemia and jaundice in newborns, putting infants at risk for acute bilirubin encephalopathy within the first few days of life that may result in subsequent death or kernicterus spectrum disorder[82]. Kernicterus spectrum disorder is manifest by hearing deficits, behavior problems, and long-lasting neurologic damage[82]. G6PD deficiency can also cause morbidity in persons in receipt of antimalarials such as dapsone by causing haemolysis and hemoglobinuria. These factors contribute to the overall public health burden in this condition[82]. G6PD prevents haemolysis and cell damage in the setting of oxidant stress by the production of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) as seen in the figure 3.4 below.

In patient with certain variants of G6PD deficiency, haemolytic anaemia can develop due to oxidant stress during the neonatal period, infection, or such exogenous agents as fava beans and certain medication[82].

2.2.1 Inheritance of Glucose-6-Phosphate Dehydrogenase Deficiency

G6PD deficiency is inherited all because it is passed down from parents through their genes to their offsprings similar to that of haemophilia and color blindness[94]. It is also a condition that is considered to be X-linked recessive since the mutated gene that causes the disorder is located on the X chromosome, one of the two sex chromosomes in each cell.

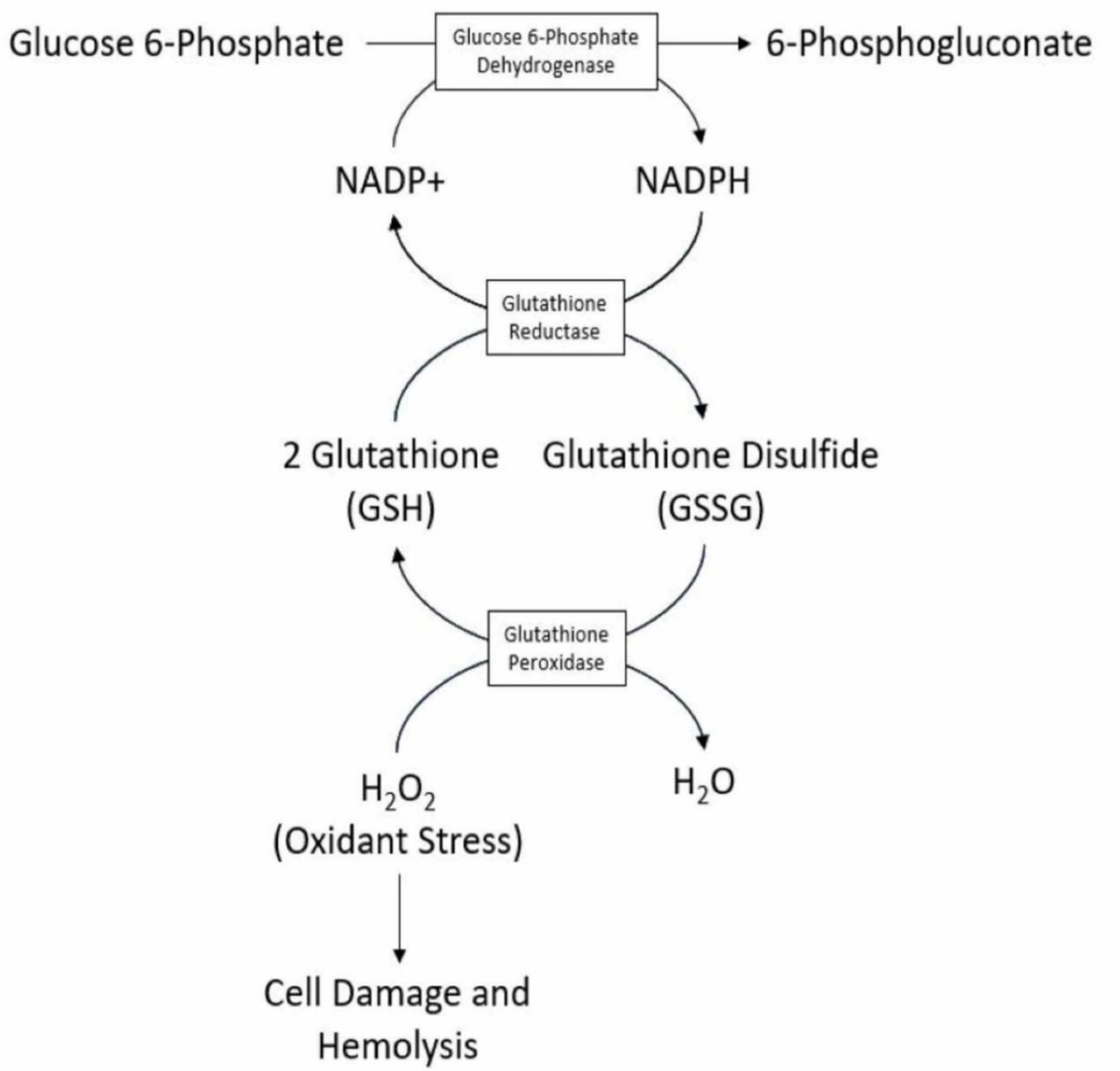

Figure 2.7 Mechanism of G6PD in preventing haemolysis in the setting of oxidant[82].

G6PD deficiency occurs most often in males with one X chromosome (and one Y chromosome) and rare in females[101]. have two X chromosomes, so if they have a mutation on one of them, they still have one X chromosome without the mutation. Females with one X chromosome mutation are known as carriers (heterogeneous) and are usually unaffected[82]. However, females can be affected if they have a mutation in both copies (homozygous) of the G6PD gene, Males have only one X chromosome and females have two copies of the X chromosome. A characteristic of X-linked inheritance is that fathers cannot pass X-linked traits to their sons but all daughters will be a carrier[82]. Mothers who carry one copy of the gene can pass G6PD deficiency to their children. The risk to each child depends on whether the child is male or female.

a) Each son has a 50% chance to be unaffected, and a 50% chance to be affected.

b) Each daughter has a 50% chance to be unaffected, and a 50% chance to be a carrier[106].

Males who get the gene (hemizygous) have G6PD deficiency. While females who get the gene are carriers (heterogeneous). They often do not have symptoms. But they can pass the gene onto their children.

In females, who have two copies of the X chromosome, one altered copy of the G6PD gene in each cell can lead to less severe features of the condition or may cause no signs or symptoms at all[6]. However, many females with one altered copy of this gene have glucose-6-phosphate dehydrogenase deficiency similar to affected males because the X chromosome with the normal copy of the G6PD gene is turned off through a process called X-inactivation[82]. in embryonic development in females, one of the two X chromosomes is permanently inactivated in somatic cells (cells other than egg and sperm cells). X-chromosome in each body cell. Usually X-inactivation occurs randomly, such that each

inactivation ensures that females, like males, have only one active copy of the X. X chromosome is active in about half of the body cells. Sometimes X-inactivation is not random, and one X chromosome is active in more than half of cells. When X-inactivation does not occur randomly, it is called skewed X-inactivation[107].

Research shows that females with glucose-6-phosphate dehydrogenase deficiency caused by mutation of the G6PD gene often have skewed X-inactivation, which results in the inactivation of the X chromosome with the normal copy of the G6PD gene in most cells of the body. This skewed X-inactivation causes the chromosome with the mutated G6PD gene to be expressed in more than half of cells. As a result, not enough normal glucose-6-phosphate dehydrogenase enzyme is produced, leading to haemolytic anaemia and other signs and symptoms of glucose-6-phosphate dehydrogenase deficiency[96].

2.2.2 Factors that Triggers Haemolysis in G6PD Deficiency

Many substances are potentially harmful to people with G6PD deficiency. Variation in response to these substances makes individual predictions difficult[82]. The following are factors that trigger haemolysis in G6PD deficiency individual;

Fava Beans

Vicia faba, also known in the culinary sense as the broad bean, fava bean, or faba bean, is a species of vetch as a flowering plant in the pea and bean family *Fabaceae*. It is widely cultivated as a crop for human consumption, and also as a cover crop[82]. Varieties with smaller, harder seeds that are fed to horses or other animals are called field bean, tic bean or tick bean. Horse bean, *Vicia faba var. equina Pers*., is a variety recognized as an accepted name. Broad beans have a long tradition of cultivation in Old World agriculture, being among the most ancient plants in cultivation and also among the easiest to grow[82]. Along

with lentils, peas, and chickpeas, they are believed to have become part of the eastern Mediterranean diet around 6000 BCE or earlier, however their wild ancestor has not been identified and their origin is unknown. Some people suffer from favism, a haemolytic response to the consumption of broad beans, a condition linked to a metabolism disorder known as G6PDD[32]. Otherwise the beans, with the outer seed coat removed, can be eaten raw, other than beans, or cooked. In young plants, the outer seed coat can be eaten, and in very young plants, the seed pod can be eaten[109.]

Acute development of symptoms such as fever, headache, abdominal pain and severe anemia after ingestion of fava beans is known as favism. All patients with favism are G6PD deficient[82]. However, not all G6PD-deficient subjects are sensitive to fava beans, and even those who are sensitive show striking variability from one exposure to the next[111]. The reason for this discrepancy is not clear, and it seems likely that one or more factors in addition to G6PD deficiency are required for the development of favism and to determine the severity of the individual attack[82]. Other foods that some persons with G6PD deficiency may prefer to avoid include the following red wine, all legumes, blueberries, soya products and tonic water.

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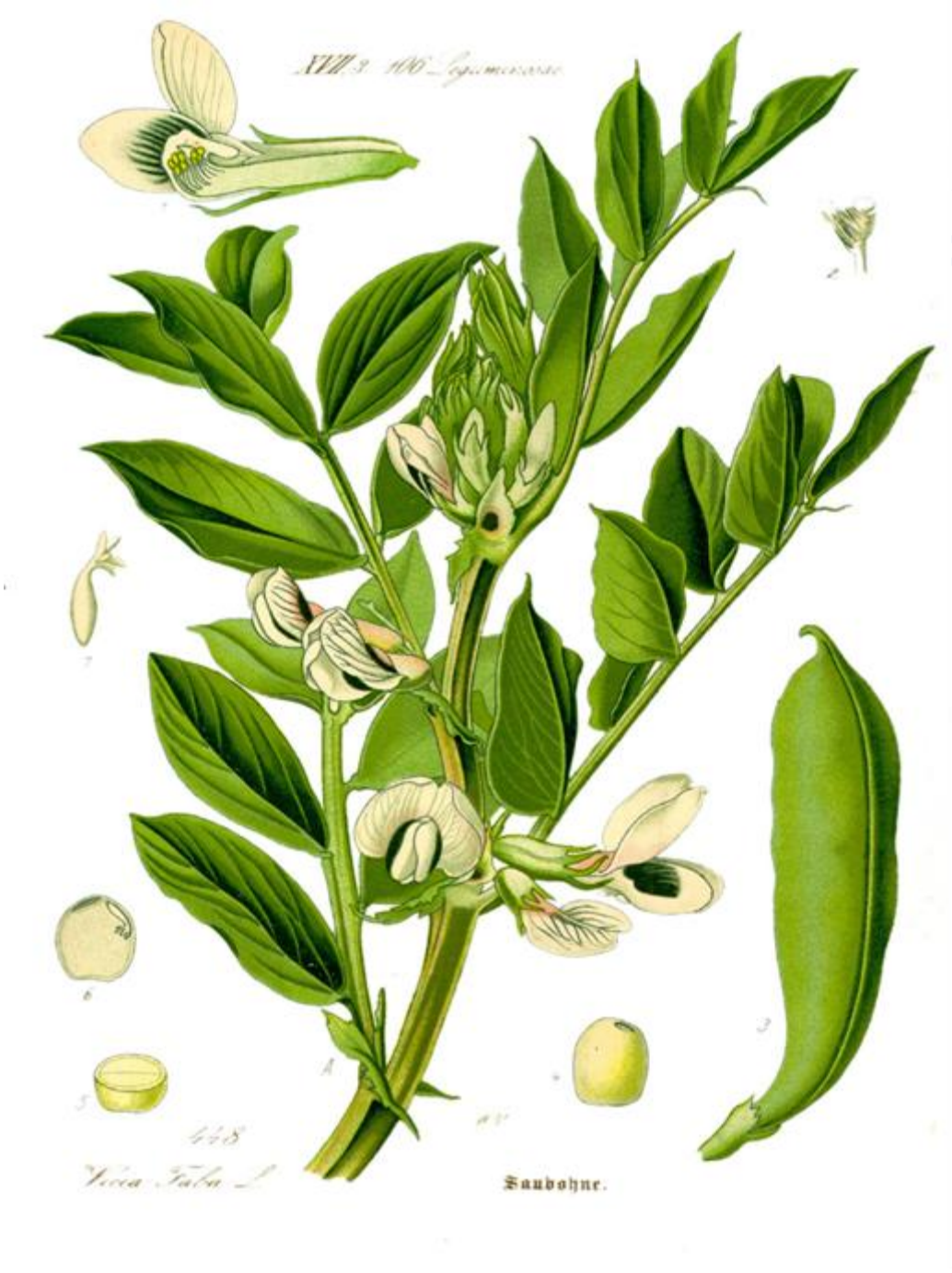

Figure 2.8 Illustration of Vicia faba in flower[110].

