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# A STUDY OF COAGULATION PROFILE IN SICKLE CELL ANAEMIA PATIENTS WITH AND WITHOUT CHRONIC LEG ULCERS AT THE GHANA INSTITUTE OF CLINICAL GENETICS, ACCRA

**BY DAVID SEBBIE SACKEY**
**ID: 10363138**

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**COLLEGE OF HEALTH SCIENCES**

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# DECLARATION

I, DAVID SEBBIE SACKEY of the Department of Haematology, School of Biomedical and Allied Health Sciences, College of Health Sciences, do hereby declare that this thesis is original and was duly carried out by me and results obtained therein are the true reflection of the work and supervised by the supervisors below.

Student: Signature: _____ Date: _____

**David Sebbie Sackey**

Supervisors:

Signature: _____ Date: _____

**Edeghonghon Olayemi, MB BS, MSc, FWACP**

Senior Lecturer, Department of Haematology, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana

Signature: _____ Date: _____

**Yvonne Dei-Adomakoh, MB BS, FWACP**

Lecturer, Department of Haematology, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana

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# DEDICATION

I dedicated this project to the God Almighty and in honour of my lovely wife, Mrs Lydia Adwoa Sackey.

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Table of Contents

DECLARATION ..... II
DEDICATION ..... III
ACKNOWLEDGEMENT ..... IV
LIST OF ABBREVIATIONS ..... VII
ABSTRACT ..... X
CHAPTER ONE ..... 1
1.0 INTRODUCTION ..... 1
1.1 Background ..... 1
1.2 Problem Statement ..... 4
1.3 Justification ..... 4
1.4 Aim ..... 5
1.5 Hypothesis ..... 5
1.6 Objectives ..... 5
CHAPTER TWO ..... 6
2.0 LITERATURE REVIEW ..... 6
2.1 Sickle Cell Disease ..... 6
2.2 Epidemiology ..... 8
2.3 Global Burden of SCD ..... 10
2.4 Clinical Features of SCD ..... 11
*2.4.1 Sickle Cell Anaemia* ..... 12
*2.4.2 Sickle Cell Crisis* ..... 13
*2.4.3 Leg Ulcer in SCD Patients* ..... 13
*2.4.4 Priapism* ..... 16
*2.5.1 Vaso-occlusion* ..... 20
*2.5.2 Coagulation* ..... 23
2.6 Management of Sickle Cell Anaemia ..... 33
2.7 Management of Leg Ulcer ..... 35
2.8 Prevention of SCD ..... 37
3.0 SUBJECTS AND METHODS ..... 38
3.1 Study design ..... 38

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3.2 Setting ..... 38
3.3 Study subjects ..... 38
3.5 Sample size and power calculation ..... 39
3.6 Methods for Data and Sample Collection, Processing and Analysis ..... 40
CHAPTER FOUR ..... 47
4.0 RESULTS ..... 47
4.1 General characteristics of study subjects ..... 47
4.2 Coagulation profile of study subjects ..... 48
CHAPTER FIVE ..... 53
5.0 DISCUSSION, LIMITATION RECOMMENDATION AND CONCLUSION ..... 53
5.1 D-dimer Levels in Subject Groups ..... 53
5.2 Fibrinogen levels in subject groups ..... 54
5.4 Platelets count, Age, Hb levels, Duration and Frequency of Leg ulcers ..... 57
5.5 Limitation of the Study ..... 59
5.6 Conclusion ..... 59
5.7 Recommendation ..... 60
Appendix G ..... 93
Appendix H ..... 96
Appendix K ..... 101
Consent Form ..... 105

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Adenine |
| ANOVA | Analysis of Variance |
| APA | Antiphospholipid Antibody |
| APTT | Activated Partial Thromboplastin Time |
| $CaCl_2$ | Calcium Chloride |
| CLU | Chronic Leg Ulcer |
| CP | Coagulation Profile |
| CVI | Chronic Venous Insufficiency |
| DD | D-dimer |
| DIC | Disseminated intravascular coagulation |
| DNA | Deoxyribonucleic Acid |
| DVT | Deep Vein Thrombosis |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| EPRC | Ethical and Protocol Review Committee |
| FBC | Full Blood Count |
| FDPs | Fibrin Degradation Products |
| FIB | Fibrinogen |
| FVIII | Factor VIII |
| G | Glutamine |
| g/dl | grams per decilitre |

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| | |
|---|---|
| GICG | Ghana Institute of Clinical Genetics |
| GLU | Glutamic Acid |
| Hb | Haemoglobin |
| HbAA | Normal Haemoglobin |
| HbAS | Sickle Cell Trait |
| ICAM-1 | Intercellular Adhesion Molecule (ICAM)-1 |
| INR | International Normalised Ratio |
| ISI | International Sensitivity Index |
| KBTH | Korle-Bu Teaching Hospital |
| LA | Lupus Anticoagulant |
| LU | Leg Ulcer |
| mg/dl | Milligram per deciliter |
| NF kB | Nuclear Factor κB |
| NO | Nitric Oxide |
| PE | Pulmonary Embolism |
| PF1+2 | Prothrombin Fragments 1 and 2 |
| PH | Pulmonary Hypertension |
| PLT | Platelet |
| PS | Phosphatidyl Serine |
| PT | Prothrombin Time |
| RBC | Red Blood Cell |

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| | |
|---|---|
| ROS | Reactive Oxygen Species |
| SCA | Sickle Cell Anaemia |
| SCD | Sickle Cell Disease |
| SD | Standard Deviation |
| Sec | Seconds |
| SPSS | Statistical Package for the Social Sciences |
| T | Thymine |
| TAT | Thrombin-antithrombin |
| TF | Tissue Factor |
| t-PA | Tissue plasminogen activator |
| TT | Thrombin Time |
| VAL | Valine |
| VALCO | Volta Aluminium Company |
| VCAM-1 | Vascular Cell Adhesion Molecule |
| VO | Vaso-occlusion |
| VTE | Venous Thromboembolism |
| vWF | von Willebrand Factor |
| WBC | White Blood Cell |
| WHO | World Health Organization |

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## ABSTRACT

**Background**: Sickle Cell Anaemia (SCA), the most common form of sickle cell disease (SCD), is associated with elevation of thrombotic factors and depression of anticoagulants, resulting in venous thrombotic events. Sickle Cell Anaemia patients suffer from recurrent, painful, slow-to-heal leg ulcers. Chronic leg ulcer (CLU) in sickle cell anaemia, may not lead to fatal outcomes though, but is associated with aesthetic deformity and reduced quality of life. The pathogenesis of CLU in SCA remains elusive, hampering therapeutic development. Activation of coagulation factors has been shown to be a feature of SCA patients with chronic leg ulcer, with rates of thrombin and fibrin formation constantly higher than normal. Several studies have reported the role of coagulation factors in chronic leg ulcers in SCA, mainly in western countries; there is paucity of data in indigenous Africans with SCA, living in sub-Saharan Africa.

**Aim**: The aim of the study was to determine if there is an association between coagulation profile abnormalities and chronic leg ulcer among SCA patients in Accra**.**

**Methods**: In this study one hundred and forty-five (145) subjects were assessed to determine their coagulation profile, full blood count and haemoglobin electrophoresis. Fifty (50) SCA with CLU and fifty (50) SCA without Leg Ulcer were recruited from the Ghana Institute of Clinical Genetics, Accra, whilst forty five (45) control subjects with haemoglobin AA were recruited from voluntary blood donors at the National Blood Transfusion Centre, Accra, Ghana. Venous blood sample was collected from each participant for coagulation analysis, Full Blood Count and Hb Electrophoresis. Tests of coagulation analyzed were, prothrombin time (PT), activated partial thromboplastin time (APTT), d-dimer concentration and fibrinogen.

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**Results:** Sickle Cell Anaemia patients with & without CLU had increased platelet counts (478.12±177.32 & 424.20±169.08 vs. 226.28±53.18, p<0.001), shorter APTT (31.27±6.16 & 34.51±6.80 vs. 35.20±6.06, p=0.006) and marginally prolonged PT (16.02±2.57, & 16.04±1.68 vrs 14.91±1.24, p=0.006) than HbAA controls. Also as the APTT approaches normal, the Hb concentration of the subjects seem to improve (r=0.185, p=0.026). Again, as the Prothrombin Time approaches normal, the Hb level also seem to improve (r= -0.233, p=0.005). The Prothrombin Time increased with increasing platelet counts (r=0.241, p=0.004). A similar correlation between the Prothrombin Time and Platelets count is seen among the female subjects (r=0.299, p=0.030). With respect to d-dimer levels, SCA with CLU had higher levels than the Hb AA controls (1.56±2.90 vs. 0.52±0.46, p=0.007); levels were however not significantly different between SCA without LU and Hb AA controls (1.04±1.56, p=0.650). D-dimer levels negatively correlated with the Hb levels (r= -0.233, p=0.005). Fibrinogen levels were not significantly different among the study groups. The history of recurrence of the leg ulcers correlated negatively with the platelet counts (r= -0.311, p=0.028). The analysis also seem to suggest that the longer the duration of leg ulcer, the lower the Hb drops from normal levels (r= -0.331, p=0.021).

**Conclusion**: The findings of this study show that there is general trend towards activation of coagulation in sickle cell anaemia patients with chronic leg ulcers. It is recommended that APTT, d-dimer levels, platelet counts and Hb should be monitored especially in SCA patients without leg ulcers. This may help identify those who are predisposed to leg ulcers. Patients with CLU could also be monitored with the same markers to monitor progress of healing to further substantiate the validity of these markers.

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# CHAPTER ONE

# 1.0 INTRODUCTION

## 1.1 Background

Sickle Cell Disease (SCD) is a common genetic disorders, with sickle cell anaemia (SCA) i.e. haemoglobin SS being the commonest type (Weatherall, 2005). Sickle Cell Anaemia is a widespread haemoglobinopathy occurring throughout the world. Its prevalence in Ghana is about 2.0% (Konotey-Ahulu, 1991). Herrick first described the characteristic sickle-shaped erythrocytes in 1910, and understanding of the disease has gradually increased since then. Pauling and colleagues identified electrophoretic abnormalities in sickle haemoglobin (HbS) and coined the term "molecular disease" in 1949 (Pauling et al., 1949). The haemoglobin biophysics and genetics underlying the disease have been extensively studied and have helped the understanding of other molecular diseases. However, clinical management of sickle cell disease is still basic and, although some evidence lends support to the use of blood transfusion and hydroxycarbamide in some circumstances, no drugs have been developed that specifically target the pathophysiology of this disease (Rees et al., 2010).

People with sickle cell disease are those who have haemoglobin S and another Hb variant, for example; SCA, HbSC, HbSβthal that cause the characteristic clinical syndrome such as vaso-occlusive pain crisis and haemolytic anaemia, whereas sickle-cell anaemia, the most common form of sickle cell disease, refers specifically to homozygosity for the βS allele. In populations of African origin, sickle cell anaemia typically accounts for 70% of cases of sickle cell disease, with the remainder having haemoglobin SC disease (HbSC disease), HbSβthal, HbS with hereditary persistence of foetal haemoglobin (SHPFH) etc., (Nagel et al., 2003).

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The clinical manifestations of sickle cell disease are diverse (Minniti et al., 2010a). Complications of SCD include leg ulcers which are relatively common. In some populations, SCD leg ulcers occur in areas with less subcutaneous fat, thin skin and with decreased blood flow (Trent and Kirsner, 2004). The commonest sites are the medial and lateral malleoli, often becoming circumferential if not controlled early; the medial malleolus is more commonly involved than the lateral malleolus. Less common sites are the anterior tibial area, dorsum of the foot, and Achilles tendon (Serjeant et al., 2005).

The pathogenesis of chronic ulcers in SCD is complex, and may include the following; mechanical obstruction by dense sickled red cells, venous incompetence, bacterial infections, abnormal autonomic control with excessive vasoconstriction when in the dependent position, in situ thrombosis, lupus anticoagulant, anaemia with decrease in oxygen carrying capacity, and decreased nitric oxide bioavailability leading to impaired endothelial function have all been proposed as potential contributing factors (Mack and Kato, 2006).

The prevalence of leg ulceration varies, being low before age 10 years (Minniti et al., 2010a). Its geographical distribution is also variable, affecting 75% of Haemoglobin SS patients in Jamaica but only 8–10% of North American patients (Ladizinski et al., 2012). These ulcers are characterized by an indolent, intractable course, typically healing up to 16 times slower than venous ulcers (Gordon and Bui, 2003). Approximately 97% of healed sickle cell ulcers will recur in less than 1 year (Ladizinski et al., 2012). Due to the recalcitrant nature of these ulcers, patients may experience significant disfigurement, social isolation, and loss of income (Ladizinski et al., 2012).

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In Ghana, the prevalence of SCD leg ulcers was estimated in the late 1970s to be 10.2% (Konotey-Ahulu, 1974). The only prospective study about the natural history of SCD leg ulcer in Ghana was reported in early 1990s. In this report, 25 SCD patients with leg ulcers were studied for 3 years of which most subjects were SCA. Sixty percent (60%) reported that the leg ulcer began as a painful blister or result of an unknown event. The mean duration of the leg ulcer in the study was 3.6 years (Ankra-Badu, 1992).

A large number of coagulation abnormalities have been described in this disorder. Thus, activation of coagulation has been shown to be a feature of sickle cell anaemia patients with chronic leg ulcer, with rates of thrombin and fibrin formation constantly higher than normal. Levels of plasma fibrinogen, factor VIII and D-dimers were also found to be increased (Nsiri et al., 1996a). Prothrombin fragment 1.2 (F1.2), which is a plasma marker of thrombin generation is elevated both in sickle cell anaemia patients in crisis and in steady state (Westerman et al., 1999). It is significantly associated with phosphatidyl serine positive red blood cell in patients with sickle cell anaemia, providing evidence for the role of phosphatidyl serine exposure in coagulation activation (Setty et al., 2001). Similar elevations in plasma levels of thrombin–antithrombin complexes and D-dimer, a marker of increased fibrinolysis, are observed in SCA (Setty et al., 2001).

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### 1.2 Problem Statement

The refractory nature of chronic leg ulcers in sickle cell disease (SCD) affects the quality of life and productivity of those persons afflicted. This, in combination with the high costs of long-term therapy, makes SCD leg ulcers a major health problem among sickle cell anaemia patients in Ghana. Management of sickle cell leg ulcer is based on understanding pathophysiologic abnormalities. In recent years, identifying prognostic factors for healing and developing novel therapeutic approaches for venous ulcers have offered valuable tools for the management of patients with this disorder (De Araujo et al., 2003).

Unpublished records at the Ghana Institute of Clinical Genetics, Korle-Bu, show that a number of SCA patients with chronic leg ulcers have dropped out of school and others lost their jobs probably due to stigmatization. Changes in coagulation pattern and coagulation product levels may be associated with the development of leg ulcers. Currently, there is no data to confirm or refute this hypothesis among SCA patients. A comparison of the coagulation profile among SCA patients with and without leg ulcers may provide baseline information on how to manage the affected group.

### 1.3 Justification

Little research has been carried out on chronic leg ulcers in SCA patients in Ghana. This study is designed to determine if there is an association between coagulation abnormalities and chronic leg ulcer in SCA patients. Research into this area could provide useful information on the relevance of recommending routine coagulation assay which may help in prevention and proper management of chronic leg ulcers in SCA patients. It may also provide a knowledge base and background information for further research on chronic leg ulcers in SCA patients.

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### 1.4 Aim

The aim of the study is to determine if there is an association between coagulation abnormalities and chronic leg ulcer among SCA patients in Accra.

### 1.5 Hypothesis

There is no association between coagulation abnormalities and chronic leg ulcers in SCA subjects.

### 1.6 Objectives

1. To compare the activated partial thromboplastin time among SCA patients with leg ulcer and those without leg ulcer as well as HbAA genotype controls.
2. To compare the levels of fibrinogen among SCA patients with and without leg ulcer as well as non-sickle cell individuals with HbAA genotype.
3. To compare the levels of D-dimers among SCA patients with and without leg ulcers as well as non-sickle individuals of HbAA genotype.

