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**Quantization of Hemoglobin Types among sickle Cell Anemia
Patients Attending Medical Army Hospital in Khartoum State**

**تحديد كمية انواع خضاب الدم المختلفة لدى مرضي الانيميا
المنجلية بالمستشفى العسكري بولاية الخرطوم**

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الآية

بسم الله الرحمن الرحيم

﴿اللَّهُ نُورُ السَّمَاوَاتِ وَالْأَرْضِ ۚ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ ۚ الْمِصْبَاحُ فِي زُجَاجَةٍ ۚ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ دُرِّيٌّ يُوقَدُ مِنْ شَجَرَةٍ مُبَارَكَةٍ زَيْتُونَةٍ لَا شَرْقِيَّةٍ وَلَا غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ ۚ نُورٌ عَلَى نُورٍ ۚ يَهْدِي اللَّهُ لِنُورِهِ مَن يَشَاءُ ۚ وَيَضْرِبُ اللَّهُ الْأَمْثَالَ لِلنَّاسِ ۚ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ﴾

صدق الله العظيم

سورة النور الآية (35)

Dedication

This work is dedicated to the soul of my lovely second mother (grand mother) for her unstoppable supports when she was with me, may the Almighty Allah open the pearly gates of the heavens and paradise for her.

To my lovely mother for her encouragement.

To my kind uncle for his large support.

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ABSTRACT

This is prospective, analytical and cross- sectional study carried out in Medical Army hospital in Khartoum State, during April 2015 - July 2015.

Forty sickle cell disease patients were studied, 18 were males and 22 were females, their ages 1-13 years, 23(58%) were sickle cell anemia (HbSS), 17(42%) were sickle cell trait (HbAS) as known cases by hemoglobin electrophoresis.

The aim of this study to measure the level different hemoglobin types in the study group and comparison of this hemoglobin types in both homozygote and heterozygote states of sickle cell disease patients.

Estimation hemoglobin types were carried out by CAPILLARYS 2 FLEX-PIERCING instrument and the results were analyzed by SPSS computer programme.

The result revealed that the level of HbF in sickle cell disease patients is (7.2 ± 4.9) where as the level of HbA ,HbA2 and HbS were (78.5 ± 19.2), (3.1 ± 0.64) and (11.2 ± 21.5).

The level of HbS is the higher indicate that most patients have large level of HbS which is the diagnostic hemoglobin for sickle cell anemia.

The mean of HbF is increased because all patients given hydroxyurea and other supportive treatment. HbA2 show normal value, while HbA is decreased.

HbF, HbS, HbA2 and HbA (%) show different means and std Deviations in males and females as HbF % in males (6.3 ± 3.5) while in females (8.1 ± 5.7), HbS % in males (75.4 ± 24.6) while in females (80.9 ± 13.3), HbA2 (%) in males (3.3 ± 0.66) while in females (2.9 ± 0.60) and HbA (%) in males (14.9 ± 27.5) while in females (8.2 ± 15.0).

The mean and standard deviation of different hemoglobin types in homozygous patients (SS) and heterozygous patients (AS) are HbF (%) in (HbSS) patients (9.7 ± 4.5) while in (HbAS) patients (3.9 ± 3.3).

Fetal Hb is higher in homozygous than heterozygous, it is concluded that sickle cell anemia patients HbF level more than sickle cell trait patients.

HbS (%) in (HbSS) patients (87.1 ± 4.3) while in (HbAS) patients (66.7 ± 24.8) there is a difference in HbS in homozygous and heterozygous patients, sickle Hb is higher in homozygous than heterozygous and this is because homozygous have two copies of HbS while heterozygous have just one copy of HbS.

HbA2 (%) in (HbSS) patients (3.3 ± 0.66) while in (HbAS) patients (2.9 ± 0.56), means no difference in HbA2 in homozygous and heterozygous states.

HbA (%) in (HbSS) patients ($.000 \pm .000$) while in (HbAS) patients (26.4 ± 26.5), adult Hb is higher in heterozygous than homozygous and this is because homozygous have no HbA gene while heterozygous have one copy of HbA gene.

Correlation between HbF (%) and age (years) among the study group show Strong negative correlation.

Correlation between HbS (%) and age (years) among the study group show Strong negative correlation.

Correlation between HbA2 (%) and age (years) among the study group show Weak negative correlation.

Correlation between HbA (%) and age (years) among the study group show positive correlation.

مستخلص البحث

هذه دراسة وصفية ، تحليلية ومقطعية أجريت في المستشفى العسكري في الفترة ما بين ابريل الى يوليو 2015م.

وقد كان عددمرضى فقر الدم المنجلي أربعون مريضاً 18 منهم ذكور بينما 22 من الإناث تتراوح اعمارهم بين سنة إلى 13 سنة، 23 (58%) منهم مصابون بالأنيميا المنجلية الموروثة، 17 (42%) منهم حاملو العامل غير المصابين بالأنيميا المنجلية تم تحديدهم بتحليل انواع خضاب الدم.

هدفت هذه الدراسة الى قياس مستويات خضاب الدم المختلفه عند مرضي الأنيميا المنجلية بالإضافة الي مقارنة مستوي هذه الأنواع المختلفه عند مرضي الأنيميا المنجلية وعند حاملو العامل غير المصابين بالأنيميا المنجلية.

تم قياس مستويات خضاب الدم المختلفه عن طريق جهاز التحليل الشعري ، وقد تم تحليل النتائج بواسطة برنامج الحزم الإحصائية للمجتمع .

وقد أظهرت النتائج أن مستوي خضاب الدم الجنيني عند جميع المرضى (7.2 ± 4.9) ، مستوي خضاب الدم المنجلي، مستوي خضاب الدم النضجي الثاني ومستوي خضاب الدم النضجي هي (19.2 ± 78.5) ، (0.64 ± 3.1) و (21.5 ± 11.2) بالترتيب.

نسبة خضاب الدم المنجلي هي الاكثر مما يعني ان معظم المرضى هم من مصابي الأنيميا المنجلية ، ويعتبر هذا النوع من خضاب الدم الفحص التأكيدي للأنيميا المنجلية.

نسبة خضاب الدم الجنيني مرتفعه لأن جميع المرضى اعطوا هيدروكسي يوريا كعلاج وغيره من الأدوية الداعمة. نسبة خضاب الدم الجنيني الثاني طبيعیه، في حين انخفضت نسبة خضاب الدم النضجي.

المتوسطات والانحرافات المعيارية لانواع خضاب الدم المختلفه في الذكور والإناث كالآتي
خضاب الدم الجنيني في الذكور (3.5 ± 6.3) بينما في الإناث (5.7 ± 8.1) ، خضاب الدم المنجلي في الذكور (24.6 ± 75.4) بينما في الإناث (13.3 ± 80.9) ، خضاب الدم النضجي الثاني في الذكور (0.66 ± 3.3) بينما في الإناث (0.60 ± 2.9) و خضاب الدم النضجي في الذكور (27.5 ± 14.9) بينما في الإناث (8.2 ± 15.0) .

المتوسط والانحراف المعياري لانواع خضاب الدم المختلفه في المصابين بالأنيميا المنجلية وحاملو العامل غير المصابين بالأنيميا المنجلية كالآتي :

خضاب الدم الجنيني عند مرضي الأنيميا المنجلية (4.5 ± 9.7)، بينما عند حاملي العامل غير المصابين (3.3 ± 3.9). خضاب الدم المنجلي عند المرضي (3.4 ± 87.1)، بينما عند حاملي العامل (24.8 ± 66.7). نسبة خضاب الدم الجنيني اعلي عند مرضي الأنيميا المنجلية منه عند حاملي العامل غير المصابين، وايضا نسبة خضاب الدم المنجلي اعلي عند مرضي الأنيميا المنجلية لأنهم تورثوا جينين مصابين من كلتا الابوين. خضاب الدم النضجي الثاني عند مرضي الأنيميا المنجلية (0.66 ± 3.3)، بينما عند حاملي العامل غير المصابين (0.56 ± 2.9). لا يوجد فرق بين بة خضاب الدم الثاني عند المصابين وغير المصابين. خضاب الدم النضجي عند مرضي الأنيميا المنجلية ($000. \pm 000$)، بينما عند حاملي العامل (26.5 ± 24.6). نسبة خضاب الدم النضجي اعلي عند حاملي العامل غير المصابين لان مرضي الأنيميا المنجلية ليس لديهم خضاب الدم النضجي، بينما حاملي العامل غير المصابين لديهم نسخة واحدة من خضاب الدم النضجي. العلاقة بين نسبة خضاب الدم الجنيني وعمر المريض علاقة عكسية قوية، وايضا علاقة خضاب الدم المنجلي وعمر المريض علاقة عكسية قوية. العلاقة بين نسبة خضاب الدم النضجي الثاني وعمر المريض علاقة عكسية ضعيفة، بينما العلاقة بين خضاب الدم النضجي وعمر المريض علاقة طردية.

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Abbreviations

2, 3-BPG: 2, 3-bisphosphoglycerate

2, 3-DPG: 2, 3-disphosphoglycerate

A: Adenine

ACS: Acute Chest Syndrome

D.W: Distilled Water

EDTA: Ethylene Diamine Tetra Acetic acid

G6PD: Glucose 6 Phosphate Dehydrogenase

Hb: Hemoglobin

HbA: Adult Hemoglobin

HbA2: Adult Hemoglobin 2

HbAS: Heterozygous Sickle Hemoglobin

HbC: Crystallised Hemoglobin

HbF: Foetal Hemoglobin

HbG_S: Sickle Hemoglobin

HbS: Sickle Hemoglobin

HbSS: Homozygous Sickle Hemoglobin

HDAC: Histone Deacetylase

HbG_{SC}: Crystallised Sickling Hemoglobin

HLA: Human Leukocyte Antigen

HPFH: Hereditary Persistence of Fetal Hemoglobin

PCA: patient-Controlled Analgesia

QTL: Quantitative Trait Loc

RBC: Red Blood Cell

SCA: Sickle Cell Anaemia

SCD: Sickle Cell Disease

SNP: Single Nucleotide Polymorphism

SPSS: Statistical Package for Social Science

T: Thymine

TCD: Transcranial Doppler

β -globin gene: Beta globin gene

Chapter One

Introduction and Literature review

Introduction and Literature review

1.1 Sickle-cell disease:

Sickle-cell disease (SCD), also known as sickle-cell anemia (SCA) and drepanocytosis, is a hereditary blood disorder, characterized by an abnormality in the oxygen-carrying hemoglobin molecule in red blood cells. This leads to a propensity for the cells to assume an abnormal, rigid, sickle-like shape under certain circumstances. Sickle-cell disease is associated with a number of acute and chronic health problems, such as severe infections, attacks of severe pain ("sickle-cell crisis"), and stroke, and there is an increased risk of death (Naghavi,2013).

Sickle-cell disease occurs when a person inherits two abnormal copies of the hemoglobin gene, one from each parent. Several subtypes exist, depending on the exact mutation in each hemoglobin gene. A person with a single abnormal copy does not experience symptoms and is said to have sickle-cell trait. Such people are also referred to as carriers(Naghavi, 2013).

The complications of sickle-cell disease can be prevented to a large extent with vaccination, preventive antibiotics, blood transfusion, and the drug hydroxyurea/hydroxycarbamide. A small proportion requires a transplant of bone marrow cells (Naghavi,2013).

Almost 300,000 children are born with a form of sickle-cell disease every year, mostly in sub-Saharan Africa, but also in other parts of the world such as the WestIndies and in people of African origin elsewhere in the world. In 2013 it resulted in 176,000 deaths up from 113,000 deaths in 1990 (Naghavi,2013).The condition was first described in the medical literature by the American physician James BHerrick in 1910, and in the 1940s and 1950s contributions by Nobel prize-winner LinusPauling made it the first disease where the exact genetic and molecular defect was elucidated (Naghavi,2013).

1.1.1 History:

The first modern report of sickle-cell disease may have been in 1846, where the autopsy of an executed runaway slave was discussed; the key finding was the absence of the spleen (Lebby, 1846; Ballaset *al.*, 2012). There were also reports amongst African slaves in the United States exhibiting resistance to malaria but being prone to leg ulcers (Ballaset *al.*, 2012). The abnormal characteristics of the red blood cells, which later lent their name to the condition, was first described by Ernest Edward Irons (1877–1959), intern to the Chicago cardiologist and professor of medicine James B. Herrick (1861–1954), in 1910 (Herrick ,1910; Savitt and Goldberg , 1989). Irons saw "peculiar elongated and sickle-shaped" cells in the blood of a man named Walter Clement Noel, a 20-year-old first-year dental student from Grenada. Noel had been admitted to the Chicago Presbyterian Hospital in December 1904 suffering from anemia (Herrick, 1910; Savitt and Goldberg, 1989). Noel was readmitted several times over the next three years for "muscular rheumatism" and "bilious attacks" but completed his studies and returned to the capital of Grenada (St. George's) to practice dentistry , He died of pneumonia in 1916(Savitt and Goldberg , 1989; Serjeant , 2010).

Shortly after the report by Herrick, another case appeared in the Virginia Medical Semi-Monthly with the same title, "Peculiar Elongated and Sickle-Shaped Red Blood Corpuscles in a Case of Severe Anemia (Washburn, 1911). In the later description by Verne Mason in 1922, the name "sickle cell anemia" is first used (Serjeant, 2010; Mason, 1922). Childhood problems related to sickle cells disease were not reported until the 1930s, despite the fact that this cannot have been uncommon in African-American populations (Ballaset *al.*, 2012).