Drugs

Drugs that pose a possible risk in some persons with G6PD deficiency include the following:

(i) Antimalarial; antimalarial drugs that can cause acute haemolysis in people with G6PD deficiency includes primaquine (PQ), an 8-aminoquinoline, which is currently considered the most effective drug to prevent Plasmodium vivax clinical relapses as well as in Plasmodium falciparum infections to prevent gametocyte development and further parasite transmission to mosquitoes[82]. However, PQ use has been restricted because it can trigger variable degrees of haemolysis in individuals with G6PD deficiency, depending on the variant type. Due to intensification of malaria control programmes worldwide and the perspectives for malaria elimination in some regions where PQ could be massively deployed, it is important to better characterize prevalence of G6PD in malaria endemic communities in these area[82].

People with G6PD deficiency are about 20 to 30 times more sensitive to the haemolytic activity of primaquine than the people with normal G6PD[82]. Mechanism for induction of haemolytic anaemia was studied in rats. Toxic metabolites of primaquine cause reduction of GSH in red blood cells, formation of methemoglobin and Heinz bodies that induce haemolytic anaemia[82]. The primaquine metabolite, 6-methoxy-8-hydroxylaminoquinoline after N-hydroxylation from 6-methoxy-8-aminoquinoline, by peroxidation of lipids in red blood cells with significant GSH and oxidation of protein in GSH-depleted red blood cells can induce hemolytic response[82]. Other antimalarial drugs include pamaquine, mepacrine, quinine and chloroquine. There is evidence that other antimalarials may also exacerbate G6PD deficiency, but only at higher doses[82].

(ii) Rasburicase (brand name Elitek) is a uric oxidase used to treat the high levels of uric acid that are associated with tumor lysis syndrome (TLS)[82]. The rare cause of methemoglobinemia is a deficiency of antioxidant enzymes such as cytochrome b5 reductase 3[82]. The use of rasburicase is contraindicated in individuals known to have G6PD deficiency, and individuals at risk of G6PD deficiency should be screened before starting rasburicase therapy. This is because an oxidizing agent, hydrogen peroxide, is produced during the conversion of uric acid to allantoin[82].

Examples of other drugs are;

(iii) Sulphonamides; Sulphanilamide, Sulphacetamide, Sulphamethoxypyridazine (Lederkyn), Sulphisoxazole (Gantrisin), Sulphafurazole.some sulfonamides, such as sulfadiazine, have been found not to be hemolytic in many G6PD-deficient individuals.

(iv) Nitrofurans; Nitrofurantoin (Furadantin), Furazolidone (Furazone), Nitrofurazone (Furacin)[82].

(v) Antipyretics and analgesics; Acetylsalicylic acid (aspirin) is often used to treat pain or inflammation. Some people even take aspirin daily to prevent heart disease. But people with G6PD deficiency should not take it[82]. Other analgesics are; Acetanilide, Acetophenetidin (phenacetin), Aminopyrine (Pyramidon), Antipyrine[82].

(vi) Sulfones; Sulfoxone (Diazone), Thiazolsulfone (Promizole), Diaminodiphenyl sulphone (DDS)[82].

(vii) Vitamin K: Vitamin K is a fat-soluble vitamin with a key role in the synthesis of clotting factors. As vitamin K may decrease glutathione concentrations in normal infant erythrocytes, it is suggested that it may trigger haemolysis in G6PD deficient infants.

(viii) Vitamin C (ascorbic acid) is well known as an antioxidant as it is able to donate an electron to reduce oxidi[82]zing radicals and may be protective in G6PD deficiency at slightly supraphysiologic levels[82]. Though high doses of vitamin C have been shown to cause little sequela in healthy patients, case reports have documented high doses of vitamin C causing hemolysis in G6PD-deficient patients. Studies have shown that high doses of ascorbic acid decrease the function and survival of G6PD-deficient red blood cells as well[82].

Diagram (figure 3.6) showing red cell response to oxidative damage from drugs. A; In glucose 6-phosphate dehydrogenase (G6PD) normal red cells, hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) are detoxified by glutathione (GSH) peroxidase, which ultimately depends on G6PD activity for the continued regeneration of GSH. B; When G6PD-deficient red cells are exposed to an oxidative challenge GSH will be rapidly exhausted. As a result, H_2O_2 and other ROS are not detoxified: methaemoglobin is allowed to build up and, more seriously, sulphydryl groups in haemoglobin are attacked, resulting in the formation of Heinz bodies, damage to the membrane and, eventually, the destruction of red cells through both intravascular and extravascular mechanisms[82].

Other substances that can trigger haemolysis are:

a. Naphthalene is an organic compound with formula $C_{10}H_8$. It is the simplest polycyclic aromatic hydrocarbon, and is a white crystalline solid with a characteristic odor that is detectable at concentrations as low as 0.08 ppm by mass[82]. As an aromatic hydrocarbon, naphthalene's structure consists of a fused pair of benzene rings. It is best known as the main ingredient of traditional mothballs[132]. Exposure to large amounts of naphthalene may damage or destroy red blood cells, most commonly in people with the inherited condition known as glucose-6-phosphate dehydrogenase (G6PD) deficiency. Humans, in particular children, have

developed the condition known as haemolytic anaemia, after ingesting mothballs or deodorant blocks containing naphthalene[82].

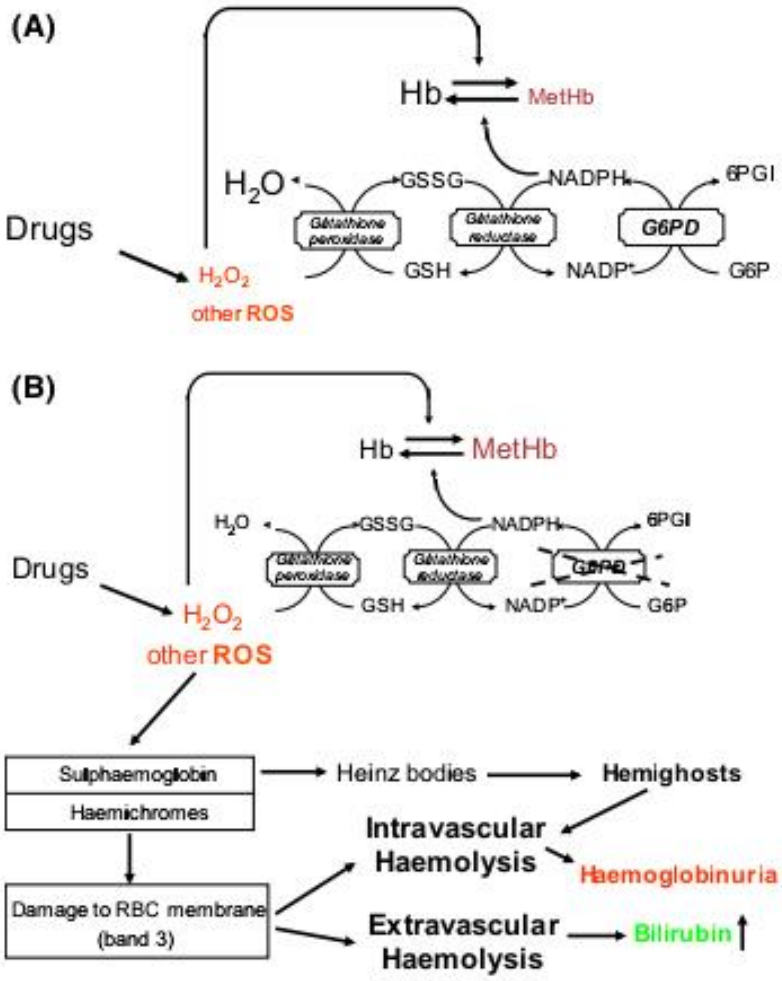

Figure 2.9 Diagram showing red cells response to oxidative damage from drugs[132].

b. Methylene blue, is a salt used as a dye and as a medication. Methylene blue is a thiazine dye. It works by converting the ferric iron in haemoglobin to ferrous iron. Methylene blue is contraindicated in patients with G6PD deficiency. Because it requires G6PD to work, it is ineffective in G6PD-deficient patients with methemoglobinemia. Additionally, methylene blue

administration may cause haemolysis in these patients[133].

Infection

This is the most commonly cited cause of acute haemolytic anaemia in patients with G6PD deficiency, particularly in those with severe deficiency and cytomegalovirus, hepatitis A and B, pneumonia, or typhoid fever[89]. Infection, whether bacterial, viral, or fungal, produces reactive oxygen species through the inflammatory response, to which deficient cells are particularly susceptible[82]. Haemolytic anaemia caused by infection and subsequent medication is a clinically important concern in patients with G6PD deficiency[82]. G6PD deficiency also may increase susceptibility to infection through these mechanisms: in leukocytes, a decrease in reactive oxygen species production within the pentose phosphate pathway reduces the microbicidal activity of phagocytes[56]. Impaired formation of reactive oxygen species-dependent neutrophil extracellular traps reduces extracellular defense mechanisms against bacterial and fungal pathogens[82].

Henna

Henna, obtained from the crushed leaves of *Lawsonia alba*, has been used for centuries to dye the skin, hair and nails in many countries in south-east Asia and the Middle East[82]. The

application of henna has resulted in life-threatening episodes of haemolytic anaemia in individuals with a genetic deficiency in G6PD activity. The use of L. alba (henna) for medicinal and cosmetic purposes is part of ancient and modern cultures and traditions of North Africa and Asia[82]. In some countries, it is used in ceremonial and social events, such as weddings and circumcisions, where henna is applied to the skin to create various patterns[138]. It is a commonly used traditional cosmetic agent, which also holds medical potentials and is used to treat skin lesions including seborrheic dermatitis or fungal infections and also has possible anti-inflammatory effects. The active dye ingredient in henna, lawsone (2-hydroxy-1,4-naphthoquinone) has been implicated as a cause of henna-induced haemolytic anaemia because of its structural similarity to other ortho-substituted 1,4-naphthoquinones, such as menadione, which are known to induce oxidative injury in red blood cells. Henna extract, or lawsone, were found to induce severe haemolytic anaemia in various animal models after exposure of the skin or ingestion. This reaction is associated with oxidative damage to erythrocytes[82].

There are few reports of life-threatening haemolysis in G6PD-deficient patients due to henna application; most of the cases reported were young infants or neonates. The oldest case of an 11-year-old boy with henna-induced haemolysis from Turkey was reported. This G6PD-deficient patient had henna applied to the whole body to treat psoriatic skin lesions[82]. Lawsone causes haemolysis in a dose-dependent manner, as evidenced by decreased levels of haematocrit and haemoglobin and by histopathological changes in the spleen, liver and kidney[82]. A 27-day-old boy who developed haemolytic anaemia and acute renal failure following extensive topical application of henna was reported. Intravascular haemolysis of various causes can result in acute tubular necrosis due to haemoglobinuria leading to acute

renal failure[82]. However, there are likely many children, such as our case, with undiagnosed G6PD deficiency. Therefore, the use of henna should be discouraged in infants in general and in G6PD-deficient individuals of any age. Additionally, public health education and universal G6PD screening are warranted to prevent this potentially lethal haemolytic crisis[82].

2.2.3 Molecular Characterization of G6PD Variants

The G6PD gene exhibits remarkable polymorphism in human populations and more than 400 variants of G6PD have been described based on their biochemical and physicochemical characteristics while some variants are not associated with significantly reduced enzyme activity in erythrocytes[82]. However, there are numerous mutations found in the G6PD gene, which are geographically isolated and cause a deficiency of the enzyme in erythrocytes[82]. Almost all G6PD deficiencies are caused by a point mutation of the genomic DNA resulting in an amino acid substitution[82].

An up to date report of around 186 mutations at DNA level indicating that this disease is heterogeneous: among these, 159 (85.4%) are single nucleotide substitutions (missense variants), 15 (8.0%) are multiple mutations (two or more substitutions), 10 (5.3%) are deletions, and two (1.0%) are mutations affecting introns. Based on mutation type, 23 have a variation in single nucleotide substitutions (missense variants), one has a deletion, four belong to multiple mutations, and three are novel mutants that affect the introns of gene[82]. The approximately 186 clinically relevant mutations reported for the G6PD gene encode deficient variants of the physiologically normal enzyme. The normal (wild-type allele) copy of the G6PD gene is known as G6PD B, and is found in most Caucasians, Asians, and Blacks. The World Health Organization categorized G6PD variants into 5 classes according to the level of enzyme activity and severity of haemolysis[82].

Class I variants, such as G6PD volendam (C514T), characterize severe deficiency and are associated with chronic nonspherocytic haemolytic anaemia.

Class II variants, such as G6PD Mediterranean (C563T), have enzyme activity values lower than 10%, leading to more frequent symptoms and crises.

Class III variants, including G6PD A (A376G), A- (G202A/A376G), G6PD Asahi (G202A), the enzyme activity ranges from 10 to 60%, and patients are usually asymptomatic, but the clinical relevance is the expression of medication-induced haemolytic crises.