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# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Sickle Cell Disease

Sickle cell disease (SCD) is a common monogenetic disorder, which is characterized by the production of abnormal haemoglobin called haemoglobin S. There are diverse clinical manifestations of SCD, with leg ulcers being relatively common and can be disabling (Minniti et al., 2010a). Leg ulcerations have been recognized as a major complication of SCD since the early twentieth century, the first SCD patient to be described in North America in 1910 had chronic leg ulcerations (Herrick, 1910). Sickle cell disease is described as the first identified "molecular" disease (Itano and Neel, 1950). Its underlying cause is as a result of autosomal recessive inheritance of two copies of a β-globin gene mutant, provided by each parent. There is a DNA substitution in the gene coding the human β-globin subunit at the $17^{th}$ position in which adenine is replaced by thymine (Rees et al., 2010). These replacement of adenine by thymine (GAG→GTG) therefore, leads to an amino acid substitution from glutamic acid to valine at the position six in the β-globin chain of haemoglobin A, resulting in a haemoglobin called haemoglobin S (Platt, 2000)

In the homozygous sickle cell disease (HbSS), both abnormal haemoglobins are HbS. Normal adult haemoglobin is made from a combination of 2β-globin protein chains with 2α-globin chains and haem. The β1-globin gene is located on the short arm of chromosome 11. Approximately 150 diseases have been linked to this same chromosome 11 for example, Usher syndrome type 1, Bardet-Biedl syndrome and Schizophrenia (Rees et al., 2010). In sickle cell disease, two abnormal allemorphic haemoglobin genes are inherited, of which at least one must

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be the sickle haemoglobin (HbS). People who have sickle cell disease are born with one copy of the mutation that causes the HbS and one copy of another abnormal haemoglobin allele. This include sickle-haemoglobin C disease (HbSC), sickle beta-plus-thalassaemia (HbS/$\beta^{+}$) and sickle beta-zero-thalassaemia (HbS/$\beta^{0}$) genes, one from each parent. Sickle cell trait (HbAS), results from the inheritance of a normal haemoglobin gene (HbA) from one parent and an abnormal mutated β-globin gene, the sickle haemoglobin gene (HbS) from the other parent. People who have sickle cell trait do not develop sickle cell disease, but they are "carriers" who can pass the abnormal gene on to their children (Ashley-Koch et al., 2000).

Herrick was the first to describe the disease (SCD). In 1910, he described the case of a young black student from the West Indies with severe anaemia which is characterized by peculiar elongated and sickle-shaped red blood corpuscles. Herrick also noted a slightly increased volume of urine of low specific gravity and thus observed a common feature of sickle cell nephropathy (Herrick, 1910).

Periodic, self-limited episodes of excruciating musculoskeletal pain punctuate the lives of patients with sickle cell anaemia. Often referred to as "crises" these episodes are the principal cause of morbidity among these patients. Although Western observers named SCA for its curious microscopic morphologic features, African cultures have named it for its painful episodes. In the Ga language of Ghana, for example, the disease was, and still called chwechweechwe – "relentless, repetitive chewing", Ahotutuo (Twi-Ghana), Nuidudui (Ewe-Ghana/Togo), Amosane (Hausa-Northern Nigeria), Aromolegun (Yoruba-Nigeria) (Konotey-Ahulu, 1974).

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## 2.2 Epidemiology

Sickle cell disease is found globally with the highest frequency in sub-Saharan Africa, the Indian – Sub continent and Middle-East. This might probably be due to the survival advantage in malaria endemic areas and subsequent migrations (Weatherall and Clegg, 2001). At least four haplotypes of the mutant β-gene that codes for the production of the haemoglobin S have been identified in Africa. These haplotypes are set of genes known as the polymorphic restriction endonuclease sites, found in and around the mutant of the sickle cell gene. The presence of these different types of haplotype specific to different regions globally suggests that the sickle gene mutation occurred on multiple occasions in the equatorial part of Africa and the South-Western part of Asia (Padmos et al., 1991).

The various sickle cell haplotypes found in Africa are the Senegal in the Atlantic West Africa above the Niger River, Benin in Central West Africa, Bantu in Central Africa and Cameroon in the West Africa as shown in figure 1 (Rosa et al., 2007). The other haplotype is found in the Asia known as the Arab-India haplotype (Flint et al., 1998). The origin of this haplotype is probably from the Indus Valley Harappa culture, which was spread to the Eastern part of the Arab peninsula through gene flow to the Eastern province of Saudi Arabia, Bahrain, Kuwait, and Oman (Perriene et al., 1978, Stuart and Nagel, 2004).

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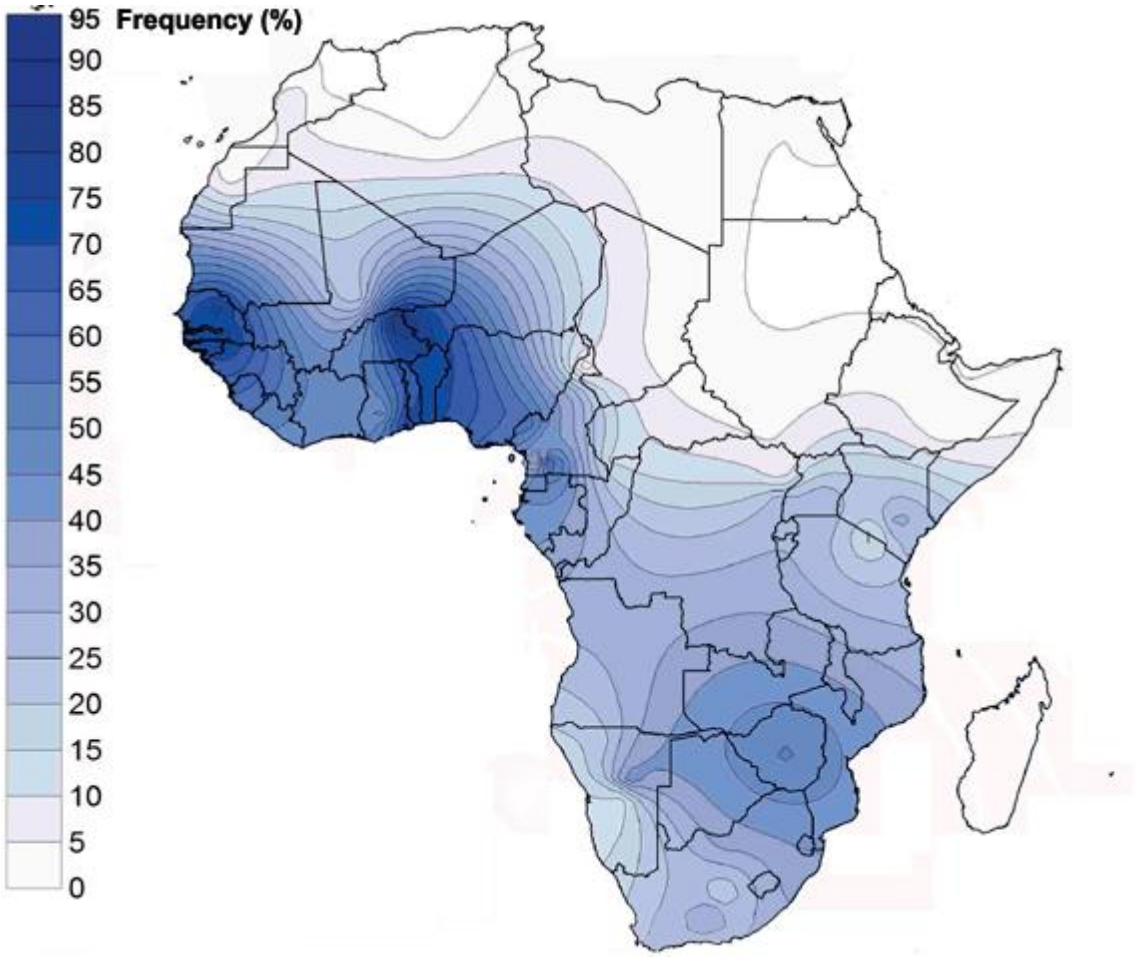


**Figure 1: Africa spatial distribution of hyplotypes of Hb Gene (Rosa et al., 2007)**

**Frequency scale (in percentage) is shown on the left**

The severity of sickle cell disease is usually influenced by the haplotype; the most severe disease is associated with the Bantu haplotype whilst the least is associated with the Senegal haplotype. The influence of these haplotypes on complication rates in individuals with sickle cell trait is yet to be determined (Powars et al., 1994).

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Sickle cell disease affects about 72 000 people in the United State and where more than 2 million people are carriers (Creary et al., 2007b) in the United State, mortality has been reduced dramatically with new-born screening and better management. However, there is a higher rate of mortality among individuals with SCD, with reports suggesting that if not treated most children with the disease in Africa will die in their early life due to low availability of comprehensive medical care (Makani et al., 2007). While, most children affected with sickle cell anaemia survive in high-income countries, a large proportion of those born in low-income countries die before the age of five (Modell and Darlison, 2008). About 2.0% of babies born in Ghana are affected by sickle cell anaemia (Konotey-Ahulu, 1999), and another study conducted in 2008 showed that the infant rate of sickle cell anaemia is 1.9% (Ohene-Frempong et al., 2008). This is similar to a study reported from Nigeria with more than 2.0% of all babies born to Nigerian parents having sickle cell anaemia (Akinyanju, 2001).

### 2.3 Global Burden of SCD

Most children with sickle cell anaemia who are not offered care die in early life, however, certain basic steps including neonatal diagnosis, prophylactic anti-malaria and antibiotics, access to hospital treatment when needed, and information and support from families greatly improve quality and length of life (Serjeant, 1997). Similarly, life expectancy of sickle cell anaemia patients who are regularly treated with blood transfusion and iron-chelation therapy, or bone-marrow transplantation, is approaching normal (Borgna-Pignatti et al., 2004).

The World Health Organization (WHO) has recommended that screening and genetic counselling for sickle cell disorders should be an integral part of healthcare programmes in countries which are most affected (Odame et al., 2011). As a result of kinetics of inherited

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disorders such as sickle cell disease, the present and future public health challenges are in two dimensions. Countries with high levels of haemoglobin S need to be prepared to provide health care for patients with sickle cell anaemia. With substantial decreases in mortality of children below 5 years of age in many low-income countries (Rajaratnam et al., 2010), homozygous neonates who might have previously died without being diagnosed are now more likely to survive to adulthood. The extent of the problem posed by this condition needs to be known by these countries so that they can have efficient and affordable treatment policy plans to reduce the morbidity of these patients during their lifetime (Williams et al., 2009). Secondly, since people with sickle cell trait are usually asymptomatic, they need to be educated about their status to prevent the future birth of homozygous children, as well as to avoid psychosocial issues such as misconceptions or stigmatization (Dyson and Atkin, 2012).

### 2.4 Clinical Features of SCD

Sickle-cell disease is a multisystem disease, associated with episodes of acute illness and progressive organ damage, and is one of the most common severe monogenic disorders worldwide (Weatherall et al., 2005). The clinical outcome of SCD vary greatly from mild to complicated severe conditions which is associated with risk of early mortality and multiple organ damage (Steinberg, 2008b). The clinical manifestations of this disease are numerous; these include leg ulcer, pain crisis, vaso-occlusion, haemolysis, pulmonary hypertension, tissue ischemia, impaired blood flow as a result of intravascular sickling in vessels, inflammation, acute chest syndrome, priapism, cerebro vascular accident, and dactylitis (Steinberg, 2005).

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Acute chest syndrome is known to be a leading cause of death and hospitalization among sickle cell disease patients and even though there is increased awareness of its diagnosis, its optimal treatment is still unknown (Vichinsky et al., 2000). The frequency of certain other diseases such as recurrent pulmonary disease and resultant chronic lung disease tends to increase with age. About half the sickle cell disease patients who developed acute chest syndromes are also admitted for other clinical symptoms within three days after hospitalization (Castro et al., 1994)

### *2.4.1 Sickle Cell Anaemia*

Sickle cell anaemia patients have a shorter red blood cell life span of about twenty (20) days compared with the normal red blood cells that survive for between ninety (90) to one hundred and twenty (120) days. This is due to a premature breakdown of the red blood cells in sickle cell anaemia which **is** an important consequence of haemoglobin S polymerization (Wood et al., 2008). The presence of a high level of haemoglobin S in the red blood cell is a major contributory factor to the pathophysiological development of sickle cell anaemia which can occur in three different ways (Platt, 2008). Under low oxygen tension, the red blood cells in sickle cell anaemia patients lose their deformability leading to ischaemia and vascular occlusion causing a painful crisis. There is also membrane damage which reduces the life span of the red blood cell, leading to chronic intravascular and extravascular haemolysis. The intravascular haemolysis therefore leads to a reduced availability of nitric oxide, increased vascular tone, and pulmonary-artery hypertension (Gladwin et al., 2004a). Subsequently, the damaged red blood cells would develop abnormal surfaces which result in an increased adherence to and damage of vascular endothelium (Platt, 2008). Adhesion receptors identified include α4β1 integrin and CD36, exclusively present on stress reticulocytes, and CD47 on mature RBCs. More recently,

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Lu/BCAM, the unique RBC receptor for laminin, and ICAM-4 are being investigated (Cartron & Elion, 2008). This process therefore provokes a proliferative lesion involving white cells, platelets, smooth-muscle cells, cytokines, growth factors, and coagulation proteins. This also enhances acute vaso-occlusion (Platt, 2008).

### *2.4.2 Sickle Cell Crisis*

Sickle cell crises are normally accompanied by an acute excruciating pain in sickle cell anaemia patients secondary to vaso-occlusion which is the cause of most hospitalization (Yale et al., 2000). This pain can begin suddenly and last several hours to several days. A steady state of a sickle cell anaemia patient is described as the duration of which the SCA patient **is** stable and free of painful crisis for at least three weeks since the last clinical event and more than three months since the last blood transfusion (Akinola et al., 1992). Sickle cell disease and its complications can be classified in four main areas; the haemolytic anaemia and its sequelae, the pain syndromes, the organ damage or failure, and the co-morbid conditions (Ballas, 2007).

### *2.4.3 Leg Ulcer in SCD Patients*

Sickle cell anaemia (HbSS) patients are more likely to develop a leg ulcer than those with other genotypes (Ankra-Badu, 1992). Chronic leg ulcers can be defined as loss of skin on the part of the lower limb that takes more than six (6) weeks to heal (Dale et al., 1983). The exact cause of sickle cell leg ulcer is unclear; leg ulcers generally occur in areas with less subcutaneous fat, thin skin, and with decreased blood flow (Trent and Kirsner, 2004). The commonest sites are the medial and lateral malleoli (ankles), often becoming circumferential if not controlled early. The medial malleolus is more commonly involved than the lateral malleolus. Less common sites are the anterior tibial area, dorsum of the foot, and Achilles tendon (Serjeant et al., 2005). Chronic leg ulcer in sickle cell anaemia is associated with the intensity of haemolysis of the red blood cell

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in the vessel (intravascular haemolysis) or elsewhere in the body (extravascular haemolysis) (Nolan et al., 2006)

Venographic studies have shown that venous insufficiency is not a primary cause of sickle cell ulcerations. Instead, it is the arteriovenous shunting which has been postulated as a factor in ulcer formation. This shunting deprives the skin of oxygen, promoting ulceration. Furthermore, trauma, infection and inflammation have all been cited as potential causes of lower extremity ulcerations in SCD. It is the combination of these factors, along with the interrupted microcirculation that contribute to the pathophysiology of lower extremity ulcerations in patients with SCD (Rees et al., 2010).

Venous hypertension results from either valve dysfunction with venous reflux, venous thrombosis causing outflow obstruction, or a combination of the two processes. The persistent venous hypertension leads to an inflammatory response by leukocytes, which in turn initiates a cascade of cytokine activity such as interlukin-1β released primarily by monocytes during inflammation. Various leukocytes mobilise in the dermis from stimulation of specific adhesion molecules on endothelial cells, cytokines and metalloproteinases are expressed leading to cellular and tissue dysfunction resulting in dermal changes observed in sickle cell anaemia with leg ulcers. Similarly, when there is thrombosis in the arteries, it prevents blood flow to the capillary bed causing infarction which prolongs wound healing (Rees et al., 2010). A study conducted in Nigeria showed that sickle cell anaemia patients with leg ulcers are more likely to have lupus anticoagulant than those without leg ulcers; SCA patients with chronic leg ulcers had a significantly longer kaolin clotting test than those without chronic leg ulcers (Olayemi and Bazuaye, 2009).

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Leg ulcers may be classified as acute or chronic according to their duration, however, there is no consensus as to a specific length of time to define chronicity of the ulcer. An acute ulcer usually should heal in less than a month. Among chronic ulcers, duration of six months seems to define the most recalcitrant ulcers. It is not uncommon for ulcers to last many years, often healing and recurring repeatedly. Chronic ulcer of the lower legs is a relatively common condition amongst adults; one that causes pain, social distress and results in considerable healthcare and personal costs. A correct diagnosis is essential to avoid inappropriate or wrong treatment that may delay the healing of the ulcer, cause deterioration of the ulcer, or harm the patient. (Herber et al., 2007).