The Memphis physician Lemuel Diggs, a prolific researcher into sickle cell disease, first introduced the distinction between sickle cell disease and trait in 1933, although it took until 1949 until the genetic characteristics were elucidated

by James V. Neel and E.A. Beet (Serjeant, 2010). 1949 was the year when Linus Pauling described the unusual chemical behavior of hemoglobin S, and attributed this to an abnormality in the molecule itself (Serjeant , 2010; Pauling and Itano ,1949).The actual molecular change in HbS was described in the late 1950s by Vernon Ingram (Serjeant , 2010). The late 1940s and early 1950s saw further understanding in the link between malaria and sickle cell disease. In 1954, the introduction of hemoglobin electrophoresis allowed the discovery of particular subtypes, such as HbSC disease (Serjeant , 2010).

The 1990s saw the development of hydroxycarbamide, and reports of cure through bone marrow transplantation appeared in 2007 (Serjeant, 2010).

Gene therapy:

In 2001 it was reported that sickle-cell disease had been successfully treated in mice using gene therapy (Pawliuk *et al.*, 2001; Wilson and Jennifer Fisher, 2002).

1.1.2 SCD in Sudan:

The first report of presence of sickle cell anemia in Sudan was in (1926) by Archibald further studies have shown that they found the disease in Western Sudan especially among the Messeria tribe in Kordofan state, with prevalence rate up to 30%. Southern with prevalence rate up to 18% among the southern Nylotes, Blue Nile and Sinnar state. East central Sudan with prevalence rate up to 5% among the indigenous population and up to 16% among Sudanese tribes (Plaitet *et al.*, 1991).

1.1.3 Signs and symptoms:

Sickle-cells in human blood, both normal red blood cells and sickle-shaped cells are present.

Normal blood cells next to a sickle-blood cell, colored scanning electron microscope image.

Sickle-cell disease may lead to various acute and chronic complications, several of which have a high mortality rate(Yawnet *al.*, 2014).

Sickle-cell crisis:

The terms "sickle-cell crisis" or "sickling crisis" may be used to describe several independent acute conditions occurring in patients with SCD. SCD results in anemia and crises that could be of many types including the vaso-occlusive crisis, aplastic crisis, sequestration crisis, haemolytic crisis, and others. Most episodes of sickle-cell crises last between five and seven days(Lennette,2000). Although infection, dehydration, and acidosis (all of which favor sickling) can act as triggers, in most instances, no predisposing cause is identified (Kumaret *al.*,2009).

Vaso-occlusive crisis:

The vaso-occlusive crisis is caused by sickle-shaped red blood cells that obstruct capillaries and restrict blood flow to an organ resulting in ischemia, pain, necrosis, and often organ damage. The frequency, severity, and duration of these crises vary considerably. Painful crises are treated with hydration, analgesics, and blood transfusion; pain management requires opioid administration at regular intervals until the crisis has settled. For milder crises, a subgroup of patients manages on NSAIDs (such as diclofenac or naproxen). For more severe crises, most patients require inpatient management for intravenous opioids; patient-controlled analgesia devices are commonly used in this setting. Vaso-occlusive crisis involving organs such as the penis(Olujohungbe and Burnett, 2013).or lungs are considered an emergency and treated with red-blood cell transfusions. Incentive spirometry, a technique to encourage deep breathing to minimise the development of atelectasis, is recommended.(Glassberg, 2011).

Splenic sequestration crisis:

Because of its narrow vessels and function in clearing defective red blood cells, the spleen is frequently affected.(Anie and Green, 2012) It is usually

infarcted before the end of childhood in individuals suffering from sickle-cell anemia. This spleen damage increases the risk of infection from encapsulated organisms (Pearson, 1977; Wong *et al.*, 1992). Preventive antibiotics and vaccinations are recommended for those lacking proper spleen function (Khatibet *al.*, 2009).

Splenic sequestration crises are acute, painful enlargements of the spleen, caused by intrasplenic trapping of red cells and resulting in a precipitous fall in hemoglobin levels with the potential for hypovolemic shock. Sequestration crises are considered an emergency. If not treated, patients may die within 1–2 hours due to circulatory failure. Management is supportive, sometimes with blood transfusion. These crises are transient; they continue for 3–4 hours and may last for one day (Khatibet *al.*, 2009).

Acute chest syndrome:

Acute chest syndrome (ACS) is defined by at least two of the following signs or symptoms: chest pain, fever, pulmonary infiltrate or focal abnormality, respiratory symptoms, or hypoxemia (Glassberg, 2011). It is the second-most common complication and it accounts for about 25% of deaths in patients with SCD, majority of cases present with vaso-occlusive crises then they develop ACS (MekontsoDessapet *al.*., 2008; Paul *et al.*., 2011). Nevertheless, about 80% of patients have vaso-occlusive crises during ACS (MekontsoDessapet *al.*, 2008; Paulet *al.*, 2011).

Aplastic crisis:

Aplastic crises are acute worsenings of the patient's baseline anemia, producing pale appearance, fast heart (tachycardia) rate, and fatigue. This crisis is normally triggered by parvovirus B19, which directly affects production of red blood cells by invading the red cell precursors and multiplying in and destroying them (Kumar *et al.*., 2009). Parvovirus infection almost completely prevents red blood cell

production for two to three days. In normal individuals, this is of little consequence, but the shortened red cell life of SCD patients results in an abrupt, life-threatening situation. Reticulocyte counts drop dramatically during the disease (causing reticulocytopenia), and the rapid turnover of red cells leads to the drop in hemoglobin. This crisis takes 4 days to one week to disappear. Most patients can be managed supportively; some need blood transfusion (Slavovet *et al.*, 2011).

Haemolytic crisis:

Haemolytic crises are acute accelerated drops in hemoglobin level. The red blood cells break down at a faster rate. This is particularly common in patients with coexistent G6PD deficiency (Balgir, 2012). Management is supportive, sometimes with blood transfusions (Glassberg, 2011).

Other:

One of the earliest clinical manifestations is dactylitis, presenting as early as six months of age, and may occur in children with sickle-cell trait (Jadavji and Prober, 1985). The crisis can last up to a month (Worrall and Butera, 1976). Another recognized type of sickle crisis, acute chest syndrome, is characterized by fever, chest pain, difficulty breathing, and pulmonary infiltrate on a chest X-ray. Given that pneumonia and sickling in the lung can both produce these symptoms, the patient is treated for both conditions (Miller, 2011). It can be triggered by painful crisis, respiratory infection, bone-marrow embolisation, or possibly by atelectasis, opiate administration, or surgery (Miller, 2011).

1.1.4 Genetics:

Normally, humans have hemoglobin A, which consists of two alpha and two beta chains, hemoglobin A₂, which consists of two alpha and two delta chains, and hemoglobin F, consisting of two alpha and two gamma chains in their bodies. Of

these, hemoglobin F dominates until about 6 weeks of age then A dominates throughout life (Green *et al.*, 1993).

The types of hemoglobin a person makes in the red blood cells depend on what hemoglobin genes are inherited from her or his parents. If one parent has sickle-cell anemia and the other has sickle-cell trait, then the child has a 50% chance of having sickle-cell disease and a 50% chance of having sickle-cell trait. When both parents have sickle-cell trait, a child has a 25% chance of sickle-cell disease, 25% do not carry any sickle-cell alleles, and 50% have the heterozygous condition (Green *et al.*, 1993).

Sickle-cell gene mutation probably arose spontaneously in different geographic areas, as suggested by restriction endonuclease analysis. These variants are known as Cameroon, Senegal, Benin, Bantu, and Saudi-Asian. Their clinical importance is because some are associated with higher HbF levels, e.g., Senegal and Saudi-Asian variants, and tend to have milder disease (Green *et al.*, 1993).

In people heterozygous for HgbS (carriers of sickling hemoglobin), the polymerization problems are minor, because the normal allele is able to produce over 50% of the hemoglobin. In people homozygous for HgbS, the presence of long-chain polymers of HbS distort the shape of the red blood cell from a smooth doughnut-like shape to ragged and full of spikes, making it fragile and susceptible to breaking within capillaries. Carriers have symptoms only if they are deprived of oxygen (for example, while climbing a mountain) or while severely dehydrated. The sickle-cell disease occurs when the sixth amino acid, glutamic acid, is replaced by valine to change its structure and function; as such, sickle-cell anemia is also known as E6V. Valine is hydrophobic, causing the hemoglobin to collapse on itself occasionally. The structure is not changed otherwise. When enough hemoglobin collapses on itself the red blood cells become sickle-shaped (Allison, 2009).

The gene defect is a known mutation of a single nucleotide (single-nucleotide polymorphism - SNP) (A to T) of the β -globin gene, which results in glutamic acid being substituted by valine at position 6. Note, historic numbering put this glutamic acid residue at position 6 due to skipping the methionine start codon in protein amino acid position numbering. Current nomenclature calls for counting the methionine as the first amino acid, resulting in the glutamic acid residue falling at position 7. Many references still refer to position 6 and both should likely be referenced for clarity. Hemoglobin S with this mutation is referred to as HbS, as opposed to the normal adult HbA. The genetic disorder is due to the mutation of a single nucleotide, from a GAG to GTG codon on the coding strand, which is transcribed from the template strand into a GUG codon. This is normally a benign mutation, causing no apparent effects on the secondary, tertiary, or quaternary structures of hemoglobin in conditions of normal oxygen concentration. What it does allow for, under conditions of low oxygen concentration, is the polymerization of the HbS itself. The deoxy form of hemoglobin exposes a hydrophobic patch on the protein between the E and F helices (Allison, 2009). The hydrophobic side chain of the valine residue at position 6 of the beta chain in hemoglobin is able to associate with the hydrophobic patch, causing hemoglobin S molecules to aggregate and form fibrous precipitates (Allison, 2009).

The allele responsible for sickle-cell anemia can be found on the short arm of chromosome 11, more specifically 11p15. A person who receives the defective gene from both father and mother develops the disease; a person who receives one defective and one healthy allele remains healthy, but can pass on the disease and is known as a carrier or heterozygote. Heterozygotes are still able to contract malaria, but their symptoms are generally less severe (Allison, 2009).

In the USA, with no endemic malaria, the prevalence of sickle-cell anemia among blacks is lower (about 0.25%) than in West Africa (about 4.0%) and is falling (Lesi and Bassey, 1972).

Without endemic malaria, the sickle-cell mutation is purely disadvantageous, and tends to decline in the affected population by natural selection, and now artificially through prenatal genetic screening. However, the African American community descends from a significant admixture of several African and non-African ethnic groups, and also represents the descendants of survivors of slavery and the slave trade. Thus, a lower degree of endogamy and, particularly, abnormally high health-selective pressure through slavery may be the most plausible explanations for the lower prevalence of sickle-cell anemia (and, possibly, other genetic diseases) among African Americans compared to sub-Saharan Africans. Another factor that limits the spread of sickle-cell genes in North America is the absence of cultural proclivities to polygamy, which allows affected males to continue to seek unaffected children with multiple partners (Lesi and Bassey, 1972).

1.1.5 Pathophysiology:

The loss of red blood cell elasticity is central to the pathophysiology of sickle-cell disease. Normal red blood cells are quite elastic, which allows the cells to deform to pass through capillaries. In sickle-cell disease, low-oxygen tension promotes red blood cell sickling and repeated episodes of sickling damage the cell membrane and decreases the cell's elasticity. These cells fail to return to normal shape when normal oxygen tension is restored. As a consequence, these rigid blood cells are unable to deform as they pass through narrow capillaries, leading to vessel occlusion and ischemia (Kumar, 2009).

The actual anemia of the illness is caused by hemolysis, the destruction of the red cells, because of their shape. Although the bone marrow attempts to compensate by creating new red cells, it does not match the rate of destruction (Kumar, 2009).

1.1.6 Diagnosis:

In HbSS, the complete blood count reveals hemoglobin levels in the range of 6–8 g/dl with a high reticulocyte count (as the bone marrow compensates for the destruction of sickled cells by producing more red blood cells). In other forms of sickle-cell disease, Hb levels tend to be higher. A blood film may show features of hyposplenism (target cells and Howell-Jolly bodies) (Clarke and Higgins, 2000).

Sickling of the red blood cells, on a blood film, can be induced by the addition of sodium metabisulfite. The presence of sickle hemoglobin can also be demonstrated with the "sickle solubility test". A mixture of hemoglobin S (HbS) in a reducing solution (such as sodium dithionite) gives a turbid appearance, whereas normal Hb gives a clear solution (Clarke and Higgins, 2000).

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

People who are known carriers of the disease often undergo genetic counseling before they have a child. A test to see if an unborn child has the disease takes either a blood sample from the fetus or a sample of amniotic fluid. Since taking a blood sample from a fetus has greater risks, the latter test is usually used. Neonatal screening provides not only a method of early detection for individuals with sickle-

cell disease, but also allows for identification of the groups of people that carry the sickle cell trait (Leeet *al.*, 2000).

1.1.7 Management:

Folic acid and penicillin:

Children born with sickle-cell disease undergo close observation by the pediatrician and require management by a hematologist to assure they remain healthy. These patients take a 1 mg dose of folic acid daily for life. From birth to five years of age, they also have to take penicillin daily due to the immature immune system that makes them more prone to early childhood illnesses (Oniyangi and Omari, 2006).

Malaria chemoprophylaxis:

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

Vaso-occlusive crisis:

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

effective agent that doctors frequently prescribe to help control itching associated with the use of opioids (Aldrich and Nagel, 1998).