Class IV variants, such as G6PD São Borja (G337A), have 60 to 150% of normal enzyme activity, with no clinical manifestations[82].

Class V variants have increased enzyme activity (>150%) and were described only once (G6PD Hektoen), in 1969.

The most common phenotypes are G6PD A (genotype A376G), which has normal enzyme activity; G6PD A- (genotype G202A), which presents from 10 to 60% of the normal enzyme activity; and B- or G6PD Mediterranean (genotype C563T), with 7% of normal activity[82].

Taking account of these new variants included in this review, the number of reported G6PD mutations is 217, being as follows: 182 (83.9%) are single nucleotide substitutions (missense variants), 19 (8.7%) are multiple mutations (two or more substitutions), 11 (5.1%) are deletions, and five (2.3%) mutations affect the introns[82]. Interestingly, there are 16 mutations corresponding to single nucleotide substitutions (missense variants) that were previously reported before in Minucchi's review and that were not considered as the G6PD Class I mutants: Zacatecas (Arg257Leu; exon 7), Palermo (Arg257Met, exon 7), Hamburg (Pro276Leu, exon 8), Veracruz (Arg365His, exon 10). Yucatan (Lys429Glu, exon 10), Tennessee (Leu422Val, exon 10), and one deletion named G6PD Taif (174Gly, exon 6)[82].

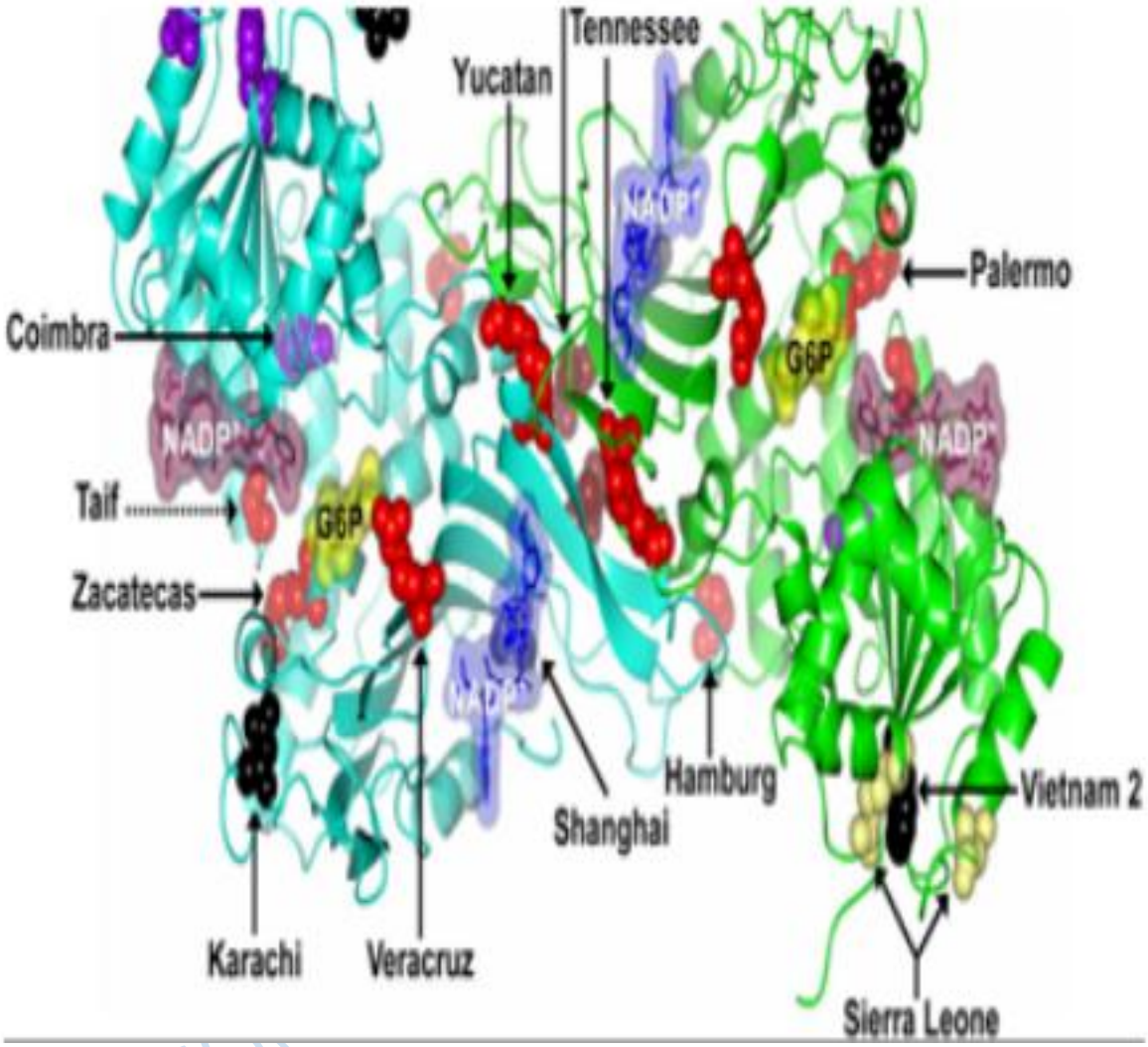

Figure 2.10 Structure of human WT G6PD enzy me (PDB entries 2BHL and 2BH9)[82].

The Class I G6PD Zacatecas mutant (Arg257Leu) was detected in a 12-year-old boy from the Mexican state of Zacatecas with neonatal jaundice and haemolytic crisis. This variant has a substitution of guanine for thymine (G > T) at nucleotide 770 (exon 7) leading to the replacement of arginine by leucine 257 (R→L) that, according to tridimensional structure is located at a distance of ~9 Å from the substrate-binding site of the β-D-glucose-6-phosphate (G6P)[151]. It is important to mention that this mutation is located in the same codon as the G6PD Wayne (R257G) that was previously characterized as a Class I mutant. However, Monteiro's review classified at theG6PD Zacatecas mutation as a Class II mutant[152]. G6PD Veracruz mutation was detected in anonymous blood samples from blood donors and has a substitution of guanine for adenine G1094A that changes the amino acid Arg 365 to His. The G6PD Yucatan mutation presents a shift of adenine for guanine (nt 1285A > G, K429E) and is located in exon 10. All these mutants were identified in a project performed to determine the molecular basis of G6PD deficiency in Mexico, including nearly 5000 individuals from the general population and patients with haemolytic anaemia belonging to at least 14 States fromMexico[153].

The figure 3.7 above indicates the location of Class I–IV mutations (missense variants) in the dimer (solid arrows). The Class I, II, III, and IV mutations are shown as red, purple, yellow, and brown spheres, respectively. The unnamed reported class mutations are shown as black spheres. Note that although the mutants are located on equivalent positions of G6PD dimer, Zacatecas, Palermo, Bahia, Vietnam 1 and 2, Sierra Leone, and San Luis Potosí mutants are shown in only one of the monomers. Also note that the Taif mutant is a deletion (dotted arrow) and Vietnam 1 mutant is represented by Val27 residue, since no electron density was

observed for the 26 N-terminal residues where the Vietnam 1 mutant is located (dotted arrow).

G6PD Palermo mutation presents a transversion of the nucleotides C769A and G770T in exon 7 and replaces arginine for methionine in codon 257, which may lead to changes in the protein structure causing chronic haemolytic anaemia[153]. The Class I G6PD Hamburg mutant involving a substitution of cytocine for thymine (C > T) at nucleotide 827 (exon 8) (Pro276Leu) and was detected in a Caucasian neonate with chronic non-spherocytic haemolytic anaemia[82]. Finally, G6PD Taif mutation has three base deletions at position 516–518 in exon 6 resulting in the loss of the amino acid Gly174. This mutation is unique because it is the only known deletion identified in this region that causes chronic haemolytic anaemia[153]. Besides the mutants described by Minucchi's review, include the following new variants:

Class II G6PD Bahia (Phe66Thr, exon 4), San Luis Potosi (Asn126Tyr; exon 5), Coimbra (Arg198His, exon 6),

Class III variant with double mutation; G6PD Sierra Leone (Arg104His, Asn126Asp, exon 5),

Class IV G6PD San Paulo mutant (Ile220Met, exon 7)[82].

Finally, there four mutants that did not classified according to their residual enzyme activity: G6PD Shanghai (Ala231Pro, exon 7), G6PD Karachi (Asp325Asn, exon 9), G6PD Vietnam 1 (Glu3Lys, exon 2), and G6PD Vietnam 2 (Phe66Cys, exon 4)[82].

The G6PD San Luis Potosi has a punctual mutation of adenine to thymine A376T that generates the substitution of 126 Asn to Tyr and was detected in an anonymous blood samples[82]. Blood sample from the subject with this variant showed a decreased fluorescence

in the screening test for G6PD deficiency and 47% of residual red cell enzyme activity versus those observed in their correspondent control blood samples from non-deficient subjects[82]. Whereas, G6PD Coimbra mutation detected among the tribal groups of the Nilgiris in Southern India, suffers a substitution of guanine for adenine (593G > A) on exon 6 switching Arg 198 by His was reported. G6PD Coimbra is very close to the G6PD Mediterranean in exon 6 and has similar kinetic properties[82]. Other mutation of the Class II G6PD Bahia mutant was reported and this mutant has a substitution of thymine for adenine (T > A) at nucleotide 197 (exon 4) (Phe66Thr) and was found in five neonates of Salvador in the Northeastern Brazilian estate Bahia[82]. The Class III G6PD Sierra Leona variant, which has double mutation in exon 5 (311G > A and 376A > G) leading to the change of amino acid 104 Arg → His and 126 Asn → Asp, respectively was as well reported. This mutation drastically reduces G6PD activity[82]. In addition, a novel Class IV G6PD Sao Paulo variant 660C > G was detected in exon 7, this mutation led to replacement of isoleucine by methionine (I220M) whose location is near the dimer interface. It is interesting to note that this mutation was detected among adults[82].

Additionally, the four mutants that were not described by Minucchi's review and that have not been classified according to their residual enzyme activity are the mutants G6PD Vietnam 1 (G7A; Glu3Lys) and Vietnam 2 (T197G; Phe66Cys) detected in the Vietnamese population with haemoglobinuria[82]. The G6PD Shanghai is a novel missense mutation (G691C) in exon 7 of the G6PD producing an Ala231Pro substitution and leading to significantly decreased of G6PD activity in red blood cells[163]. Finally, the G6PD Karachi mutant was detected in the Pakistani population with a change of guanine by adenine G > A in the nucleotide 973 exon 9, with a predicted amino acid change of Asp325Asn

(Asp325Asn)[82]. More recently, the Class I G6PD Merlo and Quilmes mutants were found in Argentine pediatric boys with chronic non-spherocytic hemolytic anemia. G6PD Quilmes Variant 995C > A was found in exon 9 and determines the Ser332Phe amino acid change. Variant Merlo 1226C > A, is located in exon 10 and is associated with the Pro409Gln amino acid change[82]. The unnamed mutant (Pro396Arg, exon 10) was identified in a 20-month-old boy with haemolytic anaemia whose enzymatic activity was severely decreased[82].

In addition, G6PD Herlev variant 592C > A (Arg198Ser) has been describe to be associated with severe enzyme deficiency, prolonged neonatal hyperbilirubinemia, and non-spherocytic haemolytic anaemia in a Danish descent male infant who on the second day of life developed jaundice peaking at 67 h that decreased upon application of double-sided phototherapy[82]. Recently, a novel unnamed mutation (1088A > T) was identified in a male infant patient aged 16month-old that predicted an Asn → Ile substitution at codon 363[82]. The variant caused by this mutation had reduced enzymatic activity, belonging to WHO Class I. Another two double mutants in Thai population were described; 6PDViangchan + Mahidol and G6PD Viangchan + Union. These double mutants should be classified as a severe G6PD deficiency[82]. However, clinical data is also required to classify them in a Class I or Class II G6PD deficiency[82].

Later, two new variants were characterized on exon 9 and were found during the screening of a large cohort of G6PD deficient patients that was performed in Tunisia[82]. The first one was named G6PD Nefza and carries the c.968T > C: p.323 Leu → Pro mutation, this variant was found in an 18-year-old male referred to the laboratory for haemolytic anaemia[82]. The second mutation, named G6PD Tunis carries the c.920A > C; p.307Gln → Pro mutation, and was found in a 54-year-old female presenting hemolytic anemia and paleness[82]. Furthermore, the

presence of a novel mutant G6PD Mexico DF that was identified in a Mexican individual from the northern Mexico; which presents the substitution of 193A > G that changes the amino acid threonine for alanine at position 65 (exon 4) has been reported[82]. The Thr65Ala mutation might lead to an unstable coenzyme union site region of the enzyme, due to the combined effect of the suppression of a peptide backbone H-bond and by disrupting the domain packing. Furthermore, there is also a report that the G6PD Gaza mutant (unclassified according to their residual enzyme activity) that was found in a Palestinian girl (38 months old) as a heterozygous genotype, presenting acute haemolytic anaemia and G6PD deficiency. This variant is characterized by a G > A transition mutation at nucleotide 536, which changes serine 179 to asparagine[82].

