

Chapter One

Introduction

1.1 Introduction:

Sickle cell disease (SCD) is a group of autosomal recessive genetic blood disorders characterized by a single nucleotide substitution (GTG>GAG in the sixth codon of the β -globin gene). Under low oxygen tension, the resultant abnormal hemoglobin S polymerizes and causes rigid and sickle-shaped red blood cells (Rees *et al.*, 2010).

In sub-Saharan Africa, the prevalence of sickle cell trait ranges between 5% and 40%, and >230,000 (0.74% of total birth) infants are born with sickle anemia every year (Weatherall *et al.*, 2006).

Rates of SCA and trait varied in different areas in Sudan with the highest rates reported from Western and Eastern Sudan where about 1% of children born in Western Sudan is at risk of having SCD (Majdi and Hanan, 2014).

Sickle cell disease is presented with many manifestations but vaso-occlusive crisis is the main clinical manifestation accompanied by haemolysis and cause hospitalization, requiring blood transfusion, organ damage and death. Moreover, stroke is the most serious complication and the major cause of physical disability and cognitive impairment. In Africa, the life expectancy of patients with sickle cell disease is lesser than 20 years, and those less than 5 years of age are at the highest risk of death (Daak *et al.*, 2013).

SCA is a major cause of morbidity and mortality in Africa where there is no readily available effective treatment (Akinsegun, 2012).

It was thought that haemolytic and vaso-occlusive crisis is caused by a mechanical obstruction of small blood vessels by rigidly distorted (sickled) red blood cells. However, the blood cells of patients with sickle cell disease

have a tendency to adhere to vascular endothelium, and there is a correlation between blood cell–vessel wall adhesive interactions and vaso-occlusive crisis (Hogan *et al.*, 2006, Kirkham, 2007, Ballas *et al.*, 2010). These findings have led to the current postulation that an enhanced tendency of red blood cells (sickled and non-sickled) to adhere to vascular endothelium and activation of platelets and leukocytes are the primary causative factors of haemolysis and vaso-occlusion (Frenette *et al.*, 2007, Okpala *et al.*, 2011). It has been reported that many modifiers such like supplementation with certain supplements tend to reduce the frequency of pain episodes requiring hospital presentation and the number of sickle cell crises (Daak *et al.*, 2013).

It was found subsequently that sickle red cells, and in particular sickle reticulocytes, express a number of adhesion receptors including the integrins which are molecules that play intricate functions in recognition of inter-cellular and cellular-matrix communication. Selectins are a family of transmembrane molecules, expressed on the surface of leukocytes and activated endothelial cells, these molecules generate ligand-specific outside-in signals to modulate neutrophil apoptosis, a critical control point in the resolution of inflammation (Daak *et al.*, 2013).

The selectin family of adhesion molecules mediates the initial attachment of leukocytes to venular endothelial cells before their firm adhesion and diapedesis at sites of tissue injury and inflammation. These receptors regulate inflammatory processes. Selectin-directed therapeutic agents are now proven to be effective in blocking many of the pathological effects resulting from leukocyte entry into sites of inflammation (Okpala, 2006).

This study aimed at studying integrin alpha-2 (*ITGA2*) C807T and L-selectin (*SELL*) P213S allele's polymorphism and their association with clinical severity of sickle cell disease among Sudanese patients.

Chapter two

Literature review

2.1 Sickle cell disease:

Sickle cell disease (SCD), also known as sickle cell anemia, is a serious disease in which the body makes an altered form of hemoglobin, the protein in red blood cells that carries oxygen throughout the body. This genetic alteration causes the body to produce abnormal sickle- or crescent-shaped red blood cells. Homozygous sickle cell anemia (Hb SS) is the most common (HbS) is insoluble and forms crystals when exposed to low oxygen tension. Deoxygenated sickle hemoglobin polymerizes into long fibers, each consisting of seven intertwined double strands with cross-linking. The red cells sickle and may block different areas of the microcirculation or large vessels cause infarcts of various organs. The sickle globin abnormality is caused by substitution of valine for glutamic acid in position 6 in the chain. It is very widespread and is found in up to one in four West Africans (Kato *et al.*, 2009).

While the doubly heterozygote conditions also cause sickling disease which SCD variants include hemoglobin SC, a heterozygous combination of HbS and hemoglobin C, and hemoglobin S and α^2 -thalassemia, these conditions cause SCD, although the symptoms and complications may be less severe than those in the homozygous condition (Akinsegun *et al.*, 2012).

2.1.1 Epidemiology of SCD:

SCD is most common in people whose families come from Africa, South or Central America (especially Panama), Caribbean islands, Mediterranean countries (such as Turkey, Greece, and Italy), India, and Saudi Arabia. In the

United States, it is estimated that SCD affects about 70,000 to 100,000 people, primarily African Americans. The disease occurs in about 1 out of every 500 African American births. The disease occurs in 1 out of every 36,000 Hispanic American births (Akinsegun *et al.*, 2012).

The available data reported the wide range of SC disease frequencies in different areas of Sudan ranging from 0.8% in central Sudan to 30.4% in Western Sudan. The Messeryia tribe (a branch of the Baggara tribes) in Kordofan and Darfur showed the highest rate of sickle cell disease where it is estimated that one in every 123 children born is at risk of having SCD. While In the Blue Nile area, where groups of indigenous population live, the prevalence ranges from 0-5% in addition to a rate of 16% among some immigrant tribes from western Sudan and West Africa in the area (Majdi and Hanan, 2014).

2.1.2 Pathophysiology of SCD:

SCD is characterized by the production of hemoglobin S (HbS) in red blood cells (RBC). Hb S polymerizes when deoxygenated, causing the cells to become rigid and adopt a sickled state, as well as making cells more fragile and susceptible to rupture therefore leading to hemolysis (Chinegwundoh and Anie, 2015).

Intravascular hemolysis has significant consequences; hemoglobin (Hb) released into the plasma results in endothelium-derived nitric oxide (NO) depletion, contribute significantly to vascular oxidative stress and endothelial cell activation. Activated endothelium produces inflammatory cytokines and expresses adhesion molecules on its surface, resulting in leukocyte capture, and RBC and platelet adhesion to the vascular wall. Inflammatory mediator production from activated platelets, leukocytes and endothelial cells results in the inflammatory state that is associated with SCD and that drives recurrent

vaso-occlusive processes, which result from leukocyte and RBC adhesion to the endothelium, leading to reduced blood flow, diminished oxygen concentrations, consequent red cell sickling and eventually the occlusion of vessels. Early investigational therapies under development for SCD use a pathophysiological-based approach to abolish or reduce one or more of the mechanisms that contribute to this disease's complex pathophysiology (Chinegwundoh and Anie, 2015).

2.1.3 Inheritance of SCD:

SCD is an inherited disease, people who have the disease inherit two copies of the sickle cell gene one from each parent. If a person inherits only one copy of the sickle cell gene (from one parent), he or she will have sickle cell trait. Sickle cell trait is different from SCD. People who have sickle cell trait do not have the disease (carrier), but they have one of the genes that cause it. Like people who have SCD, people who have sickle cell trait can pass the gene to their children (Rumaney *et al.*, 2014).

2.1.4 Clinical features of SCD:

Clinical features are of a severe haemolytic anemia punctuated by crises. The symptoms of anemia are often mild in relation to the severity of the anemia because Hb S gives up oxygen (O₂) to tissues relatively easily compared with Hb A, its O₂ dissociation curve being shifted to the right (Hoff brand *et al.*, 2006).

2.1.5 Common complications and crises:

The clinical expression of Hb SS is very variable, some patients having an almost normal life, free of crises but others develop severe crises even as infants and may die in early childhood or as young adults. Older children with sickle cell disease and VOC have increased hospitalizations rates, age effect should be considered when measuring the effect of an intervention on hospital

utilization. Ulcers of the lower legs are common; as a result of vascular stasis and local ischemia. The spleen is enlarged in infancy and early childhood but later is often reduced in size as a result of infarcts (autosplenectomy) (Panepinto *et al.*, 2005, Hoff brand *et al.*, 2006).

2.1.5.1 Painful vaso-occlusive crises:

These are the most frequent and are precipitated by such factors as infection, acidosis, dehydration or deoxygenation (e.g. altitude, operations, obstetric delivery, stasis of the circulation, exposure to cold, violent exercise). Infarcts can occur in a variety of organs including the bones (hips, shoulders and vertebrae are commonly affected) the lungs and the spleen. The most serious vasoocclusive crisis is of the brain (a stroke occurs in 7% of all patients) or spinal cord. Transcranial Doppler ultrasonography detects abnormal blood flow indicative of arterial stenosis. This predicts for strokes in children. This can be largely prevented by regular blood transfusions in these cases. The 'hand-foot' syndrome (painful dactylitis caused by infarcts of the small bones) is frequently the first presentation of the disease and may lead to digits of varying lengths (Du *et al.*, 2015).

For example, based on data from the Cooperative Study of Sickle Cell Disease (CSSCD), in which the circumstances of death were examined in 209 patients who were over 20 years of age when they died, 22% of deaths occurred during a pain episode. Acute chest episodes were temporally related to hospitalization for pain in 77% of patients who had them, and individuals older than 20 years of age with a higher rate of painful episodes had an increased risk of premature death when compared to those with a lower rate of pain (Steinberg *et al.*, 2008).

Painful events are unpredictable and often severe resulting in repeated hospitalizations, missed days of school or work, and very poor health-related

quality of life as well as an increased mortality rate. Furthermore, data suggest that nearly every day, children, adolescents and adults with sickle cell disease all suffer from pain that is intense enough to disrupt day to day functioning. Despite how common and widespread this complication is, there are few treatment options to prevent the development of these events and most are managed with traditional supportive care measures that have not markedly changed in decades. The pain which occurs can be acute or chronic, it varies among individuals in its frequency and intensity, and it is the primary cause of hospitalization in patients with SCD. Common triggers for vaso-occlusive crises include dehydration, infection, extreme temperature, and emotional stress. However, often no identifiable cause is found and pain often occurs without warning (Kato *et al.*, 2009).

2.1.5.2 Bacteremia and sepsis:

Children with sickle cell disease are at increased risk for bacteremia that can result in sepsis and death; due in large part to functional asplenia that develops over time in these children. In developed countries and mainly in Africa, the most common organisms involved include *Streptococcus pneumoniae*, *Salmonella* species, and *Haemophilus influenza* (Williams *et al.*, 2009).

2.1.5.3 Acute chest syndrome:

The specific definition of what constitutes acute chest syndrome (ACS) varies but usually refers to a new pulmonary infiltrate accompanied by fever and/or symptoms or signs of respiratory distress in a patient with sickle cell disease (SCD). It is a relatively common cause of frequent hospitalizations and death and a common indication for transfusion and treatment with hydroxyurea. Several studies suggest that the case fatality rate is lower in children (1.1–1.5%) than adults (4.3–9%), but ACS accounts for a significant proportion of mortality in both groups. Over half of the patients who developed ACS were

hospitalized for another reason prior to developing ACS, usually a vaso-occlusive painful crisis. The etiology of ACS is multi-factorial and not completely understood. Previous studies have shown that infection, fat emboli, and pulmonary infarction are all commonly associated with the development of ACS but many episodes of ACS develop without an obvious cause (Steinberg *et al.*, 2008).

Treatment usually involves antimicrobials to cover both common causes of pneumonia such as *Streptococcus pneumoniae* and *Chlamydia pneumoniae* as well as atypical pathogens such as mycoplasma. If there is a history of asthma, bronchodilators and corticosteroids may be used during an acute chest syndrome event. However, use of corticosteroids may prolong hospitalization or lead to readmission. In addition to these measures, red blood cell transfusion is often used as supportive treatment during an acute chest syndrome event (Kato *et al.*, 2009).

2.1.5.4 Visceral sequestration crises:

These are caused by sickling within organs and pooling of blood, often with a severe exacerbation of anemia. The acute sickle chest syndrome is a feared complication and the most common cause of death after puberty. It presents with dyspnea, falling arterial P_{O_2} , chest pain and pulmonary infiltrates on chest X-ray. Treatment is with analgesia, oxygen, exchange transfusion and ventilatory support if necessary. Hepatic and girdle sequestration crises and splenic sequestration may lead to severe illness requiring exchange transfusions. Splenic sequestration is typically seen in infants and presents with an enlarging spleen, falling hemoglobin and abdominal pain. Treatment is with transfusion and patients must be monitored at regular intervals as progression may be rapid. Attacks tend to be recurrent and splenectomy is often needed (Berry *et al.*, 2007).

2.1.5.5 Pulmonary hypertension:

The prevalence of pulmonary hypertension in adults with sickle cell disease is 25-32% in both the United States and Africa. The use of echocardiogram to detect high tricuspid regurgitant velocity as a marker of increased systolic pulmonary artery pressure has been increasingly used over the last 5 years leading to the recognition that pulmonary hypertension is common in sickle cell disease and is associated with an increased risk of death (Gladwin *et al.*, 2004).

2.1.5.6 Central nervous system disease:

Central nervous system disease is common in sickle cell disease and usually manifests as stroke and/or vasculopathy in those with the disease. Overt stroke occurs in up to 10% of children with the disease and usually involves large cerebral vessels that affect large regions of the brain. Without treatment, there is a high risk of recurrence. With transfusion therapy, this risk remains substantial at 22%. Silent strokes, defined as an infarct on imaging studies with a normal neurological examination, occurs in at least 22% of those with sickle cell disease. Over the last decade much has been learned about cerebral vasculopathy given the advent of newer imaging modalities. Children suffer cognitive impairment from stroke that impacts their academic achievement. In addition, they may suffer physical limitations related to the stroke such as hemiparesis (Kato *et al.*, 2009).

2.1.5.7 Priapism:

Priapism is a condition in which a penis remains erect for hours in the absence of stimulation or after stimulation has ended. Priapism is another vaso-occlusive event that occurs in patients with sickle cell disease. Priapism is not uncommon for males with sickle cell disease with a probability of having at least one episode by age 20 of 89% and an average age of 12 years for the first

episode. The frequency in adults with sickle cell disease ranges from 30-45% (Rogers, 2005).