Acute chest crisis:

Management is similar to vaso-occlusive crisis, with the addition of antibiotics (usually a quinolone or macrolide, since cell wall-deficient ["atypical"] bacteria are thought to contribute to the syndrome (Aldrich and Nagel, 1998). oxygen supplementation for hypoxia, and close observation. Should the pulmonary infiltrate worsen or the oxygen requirements increase, simple blood transfusion or exchange transfusion is indicated. The latter involves the exchange of a significant portion of the patient's red cell mass for normal red cells, which decreases the percent of hemoglobin S in the patient's blood. The patient with suspected acute chest syndrome should be admitted to the hospital with worsening A-a gradient an indication for ICU admission (Glassberg, 2011).

Hydroxyurea:

The first approved drug for the causative treatment of sickle-cell anemia, hydroxyurea, was shown to decrease the number and severity of attacks in a study in 1995 (Charache *et al.*, 1995) and shown to possibly increase survival time in a study in 2003 (Steinberg *et al.*, 2003). This is achieved, in part, by reactivating fetal hemoglobin production in place of the hemoglobin S that causes sickle-cell anemia. Hydroxyurea had previously been used as a chemotherapy agent, and there is some concern that long-term use may be harmful, but this risk has been shown to be either absent or very small and it is likely that the benefits outweigh the risks (Yawn *et al.*, 2014; Platt, 2008).

Transfusion therapy:

Blood transfusions are often used in the management of sickle-cell disease in acute cases and to prevent complications by decreasing the number of red blood cells (RBC) that can sickle by adding normal red blood cells (Drasare *et al.*, 2011).

In children prophylactic chronic red blood cell (RBC) transfusion therapy has been shown to be efficacious to a certain extent in reducing the risk of first stroke or silent stroke when transcranial Doppler (TCD) ultrasonography shows abnormal increased cerebral blood flow velocities. In those who have sustained a prior stroke event it also reduces the risk of recurrent stroke and additional silent strokes (Gyanget *al.*, 2011; Mirreet *al.*, 2010).

Bone marrow transplants:

Bone marrow transplants have proven effective in children. Bone marrow transplants are the only known cure for SCD (Walters *et al.*, 1996). However, bone marrow transplants are difficult to obtain because of the specific HLA typing necessary. Ideally, a twin family member (syngeneic) or close relative (allogeneic) would donate the bone marrow necessary for transplantation (Walters *et al.*, 1996).

1.1.8 Prognosis:

About 90% of patients survive to age 20, and close to 50% survive beyond the fifth decade (Kumar *et al.*, 2009). In 2001, according to one study performed in Jamaica, the estimated mean survival for sickle-cell patients was 53 years old for men and 58 years old for women with homozygous SCD (Wierenga *et al.*, 2001).

1.1.9 Complications:

Sickle-cell anemia can lead to various complications, including:

- Increased risk of severe bacterial infections due to loss of functioning spleen tissue (and comparable to the risk of infections after having the spleen removed surgically). These infections are typically caused by encapsulated organisms such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. Daily penicillin prophylaxis is the most commonly used treatment during childhood, with some hematologists continuing treatment indefinitely.

Patients benefit today from routine vaccination for S. pneumonia(Kavanaghet *al.*, 2011).

- Stroke, which can result from a progressive narrowing of blood vessels, prevents oxygen from reaching the brain. Cerebral infarction occurs in children and cerebral hemorrhage in adults(Adams *et al.*, 2001; Adam, 2007).
- Silent stroke causes no immediate symptoms, but is associated with damage to the brain. Silent stroke is probably five times as common as symptomatic stroke. About 10–15% of children with SCD suffer strokes, with silent strokes predominating in the younger patients (Adams *et al.*, 2001; Adam, 2007).
- Cholelithiasis (gallstones) and cholecystitis may result from excessive bilirubin production and precipitation due to prolonged hemolysis(Martí-Carvajalet *al.*, 2004).
- Avascular necrosis (aseptic bone necrosis) of the hip and other major joints may occur as a result of ischemia(Martí-Carvajalet *al.*, 2004).
- Decreased immune reactions due to hyposplenism (malfunctioning of the spleen) (Kenny *et al.*, 1980).
- Priapism and infarction of the penis (Chrouseret *al.*, 2011).
- Osteomyelitis (bacterial bone infection), the most common cause of osteomyelitis in SCD is Salmonella(especially the atypical serotypes Salmonella typhimurium, Salmonella enteritidis, Salmonella choleraesuis and Salmonella paratyphi B), followed by Staphylococcus aureus and Gram-negative enteric bacilli perhaps because intravascular sickling of the bowel leads to patchy ischaemic infarction (Almeida and Roberts, 2005).
- Opioid tolerance can occur as a normal, physiologic response to the therapeutic use of opiates. Addiction to opiates occurs no more commonly

among individuals with sickle-cell disease than among other individuals treated with opiates for other reasons (Rudge, 1991).

- Acute papillary necrosis in the kidneys (Rudge, 1991).
- Leg ulcers (Rudge, 1991).
- In eyes, background retinopathy, proliferative retinopathy, vitreous hemorrhages, and retinal detachments can result in blindness (Elagouz *Met al.*, 2010).
- During pregnancy, intrauterine growth retardation, spontaneous abortion, and pre-eclampsia (Smith *et al.*, 2008).
- Chronic pain: Even in the absence of acute vaso-occlusive pain, many patients have unreported chronic pain (Smith *et al.*, 2008).
- Pulmonary hypertension (increased pressure on the pulmonary artery) can lead to strain on the right ventricle and a risk of heart failure; typical symptoms are shortness of breath, decreased exercise tolerance, and episodes of syncope. 21% of children and 30% of adults have evidence of pulmonary hypertension when tested; this is associated with reduced walking distance and increased mortality (Caughey *et al.*, 2015).
- Chronic kidney failure due to sickle-cell nephropathy manifests itself with hypertension, protein loss in the urine, loss of red blood cells in urine and worsened anemia. If it progresses to end-stage renal failure, it carries a poor prognosis (Powar *et al.*, 1991).

1.1.10 Epidemiology:

The highest frequency of sickle cell disease is found in tropical regions, particularly sub-Saharan Africa, tribal regions of India and the Middle-East (Weatherall and Clegg, 2001). Migration of substantial populations from these high prevalence areas to low prevalence countries in Europe has dramatically

increased in recent decades and in some European countries sickle-cell disease has now overtaken more familiar genetic conditions such as hemophilia and cystic fibrosis(Roberts and de Montalembert , 2007). In 2013 it resulted in 176,000 deaths due to SCD up from 113,000 deaths in 1990(Naghavi,2013).

Sickle-cell disease occurs more commonly among people whose ancestors lived in tropical and sub-tropical sub-Saharan regions where malaria is or was common.

Where malaria is common, carrying a single sickle-cell allele (trait) confers a selective advantage in other words, being a heterozygote is advantageous(Wellemset *al.*, 2009).

Specifically, humans with one of the two alleles of sickle-cell disease show less severe symptoms when infected with malaria (Wellemset *al.*, 2009).

Africa:

Three quarters of sickle-cell cases occur in Africa. A recent WHO report estimated that around 2% of newborns in Nigeria were affected by sickle cell anemia, giving a total of 150,000 affected children born every year in Nigeria alone. The carrier frequency ranges between 10% and 40% across equatorial Africa, decreasing to 1–2% on the north African coast and <1% in South Africa (Isahet *al.*, 2013). There have been studies in Africa that show a significant decrease in infant mortality rate, ages 2–16 months, because of the sickle-cell trait. This happened in predominant areas of malarial cases(Aidooet *al.*, 2002).

United States:

The prevalence of the disease in the United States is approximately 1 in 5,000, mostly affecting Americans of Sub-Saharan African descent, according to the National Institutes of Health In (Cares, 2003). the United States, about one out of 500 African-American children and one in every 36,000 Hispanic-American children have sickle-cell anemia (Cares, 2003). Most infants with SCD born in the United States are now identified by routine neonatal screening. Forty-four states

along with the District of Columbia, Puerto Rico and the Virgin Islands currently provide universal neonatal screening for SCD(Pass *et al.*, 2000). It is estimated that 2.5 million Americans are heterozygous carriers for the sickle-cell trait (Cinnchinsky *et al.*, 2011).

France:

As a result of population growth in African-Caribbean regions of overseas France and immigration from North and sub-Saharan Africa to mainland France, sickle-cell disease has become a major health problem in France (Bardakdjian and Wajcman , 2004). SCD has become the most common genetic disease in the country, with an overall birth prevalence of 1/2,415 in mainland France, ahead of phenylketonuria (1/10,862), congenital hypothyroidism (1/3,132), congenital adrenal hyperplasia (1/19,008) and cystic fibrosis (1/5,014) for the same reference period. In 2010, 31.5% of all newborns in mainland France (253,466 out of 805,958) were screened for SCD (this percentage was 19% in 2000). 341 newborns with SCD and 8,744 heterozygous carriers were found representing 1.1% of all newborns in mainland France. The Paris metropolitan district (Île-de-France) is the region that accounts for the largest number of newborns screened for SCD (60% in 2010). The second largest number of at-risk is in Provence-Alpes-Côte d'Azur at nearly 43.2% and the lowest number is in Brittany at 5.5% (Bardakdjian-Michau,*et al.*, 2009;Bardakdjian-Michau, 2012).

Middle East:

In Saudi Arabia about 4.2% of the population carry the sickle-cell trait and 0.26% have sickle-cell disease. The highest prevalence is in the Eastern province where approximately 17% of the population carry the gene and 1.2% have sickle-cell disease (Jastaniah , 2011). In 2005 in Saudi Arabia a mandatory pre-marital test including HB electrophoresis was launched and aimed to decrease the incidence of SCD and thalassemia(Memish and Saeedi, 2011).

India and Nepal:

Sickle-cell disease is common in the tribal people of central India who share a genetic linkage with the African race, where the prevalence has ranged from 9.4 to 22.2% in endemic areas of Madhya Pradesh, Rajasthan and Chhattisgarh (Awasthy *et al.*, 2008).

Caribbean Islands:

In Jamaica, 10% of the population carries the sickle-cell gene, making it the most prevalent genetic disorder in the country (Asnani *et al.*, 2011).

1.2 Hemoglobin Types in humans:-

Hemoglobin variants are a part of the normal embryonic and fetal development, but may also be pathologic mutant forms of hemoglobin in a population, caused by variations in genetics. Some well-known hemoglobin variants such as sickle-cell anemia are responsible for diseases, and are considered hemoglobinopathies. Other variants cause no detectable pathology, and are thus considered non-pathological variants (Schneider *et al.*, 1976; Huisman, 1996).

1.2.1 In the embryo:

- Gower 1 ($\zeta_2\epsilon_2$)
- Gower 2 ($\alpha_2\epsilon_2$)
- Hemoglobin Portland I ($\zeta_2\gamma_2$)
- Hemoglobin Portland II ($\zeta_2\beta_2$) (Schneider *et al.*, 1976; Huisman, 1996).

1.2.2 In the fetus:

- Hemoglobin F ($\alpha_2\gamma_2$) (Schneider *et al.*, 1976; Huisman, 1996).

1.2.3 After birth:

- Hemoglobin A ($\alpha_2\beta_2$) – The most common with a normal amount over 95%
- Hemoglobin A₂ ($\alpha_2\delta_2$) – δ chain synthesis begins late in the third trimester and, in adults, it has a normal range of 1.5–3.5%

- Hemoglobin F ($\alpha_2\gamma_2$) – In adults Hemoglobin F is restricted to a limited population of red cells called F-cells. However, the level of Hb F can be elevated in persons with sickle-cell disease and beta-thalassemia (Schneider *et al.*, 1976; Huisman, 1996).

1.2.4 Variant forms that cause disease:

- Hemoglobin D-Punjab – ($\alpha_2\beta^D_2$) – A variant form of hemoglobin.
- Hemoglobin H (β_4) – A variant form of hemoglobin, formed by a tetramer of β chains, which may be present in variants of α thalassemia.
- Hemoglobin Barts (γ_4) – A variant form of hemoglobin, formed by a tetramer of γ chains, which may be present in variants of α thalassemia.
- Hemoglobin S ($\alpha_2\beta^S_2$) – A variant form of hemoglobin found in people with sickle cell disease. There is a variation in the β -chain gene, causing a change in the properties of hemoglobin, which results in sickling of red blood cells.
- Hemoglobin C ($\alpha_2\beta^C_2$) – Another variant due to a variation in the β -chain gene. This variant causes a mild chronic hemolytic anemia.
- Hemoglobin E ($\alpha_2\beta^E_2$) – Another variant due to a variation in the β -chain gene. This variant causes a mild chronic hemolytic anemia.
- Hemoglobin AS – A heterozygous form causing sickle cell trait with one adult gene and one sickle cell disease gene
- Hemoglobin SC disease – A compound heterozygous form with one sickle gene and another encoding Hemoglobin C (Schneider *et al.*, 1976; Huisman, 1996).

1.3 Hemoglobin types in Sickle Cell disease patients:

1.3.1 Adult hemoglobin (HbA):

Hemoglobin A (HbA), also known as adult hemoglobin or $\alpha_2\beta_2$, is the most common human hemoglobin tetramer, comprising over 97% of the total red blood

cell hemoglobin. It consists of two alpha chains and two beta chains (Naghavi, 2013).