Besides, the G6PD Qingzhen which is a mutation affecting the introns, was detected in a 21/2years old male patient of the Yi ethnic group in the Qingzhen city, Guizhou province, in China. The patient presented acute jaundice, anemia, wine urine, emesis, low-grade fever, and the occasional headache. Mutation analysis of G6PD revealed that the patient had a novel subtle splice-site mutation (IVS5-1G > A)[82]. In addition, two mutations affecting introns were reported where the first was the G6PD mutation IVS-V 655C > T, found in four female subjects with mild deficiency of class III variant. The second mutation affecting the introns was the IVS-VIII 43G > A found in three male subjects with a mild deficiency of the class III variant. It is interesting to note that all known mutations have been found to affect the coding regions of the gene and none described in the regulatory regions. Because mutations can alter the cellular process that generally trigger a metabolic disease[168].

2.2.4 Clinical Manifestations of Glucose-6-Phosphate Dehydrogenase Deficiency

The degree of G6PD deficiency determines the clinical expression of the disorder. Individuals with minimally reduced enzyme levels do not experience haemolysis. Others with a greater degree of deficiency have episodes of brisk haemolysis triggered by infections, taking drugs and some chemicals that increase oxidative stress or ingesting fava beans[82]. Haemolysis due to oxidant stresses are usually self-limiting within 8 to 14 days due to the compensatory production of young red blood cells with high levels of G6PD[82]. Haemolytic anaemia occurs when red blood cells are destroyed faster than the body can replace them[82]. This type of anemia leads to paleness, yellowing of the skin and whites of the eyes (jaundice), dark urine, fatigue, shortness of breath, enlarged spleen, and a rapid heart rate. Skin ulcers are uncommon but may occur in people with severe G6PD deficiency[178].

Patients with severe G6PD deficiency have chronic haemolysis and are often thought to have non-spherocytic haemolytic anaemia[82]. G6PD-deficient individuals are entirely asymptomatic and the overwhelming majority of the remainder only develop symptoms in response to oxidant stress. The commmonest clinical manifestations are neonatal jaundice and acute haemolytic anaemia related to drugs, infection or ingestion of fava beans[180]. The critical role of environmental precipitants has been recognized since the earliest descriptions of G6PD deficiency. Pythagoras is said to have warned his disciples against the dangers of eating fava beans (*Vicia faba*; broad beans)[82]. Observant practitioners had noticed that favism appeared to 'run in families'[82]. It was also clear that only some individuals were susceptible to haemolytic anaemia caused by drugs before the discovery in Chicago that primaquine-sensitive people had a very low level of G6PD activity in their red cells[82].

Mechanism of haemolysis

The detailed mechanism of haemolysis is not fully known but it undoubtedly results from the inability of G6PD-deficient red cells to withstand the oxidative damage produced, directly or indirectly by an exogenous trigger. The identity of the G6PD variant, and hence the residual enzyme activity, is clearly an important variable[82]. Residual activity is below a critical level in Class 1, NADPH production is inadequate for the steady-state requirements of the red cell, and chronic non-spherocytic haemolytic anaemia (CNSHA) results. Neonatal erythrocytes have depressed levels of vitamin E, glutathione reductase and catalase, making them more susceptible to oxidant haemolysis[82].

Certain drugs and infectious agents (for example, influenza A virus) stimulate the hexose monophosphate shunt pathway in normal red cells, indicating that in their presence increased NADPH production is required. Hydrogen peroxide is generated by activated polymorphonuclear neutrophils[82]. Based on studies of the effect of fractionated extracts on erythrocyte metabolism, the toxic components of fava beans have been suggested to be the pyrimidine aglycones, divicine and isouramil in combination with ascorbic acid[82].

A reasonable model for all of these situations is that the red cell GSH level becomes so low that critical sulphydryl groups in some key proteins are not maintained in reduced form, and intramolecular or intermolecular disulphides are formed[16]. Such aggregates decrease red cell deformability, and they may alter the cell surface sufficiently to make it recognizable by macrophages as abnormal (much like an aged red cell), thus leading to extravascular haemolysis within the reticuloendothelial system[82]. Disturbed erythrocyte calcium homeostasis (specifically, reduced activity of the membrane Ca^{2+}-ATPase, leading to increased intraerythrocytic calcium and decreased intraerythrocytic potassium) has been suggested to mediate activation of proteolytic activity within erythrocytes of favic subjects[82].

Chronic non-spherocytic haemolytic anaemia Some G6PD variants are characterized by overt chronic haemolytic anaemia, which is further exacerbated by oxidant stress. Such variants have been described (almost invariably in males within a single kindred) in many parts of the world, regardless of whether the common types of G6PD deficiency are endemic in the region[157]. Most patients present with or give a history of neonatal jaundice, often requiring exchange transfusion and go on to develop infection and drug-induced haemolysis[82]. Gallstones may be a prominent feature and splenomegaly is usually present, G6PD activity is low in all tissues and, in rare cases, deficiency in granulocytes is associated with granulocyte dys- function and haemolysis is worsened by increased susceptibility to infection[82].

Drug-induced Haemolytic Anaemia

A critical analysis of the data whereby individual drugs have been implicated in the causation of haemolysis in G6PD-deficient subjects has been conducted by Beutler who uncovered a discrepancy between the relatively small list of drugs for which there is strong evidence linking them to haemolytic anaemia and a much larger list of agents for which the evidence is less secure[82]. The degree of haemolysis is also influenced by the activity of the host G6PD activity, the dose and duration of therapy, and the presence of additional oxidant stress, for example, infection. Furthermore, clinical and haematological assessment of haemolysis has notoriously low sensitivity, in that even a two- to three-fold increase in red cell destruction may not produce a significant anaemia or reticulocytosis. Clinical haemolysis and jaundice typically begin within 2 to 3 days of starting the drug[82].

There is genetic heterogeneity of G6PD deficiency which means that a drug found to be safe in some deficient subjects may not necessarily safe in all. The risk and severity of haemolysis is usually dose related[82]. Quinine, chloroquine and quinidine are all acceptable for the

treatment of acute malaria, and chloroquine, mefloquine, halofantrine proguanil and pyrimethamine (but not Maloprim, which contains dapsone, or Fansidar, which contains a sulphonamide) are acceptable for malaria prophylaxis[82]. The haemolysis is largely intravascular and it is characteristically associated with haemoglobinuria. The anaemia worsens until the seventh to eighth day, a reticulocyte response then sets in, and the haemoglobin level begins to recover on the eighth to tenth day[82]. Invitro tests have been developed aiming to predict whether a drug will cause haemolysis in vivo and they should be carried out before a new drug is introduced to a population in which G6PD deficiency is prevalet[196].

Infection-induced Haemolysis

Infection is probably the most common cause of haemolysis in subjects with G6PD deficiency. Numerous bacterial, viral and rickettsial infections have been reported as precipitants, but particularly important are infectious hepatitis, pneumoniaand typhoid fever[82]. Viral infections affecting either the upper respiratory tract or the gastrointestinal tract are reported to cause more severe haemolysis than bacterial infections in G6PD-deficient children. Haemolysis is again largely intravascular and renal failure is a well-recognized complication in adults, whereas it is rare in children[197].

Favism

All patients with favism are G6PD deficient; however, not all G6PD deficient subjects are sensitive to fava beans, and even those who are sensitive show striking variability from one exposure to the next[82]. The reason for this discrepancy is not clear, and it seems likely that one or more factors in addition to G6PD deficiency are required for the development of favism and to determine the severity of the individual attack[82]. Clinical favism presents

characteristically with sudden onset of acute haemolytic anaemia within 24-48 hours of ingestion of the beans. Pallor, jaundice and haemoglobinuria are the hallmarks[199]. Acute renal failure may supervene in adults, but it is very rare in children; however, fatalities in children were not uncommon prior to the availability of transfusion therapy[199].

The highest incidence is in boys aged 2-6 years. It is well documented that heterozygous girls are affected, although the condition is usually milder in these subjects." Favism occurs after ingestion of fresh, dried or frozen beans, but fresh beans are by far the commonest offender and therefore favism is commonest during the spring season[157]. Haemolysis in breast-fed babies whose mothers have eaten fava beans is well documented. Experience in Sardinia has demonstrated the value of neonatal screening and health education in reducing the incidence of favism within that community[155]. It has been widely held that favism is only associated with the more severely deficient amongst the polymorphic variants of G6PD (particularly G6PD Mediterranean); and specifically that G6PDA- in not associated with favism. This is not correct, as typical attacks of favism have been well documented in subjects of African origin with the A-variant[82].

Neonatal Jaundice

G6PD deficiency is the commonest red cell enzymopathy to cause neonatal haemolysis and jaundice[82]. The best population data are available from West Africa, the Mediterranean and the Far East (for example, Thailand) and it is clear that perhaps as many as one third of all males with neonatal jaundice have G6PD deficiency, and a similar proportion of male children with G6PD deficiency develop neonatal jaundice (NNJ)[82]. Kernicterus has been described in all population groups. G6PD deficiency is a less frequent cause of NNJ among subjects of African descent in the USA, and of Greek ancestry in Australia, than in the

countries of origin of these populations, although the differences are perhaps less marked than originally thought[82].

Environmental factors that may account for this include maternal exposure to oxidant drugs and use of herbal remedies that may precipitate or exacerbate NNJ. Gestational age and maturity is an important consideration, as NNJ is more common, severe and potentially harmful in premature infants[203]. Environmental factors will also affect the incidence of neonatal infection, hypoglycaemia, acidosis and the normal level of neonatal haemoglobin within a population. Cultural factors, including exposure to icterogenic agents, have been identified as important precipitants of NNJ amongst the G6PD-deficient population of Nigeria[82]. Of genetic factors, the type of G6PD variant that is prevalent within a population is likely to be relevant, and is clearly of importance with respect to unusual or sporadic variants in the USA[204].

Chronic Non-spherolytic Haemolytic Anaemia

Congenital non-spherocytic haemolytic anaemia (CNSHA) is an autosomal recessive condition that leads to a deficiency of vital enzymes required for glycolysis and red blood cell (RBC) nucleotide metabolism. It presents as a congenital haemolytic anaemia and patients also show signs of jaundice[82]. A small percentage of G6PD-deficient patients have chronic nonspherocytic haemolytic anaemia of variable severity. G6PD Brighton, G6PD Harilaou, and G6PD Serres are included in this category[82]. The patient with chronic nonspherocytic haemolytic anaemia is usually a male with a history of neonatal jaundice who may present with anaemia, unexplained jaundice, or gallstones later in life. Although they have chronic haemolysis, the patients are also vulnerable to acute oxidative damage on exposure to an oxidative agent[82].

2.2.5 Prevalence of Glucose-6-Phosphate Dehydrogenase Deficiency

The deficiency of G6PD has been recognized as the most common enzymopathy, affecting nearly 400 million people worldwide including individuals of all races and ethnic backgrounds[155]. G6PD deficiency is more commonly expressed in males compared to females with the highest prevalence rates found in Africa, the Middle East, certain parts of the Mediterranean, and certain areas in Asia. In these regions, the rate ranges from 5% to 30% of the population[156]. The severity of G6PD deficiency can vary based upon specific racial groups. Interestingly, the prevalence of G6PD deficiency correlates with the geographical distribution of malaria, leading to postulate that G6PD deficiency gives a partial protection against this infection[82]. The severe form of the disorders occurs more often in the Mediterranean population. In the United States, the incidence is much higher among the African-American population than in other sectors. The frequency of a carrier state in which one partner carries a normal gene and the other carries an abnormal variant is as high as 24% and about 10%-14% of African-American males are affected.

The prevalence of this deficiency varies around the globe, with frequencies ranging from 2% to 20% in Greece, Turkey, and Italy; but increased as much as 70% in groups of Kurdish Jews[113]. Recently, a Bayesian geo-statistical model was used to estimate the prevalence of G6PD deficiency worldwide. The results of this study showed that in Latin America (LA), the prevalence of G6PD deficiency is lower compared with other regions such as sub-Saharan Africa or Asia. In Asia, the deficiency prevalence ranges from 6.0% to 15.8%. In India, it is 10.5%, while in the Middle East the prevalence varies from 3% to 29%[82]. In Brazil, a few studies have found a prevalence between 1.7% and 6.0%[109]. In africa, the prevalence of G6PD deficiency has been reported as high as 28.1% in south-west Nigeria,

22.5% in Congo (Brazzaville), 15.7% in Mali (Bamako), 13.0% in Uganda and 9.0–15.5% in Gabon[82].

Previous reports in Nigeria showed that the prevalence of G6PD deficiency ranged from 4% to 26%. However, a study in Sokoto (Nigeria) was carried out among 118 children visiting the Emergency Pediatrics unit of Usmanu Danfodiyo University Teaching Hospital for pediatrics related care indicated G6PD deficiency of 14.4%[82]. Also, another study in Katsina among children aged 0 – 5 years reported an overall prevalence of 16.2%. It is most frequent among individuals of African descent, with a frequency ranging from 3.6%-28%[111].