#### *2.4.3.1 Aetiology of Leg Ulcer in SCD*

The factors that predispose to leg ulcers are numerous; the most common causes are the vascular disorders such as chronic venous insufficiency (CVI) or atherosclerotic disease of the arteries. Valvular incompetence in the deep veins causes the vessels to become distended and stretch to accommodate the additional blood flow. The valves are not able to effectively close, which results in reverting the blood flow, venous hypertension and lower leg oedema (Meissner et al., 2007). Patients with Sickle Cell Disease are at high risk for a variety of thrombotic complications such as vaso-occlusive crisis, ischaemic stroke and production of micro-thrombi (Prengler et al., 2002).

Cutaneous vasculitis which is the inflammation and destruction of blood vessel walls of the skin can also affect any blood vessel in any organ. It usually results from the inflammation and ischaemia of small-to medium-sized blood vessels of the skin. Current research suggests that the

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deposition of circulating immune complex arising from hypersensitivity to auto-antibodies also attributed to the pathogenesis of most types of cutaneous vasculitis (Grzeszkiewicz and Fiorentino, 2006).

### *2.4.4 Priapism*

Priapism is one of the clinical complications associated with sickle cell anaemia (Diggs, 1934), and it is a term used to describe a prolonged and persistent painful penile erection that can last from several hours to days which has no association with sexual interest or stimulation (Claudino and Fertrin, 2012). About forty five percent (45%) of males, including children, with sickle cell anaemia suffer from priapism, and the rate of resulting erectile dysfunction is more than thirty percent (30%). Permanent partial or total complete erectile dysfunction may occur if untreated (Adeyoju et al., 2002). Typically, only the corpora cavernosa are affected without involvement of the corpus spongiosum and glans (Song and Moon, 2013). One larger study conducted by Burnett et al indicated that majority of the priapism cases were without a cause but were associated with sickle cell anaemia, perineal trauma and excessive intake of alcohol or drug abuse (intracavernous self-injection of drugs, such as phentolamine, prostaglandin, and papaverine) (Burnett and Bivalacqua, 2011).

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## 2.5 Pathophysiology of Sickle Cell Disease

The loading of a very high concentration of haemoglobin into red cells requires that the protein be extraordinarily soluble. When haemoglobin S is deoxygenated, the substitution of glutamic acid for valine at position six of the β-globin chain, results in a hydrophobic interaction with the adjacent β-globin chain in the haemoglobin molecule, triggering aggregation and formation of large polymers (Ferrone and Rotter, 2004). This crystallisation also produces a polymer nucleus, which grows and fills the erythrocyte, disrupting its architecture and flexibility and promoting cellular dehydration, with physical and oxidative cellular stress (Brittenham et al., 1985). As a result of this change, RBCs form characteristic sickle-shapes and the surface of these RBCs attracts each other more readily due to their sticky surfaces, polymerizing when in reduced oxygen environment. This polymerization of deoxygenated haemoglobin S is the basic event in the molecular pathogenesis of sickle cell disease which exist in dynamic equilibrium with soluble haemoglobin tetramer, resulting in a distortion of the shape of the red cell, a marked decrease in its deformability and sickle morphology for which the disease was named (Ferrone and Rotter, 2004)

The rigidity of these cells is therefore responsible for the vaso-occlusive phenomena, the hallmark of the disease. The deoxygenated red blood cells containing haemoglobin S assume a variety of interesting shapes. Scanning cells with transmission electron microscopy reveals the presence of bundles of fibres oriented along the long axis of the cell in a banana or sickle shape, some cells assume a holly-leaf shape, bundles of haemoglobin S fibres point in the direction of each projection. The three-dimensional fibre structure has been elucidated by high-resolution electron microscopy and novel methods of image reconstruction (Dykes et al., 1979).

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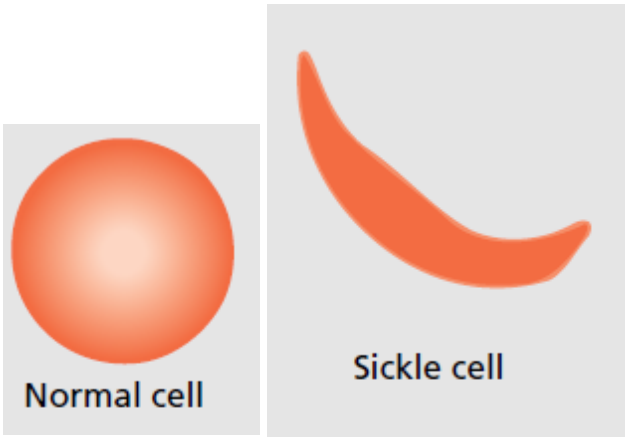


**Figure 2: Normal red blood cell and a 'sickled' red blood cell (Bain, 2004)**

The rate and the extent of HbS polymerisation are proportional to the extent and duration of haemoglobin deoxygenation and the intracellular HbS concentration. But the presence of foetal haemoglobin in the erythrocyte effectively reduces the concentration of the HbS (Noguchi et al., 1988). The main determinant of the sickle cell disease severity is the rate and extent of HbS polymerisation, which is exemplified by co-inheritance of genetic factors that modulate the intracellular HbS or foetal haemoglobin concentration. Similarly, therapeutic inhibition of the cation transport channels prevents erythrocyte dehydration and effectively reduces HbS concentration and haemolysis. These manifestations are driven by two major pathophysiological processes: vaso-occlusion with ischaemia-reperfusion injury and haemolytic anaemia (Bunn, 1997).

Haemolysis has long been known to cause anaemia which leads to fatigue, and cholelithiasis as a result of persistent and accelerated haemolysis associated with multiple transfusion, but there is now evidence that it contributes to the development of progressive vasculopathy. As patients with sickle cell disease age, they are at a high risk of vasculopathy, characterised by systemic

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and pulmonary hypertension, endothelial dysfunction, and proliferative changes in the intimal and smooth muscle of blood vessels (Gladwin et al., 2004b).

Data from epidemiological studies suggest that several complications are associated with increased rates of haemolysis: cholelithiasis, cutaneous leg ulceration, priapism, and pulmonary hypertension are associated with low steady state haemoglobin concentrations and an increased rate of intravascular haemolysis (Kato et al., 2007). An association between the development of pulmonary hypertension and the intensity of haemolytic anaemia was noted in three prospective screening studies of adults with sickle-cell disease (Ataga et al., 2006) and in paediatric studies (Dham et al., 2009).

An important disease mechanism involves the release of haemoglobin into the circulation during intravascular haemolysis. Free plasma haemoglobin generates reactive oxygen species, such as the hydroxyl and superoxide radicals (Repka and Hebbel, 1991), which is a potent scavenger of nitric oxide (Reiter et al., 2002). Nitric oxide is normally produced by the endothelium and regulates basal vasodilator tone, and inhibits platelet and haemostatic activation and transcriptional expression of nuclear factor κB (NF κB)-dependent adhesion molecules, such as vascular cell-adhesion molecule-1, intercellular cell-adhesion molecule-1, and the selectins (Palmer et al., 1988). The release of haemoglobin into the plasma during haemolysis potently inhibits endothelial nitric oxide signalling, leading to endothelial cell dysfunction and nitric oxide resistance (Gladwin, 2006).

Haemolysis also releases erythrocyte arginase-1 into plasma. Arginase metabolises plasma arginine into ornithine, decreasing the required substrate for nitric oxide synthesis and compounding the decreased bioavailability of nitric oxide in patients with sickle-cell disease

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(Morris et al., 2005). Chronic depletion of nitric oxide and arginine might also contribute to the hypercoagulable state in haemolytic diseases. Studies have shown correlations between the rate of haemolysis and levels of platelet activation and procoagulant factors in the blood (Ataga et al., 2008). Haemolysis is also associated with the formation of erythrocyte microvesicles containing phosphatidyl serine, which is an activator of tissue factor; the numbers of microvesicles are increased further by the functional asplenia present in patients with sickle-cell disease (Westerman et al., 2008).

### *2.5.1 Vaso-occlusion*

Vaso-occlusion is one of the most common painful complications in sickle cell disease which normally occurs in adults and adolescents (Yale et al., 2000). But the vaso-occlusive crisis affects almost all individuals with sickle cell disease, often beginning in the last stage of infancy and recurring throughout human life (Almeida and Roberts, 2005). The frequency, severity, and duration of vaso-occlusive crises are considerably varied and the molecular basis of its development in the sickle cell disease is unclear (Camus et al., 2012). The pathogenesis of the microvascular occlusion, which is the main cause of painful sickle cell crisis, is complex involving the sickle-shape of the red blood cell, the activation and adhesion of leucocytes, platelets activation and endothelial cells (e.g integrins, selectins and cadherins) (Ruoslahti and Pierschbacher., 1987, Frenette, 2004). This process normally occurs in virtually all organs but most common in the bone marrow, leading to a bone marrow infarction either in the medullary cavity or epiphyses due to marrow hyper-cellularity (Kim and Miller, 2002).

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There are various conditions that predispose to vaso-occlusive crisis. Dehydration which is one of the causes is mostly due to a reduced ability of the sickle cell patients to conserve water secondary to defect in renal concentration ability (Yale et al., 2000). These patients are therefore advised to always wear warm clothes during cold weather, take in adequate amounts of fluids during hot weather and if possible avoid strenuous exercises that can cause fatigue and dehydration (Yale et al., 2000). Similarly, certain adhesion molecules, for example, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, facilitate binding of sickle red blood cells and white blood cells to the vascular endothelium, may also play a major role in the development of vaso-occlusion in sickle cell disease patients (Sultana et al., 1998).

Sickle red blood cells are strongly susceptible to endothelium adherence. Although, haemoglobin S is necessary to cause vaso-occlusive episode it is not sufficient enough to initiate the process. The abnormal adhesion of human sickle red cells to vascular endothelium has been shown as a potential initiating factor in vascular obstruction in sickle cell disease (Kaul et al., 2009). Vaso-occlusion crisis normally occur when the sickle-shaped red blood cells are trapped in the small capillaries and subsequently decrease the flow of the blood. The trapping of these rigid sickle red blood cells in the capillaries leads to intravascular haemolysis resulting in anaemia. Vascular occlusion is the result of a dynamic interaction between erythrocytes and the vascular endothelium, resulting in microvascular occlusion and ischaemia, followed by restoration of blood flow, which further promotes tissue injury mediated by reperfusion. These cycles of ischaemia and reperfusion cause oxidant stress, with activation of vascular oxidases (Wood et al., 2005) and inflammatory stress, increasing expression of endothelial cell-adhesion molecules, increasing synthesis of inflammatory cytokines, and can cause leucocytosis (Belcher et al.,

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2005). Highly vascularised organs like the bone, the penis, the kidney and the lung are the main areas of disseminated vascular occlusions with a restricted blood flow causing ischaemia, pain, necrosis and often damage to the organ (Nath and Katusic, 2012).

**Renal and hepatic dysfunction in SCD**

Renal damage is almost inevitable in sickle-cell disease. There is a strong tendency for haemoglobin S to polymerise in the renal medulla, because of the low partial pressure of oxygen, the low pH, and the high osmolality causing erythrocyte dehydration. The consequent vaso-occlusion causes renal infarction with papillary necrosis, and medullary fibrosis with focal segmental glomerulosclerosis. Glomerular hyperfiltration and tubular dysfunction also occur, and are possibly associated with anaemia and increased sensitivity to prostaglandins (Scheinman, 2008). Renal dysfunction is apparent from an early age in patients with sickle-cell anaemia, with glomerular hyperfiltration apparent at 13 months (Ware et al., 2010). Microalbuminuria is common in childhood and up to 20% of adults develop nephrotic-range protein loss, with more than 3·5g proteinuria in 24 hours (Scheinman, 2008). Thirty percent (30%) of adults develop chronic renal failure, which is a contributory factor in many deaths. Other renal manifestations include haematuria, renal medullary carcinoma, and nocturnal enuresis (Scheinman, 2008).

Hepatic dysfunction is a commonly recognized complication of sickle cell disease (SCD) due to multiple factors such as intrahepatic sinusoidal sickling, bilirubin gallstones, transfusion-related hepatitis infections or excess iron deposition (Kakarala and Lindberg, 2004). Clinical evidence of hepatic dysfunction in patients with SCD was explained by trapping of sickled cells during passage through the hepatic sinusoids which are engulfed by phagocytes causing hepatomegaly

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(Beutler and Williams, 2001). Sludging and congestion of vascular beds were suggested to be the main cause of tissue ischaemia and infarction (Altintas et al., 2003). Bauer and his colleagues (1980) in their retrospective study including autopsy findings in seventy (70) sickle cell disease patients found that the spectrum of liver disease appears to be the consequence of repeated vaso-occlusive episodes. There are other studies suggesting that the main causes of liver injury in sickle cell disease patients are due to factors other than intrahepatic sickling, which was considered to be reversible, such as viral hepatitis or transfusional iron overload (Omata et al., 1986).

### *2.5.2 Coagulation*

Coagulation protects mammals from excessive loss of blood and fluids by sealing the sites of injury and restoring vascular integrity. The processes of clot formation and fibrinolysis involve highly complex series of plasma proteins in a cascade of enzyme-catalyzed reactions each of which converts an inactive zymogen precursor into an active enzyme leading to the formation of a fibrin clot (Smith, 2009). Coagulation is a complex process by which blood forms clots. It is highly conserved throughout biology and in all mammals; it involves both a cellular (platelet) and a protein (coagulation factor) component. Coagulation begins almost instantly after an injury to the blood vessel causing damaged the endothelium. Exposure of the blood to proteins such as tissue factor initiates changes to blood platelets and the plasma protein fibrinogen, a clotting factor. Platelets immediately form a plug at the site of injury; this is called primary haemostasis. Secondary haemostasis occurs simultaneously: Proteins in the plasma, called coagulation factors or clotting factors, respond in a complex cascade to form fibrin strands, which strengthen the platelet plug (Furie and Furie, 2005).

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The theory on blood coagulation has been in existence since ancient time. Johannes Müller (1801-1858) a physiologist had described fibrin (activated by fibrinogen) as the substance of a thrombus. The enzymatic process involved in the conversion of fibrinogen to fibrin through enzymes thrombin and its precursor prothrombin was first hypothesized by Alexander Schmidt (Schmidt, 1872), and the theory that thrombin is generated by the presence of tissue factor was consolidated by Paul Morawitz in 1905 (Morawitz, 1905). As early as 1890, Arthus discovered that calcium was essential in coagulation (Shapiro, 2003) and at this stage, it was known that thromboplastin (factor III) is released by damaged tissues, reacting with prothrombin (factor II), which, together with calcium (factor IV), forms thrombin, which converts fibrinogen (factor I) into fibrin (Giangrande, 2003).

Coagulation process that leads to haemostasis involves a complex set of reactions that convert fibrinogen to fibrin, which, together with platelets, form a stable thrombin. Several coagulation cascade models have been proposed, including the intrinsic and extrinsic pathway model and currently the cell-based model which describes a three phase step-wise process; initiation, amplification and propagation (Hoffman and Monroe, 2007). The tissue factor (TF) pathway of coagulation is the major physiological route by which thrombin generation is initiated in response to damage to the blood vessel. During this vessel blood injury, inactivated factor VII (FVII) leaves the circulation and comes into contact with the tissue factor expressed on tissue-factor-bearing cells forming an activated complex (TF/FVIIa) leading to initiation of the blood coagulation *in vivo* (Mackman, 2006, Monroe and Key, 2007).

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Activated factor X (FXa) then associates with FVa to form prothrombinase complexes on the tissue-factor-bearing cells after the TF/FVIIa/ complex activates small amounts of factor IX and factor X (Monroe et al., 1996). When an injury has occurred and platelets have adhered near the site of the TF-bearing cells, the activated IX can diffuse to the surface of nearby activated platelets, which can then bind to a specific platelet surface receptor, interact with its cofactor (FVIIIa), and activate FX directly on the platelet surface (Rawala-Sheikh et al., 1992). The large burst of thrombin required for effective haemostasis is formed on the platelet surface during the propagation phase.