Priapism treatment varies and consists largely of supportive measures with intravenous fluids, non-steroidal anti-inflammatory medication and opioids. A urological consultation for aspiration and irrigation of the corpora is warranted for persistent priapism and has been effective. There are few randomized trials comparing treatment options and preventive measures especially in pediatric patients (Chinegwundoh and Anie, 2015).

2.1.5.8 Renal effects in SCD:

Microalbuminuria and albuminuria are common in the more severe genotypes of sickle cell disease and can occur in up to 80% of patients resulting in a glomerulopathy. Approximately 15% of patients will advance to end stage renal disease by their third decade of life. About 25% of patients with hemoglobin SS disease have renal insufficiency defined as a reduced creatinine clearance of < 90 ml/min. There are no identified treatments that have been shown to be effective in preventing the development of end stage renal disease in patients with sickle cell disease who show evidence of kidney disease early on. However, treatment with an angiotensin-converting enzyme inhibitor may decrease microalbuminuria and proteinuria (McKie *et al.*, 2007).

2.1.5.9 Avascular necrosis:

Avascular necrosis (AVN) of the hip is a common cause of morbidity in SCD. Avascular necrosis is one of the few complications that is more common with Hb SC than Hb SS and its prevalence has been reported to be as high as 41% of adults with sickle cell disease. Its prevalence increases with age and predisposing factors include coexistent α -thalassemia trait, frequent vaso-occlusive crisis and a high Hct (Hernigou *et al.*, 2008).

2.1.5.10 Aplastic crises:

Aplastic crisis or splenic sequestration, occur as a result of infection with parvovirus or from folate deficiency and are characterized by a sudden fall in hemoglobin, usually requiring transfusion (Booth *et al.*, 2010).

1.2.1.5.11 Haemolytic crises:

These are characterized by an increased rate of haemolysis with a fall in hemoglobin but rise in reticulocytes and usually accompany a painful crisis. Hemolysis and indicators of erythrocyte survival do not correlate with acute vaso-occlusive crises in patients with sickle cell disease (Ataga *et al.*, 2011).

2.1.6 Sickle cell trait:

This is a benign condition with no anemia and normal appearance of red cells on a blood film. Hematuria is the most common symptom and is thought to be caused by minor infarcts of the renal papillae. Hb S varies from 25 to 45% of the total hemoglobin. Care must be taken with anesthesia, pregnancy and at high altitudes (Hoff brand *et al.*, 2006).

The most common of these are Hb S -thalassemia, altered sickle cell/C disease. In Hb S/-thalassemia, the MCV and MCH are lower than in homozygous Hb SS. The clinical picture is of sickle cell anemia; splenomegaly is usual. Patients with Hb SC disease have a particular tendency to thrombosis and pulmonary embolism, especially in pregnancy. In general, when compared with Hb SS disease, they have a higher incidence of retinal abnormalities, milder anemia, splenomegaly and generally a longer life expectancy. Diagnosis is made by hemoglobin electrophoresis, particularly with family studies (Rumaney *et al.*, 2014).

2.1.7 Symptoms of SCD:

SCD is a pleiotropic genetic disorder of hemoglobin that has profound multiorgan effects such as pain in the hands and feet, chest pain and shortness of breath, fever, fatigue and elevated heart rate, jaundice delayed growth or delayed puberty, leg ulcers (Ballas *et al.*, 2010).

2.1.8 Risk factors of SCD:

The most straightforward laboratory risk factor was the fetal hemoglobin level (Platt *et al.*, 1994).

The disease occurs most often among people whose ancestry can be linked to sub-Saharan Africa, South and Central America, the Caribbean, India, and the Middle East and Mediterranean regions, that means it associated with certain genes ancestry (Steinberg *et al.*, 2008).

2.1.9 Diagnosis of SCD:

The repeated sickling and unsickling damages the red cell membrane leading to irreversibly sickled red cell even when the oxygen pressure is increased thus reducing red cell life span as a result of membrane damage inducing anemia. The white blood cells and platelets are also affected by the mutation. Homozygous sickle cell disease patients have lower values of red cell parameters, but higher values of white cell and platelets counts compared to hemoglobin phenotype AA (Fitzhugh *et al.*, 2010).

2.1.9.1 Complete blood count values:

The CBC provides information on the following erythrocyte, or red blood cell (RBC), count, measure of hemoglobin (Hb), hematocrit (Hct) (percentage), mean corpuscular hemoglobin (MCH) measurement, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), leukocyte, or white blood cell (WBC), count, thrombocyte count, reticulocyte

count, and explanation of cell morphology. These values differ between steady state and during vaso-occlusive crisis, **table (2.1)** (Omoti, 2005).

2.1.9.1.1 Factors which affect CBC values in SCD:

Several factors can affect CBC values. Degree of haemolysis is inversely related to hemoglobin concentration and packed cell volume in sickle cell anemia patient. Numerous factors affect haemolysis in sickle cell anemia, percentage of irreversible sickle cell is of greatest significance. The degree of hemoglobin polymer formation, calculated from the mean corpuscular hemoglobin concentration and the relative proportion of hemoglobin fractions also correlates closely with the severity of haemolysis (Akinsegun *et al.*, 2007).

2.1.9.1.2 Hemoglobin in SCD:

Hemoglobin is the main component of the red cell its concentration within the red cell is approximately 34 g/dL. It is a red pigment with molecular weight 64.5 KD protein (globins) synthesized and conjugated iron particles (heme) to form hemoglobin (figure in appendix). Hemoglobin estimation is measurement of red dye which is a main constituent of red cells and gives its color (Maton *et al.*, 1993). The overall mean hemoglobin concentration of sickle cell disease patients according to previous studies was 7.9 ± 1.4 g/dl (Omoti, 2005).

2.1.9.1.3 Red blood cells in SCD:

The RBCs count measures the number of circulating erythrocytes. A mature RBC is a non-nucleated, biconcave disc, surrounded by a flexible membrane. Fetal (and neonatal) RBCs differ from adult RBCs in that they are larger in size, have a shorter life span, altered shape and deformability, and they contain a high fetal hemoglobin concentration (Akinsegun *et al.*, 2007). Quantitative and qualitative changes in red blood cells have been reported. Haemolysis

consequent to the damaged red cell membrane could be intravascular or extravascular. The former results from the lysis of complement-sensitive red cells and hemoglobin lost during sickling-induced membrane damage. The latter, occurs by phagocytosis of red cells that have undergone sickling and physical entrapment of rheologically compromised red cells. Increased susceptibility to mechanically induced cell fragmentation has been documented in-vitro and in sickle cell patients undergoing vigorous exercise (Akinsegun *et al.*, 2007).

2.1.9.1.4 Hematocrit in SCD:

Hematocrit is the proportion of blood volume that consists of the RBCs. It is expressed as a percentage on the CBC. Hemoglobin in blood is measured in grams per one deciliter of whole blood and is expressed as g/dL (mmol/L) on the CBC. Two conditions that can be identified by evaluating the RBC count are anemia and polycythemia. Anemia is a deficiency in the concentration of erythrocytes and hemoglobin in the blood. Anemia can be caused by acute, chronic, or iatrogenic blood loss; decreased erythrocyte production; increased destruction of erythrocytes, as with hemolysis; or shortened erythrocyte survival (Akinsegun *et al.*, 2012).

1.2.1.9.1.5 Indices in SCD:

These indices can be measured directly or calculated electronically using modern hematology analyzers. They can be useful in further classifying anemia according to the hemoglobin quantity in the RBCs or the size of the RBCs or in identifying the pathologic process causing the anemia. The erythrocyte indices include the MCV, the MCHC, and the MCH. The MCV measures the average size of circulating erythrocytes. It can help to quantify anemia as microcytic (small cells) or macrocytic (large cells). The MCHC measures the hemoglobin concentration in a given volume of red blood cells.

The RBCs can be described as normochromic, hypochromic, or hyperchromic, depending on their color, which is determined by the amount of hemoglobin present in the RBC. The MCH measures the average amount of hemoglobin per RBC in a sample of blood (Hoff brand *et al.*, 2006).

2.1.9.1.6 White blood cells in SCD:

Many complications of sickle cell disease are associated with leukocytosis although SCD is primarily a disease of the red blood cell, leucocytes, because of their sizes obstruct blood vessels more effectively than red blood cells when attached to the endothelium. The red blood cells measures 7.2 μm , while small lymphocytes measures 10 μm , neutrophils 10–14 μm , large lymphocytes 12–16 μm , monocytes 14-20 μm . Bacterial infection associated with leukocytosis is a known predisposing factor to SCD crises a high absolute neutrophil count showed relationship with clinical severity of sickle cell anemia. It is implicated in clinically overt stroke. Pathogenesis of silent cerebral infarction and acute chest syndrome has been associated with leukocytosis (Okpala, 2006).

2.1.9.1.7 Platelets in SCD:

Unlike the red and white blood cells, the clinical effects of platelets on sickle cell disease are not well established. However, an association between stroke in sickle cell disease and platelet count $> 450,000/\mu\text{l}$ has been reported qualitatively, poor platelet aggregation responses to epinephrine and ADP were also reported in sickle cell disease (Akinsegun *et al.*, 2007).

Table 2.1: Hematological values in sickle cell anemia patients in vaso-occlusive crisis compared to steady state normal values

Variable	HbSS vaso-occlusive and hemolytic crisis Mean ± S.D	HbAA normal (Mean ± S.D)
Hemoglobin (g/dl)	7.77±2.25	12.93±2.22
White blood cell count (x 10⁹/l)	13.67±7.57	5.71±0.97
Platelet count (x 10⁹/l)	352.89±144.78	304.24±61.47
Mean cell volume (fl)	85.50±8.14	84.46±5.26
Mean cell hemoglobin (pg)	28.79±2.78	30.16±2.99
Mean cell hemoglobin concentration (g/dl)	31.23 ± 1.04	33.76±3.44

HbAA: hemoglobin A, HbSS: hemoglobin S, S.D: Standard Deviation.

Table 2.2: Relation between Hct and reticulocyte maturation time in BM and PB

PCV	Bone marrow maturation time/ day	PB maturation time/ day
45	3.5	1
35	3	1.5
25	2.5	2
15	1.5	2.5

PCV: packed cell volume, PB: peripheral blood

2.1.9.2 Reticulocytes:

Reticulocyte is immature red cell contains remaining of cytoplasmic RNA result from nucleus extrude of the late normoblast, the cell mature to red cell within two days in bone marrow and within 24 hours in peripheral blood. The amount of RNA reduced with continues maturation of the cell. Reticulocyte count as percentage, corrected count, absolute count and reticulocyte production index used to assess bone marrow erythropoietic activity for detection of effective or ineffective erythropoiesis (figure in appendix) associated with anemias, **table (2.2)**(Kaul *et al.*, 2009).

Reticulocytes are typically composing about 1% of the red cells in the human body. Reticulocytes develop and mature in the red bone marrow and then circulate for about a day in the blood stream before developing into mature red blood cells. Like mature red blood cells, reticulocytes do not have a cell nucleus (Ariel *et al.*, 2012).

The reticulocyte count rises when there is a lot of blood loss or in certain diseases in which red blood cells are destroyed prematurely, such as hemolytic anemia. Also, being at high altitudes may cause reticulocytes counts to rise. They are called reticulocytes because of a reticular (mesh-like) network of ribosomal RNA that becomes visible under a microscope with certain stains such as new methylene blue. Reticulocytes appear slightly bluer than other red cells when looked at with the normal Romanowsky stain (Omoti, 2005).

The normal range of values for reticulocytes in the blood depends on the clinical situation and the laboratory, but broadly speaking is 0.5% to 1.5%. However, if a person has anemia, their reticulocyte percentage should be higher than "normal" if the bone marrow to produce new blood cells remains intact. Thus, calculating the reticulocyte production index is an important step

in understanding whether the reticulocyte count is appropriate or inappropriate to the situation (Kaul *et al.*, 2009).

The number of reticulocytes is a good indicator of bone marrow activity, because it represents recent production. This means that the reticulocyte count, and the reticulocyte production index that can be calculated from it, can be used to determine whether a production problem is contributing to the anemia, and can also be used to monitor the progress of treatment for anemia. When there is an increased production of red blood cells to overcome chronic or severe loss of mature red blood cells, such as in a haemolytic anemia, people often have a markedly high number and percentage of reticulocytes (Omoti, 2005).

2.1.9.2.1 Reticulocytes count:

The reticulocyte count is given as the percentage of red blood cells that are reticulocytes (the number of reticulocytes divided by the total number of red blood cells, multiplied by 100). Normal values may vary from laboratory to laboratory. Results are ready in 1 day. Newborns have a normal reticulocyte count of 2.5% to 6.5%. This value drops within 2 weeks to 0.5% to 2.0% (Ariel *et al.*, 2012).

A high reticulocyte count can occur after a lot of bleeding, a move to a high altitude, or certain types of anemia such as sickle cell anemia.. A low reticulocyte count can also be caused by exposure to radiation, a long- term (chronic) infection, or by certain medicines that damage the bone marrow. There are many factors may affect the reticulocyte count which include; taking medicines, such as levodopa, corticotropin, azathioprine, chloramphenicol, dactinomycin medicines to reduce a fever, medicines to treat malaria, and methotrexate and other cancer chemotherapy medicines, also getting radiation therapy, taking sulfonamide antibiotics, being pregnant,

or having a recent blood transfusion in anemia, the reticulocyte count will be abnormal because the levels of red blood cells and hemoglobin are low (Omoti, 2005, Ariel *et al.*, 2012).

2.1.9.2.2 Reticulocytes count in SCD:

Reticulocytes make up a higher percentage of the blood count in SCD, which makes the reticulocyte count falsely high. For this reason, checking the reticulocyte count along with the reticulocyte production index (RPI) when investigating for a SCA (Omoti, 2005).