G6PD gene is highly polymorphic and the frequency of these mutations varies among populations and countries. According to the World Health Organization (WHO), 7.5-10% of the world population bears one or two genes variants for G6PD deficiency and about 2.9% out of the said number are G6PD deficient[111]. For instance, Mutation S188F, sometimes called the Mediterranean mutation, is most prevalent among individuals from the Middle East[6]. Mutations C131G and G487A that were common in Dhaka, Bangladesh appear to be linked to G6PD deficiency by affecting NADP binding or disrupting the protein structure[82]. The G6PD genetic variants were relatively homogeneous in America, Africa, and western Asia compared to those in East Asia and Oceania. In North America, Africa, Yemen and Saudi Arabia, G6PD*A- variant is predominant among populations[109]. For example, two common variants occur in many African-American males. Approximately 20 to 25 percent have the near normal G6PD variant called “A+” (mutation A376G), while about 10 to 13 percent have another variant called “A-” (G202A arose on a G6PD A+ chromosome)[70]. However, in sub-Saharan Africa three variants occur with polymorphic frequencies (> 0.1%): G6PD*B, G6PD*A and G6PD*A-. G6PD*B is the wild type and the most common variant

in Africa and worldwide. G6PD*A has a single A→G substitution [82]at nucleotide number 376. It is a normal variant with about 90% of the G6PD*B enzyme activity. G6PD*A- is a deficient variant with about 8-20% of the wild type enzyme activity and caused by the A376G and G202A mutations. G6PD Mediterranean (C563T) variant is caused by the C563T mutation with less than 10% enzyme activity and found in Italy, Cyprus and in the Middle East. Another relatively common G6PD variant is found particularly among individuals of Sephardic Jewish or Sardinian descent. In addition, another somewhat common variant is present among some individuals of southern Chinese descent[70].

Endnotes

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Chapter Three

Methodology

3.1 Research Design

The study was cross-sectional design. It was undertaken between December 2021 and May 2022 to determine Glucose-6-phosphate Dehydrogenase Deficiency among Sickle cell anaemia patients in Ibadan.

3.1.1 Study Sites/Area: The study location for this research was the Sickle cell clinics at the University College Hospital and Adeoyo Hospital Ring road, Ibadan, Oyo State, Nigeria. The study area was Ibadan, one of the oldest cities in Africa. It is the capital of Oyo state and has eleven Local government area which include five urban local government areas and six semi-urban local government areas. It is the third largest populous cities in Nigeria with 3,565,108 people after Lagos and kano. It lies within latitude 7° 19' 08" and 7° 29' 25" of the equator and 3° 47' 50" and 4° 0' 22"[82]

3.1.2 Study Population: The population for the study include the sickle cell anaemia patients attending the Haematology Daycare Clinic of University College Hospital and Sickle Cell Clinic Adeoyo Hospital Ibadan. This study dealt with all males and females above 13 years of age presenting with sickle cell disease.

3.2 Ethical Consideration: Ethical clearance (with Ref. No. AD 13/479) for the conduct of this study was obtained from Oyo State Joint Ethical Review Board. Inform consent (written and verbal) was seek from respondents before the commencement of the study. Respondents was given adequate information ensuring that they understood the research purpose and protocols.

3.2.1 Inclusion Criteria

The participants included must be sickle cell individuals attending the hospital/clinic. They must be willing to give informed consent. Under 18 years intending participants must give assent while their parents/legal guidance will give consent to be part of the study. Participants must be willing to donate about 5ml of blood sample for research

Confidentiality of Data: The data was treated with utmost confidentiality and only be used for the purpose of research alone. In order to ensure anonymity of responses, names of respondents will not be written on the questionnaire.

3.3 Sample and Sampling Techniques

Questionnaires were administered to voluntary patients to collect the information about demographic data, medical challenges and status

3.2.1 Sample Size: The sample size was determined using the formulae below for sample size calculation;

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

where:

n = calculated sample size d = margin of error- 0.05

Z = level of confidence- 1.96 P – prevalence of a study = 9.6%

$$n = \frac{(1.96)^2\, 0.096(1-0.096)}{0.05 \times 0.05} = 133.35576576$$

~133 samples

10% attrition rate will be added to 133

13.3 +133=146.3

=147 participants

3.1.4 Sampling Technique: A convenient sampling method was used to recruit up to the calculated sample size.

3.1.5 Sample Collection/Processing: Ten milliliter of blood sample was collected from individual sickle cell patient at the clinic through venipuncture and dispensed in duplicate into ethylenediaminetetraacetic acid (EDTA) bottle. One of the bottles was centrifuge at 3000rpm for 15minutes and the buffy coat was removed and dispensed into eppendoff bottle which was kept at -40^{0}C till the time of extraction.

3.2 Sample Analysis

Haemoglobin Electrophoresis

Estimation of haemoglobin genotype by alkaline Hb electrophoresis using cellulose acetate paper[82].

Principle: At alkaline pH, haemoglobin is a negatively charged protein and when subjected to electrophoresis will migrate toward the anode (+). By their separation, they appear as bands representing the constituent allele

Procedure: Haemolysate (Hb solution) was prepared from the EDTA patient sample by adding 500ul of whole into 2ml of water. With the power supply disconnected to the electrophoresis machine, equal amount of trisEDTA Borate buffer (at alkaline pH of 8.6) was placed in each buffer compartment of the electrophoresis tank. After wetting the two chamber wicks with buffer, one was placed along each bridge support, ensuring that they make good contact with the buffer. The cellulose acetate membrane was soaked by lowering

it slowly into a reservoir of the buffer and left for at least 5 minutes. The cellulose acetate membrane was taking from the buffer and blotted between two layers of cleaned blotting paper. The cleaned tips applicator was loaded by depressing the tip into the same well twice and applying it first onto some clean blotting paper. The applicator was reloaded with the haemolysate and applied to the cellulose acetate membrane alongside the controls. Placement of cellulose acetate membrane across the bridges after which the power supply to the electrophoresis power pack was connected and electrophoresis was carried out at 240 volts for 30 minutes. Haemoglobin genotypes of the participants was identified and recorded.

Qualitative analysis of G6PD deficiency[82]

Principle: G6PD catalyzes a reaction in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells such as erythrocytes by maintaining the level of the reduced form of the co-enzyme nocotinamide adenine dinucleotide phosphate (NADPH).

Glucose-6- phosphate + NADP ---------→ 6-phosphogluconate + NADPH

(non-fluorescent) G6PDH (fluorescent)

Procedures:

i. 10ul of blood sample was added into a clean tube

ii. 100ul of G6PD reagent was also added into the tube.

iii. It was allowed to mix very well and then applied on the chromatographic paper in duplicate.

iv. The chromatographic paper was incubated at room temperature for 30mins.

v. Using UV lamp, visual reading (flourescence) was taking to determine the G6PD deficient and normal status.

Quantitative G6PD Assay Using Spectrophotometric Method[82]

Determination of haemoglobin concentration using hemocue machine

With the aid of applicator, the microcuvette was filled with blood through capillary action. The microcuvette was placed in the hemocue machine and the absorbance was read using hemocue photometer and the haemoglobin concentration was displayed on the monitor of the hemocue machine.

For the G6PD enzyme activity,

Reagents

0.9% sodium chloride, digitonin (R4), substrate (R3), NADP solution (R2) and buffer (R1; triethanolamine buffer EDTA)

Principle: the enzyme activity is determined by measurement of the rate of absorbance change at 340nm due to the reduction of $NADP^+$

$$G\text{-}6\text{-}P + NADP^+ \xrightarrow[G\text{-}6\text{-}PDH]{} \text{gluconate-6-P} + NADPH + H^+$$

Preparation of haemolysate:

a. 0.2ml of blood sample was placed in a centrifuge tube and washed by adding 2ml of 0.9% NaCl solution.

c. The solution was centrifuge three times for 10 minutes at centrifugation of 3000rpm.

d. The supernatant was removed and remains the haemolysate

e. The haemolysate was suspended in 0.5ml of solution 4 (digitonin) and then centrifuge again after being allowed to stand to 15 minutes at 4^0C.

f. 1ml of solution 1 (buffer), 30ul of solution 2 (NADP) and 15ul of haemolysate were mixed together and incubated for 5 minutes at 37^0C

g. 15ul of Solution 3 (substrate) was added and mixed.

h. The absorbance was taken using Stardust semi-autoanalyser spectrophotometer at 340nm wavelength the enzyme activity was calculated as mU/g haemoglobin after the reading has been taken three times and multiply the factor 33600

$$\text{G6PD activity (mU/gHb)} = \frac{\text{mU.erythrocytes per ml*100}}{\text{Hb(g/dl)}}$$

Hb(g/dl) = Haemoglobin concentration determined for each specimen

100 = factor to convert ml to dl

$$\text{Then to get G6PD activity at U/gHb} = \frac{\text{G6PD activity (mU/gHb)}}{1000}$$

The normal range is 6.97-20.50U/gHb. Any value below 6.97U/gHb is known to have G6PD deficiency.

Molecular analysis

Reagents

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

Sample treatment and DNA extraction using Da An Gene (spin column) extraction kit

i. The buffy coat was removed from the freezer and allowed to thaw. 50ul of proteinase K was added into a sterile centrifuge tube

ii. 200ul of buffy coat was added into the centrifuge tube

iii. Lysis solution of 200ul was added and the tube cover was fastened down and oscillated in vortex for 15 seconds to mix the solution sufficiently.

iv. High-speed centrifugation was conducted for 10 seconds and heated at temperature of 72^0C for 10 minutes.

v. The eluent was also preheated at 72^0C for 10 minutes and kept aside.

vi. 250ul of ethanol was added into the solution in the centrifuge tube and the cover was fastened down and oscillated in vortex for 15 seconds. The whole mixture was drawn into the spin column and centrifuge at 12,000g for 1 minutes at room temperature and the spin column is being fit into a new collection tube

vii. Inhibitor remover of 500ul was added into the spin column and centrifuge at 12,000g for 1 minutes at room temperature and the spin column is being fit into a new collection tube

viii. Deionized solution of 500µl was added into the spin column and centrifuge at 12,000g for 1 minutes at room temperature and the spin column is being fit into a new collection tube

ix. Another deionized solution of 500µl was added into the spin column and centrifuge at 12,000g for 1 minutes at room temperature and the spin column is being fit into a new collection tube. The spin column together with collection tube was centrifuge at 14,000g for 3 minutes in order to remove the residual ethanol

x. The spin column was moved out and fit into another centrifuge tube. The cover of the spin column was opened and lay aside for 2 minutes at temperature of 72^0C. Carefully, 50µl of eluent preheated at 72^0C for 10 minutes was added right above the membrane of the spin column.

xi. The tube cover was fastened down after standing for 1 minute at room temperature and centrifuge at 14,000g for 1 minutes.

The solution in the centrifuge tube is the DNA extract which was checked using nanodrop of model 2000 from thermo scientific for the concentration and purity nanodrop.

Amplification using polymerase chain reaction (PCR)

The amplification was carried out by PCR on the extracted DNA using A376G primer (5'-CCCAGGCCACCCCAGAGGAGA-3' (forward) and 5'-CGGCCCCGGACACGCTCATAG-3' (reverse)). The reaction cocktail includes 12.5µl of 2X master mix (MgCl, taq DNA polymerse DNTP), 0.5µl each of both forward and reverse primer together with template DNA of 5ul which was made up to 25µl with sterile nuclease free water of 6.5µl. To prevent evaporation, 10ul of mineral oil was also added to the mixture.

PCR condition includes an initial denaturation step at 95^0C for 10 minutes followed by 35 cycles denaturation at 96^0C for 30 seconds. Primer annealing step at 61^0C for 30 seconds and extension at 68^0C for 30 seconds, then the final extension at 72^0C for 10 minutes. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair. Integrity of the amplified fragments was checked on agarose gel

Preparation of Gel

The buffer 10X TBE was brought down to 0.5X by adding 50ml of the 10XTBE into 950ml of water. The buffer (0.5X TBE) prepared was subsequently used to prepare 2% agarose gel. Agarose gel of 4g was measured into a glass beaker and 200ml of 0.5XTBE buffer was added. The suspension was boiled in a microwave for 5 minutes and stir carefully to allow homogeneity. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5

g/ml EZ-vision (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 30 minutes to form the wells. The 0.5X TBE buffer was poured into the gel tank to barely submerge the gel.