The platelet-bound FXIa can activate more FIX to IXa. Once the platelet "tenase" complex is assembled, FX from the plasma is activated to FXa on the platelet surface. FXa and its co-factor FVa then associate to form a burst of thrombin generation from prothrombin (factor II); large enough to produce a stable fibrin clot. This large amount of thrombin generated on the platelet surface is responsible for stabilizing the haemostatic clot in more ways than just promoting fibrin polymerization (Hoffman and Monroe, 2007). The platelet-produced thrombin also stabilizes the clot by activating FXIII (Lorand, 2001), activating tissue factor pathway inhibitor (Bajzar et al., 1995), and cleaving the platelet protease-activated receptor-4 (Ofosu, 2003).

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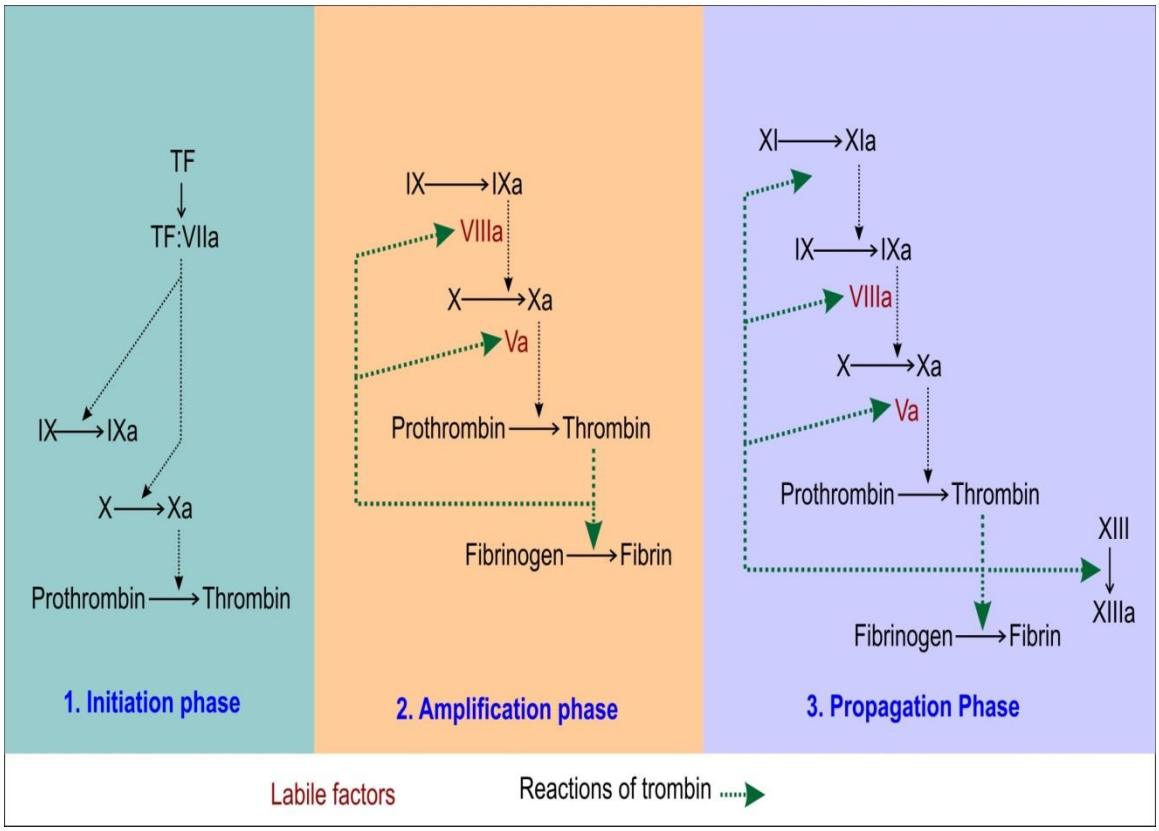


**Figure 3: Steps in a cell-based model of coagulation**

Fibrinogen is a soluble plasma glycoprotein that is transformed into highly self-adhesive fibrin monomers after thrombin cleavage (Blomback et al., 1978). The process of fibrin formation leads to the first step of D-dimer formation in which thrombin cleavage exposes a previously cryptic polymerization site on fibrinogen that promotes the binding of either another fibrinogen or a monomeric fibrin molecule. Fibrin monomers then bind to one another in an overlapping manner to form 2 thick molecules (Doolittle and Pandi, 2007). Plasma remains fluid until 25% to 30% of plasma fibrinogen is cleaved by thrombin, allowing time for fibrin to polymerize while simultaneously promoting thrombin activation of plasma factor XIII (Greenberg et al., 1988). Thrombin remains associated with fibrin, and as additional fibrin molecules polymerize, it activates plasma factor XIII bound to fibrinogen (Meh et al., 1996). The complex between

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soluble fibrin polymers, thrombin, and plasma factor XIII promotes the formation of factor XIIIa before a fibrin gel is detected (Greenberg et al., 1988).

#### *2.5.2.1 Hypercoagulability*

Historically, the pathophysiology of the clinical complications of sickle cell disease was attributed solely to the polymerization of the red blood cells into a sickle or crescent shape in the microcirculation (Bunn, 1997). However, several studies have now proved that various complex mechanisms such as hypercoagulability contribute considerably to the disease pathology. Enhanced platelet function (Foulon et al., 1993), activation of the coagulation cascade (Westerman et al., 2008), and impaired fibrinolysis are some of the mechanisms of the hypercoagulability in sickle cell disease (Ataga and Orringer, 2003). Almost every component of haemostasis, such as platelet function, the procoagulant, anticoagulant, and fibrinolytic systems is altered in sickle cell anaemia (Ataga and Key, 2007b), hence sickle cell anaemia is mostly referred to as a "hypercoagulable state (Francis, 1991b). These changes are partly due to alterations in red blood cell of the sickled structure resulting in intravascular haemolysis and externalization of highly procoagulant phosphatidyl serines on the red blood cell membrane (Chiu et al., 1981).

Phosphatidyl serine is normally found in the inner monolayer of the red blood cell membrane, whilst choline-containing phospholipids such as sphingomyelin and phosphatidylcholine are found in the outer monolayer in the plasma membrane (Devaux and Zachowski, 1994). The action of ATP dependent aminophospholipid translocase (or flipase) usually maintains the red blood cell membrane phospholipid asymmetry which transports phosphatidylserine and

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phosphatidylethanolamine from the outer membrane to the surface of the inner membrane (Devaux and Zachowski, 1994). Additionally, the activation of scramblase by an inward flow of extracellular calcium causes the movement of all phospholipids in both directions, resulting in rapid Phosphatidylserine exposure (Zwaal and Schroit, 1997). This abnormal exposure of the phosphatidylserine in sickle red blood cells may occur due to repeated cycles of sickling and un-sickling linked to polymerization and depolymerization of haemoglobin S that results in the production of terminal spicules or microvesicles with exposed Phosphatidylserine (Allan et al., 1982).

Reduced flipase activity in the red blood cell due to oxidative stress and sulphydryl modification may also results in the abnormal exposure of the Phosphatidylserine (Jong and Kuypers, 2006). The abnormal exposure of this Phosphatidylserine therefore functions as both a recognition signal for cell removal during apoptosis of nucleated cells (Fadok et al., 1992), and also a docking site for enzymatic complexes involved in coagulation and anticoagulation pathways (Zwaal and Schroit, 1997). The external exposure of the phosphatidylserine then alters the adhesive properties of the sickle red blood cell (Setty et al., 2002), and appears to be involved in the haemostatic changes which is observed in sickle cell disease (Setty et al., 2000).

#### *2.5.2.2 Coagulation Abnormalities in SCA*

A study conducted on abnormalities of coagulation in sickle cell anaemia individuals revealed that prothrombin time and activated partial thromboplastin time were either normal or prolonged in patients with sickle cell anaemia while there was a decrease in thrombin time (Nsiri et al., 1996a). The shortened levels of the thrombin time may be due to the presence of circulating

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activated clotting factors which enhance intravascular thrombin generation (Nsiri et al., 1996a). A high plasma concentration of fibrinopeptide A, thrombin-antithrombin III complexes, and prothrombin fragment 1+2 are reported evidence for increased thrombin generation in sickle cell anaemia (Francis, 1991a). However, Chinawa et al later reported prolonged levels of prothrombin time and activated partial thromboplastin time in sickle cell anaemia individuals in steady state and even more prolonged in individuals during crisis (Chinawa et al., 2013). Similarly, Olayemi et al also reported that the presence of lupus anticoagulant may lead to prolongation of some clotting factors such as prothrombin time, activated partial thromboplastin time and kaolin clotting time (Olayemi and Bazuaye, 2007)

Decreased levels of natural anticoagulant proteins are also observed in sickle cell anaemia. Levels of protein C and protein S are decreased in patients with sickle cell anaemia (Westerman et al., 1999). The reduced levels of these anticoagulant proteins may be the result of chronic consumption because of an increase in the tissue factor expression and thrombin generation and/or hepatic dysfunction (Bayazit and Kilinc, 2001). Significantly, decreased levels of these two anticoagulant proteins have been found in SCA patients who developed thrombotic strokes compared with neurologically normal SCA children (Tam, 1997). Heparin co-factor II (HCII), a circulating inhibitor of thrombin via a target enzyme specificity, which has similar properties to antithrombin, is also decreased in both SCA (Porter et al., 1993). Heparin co-factor II levels have been reported to increase following chronic blood transfusions in these patients. However, it is uncertain whether HCII deficiency is a risk factor for thrombosis (O'Driscoll et al., 1995).

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Patients with sickle cell anaemia appear to be more resistant to activated protein C than the healthy control subjects (Wright et al., 1997). This may result from an increase in circulating plasma levels of factor VIII coagulant activity, perhaps coupled with the reduction in both total and free protein S that is observed in these individuals. However, mutations in the factor V gene (G1691A or factor V Leiden) and the prothrombin gene (G20210A) are quite low in individuals of African descent (Andrade et al., 1998). Despite reports that they do not appear to be associated with thrombotic complications in patients with SCD, the low frequency of these two alleles makes it difficult to determine their possible contribution to the development of thrombotic complications in these patients (Andrade et al., 1998).

The D-dimer antigen is a unique marker of fibrin degradation that is formed by the sequential action of 3 enzymes: thrombin, factor XIIIa, and plasmin. First, thrombin cleaves fibrinogen producing fibrin monomers, which polymerize and serve as a template for factor XIIIa and plasmin formation. Secondly, the thrombin activates plasma factor XIII bound to fibrin polymers to produce the active trans-glutaminase, factor XIIIa (Westerman et al., 2002). Factor XIIIa catalyzes the formation of covalent bonds between D-domains in the polymerized fibrin. Finally, plasmin degrades the cross-linked fibrin to release fibrin degradation products and expose the D-dimer antigen. D-dimer antigen can exist on fibrin degradation products derived from soluble fibrin before its incorporation into a fibrin gel or after the fibrin clot has been degraded by plasmin. The clinical utility of D-dimer measurement has been established in some scenarios, most notably for the exclusion of venous thromboembolism (VTE) (Westerman et al., 2002).

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D-dimer assays may be used in the initial evaluation of patients suspected of having venous thromboembolism, because the exclusion of VTE cannot be made on clinical grounds alone (Wells, 2007b). The fact that only a small portion of circulating fibrinogen needs to be converted to cross-linked fibrin to generate a detectable D-dimer antigen signal after plasmin digestion in plasma confers the sensitivity required (Elms et al., 1983). Thus, a normal D-dimer in the appropriate clinical context denotes that there is no major on-going activation of intravascular coagulation, and serves as a reliable tool for the exclusion of VTE. In a large meta-analysis, Stein et al (2004) demonstrated that a negative D-dimer test by the rapid ELISA method is as diagnostically useful as a negative computed tomography (CT) or a negative compression ultrasonography study (CUS) in excluding pulmonary embolism (PE) and deep vein thrombosis (DVT), respectively (Stein et al., 2004b). However, recent surveys indicate that D-dimer assays are often not used appropriately for the exclusion of VTE in clinical practice (Arnason et al., 2007).

Data from a case-control study of 1070 black patients and a retrospective cohort study of 65,000 consecutive hospitalizations of black men suggest that individuals with sickle cell trait have higher rates of venous thromboembolic events (deep vein thrombosis or pulmonary embolism) compared with similar blacks with normal haemoglobin (Austin et al., 2007). In this study, persons with sickle cell trait had approximately a 4-fold increased risk for pulmonary embolism (odds ratio=3.9; 95% CI= 2.2-6.9) and approximately a 2-fold (odds ratio 1.8; 95% CI, 1.2-2.9) risk of combined deep vein thrombosis or pulmonary embolism (Austin et al., 2007). Compared with matched controls with normal haemoglobin, individuals with sickle cell trait have increases in the measures of coagulation activity (Westerman et al., 2002). These individuals with sickle

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cell trait had significantly higher levels of d-dimers, thrombin-antithrombin complexes, and prothrombin fragment 1.2, and their absolute blood monocyte levels were also increased (Rees et al., 2010).

#### *2.5.2.3 Coagulation in Leg Ulcer*

The formation of leg ulcer is attributed to many factors, including some abnormalities in coagulation pathways, such as high levels of Plasminogen Activated Iinhibitors-1 activity (Zollner et al., 1997), positive lupus anticoagulant (Goto et al., 2011) and platelet hyper-aggregability (Higgins et al., 1989). An in vivo chronic activation of blood coagulation is associated with leg ulcer as a clinical equivalent of end organ damage (Cacciola et al., 1990). Hypercoagulable disorders may be the cause of chronic leg ulcers, either indirectly as a consequence of venous thrombosis, or directly by thrombus formation in small arteries, arterioles, capillaries or venules (Marechal et al., 1999). Antiphospholipid syndrome, deficiency of antithrombin III, proteins C, protein S, and some abnormal clotting factors (Maessen-Visch et al., 1999), are a number of growing conditions predisposing sickle cell anaemia patients to thrombosis (Mekkes et al., 2003).

D-dimer is normally estimated to ascertain to what extent fibrin formation has been initiated or to find out whether there is any change in this process in the course of a specific therapy, inflammation or disease process (Rathbun et al., 2004). The measurement of D-dimer has been most comprehensively validated in the diagnosis and monitoring of coagulation activation in disseminated intravascular coagulation (DIC), the exclusion of venous thromboembolism in certain patient populations, and the initial evaluation of patients suspected of having deep vein thrombosis and/or pulmonary embolism (Wells, 2007a). A normal level of plasma D-dimer

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shows that there is no major on-going activation of intravascular coagulation which therefore, serves as a reliable tool for the exclusion of venous thromboembolism (Stein et al., 2004a). It has been demonstrated that a negative D-dimer test is as diagnostically useful as a negative computed tomography in excluding pulmonary embolism or a negative compression ultrasonography study for excluding deep vein thrombosis (Arnason et al., 2007).

**Figure 4: Leg Ulcer caused by Lupus Anticoagulant (Mekkes et al., 2003).**

### 2.6 Management of Sickle Cell Anaemia

Pain is an unpredictable hallmark of sickle cell disease presenting either in an acute or chronic form (Raphael and Oyeku, 2013). Despite clinical innovations in medical care and improved survival among individuals, pain is still the commonest cause for hospitalization which occurs with varying degrees of severity in different patients and its consequences are substantial, affecting both the individual and the health care system (Quinn et al., 2010). The management of sickle cell disease continues to pose a real challenge for patients, families, clinicians, scientists, and policy makers (Raphael and Oyeku, 2013). Unfortunately, there is still no cure for sickle cell

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anaemia individuals except only few selected children using stem cell transplantation (Walters et al., 1996, Gore et al., 2000).

A major worldwide therapeutic approach to sickle cell anemia has been to try to shift haemoglobin production from sickle haemoglobin to fetal hemoglobin, by changing marrow-proliferation kinetics to favor F-cell production (Platt, 2008). Induction of HbF has the ability to inhibit HbS polymerization and has therefore, for some years now become the main source of management for sickle cell anaemia patients (Poillon et al., 1993). Hydroxyurea (hydroxycarbamide), decitibine, arginine butyrate, and histone deacetylase inhibitors are the classes of HbF induction agents (Steinberg, 2008a). Hydroxyurea, a ribonucleotide reductase inhibitor, with its ability to induce high levels of HbF is the sole drug with widespread regulatory approval for the treatment of sickle cell anaemia (Charache et al., 1995). If hydroxyurea is taken correctly, it can induce higher HbF levels in most sickle cell anaemia patients (Brawley et al., 2008), and therefore reduced the frequency of hospitalization and the incidence of acute painful events, acute chest syndrome, and blood transfusion by more than forty percent (40%) (Charache et al., 1995). Blood transfusion is paramount to the management of sickle cell disease patients, to the extent that some institutions provide specialized blood-banking services to their sickle cell disease patients, unfortunately, the availability and safety of blood are the major challenges to healthcare providers especially in developing countries (Ansong et al., 2013).