2.1.9.3 Other laboratory findings in SCD:

Sickle cells and target cells occur in the blood features of splenic atrophy (e.g. Howell Jolly bodies) may also be present. Screening tests for sickling are positive when the blood is deoxygenated (e.g. with dithionate and di-sodium hydrogen phosphate $\text{Na}_2 \text{HPO}_4$). While hemoglobin electrophoresis shows Hb SS and no Hb A is detected. The amount of Hb F is variable and is usually 5-15%, larger amounts are normally associated with a milder disorder (Hoff brand *et al.*, 2006).

2.1.9.4 DNA diagnosis in SCD:

The majority of samples are obtained by chorionic villus biopsy although amniotic fluid cells are sometimes used. Techniques to sample maternal blood for fetal's cells or fetal's DNA-are being developed. The DNA is then analyzed using one of the Polymerase chain reaction (PCR) is the most commonly used technique and may be performed by using primer pairs that only amplify individual alleles ('allele-specific printing') or by using consensus primers that amplify all the alleles followed by restriction digestion to detect a particular allele. This is best illustrated by Hb S in which the enzyme D del detects the A-T change. Prenatal screening can be done if the fetus is at risk for sickle cell disease. At birth, screening is universally

performed in all SCD' states. Blood testing reveals anemia and sickle-shaped red blood cells. In affected individuals, several types of diagnostic testing may be necessary throughout their lifetimes. These include ultrasounds of the head and neck to identify those at risk for stroke, routine eye examinations to check for chronic eye disorders, and chest X-rays to assess lung structure and function (Hoff brand *et al.*, 2006).

2.1.10 Treatment of SCD:

Pain can be treated with analgesic medications (e.g., Tylenol, ibuprofen, and ketorolac), warm compresses, and hydration. In some cases, stronger pain medications, including narcotics (e.g., morphine), are required. In addition, complementary therapies, such as hypnosis, relaxation techniques, and biofeedback, may be helpful. Infection is the most common cause of death in patients with sickle cell disease. Antibiotics are often necessary when infection occurs, which may require hospitalization for intravenous delivery. The influenza and pneumococcus vaccines should routinely be used. Supplementation with folic acid should be taken in doses of 1 milligram daily. In addition, supplementation with magnesium may be helpful (Thornburg *et al.*, 2010).

Although HU has recognizably successful benefits in SCD, the search continues for agents that control other aspects of the pathophysiology of the disease for use in combination with, or as an alternative to, HU. Other current therapeutic approaches for SCD include the use of transfusion therapy to reduce HbS concentrations, particularly in children identified as at risk for stroke (Conran, 2015).

Transfusions these are also sometimes given repeatedly as prophylaxis to patients having frequent crises or who have had major organ damage (e.g. of

the brain) or show abnormal transcranial Doppler studies. The aim is to suppress Hb S production over a period of several months or even years. Iron overload, which may need iron chelation therapy, and allo-immunization against donated blood are common problems. Blood transfusions are commonly necessary to supply properly functioning red blood cells to increase oxygen-carrying capacity (Thornburg *et al.*, 2010).

In addition, several medications (e.g., hydroxyurea and erythropoietin) may be used to improve the oxygen-carrying capacity of red blood cells. Stem cell transplantation and gene therapy are experimental, but hold promise for a cure in the near future. Particular care is needed in pregnancy and anaesthesia. There is debate as to whether or not patients need transfusions with normal blood to reduce HbS levels during pregnancy or before delivery or for minor operations. Hydroxyurea is the only approved medication for the treatment of sickle cell anemia (HbSS) and is widely used in children despite an indication limited to adults (Strouse and Heeney, 2012).

2.1.11 Nutritional considerations in SCD:

Patients with sickle cell disease have increased needs for calories and micronutrients (e.g., vitamins and minerals). A diet emphasizing fruits, vegetables, whole grains, and legumes will provide a greater proportion of essential nutrients than a typical Western diet, and appropriate supplementation (one to three times the recommended intakes for most essential nutrients) can prevent deficiency. The average caloric intake of sickle cell patients is typically low, especially during flare-ups of the disease. As a result, children with sickle cell disease have impaired growth and significantly lower weight compared with unaffected individuals. A careful nutritional assessment and the addition of energy supplements are needed (Daak *et al.*, 2013).

Sickling of red blood cells increases when patients become dehydrated. Thus, it is important to maintain hydration by consuming adequate fluids. In some cases, hospitalization to receive intravenous fluids may become necessary. Blood levels of several vitamins and minerals are often low in individuals with sickle cell disease, including vitamin A and carotenoids, vitamin B6, vitamin C, vitamin E, magnesium, and zinc. This can result in a significant deficiency of antioxidants, which may increase the risk of disease flare-ups. Studies indicate that vitamin-mineral supplements of certain nutrients (vitamins C and E, zinc, and magnesium) or treatment with a combination of high-dose antioxidants can reduce the percentage of sickled red blood cells. Also supplementation with omega-3 fatty acids can improve the membranes of red blood cells and may decrease flare-ups of the disease. A small preliminary study indicated that omega-3 fatty acid supplementation with fish oil reduced the number of painful episodes requiring hospitalization (Daak *et al.*, 2013).

2.1.12 Study types in genomic research of SCD:

Genomic research has rapidly evolved during the past decades, and the scientific approach to genetic-based understanding of human disease has changed along with technological development, **figure 2.1** shows a timeline of scientific discoveries important for the study of SCD, and it has become evident that knowledge in this field has been growing faster in time and in quantity alike (Costa and Fertrin, 2010).

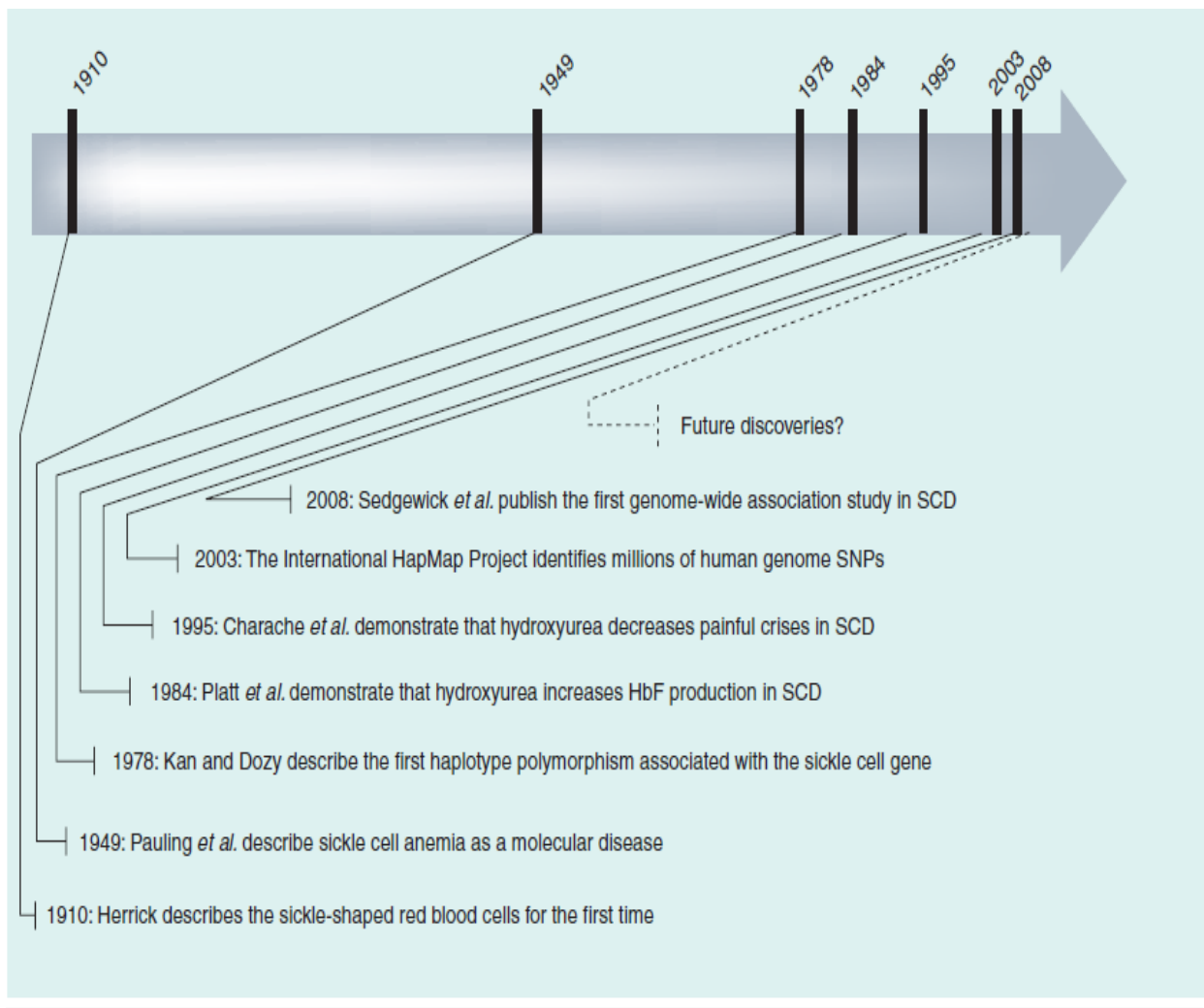


Figure (2.1) Timeline of major scientific achievements relevant to the study of SCD (Costa and Fertrin, 2010).

2.1.12.1 Clinical severity genetic overview:

Study conducted by Sebastiani *et al.*, 2010, which managed to correlate clinical severity of SCD based on a network model (composed of 25 different clinical parameters) to 40 different SNPs, including genes not previously identified as pathogenetically important in SCD. SNPs that could define regions of the genome where these genetic variations occur with statistical significance; therefore, identifying more candidate genes associated with a certain sub-phenotype, and also several other genes that are directly related to SCD pathophysiology or have certain functions (Sebastiani *et al.*, 2010, Driscoll *et al.*, 2013).

2.1.12.1.1 Severity scoring system of SCD:

For each participant, lifetime cumulative incidence of specific complications of SCD, as described by the SCD cooperative study group, **table 2.3** (Ballas *et al.*, 2010, Alabid *et al.*, 2016).

Table (2.3): Scoring System of SCD

- For number of previous transfusions per life score:
 - 0 when number is 0
 - 1 when number is 1/whole life
 - 2 when number is 2/whole life
 - 3 when number is 3/whole life
 - 4 when number is 4/whole life
 - 5 when number is more than 5/whole life
- For number of previous transfusions in last year score:
 - 0 when number is 0
 - 1 when number is 1/year
 - 2 when number is 2/year
 - 3 when number is 3/year
 - 5 when number is > 3/year
- For number of previous hospitalizations, score:
 - 0 when number is 0
 - 1 when number is 1/whole life
 - 2 when number is 2/whole life
 - 3 when number is 3/whole life
 - 4 when number is 4/whole life
 - 5 when number is more than 4/whole life
- For packed cell volume, score:
 - 0 when $\geq 24\%$
 - 1 when 18–23%
 - 2 when $< 18\%$
- For white blood cell count, score:
 - 0 when $< 11,000/\text{mm}^3$
 - 1 when between 11,000 and 15,000/ mm^3
 - 2 when $> 15,000/\text{mm}^3$
- For lifetime cumulative incidence of specific complications, score:
 - 5 when CVD is/was present, 0 when absent
 - 5 for each when gall stone, chronic leg ulcer, osteomyelitis, priapism is/was present, 0 when absent.
 - 1 when steady state is present, 0 when absent.

Modification = divide the score by 5 Over all Scores of ≤ 3 were deemed mild disease. Scores of $3 \geq 5$ were considered moderate disease, while scores > 5 were taken for severe disease.

2.2 Selectins and integrins:

The selectin family consists of three closely related cell-surface molecules with differential expression by leukocytes (L-selectin), platelets (P-selectin), and vascular endothelium (E- and P-selectin) (figures in appendix) and these selectins mediate neutrophil, monocyte, and lymphocyte rolling along the venular wall (Okpala, 2006).

Integrins; the CD11/CD18 (12 integrins) family consists of three heterodimeric cell surface receptors, CD11a/CD18, CD11b/CD18, and CD11c/CD18 (Zhou *et al.*, 2013).

Unlike that of many other integrins, 132 integrin expressions are restricted to leukocytes. CD11a and CD18 are expressed on virtually all mature leukocytes. Expression of CD11b and CD11c is restricted to phagocytes (polymorph nuclear cells and monocytes/macrophages) and large granular lymphocytes (Kaul *et al.*, 2009).

12 integrins play major roles in the immune system. They are critical for the recruitment of circulating leukocytes to inflammatory sites, in that they are responsible for the establishment of firm adhesion and trans-endothelial migration. Each receptor also has additional distinct functional profiles (Koch *et al.*, 2002).

2.2.1 Selectin and Integrin in SCD:

In a study by Aslihan, demonstrated that leukocytes play a direct role in the vascular occlusions caused by sickle cell disease and being directly engaged in the pathogenesis of vasoocclusion and concluded that the leukocytosis in SS patients is associated with increased mortality (Aslihan, 2002).

Another study by Okpala *et al.*, reported high steady-state expression of alphaMbeta2 integrin and l-selectin by leukocytes predisposes to severe manifestations, increased leukocyte adhesion molecules expression above steady-state levels could be important in the genesis of crisis (Okpala *et al.*, 2002).

Also study by Vijayan *et al.*, reported that; these proteins play an important role in the physiological and pathological processes of inflammatory reaction, immune response and thrombosis (Vijayan *et al.*, 2003).

Many studies have reported that RBCs adhesion act as a trigger that slows down the flow to fulfill the delay time requirement for sickling, the finding which may explain the established positive correlation between sickle cell adhesion to endothelium and clinical severity of SCD (Benther *et al.*, 2006).

Findings by Frenette *et al.*, have led to the current postulation that an enhanced tendency of red blood cells (sickled and non-sickled) to adhere to vascular endothelium and activation of leukocytes and platelets are the primary causative factors of vaso-occlusion and haemolysis (Frenette *et al.*, 2007).