1.3.2 Hemoglobin A2:

Hemoglobin A2 (HbA₂) is a normal variant of hemoglobin A that consists of two alpha and two delta chains ($\alpha_2\delta_2$) and is found at low levels in normal human blood. Hemoglobin A2 may be increased in beta thalassemia or in people who are heterozygous for the beta thalassemia gene (Benz EJ *et al.*, 2005).

HbA2 exists in small amounts in all adult humans (1.5-3.1% of all hemoglobin molecules) and is increased in people with Sickle-cell disease. Its biological importance is not yet known (Benz EJ *et al.*, 2005).

1.3.3 Sickle hemoglobin (HbS):

HbS is an abnormal oxygen-carrying protein hemoglobin found in red blood cells. This leads to a propensity for the cells to assume an abnormal, rigid, sickle-like shape under certain circumstances (Naghavi, 2013).

Sickle-cell disease occurs when a person inherits two abnormal copies of the hemoglobin gene, one from each parent. Several subtypes exist, depending on the exact mutation in each hemoglobin gene. A person with a single abnormal copy does not experience symptoms and is said to have sickle-cell trait. Such people are also referred to as carriers (Naghavi, 2013).

Hemoglobin S results from the substitution of a valine for glutamic acid as the sixth amino acid of the beta globin chain, which produces a hemoglobin tetramer ($\alpha_2\beta_2^S$) that is poorly soluble when deoxygenated (Embury and Vichinsky, 2006). The polymerization of deoxy hemoglobin (Hb) S is essential to vasoocclusive phenomena (Embury and Vichinsky, 2006).

1.3.4 Fetal hemoglobin (HbF):

1.3.4.1 Definition:

Fetal hemoglobin, or **foetalhaemoglobin**, (also **hemoglobin F**, **HbF**, or $\alpha_2\gamma_2$) is the main oxygentransport protein in the human fetus during the last seven months of development in the uterus and persists in the newborn until roughly 6 months old. Functionally, fetal hemoglobin differs most from adult hemoglobin in that it is able to bind oxygen with greater affinity than the adult form, giving the developing fetus better access to oxygen from the mother's bloodstream(Lanzkronet *al.*, 2008). In newborns, fetal hemoglobin is nearly completely replaced by adult hemoglobin by approximately 6 months postnatally, except in a few thalassemia cases in which there may be a delay in cessation of HbF production until 3–5 years of age. In adults, fetal hemoglobin production can be reactivated pharmacologically (Lanzkronet *al.*, 2008), which is useful in the treatment of diseases such as sickle-cell disease(Lanzkronet *al.*, 2008).

Oxygenated blood is delivered to the fetus via the umbilical vein from the placenta, which is anchored to the wall of the mother's uterus. The chorion acts as a barrier between the maternal and fetal circulation so that there is no admixture of maternal and fetal blood. Blood in the maternal circulation is delivered via open ended arterioles to the intervillous space of the chorionic plate, where it bathes the chorionic villi that carry umbilical capillary beds, thereby allowing gas exchange to occur between the maternal and fetal circulation. Deoxygenated maternal blood drains into open ended intervillousvenules to return to maternal circulation. Due to the admixture of oxygenated and deoxygenated blood, maternal blood in the intervillous space is lower in oxygen than arterial blood. As such, fetal hemoglobin must be able to bind oxygen with greater affinity than adult hemoglobin in order to compensate for the relatively lower oxygen tension of the maternal blood supplying the chorion(Berg, Jeremy *et al.*, 2002).

Fetal hemoglobin's affinity for oxygen is substantially greater than that of adult hemoglobin. Notably, the P50 value for fetal hemoglobin is lower than adult hemoglobin (i.e., the partial pressure of oxygen at which the protein is 50% saturated; lower values indicate greater affinity). The P50 of fetal hemoglobin is roughly 19 mmHg, whereas adult hemoglobin is approximately 26.8 mmHg. As a result, the "oxygen saturation curve", which plots percent saturation vs. pO_2 , is left-shifted for fetal hemoglobin as compared to adult hemoglobin (Berg, Jeremy *et al.*, 2002).

This greater affinity for oxygen is explained by the lack of fetal hemoglobin's interaction with 2, 3-bisphosphoglycerate (2,3-BPG or 2,3-DPG). In adult red blood cells, this substance decreases the affinity of hemoglobin for oxygen. 2, 3-BPG is also present in fetal red blood cells, but interacts less efficiently with fetal hemoglobin than adult hemoglobin. This is due to a change in a single amino acid (residue 143) found in the 2,3-BPG 'binding pocket': from histidine to serine, which gives rise to the greater oxygen affinity (Berg, Jeremy *et al.*, 2002). Whereas histidine is positively charged and interacts well with the negative charges found on the surface of 2,3-BPG, Serine has a neutrally charged side chain at physiological pH, and interacts less well. This change results in less binding of 2,3-BPG to fetal Hb, and as a result oxygen will bind to it with higher affinity than adult hemoglobin (Berg, Jeremy *et al.*, 2002).

For mothers to deliver oxygen to a fetus, it is necessary for the fetal hemoglobin to extract oxygen from the maternal oxygenated hemoglobin across the placenta. The higher oxygen affinity required for fetal hemoglobin is achieved by the protein subunit γ (gamma), instead of the β (beta) subunit. Because the γ subunit has fewer positive charges than the (adult) β subunit, 2, 3-BPG is less electrostatically bound to fetal hemoglobin compared to adult hemoglobin. This lowered affinity allows

for adult hemoglobin (maternal hemoglobin) to readily transfer its oxygen to the fetal bloodstream (Berg, Jeremy *et al.*, 2002).

1.3.4.2 Distribution:

After the first 10 to 12 weeks of development, the fetus' primary form of hemoglobin switches from embryonic hemoglobin to fetal hemoglobin. At birth, fetal hemoglobin comprises 50-95% of the infant's hemoglobin. These levels decline after six months as adult hemoglobin synthesis is activated while fetal hemoglobin synthesis is deactivated. Soon after, adult hemoglobin (hemoglobin A in particular) takes over as the predominant form of hemoglobin in normal children (Lanzkron *et al.*, 2008; Charache *et al.*, 1995).

Certain genetic abnormalities can cause the switch to adult hemoglobin synthesis to fail, resulting in a condition known as hereditary persistence of fetal hemoglobin (HPFH) (Lanzkron *et al.*, 2008; Charache *et al.*, 1995).

1.3.4.3 Structure and genetics:

Most types of normal hemoglobin, including hemoglobin A, hemoglobin A₂, as well as hemoglobin F, are tetramers composed of four protein subunits and four heme prosthetic groups. Whereas adult hemoglobin is composed of two α (alpha) and two β (beta) subunits, fetal hemoglobin is composed of two α subunits and two γ (gamma) subunits, and is commonly denoted as $\alpha_2\gamma_2$. Because of its presence in fetal hemoglobin, the γ subunit is commonly called the "fetal" hemoglobin subunit (Lanzkron *et al.*, 2008; Charache *et al.*, 1995).

In humans, the gamma subunit is encoded on chromosome 11, as is the beta subunit. There are two similar copies of the gamma subunit gene: γ G which has a glycine at position 136, and γ A which has an alanine. The gene that codes for the alpha subunit is located on chromosome 16 and is also present in duplicate (Lanzkron *et al.*, 2008; Charache *et al.*, 1995).

1.3.4.4 Clinical significance:

When fetal hemoglobin production is switched off after birth, normal children begin producing adult hemoglobin (HbA). Children with sickle-cell disease instead begin producing a defective form of hemoglobin called hemoglobin S which aggregates together and forms filaments that cause red blood cells to change their shape from round to sickle-shaped. These defective red blood cells have a greater tendency to stack on top of one another and block blood vessels. These invariably lead to so-called painful vaso-occlusive episodes, which are a hallmark of the disease (Lanzkron *et al.*, 2008; Charache *et al.*, 1995).

If fetal hemoglobin remains the predominant form of hemoglobin after birth, the number of painful episodes decreases in patients with sickle-cell disease (Lanzkron *et al.*, 2008; Charache *et al.*, 1995).

Hydroxyurea promotes the production of fetal hemoglobin and can thus be used to treat sickle-cell disease (Lanzkron *et al.*, 2008; Charache *et al.*, 1995). The fetal hemoglobin's reduction in the severity of the disease comes from its ability to inhibit the formation of hemoglobin aggregates within red blood cells which also containing hemoglobin S. Combination therapy with hydroxyurea and recombinant erythropoietin rather than treatment with hydroxyurea alone has been shown to further elevate hemoglobin F levels and to promote the development of HbF-containing F-cells (Rodgers *et al.*, 1993).

1.4 Fetal hemoglobin in sickle cell anemia:

Fetal hemoglobin (HbF) is the major genetic modulator of the hematologic and clinical features of sickle cell disease, an effect mediated by its exclusion from the sickle hemoglobin polymer. Fetal hemoglobin genes are genetically regulated, and the level of HbF and its distribution among sickle erythrocytes is highly variable (Steinberg *et al.*, 2001).

Some patients with sickle cell disease have exceptionally high levels of HbF that are associated with the Senegal and Saudi-Indian haplotype of the HBB-like gene cluster; some patients with different haplotypes can have similarly high HbF. In these patients, high HbF is associated with generally milder but not asymptomatic disease. Studying these persons might provide additional insights into HbF gene regulation. HbF appears to benefit some complications of disease more than others (Steinberg *et al.*, 2001).

This might be related to the premature destruction of erythrocytes that do not contain HbF, even though the total HbF concentration is high. Recent insights into HbF regulation have spurred new efforts to induce high HbF levels in sickle cell disease beyond those achievable with the current limited repertoire of HbF inducers (Steinberg *et al.*, 2001).

1.4.1 HbF decrease crises:

Appreciating the role of fetal hemoglobin (HbF; $\alpha_2\gamma_2$) in sickle cell disease started more than 60 years ago when Janet Watson confirmed that infants with sickle cell disease had few symptoms and that their deoxygenated erythrocytes took longer to sickle and did not deform as extensively as did their sickle cell trait-carrying mother's cells. She attributed these observations to high HbF levels in infant blood (Steinberg *et al.*, 2001).

Sickle hemoglobin (HbS) gelation studies showed that HbF did not interact with HbS; it was also reported that compound heterozygotes for sickle cell trait and hereditary persistence of HbF (HPFH) were clinically normal despite having a very high HbS concentration (Steinberg *et al.*, 2001).

HbF is the most powerful modulator of the clinical and hematologic features of sickle cell anemia (defined as homozygosity for glu6val in the β -globin gene or HBB). To protect against various complications of disease, different concentrations of HbF were postulated to be required, although any increment in HbF had a

beneficial effect on mortality (Powar *et al.*, 1984; Platt *et al.*, 1994). Higher HbF levels were associated with a reduced rate of acute painful episodes, fewer leg ulcers, less osteonecrosis, less frequent acute chest syndromes, and reduced disease severity (Steinberg *et al.*, 2009). However, HbF level had a weak or no clear association with priapism, urine albumin excretion, stroke and silent cerebral infarction, systemic blood pressure, and perhaps sickle vasculopathy as estimated by tricuspid regurgitant velocity (Steinberg *et al.*, 2009). The failure of HbF to modulate uniformly all complications of sickle cell disease might be related to the pathophysiologic events that impact the likelihood of developing these complications (Kato *et al.*, 2007). Many epidemiologic studies suggested that disease complications most closely linked to sickle vaso-occlusion and blood viscosity were robustly related to HbF concentration, whereas complications associated with the intensity of hemolysis were less affected (Kato *et al.*, 2007), although HbF is protective for leg ulcers, one complication closely associated with hyperhemolysis (Koshy *et al.*, 1989; Nolan *et al.*, 2006). After defining hyperhemolysis by the highest quartile of serum lactic dehydrogenase and controlling for liver disease by examining only patients with normal serum alanine aminotransferase, HbF levels were lower in patients with the highest quartile compared with the lowest quartile of hemolysis (Taylor *et al.*, 2008).

Even when total HbF levels are high, perhaps the intravascular hemolysis of erythrocytes containing little or no HbF leads to sufficient nitric oxide scavenging by plasma hemoglobin to provoke hemolysis-related complications (Jeffers *et al.*, 2006).

Sickle erythrocytes are a mixture of cells with measurable HbF (F cells) and non-F cells. F cells are long lived, do not acquire the same increment of HbS-induced damage as non-F cells, are less likely to initiate adhesive events, and are associated with protection from sickle vaso-occlusion (left arrow). The heterocellular

distribution of HbF in sickle cell anemia, even when total HbF concentrations are high at baseline or in response to hydroxyurea, means that some erythrocytes with no HbF or with suboptimal concentrations of HbF are present. Some of these cells hemolyze intravascularly liberating hemoglobin, which scavenges nitric oxide and contributes to certain vascular complications of this disease (right arrow). This might account for the failure of high HbF that is heterocellularly distributed to protect against all disease complications (Pembrey *et al.*, 1978; Padmos *et al.*, 1991).

The Saudi-Indian and Senegal haplotypes of the HBB-like globin gene complex, are associated with high HbF levels, and carriers of these haplotypes can have milder disease (Pembrey *et al.*, 1978; Padmos *et al.*, 1991). Some patients who have other HbS-associated haplotypes also have very high HbF (Pembrey *et al.*, 1978; Padmos *et al.*, 1991). Notwithstanding the high HbF levels of all these patients, acute painful episodes and other symptoms of sickle cell disease still occur, perhaps because the heterogeneous cellular distribution of HbF does not equally protect all erythrocytes from polymerization-induced damage (Pembrey *et al.*, 1978; Padmos *et al.*, 1991).