Prompt diagnosis and early treatment of malaria is recommended by World Health Organization (WHO) Roll Back Malaria programme as a standard practice for the management of sickle cell disease patient, however, anti-malaria prophylaxis is currently not being encouraged in the routine management of sickle cell disease patients (Makani et al., 2010). Sickle cell anaemia

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patients especially children are particularly prone to infection by encapsulated microorganisms such as Streptococcus pneumoniae, Haemophilus influenza B and Salmonella typhi as well as intracellular organisms like Mycoplasma pneumonia (Ramakrishnan et al., 2010).

### 2.7 Management of Leg Ulcer

Approximately, fifty percent (50%) of all patients with leg ulcer undertake self-treatment (Nelzen and Bergqvist, 1995). Treatment of leg ulcer needs a multidisciplinary approach with different medical specialists such as dermatologists, haematologist, plastic surgeons and vascular surgeons, increased healing rates with reduced recurrence has also been demonstrated by well trained nurses and other health-care providers. In some cases, primary care doctors and community nurses also provide assistance (Kesson, 1995).

Sickle cell anaemia patients with leg ulcers have a wide range of possible treatments. Many of these treatments have been considered as intervention cure among individuals with leg or foot ulcers resulting from other pathological causes. Examples of these treatments are silver-based wound dressing and topical agents for treating diabetic foot ulcers (O'Meara et al., 2013), the diabetic foot ulcers debridement (Edwards and Stapley, 2010), oral zinc for arterial and venous leg ulcers (Wilkinson and Hawke, 1998) and venous leg ulcers compression (O'Meara et al., 2009). Other factors which also need to be considered to ensure successful chronic wound healing is underlying factors such as disease, drug therapy and patient circumstance before even a particular wound dressing is applied (Boateng et al., 2008). Leg ulcers tend to be indolent and intractable, healing slowly over months or years (Minniti et al., 2010b), even with rigorous conventional care (occlusive dressing and debridement and cleansing) (Smith et al., 2011).

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Healing of chronic leg ulcer can also be delayed due to excessive production of exudates that can cause maceration of healthy skin tissue around the wound (Cutting and White, 2002). This exudate sometimes accompanies with bad smells which impact a negative social life on the sickle cell patients (Hareendran et al., 2005).

A fundamental issue which applies to any chronic ulcer, notwithstanding the site of the ulcer, is the optimum preparation of the appropriate environment for the ulcer (ulcer bed) (Velasco, 2011). Tissue removal, infection control, and moisture balance, are all aspects that need to be analyzed in the management of any chronic ulcer, which help in making appropriate decisions and preparing the ulcer bed for healing (Schultz et al., 2003). The healing of any chronic ulcer also involves a complex interaction between epidermal and dermal cells, the extracellular matrix, controlled angiogenesis, and plasma derived proteins which are coordinated by an array of cytokines and growth factors (Harding et al., 2002).

The average duration of a sickle cell leg ulcer in Ghana is three and half (3.6) years (Ankra-Badu, 1992) .This is similar in the United State, which has been reported to exceed three years with recurrence rates ranging from 25% to 52% (Koshy et al., 1989). The increased disability, reduced quality of life, absence from work and even sometimes from school, and high utilization of health care resources can severely affect the lives of sickle cell individuals with chronic leg ulcers (Halabi-Tawil et al., 2008).

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### 2.8 Prevention of SCD

There is a strong advocacy for sickle cell screening in Africa. Currently, there are cheap and simple methods for testing and diagnosing adults and newborns. New knowledge of risk factor of the disease allows a range of options, such as reduction of family size, ensuring that infants who are at risk are screened at birth, and also requesting for prenatal diagnosis and counselling (Modell and Darlison, 2008).

To prevent sickle cell disease, it is important to educate carriers (AS individuals) who are usually asymptomatic about their status to try to prevent the future birth of sickle cell anaemia children, as well as to avoid psychosocial issues such as misconceptions or stigmatisation (Dyson and Atkin, 2012). Population screening, especially the offer of testing in high school (Mitchell et al., 1996) and before marriage allows a wide range of choices and requires a minimum number of laboratory tests (Samavat and Modell, 2004).

All young people of marriageable age should be properly counselled and go through laboratory screening for Hb genotype (Akinyanju, 2010). This counselling should include discussions of contraception, complications related to pregnancy, and the importance of maintaining good health habits. Similarly, both parents, as they plan their pregnancy, should receive genetic counselling to determine their chances of having a child with sickle cell anaemia. This can easily be achieved after each partner has been tested for Hb genotype (Dauphin-McKenzie et al., 2006). It is also important that those individuals who carry the sickle cell trait become knowledgeable of their carrier status and be educated on how they can potentially pass the trait or the disease onto their offspring (Creary et al., 2007a).

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# CHAPTER THREE

# 3.0 SUBJECTS AND METHODS

## 3.1 Study design

The study design for this project was cross-sectional, involving patients who met the eligibility criteria.

## 3.2 Setting

The study was conducted at the Ghana Institute of Clinical Genetics, Korle Bu, Ghana which is a referral health facility and receives patients with sickle cell disease from across the country. The Institute was established in 1974, funded by the Ministry of Health and the Managing Trustees of Volta Aluminium Company Limited (VALCO). The adult sickle cell clinic is held every working day (Monday to Friday), it attends to patients' age 12 years and above. Average daily attendance is fifty (50). The institute has registered 24,495 patients as at the end of 2010.

## 3.3 Study subjects

Subjects for this study were both male and female sickle cell anaemia (SCA) patients who had already been diagnosed as Hb SS with Hb Electrophoresis aged 12 years and above and in a steady state with and without leg ulcers. The control subjects were age matched healthy individuals with haemoglobin AA genotypes who were recruited from voluntary blood donors at the Accra Area Blood Transfusion Centre of the National Blood Service, Ghana. Equal numbers were recruited (from May 2013 – August 2013) for the study.

Written informed consent was sought from the subject's or guardians/parents. Approval for this study was sought from the University of Ghana Medical School (College of Health Sciences) Ethical and Protocol Review Committee.

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### 3.4 Eligibility Criteria

| *3.4.1 Inclusion Criteria* | *3.4.2 Exclusion Criteria* |
|---|---|
| All consenting adult (≥ 12 years) sickle cell anaemia patients with chronic leg ulcer and those without leg ulcer in a steady state<br><br>Subjects with haemoglobin AA genotype as controls. | Patients on anticoagulant medication, oral contraceptives, aspirin and pregnancy. |

### 3.5 Sample size and power calculation

The formula below gives the computation of minimum sample size required to achieve the needed effect size of 1-β (power of 80%) at two-sided 95% significant level (α=0.05), z is the standard score for confidence level, with the mean levels of the analyte being assayed between SCD patients and controls as $\mu_1$ and $\mu_0$ respectively, and a standard deviation of σ.

$$n = \frac{2\left(z_{1-\alpha/2} + z_{1-\beta}\right)^2}{\left(\frac{\mu_0 - \mu_1}{\sigma}\right)^2}$$

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| Parameter | Hypothesized effect | α | Power | n |
|---|---|---|---|---|
| d-dimer | 0.92 | 0.05 | 80 | 25 |
| Fibrinogen | 0.80 | 0.05 | 80 | 45 |

### 3.6 Methods for Data and Sample Collection, Processing and Analysis

#### *3.6.1 Data and Blood Sample Collection*

After a voluntary informed consent was obtained from the participants, a questionnaire (Appendix A) was then administered for demographic data such as age and sex. The following relevant information were also obtained and recorded on the data sheet as shown in appendix A; duration of the ulcer, the number of times the ulcer reoccurred and frequency of dressing the wound. Additionally, other clinical information relevant to the study was also taken from the patient's folder.

A 7.5ml venous blood sample (well taken with minimum of stasis) was collected from the antecubital fossa area of each participant and divided into two (2) portions; 4.5ml into 0.5ml of 0.109M trisodium citrate tube (9 part of the blood to 1part of the citrate) for coagulation analysis and 3.0ml into ethylenediamine tetra-acetic acid (EDTA) tube. Samples in the EDTA tubes were used for Full Blood Count (FBC) using the principles of ABX Micros ES60 OT/CT; a 3-part automated haematology analyser, qualitative sickle slide test and Hb Electrophoresis using Cellulose Acetate Method.

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Blood samples were collected from hundred (100) sickle cell anaemia patients at the Ghana Institute of Clinical Genetics, Korle-Bu and forty five (45) control blood samples from healthy voluntary blood donors at the Accra Area Blood Centre of the National Blood Service, Ghana.

A volume of 4.5ml of the venous blood (well taken with minimum of stasis) was dispensed into a plastic tube containing 0.5ml of the 0.109M tri-sodium citrate tube and mixed gently. This gives a concentration of 1:10 dilution; i.e. nine (9) part of blood to one (1) part of the citrate. These samples in the tri-sodium citrate tubes were centrifuged at 3000g for 15 minutes (to obtain platelet poor plasma) without delay. For the purpose of the study, gender, study Identification Number (ID), Pathological Number, date of sample collection and time of sample collection were used both on the sample bottle and the patient designed form. The plasma samples were aliquoted into eppendorf tubes and stored immediately at -80℃ for the coagulation assays

The details procedure for the blood sample collection and processing is shown in appendix B.

### 3.6.2 Blood Sample Analysis

#### *3. 6.2.1 Plasma Analysis*

The plasma samples after removing from the freezer at -80$^{o}$C were allowed to thaw at room temperature and incubated at 37$^{o}$C prior to testing. All the procedures were performed following the manufacturer's instructions. The reagents and equipment were Helena Biosciences Products from the United Kingdom as shown in appendix C. HumaClot Duo$^{plus}$ is a 2-channel photo-optical semi-autoanalyser instrument that offers clotting, chromogenic and immunoturbidimetric testing capabilities which was used to analyse all the plasma samples.

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#### *3.6.2.1.1 Prothrombin Time (PT)*

The prothrombin time test is defined as the coagulation time measured in seconds of a mixture of platelet-poor plasma, tissue factor (thromboplastin) and calcium chloride as a means to investigate coagulopathy. Prolongation of the test indicates either congenital or acquired disorder that affects extrinsic coagulation factors; FI, FII, FV, FVII and FX.

**Principle**

The PT test measures the clotting time of recalcified plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and calcium. Tissue thromboplastin, in the presence of calcium ions, is an activator which initiates the extrinsic pathway of coagulation. When a mixture of tissue thromboplastin and calcium ions is added to normal citrated plasma, the clotting mechanism is activated, leading to a fibrin clot. If a deficiency exists within the extrinsic pathway, the time required for clot formation will be prolonged depending on the severity of the deficiency. The details of the procedure is shown in appendix D

#### *3.6.2.1.2 Activated Partial Thromboplastin Time (APTT)*

The APTT Test is used to detect the disorders of the intrinsic coagulation pathway. Apart from FVII, APTT test is sensitive to the deficiency of all plasma clotting factors; however, it is mainly used to detect deficiencies in Factors VIII, IX, XI, XII, High Molecular Weight Kininogen (HMWK) and Prekallikrein (Fretcher Factor).

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**Principle**

APTT measures the clotting time of plasma after the activation of contact factors and this is done by the addition of the APTT reagent to the test sample. The APTT reagent contains a plasma activator and a phospholipid in which the phospholipid serves as a substitute for platelets. The mixture is incubated for 3 minutes at 37$^{o}$C for activation to occur. This is then followed by the addition of a pre-warmed (at 37$^{o}$C) 0.025M calcium chloride and clot formation is timed. The test procedure is shown in appendix E.

***3.6.2.1.3 Thrombin Time (TT)***

The Thrombin Test is sensitive to the concentration and reaction of fibrinogen and by the presence of inhibitory substances, including fibrinogen/fibrin degradation products (FDPs). The clotting time and the appearance of the clot are equally informative. The thrombin time test was performed as part of the Clauss method for the determination of fibrinogen.

**Principle**

The thrombin time test is a simple test to screen for conditions that can interfere with the conversion of fibrinogen to fibrin. Bovine thrombin is added to undiluted plasma and the clotting time is measured. The procedure of the test is shown in appendix F.

***3.6.2.1.4 Fibrinogen Quantitation by Clauss***

Clauss Fibrinogen 100 is intended for the quantitative determination of human plasma fibrinogen. Fibrinogen is a plasma protein which is converted from a soluble to an insoluble polymer by the action of thrombin, resulting in the formation of a fibrin clot. The determination of plasma fibrinogen for diagnosis, treatment monitoring and prognosis of various haemorrhagic

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disorders is widely accepted as a useful test. Levels of fibrinogen can be increased as a result of inflammation, pregnancy or oral contraceptive use.

**Principle**

Thrombin (100 NIH/mL) is added to a 1:10 pre-diluted plasma sample; the plasma must be diluted to give a low level of any inhibitors (e.g. Fibrin Degradation Products and Heparin). The measured clotting time is inversely proportional to the fibrinogen concentration in the sample. A strong thrombin solution must be used so that the clotting time over a wide range is independent of the thrombin concentration. The test procedure is shown in appendix G.

***3.6.2.1.5 D-dimer by Auto blue***

The auto blue d-dimer is an immunoturbidimetric assay used for quantitative determination of the Fibrin Degradation Products (FDPs) that contain D-dimer in human plasma.

**Principle**

Fibrin fragments containing D-dimer antigen are always present as a result of plasmin degradation of Factor XIIIa cross-linked fibrin. Auto blue D-dimer 400 is a test that utilizes antibody coated latex particles. These latex particles are coated with monoclonal antibody that reacts with fibrin D-dimer or fragment D of fibrin. The antibody has no cross reactivity with fibrinogen and therefore allows the measurement of the D-dimer in the plasma sample. In the presence of D-dimer, the particles agglutinate and turbidity increases. The high levels of the turbidity would therefore cause the increase in scattered light which is proportional to the amount of D-dimer in the plasma. The test procedure is shown in appendix H.

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### *3.6.2.2 Whole Blood Analysis*

The whole blood was used to analyse a full blood cell count and sickle slide test. The full blood count includes Haemoglobin levels and platelet counts using the ABX Micros ES60 OT/CT; a 3-part auto analyser. The ABX Micros ES60 OT/CT is a full automated haematology analyser used for blood cell count. Its performance doesn't require any manual operations for aspirating, dilutions, measuring, and calculations of the blood cells. The cells are measured by an electronic impedance variation principle. The principle of the analyser is shown in appendix J. Sickle slide test for detecting the presence of Haemoglobin S depends on the decreased solubility of the haemoglobin at low oxygen tensions. It does not however, differentiate between a sickle cell disease and a sickle cell trait

**Principle of Sickle Test**

Under low oxygen tension, red blood cells containing haemoglobin S assume the characteristic sickle shape. Two percent (2%) Sodium Metabisulphite is a reducing agent which reduces oxygen tension. An equal volume of 2% Sodium Metabisulphite solution is added to a whole blood and the mixture is covered with a cover slide and incubated at room temperature for a minimum of one (1) hour. The reducing agent deoxygenates the haemoglobin in the red blood cells providing the conditions for the cells containing HbS to sickle when viewed under the microscope. The procedure of the test is shown in appendix K.

### *3.6.2.3 Haemoglobin Electrophoresis*

Haemoglobin electrophoresis is used to separate and identify the different haemoglobins by their mobility within an electric field. Haemoglobin variants are separated at different levels due to differences in their surface electrical charge as determined by their amino acid structure.

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Cellulose Acetate Paper Electrophoresis is a simple, reliable and rapid method for the detection of most common clinically important haemoglobin variants

**Principle**

Normal haemoglobin A and the various abnormal haemoglobins separate at different rates on an electrophoretic strip allowing their haemoglobins to be distinguished from each other and to specifically identify. In a high alkaline medium (pH 8.4-8.6), haemoglobin is a negatively charged protein and when subjected to electrophoresis will migrate from the negative electrode (cathode) toward the positively charged electrode (anode). The test procedure is shown in appendix L.

### 3.7 Statistical Analysis

The data was summarised in tabular form as means ± standard deviation for continuous variables and percentages for categorical variables. The mean difference across various categories of subjects variables were analysed using ANOVA, with Bonferroni posthoc analysis for pairwise comparison. The association between clinical parameters of study subjects and coagulation profile parameters were analysed using Spearman's correlation, and depicted graphically using scatter plot. All the analysis was done with Statistical Package Social Sciences (SPSS) software, version 20 (IBM Incorporated). A p-value less than 0.05 was considered statistically significant.