But Ugochukwu *et al.*, found that there was high expression of L-selectin (*SELL*) and alphaMbeta2 integrin (*ITGA2*) on leukocytes in patients with complications of sickle cell disease but there was no association between any of these gene polymorphisms and high expression of L-selectin by leukocytes, or the development of complications in SCD (Ugochukwu *et al.*, 2008).

Many studies have associated *ITGA2* and *SELL* genes polymorphisms with vaso-occlusion (Payne *et. al.*, 2008, Manginas *et. al.*, 2008, and Giusti *et. al.*, 2008)

In contrast the integrin alpha-2 (*ITGA2*) gene polymorphism was associated with ischemic stroke (Wei *et al.*, 2009).

Study by Okpala *et al.*, showed that early symptomatic improvement that follows Hydroxyurea therapy is mediated via mechanisms independent of increased HbF, and may involve reduced adhesion molecules expression in leukocytes and other treatment modalities that reduce leukocyte adhesion molecules expression might also confer clinical benefit (Okpala *et al.*, 2011).

A study in Sudan by Daak *et al.*, reported that more than 10% of patients with sickle cell anemia develop overt stroke, and 22% show evidence of silent cerebral infarction (Daak *et al.*, 2013).

2.3 Rationale:

Selectins and integrins approved to mediate the adhesion of WBCs in many inflammatory conditions. In sickle cell disease (SCD) the most characteristic manifestation, however, is the vaso-occlusive crisis and appear to result from blockage of blood flow by SS RBCs and WBCs.

This event accounts for most of the morbidity and mortality associated with this condition.

A number of inherited and acquired factors are found to influence the pathogenesis of the clinical symptoms of SCD but still a huge controversy exists about the key modifiers of the clinical severity of the disease, although all patients with SCD share a specific, invariant genotypic mutation, the variability in the clinical severity is astounding.

Up to the date in Sudan, all previous researches and observations on SCD, agreed on presence of a severe type of the disease, and according to my knowledge there was no well-powered study has been performed to determine allelic polymorphism of the integrin and selectin genes, to explain the variations in the clinical severity, and biochemical indications of the disease among the patients in Sudan.

This study may provide a possible way to connect selectin and integrin genes polymorphism with clinical severity in sickle cell disease, and may reveal different results because the disease itself is affected by many genetic and environmental factors. The outcome of this study, may help in developing a novel therapy based on individualized genetic makeup, to reduce haemolysis, vaso-occlusive events and associated pathogenic complications, and reduce incidence of premature birth, maternal and neonatal morbidity and mortality.

2.4 Objectives:

2.4.1 General objective:

To study integrin alpha-2 (*ITGA2*) C807T and L-selectin (*SELL*) P213S allele's polymorphism and their association with clinical severity of sickle cell disease among Sudanese patients.

2.4.2 Specific objectives:

1. To detect integrin alpha-2 (*ITGA2*) and L-selectin (*SELL*) genes allelic polymorphisms among study groups.
2. To detect the association of integrin and selectin genes polymorphisms with clinical severity among SCD Sudanese patients.
3. To correlate between integrin and selectin genes polymorphisms and clinical indicators and modifiers (medications and supplements).
4. To correlate patient's episodes of painful sequestration, hospitalization rate, blood transfusion rate and other indicators of the clinical severity among study group.
5. To measure patients' and control hematologic markers (CBC, PBP, and retics count) and correlate them to clinical severity.
6. To determine the bone marrow erythropoietic activity among patients with SCD.

Chapter three

Material and methods

3.1 Study design:

This was analytical, hospital-based, case-control study.

3.2 Study setting and time line:

The study was conducted at outpatient sickle cell disease referral clinic, and Gaafar Ibn-Auf Paediatric Tertiary Hospital from June 2015 to June 2017.

3.3 Study population:

Sickle cell disease patients were selected in this study based on inclusion and exclusion criteria

3.3.1 Inclusion criteria:

Patients (cases) of any age from different ethnic backgrounds and comparable clinical status were included in this study; with HbSS, phenotypic characteristic which was confirmed by cellulose acetate electrophoresis at pH 8.5 and correlated to the clinical investigation and records.

Apparently healthy individuals as controls were selected to be similar as sex, age group and residence with the patients.

3.3.2 Exclusion criteria:

Other hemoglobinopathies, presence of other chronic diseases, blood transfusion in the last two months, and pregnancy were excluded.

3.4 Sample size:

The sample size in this study was calculated for each category (on average) to give a maximum of error (0.05) with a probability of ($\alpha = 0.05$). The formula bellow was used.

$$n = \frac{z^2 \cdot p \cdot q}{d^2}$$

$$N = \frac{(1.96)^2 \times (0.084) \times (0.916)}{(0.05)^2} = 118$$

z = the value in normal curve corresponding to level of confidence 95% = 1.96

p = probability prevalence in the community is (average prevalence reported in Khartoum 8.4% (Majdi and Hanan, 2014)

$q = (1-p) = 1-0.084= 0.916$

d = margin of error = 0.05= 118 as cases and 118 as control

Total of 245 samples; 133 cases and 112 apparently healthy individuals aged matched as control group were selected according to inclusion and exclusion criteria.

3.5 Data collection and severity scoring:

Clinical data were collected by enclosed questionnaire and recording forms and authorized clinician who carries out the clinical examination.

Parameters such as cerebrovascular disease (CVD), acute chest syndrome (ACS), gall stones, osteomyelitis, chronic leg ulcer, and priapism, were documented. Events were confirmed by review of past medical histories and by checking the medical records of all patient.

3.6. Sample collection and processing:

The blood samples were collected during outpatient department visit. Using disposable sterile syringe from anti-cubital fossa following cleaning with anti-septic alcohol pads, venous blood sample of 5mls was collected from both patients and controls into two EDTA bottles for an aliquot was used to determine complete blood counts (CBC) within 2 hours of collection while the remainder was used to prepare haemolysate for haemoglobin

electrophoresis and for reticulocyte count, and the other one for DNA extraction.

Processing, full blood count analysis was done on the same day of collection using Sysmex KN-21 N, (manufactured by Sysmex Corporation Kobe, Japan) a three- part auto analyzer able to run 19 parameters per sample haematological parameter with high accuracy and precision. Small portion of sample used for reticulocyte count within maximum two hours of sample collection.

From each subject, 3 mL of whole blood collected in (EDTA) containers were used to extract DNA using a small amount of whole blood genomic DNA extraction kit quickly, according to manufacturer's instructions and then were stored at -20°C till use.

The detection of integrin alpha-2 (*ITGA2*) and L-selectin (*SELL*) genes was based on examination of the size of the (PCR) products following DNA amplification of the target sequence of the *ITGA2* and *SELL* genes.

3.6.1 Complete blood count (CBC):

For each sample of blood, the following hematologic variables: red blood cells (RBC), hematocrit (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin(MCHC), absolute neutrophils count (ANC) and absolute lymphocytes count (ALC) were determined in an automated counter.

3.6.1.1 Automated method:

Principally Sysmex analyzer is based on the electronic resistance (impedance) detection method for counting and sizing recognition of the leukocytes erythrocyte, and platelet using three hydraulic systems for, WBC, RBC, platelet and hemoglobin, and display the results on the liquid crystal displayer (LCD) with histogram and printed out the results in thermal paper. The

erythrocyte morphology was assessed by light microscopy in Leishman's stain films.

3.6.1.2 Reagents and materials:

Commercial close system reagents were provided by Sysmex KX-21N operators and **Consist of:**

Cell pack and stromatolyser and detergent and Cell cleaner.

3.6.1.3 Principle of hematological analyzer:

Measurement of blood cells (RBCs, WBCs, & Platelet), and hemoglobin concentration obtained by aspiration of small volume of well mixed K₃EDTA blood by sample probe and mixed with isotonic diluents in nebulizer. Diluted mixture aspiration delivered to RBC aperture bath for providing information about RBC and platelet based on cell sizes, particles of 2 to 20 fl counted as platelets, above 36fl counted as red cells. Some portion of aspirated mixture induced into WBC bath in which hemolytic reagent (Stromatolyzer) was added automatically to measure hemoglobin concentration in build calorimeter, based on cyanomethmoglobin method (HiCN). Blood cells counted and size information generated in triplicate pulses according to electronic conductivity, and translated into digital number using in build calculator programmed and designed for that RBC, WBC counts., hence three values were directly measured (RBC, WBC, Hb), and displayed on (LCD). Other values of red cell indices, platelet counts, leukocyte differential and absolute count calculated from given information and automated constructed histograms, the result printed out according to the setting mode.

3.6.2 Thin blood films and staining technique:

Principle: drop of well mixed blood placed on clean stationary slide, thin blood films were made by manual proper spreader slide, then air dried fixed thin films were placed on a flat staining rack, some portion of Leishman's stain was added, after two minutes twice volume of buffer was added to the stain and mixed well, diluted stain allowed for eight minutes and washed off in tap water to obtain stained thin blood films used for blood cell morphological studies.

3.6.2 .1 Reagents and materials:

Stationary slides, spreader slides, Leishman's stain., oil immersion, microscope and differential cell counter.

3.6.3 Reticulocyte count:

A sample of blood is stained with a supravital dye that marks reticulocytes. An increased number of reticulocytes is seen when the marrow is churning out RBCs at excessive speed (presumably to make up for those lost to hemolysis or hemorrhage). Most labs will report the result of the reticulocyte count in percent of all RBCs counted. At Hermann the normal range is 0.5-1.5 %. Making clinical decisions based on this raw count is somewhat fallacious. For instance: A normal person with an RBC count of 5,000,000 / μ L and an absolute reticulocyte count of 50,000 / μ L would have a relative retics count of 1.0%. An anemic person with 2,000,000 RBCs/ μ L and the same 50,000 retics/ μ L would have an apparently "abnormal" relative retic count of 2.5 % and could be misdiagnosed as having high turnover. Clearly, one needs to find some way to correct the raw retic count so as to avoid this problem. One can easily calculate the absolute retic count (in cells/ μ L) by multiplying the RBC count by the relative retic count. The normal range for the absolute retic count is 50,000-90,000 / μ L. Another parameter that has

found popularity is the reticulocyte production index (RPI) (Ariel *et al.*, 2012).

3.6.3.1 Principle:

Ribosomal RNA of the reticulocyte stained supravivally and reticulocyte recognized microscopically as non-nucleated erythrocyte that contains two or more blue granulofilamentuos materials.

3.6.3.2 Requirements:

EDTA blood, reticulocyte stain (1% new methylene blue and 1% brilliant cresyl blue, test tube, water bath, slides and microscope.

3.6.3.3 Procedure:

Whole blood and stain were mixed as equal volume in small test tube.

Then were incubated at room temperature for 20 minutes, and remixed two thin films were made from homogenous mixture.

Then with oil immersion 1000 red cell and reticulocytes were counted and then reticulocytes were calculated using following formula:

(Retics %) calculated as follows:

$$\text{Retics\%} = \frac{\text{NO of reticulocytes} \times 100}{1000 \text{ RBC observed}}$$

3.6.3.4 Reference range:

According to age for adult = 0.2% - 2% and for neonates up to 6%

3.6.3.5 Absolute reticulocyte count (ARC):

Absolute retics count reflects the actual number of reticulocytes in one liter of whole blood used for assessment of bone marrow transplanted patients and patients on chemotherapy.

(ARC) calculated as follows:

$$\text{ARC} = \frac{\text{Reticulocyte\%} \times \text{RBC Count}}{100}$$

Reference range: = 10 -110 x 10⁹/L.

3.6.3.6 Corrected reticulocyte count:

The CRC corrects the observed reticulocyte count to a normal Hct of 0.45 L/L for male and 0.42 L/L for female correct for degree of anemia. It is used when Hct less than 0.35L/L

(CRC) calculated as follows:

$$\text{CRC} = \frac{\text{Retic\%} \times \text{pt. Hct (L/L)}}{\text{Normal Hct L/L}}$$

3.6.3.7 Reticulocyte production index (RPI):

Its known as shift correction that provides refinement of the CRC and used to assess of bone marrow erythropoiesis, the count proportional to retics maturation time in the bone marrow may be shortened from usual 3.5 days to as little as 1 day allowing released of shift cells with polychromatic appearances, so (RPI) is correction for both the Hct and maturation time in the peripheral blood.

(RPI) calculated as follows:

$$\text{RPI} = \frac{\text{CRC}}{\text{Maturation time in peripheral blood}}$$

Reference range:

It is equal 1 when PCV is 0.40 or greater with normal maturation time 1 day
RPI > 2 indicates adequate bone marrow response to anemia.

RPI = 2 indicates moderate bone marrow response to anemia.

RPI < 2 indicates inadequate bone marrow response to anemia and refers as anemia of ineffective erythropoiesis. Rather refer to less haemolytic events.

3.6.4 Hemoglobin electrophoresis:

Method used for separation of hemoglobin accordingly to net charge structure for diagnosis of hemoglobinopathies.

3.6.4.1 Method:

Cellulose acetate electrophoresis at alkaline pH 8.6

3.6.4.2 Principle: Hb is negatively charge when placed at cathode (-) will migrate toward anode (+) the migration bases on Hb structure and net charge. Hence some hemoglobin's are slower others are faster in mobile

3.6.4.3 Requirements:

3.6.4.3.1 Equipment:

Electrophoresis apparatus consist of the following:

Electrophoresis tank consists of two chambers, plastic bridge gap set at 7cmm, two electric wire and cover, two electrodes with two electric pins, power supply, applicator of sample loading, and filter paper for blotting.

3.6.4.3.2 Reagents:

Tris/ EDTA/borate (TEB) buffer, ponceau S stain, and 3% glacial acetic acid.

3.6.4.4 Procedure:

Bridge gap was adjusted and each chamber was filled with TEB buffer up to the line, then wicks was placed on the plastic bridge.

Straight original line was drawn at 10 cm distance from one end and C, T1, T2, and T3, were labelled and then the paper was socked in the buffer for 5 minutes.

After that the paper was blotted between two filter papers to remove of excess buffer.