Digestion with the use of restriction enzymes[82]

The digestion mix was prepared with 10ul of amplicon, 1ul of fokI restriction enzyme (Inqaba Biotec West Africa Ltd) along with 5µl of buffer and made up to 20µl with nuclease-free water of 4µl. The digestion process was carried out at 37^0C for 2 hours after denaturation at 65^0C for 20 minutes

Gel Electrophoresis

Two microliters (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 8µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

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Chapter Four

Results and Discussion of Findings

4.1 Demographic Data Analysis

The total number of participants recruited for this study is 147. Out of which 86 (58.5%) are female while 61 (41.5%) are male. From the sex distribution, it shows that the percentage of female is higher than the male. The age range is between 10-60years with aged 16–21 years (38.5%) being the highest follow by those who were 22-27 years (21.7%) then those that are above 51 years being the lowest (1.5%) among the age group. The mean age is 23 ± 0.6 (SE). Table 4.1 shows the distribution of exposure to agents that can cause haemolysis among G6PD deficient among sickle cell disease patients. From this study, 23 (27.1%) of the female among the participants (sickle cell disease patients) were exposed to camphor while 12 (20.0%) of the male are being exposed giving rise to a total number of 35 (24.1%) participants that were exposed to camphor. The female being exposed to metholathum are 23(27.1%) while the male is 13 (21.7%) making a total number of 36 (24.8%) being exposed to metholathum. The total number of participants exposed to naphthalene are 8 (5.5%) out of which 5 (5.9%) were female and 3 (5.0%) were male.

The total number of those that were being exposed to both camphor and naphthalene were 2 (1.4%) with only 1 (1.2%) among the female and 1 (1.7%) among the male. haemolysis among G6PD deficient) among sickle cell disease patients. The total number of participants being exposed to metholathum and naphthalene were 5 (3.4%) with 2 (2.4%) female and 3 (5.0%) male. The participants being exposed to camphor, metholathum and naphthalene is 1

(0.7%) with 1 (1.2%) female and none among the male. Those participants which were not exposed to any of the three agents were 58 (40.0%) with 30 (35.3%) female and 28 (46.7%) male.

4.2 Presentation of Data

Laboratory data of the participants

The statistical analysis of the haemoglobin variants among sickle cell disease patients present in this study (table 4.2) has showed that the homozygous haemoglobin SS to be 82 (95.3%) among the female and 60 (98.4%) among the male with a total number of 142 (96.6%) among all 147 participants. The total number of heterozygous haemoglobin SC were 4 (2.7%) with 3 (3.5%) female and 1 (1.6%) male. From this study, none of the male participants were homozygous haemoglobin CC while only only 1 (1.2%) female was homozygous haemoglobin CC among the participants.

Table 4.4 showed the frequency of glucose-6-phosphate dehydrogenase status among the sickle cell disease patients. Using fluorescent spot test (FST), those that showed full deficiency among the participants using were 21 (14.3%) with 3 (3.5%) among the female and 18 (29.5%) among the male participants. The total number of participants that showed partial deficiency for glucose-6-phosphate dehydrogenase were 11 (7.5%) with 11 (12.8%) among the female and none among the male. Those that came out to have normal glucose-6-phosphate dehydrogenase among the participants were 72 (83.7%) female and 43 (70.5%) male with a total number of 115 (78.2%) among all the 147 participants.

Figure 4.6 shows the image of gel electrophoresis showing band size of 308bp of G6PD A variant (A376G). The point labelled L indicate the ladder (100bp) which serves as control to

determine the band size of the gene. Sample 1 and 2 have no band because the gene specific for the primer is not present in the samples while other samples have the G6PD A variant.

Figure 4.9 shows the gel electrophoresis image after digestion with of fokI restriction enzyme. FokI restriction enzyme is enzyme that is specific for the digestion of the amplicon of G6PD A variant (rs1050829) after the amplification using (PCR). From figure 4.9, the gel image showed that there is no gene polymorphism of G6PD A (rs1050829) among the participants.

Table 4.1: Exposure to agents (that can cause haemolysis among G6PD deficient) among sickle cell disease patients.

Variable	F (N=86)	M (N=61)	Total (N=147)	p value
Exposure.to.agents				0.703
C	23 (27.1%)	12 (20.0%)	35 (24.1%)	
M	23 (27.1%)	13 (21.7%)	36 (24.8%)	
N	5 (5.9%)	3 (5.0%)	8 (5.5%)	
C,M	1 (1.2%)	1 (1.7%)	2 (1.4%)	
M,N	2 (2.4%)	3 (5.0%)	5 (3.4%)	
C,M,N	1 (1.2%)	0 (0.0%)	1 (0.7%)	
None	30 (35.3%)	28 (46.7%)	58 (40.0%)	

Source, Laboratory 2022.

chi-squared = 3.8639, df = 7,

Abbreviations: F-female

M-male

C- camphor,

M- metholathum,

N- naphtalene

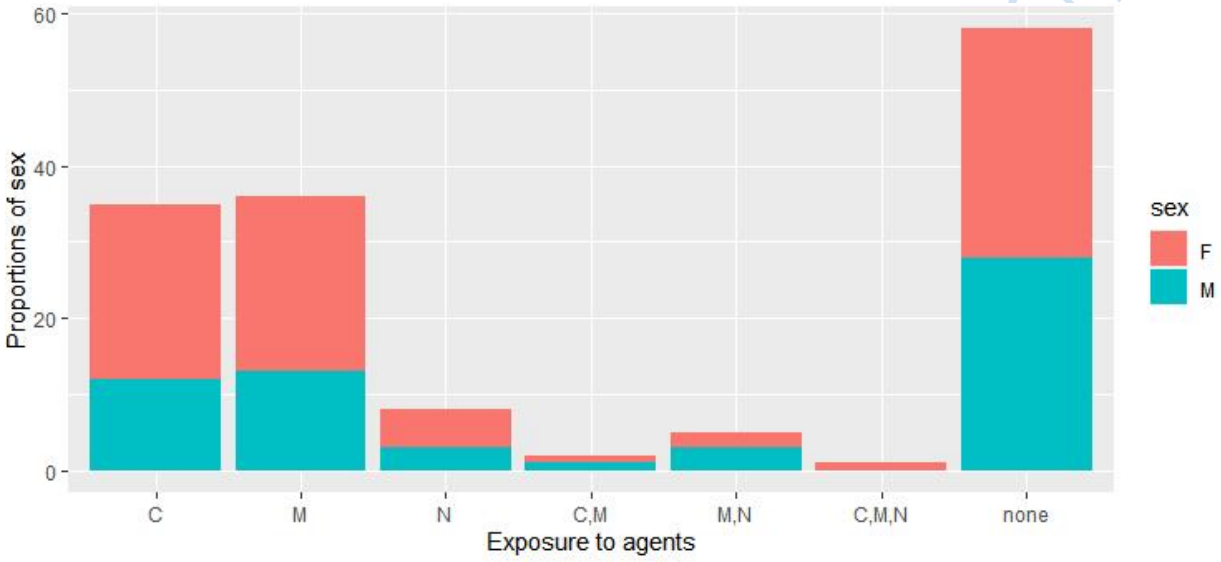

Figure 4.1: Exposure of agents to agents that can trigger haemolysis

Source, Laboratory 2022.

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Abbreviations:

F-female

M-male

C- camphor,

M- metholathum,

N- naphtalene

Table 4.2: The frequency of haemoglobin variants among sickle cell anaemia patients

variables	F (N=86)	M (N=61)	Total (N=147)	p value
Genotype				0.551
SS	82 (95.3%)	60 (98.4%)	142 (96.6%)	
SC	3 (3.5%)	1 (1.6%)	4 (2.7%)	
CC	1 (1.2%)	0 (0.0%)	1 (0.7%)	

Source, Laboratory 2022.

Chi -squared = 1.1912, df = 2, p-value = 0.5512

SS- homozygous haemoglobin S

SC- heterozygous haemoglobin S and C

CC- homozygous haemoglobin C

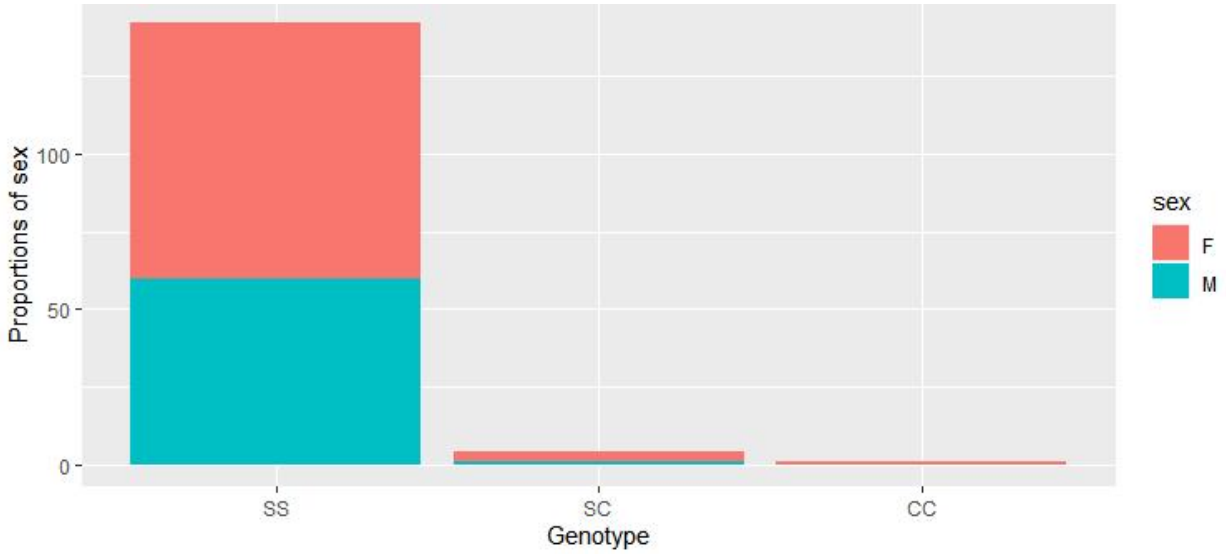

Figure 4.2: Haemoglobin electrophoresis with respect to sex

Source, Laboratory 2022.

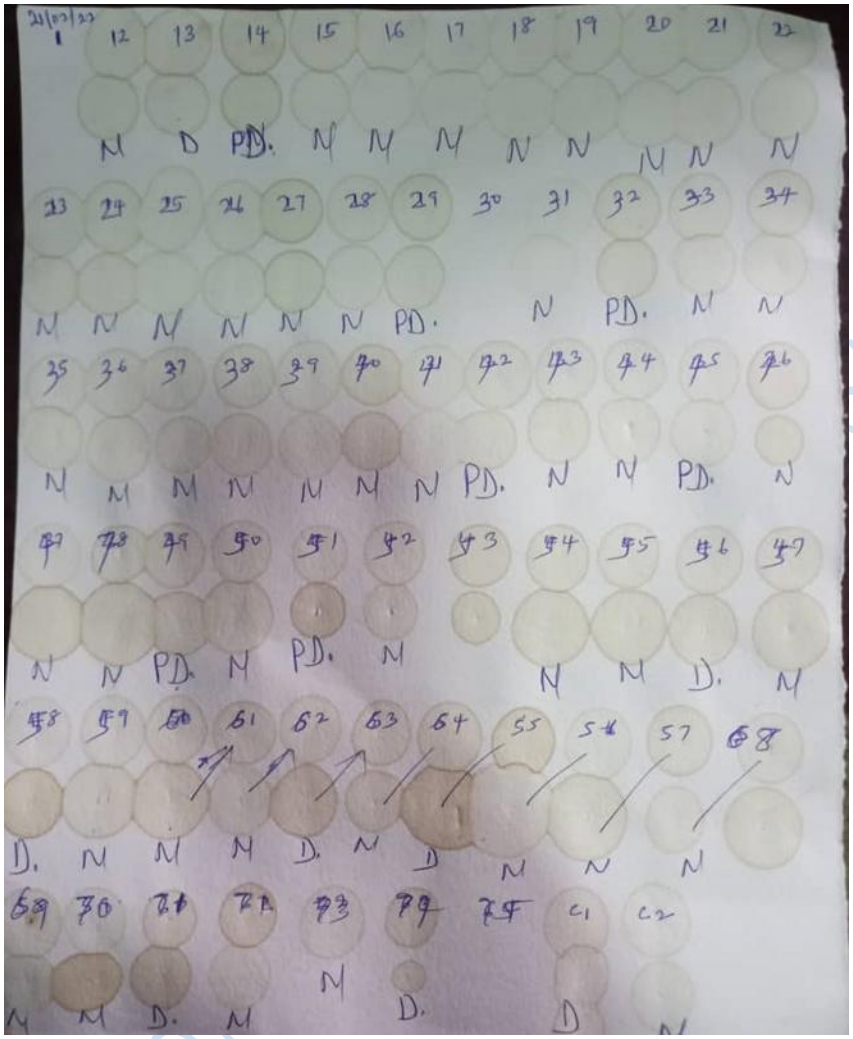

Figure 4.3: Photograph of the result of fluorescent spot test showing PD, D, and N

Source, Laboratory 2022

PD- Partial G6PD deficiency

D- Full G6PD deficiency

N- Normal G6PD

Table 4.3: Frequency G6PD status among the sickle cell anaemia patients

Variable	F (N=86)	M (N=61)	Total (N=147)	p value
G6PD.status				< 0.001
D	3 (3.5%)	18 (29.5%)	21 (14.3%)	
N	72 (83.7%)	43 (70.5%)	115 (78.2%)	
PD	11 (12.8%)	0 (0.0%)	11 (7.5%)	

Source, Laboratory 2022.