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# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 General characteristics of study subjects

A total of 145 subjects were enrolled into the study; comprising 50 subjects with sickle cell anaemia and chronic leg ulcer (SCA with CLU), 50 subjects with sickle cell anaemia but no leg ulcer (SCA without LU) and 45 subjects with haemoglobin AA genotype (controls: 5 outliers removed because the test results had values way out of what is expected for healthy individuals). The gender distribution, mean age and haemoglobin levels are shown in Table 1. There is significant difference between the gender distributions among the various groups of study subjects; there are more males in the control group than females. With respect to the age distribution, there was also a significant difference in mean ages across various groups. The SCA with CLU subjects had the lowest levels of haemoglobin (7.30 g/dl), followed by SCA without LU (7.99 g/dl), with the controls having the highest (14.01 g/dl). Posthoc (Bonferroni) analysis however showed a significant difference in the mean haemoglobin levels between the SCA with CLU and SCA without LU subjects (p=0.038). Further, posthoc analyses of the mean ages showed no significant difference between SCA with CLU and controls (p=0.989), but the SCA without LU subjects were younger than the SCA with CLU and control subjects (p<0.003).

**Table1. General characteristics of study and leg ulcer subjects**

| Group Characteristics | SCA with CLU | SCA without LU | Controls | P |
|---|---|---|---|---|
| Gender (M/F) | 29/21 | 22/28 | 41/4 | <0.001 |
| Age (years) | 31.26±7.87 | 25.14±10.24 | 33.02±7.91 | <0.001 |
| Haemoglobin(g/dl) | 7.30±1.42 | 7.99±1.39 | 14.01±1.29 | <0.001 |

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The SCA subjects with leg ulcer were aged 14 to 56 years. From the information provided by the subjects, the earliest age at which ulceration of the leg occurred in this study population was 8 years and the latest time at which one had the ulceration was 49 years. The mean age, however, of the subjects with leg ulcers was 22 years. Furthermore, the majority (greater than 60%) of these subjects first suffered ulceration of the leg after the age of 20 years. The frequency seemingly increasing with increasing in age.

### 4.2 Coagulation profile of study subjects

Table 2 shows the mean platelets count, Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) for the various categories of study subjects analysed using ANOVA. There was a significant difference in the mean values for Platelet count, APTT and PT among the study groups ($P < 0.01$). Posthoc (Bonferroni) analyses among the groups showed significant differences in the mean values between the study groups: SCA subjects with CLU subjects had a significantly shortened APTT than those without LU ($p=0.035$) and the control subjects ($p = 0.009$). However, there was no significant difference observed between the SCA subjects without LU and Control subjects ($p = 0.605$). With respect to Platelet counts, there was no significant difference in the mean counts among SCA with CLU and SCA without LU ($p = 0.206$), however, both had significantly higher mean counts compared to the Controls ($p<0.001$).

**Table2. Coagulation markers of study subjects**

| Parameter | SCA with CLU | SCA without LU | Control | P-value | Ref. Range |
|---|---|---|---|---|---|
| Platelet ($\times 10^9$/L) | 478.12±177.32 | 424.20±169.08 | 226.28±53.18 | <0.001 | 150 – 400 |
| PT (Sec) | 16.02±2.57 | 16.04±1.68 | 14.91±1.24 | 0.006 | 12 – 16 |
| APTT (Sec) | 31.27±6.16 | 34.51±6.80 | 35.20±6.06 | 0.006 | 26 – 36 |

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There was also a significant difference observed among the groups with respect to the mean Prothrombin Time (p=0.006). Posthoc (Bonferroni) analyses showed no difference amongst the SCA subjects with CLU and those without LU (p=1.000). There was however, significant differences in the mean Prothrombin Time between SCA with CLU and Control group (p=0.017) and the SCA without LU and Control group (p=0.014)

In Table 3 below, ANOVA analyses showed significant difference in the mean d-dimer levels (p=0.037) but not fibrinogen levels (p=0.127) among the various categories of subjects. Posthoc (Bonferroni) analyses for d-dimer levels showed no significant difference between the levels seen in the SCA subjects without LU and those with CLU (p=0.559) as well as between the SCA subjects without LU and control subjects (p=0.588). However, there was significant difference in the d-dimer levels between SCA subjects with CLU and controls (p=0.032). The ANOVA also did not show any significant difference in fibrinogen levels among the study groups (p=0.127).

**Table3. Levels of fibrinogen and d-dimers among study subjects**

| Characteristics | SCA with CLU (n=50) | SCA without LU (n=50) | Control (n=45) | P-Value | Reference Range |
|---|---|---|---|---|---|
| Fibrinogen (mg/dl) | 314.30±109.83 | 284.90±83.46 | 276.89±88.02 | 0.127 | 150 – 350 |
| D-dimer (µg/ml) | 1.56±2.90 | 1.04±1.56 | 0.52±0.46 | 0.037 | 0 – 0.5 |

Data presented as mean ± standard deviation.

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The association between the characteristics of the study subjects and coagulation markers are shown in Table 4. The frequency of recurrence of the leg ulcers correlated negatively with the platelet counts (r= -0.311, p=0.028). The correlation also indicates that the duration of the leg ulcer reduced with higher haemoglobin levels (r= -0.331, p=0.021). There was a positive correlation between age and duration of the leg ulcer (r=0.351, p=0.015).

**Table 4. Association between patients' characteristics and coagulation profile**

| Parameter | | Age | Duration of LU | Frequency of LU | Hb | Platelet |
|---|---|---|---|---|---|---|
| Age | r | 1.000 | 0.351* | 0.108 | 0.182* | -0.349** |
| | p | | 0.015 | 0.455 | 0.028 | 0.000 |
| Hb | r | 0.182* | -0.331* | -0.204 | 1.000 | -0.664** |
| | p | 0.028 | 0.021 | 0.156 | | 0.000 |
| Fibrinogen | r | 0.096 | 0.131 | -0.104 | -0.008 | -0.037 |
| | p | 0.253 | 0.374 | 0.471 | 0.922 | 0.659 |
| D-dimer | r | 0.065 | -0.062 | -0.024 | -0.233** | 0.126 |
| | p | 0.440 | 0.676 | 0.867 | 0.005 | 0.130 |
| PT | r | -0.076 | 0.035 | -0.162 | -0.233** | 0.241** |
| | p | 0.360 | 0.815 | 0.260 | 0.005 | 0.004 |
| APTT | r | -0.096 | -0.181 | -0.086 | 0.185* | -0.120 |
| | p | 0.253 | 0.219 | 0.552 | 0.026 | 0.149 |
| Platelets | r | -0.349** | -0.166 | -0.311* | -0.664** | 1.000 |
| | p | 0.000 | 0.261 | 0.028 | 0.000 | |

r, coefficient of correlation; LU, leg ulcer; Hb, haemoglobin; PT, prothrombin time; APTT, activated partial thromboplastin time, *Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

There was a negative correlation between Hb level and platelet counts (r= -0.664, p<0.001), D-dimer levels (r= -0.233, p=0.005) and the Prothrombin Time (r= -0.233, p=0.005). Prothrombin

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Time correlated positively with platelet counts (r=0.241, p=0.004) among the subjects but particularly among the female subjects (r=0.299, p=0.030) but not the males. Also the Hb of the subjects seem to increase as the APTT approaches normal (r=0.185, p=0.026).

**Sub-group Analysis of the Relationship between platelet, fibrinogen and d-dimer**

Figure 5 below depicts the relationship between platelets and fibrinogen in various study groups. There was no significant correlation between platelet counts and fibrinogen level in the SCA with CLU subjects (r= -0.118, p=0.415), SCA without LU subjects (r= -0.157, p=0.276) as well as control subjects (r= -0.128, p=0.401).

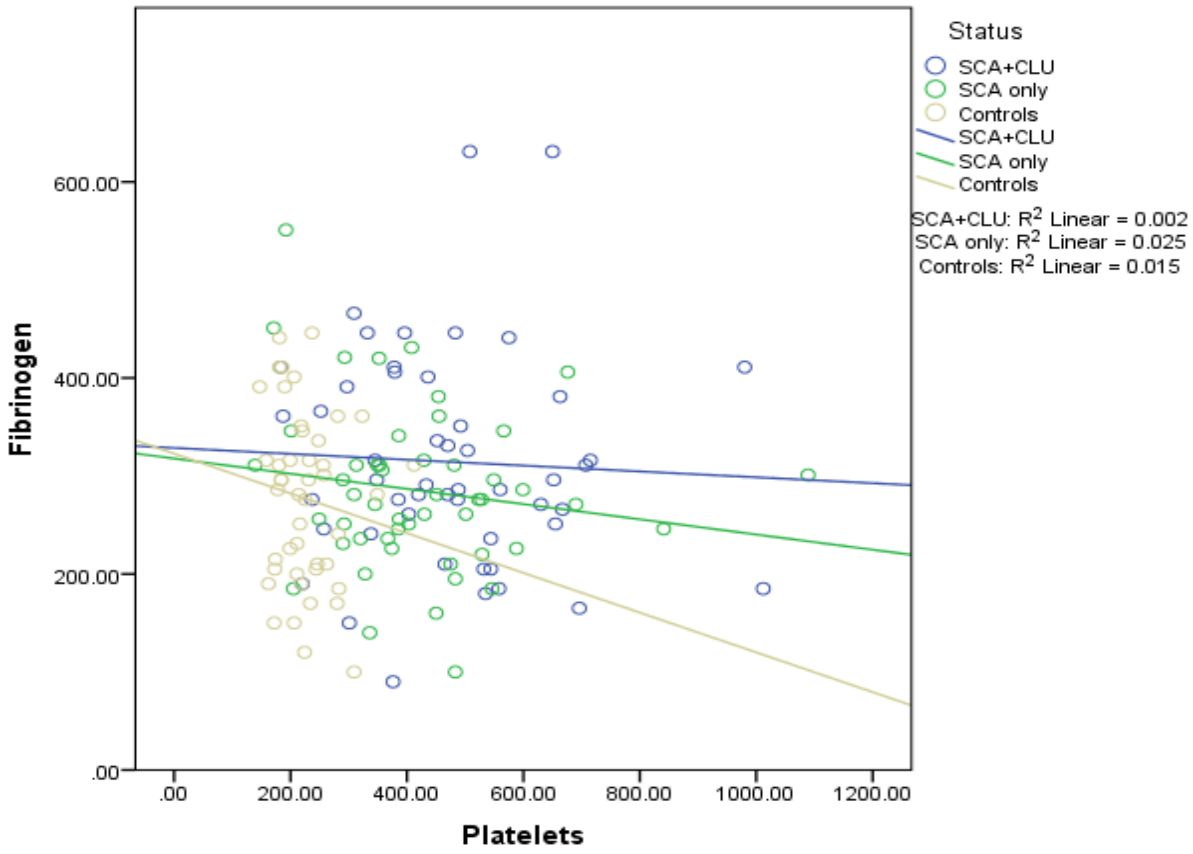


**Figure 5: Relationship between Platelets and Fibrinogen**

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Figure 6 shows the relationship between platelets and d-dimer levels in various study groups. There was also no significant correlation between the platelets and d-dimer in SCA with CLU subjects (r= -0.108, p=0.457) and control subjects (r=-0.119, p=0.436); but significant association was found in SCA without LU subjects (r=0.343, p=0.015).

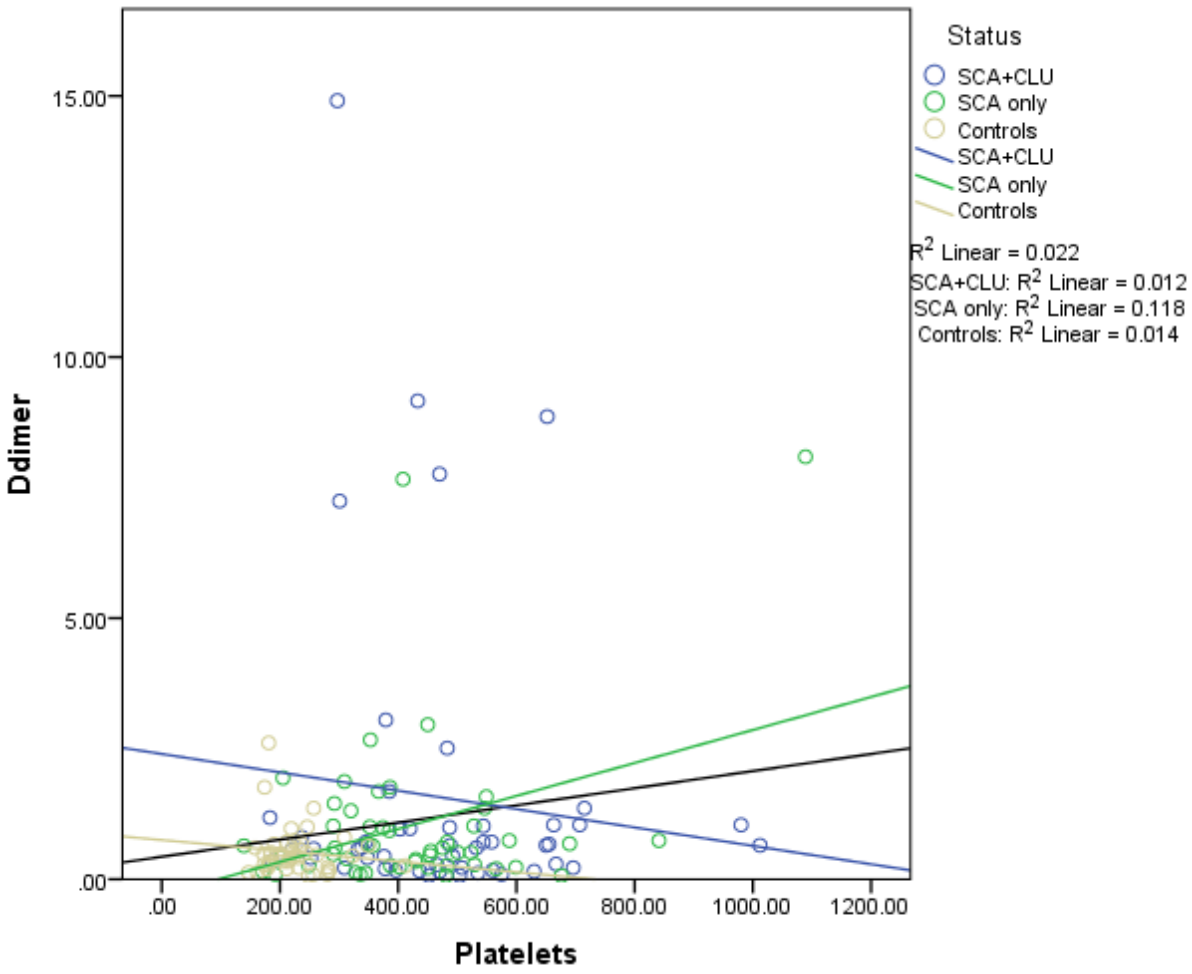


**Figure 6: Relationship between Platelets and D-Dimer**

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# CHAPTER FIVE

## 5.0 DISCUSSION, LIMITATION RECOMMENDATION AND CONCLUSION

This study aimed to determine the association, if any, between coagulation abnormalities and the occurrence of chronic leg ulcer among SCA patients. Fifty (50) SCA patients with CLUs, 50 SCA patients without LUs and 45 Hb AA subjects were used as controls for this study.

Data from the study suggests that CLUs in SCA patients occur at a relatively older age and agrees with the report by Minniti et al, that though the prevalence of leg ulcers is usually low, minority of cases occur below the age of 10 years (Minniti et al., 2010b). Also, SCA subjects with CLU had significantly lower levels of haemoglobin compared to the SCA subjects without LU. This may suggest that the events surrounding the development and sustenance of the ulcer predisposes sufferers to an increased level of haemolysis and consequently a reduced haemoglobin level relative to subjects with only SCA without LU.

### 5.1 D-dimer Levels in Subject Groups

The findings in this study indicate that d-dimer levels were elevated in sickle cell patients with chronic leg ulcers compared to the controls. D-dimer levels have been reported to be elevated in HbSS patients compared to HbAA controls. For instance in United Kingdom, Mohan et al. (Mohan et al., 2005) reported elevated d-dimer levels for HbSS subjects compared to those with HbSC and HbAA.