Then using clean applicator control and samples hemolysate were loaded at right location exactly on origin line at cathode end, and paper was placed across the bridges with corrected position.

Then the cover switch was placed on and electrophoresis at 350V for 25 minutes was adjusted.

After 25 minutes the paper was transferred for staining with ponceau S for seconds to 5 minutes.

Then rinsed in distaining solution several times, and then blotted with filter paper and air dried, then Interpretation done visually and the results were given.

3.6.4.5 Comment:

Hemoglobin of the hemolysate adjusted 8-12gm/dl and hemoglobin mobile to anode (+) when placed at cathode (-) in order of C, S, F, A, H, while C, E, O Arab and A2 yield one band. But S, D, G, lepore yield one band, and for A band is 3mm distances from F band, then A band is 8 distances from S band, and A band 16 distances from C band.

Membrane or cellulose acetate papers were used.

Gel and citrate agar acid electrophoresis were give restricted separated bands.

Cellulose acetate membrane or paper alkaline electrophoresis is satisfactory for separation of hemoglobin's C, S, F, and A.

3.6.5 The PCR requirements and procedure:

Determination of *ITGA2* and *SELL* genotypes were performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, using PCR different protocols, and then PCR amplification products were digested with restriction enzymes to identify polymorphisms.

The genotypes have been established after ethidium bromide staining (Xu-Guang *et al.*, 2005, Costa and Fertrin, 2010).

3.6.5.1 Genomic DNA extraction:

From each subject, 3 mL of whole blood collected in (EDTA) containers were used to extract genomic DNA using a small amount of whole blood quickly with (innuPREP) whole blood DNA (Mini Genomic DNA extraction Kit) according to manufacturer's instructions and then were stored at -20°C till use.

3.6.5.2 Primer design and PCR amplification:

It is designed by primer 3 with aid of computer software (Serial Cloner 2.6 program) (Image in appendix).

For **ITGA 2** each amplification reaction (total volume 25 µl L) contained the primer sequences for detecting the polymorphism 5'-TTCAGCTCTCAGCCAGCTTC-3' (forward primer) and 5'-TGCACTGAATCCCCTTGTGA-3' (reverse primer) (designed by primer 3 with aid of computer software (serial cloner 2.6 program). 1 µl of forward primer, 1 µl of reverse primer and 2 µl of DNA were added to the other PCR components needed for the reaction (Maxime PCR PreMix, i-TagiNtRON BIOTECHNOLOGY South Korea)

3.6.5.3 Restriction digestion and DNA genotyping:

PCR amplification products were digested with restriction enzymes to identify polymorphisms; 3 µl products were digested with *AvaII* (Thermo-Fisher, Waltham, Massachusetts, USA) followed by PAGE (8%). The genotypes have been established after ethidium bromide staining. To identify *ITGA2*, reactions were performed at 37°C for 3 hours, and then products were identified by electrophoresis on an 8% polyacrylamide gel stained with ethidium bromide. A gel documentation imaging system was used to visualize bands.

3.6.5.4 Protocols for selectin genotyping:

The Pro213Ser (P213S) *SELL* (rs4987310) polymorphism genotyping has been achieved by PCR-RFLP technique, using the following protocol: each amplification reaction (total volume 25 µl L) contained, the primer sequence for detecting the polymorphism were 5'-TGATTCAGTGTGAGCCTTTG -3' (forward primer) and 5'-CTTGACAGGTTGGTTCTG-3' (reverse primer), 1µl of forward primer, 1ul of reverse primer and 2 µl of DNA is added to the other PCR components needed for the reaction (Maxime PCR PreMix, i-Tag), with following protocol:

Initial denaturation in 95°C for 5 min.

Denaturation in 94°C for 30 sec.

Annealing in 54°C for 30 sec,

Extension in 72°C for 30 sec, 30 cycles

Final elongation in 72°C for 5 min.

The genotypes were determined by digestion of each amplicon (5 µl) with 5U of (*HphI* Thermo fisher Scientific Waltham, Massachusetts, USA) followed by PAGE (8%) (Xu-Guang et. al, 2005)

3.6.5.5 Protocols for integrin genotyping:

The detection of integrin alpha-2, (*ITGA2*) genes was based on examination of the size of the polymerase chain reaction (PCR) products. The *ITGA2* C807T (rs1126643) polymorphism genotyping has been achieved by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, using PCR following protocol

Initial denaturation in 95°C for 5 min,

Denaturation in 94°C for 1 min.

Annealing in 60°C for 1 min.

Extension in 72°C for 1 min, 30 cycles

Final elongation in 72°C for 10 min

The genotypes were determined by digestion of 3 µl products with (*AvaII* Thermo fisher Scientific Waltham, Massachusetts, USA) followed by PAGE (8%).

3.7 Result interpretation:

All quality control measures were adopted during specimen collection and processing. All results were interpreted according to normal and reference values obtained from control group was compared to cases group. Control ladder was run synchronized with each batch in order to determine the gene base pair sequence. The results of automated hematology analyzer were compared to stained thin blood films.

3.8 Statistical analysis:

Data analysis was performed using Statistical Package for Social Sciences (SPSS) IBM analytics US, for Windows software version 23.0. Means, standard deviations (SD), and percentages were determined. Means \pm SD were compared using independent t-test or one-way analysis of variance (ANOVA) as appropriate. Ratios were compared using the Pearson Chi squared (χ^2) test. The odds ratio (OR) was used in order to compare distributions of alleles and genotypes between patients and healthy individuals. Comparative analysis of means was performed with significant at level (P. value < 0.05), and the results were presented in form of figures and tables.

3.9 Ethical consideration:

This study poses no physical risk to participants through an interview of 5 minutes. Personal information was obtained and each participant was given a personal serial number.

All collected data were secured in computer protected by password and used only for research purposes for confidentiality. Informed written consent from patients or their guardians (in case of children or patients with mental disability).

The local ethics committee at Sudan University of Science and Technology and Khartoum State Ministry of Health, then Gaafar Ibn-Auf Paediatric Tertiary Hospital approved the research conducted in accordance with world medical association (WMA) Declaration of Helsinki (2008).

Chapter four

Results

This analytical, hospital-based, case-control study, was conducted at outpatient sickle cell disease referral clinic and Gaafar Ibn-Auf Paediatric Tertiary Hospital from June 2015 to June 2017.

A total of 245 samples; 133 cases and 112 apparently healthy individuals age-matched as control group were collected according to inclusion and exclusion criteria.

All clinical parameters of the subjects were recorded before polymorphism genotyping. The age of respondents ranged from 1 to 37 years; 7 years is most common age group as in **tables 4.1** which shows demographic data distribution for study population. Original home frequency distribution for patients explained in **table 4.2**. In **tables 4.3** there are hematologic markers for study population, while hematological values are shown in **table 4.4** and clinical severity parameters in **table 4.5**.

Table 4.6 shows the correlations between SCD clinical severity and other disease modifiers. Frequency distribution of bone marrow erythropoietic activity as shown in **figure 4. 1**.

Figure 4.2 shows genotyping of the L selectin P213S polymorphism while the genotypes distribution explained in **tables 4.7**.

Figure 4.8, shows genotyping of the *ITGA2* C807T polymorphism.

Frequencies genotype for *ITGA2* C807T among studied patients and healthy controls are shown in **tables 4.9**

Table 4.10 shows the *ITGA2* gene allele's expression with clinical severity of SCD among Sudanese patients.

Table 4.1: Demographic data for the study population

	Case: 133		Control : 112
Sex frequency (N & %)	Female	76 57.1 %	62 55.4 %
	Male	57 42.9 %	50 44.6 %
Age (mean years)		7.2	7.3
Income	92% low income		50%
F/M education	9.8 % reach university level		80%
School attendance	70% irregular schooling		10%
Consanguinity	89% relatives		23%
History of SCD	51 % diagnosed on first year		Healthy

N= number

Table 4.2: Original home frequency for the studied patients

Tribe origin	Center	West	East	North	South
N	57	45	8	0	23
%	42.9%	33.8%	6.0%	0.0%	17.3%

Table 4.3: Mean \pm SD of hematological markers among the apparently healthy individuals and patient with SCD

Test	Sample	Mean \pm S.D	P.value*
RBCs X10 ³ C/mm ³	Healthy individuals	4.27 \pm 0.453	0.000
	Patients with SCD	2.702 \pm 0.540	
Hct %	Healthy individuals	34.6 \pm 2.97	0.003
	Patients with SCD	23.56 \pm 9.5	
Retics %	Healthy individuals	2.61 \pm 0.88	0.000
	Patients with SCD	11.88 \pm 9.508	
CRC %	Healthy individuals	2.2847 \pm 0.705	0.000
	Patients with SCD	6.6605 \pm 5.189	
RPI	Healthy individuals	1.7319 \pm .498	0.000
	Patients with SCD	3.5424 \pm 2.625	
ARC ($\times 10^9/L$)	Healthy individuals	10.927 \pm 3.298	0.000
	Patients with SCD	30.274 \pm 21.251	

*Both were compared using independent sample T-test; (P.value< 0.05).

Table 4.4: Correlation between laboratory findings and disease severity among Sudanese patients with SCD

Laboratory parameters	Severity of disease			P.value
	Mild disease N=14 (mean)	Moderate disease N=35 (mean)	Severe disease N=84 (mean)	
TWBCs ($\times 10^9/L$)	12.2	13.8	15.1	0.007
Neutrophils ($\times 10^9/L$)	6.8	7.0	7.5	0.000
Lymphocytes ($\times 10^9/L$)	5.9	5.7	5.1	0.001
RBCs ($\times 10^{12}/L$)	2.6	2.7	2.3	0.045
Haemoglobin (g/dL)	7.0	8.2	7.3	0.000
Hematocrit (%)	22.7	22.9	21.3	0.034
MCV (fL)	89	86.3	94	0.017
MCH (pg)	32.4	30.7	34.3	0.000
MCHC (g/dL)	35.8	35.8	36.5	0.001
Platelets ($\times 10^9/L$)	572.6	450	525	0.044

Table 4.5: Clinical severity of sickle cell disease among study population

Clinical Data		Severity of disease			Total	P.value
		Mild	Moderate	Severe		
Gender	Female	7	23	46	76	0.456
	Male	7	12	38	57	
Age (mean) years		7.2	8.2	6.8	7.2	0.001
Stroke		2	18	67	87	0.000
Sequestration/ Vasocclusive		4	21	81	106	0.000
Hemolytic crisis		14	35	84	133	*a
Leg ulcer		0	1	3	4	0.768
Muco-skeletal pain		9	35	79	123	0.000
Acute chest syndrome		8	23	76	107	0.001

*Bolted values indicate statistical significance. *a No statistics are computed because hemolytic crisis is a constant

Table 4.6: Correlation between SCD clinical severity and other diseases modifiers among Sudanese patients.

Modifiers		Severity of disease			P.value*
		Mild	Moderate	Severe	
Chronic diseases	No	6	12	5	0.023
	Yes	8	23	79	
Hydroxyurea	No	6	6	56	0.000
	Yes	8	29	28	
Omega-3 supplementation	No	1	9	70	0.000
	Yes	13	26	14	
Folic acid supplementation	No	10	20	59	0.421
	Yes	4	15	25	
Blood transfusion	No	7	18	68	0.364
	Yes	7	17	16	
Total		14	35	84	Total
					133

*Made by using chi-square test; (P.value set to level < 0.05), bolded are significant.

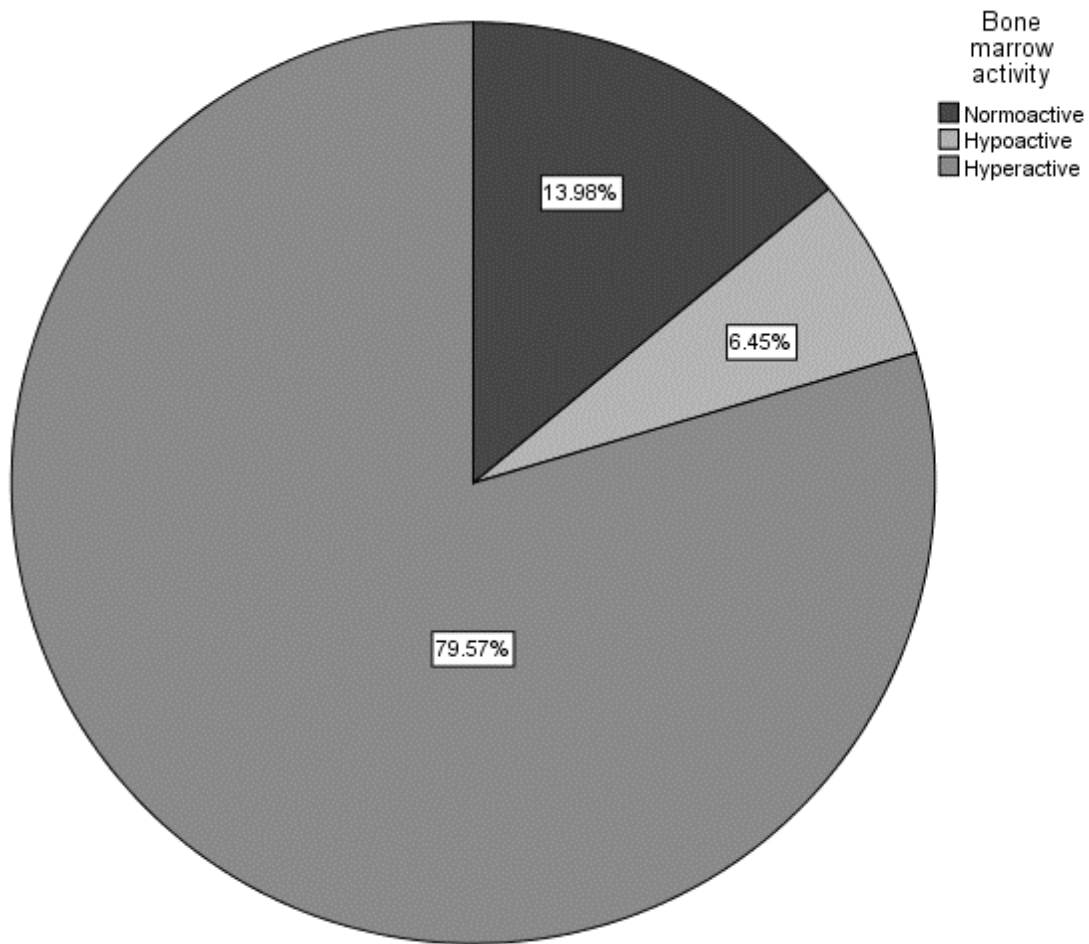


Figure 4.1: Frequency distribution of bone marrow erythropoietic activity among Sudanese patients with SCD