In contrast, persons who are compound heterozygotes for HbS and gene deletion HPFH, where HbF is more evenly apportioned among erythrocytes or pancellularly distributed, are clinically asymptomatic with nearly normal hemoglobin levels (Pembrey *et al.*, 1978; Padmos *et al.*, 1991).

1.4.2 HbF and the retardation of HbS polymerization:

HbF is composed of 2 α -globin polypeptide chains and 2 γ -globin chains. The γ -globin chains are encoded by 2 nearly identical genes (HBG2 and HBG1) within the β -globin gene-like cluster on chromosome 11p that differ by a glycine or alanine residue at amino acid position γ 136. $^G\gamma$ - and $^A\gamma$ -globins have similar effects on HbS polymerization (Adachi *et al.*, 1990), with the rapid decrease in the numbers of circulating fetal erythrocytes, the ratio of $^G\gamma$ to $^A\gamma$ -globin falls from 0.7

at birth to 0.4 at age 5 months. This is accompanied by a progressive decline in the number of erythrocytes with measurable HbF, called F cells. In normal adults, HbF is less than 1% of total hemoglobin and is distributed unevenly among erythrocytes (Steinberg *et al.*, 2009). HbF levels in sickle cell anemia range between 5% and 8%, In African Americans with sickle cell anemia, 2% to 80% of erythrocytes were F cells compared with $2.8\% \pm 1.6\%$ in normal African Americans (Steinberg *et al.*, 2009). Sickle cell trait carriers have a mean HbF of 1.4% and $14.1\% \pm 7.5\%$ F cells (Steinberg *et al.*, 2009). In sickle cell anemia, F cells survive longer than non-F cells, and this depends on the amount of HbF/F cells (Steinberg *et al.*, 2009).

A high correlation ($R^2 = 0.967$) is present between the number of F cells and the percentage of HbF (Steinberg *et al.*, 1997). The pathophysiology of sickle cell disease is dependent on the polymerization of deoxy sickle hemoglobin (Steinberg *et al.*, 2009). Increased levels of HbF retard this process. HbF reduces HbS concentration, but more importantly, both HbF and its mixed hybrid tetramer ($\alpha 2\beta^S\gamma$) cannot enter the deoxy sickle hemoglobin polymer phase (Steinberg *et al.*, 2009). In contrast, the hybrid tetramer-containing β^S and β^A chains has only half the probability of entering the polymer as the HbS molecule, hence the special value of HbF compared with other hemoglobins (Steinberg *et al.*, 2009). The antipolymerization effect of HbF resides primarily in HBG (both γ -globin genes) residues glycine $\gamma 87$ and aspartic acid $\gamma 80$ (Steinberg *et al.*, 2009). By inhibiting the tendency of deoxy sickle hemoglobin to polymerize, sufficient HbF thwarts the cellular damage evoked by HbS polymer (Steinberg *et al.*, 2009).

Engineering recombinant HbF and HbA by adding additional substitutions can enhance the capacity of the molecule to inhibit polymerization, an approach exploited when devising vectors for gene therapy. Conversely, the natural mutant hemoglobin, HbS-Antilles (HBB glu6val; val23ile), has enhanced polymerization tendencies; contrasted with persons with sickle cell trait, heterozygotes with this

variant are symptomatic and homozygotes have severe sickle cell disease (Steinberg *et al.*, 2009).

1.4.3 Genetic basis of HbF regulation:

Globin gene switching:

HbF is the predominant hemoglobin from early gestation until 1 to 2 months postnatally when adult HbA predominates (Steinberg *et al.*, 2009). Although erythroid precursors of normal adults express HbG at a low level (Stamatoyannopoulos and Papayannopoulou, 1981), stress erythropoiesis is associated with increased HbF (Blau *et al.*, 1993; Stamatoyannopoulos *et al.*, 1987). A stochastic model posits that the increase in HbF is the result of recruitment of erythroid progenitor cells that prematurely undergo terminal differentiation and are committed to producing γ -globin (Stamatoyannopoulos, 2005; Stamatoyannopoulos *et al.*, 1981). The stress signal transduction model suggests that cytokines, such as erythropoietin, stem cell factor, and transforming growth factor- β (Bhanu *et al.*, 2005), initiate downstream intracellular signaling pathways that activate HbG expression and is the premise on which HbF induction by cytostatic agents was based (Mabaera *et al.*, 2008).

In sickle cell anemia, there is a delayed switch from HbG to HbB expression, and the replacement of HbF by HbS, and HbF levels remain above normal in most patients. The mechanism accounting for this is unknown but might reflect the slower centripetal regression of red, or hematopoietic marrow to the axial skeleton in the presence of expanded erythropoiesis that is the result of sustained hemolysis (Steinberg *et al.*, 2009; Weatherall and Clegg, 2001).

The goal of HbF-inducing treatments is to reverse this switch to the largest degree possible. Compound heterozygotes with HbSC disease and HbS- β^+ thalassemia, who usually have lower levels of hemolysis, most often have HbF levels near normal or only slightly increased (Steinberg *et al.*, 2009; Weatherall and Clegg

,2001). This could be a result of less intense hemolysis or a lack of those genetic modulators linked to the HbS gene (Wilber *et al.*, 2011).

1.4.4 Haplotypes of the HBB gene-like cluster:

The HbS β -globin gene is found on 4 or 5 common haplotypes reflecting its regions of origin in Africa, the Middle East, and the Indian subcontinent (Embury *et al.*, 1994; Lapoumeroulie *et al.*, 1992). Patients with a Bantu haplotype have the lowest HbF and those with a Senegal or Saudi-Indian haplotype have the highest; persons with a Benin haplotype have HbF levels that are intermediate (Nagel *et al.*, 1987; Labie *et al.*, 1985). Carriers of all haplotypes have considerable variance in HbF levels, suggesting the importance of other quantitative trait loci (QTL) modulating HBG expression (Embury *et al.*, 1994).

1.4.5 HbF-inducing agents:

1.4.5.1 Hydroxyurea:

The beneficial effects of high HbF in sickle cell anemia and in β -thalassemia where HbF can substitute for HbA launched an effort to find drugs capable of increasing HbF levels. The DNA-hypomethylating agent 5-azacytidine was used to induce HbF in anemic baboons, a species whose hemoglobin composition and regulation are nearly identical to those of humans. Based on the impressive results of these preclinical studies, this agent was used in sickle cell anemia and β -thalassemia with promising results; however, continued trials were abandoned because of potential carcinogenicity. In these studies, it was unclear whether HbF induction was the result of HBG hypomethylation or the cytotoxic effects of 5'-azacitidine (Steinberg *et al.*, 2001). Therefore, trials of hydroxyurea, an S-phase specific agent without primary hypomethylating activity, with a long history of use in myeloproliferative disorders and with tolerable side effects, were started. The culmination of this work was the Multicenter Study of Hydroxyurea, a double-

blind, placebo-controlled study of patients with symptomatic sickle cell disease (Charache *et al.*, 1995; Voskaridou *et al.*, 2010).

Patients randomized to hydroxyurea had fewer pain episodes, less acute chest syndrome, and a lower transfusion requirement than placebo-treated cases, and this agent rapidly received approval for use in sickle cell anemia in the United States and elsewhere (Charache *et al.*, 1995; Voskaridou *et al.*, 2010). How therapeutic induction of HbF affects less common complications, such as priapism, leg ulcers, pulmonary vasculopathy, and stroke, is not known. The average increment in HbF achieved in the Multicenter Study of Hydroxyurea was only 3.6% over the baseline level of 5.1%, but other studies using different dosing regimens found higher increases in HbF (Charache *et al.*, 1995; Voskaridou *et al.*, 2010). Long-term follow-up studies of Multicenter Study of Hydroxyurea patients suggested that mortality was reduced in patients who took this drug and that side effects were minimal (Voskaridou *et al.*, 2010; Brawley *et al.*, 2008); other studies confirmed the benefits of hydroxyurea (Brawley *et al.*, 2008). A trial of hydroxyurea in babies has been completed. Although the trial's primary endpoints of preservation of renal and splenic function were not met during the relatively brief observation period, treated patients had less pain, higher hemoglobin concentrations, increased HbF, and reduced leukocyte counts, with minimal short-term toxicity (Wang *et al.*, 2011). The long-term effects of hydroxyurea begun in the neonatal period will require careful follow-up (Wang *et al.*, 2011).

1.4.5.2 Other HbF-inducing agents:

Not all patients respond to hydroxyurea, and the erythrocytic distribution of HbF in treated patients is heterocellular (Platt *et al.*, 1984). Among responders, the increment in HbF is variable, suggesting the need for additional agents capable of inducing HbF and perhaps broadening its cellular distribution. One class of promising agents are histone deacetylase (HDAC) inhibitors whose inhibition is

associated with increased expression of HbG. Arginine butyrate, a short chain fatty acid with HDAC inhibitory activity used as single agent or with hydroxyurea, has been associated with increases in HbF (Atweh *et al.*, 1999; Sutton *et al.*, 1998). However, a pulsed, or intermittent, dosing regimen was necessary to avoid cytotoxicity from butyrate while retaining the targeted promoter activation (Hebbel *et al.*, 2010).

Another oral short chain fatty acid derivative, sodium 2,2dimethylbutyrate, showed HbF induction in thalassemia in early phase clinical trials (Hebbel *et al.*, 2010). Suberoylanilidehydroxamic acid (Vorinostat), an orally available agent approved for treatment of cutaneous T-cell lymphoma, is an HDAC inhibitor that induced HbF expression in K562 cells (Hebbel *et al.*, 2010). A phase 1/2 trial of this agent in sickle cell anemia is presently enrolling patients (Bradner *et al.*, 2010).

High throughput screening studies with follow-up of promising candidates have suggested that strong inhibitors of HDAC1 and HDAC2 were associated with substantial increments in both HbG expression and HbF in vitro (Bradner *et al.*, 2010). BCL11A has been shown to interact with HDAC1 and HDAC2 (Sankaran *et al.*, 2008), Decitabine (5-aza-2'-deoxycytidine), a less toxic and perhaps noncarcinogenic deoxynucleotide, is also associated with DNA hypomethylation and has been used as single-agent therapy and with hydroxyurea in a small number of patients with sickle cell anemia (Sauntharajah *et al.*, 2003).

The recent studies of BCL11A and HMIP have stimulated a search for new agents that might act by modulating the expression of these genes and their signaling pathways to augment HbF expression in the β -hemoglobinopathies (Bauer and Orkin, 2011).

In conclusion, HbF has beneficial effects in sickle cell anemia. The contrast between asymptomatic persons with HbS-gene deletion HPFH and symptomatic patients with sickle cell anemia with similarly high HbF levels suggests that, if it

were possible to induce high HbF levels in most sickle erythrocytes and if this could be done before organ damage occurs, one might expect the disease to be “cured.” Presently, this is not possible, but a better understanding of how HbF levels are modulated might suggest new therapeutic approaches and combinations of HbF-inducing agents that could allow this goal to be met (Bauer and Orkin, 2011).

1.4.6 Previous studies in different geographic areas:

In Jamaica, Saudi Arabia and Orissa state India, their result about HbF were+ (0.4 – 33.2%, 4.9 – 22.1%, 4.6 – 31.5%) respectively(Noelken *et al.*, 1996).

In some African population such as Zairian and American black (6.5% and 6.1% respectively(Keren , 2003), However the mean HbF concentration found among sickle cell patient as reported for Kuwait Arabs (23.1%) , Iraninas (18%) (Ashley *et al.*, 2000).

1.4.7 Sickle cell disease and unusually high HbF:

HbF in African Americans:

We define an “HbSF” phenotype as an HbF concentration of at least 10% in sickle cell anemia patients 4 years of age or older, the time by which HbF levels stabilize (Gibney *et al.*, 2008; Solovieff *et al.*, 2010). After excluding the known causes of the HbSF phenotype, such as point mutations in the HBG promoters or large deletions within the HBB-like globin gene cluster, the molecular basis of this phenotype was studied in African Americans (Galanello *et al.*, 1990; Kulozik *et al.*, 1988).

HbF in Saudi Arabian sickle cell anemia:

Sickle cell anemia in Saudi Arabia has population concentrations in the Southwestern and Eastern Provinces. Most Eastern Province patients carry the Saudi-Indian β -globin gene-like cluster haplotype and have very high levels of

HbF. Southwestern Province patients have typical African-derived haplotypes and lower HbF levels, albeit higher than comparable haplotype groups of African descent (Padmoset *al.*,1991; Acquaye*et al.*, 1985; Al-Jam'a*et al.*, 2000) .

Southwestern Province:

The HbS gene in Saudi patients from the Southwestern Province was introduced from Africa and is present on typical African HBB haplotypes. Nevertheless, patients differ from African Americans phenotypically and have fewer episodes of stroke, priapism, and leg ulcers and a higher prevalence of splenomegaly (Padmoset *al.*,1991; Acquaye*et al.*, 1985; Al-Jam'a*et al.*, 2000) .

Eastern Province:

In the Eastern Province of Saudi Arabia, sickle cell anemia is usually associated with the Saudi-Indian (sometimes called Arab-Indian) HBB-gene cluster haplotype, high levels of HbF, and a milder, but not asymptomatic clinical course (Pembrey*et al.*, 1978; El-Hazmi , 1992; Padmoset *al.*,1991; Perrine *et al.*, 1972).