There was no significant difference in the mean d-dimer levels between the SCA subjects without CLU (1.04 mg/ml) and those with CLU (1.56 mg/ml) subjects. The two SCA groups were in the steady state and values recorded for d-dimer in this study are comparable to those reported by Fakunle and his colleagues who reported a mean steady state d-dimer level of 1.32 mg/ml (Fakunle et al., 2012). There was as well a non-significant difference observed in the

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mean d-dimer levels of SCA without LU and control subjects (p=0.559). This contrasted the study by Fakunle et al, 2012 who found a significant difference in d-dimer levels of steady state SCA patients (1320.00 ng/mL) from Hb AA (73.59 ng/mL) control subjects. There was however a significant difference among the d-dimer levels of SCA with CLU and controls (p=0.032). From this we may say in agreement to the study by Fakunle et al, 2012 that in steady state, SCA with and without leg ulcers have an activated coagulation and fibrinolytic systems. The activation of coagulation has been reported to be in response to increased haemolysis in sickle cell patients, especially in those with chronic leg ulcer. An increased level of d-dimer is an indication of increased fibrin formation and an active fibrinolytic system.

This study also showed an inverse relationship between d-dimer levels and the Hb concentration of study subjects. Idell, has described how fibrin deposition together with platelet aggregation could lead to microangiopathy and consequently result in intravascular haemolysis which could reduce Hb levels (Idell, 2003). This presents with many schistocytes in a blood film.

**5.2 Fibrinogen levels in subject groups**

Abnormalities in fibrinogen level and/or function are well established risk factors for venous thromboembolism. An elevated plasma fibrinogen level significantly increases the risk of venous thromboembolism; this risk is concentration-dependent and present across the various genders (van Hylckama Vlieg and Rosendaal, 2003).

Nsiri et al, 1996 citing Girot (1992) reported that fibrinogen levels are increased in SCD (Nsiri et al., 1996b). Values from this study although normal were similar (p=0.127) across the study groups. There was also no significant difference in the mean fibrinogen level between the study

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groups. Levels of fibrinogen in our study subjects including controls were significantly lower in both SCA with CLU (p=0.0051) and SCA without LU subjects (p=0.0003) compared to values recorded by Buseri et al., (2006) among steady state Hb SS patients in Nigeria. It is however, not clear why that difference. The control (Hb AA) group from this study showed similar fibrinogen level compared to the Hb AA group (p=0.590) from the study by Buseri and colleagues (Buseri et al., 2006).

Nsiri et at, (1996) in contrast to the reports from Nigeria and this study reported significantly lower fibrinogen (Hb SS in steady state 96.5 ± 17.0 mg/dl, $p < 0.001$ and Hb AA 102.5 ± 11.5 mg/dl, $p < 0.001$) levels. One of the reasons for the low fibrinogen levels may have resulted from their lower sample size (n=12) (Nsiri et al., 1996b). This phenomenon will have to be researched to find out what accounts for the differences.

Raised fibrinogen levels have been implicated in the development and progress of leg ulcers. This happens in the event of venous hypertension when the vessel walls are stretched allowing the leakage of waste products of fibrinogen as well as other blood proteins into the surrounding tissues causing venous eczema and provided other factors (leg ulcer allergens especially) come to play in which one may end up with a leg ulcer (Dowsett and Newton, 2005).

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### 5.3 APTT and PT in Subjects

Sickle cell anaemia subjects with chronic leg ulcer had a significantly shortened APTT compared to those without LU (p=0.014) and controls (p=0.002). However, the APTT for the SCA subjects without LU was similar to that of the Hb AA controls (p=0.605). This is in keeping with the assertion that sickle cell anaemia with leg ulcer is characterised by a hypercoagulable state. The finding from this study however is in contrast to findings from other studies. In the study by Buseri et al, (2006), a significantly prolonged APTT was recorded (46.0 ± 9.6secs, $p < 0.001$) among steady state Hb SS patients. The control subjects as well showed a significantly prolonged APTT (41.0 ± 3.7, $p < 0.001$), compared to the HbAA controls from this study. This difference ought to be researched. Management of these patients could be directed towards preventive more than curative measures. In contrast to the study in Nigeria by Buseri et al, values provided for the APTT per the study by Nsiri et al, (1996) in Tunisia was 34.4 ± 4.2, ($p> 0.05$) and was quite similar to the values provided per this study among SCA subjects without leg ulcer. The differences in APTT values could have resulted from the definition criteria for "Steady State in SCD". Also from this study it was realised that as the APTT approaches normal, the Hb of the subjects also approaches normal levels (r=0.185, p=0.026). This may suggest that red cell destruction resulting from mechanical damage from fibrin deposition in the microvasculature reduces as the APTT comes to normal and hence an improved Hb in the subjects.

Prothrombin time (PT) values recorded among the SCA subjects with CLU and those without LU seem to be borderline high while that of the controls are conveniently within reference limits. The differences in mean values of the study groups was statistically significant (p=0.006). This

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agreed with a study in Enugu, Nigeria with a sample size of 50 individuals, reported a prolonged prothrombin time compared to HbAA controls (Chinawa et al., 2013). For sickle cell anaemia subjects with CLU, the prolonged PT seem to suggest that the formation of a clot at a site of vessel wall injury may be somehow compromised and that may have contributed to the pathology of CLU

The prothrombin Time tends to be prolonged with higher platelet counts ($r=0.241$, $p=0.004$). A similar correlation between the Prothrombin Time and Platelets count was seen among the female subjects ($r=0.299$, $p=0.030$) but not the males. These findings calls for further investigation especially regarding levels as well as functional integrity of FVII among these groups of SCA subjects since the PT provides both tissue thromboplastin and calcium. This investigation must also consider gender variations if any.

In conclusion, one may say that a shortened APTT in Ghanaian SCA subjects suggests a higher degree of hypercoagulability compare to what has been reported by earlier studies and must be further investigated.

**5.4 Platelets count, Age, Hb levels, Duration and Frequency of Leg ulcers**

Platelet counts were also significantly increased (mild to moderate) above the normal in both SCA subjects with CLU and those without LU ($p<0.001$) compared to the Hb AA controls. The difference, however, was not significant ($p= 0.123$) comparing counts between the SCA subjects with CLU and those without LU. Platelets are reportedly activated in SCD during the steady state and further activation is seen during acute pain episodes (Ataga et al., 2012). It has been reported also that circulating platelets in sickle cell patients are chronically activated and platelet aggregation is increased (Kenny et al., 1980, Westwick et al., 1983). This may be attributed to

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the increased numbers of young, metabolically active platelets or increased plasma levels of platelets agonists, such as epinephrine, adenosine diphosphate or thrombin, in the blood of sickle cell patients (Ataga and Key, 2007a). Conditions such as those mentioned above might contribute to hypercoagulability in such patients and it has been reported by Marechal et al., 1999 hypercoagulable disorders may be the cause of chronic leg ulcers, either indirectly as a consequence of venous thrombosis, or directly by thrombus formation in small arteries, arterioles, capillaries or venules (Marechal et al., 1999).

The history of recurrence of the leg ulcers correlated negatively with the platelet counts ($r = -0.311$, $p=0.028$). One cannot make much of a meaning of this correlation since this study is cross-sectional. A prospective study will best explain the link between the recurrence of the leg ulcer and platelet count. The platelet counts also correlated negatively with age of the subjects ($r = -0.349$, $p<0.001$) and may suggest that in our subjects, platelet counts decreased relatively with increasing age. The study also showed a positive correlation between age and the duration of the leg ulcer ($r=0.351$, $p=0.015$). The above points seem to suggest that the ulceration of the leg among the subjects occurred at a relatively younger age and becomes chronic taking most often than not several years to heal. The analysis also seem to suggest that as the leg ulcer becomes chronic, patients Hb reduces further from normal levels ($r = -0.331$, $p=0.021$) and may further suggest that an event initiates the ulceration of a leg in our subjects and as that event becomes chronic, it results in further destruction of RBCs and consequently reducing the haemoglobin level.

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### 5.5 Limitation of the Study

The study design could only infer association but not causality. Funding challenges limited the expansion of the study to consider other sickle cell phenotypes as well as measure levels of specific coagulation and fibrinolytic markers as well as assess the role of anti-coagulation factors in the development and sustenance of leg ulcers in our subjects.

### 5.6 Conclusion

Chronic leg ulcer in SCA patients seem to occur at a relatively older age with a lower frequency below age 10 years. Sickle Cell Anaemia patients with chronic leg ulcers have a significantly lower Hb compared to those without leg ulcers. D-dimer levels were significantly higher in SCA with CLU subjects compared to controls. The negative correlation between D-dimer and Hb seem to suggest that when fibrin formation reduces towards normal the Hb tends to improve. Fibrinogen levels were similar among the groups.

The APTT was significantly shortened in this study for the SCA with CLU and SCA without LU subjects compared to what has been reported in other studies. It is however, suspected that differences in the interpretation of the 'Steady State" among the various studies might have contributed to this phenomenon. However, the Hb of subjects seems to improve as the APTT approached normal. Strangely, it was observed that the PT was prolonged with higher platelet counts particularly in the female subjects suggesting a compromised platelet function. For Sickle Cell Anaemia subjects with CLU, the prolonged PT seem to suggest that the formation of a clot at a site of vessel wall injury may be somehow compromised and that may have contributed to the pathology of CLU. It was however realised that the APTT was shortened among the SCA subjects and may suggest a hypercoagulable state among our subjects.

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The shortened APTT indicates a highly active coagulation system and thus a hypercoagulable state which could lead to accelerated vessel thrombosis. Once there is thrombosis it could lead to congestion and subsequently venous hypertension which can facilitate the development and sustenance of a leg ulcer.

Although platelet counts were increased among the SCA subjects there was no significant difference among the two SCA groups suggesting equal thrombotic tendencies for the subjects. The frequency of recurrence of leg ulcers was influenced by the platelet count with increased counts playing to one's advantage. Also the age at which one gets the ulcer seems to influence the time of healing with relatively younger subjects healing faster. Also, the ulcer tends to heal faster with a much improved Hb level.

### 5.7 Recommendation

1. Future research should consider a longitudinal study design for SCA patients to assess the actual contribution of coagulation factors to the development and sustenance of leg ulcers.
2. The levels of markers of coagulation can be measured in crisis state and compared to steady state in order to determine the role of coagulation in resurgence of crisis.
3. Assessment of anticoagulants could be done in addition to procoagulant factors to determine and assess relative role of activation of coagulation in sickle cell patients with chronic leg ulcers.
4. Specific procoagulants that influence the APTT will have to be assessed in order to better understand the aetiology of the shortened APTT in SCA subjects.

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5. The difference in the levels of some of the determinants of the coagulation activity in SCA patients per this study in relation to other studies within the West Africa sub-region as well as North Africa needs further research.
6. Determination of HbS haplotypes as well as the single nucleotide polymorphisms within a particular haplotype in relation to this study could be considered in future research and may lead us to some answers.
7. It may be suggested per the findings of this study that APTT, d-dimer levels, platelet counts and Hb could be monitored periodically especially in SCA patients who have not developed leg ulcers to aid in preventive management of these patients. Patients with CLU could also be monitored with the same markers to monitor progress of healing to confirm the reliability of these markers.

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## APPENDIX A:

**QUESTIONNAIRE**

**Name** …… **Folder No**: ……

**Study No**.: ……..

**Demographic data**

**Date of Birth**:……..**or Estimated**

**age**:……**years**

**Gender** …… **Residence** ……

**Blood Pressure** …….

1. How long have you had this leg ulcer?

   [a] …….. Years [b]…….months [c] ……..weeks

2. When was the first time you had the leg ulcer?

   ----------

3. How many times has it reoccurred?

   ……

4. Do you come regularly to dress your wound?

   [a] Yes [b]No

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## Appendix B:

### Blood Sample Collection and Processing

After gloves were worn, 7.5mls of venous blood sample was collected from the anticubital fossa or the back of the hand using a disposable 10.0ml syringes and 21G needles. To do this, the site of the venepuncture was cleansed with 70% alcohol and a tourniquet was tied around the upper arm to apply a little pressure and restrict blood flow through the vein causing the vein below the tourniquet to distend. The needle was inserted into the vein and the tourniquet was released as soon as blood begins to flow into the syringe. The needle was removed from the puncture and a dry cotton wool was placed over the puncture site and pressure applied to stop the bleeding. Three millilitres of blood was dispensed into a vacuum sample tube containing Ethylene Diamine Tetra Acetic acid (EDTA) anticoagulant and 4.5ml into 0.5ml of the 0.109M trisodium citrate tube and mixed gently. For the purpose of the study, gender, study Identification Number (ID), Pathological Number, date of sample collection and time of sample collection were used both on the sample bottle and the patient designed form.

The samples in the trisodium citrate tubes were centrifuged at 3000g for 15 minutes without delay. The plasma samples were aliquoted into eppendorf tubes and stored at -80℃ for the coagulation assays. The three millilitres whole blood samples in the EDTA tubes were analysed immediately for sickle slide test and full blood count and Hb electrophoresis on cellulose acetate paper at alkaline pH of 8.4 – 8.6.

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## Appendix C:

**Equipment**

1) HumaClot Duo$^{plus}$ Coagulometer.
2) Auto pipettes (20ul-1000ul).
3) Coagulation Cuvettes of manufacturer's specifications.
4) Gloves
5) Waste collector-for used disposables.
6) Bench top Laboratory centrifuge
7) A refrigerator
8) A blunt-nosed thumb forceps
9) Test tubes (12 × 72 mm)
10) Test tube rack
11) Marker for labeling

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## Appendix D

### Test Procedure for PT

### Reagents

Control plasma sample

Thromboplastin LI

0.025M Calcium Chloride

Distilled Water

### Procedure

A 0.1ml of platelet-poor plasma of the test sample in duplicate was pipetted into analyser's pre-warmed coagulation cuvette at 37$^{o}$C and the timer key was pressed to start the stop-watch for 3 minutes.

1) 0.1ml of the control sample in duplicate was also pipetted into the analyser's pre-warmed coagulation cuvette at 37$^{o}$C and timer key was pressed to start the stop-watch for 3 minutes.
2) Cuvettes (both the test sample and the control sample) were transferred to the measuring positions in the analyser.
3) 0.2ml of a freshly prepared equal volume of the thromboplastin and the calcium chloride was added to both the test samples and the controls. Simultaneously, the "Optic key 1" was pressed twice to displayed the "Active" message showing that the channel 1 is ready to start the reaction
4) When test is complete, results are displayed on the screen. Results are displayed in an order of patient's time, measured in seconds and in INR. Control sample results are also displayed in the same order.

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Each tube was handled as below;

| Tube | Test | Control |
|---|---|---|
| Patient's Sample | 0.1 ml | - |
| Control Sample | - | 0.1 ml |
| Thromboplastin with $CaCl_2$ | 0.2 ml | 0.2 ml |

ISI=International Sensitivity Index.

INR= International Normalization Ratio

**Normal Reference Ranges:** From reagent manual

PT = 12 - 16 Sec.

Control result = 14.0 Sec

Normal INR = 1.0 - 1.5,

Anticoagulant therapy INR = 2.0 – 3.5

ISI = 1.11

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## Appendix E:

### Test Procedure for APTT

### Reagents

APTT Si L Minus

Calcium chloride, 0.025M

Control sample

Distilled Water

### Procedure

1) The reagents and the coagulation cuvettes were pre-warmed at 37$^{o}$C on the analyzer.
2) 0.1ml of platelet-poor patient's plasma in duplicate was delivered into the analyser's pre-warmed coagulation cuvettes at 37$^{o}$C for 2 minutes.
3) 0.1ml of the control sample in duplicate was also pipetted into the analyser's pre-warmed coagulation cuvette at 37$^{o}$C for 2 minutes
4) 0.1ml of the pre-warmed APTT Si L Minus was added to the test plasma and the control plasma and incubated for exactly 5 minutes at 37$^{o}$C.
5) Cuvettes of both the test sample and the control sample were transferred into the measuring position on the analyser.
6) 0.1ml of the pre-warmed 0.025M calcium chloride was added to both the test samples and the control samples. Simultaneously, the "Optic 1" key was pressed twice to display the "Active" message showing that the channel 1 is ready to start the reaction
7) When the test is complete, the result is then displayed on the analyser screen.