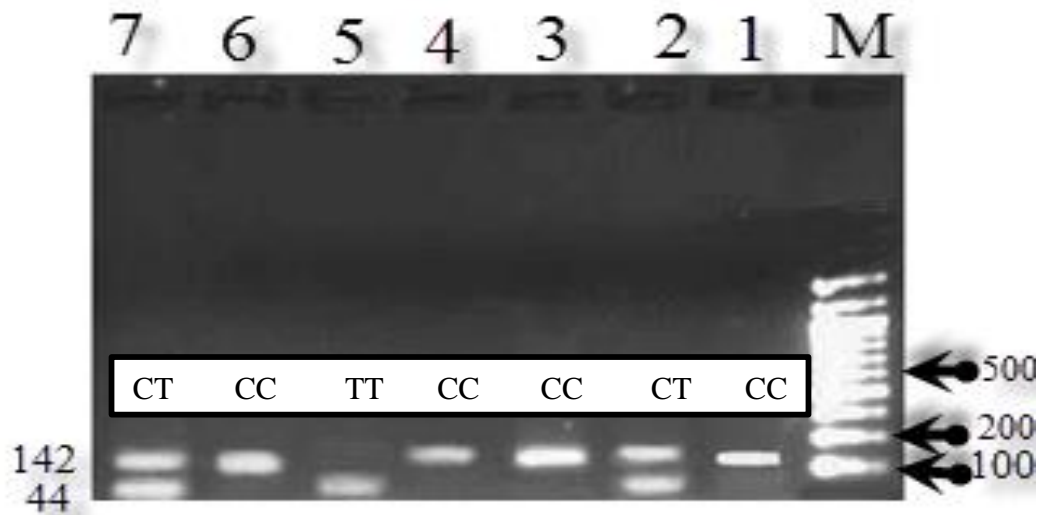


Figure 4.2: Genotyping of the *SELL* P213S polymorphism among Sudanese patients with SCD*

*(Lanes M: DNA leader 100 bp, lane 1: control sample, 2&7 (PS /CT) genotype; lanes 3, 4&6: (PP /CC) genotype - lanes 5: (SS /TT) genotype).

The presence of CT (213Ser) allele generates a restriction site for *Hph I* enzyme and the amplicon digestion generates two fragments of 142 bp and 44 bp. When CC (Pro213) allele is present, the restriction site is not created, the amplicon is not digested and it maintains its size of 186 bp, while presence of homozygous TT alleles generates two restriction sites (44bp and 142 bp was cut into two fragment appeared in same position).

Table 4.7: Distribution of L-selectin gene genotypes among studied patients and healthy controls

Sample		Frequency	Percent %	P.value
Patients**	TT	6	4.5	0.000
	CC	49	36.8	
	CT	78	58.7	
Controls**	TT	3	2.7	
	CC	73	65.2	
	CT	14	12.5	

**All patients' samples gave positive result for gene expression, 22 control samples represented 19.6 % gave negative results.

Table 4.8: L-selectin gene allele's expression with clinical severity among

Alleles%	Degree of clinical severity			P. value*
	Mild severity	Moderate severity	Severe cases	
PS(CT)	21.9	4	74.1	0.000
PP(CC)	7.2	82.7	10.1	
SS (TT)	70.9	13.3	15.8	

Sudanese patients with SCD

* The allele's % compared using chi-squared test.

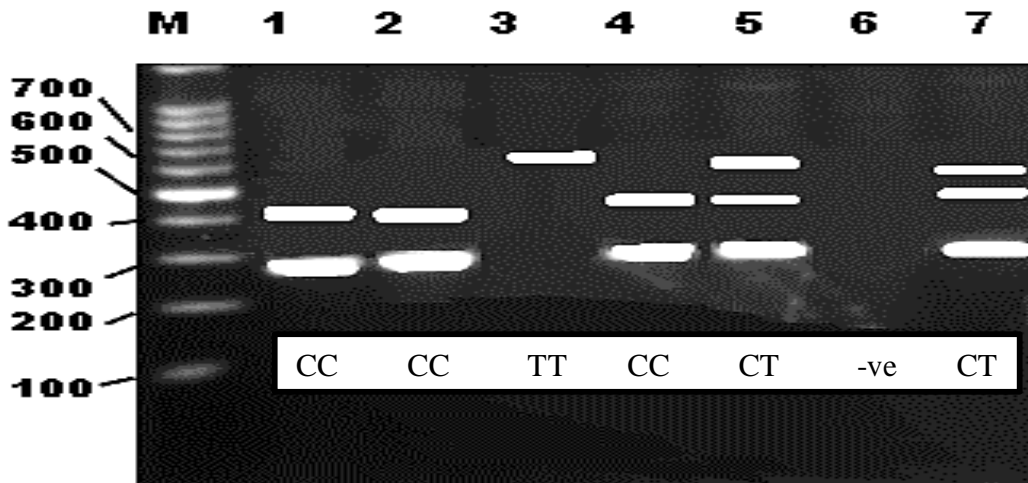


Figure 4.3: Genotyping of the *ITGA2* C807T polymorphism among Sudanese patients with SCD*

*Lane M: DNA marker; lanes 1, 2, and 4: CC genotype; lanes 3: TT genotype; lanes 5 and 7: CT genotype and; lanes 6 is negative control

RFLP for the *ITGA2* C807T polymorphism produced three possible allele combinations: CC (two bands, 414 bp and 275 bp, CT (three bands, 689 bp, 414 bp, and 275 bp; or TT (one band, 689 bp), as shown.

Table 4.9: Frequencies genotype for *ITGA2* (C807T) among studied patients and healthy controls

Sample		Frequency	Percent%	P. value
Patients**	CC	17	12.8	0.0021
	CT	23	17.3	
	TT	93	69.9	
Controls**	CC	91	81.3	
	CT	12	10.6	
	TT	7	6.3	

**All patients' samples gave positive result for gene expression, 2 control samples represented 1.8% gave negative results.

Table 4.10: *ITGA2* gene allele's expression with clinical severity of SCD among Sudanese patients

Alleles%	Degree of clinical severity			P. value *
	Mild severity	Moderate severity	Severe cases	
TT	12	18	70	0.000
CT	0	72	28	
CC	88	10	2	

* The allele's % compared using chi-squared test.

Chapter five

Discussion, Conclusion and Recommendation

5.1 Discussion:

In this study *ITGA2* C807T and L- Selectin P213S genes allelic polymorphisms have been determined in 133 SCD patients and 112 healthy controls among Sudanese population. Those patients had median age about 7 years, 50% of them were diagnosed early at first year of their childhood as obviously genetic disorder.

This study provided an evidences for socioeconomic impacts on SCD; in which there were more than 90% of patient's families were found to be with low income. Children are irregular or younger for schooling and only 10% of them reach to university level, these result agree with Daak *et al.*, study done in 2013 in Sudan, in which a proof given for socioeconomic effects on SCD. The largest percentage of patient's original home found in the center, 42.8% which is actually reflects the immigration of different tribes from west 33.8%, south 17%, and east 8% toward the capital Khartoum. As SCD is a hereditary disorder so no doubt consanguinity marriage will affect in children; here in this study more than 90% of families are tend to have consanguinity marriage which agree with all previous studies that connected relative marriages.

Hematologic markers and CBC are more useful markers for assessment of erythropoietic bone marrow activity. In patients with SCD, the hemolytic markers results were proportional reflect the degree of hemolysis and revealed lower result when compared to controls ($P=0.000$) and when compared between severity degrees (Hb, RBCs, ARC, CRC and Retics%, RPI all of them with P . values $=0.000$) and ($P=0.003$) for Hct. These results are totally agree with study done by Omoti on 2005, in which these parameters compared

in healthy and SCD patients with different severities and reported lower hemolytic values in patients (P=0.000).

In this study about 14 patients were mild cases, 35 with moderate severity, and 84 were with severe situations represented by vaso-occlusion and sequestration for 79 % of the SCD patients and those without crisis about 21% of all selected, hemolytic crisis were detected in all cases, so hematologic parameters were significantly different between severity degrees as well as between cases and control, obviously in Plts (P=0.044) and in WBCs (P=0.007) and their differential count which were found to have a major role in severity status of SCD. These results agree with Aslihan 2002, study in which revealed contribution of WBCs to severity of disease. Then later on were more explained in study by (Vijayan *et al.*, 2003, Benter *et al.*, 2006 and Frenette *et al.*, 2007) in their studies were connected these leukocyte adhesive interactions to blood vessel occlusion which is the major mechanism of organ damage in SCD.

The allelic polymorphisms frequencies of both selectins and integrins were found to be significantly different between patients and controls groups, (P.value = 0.000 and 0.0021) for selectins and integrins respectively and even some controls gave negative results for expression of both genes. The SNPs analyzed in this study showed that huge association of these gene polymorphism and SCD complications in Sudanese patients agree with Okpala *et al.*, 2011, who reported significant association between leucocytes adhesion molecules genes polymorphisms with vaso-occlusion and disagreed with Ugochukwu *et al.*, 2008 study which reported no significant association between these gene polymorphisms and high expression of L-selectin by leukocytes or the development of complications in SCD.

The results in this study indicated that the P213S polymorphism of L-selectin gene and the C807T polymorphism of *ITGA2* may contribute to susceptibility to vaso-occlusive crisis and more severe situation in the Sudanese population, and showed possible contribution of this polymorphisms to the increased risk for severe complication of SCD in patients with stroke, the results above agreed with studies have associated *ITGA2* polymorphisms with vaso-occlusion by Payne *et al.*, 2008, Manginas *et al.*, 2008, and Giusti *et. al.*, 2008, those were connected the polymorphism to different degrees of clinical severity.

Further, the results agree with Wei *et al.*, 2009, who reported that *ITGA2* C807T polymorphism affects the clinical severity degrees. As shown in the results; the TT levels were significantly higher in patients with severe cases about 70% when compared with patient carrying the CT allele 17.3 % and there was higher level of CC allele in mild cases. Therefore, individuals with increased TT allele, were 5.4 times more likely to suffer from hemolytic crisis, vaso-occlusive and ischemic stroke rather than patients with the C allele. But in P213S polymorphism for *SELL*, the results showed that the higher allele percentage was CT (PS), about 58.7% which also accounted for about 74.1% of severe cases of SCD with (P.value =0.000) which agreed with Steinberg *et al.*, 2008 who connected these genes high expression with clinical severity and disagreed with study by Sebastiani *et al.*, 2010, which managed to correlate clinical severity of SCD based on genes not previously identified as pathogenetically important in SCD.

Presence of disease severity modifiers is very clear with statistically significant levels of P=0.023 when correlating patient chronic disease which had a worse effect on accelerating SCD severity. On the other hand, supplementations with either supplements such as omega-3 fatty acid or

conventional treatment with hydroxyurea had adverse effect on disease severity ($P=0.000$), but supplementation with folic acid were found had no effect on disease severity modification ($P=0.421$), while blood transfusion had no clear correlation on clinical severity; and this may due to multiple blood transfusion those patients with SCD received in their lives, eventually will affect the clinical situation in many means despite any inconveniences. These findings agree with Kato *et al.*, 2009 and Daak *et al.*, 2013, in which their studies correlated diseases modifiers to clinical severity of SCD.

Limitation of this study:

Due to the relatively limited number of patients, only the allelic positivity test and the heterozygous and homozygous condition for (P213S) (PP, PS and SS) have reached the statistical significance for increasing the risk, regarding all possibilities.

And because findings of correlations between selectins and integrins genes polymorphisms and vaso-occlusion and ischemic stroke have been conflicting, so only the *ITGA2* C807T and *SELL* P213S polymorphisms, are not enough to understanding of the potential mechanistic contribution of these genes.

5.2 Conclusion:

- Gene polymorphisms of *ITGA2* C807T in particular, the T allele may be a hereditary susceptibility allele for development of vaso-occlusive crisis, cerebral stroke and more severe complications in Sudanese patients with SCD.
- The polymorphism of (C807T) *ITGA2* gene may contribute to susceptibility to more severe states and complications in the Sudanese population.
- L- Selectin (P213S) *SELL* gene polymorphisms will predispose to high leukocyte expression of L-selectin, or development of complications and severity course of the disease in Sudanese patients with SCD.
- Hematological variables are totally affected by the degree of SCD severity.
- SCD is affected by and effect on socioeconomic status.
- There was a correlation between SCD clinical severity and other chronic disease, supplementation and other treatments which are regarded as disease modifiers.
- Patients under regular treatment showed hypoactive to normo-active bone marrow in contrast to patients who usually do not take treatments or supplements.
- This study also reflected a suppression of normal bone marrow activity in patients underwent omega-3 therapy and other supplementation.

5.3 Recommendations:

- Any genetic studies for patients with SCD should include *ITGA2* C807T and *SELL* P213S in particular.
- Including wide variety of genes and sub-genes in such types of studies and investigations in future and focusing in DNA sequencing to identify novel alleles affecting SCD severity.
- Investigation of new therapeutic approaches of SCD including targeted integrins and selectins genes.
- Searching beyond the key modifiers of SCD.
- Using of RPI as indicative marker for hemolysis and bone marrow activity in patients with SCD.
- Establishing treatment protocols for SCD based on the individualized basic hematologic parameters.
- Follow up of patients with SCD is very important after primary admission.
- Clinical studies are still required to confirm the full potential of these different approaches of genes polymorphism for SCD therapy.