X-squared = 25.514, df = 2,

D- full deficiency of G6PD enzymes

N- normal (not deficient of G6PD enzymes)

PD- partial deficiency of G6PD enzymes

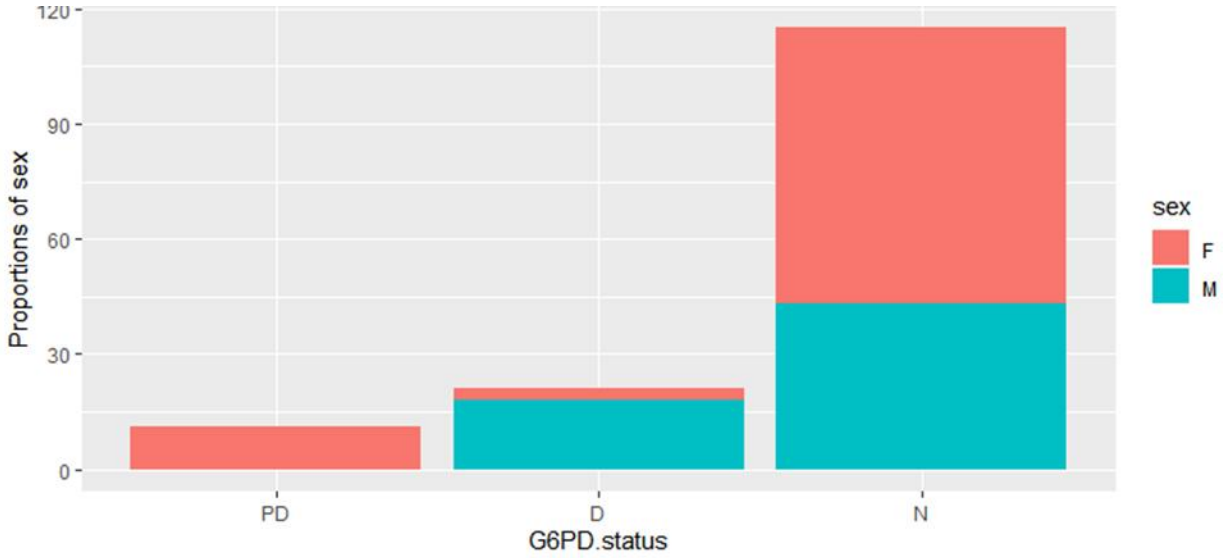

Figure 4.4: Bar chart of G6PD status with respect to sex

Source, Laboratory 2022.

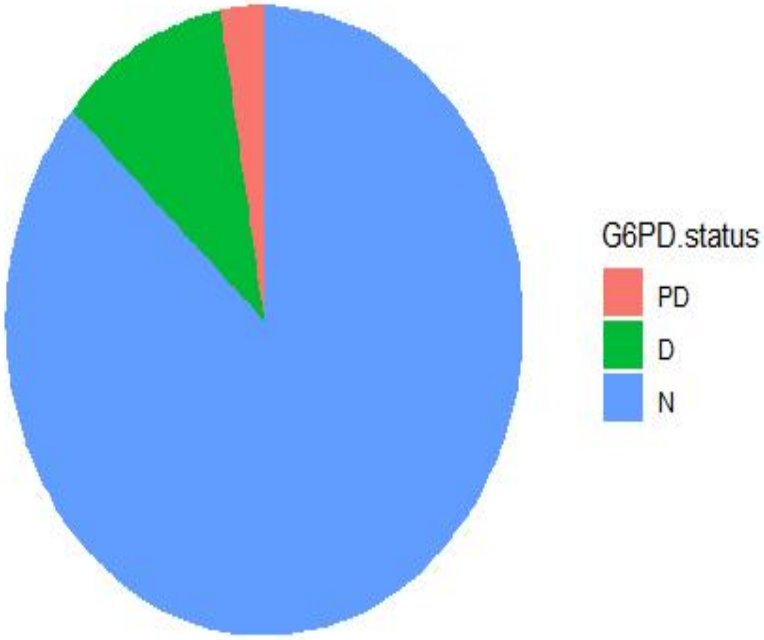

Figure 4.5: Pie chart showing the frequency of G6PD status

Source, Laboratory 2022.

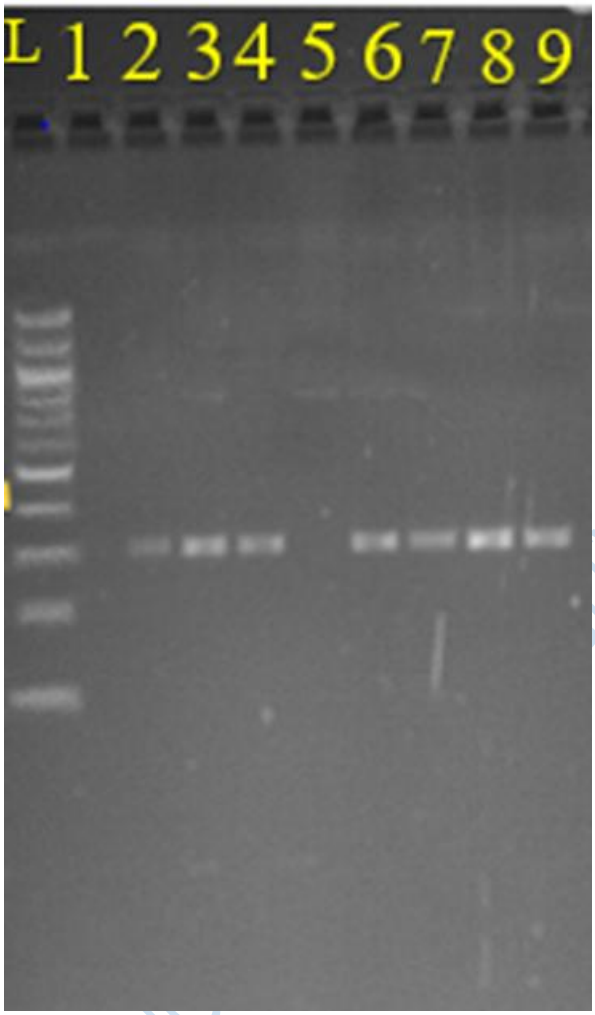

Figure 4.6: Image of gel electrophoresis showing band size of 308bp of G6PD A variant (A376G)

Source, Laboratory 2022.

Table 4.4: Frequency of A376G variant and haemoglobin genotype

	A376G (N=32)	none (N=115)	Total (N=147)	p value
Genotype				0.335
CC	0 (0.0%)	1 (0.9%)	1 (0.7%)	
SC	2 (6.2%)	2 (1.7%)	4 (2.7%)	
SS	30 (93.8%)	112 (97.4%)	142 (96.6%)	
sex				0.055
F	14 (43.8%)	72 (62.6%)	86 (58.5%)	
M	18 (56.2%)	43 (37.4%)	61 (41.5%)	

Source, Laboratory 2022.

Band.A376G and sex

Chi-squared = 2.9317, df = 1,

Band.A376G and Genotype

Chi-squared = 2.1846, df = 2,

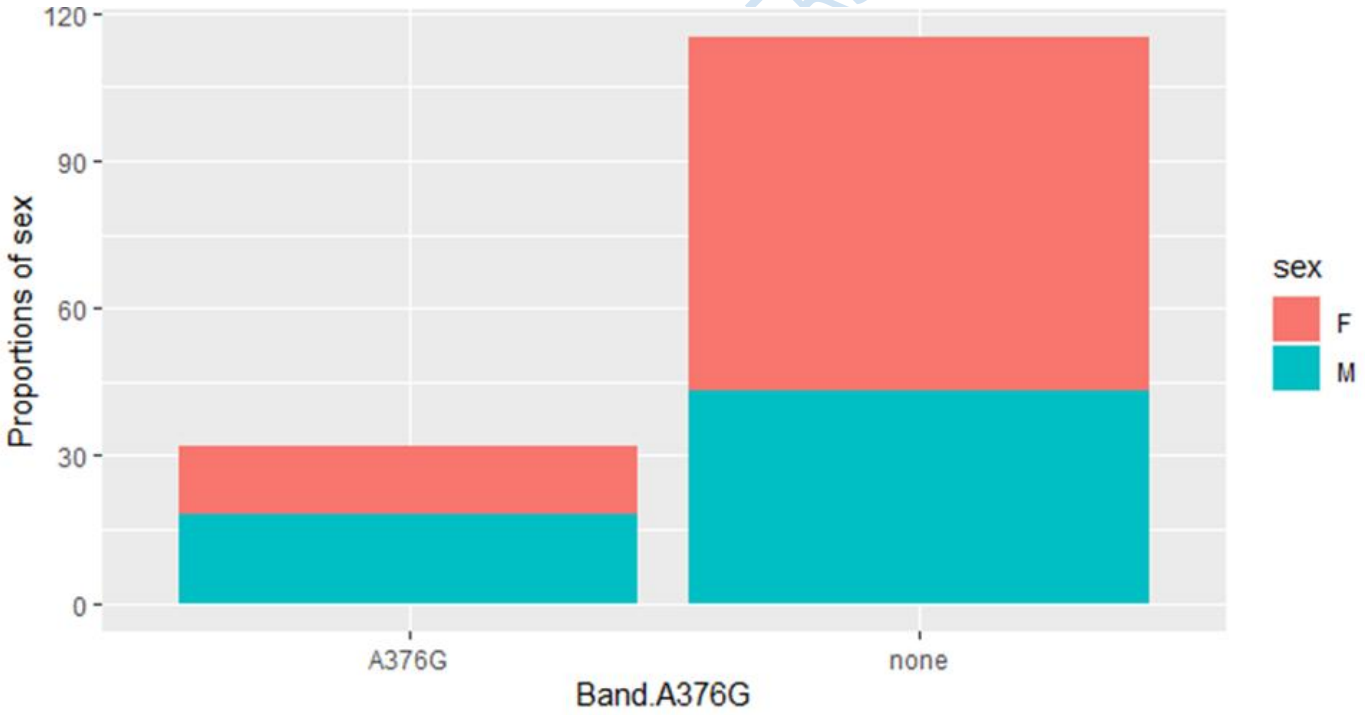

Figure 4.7: Band A376G (G6PD A variant) with respect to sex

Source, Laboratory 2022.

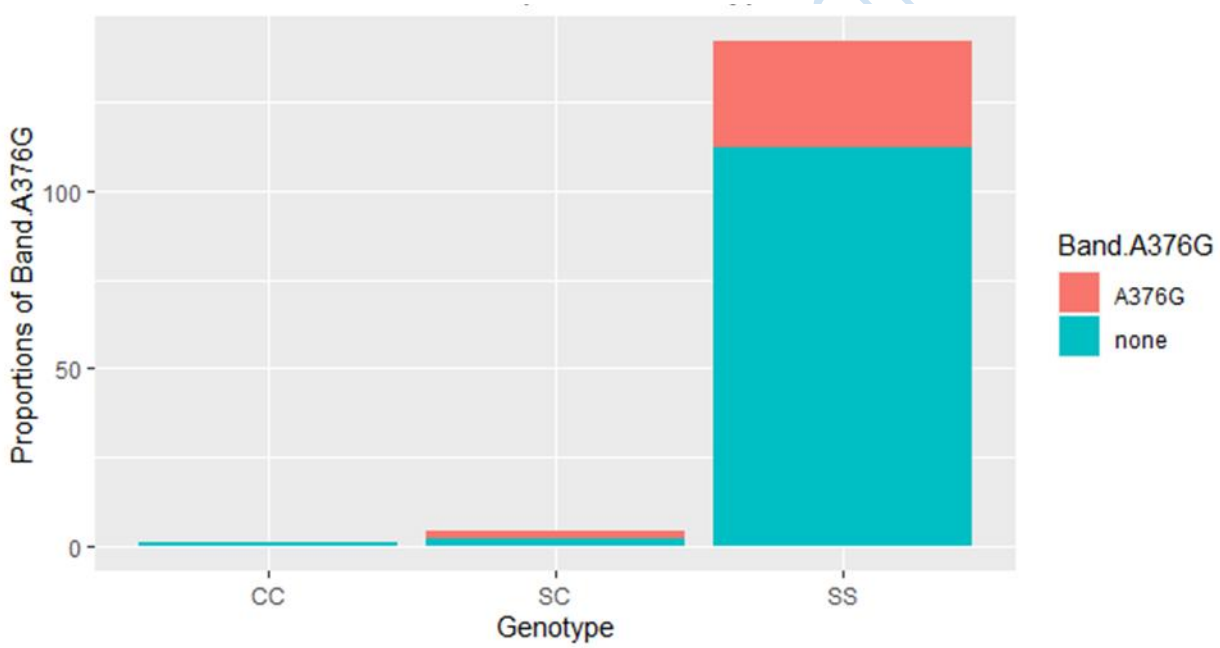

Figure 4.8: Plot of graph showing Band A376G with respect to genotype

Source, Laboratory 2022.