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Each tube was handled as below;

| Tube | Test | Control |
|---|---|---|
| Patient's Plasma | 0.1 ml | - |
| Control Plasma | - | 0.1 ml |
| aPTT Reagent | 0.1 ml | 0.1 ml |
| Calcium Chloride | 0.1 ml | 0.1 ml |

**Normal Reference Range**

APTT = 26 – 36 Seconds

Control Result = 30 Seconds

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## Appendix F

### Test Procedure for Thrombin Time

### Reagents

Thrombin solution

Control Sample

Distilled Water

### Procedure

1) 0.2ml of platelet-poor plasma of the test sample in duplicate was pipette into analyser's pre-warmed coagulation cuvette at 37$^{o}$C and timer key was pressed to start the stop-watch for 3 minutes.
2) 0.2ml of the control sample in duplicate was also pipetted into the analyser's pre-warmed coagulation cuvette at 37$^{o}$C and timer key was pressed to start the stop-watch for 3 minutes.
3) Cuvettes (both the test sample and the control sample) were transferred into the measuring position in the analyser.
4) 0.1ml of the Bovine Thrombin Solution was added to both the test samples and control samples. Simultaneously, the "Optic 1" key was pressed twice to displayed the "Active" message showing that the channel 1 is ready to start the reaction
5) When test is complete, results are displayed on the screen in seconds.

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Each tube was handled as below;

| Tube | Test | Control |
|---|---|---|
| Patient's Sample | 0.2 ml | - |
| Control Sample | - | 0.2 ml |
| Bovine Thrombin Solution | 0.1 ml | 0.1 ml |

**Normal Reference Ranges**

Thrombin Time = 12 – 15 Seconds

Control result = 13 Seconds

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## Appendix G

**Test Procedure for Fibrinogen**

**Reagents**

Thrombin 100 NIH/Ml

Fibrinogen Calibrator

Owren's Buffer

Kaolin Suspension

Distilled Water

Specific Assayed Control N

Specific Assayed Control A

**Procedure**

1. **Preparation of Calibration/ Standard curve**

a. The fibrinogen calibrator was reconstituted with exactly 1.0ml of distilled water. The content was mixed gently and allowed to stand for 10minutes.

b. The thrombin reagent 100 NIH U/mL was reconstituted with exactly 2.0mls of distilled water and allowed to stand for 15minutes.

c. Five (5) test tubes (1-5) 10 x 75 mm were labelled. Each test was performed in duplicate

d. Serial dilutions were prepared in duplicates in Owren's buffer to prepare a standard curve as shown in the table below.

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Plasma Dilution for Clauss Fibrinogen 100

| **Tube No.** | **Fibrinogen Calibrator (ml)** | **Owren's Buffer (ml)** | **Dilution** |
|---|---|---|---|
| 1 | 0.2 | 0.8 | 1:5 |
| 2 | 0.1 | 0.9 | 1:10 |
| 3 | 0.1 | 1.9 | 1:20 |
| 4 | 0.1 | 2.9 | 1:30 |
| 5 | 0.1 | 3.9 | 1:40 |

| Tube No: | $T_1$ (S) | $T_2$(S) | Average (S) | Dilution | Conc. (mg/dl) |
|---|---|---|---|---|---|
| 1 | 14.00 | 12.40 | 13.20 | 1 : 5 | 556 |
| 2 | 24.20 | 23.50 | 23.85 | 1 : 10 | 278 |
| 3 | 27.60 | 29.10 | 28.35 | 1 : 20 | 139 |
| 4 | 50.20 | 48.30 | 49.25 | 1 : 30 | 92 |
| 5 | 43.60 | 47.50 | 45.55 | 1 : 40 | 70 |

Each of the diluted Fibrinogen calibrator solution was treated with the Thrombin Reagent.

a. 0.2ml each of the diluted Fibrinogen Calibrator solution was dispensed into a coagulation cuvette and the timer key was simultaneously pressed to start the stopped watch.
b. The solution was pre-warmed for 2minutes at $37^{o}$C.
c. The cuvette was then transferred to the measuring position
d. The "Optic 1" key was pressed twice to display the "Active" message showing that the channel 1 is ready to start the reaction

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e. 0.1 ml of thrombin reagent (100 NIH U/mL) was then added simultaneously to the test cuvette

f. Once clots are detected, result output will be shown on the displayed screen in seconds and in mg/dl.

g. Each test was performed in duplicate.

The assigned fibrinogen calibrator plasma value which was used to prepare the standard curve was 278mg/dl. This value corresponds to 100% activity and equals to the 1:10 dilution.

The 1:5 dilution equals to 556mg/dl and corresponds to twice as concentrated.

The 1:20 dilution equals to 139mg/dl

The 1:30 dilution equals to 92mg/dl and

The 1:40 dilution equal to 70mg/dl

These calibration data were then entered in the setup test menu for the standard curve.

2. **Patients and control Sample Preparation**

   a. 1:10 dilutions of the patient plasma and control plasma were prepared in Owren's buffer.

   b. The mixture was mixed without shaking.

3. **Testing**

   a. All tests were performed in duplicate.

   b. 0.2 ml of patient plasma dilution was pipetted into the coagulation cuvette and incubated for 3 minutes.

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c. 0.1 ml of thrombin reagent (15-30$^{o}$C) was added and the "Optic 1" key was pressed twice to display the "Active" message showing that the channel 1 is ready to start the reaction.

d. The results are displayed on the screen when clots are formed.

e. The control sample was also treated the same

**Normal Reference Range: 150 – 350 mg/dl**

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## Appendix H

**Test Procedure for D-Dimer**

**Reagents**

D-dimer Blue Latex

D-dimer Blue Buffer

D-dimer Diluent

D-dimer Calibrator

D-dimer Control High

D-dimer Control Low

**Calibration Curve**

| Tube No: | D-dimer Calibrator | D-dimer Diluent | Dilution |
|---|---|---|---|
| 1 | 0.2 | - | Undiluted (Neat) |
| 2 | 0.1 | 0.9 | 1:10 |
| 3 | 0.1 | 1.9 | 1:20 |
| 4 | 0.1 | 2.9 | 1:30 |
| 5 | 0.1 | 3.9 | 1:40 |

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| Tube | $E_1$ | $E_2$ | Average E | Calibrator (U) Concentration | U/ E |
|---|---|---|---|---|---|
| 1 | 0.194 | 0.196 | 0.195 | 2.888 | 14.81 |
| 2 | 0.107 | 0.104 | 0.103 | 1.444 | 14.02 |
| 3 | 0.060 | 0.060 | 0.060 | 0.963 | 16.04 |
| 4 | 0.028 | 0.024 | 0.026 | 0.722 | 27.77 |
| 5 | 0.000 | 0.000 | 0.000 | 0.577 | 0.00 |

U = Universal Unit / mL

E = Optical Density

Factor (F) = 14.5ug/ml

**Normal Reference Range:** 0 - 0.5µg/mL or <500µg/L (Perrier et al., 1997)

**Test Procedure**

a. All tests were performed in duplicate

b. 0.025ml of the plasma sample was pipetted into the coagulation cuvette

c. 0.025ml of the controls (High and Low) were pipetted into the coagulation cuvette

d. 0.100ml of the reaction buffer was added to each of the contents

e. The mixtures were incubated for 2 – 10 minutes.

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f. Each cuvette was transferred to the measuring position.

g. 0.050ml of the pre-warmed latex reagent was added and the “Optic 1” key was pressed twice to display the “Active” message showing that the channel 1 is ready to start the reaction

h. Using the pipette, the content was well mixed repeatedly for 15 times.

i. The results were displayed on the screen in both Optical Density (OD) and in Concentration

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## Appendix J

### Test Procedure for 3part Haematology Autoanalyser

### ABX Micros ES60 OT/CT

The ABX Micros ES60 OT/CT is a full automated haematology analyser used for blood cell count. Its performance doesn't require any manual operations for aspirating, dilutions, measuring, and calculations of the blood cells. The cells are measured by an electronic impedance variation principle. This means that an electronic field is generated around the micro-aperture in which the blood cells pass through. Inside the detector, the sampling needle (nozzle) is positioned in front of the aperture and in line with the centre.

Firstly, the samples were well mixed gently and thoroughly using a mechanical mixer. The blood collection tube cap was removed and the sample placed beneath the nozzle. The sample tube was then raised up so that the sampling needle lowers into the blood and simultaneously pressed the manual sample bar. 28.3µl of the blood sample is then aspirated through the aperture centre into a conical chamber.

The cells create a resistance (impedence) in the electronic field between two electrodes as they pass through the calibrated micro-aperture. Since the current is constant and remains unchanged, the larger the cell is, the "more" resistance it has. The smaller the cell is, the "less" resistance it has. The voltage which measures the cells is proportional to the cell size. The larger the cell, the higher the voltage will be. The smaller the cell, the lower the voltage will be.

This in turn causes an electronic pulse to be generated which is amplified, measured and then mathematically calculated to create a numerical value. When the analysis is completed, the "Sample analysis" dialog box is closed and results are automatically displayed in the "Result display" screen.

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**Quality Control**

Daily quality control is always performed on three (3) levels of control blood material (low, normal and high) to verify that the instrument is performing within the specified ranges of the quality control material before any test blood sample is analysed.

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## Appendix K

### Test Procedure for Sickle Slide Test

### Reagents and Equipments

Microscope

Microscope slides

Cover slides

Pasteur pipette

2% Sodium Metabisulphite

Distilled Water

### Test Procedure

1. One drop of the test whole blood sample was added to one drop of a freshly prepared 2% sodium metabisulphite
2. Using the edge of the cover slide the mixture was mixed thoroughly and gently.
3. The cover glass was placed over the mixture on the slide.
4. The mixture was examined microscopically after one hour for sickle cells using the high power lens of the microscope (×40 objectives).

### Control

A known abnormal blood sample containing haemoglobin S (Positive Control) and a known normal blood sample containing haemoglobin AA (Negative Control) were run alongside with each batch of test.

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## Appendix L:

**Test Procedure for Hb Electrophoresis**

**Equipments and Reagents**

Electrophoresis Tank

Power Pack

Cellulose Acetate Membranes

Applicator

Staining Equipment

Tris Buffer

Applicator Well

Control Haemolysates

Wetting Agent

Distilled water

**Haemolysate preparation**

i. Each test sample was centrifuged at 3,000rpm for 5mins and the plasma was removed by careful aspiration leaving the packed red blood cells
ii. The packed red blood cells were re-suspended in a physiological saline and washed 3-4 times at 3,000rpm for 5minutes to get rid of any protein component that might interfere with the migration of the haemoglobin variants.
iii. The washed red blood cells were haemolysed by mixing 2 volumes of the red blood cells to 1volume of distilled (deionized) water.
iv. One volume of carbon tetrachloride was added to the mixture and was shaken vigorously for several minutes for proper mixing.
v. The mixture was then centrifuged at 3,000rpm for 30mins.
vi. The clear supernatant haemolysate was then transferred to a clean sample container.

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**Procedure**

1. Each test sample was taken through a process to obtain a haemolysate sample.
2. 100ml of the Tris buffer was poured into each of the chambers of the tank.
3. The Cellulose Acetate Paper was placed in the Tris buffer by lowering it slowly (to avoid bubbles) into the buffer for at least 5 minutes before use.
4. With the aid of the capillary tube the sample well plate was filled with the control sample (haemolysate with A, S, C and F variants) as well as the test samples.
5. The cellulose acetate paper was removed from the buffer and blotted between two layers of blotting paper to reduce excess buffer.
6. The applicator was loaded with the samples and applied onto the cellulose acetate paper.
7. The loaded cellulose acetate paper was placed across the two bridges with samples on top of the paper in which the starting point was closer to the negatively charged electrode (cathode) whilst the blank part was closer to the positively charged electrode (anode).
8. The tank was connected to a power pack and voltage was set between 250–350volts and allowed to run for 45 minutes
9. At the end of the 45 minutes, the haemoglobin variants were separated clearly and the test sample results were recorded by comparing with the control sample results.
10. The cellulose cellulose acetate paper was transferred into a square flat bottom staining container and was stained with Ponceau S. This was done by immersing the cellulose acetate paper in the Ponceau stain for 5minutes. The excess stain was then washed for 5minutes in 5% acetic acid and blotted with a clean blotting paper and leave to dry.

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**Consent Form**

Title: A study of Coagulation Profile in Sickle Cell Anaemia Patients with and without Chronic Leg Ulcers at the Ghana Institute of Clinical Genetics, Accra.

Principal Investigator: David Sackey, BSc. Department of Haematology, Box 4236, Accra.

**Information:** (To be read or translated to patient in their own mother tongue)

*Dear Volunteer*

This consent form contains information about the research entitled; *A study of Coagulation Profile in Sickle Cell Anaemia Patients with and without Chronic Leg Ulcers in Ghana.* In order to be sure that you are informed about participating this study, we are asking you to read (or have read to you) this Consent Form. You will also be asked to sign it (or make your mark **in the presence of** a witness). We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understand.

**Why this study is planned**

You are being asked to participate in the above study in order to find out factors in the blood that may prone sickle cell disease patients to develop sores on the leg (leg ulcer) that is difficult to heal. Sickle cell disease is a condition in which the hereditary material in the blood cells is modified, making those with this condition developing body pains, blood shortages and unhealing leg sores. These sores on the legs of sickle cell patients are difficult to treat and might reoccur later when it's even healed. We do not understand fully the factors that predispose sickle patients to the development of leg sores. To understand this problem we need to study sickle cell patients with leg sores and compare them to sickle cell patients without leg sores as well as individuals without sickle cell disease. If we can find the answer to this problem, we hope to be able to suggest new ways to prevent and manage leg sores in sickle cell patients.

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**General Information and your part in the study**

For you to qualify to be part of this study, you should be 12years or more. If you agree to be in the study, we will collect 7.5ml of venous blood sample for laboratory analysis. When your blood sample is taken, you will not be identified as being in this research.

**Possible Benefits:** There are no direct benefits to you from this study. However, your participation may help us develop better management strategy for leg sores in sickle cell disease.

**Possible Risks:** The amount of blood collected **will not have adverse effect on your health**, although there may be a slight pain and bruising at the bleeding site. All subjects will receive appropriate treatment as necessary. Sterile techniques and disposable, single-use equipment will be used at all times.

**Withdrawal from study**

We would like to stress that this study is strictly voluntary. Should you decide not to participate; it will have no consequences for you. If you decide at any point during the study that you do not wish to participate any further, you are free to terminate the participation, effective immediately. Any such decision will be respected without any further discussion. Your decision will not affect the health care you would normally receive.

**Confidentiality**

All information gathered would be treated in strict confidentiality. We will protect information about your taking part in this research to the best of our ability. You will not be named in any reports. However, the staff of the Korle-Bu Central Laboratory may sometimes look at your research records. If you have any questions, please feel free to ask the physician or researcher in charge.

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**Contacts:** If you ever have any questions about the research study or study-related problems, you may contact the following: Mr. David Sackey (0243615231), at the Department of Haematology. For questions about the ethical aspects of this study or your rights as a volunteer, you may contact the investigator, Mr. David Sackey of Haematology, University of Ghana Medical School.

**Your rights as a participant**

This research has been reviewed and approved by the University of Ghana Medical School Ethical Review Board. An Ethical Review Board or Ethical Committee is a committee that reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant, you may contact the investigator, Mr. David Sackey (0243615231) of Haematology, University of Ghana Medical School.

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**VOLUNTEER AGREEMENT**

The above document describing the benefits, risks and procedures for the research title: *Coagulation Profile of Sickle Cell patients with chronic Leg Ulcers in Ghanai*ans been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

---------- ----------

Date Signature or Thumbprint of volunteer

**If volunteer cannot read the form himself/herself, a witness must sign here:**

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

---------- ----------

Date Signature or Thumbprint of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

---------- ----------

Date Signature Person who obtained Consent

University of Ghana http://ugspace.ug.edu.gh

**UNIVERSITY OF GHANA MEDICAL SCHOOL**
**COLLEGE OF HEALTH SCIENCES**
*ACADEMIC AFFAIRS OFFICE*

**Phone: +233-0302-666987-8**
**Fax: +233-0302-663062**
**E-mail: academic.ugms@chs.edu.gh**
**My Ref. No: MS-AA/C.2/Vol.16[A]**

**Your Ref. No.**

**P O Box 4236**
**Accra**
**Ghana**

18th April, 2013

David Sebbie Sackey
Dept. of Haematology
UGMS

**ETHICAL CLEARANCE**

Protocol Identification Number: MS-Et/M.7 – P 4.3 /2012-13

The Ethical and Protocol Review Committee of the University of Ghana Medical School on 18h April, 2013 unanimously approved your research proposal.

TITLE OF PROTOCOL:"**Coagulation Profile of Sickle Cell Patients with Chronic Leg Ulcers at the Centre for Clinical Genetics, Korle-Bu Teaching Hospital – Accra.**"

PRINCIPAL INVESTIGATOR: David Sebbie Sackey

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed:.....
PROFESSOR JENNIFER WELBECK
(CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: Dean
Head of Department
Research Office