As in Southwestern patients, splenomegaly is common and stroke and leg ulcers are rare. The rarity of stroke might be a result of the higher hemoglobin concentration and a high incidence of α -thalassemia; little information on the incidence of pulmonary vasculopathy is available. Sickle cell trait carriers or persons with HbA and the Saudi-Indian haplotype did not have high HbF, but the cultured erythroblasts of sickle cell trait patients with the Saudi-Indian haplotype made increased amounts of HbF, suggesting that the kinetics of erythropoiesis played a role in the expression of the high HbF determinant (Miller *et al.*, 1986; Miller *et al.*,1987; Miller *et al.*,1987).

1.4.8 Previous studies (HbF level of SCD) in Sudan:

Study done by Khair F.M (Khartoum University) at 2002 in Sudan was found sickle cell anemic patient HbF level about (7+or – 5.6%), while sickle cell trait patient had (0.8 + or - 0.5) and sickle cell HbSF was found HbF level (17.4 +or –

4.2%) in other research sickle cell anemic patient had (9.2 + or -4.9%) of HbF level(Khair, 2002).

As reported by this research that sickle cell anemic patient in their first decade of life (0_10 years) had higher HbF (8.2+ or – 5.8) than patient in old age group (above 10 years) HbF level (2.7 + or – 1.1%) (Khair, 2002).

Gender also factor rate of HbF level in female (8 + or -2.4%) sickle cell anemia is associated with higher HbF concentration than male (5.6 + or – 3.1%)(Khair, 2002).

Drug inducing HbF have effect also been stimulating the formation of HbF(Khair, 2002).

The mean HbF level among sickle cell Sudanese as reported by previous research was (9%) (Khair, 2002).

1.5 Rationale:

Sickle cell anemia one of the major diseases in Sudan which cause morbidity and mortality. Patients have different types of hemoglobin, the most significant is HbS which do polymerization at low oxygen tension and cause many symptoms.

Some patients inherited defective genes from both parents (HbSS) and others inherited one defective and one normal gene (HbAS) those patients have adult hemoglobin (HbA), also sicklers have adult hemoglobin 2 (HbA2).

Sickler hemoglobin is responsible of most of severity, when patient is sickle cell trait has one normal adult hemoglobin and one defective hemoglobin symptoms are less.

Hemoglobin A2 may be increase or normal in sicklers and this hemoglobin has no significant effect in increase or decrease severity of symptoms, but when hemoglobin A2 in its normal range this will exclude heterozygosity between sickle cell anemia and other hemoglobin diseases.

Fetal hemoglobin also may present in sicklers in different quantities, HbF is beneficial Hb in sicklers because it decrease sickle cell anemia crises.

Fetal hemoglobin is given to patients to improve quality of their life and decrease sickle cell anemia crises, hydroxyurea is the most important treatment use to increase level of fetal hemoglobin in sickle cell disease patients, other fetal hemoglobin inducing agents also used when patients not respond to hydroxyurea, one of this agents are histone deacetylase (HDAC) inhibitors.

Fetal hemoglobin decrease sickle cell disease crises because of it's high oxygen affinity which an effect mediated by its exclusion from the sickle hemoglobin polymer.

This study measure the level of different hemoglobin types in sickle cell disease patients attended medical Army hospital in Khartoum state.

Also compare the level of this hemoglobin types in males and females, also in homozygous and heterozygous states of sickle cell disease.

Correlation of different hemoglobin types with age is also done.

1.6 Objectives:

General objective:

To quantify the level of Hemoglobin types among sickle cell disease patients.

Specific objectives:

- 1- To measure level of hemoglobin F quantitatively by capillary electrophoresis among sicklers.
- 2- To measure level of hemoglobin S among sicklers.
- 3- To measure level of hemoglobin A2 among sicklers.
- 4- To measure the level of hemoglobin A among sicklers.
- 5- To compare level of different hemoglobin types level in both genders and also in homozygous and heterozygous states of sickle cell disease.
- 6- To compare different hemoglobin types level with age.

Chapter Two

Materials and Method

Materials and Method

2.1 Study design:

This is a prospective, analytical and cross sectional study.

2.2 Study area and period:

This study was done in Khartoum state during the period April 2015 to July 2015.

2.3 Study population:

The study was performed in Sudanese patients with sickle cell disease as study population.

2.3.1 Inclusion criteria:

Sickle cell disease patients.

2.3.2 Exclusion Criteria:

No Sickle cell disease.

2.4 Sample size and sampling technique:

According to the following formula the sample size :-

$$no = \frac{z^2 Pq}{d^2}$$

n = Sample size

z = the normal standard deviate

p = the frequency of occurrence of an event

q = the frequency of non occurrence of an event

d = degree of precision

Due to limitations of our resources only forty Sudanese sickle cell disease patients as study group.

2.5 Storage of samples:

Samples were stored at 2-8 °C for seven days.

2.6 Sample preparation:

-Whole blood samples used directly.

-all the tubes checked that were containing minimum 1 ml of blood and perfectly closed.

-Samples stored at 2-8°C for one week vortexed for 5 seconds.

2.7 Equipment and accessories required:

1. CAPILLARYS 2 FLEX-PIERCING System SEBIA(manufacturing in France).

2. Sample racks supplied with CAPILLARYS 2 FLEX-PIERCING.

3. CAPILLARYS 2 FLEX-PIERCING racks for tubes 11 mm.

4. Container Kit supplied with CAPILLARYS 2 FLEX-PIERCING: Rinse (to fill with distilled or demineralized water), wash solution and waste container.

5. Collection tubes with 13-16 mm diameter, 75-100 mm high or from 1.5 ml micro tubes positioned on the primary sample tubes, tubes with caps.

(Sample volume 20 to 40 micro liter).

6. Tubes and caps for Controls (20 units) or (500 units), conical tubes and their caps to analyze blood controls with the CAPILLARYS 2 FLEX-PIERCING instrument.

7. Wedge adapters for tubes for controls SEBIA, 10 units (or supplied with CAPILLARYS 2 FLEX-PIERCING).

8. Boxes for controls storage, 2 boxes for storage of dilution segments containing hemolyzed Control.

2.8 Principle of the test:

The hemoglobin spatial structure and other molecular properties (like that of all proteins) depend on the nature and the sequence of the amino acids constituting the chains (Landers, 1995; Livingstone, 1986; Schneider, 1978).

Substitution of amino acids by mutation is responsible for formation of hemoglobin variants which have different surface charge and consequently different electrophoretic mobilities, which also depend on the pH and ionic strength of the buffer (Landers, 1995; Livingstone, 1986; Schneider, 1978).

Hemoglobin electrophoresis is a well established technique routinely used in clinical laboratories for screening samples for hemoglobin abnormalities (Bardakdjian-Michau *et al.*, 2003; Fairbanks, 1980; Galacteros, 1986; Hempe *et al.*, 1997; Oda *et al.*, 1997).

CAPILLARYS 2 is an automated, multitasking capillary electrophoresis system using 8 capillary tubes for multiple and simultaneous hands-free electrophoretic separation at high speed.

The CAPILLARYS 2 provides fully automated electrophoresis sequencing, from the primary sample tube right through to the final electrophoretic profile: sample identification, sample dilution, capillary cleansing, sample injection in to the capillaries, migration, detection, results processing and transfer over computer network .

In many respects, the methodology can be considered as an intermediary type of technique between classical zone electrophoresis and liquid chromatography (Krauss *et al.*, 1986; Maier-Redelsberger and Girot, 1989).

The CAPILLARYS 2 FLEX-PIERCING instrument uses the principle of capillary electrophoresis in free solution. With this technique, charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific

pH. Separation also occurs according to the electrolyte pH and electro osmotic flow (Huisman and Jonxis, 1977).

A sample dilution with hemolysing solution is prepared and injected by aspiration at the anodic end of the capillary. A high voltage protein separation is then performed and direct detection of the hemoglobin types is made at 415 nm at the cathodic end of the capillary. Before each run, the capillaries are washed with a wash solution and prepared for the next analysis with buffer.

The hemoglobin, separated in silica capillaries, are directly and specifically detected at an absorbance wave length of 415 nm which is specific to hemoglobin. The resulting electrophoregrams are evaluated visually for pattern abnormalities. Direct detection provides accurate relative quantification of individual hemoglobin fraction, with particular interest, such as A₂ hemoglobin for β thalassemia diagnosis. In addition, the high resolution of this procedure should allow the identification of hemoglobin variants, in particular, to differentiate hemoglobin S from D, and E from C.

The hemoglobin A₂ quantification can also be performed when hemoglobin E is present.

By using alkaline pH buffer, normal and abnormal (or variant) hemoglobin are detected in the following order, from cathode to anode: δ A'₂ (A₂ variant), C, A₂/O-Arab, E, S, D, G-Philadelphia, F, A, Hope, Bart's, J, N-Baltimore and H. The carbonic anhydrase is not visualized on the hemoglobin electrophoretic patterns, this permit to identify hemoglobin A₂ variants in this migration zone.

2.9 Reagents:

1. Normal Hb A2 control:

Composition:

The normal Hb A2 control is obtained from a pool of normal human blood samples. The normal Hb A2 control is in a stabilized lyophilized form.

Use:

The normal Hb A2 control is designed for the migration control before starting a new analysis sequence, after the analyses of 10 successive sample racks and at the end of an analysis sequence, and for the quality control of human hemoglobin A2 quantification with CAPILLARYS HEMOGLOBIN(E) electrophoresis procedure performed with the CAPILLARYS 2 FLEX-PIERCING instrument.

Reconstitute each normal Hb A2 control vial with the exact volume of distilled or water, as indicated in the package insert of the normal Hb A2 control.

Allow to stand for 30 minutes and mix gently (avoid formation of foam).

NOTE: The precision of the reconstitution volume to be maintained is $\pm 1.0\%$.

2. Distilled or demineralized water:

Use:

For rinsing capillaries in automated instrument CAPILLARYS 2 FLEX-PIERCING, SEBIA, for capillary electrophoresis deionized.

It is recommended to use filtered distilled or demineralized water (on a filter with a porosity $\leq 0.45 \mu\text{m}$).

3. Capiclean:

Composition:

The vial of capiclean concentrated solution (25 ml) contains: proteolytic enzymes, surfactants and additives non-hazardous at concentrations used, necessary for optimum performances.

Use:

For sample probe cleaning in automated instrument CAPILLARYS 2 FLEX-PIERCING, SEBIA, for capillary electrophoresis, during the capiclean cleaning sequence.

4. Sodium hypochlorite solution (for sample probe cleaning):

Preparation:

Prepare a sodium hypochlorite solution (2 % to 3 % chloride) by diluting 250 ml 9.6% chloride concentrated solution to 1 liter with cold distilled or deionized water.

Use:

For the sample probe cleaning in the CAPILLARYS 2 FLEX-PIERCING instrument (weekly maintenance in order to eliminate adsorbed proteins from the probe).

5. CAPILLARYS / Minicap wash solution:

Preparation:

Each vial of the stock wash solution (2 vials, 75 ml) should be diluted up to 750 ml with distilled or deionized water.

After dilution, the wash solution contains an alkaline solution pH \approx 12.

Use:

additional reagent is needed when the number of tests in series is below 40.

6. Trace buffer PH (8.4):

Preparation:

- hydroxymethylamin
- phosphoric acid (3.4)
- EDTA

Which dissolve in 1 liter distilled water.

7. Diluted acetic acid 3% (3ml +97 ml D.W)

8. Poncea red stain 5% (5 g + 100 D.W)

2.10 Procedure:

The CAPILLARYS 2 FLEX-PIERCING instrument is a multiparameter instrument for hemoglobin analysis on parallel capillaries. The hemoglobin assay uses 8 capillaries to run the samples.

The sequence of automated steps is as follows:

- Bar code reading of sample tubes (for up to 8 tubes) and sample racks.
- Mixing of blood samples before analysis.
- Sample hemolysis and dilution from primary tubes into dilution segments.
- Capillary washing.
- Injection of hemolyzed samples.
- Hemoglobin separation and direct detection of the separated hemoglobin on capillaries.

The manual steps include:

- Placement of sample tubes (with caps) in sample racks in positions 1 to 8;
- Placement of new dilution segments in sample-racks;
- Placement of racks on the CAPILLARYS 2 FLEX-PIERCING instrument;
- Removal of sample-racks after analysis.

CAREFULLY READ THE CAPILLARYS 2 FLEX-PIERCING INSTRUCTION MANUAL.

Preparation of capillary analysis:

1. Switch on CAPILLARYS 2 FLEX-PIERCING instrument and computer.
2. Set up the software, enter and the instrument automatically starts.
3. The CAPILLARYSHEMOGLOBIN(E) kit is intended to run with "HEMOGLOBIN(E)" analysis program from the CAPILLARYS 2 FLEX-PIERCING instrument. To select "HEMOGLOBIN(E)" analysis program and place the CAPILLARYS HEMOGLOBIN(E) buffer and hemolyzing solution vials in the instrument, please read carefully the CAPILLARYS 2 FLEX-PIERCING instruction manual.
4. The sample rack contains 8 positions for sample tubes. Place up to 8 capped sample tubes with whole blood on each sample rack (positions 1 to 8); the bar code of each tube must be visible in the openings of the sample rack.
5. Position a new dilution segment on each sample rack. The sample rack will be ejected if the segment is missing.
6. Slide the complete sample carrier(s) into the CAPILLARYS 2 FLEX-PIERCING instrument through the opening in the middle of the instrument.
Up to 13 sample racks can be introduced successively and continuously into the instrument. When analyzing a control blood sample, it is advised to use the sample rack No. F0 intended for control blood sample with specific tubes, caps and the wedge adapter for tubes for controls.
7. Remove analyzed sample racks from the plate on the left side of the instrument.
8. Take off carefully used dilution segments from the sample rack and discard them.