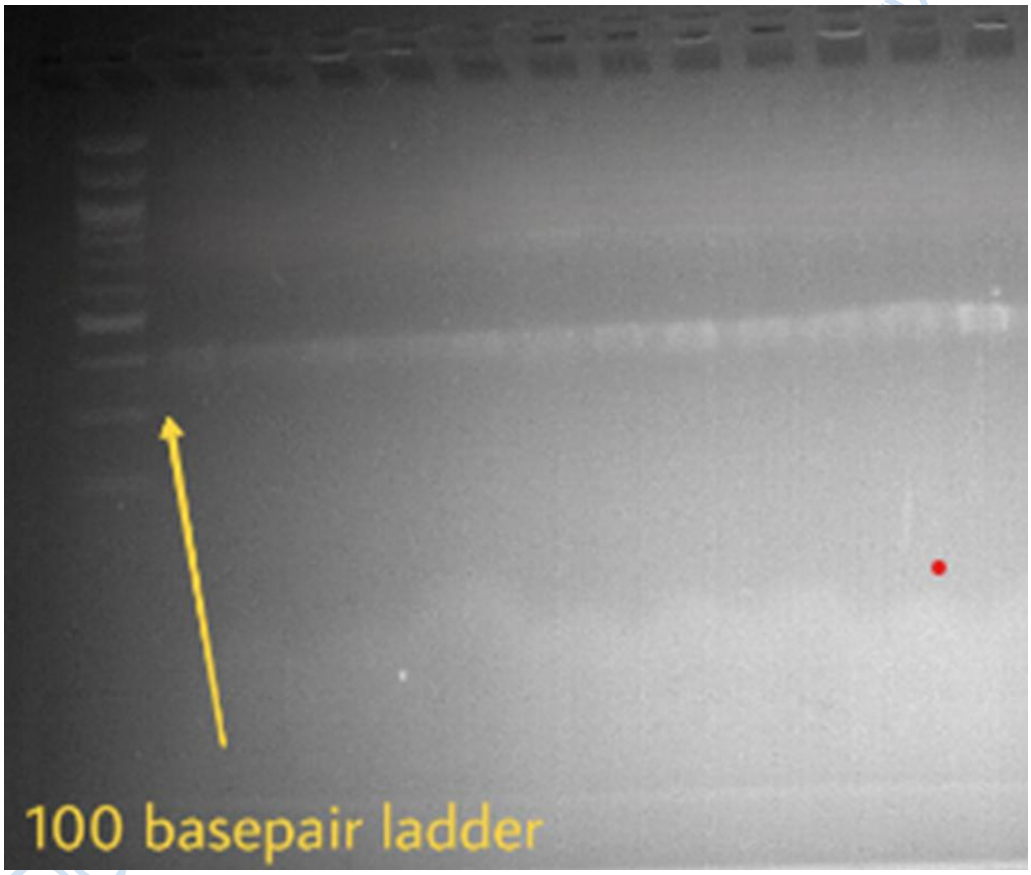

Figure 4.9: Gel electrophoresis image after digestion with of fokI restriction enzyme

Source, Laboratory 2022.

4.2.1 Research Questions

(a) What is the prevalence of haemoglobin variants present among sickle cell anaemia patients?

(b) What is the G6PD status of the sickle cell anaemia patients?

(c) Which of the G6PD variants are present among G6PD-deficient SCA patients?

(d) What is the gene polymorphism of the G6PD variant present among different haemoglobin variants?

4.3 Discussion of Findings

From this study, homozygous haemoglobin S (HbSS) has the highest frequency (96.6%0 among the participants followed by heterozyzous haemoglobin SC (HbSC) 2.7% and homozygous haemoglobin CC (HbCC) 0.7%. being the lowest among all the participants. However, a previous report among students in Ladoke Akintola University of Technology Ogbomoso, Nigeria observed 0.18% HbCC and 0.80% of HbSC which is lower compare to the result from this study[82]. Likewise, the same report from Anambra State, Nigeria indicated HbCC prevalence of 0.01% and also prevalence of 1.1% of HbSC was observed among the Yoruba ethnic nationality in Ibadan, South Western Nigeria[82].

The prevalence of those showing normal G6PD are more than those that are deficient using fluorescent spot test (FST). Among those that were deficient, full deficiency occur to be more than those showing partial deficiency among the deficient individual. The prevalent of G6PD in this study is lower than what was obtained in another study in Ghana where G6PD

deficiency was detected in 43 (35.83%) of the sickle cell disease individual made up of 16 (13.33%) males and 27 (22.50%) females of whom 17 (14.17%) had partial deficiency and 10 (8.33%) full deficiency[82]. Also, from a study carried out in Burkina Faso where there was G6PD deficiency in 20 (27.03%) out of 74 patients with major sickle cell disease (MSCS) and 26 (18.57%) out of 114 individuals with sickle cell trait[82].

Using estimates of the biochemical activity of G6PD enzymes, this study found the prevalence of 21.8% G6PD deficiency among sickle cell anaemia patients to be within the range found in various national and international studies. From previous studies, the prevalence of G6PD deficiency in SCA patients ranged from 12% to 27.3%[82]. The rates vary with study subject characteristics such as disease state (VOC or steady-state), geographical location, method of estimation, and cut-off level of G6PD activity used to define deficiency state among others. A relatively lower rate was among children less than 15 years old (15.3%) and they observed ethnic variations with SCA patients of Yoruba ethnicity bearing the highest burden of 16.9%[82]. The index study was in the adult population and also conducted in Southwestern Nigeria with predominant Yoruba participants. G6PD deficiency detected in males with SCD in Nigeria was 2–24%, also 2-25% among males in Kenya, and about 24% in Ghanaian males[82]. In Ibadan, in Nigeria, G6PD deficiency was detected in 16 males out of 100 male participants with SCD, also in 25%, 24%, and 19% among 56, 54, and 24 sickle cell anemia patients in Chicago, New York, and Los Angeles, respectively, in USA. Again, 22% out of 41 sickle cell anaemia patients were found to be G6PD-deficient in Lubumbashi, Zaire[82].

The prevalence of G6PD A variant (rs1050829, 376 A→G, chrX:154535277; 156 asn → asp) obtained in this study is 21.8% which is similar to the one obtained in a study whereby it was stated that A376G was detected in 23.26% (20/86 patients) of individuals, whereas G202A and C563T were absent. In another study which has a lower prevalence showed that 272 (87.5%) out of 311 clinically determined malaria patients carried the normal G6PD allele while 39 (12.5%) carried the G6PD A (A376G) variant with the absence of G6PD A- and G6PD Mediterranean variants in the study[82].

Endnote

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Chapter Five

Conclusion

5.1 Summary of the Findings

As earlier mentioned, this study has helped to found out that homozygous haemoglobin S (HbSS) has the highest prevalence among the haemoglobin variants followed by heterozygous haemoglobin SC while homozygous haemoglobin CC has the least among the participants. This has shown that most patients that attend the selected sickle cell clinic are HbSS and special care has to be taking in selecting the medication to be given to them.

G6PD deficiency is most prevalent among the participants that have HbSS variant and thus they are likely to have double disadvantages of haemolytic crisis. There can be increase in the breaking down of red blood cells of those that are HbSS because many of them are G6PD deficient. They are also likely to be exposed to some agents that can trigger haemolysis in

them if they are not aware of their G6PD status. For instant, mentholathum and naphthalene used when they have pain can as well result into lysis of their red blood cells.

It was also observed from the study that all that were G6PD deficient has G6PD A variant gene (rs1050829, 376 A→G, chrX:154535277; 156 asn → asp) which has 308bp. No G6PD A gene polymorphism were observed among those that shown G6PD deficiency (both partial and full) after the digestion with of fokI restriction enzyme using restriction fragment length polymorphism (RFLP) PCR.

Since none of them shown G6PD A gene polymorphism, then there was no G6PD A variant gene polymorphism observed among the various haemoglobin variants (HbSS, HbSC and HbCC) present in the study.

5.2 Conclusion

This study had shown that homozygous haemoglobin S (HbSS) has the highest prevalence among the haemoglobin variants while homozygous haemoglobin CC has the least among the participants.

From this study, G6PD deficiency is most prevalent among the participants that have HbSS variant and thus they are likely to have double disadvantages of haemolytic crisis.

No G6PD A gene polymorphism were observed among those that shown G6PD deficiency (both partial and full).

Since none of them shown G6PD A gene polymorphism, then there was no G6PD A variant gene polymorphism observed among the various haemoglobin variants (HbSS, HbSC and HbCC).

The homozygous haemoglobin S (HbSS) has the highest prevalence among the haemoglobin variants and majority of them as well have G6PD deficiency among all other haemoglobin variants present in the study. SCA and G6PD deficiency are both inheritable diseases of the red blood cells that can co-exist in an individual. The impact of comorbidity has been reported in various studies but their findings were not consistent. This study using biochemical activity activities of red cell enzyme found the prevalence of 21.8% G6PD deficiency in SCA patients. G6PD A variant (A376G) mutation being the commonest variant and was the only one observed in this study which confer approximately 85% enzyme activity of a non-deficient individual. Though any other variants that will have significant clinical manifestation of G6PD-related haemolysis or appearing to confer resistance to malaria must have the G6PD A variant mutation plus any other mutation which may include a202g

. The G6PD deficient individuals should be counsel including proper guidelines and orientation of their status to ensure avoidance of agents, medications and any other things that can trigger haemolysis in them.

5.3 Recommendations

(i) With this study I recommend that there should be a point of care (POC) method for screening of everyone for glucose-6-phosphate dehydrogenase in all levels of health sector.

(ii) The health care educator should ensure orientation of any individual discovered to be deficient whether full deficiency or partial deficiency on what they should avoid which may trigger haemolysis in them.

(iii) Furthermore, awareness should be created in all our media houses to sensitize the society on the hazard and the repercurssion on the ignorance of this disorder.

(iv) Governments parastatal, non-governmental organization and phylantropis should subsidize the screening and management of these disorders

(v) The screening of glucose-6-phosphate dehydrogenase should also be included in the neonatal test before discharge of the neonate.

5.4 Contribution to Knowledge

Through this study, it has been established that the triggered agents (camphor, naphthalene and metholathum) which are commonly used in our environment would elicit breakdown of red blood cells in the individual that are glucose-6-phosphate dehydrogenase deficient which could complicate the haemolytic crisis among sickle cell patients.

This study has allowed us to determine the various haemoglobin variants among the sickle cell anaemia patients attending the selected sickle cell clinics in Ibadan.

With this study, we have been able to assess the glucose-6-phosphate dehydrogenase status of the sickle cell anaemia patients attending the selected sickle cell clinics in Ibadan.

This study also helped us to determine the gene responsible for glucose-6-phosphate dehydrogenase deficiency among the sickle cell anaemia in the selected sickle cell clinics in Ibadan.

5.5 Suggested Areas for Further Research Work

There are other agents that trigger haemolysis among individual that are glucose-6-phosphate dehydrogenase deficient, as a result of this, the severity of the haemolysis expressed can be looked into in further research.

Assessment of other variants of glucose-6-phosphate dehydrogenase enzyme in our area which include G6PD A- and G6PD Mediterranean could also be studied.

World health organization has been able to classify the various glucose-6-phosphate dehydrogenase variant therefore further work can be done to categorize the variant present in our environment.

Bio-data

A. Personal Data

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2. Date and Place of Birth: 16/09/1984 and Place of Birth: Ibadan

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4. Name and Address of Next of Kin: Kharashi Abideen Titilope, Plot 6&7 Suara Yusuf Street, Iwo Road, Ibadan.

B. Educational Background

Educational Institutions Attended with Dates and Qualification

i. St. Demonstration Pry School, Molete, Ibadan. (1995)
PSLC

ii. Ikolaba Grammar School, Agodi, Ibadan. (2001)
SSCE

iii. Ladoke Akintola University of Technology, Ogbomoso, Nigeria. (2008)
B.MLS

iv. Nigeria Youth Service Corps. (2010)
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C. Working Experience with Dates

Praise Medical Diagnostic, Yemetu, Ibadan.
2011

University College Hospital, Ibadan.
2014

D. Awards and fellowships (if any) Nil

E. Membership of Academic Professional Bodies

Member, Association of Medical Laboratory Scientist of Nigeria (AMLSN)
2008

Associate, Medical Laboratory Science Council of Nigeria (MLSCN)
2008

F. Publication (if any)

1. Thesis/Dissertation Nil

2. Book/Monographs Nil

3. Scholarly Articles Nil

4. Notable Scholar or Professional Accomplishments Nil

5. Major Conferences/Workshops attended

3rd Internatioal Conference of the Faculty of Natural and Applied Sciences
Lead City University Ibadan, Nigeria.
2022

6. Date ……. Signature …….

University Compliance Certification

This is to certify that the Thesis of **Kharashi-Kareem Bilikis** with Matriculation Number **LCU/PGD/001514**, in the Department of Biological Sciences, Faculty of Natural and Applied Sciences, Lead City University, Ibadan, Nigeria is in full compliance with the approved University format and style.

Date _____

Signature _____