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Appendixes

Questionnaire

Sudan University of Science and Technology
College of Graduate Studies

Association of Integrin and Selectin genes Polymorphisms with
 Clinical severity of Sickle Cell disease among Sudanese Patients

Date:...../...../2015 Sample serial No:

Patient files No:

Age:.....years

Gender : Male: Female:

Residence:..... Class:.....

Age when final diagnosed as SCD:..... years

Another Chronic disease?: Yes: No:

If yes:.....

Did you take Hydroxyurea?: Yes: No:

	Father	Mother
Age		
Tribe		
Occupation		
Residence		
Education		
Income /month		
History of SCD		

Consanguinity and family tree:
 First degree: Second: From the same tribe: Not related:

School attendance:

Regular	Moderately regular	Irregular	Stopped school	Younger for school
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Past history:

Clinical cases:	Yes	No	No.of times
Sequestration crisis			
Haemolytic crisis			
Acute chest syndrome			
Musco-skeletal pain			
Leg ulcer			
Stroke			
Gall bladder stone/s			
Renal failure			

Episodes:	Once	Twice	3	4	5	6	7	7-15	>15
In the past year how many times have you experienced <u>severe pain</u> leading to hospital presentation?									
In the past how many times have you been admitted due to SCD?									
In the past year, what the total number of days have you been admitted due to SCD?									
How many times you have received blood due SCD?									

Medicines and supplements	Not on	daily	3/week	2/week	1/month	Others
How often you take omega-3						
How often you take folic acid						
How often you take multi-vitamins						
How often you take other medicines						
Notes: medications:						

إستمارة موافقة الشخص المشارك في البحث أو من ينوب عنه

أنا الباحث: علاء الدين مساعد محمد الزبير, كلية الدراسات العليا , جامعة السودان للعلوم والتكنولوجيا, أقوم ببحث أو دراسة للربط بين تعدد أشكال جينات الانتغرين والسليكتين مع شدة الحالة السريرية لدى المرضى السودانيين المصابين بالانيميا المنجلية)

(Association of Integrin and Selectin genes Polymorphisms with Clinical Severity of Sickle Cell Disease among Sudanese Patients)

لقد تم إختيارك لتشارك في هذا البحث أنت (أو من تنوب عنه) ومعك عدد آخر من المشاركين. نتوقع بمشاركتك أنت والمشاركين الآخرين أن نتحصل على نتائج تفيد فهم طبيعة هذا المرض وتشخيصه المبكر وقد يعود البحث بفوائد على كل من المشارك نفسه أو المجتمع أو مقدمي الخدمات الصحية .

خلال هذه الدراسة سأقوم بأخذ معلومات عنك وعن المرض و أخذ عينة دم من الوريد حوالي 5مليتر، ثم إجراء عد كامل للدم وعد للخلايا الشبكية و نحتاج لعمل افلام مصبوغة و دراسة جينية للكروموسومات من عينة الدم.

الإجراء الذي سأقوم به تجاهك ليس به أية مخاطر أو أعراض جانبية على المشارك أو من ينوب عنه.

ونحن إذ نأمل في مشاركتك معنا في هذا البحث , نؤكد لك على سرية المعلومات و الوثائق الخاصة بك , و أنه لن يطلع عليها إلا الباحث المعنى و لجنة أخلاقيات البحوث الصحية القومية أو المحلية.

و سوف نخبرك بنتيجة الفحوصات عن طريق طبيبك المعالج , لن نجمع منك أى عينات أخرى , أما العينات التي يتم جمعها سوف تستعمل لغرض هذه الدراسة فقط. و نود أن نشير كذلك إلى أن المشاركة في البحث طوعية وأن رفضك للمشاركة في البحث لا تفقدك الحق في أى فوائد من البحث , مع التأكيد على أنه لن يتم منحك أي قيمة نقدية مقابل المشاركة في هذا البحث و أنه بمشاركتك ستكون أحد المتطوعين الذين يشملهم البحث.

إذا كان لديك أى سؤال أو إستفسار يخص البحث يمكنك الإتصال على الباحث أعلاه في رقم

جواله:0914170073

فورم إقرار موافقة المشارك في البحث

إقرار المشارك :

لقد إطلعت على المعلومات الحالية والتي تم شرحها لي وأتيت لي طرح الأسئلة عنها كيفما شئت , و قد تلقيت الإجابات الوافية عن كل الأسئلة , و أنا أقر بالموافقة (أو أقر عن من انوب عنه) على المشاركة طوعية في هذه الدراسة و أعلم بحقى في التوقف عن المشاركة في أى وقت دون أن يؤثر ذلك على حقوقى فى تلقى العناية الطبية اللازمة فى أى وقت .

رمز المشارك.....

إسم المشارك:

توقيع المشارك.....

رمز من ينوب عن المشارك (في حال الطفل أو المعاق ذهنياًإلخ)

توقيع من ينوب عن المشارك شرعاً.....

عنوان من ينوب عن المشارك:.....

في حال عدم قدرة المشارك على قراءة الإقرار ويحتاج إلى من يشرح أو يترجم له:

إسم الشارح (المترجم)

عنوان الشارح أو (المترجم):

توقيع الشارح أو (المترجم):

توقيع الباحث:.....

التاريخ:.....

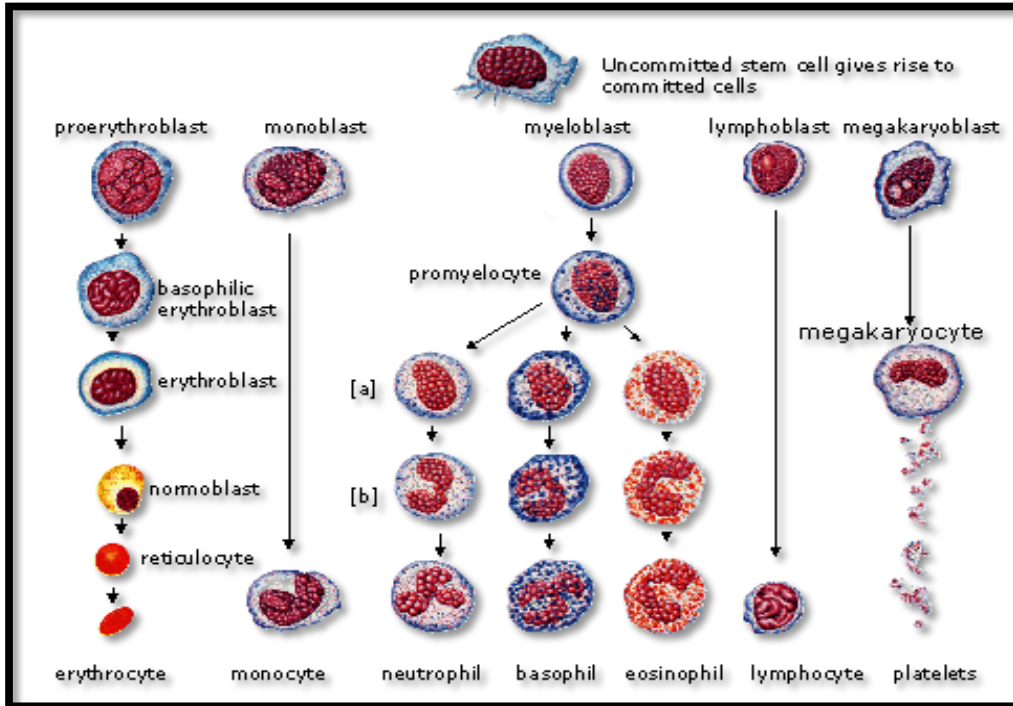


Figure: Hematopoiesis (erythropoiesis, leucopoiesis, and thrombopoiesis).

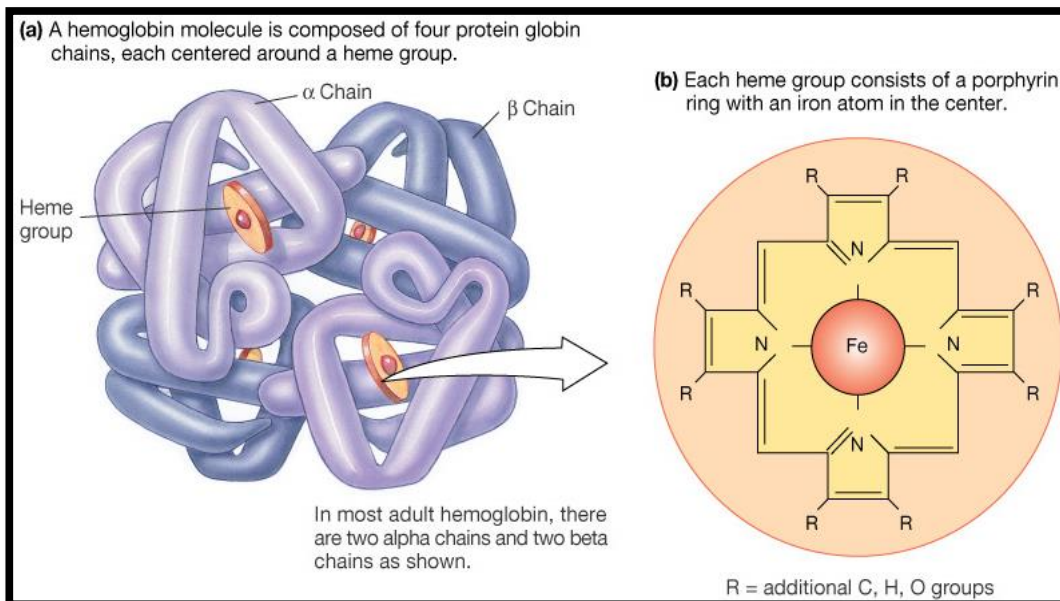


Figure: Hemoglobin molecule structure.



Image: Automated hematology analyzer (Sysmex KX-21N)



Figure Techne TC-412 Thermal Cycler keison UK

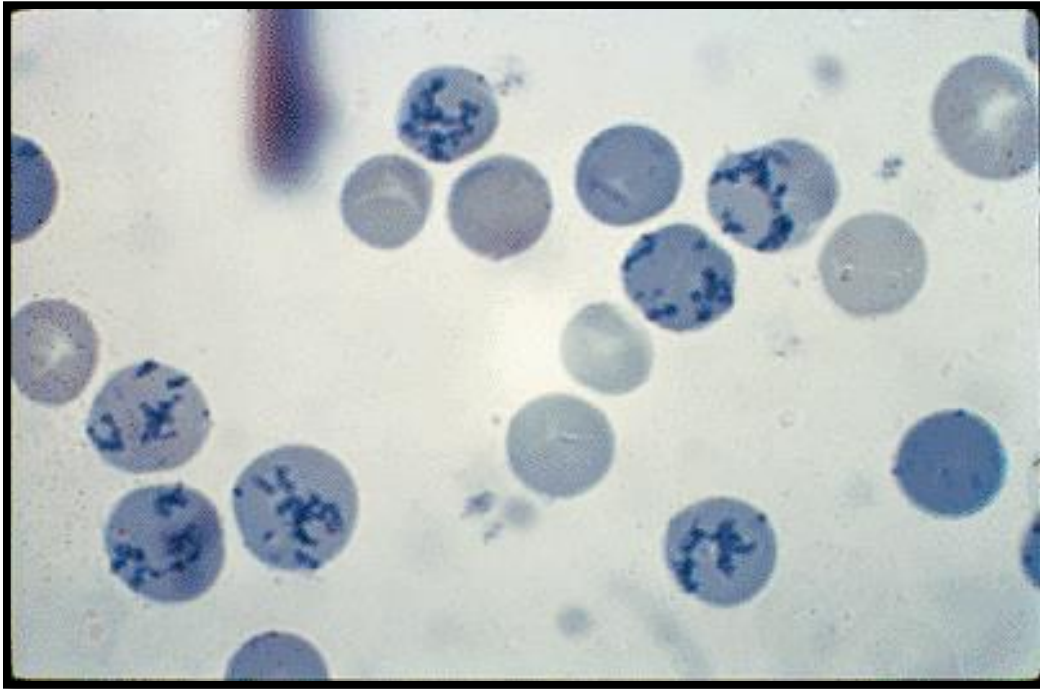


Image: Reticulocyte count using X100 oil immersion lens, whole blood sampled stained with supra-vital stain 1% brilliant cresyl-blue

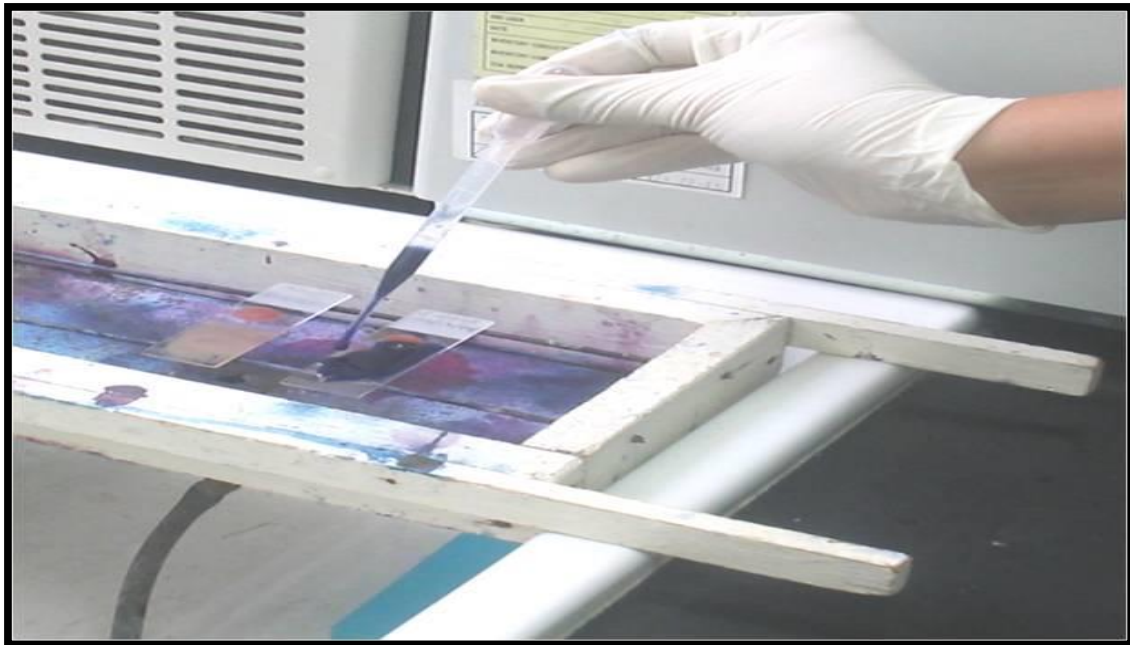


Image: Staining procedure of blood film; using ready to leishman's stain



Image: Normal red blood cell (right) versus sickle cell.