WARNING: Dilution segments with biological samples have to be handled with care.

Dilution-Migration-Description of the automated steps:

1. Bar codes are read on both sample tubes and sample racks.
2. Mixing of tubes.
3. Samples are diluted in hemolysing solution and the sample probe is rinsed after each sample.
4. Capillaries are washed.
5. Diluted samples are injected into capillaries.
6. Migration is carried out under constant voltage for about 8 minutes and the temperature is controlled by Peltier effect.
7. Hemoglobins are detected directly by scanning at 415 nm and an electrophoretic profile appears on the screen of the instrument.

2.11 Result analysis in CAPILLARYS 2 FLEX-PIERCING:

At the end of the analysis, relative quantification of individual hemoglobin fractions is performed automatically and profiles can be analyzed; the hemoglobin fractions, HbA, HbF and HbA2 are automatically identified; the HbA fraction is adjusted in the middle of the review window. The resulting electrophoregrams are evaluated visually for pattern abnormalities.

The potential positions of the different hemoglobin variants (identified in zones called Z1 to Z15) are shown on the screen of the instrument and indicated on the result ticket. The table in paragraph "Interpretation" shows known variants which may be present in each corresponding zone.

When the software identifies a hemoglobin fraction in a defined zone, the name of this zone is framed. Patterns are automatically adjusted with regard to HbA and HbA2 fractions to facilitate their interpretation:

- when HbA and/or HbA2 fractions are not detected on an electrophoretic pattern, a yellow warning signal appears, the adjustment is performed using the position of the HbA fraction on the two previous patterns obtained with the same capillary; then, there is no fraction identified (except when HbC is detected: in this case, HbA2 and HbC fractions are identified);
- when HbF is detected on an electrophoretic pattern, without any detection of HbA, the yellow warning signal does not appear, the adjustment is then performed using the position of the HbF fraction, and HbF and/or HbA and/or HbA2 fractions are identified;
- when the adjustment is not possible, a red warning signal appears, HbF and HbA2 fractions are then not identified (Call SEBIA);
- when optical density (OD) is insufficient on a migration control electrophoretic pattern (obtained with the Normal HbA2 Control, identified with its bar code label on the sample rack No.(0)), a warning message is displayed in order to consider or remove this analysis for the determination of HbA fraction position. Then, a purple warning signal appears on the review window and HbA and HbA2 fractions are not identified.

In all cases, the different migration zones (Z1 to Z15) do not appear neither on the screen of the instrument, nor on the ticket result.

On the electrophoretic pattern, the curves of HbA2 and HbC fractions, are calculated and redrawn by fitting with adjustment (or fitted) and are overlaid with the native curve. This display allows the HbA2 fraction quantification if HbC is present in the sample.

WARNING: In some cases of hemoglobin C (homozygous) or after a technical problem, the hemoglobins A2 and C are not fitted; these fractions are then under-quantified. It is then recommended to quantify the HbA2 fraction by using another technique.

Values:

Direct detection at 415 nm in capillaries yields relative concentrations (percentages) of individual hemoglobin zones.

Reference values for individual major electrophoretic hemoglobin zones in the CAPILLARYS 2 FLEX-PIERCING instrument have been established from a healthy population of 113 adults (men and women) with normal hemoglobin values using HPLC technique:

Hemoglobin A: comprised between 96.7 and 97.8 %.

Hemoglobin F: ≤ 0.5 %.

Hemoglobin A2: comprised between 2.2 and 3.2%.

2.12 Qualitative abnormalities (Hemoglobinopathies):

Most hemoglobinopathies are due to substitution by mutation of a single amino acid in one of the four types of polypeptide chains (Bardakdjian-Michauet *al.*, 2003; Fairbanks, 1980; Hempe, 1997; Landers, 1995; Odaet *al.*, 1997). The clinical significance of such a change depends on the type of amino acid and the site involved (Schneider, 1978). In clinically significant disease, either the α -chain or the β -chain is affected. More than 1400 variants of adult hemoglobin have been described (Jellumet *al.*, 1997; Vovanet *al.*, 1985). The first abnormal hemoglobins studied and the most frequently occurring have an altered net electric charge, leading to an easy detection by electrophoresis. There are five main abnormal hemoglobins which present a particular clinical interest: S, C, E, O-Arab and D.

2.13 Hemoglobin S:

Hemoglobin S is the most frequent. It is due to the replacement of one glutamic acid (an acidic amino acid No. 6) of the β -chain by valine (a neutral amino acid), when compared to HbA, its isoelectric point is elevated and its total negative

charge decreased with the analysis pH. Its electrophoretic mobility is therefore increased in the capillary and this hemoglobin is faster than A fraction. With alkaline buffered CAPILLARYS HEMOGLOBIN(E) procedure, hemoglobin S migrates between A and A2 fractions, next to HbA2.

Chapter Three

Result

Result

Demographic results:

40 sickle cell disease patients were studied, 18 patients were males and 22 patients were females, aged from 1 year to 13 years. 23 patients were homozygous (HbSS) and 17 patients were heterozygous (HbAS) as known cases.

Different hemoglobin types were measured by capillary electrophoresis, and results were analyzed by SPSS computer programme.

Mean and standard Deviation of different Hb types (HbF, HbS, HbA₂ and HbA) were obtained, comparison between Hb types with gender and homozygous and heterozygous states, Correlation of this types with age also obtained.

Results as following:

Table (3-1): Mean and standard Deviation of different hemoglobin types

Variable	Mean \pm std Deviation
HbF (%)	7.2 \pm 4.9
HbS (%)	78.5 \pm 19.2
HbA ₂ (%)	3.1 \pm 0.64
HbA (%)	11.2 \pm 21.5

Table (3-2): Comparison of different hemoglobin types in both genders

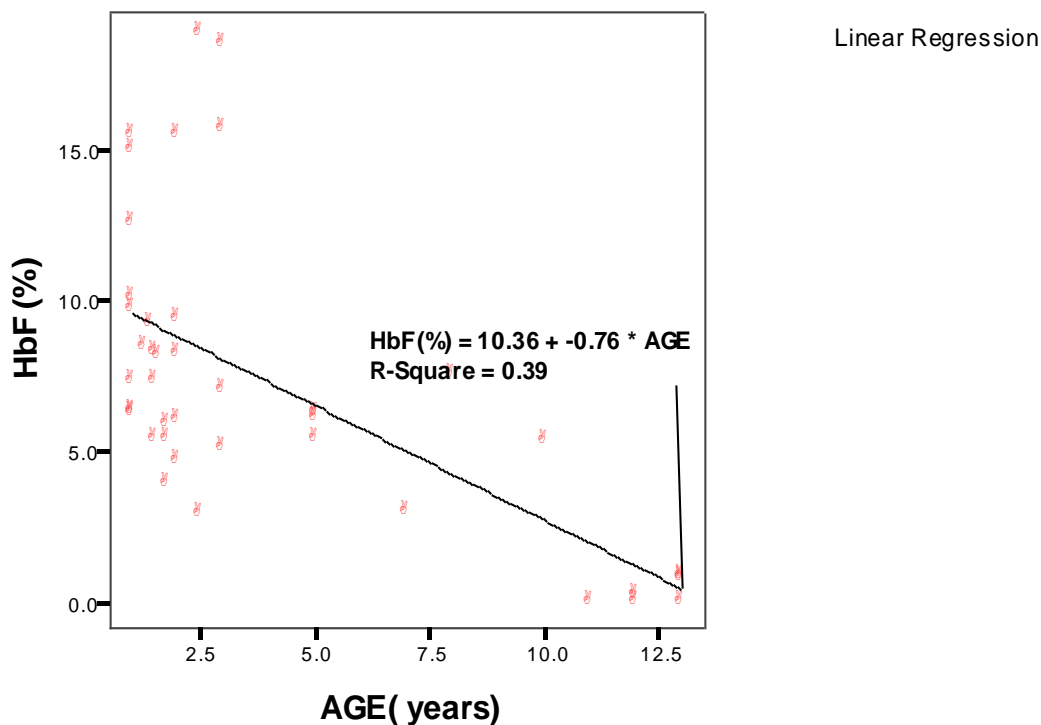
Variable	Male(18) Mean \pm std Deviation	Female(22) Mean \pm std Deviation	P.value
HbF(%)	6.3 \pm 3.5	8.1 \pm 5.7	0.218
HbS(%)	75.4 \pm 24.6	80.9 \pm 13.3	0.370
HbA ₂ (%)	3.3 \pm 0.66	2.9 \pm 0.60	0.164
HbA(%)	14.9 \pm 27.5	8.2 \pm 15.0	0.327

Independent T- Test was used, P value significant at ≤ 0.05

Table (3-3): Comparison of different hemoglobin types in both homozygous and heterozygous states of Sickle Cell disease

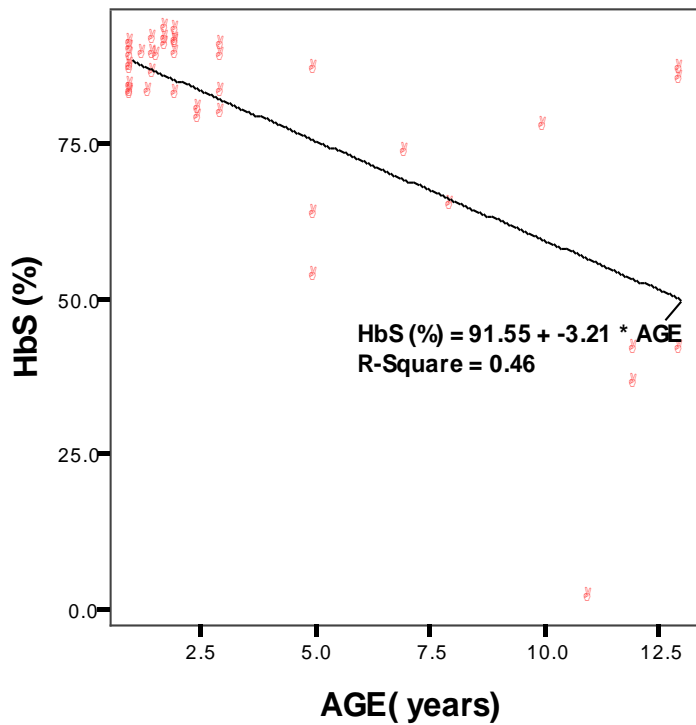
Variable	HbSS (23) Mean \pm std Deviation	HbAS (17) Mean \pm std Deviation	P value
HbF(%)	9.7 \pm 4.5	3.9 \pm 3.3	0.000
HbS(%)	87.1 \pm 4.3	66.7 \pm 24.8	0.004
HbA2 (%)	3.3 \pm 0.66	2.9 \pm 0.56	0.105
HbA(%)	.000 \pm .000	26.4 \pm 26.5	0.001

Independent T- Test was used, P value significant at ≤ 0.05



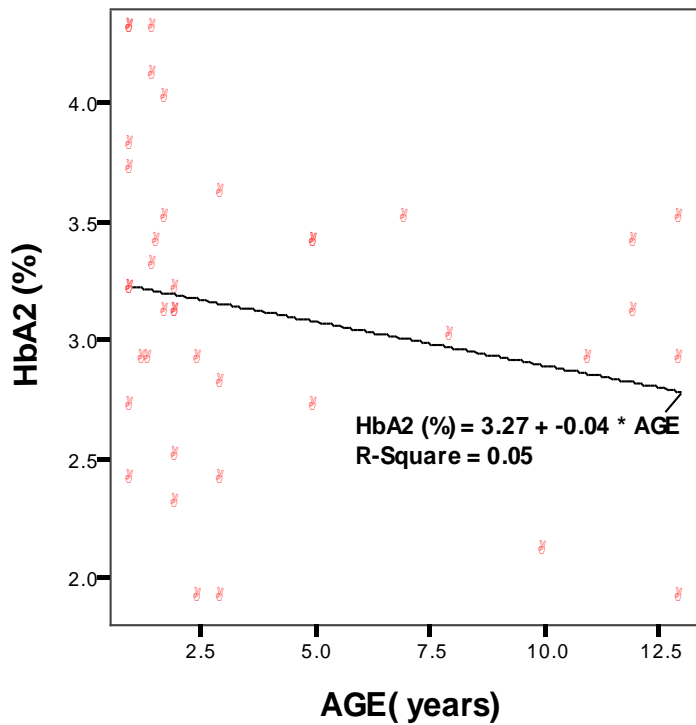
Figure(3-1):Correlation between HbF (%) and age (years) among the study group

Strong negative correlation ($p = .000, r = -.623^{**}$). Correlation is significant at 0.01 level.



Linear Regression

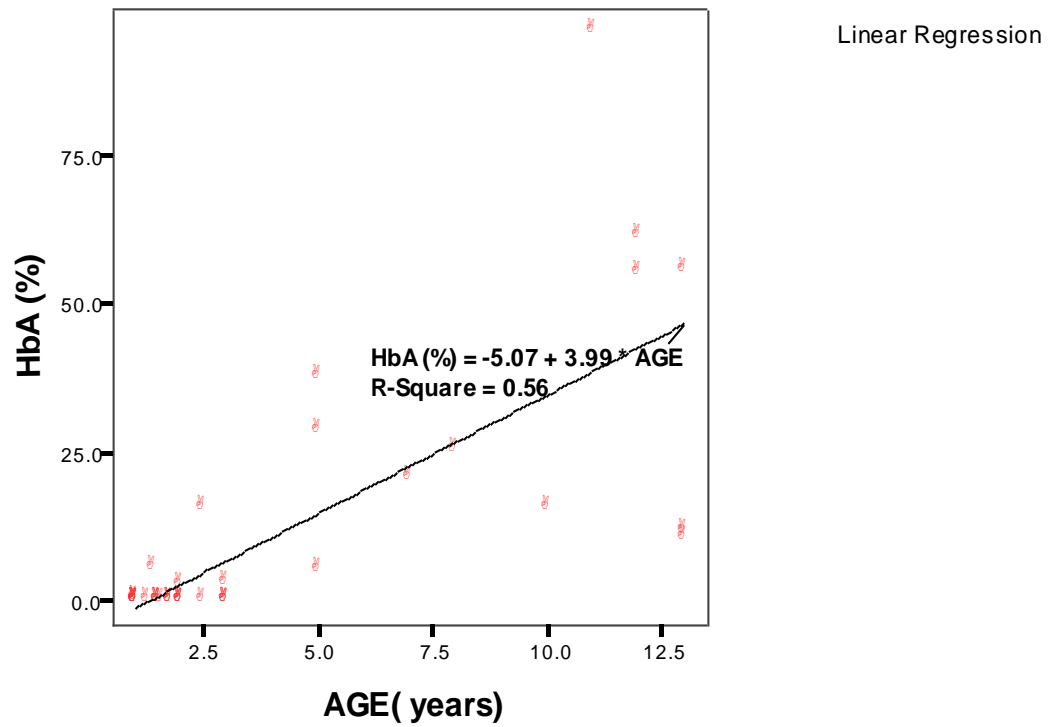
Figure(3-2): Correlation between HbS (%) and age (years) among the study group
 Strong negative correlation ($p = .000$, $r = -.676^{**}$). Correlation is significant at 0.01 level.



Linear Regression

Figure(3-3): Correlation between HbA2 (%) and age (years) among the study group

Weak negative correlation ($p = .148$, $r = -.233$).



Figure(3-4): Correlation between HbA (%) and age (years) among the study group
Positive correlation ($r = .750$, $p = .000$).Correlation is significant at 0.01 level.

Chapter Four

Discussion, Conclusion and Recommendations

Discussion

This study was conducted to determine level of different hemoglobin types in sickle cell anemia patients.

Forty blood samples were analyzed during the period April 2015 to July 2015 in Khartoum state, 18 patients were males and 22 patients were females, 23 patients were homozygous (HbSS) and 17 patients were heterozygous (HbAS) as known cases.

Hemoglobin types levels measured by capillary electrophoresis and result analyzed by SPSS computer programme.

The mean and standard deviation of HbF, HbS, HbA₂ and HbA in percentages are (7.2 ± 4.9), (78.5 ± 19.2), (3.1 ± 0.64) and (11.2 ± 21.5) respectively as in table (3-1).

The mean of HbS is the larger indicate that most patients have large level of HbS which is the diagnostic hemoglobin for sickle cell anemia.

The mean of HbF is increased because all patients given hydroxyurea and other supportive treatment. HbA₂ show normal value, while HbA_{1c} is decreased.

HbF, HbS, HbA₂ and HbA (%) show different means and standard deviations in males and females as HbF % in males (6.3 ± 3.5) while in females (8.1 ± 5.7) P value (0.218), HbS % in males (75.4 ± 24.6) while in females (80.9 ± 13.3) P value (0.370), HbA₂ (%) in males (3.3 ± 0.66) while in females (2.9 ± 0.60) P value (0.164) and HbA (%) in males (14.9 ± 27.5) while in females (8.2 ± 15.0) P value (0.327) as in table (3-2). P value insignificant in all.

The mean and standard deviation of different hemoglobin types in homozygous patients (SS) and heterozygous patients (AS) are HbF (%) in (HbSS) patients (9.7 ± 4.5) while in (HbAS) patients (3.9 ± 3.3) P value (0.000) which is significant value

means the difference of HbF in homozygous and heterozygous patients is true difference that show fetal Hb is higher in homozygous than heterozygous.

HbS (%) in (HbSS) patients (87.1 ± 4.3) while in (HbAS) patients (66.7 ± 24.8) P value (0.004)) which is significant value means the difference in HbS in homozygous and heterozygous patients is true difference that show sickle Hb is higher in homozygous than heterozygous and this is because homozygous have two copies of HbS while heterozygous have just one copy of HbS.

HbA2 (%) in (HbSS) patients (3.3 ± 0.66) while in (HbAS) patients (2.9 ± 0.56) P value (0.105) insignificant value.

HbA (%) in (HbSS) patients ($.000 \pm .000$) while in (HbAS) patients (26.4 ± 26.5) P value (0.001) which is significant value means the difference in HbA in homozygous and heterozygous patients is true difference that show adult Hb is higher in heterozygous than homozygous and this is because homozygous have no HbA gene while heterozygous have one copy of HbA gene (shown in table 3-3).

Correlation between HbF (%) and age (years) among the study group show Strong negative correlation as in figure (1) ($p = .000, r = -.623^{**}$). Correlation is significant at 0.01 level.

Correlation between HbS (%) and age (years) among the study group show Strong negative correlation as in figure (2) ($p = .000, r = -.676^{**}$). Correlation is significant at 0.01 level.

Correlation between HbA2 (%) and age (years) among the study group show weak negative correlation as in figure (3-3) ($p = .148, r = -.233$).

Correlation between HbA (%) and age (years) among the study group show positive correlation as in figure (3-4) ($r = .750, p = .000$). Correlation is significant at 0.01 level.

This study similar to study which done by Khair F.M (Khartoum University) at 2002 in Sudan was found sickle cell anemic patient HbF level about ($7 \pm 5.6\%$),

while sickle cell trait patient had (0.8 + or - 0.5), in other research sickle cell anemic patient had (9.2 + or -4.9%) of HbF level(Khair, 2002).

As reported by this research that sickle cell anemic patient in their first decade of life (0-10 years) had higher HbF (8.2+ or – 5.8) than patient in old age group (above 10 years) HbF level (2.7 + or – 1.1%) (Khair, 2002).

Gender also factor rate of HbF level in female (8 + or -2.4%) sickle cell anemia is associated with higher HbF concentration than male (5.6 + or – 3.1%)(Khair, 2002).

Drug inducing HbF have effect also been stimulating the formation of HbF(Khair, 2002).

The mean HbF level among sickle cell Sudanese as reported by previous research was (9%) (Khair, 2002).

In another study by Akinsheye (Akinsheye *et al.*, 2011) fetal hemoglobin is the major genetic modulator of the hematologic and clinical features of sickle cell disease, an effect mediated by its exclusion from the sickle hemoglobin polymer. Fetal hemoglobin genes are genetically regulated, and the level of HbF and its distribution among sickle erythrocytes is highly variable. Some patients with sickle cell disease have exceptionally high levels of HbF that are associated with the Senegal and Saudi-Indian haplotype of the *HBB*-like gene cluster; some patients with different haplotypes can have similarly high HbF. In these patients, high HbF is associated with generally milder but not asymptomatic disease. Studying these persons might provide additional insights into HbF gene regulation. HbF appears to benefit some complications of disease more than others. This might be related to the premature destruction of erythrocytes that do not contain HbF, even though the total HbF concentration is high. Recent insights into HbF regulation have spurred new efforts to induce high HbF levels in sickle cell disease beyond those

achievable with the current limited repertoire of HbF inducers (Akinsheye *et al.*, 2011).

Hemoglobin F (HbF) has been a useful criterion in predicting the clinical severity of sickle cell disease (SCD) as studied by Kotilaat (2000). Thus different treatment modalities are geared towards raising its level. This study estimated HbF levels in sickle cell anemia patients. HbF levels were then compared with clinical parameters such as the average number of bone pain crisis per year, transfusion requirement, enlargement of both the spleen and liver and the hematocrit level. The mean HbF value was $7.4 \pm 3.6\%$. Males recorded a higher mean level than females $7.6 \pm 3.9\%$, and $6.7 \pm 3.6\%$ respectively, ($P > 0.05$). HbF of 7.4% was used to divide the patients into two broad groups. Patients with HbF of more than 7.4% were older compared to those with less than 7.4% ($P > 0.5$), the former group was also less transfusion dependent ($P > 0.05$) even though their hematocrit was not significantly different ($P > 0.05$) from those with HbF of $< 7.4\%$. The patients with higher HbF levels are also more likely to retain their spleen longer than their counterpart with lower values. It appears that clinical severity has a relationship with HbF values even though most were not statistically significant. There is a need for larger studies to study this relationship more closely (Kotila *et al.*, 2000). HbA2 is raised in Sickle-beta + thalassemia (Hb S- β^+ thal) and Sickle-beta 0 thalassemia (Hb S- β^0 thal), but not in sickle cell anemia (Embury, 2005).

Conclusion:

- HbF in sickle cell anemia patients was found to be 7.2% (raised).
- HbS in sickle cell anemia patients was found to be 78.5%.
- HbA2 in sickle cell anemia patients was found to be 3.1% (normal).
- HbA in sickle cell anemia patients was found to be 11.2% (decreased).
- HbF in males (6.3%), in females (8.1%) (increase in both gender, minimal difference between two gender).
- HbS in males (75.4%), in females (80.9%) (increase in both gender with minimal difference).
- HbA2 in males (3.3%), in females (2.9%) (normal in both).
- HbA in males (14.9%), in females (8.2%) (decrease in both gender).
- HbF in homozygous patients (SS) was found to be (9.7%), HbF in heterozygous patients (AS) was found to be (3.9%). The level of HbF in homozygous patients is more than in heterozygous patients.
- HbS in homozygous patients (SS) was found to be (87.1%), HbS in heterozygous patients (AS) was found to be (66.7%). The level of HbS in homozygous patients is more than in heterozygous patients.
- HbA2 in homozygous patients (SS) was found to be (3.3%), HbA2 in heterozygous patients (AS) was found to be (2.9%) (normal in both states).
- HbA in homozygous patients (SS) was found to be (.000%), HbA in heterozygous patients (AS) was found to be (24.6%) (decrease).

Recommendations:

- 1- Further research to determine the role of HbF in decrease crises and improve sicklers life.
- 2- Further research for drugs that can be introduced to raise the level of HbF.
- 3- Patients must given folic acid and pencillin.

Chapter Five

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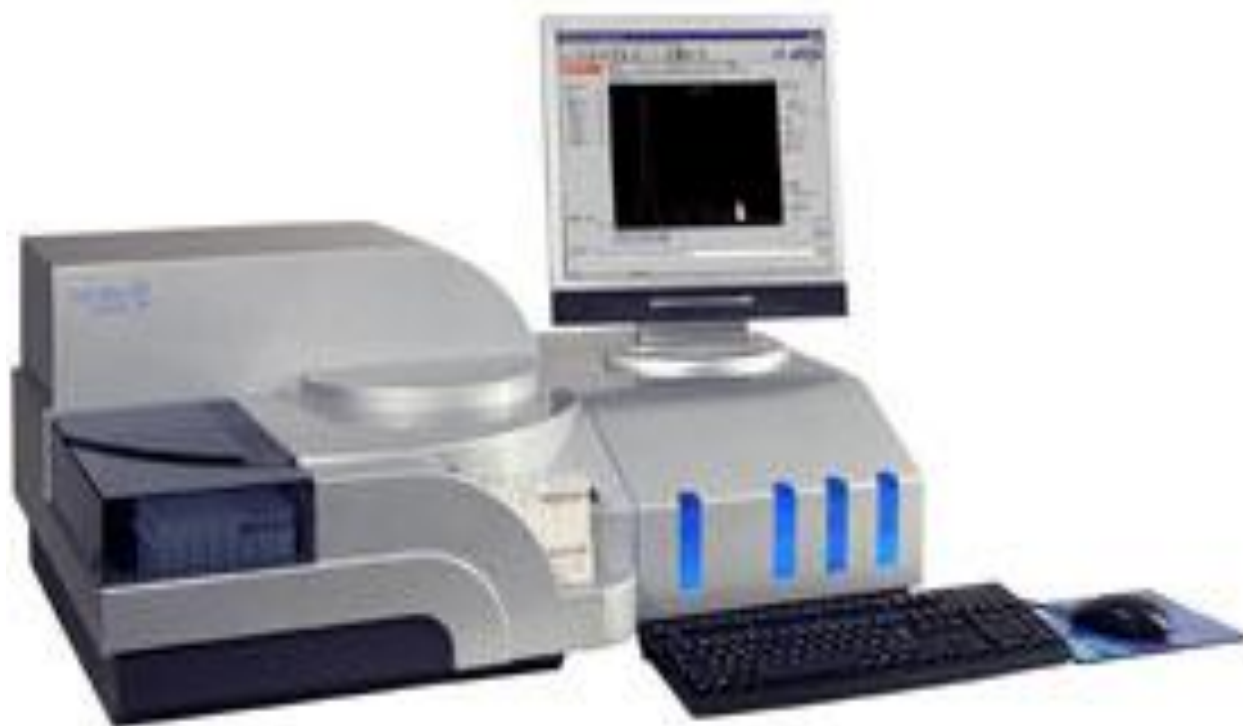
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Appendices



Appendix (1): CAPILLARYS 2 FLEX-PIERCING Instrument