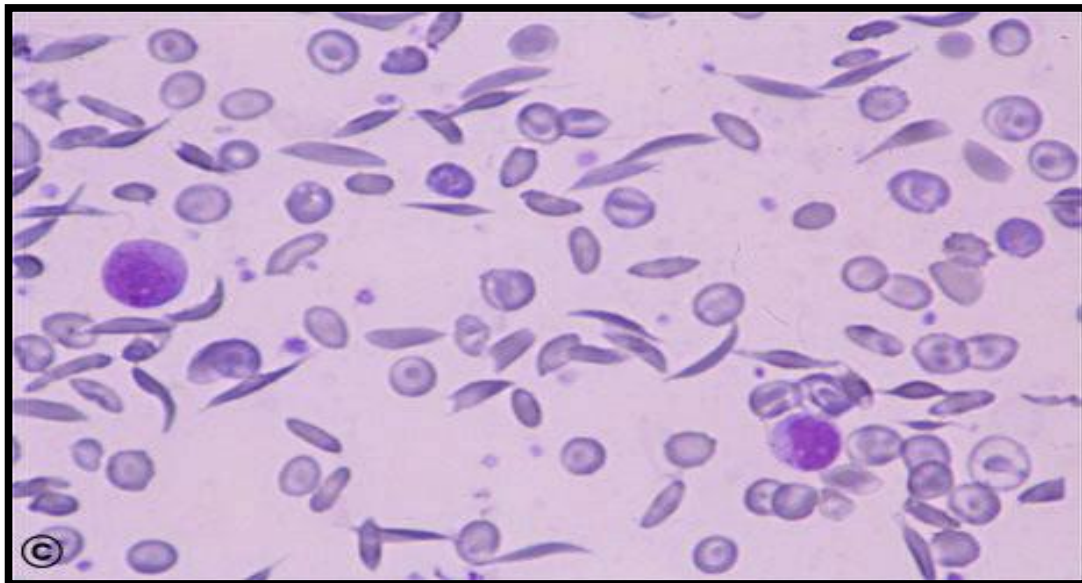


Image: This is sickle cell anemia in sickle cell crisis. The abnormal hemoglobin SS is prone to crystallization when oxygen tension is low, and the RBC's change shape to long crescent shape.

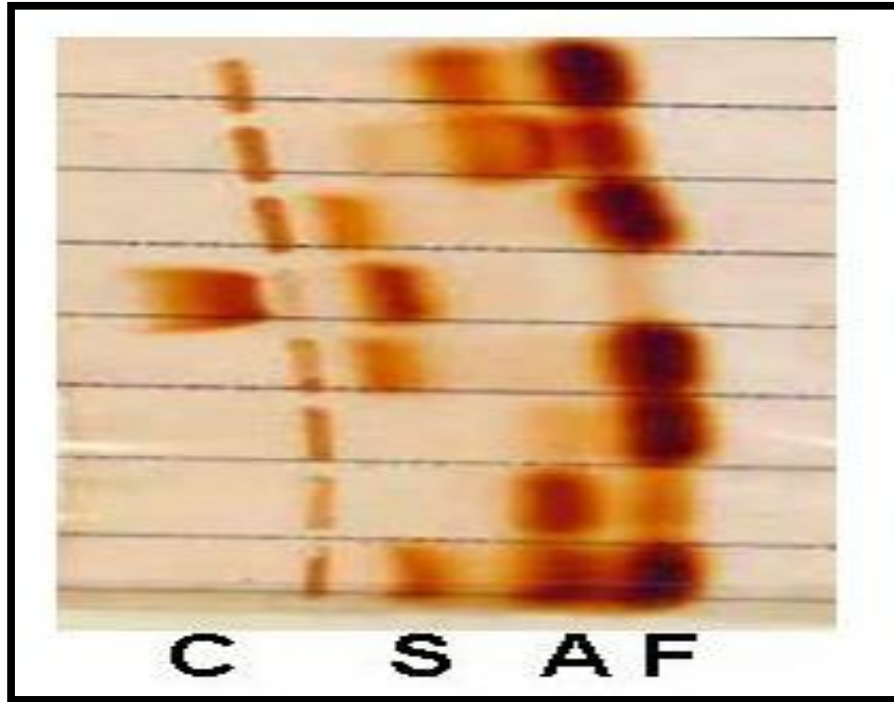


Image: Cellulose acetate electrophoresis at alkaline pH 8.6



**Image: Maxime PCR PreMix, i-Tag
iNtRON BIOTECHNOLOGY (South Korea)**



Image: Sickle Cell Anemia crisis; vaso-occlusive crisis (dactylitis x-ray)



Image: Sickle Cell Anemia crisis; gallbladder (left) stones & leg ulcer

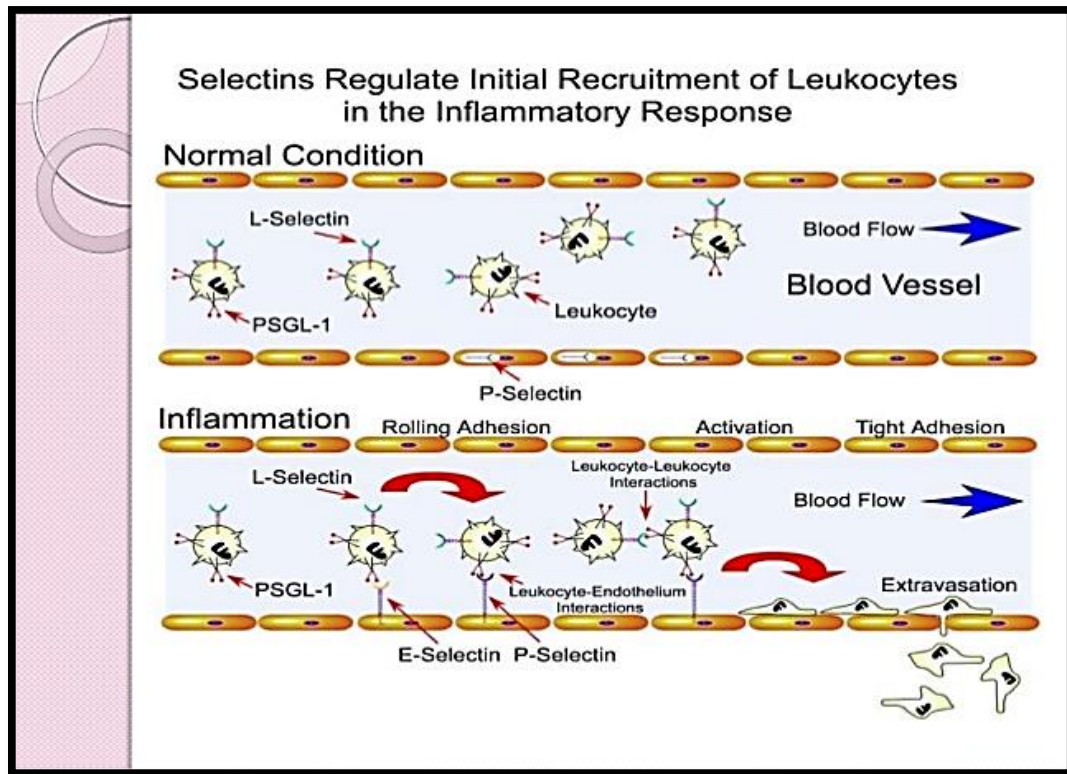


Figure: E, P, and L Selectins adhesion molecules mediates the initial attachment of leukocytes to venular endothelial cells

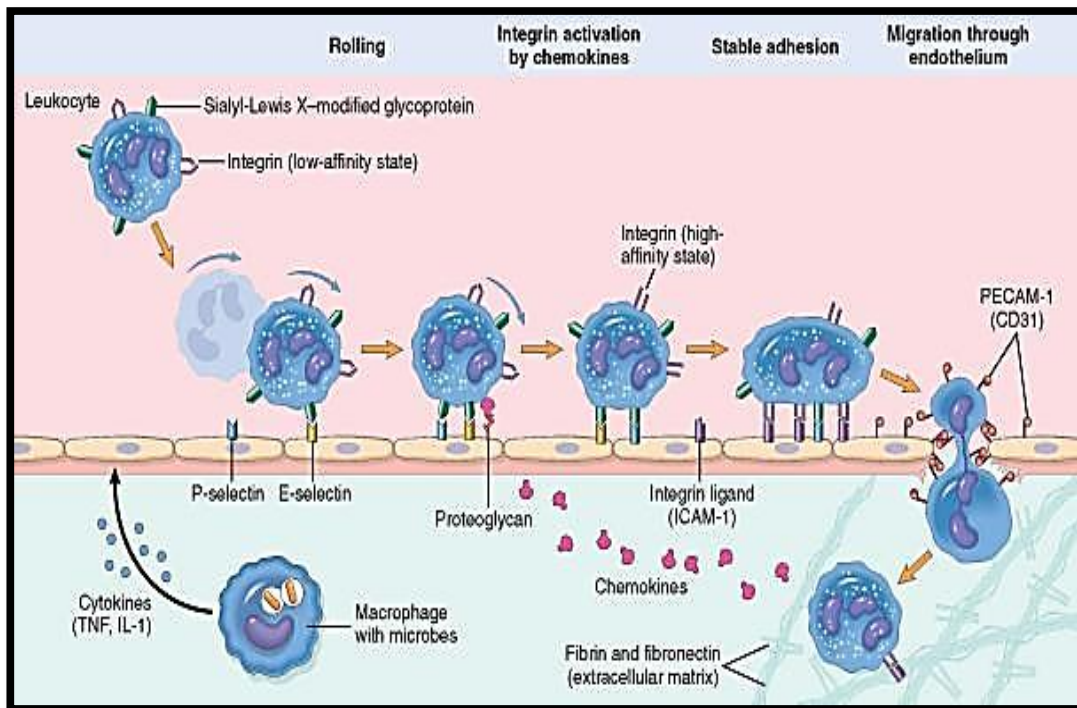


Figure: Integrins heterodimeric transmembrane glycoprotein



Figure: innuPREP) whole Blood DNA (Mini Genomic DNA extraction Kit)

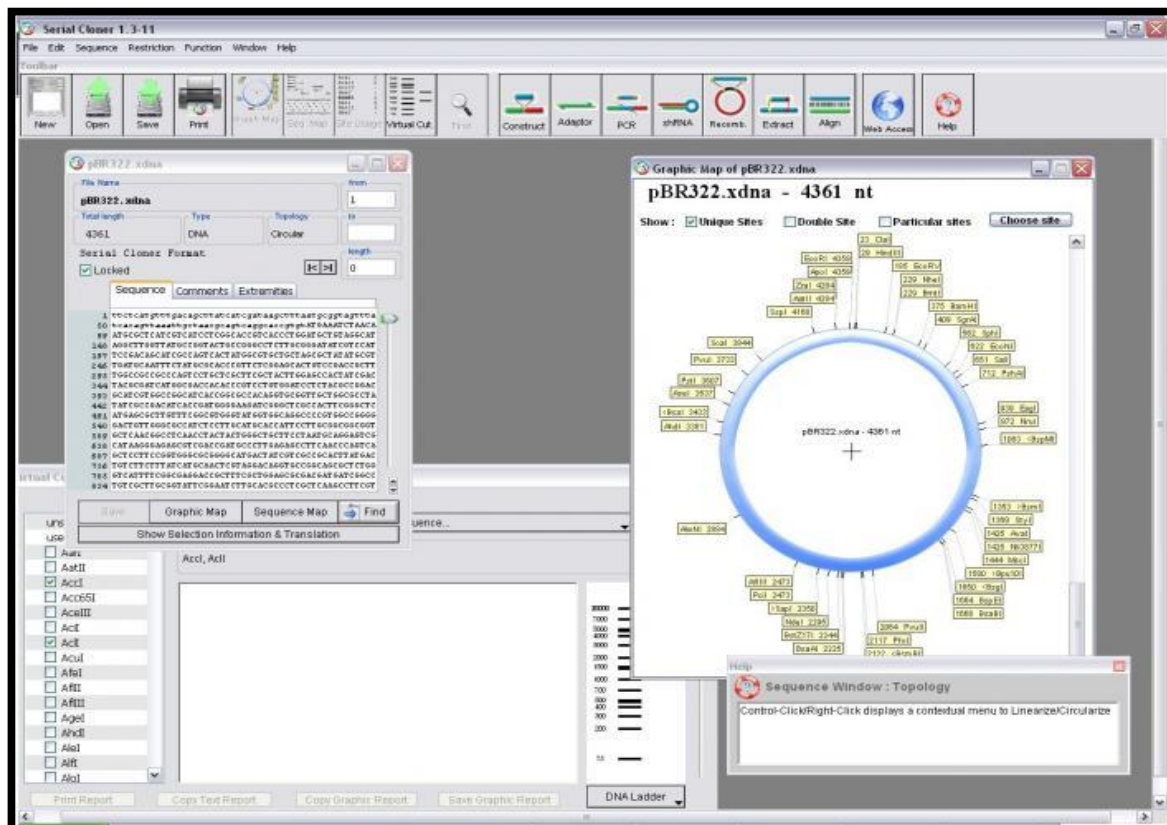


Image: Computer software (Serial Cloner 2.6 program)